FUSOKINE DESIGN AS NOVEL THERAPEUTIC STRATEGY FOR IMMUNOSUPPRESSION

Moutih Rafei

Division of Experimental Medicine, Department of Medicine

Faculty of Medicine, McGill University

Montreal, Quebec, Canada

January 2009

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirement of the degree of

Doctor of Philosophy (Ph.D.)

Your file Votre référence
ISBN: 978-0-494-66662-3
Our file Notre référence
ISBN: 978-0-494-66662-3

ABSTRACT

The societal burden of autoimmune diseases and donor organ transplant rejection in developed countries reflects the lack of effective immune suppressive drugs. The main objective of my thesis was to develop novel fusion proteins targeting receptors linked to autoimmunity; strategies that will allow the suppression of autoreactive cells while sparing resting lymphocytes. Interleukin (IL) 15 has been demonstrated to exert its effects mainly on activated T-cells triggered via their T-cell receptor (TCR). Since we found that the fusion of granulocyte-macrophage colony stimulating factor (GMCSF) to IL15 – aka GIFT15 - paradoxically leads to aberrant signalling downstream of the IL15R and blocks interferon (IFN)-gamma secretion in a mixed lymphocyte reaction (MLR), we hypothesized to use this fusokine in proof-of-principle cell transplantation models and shown that GIFT15 can indeed block the rejection of allogeneic and xenogeneic cells in immunocompetent mice. Additionally, we found that ex vivo GIFT15 treatment of mouse splenocytes lead to the generation of regulatory B-cells (Bregs). These Bregs express high levels of MHCII, IL10 and are capable to block antigen (Ag)-presentation *in vitro* as third party bystander cells. Moreover, a single injection of these GIFT15-generated Bregs in mice with pre-developed experimental autoimmune encephalomyelitis (EAE) leads to long lasting remission of disease.

Along those lines, we also found that mesenchymal stromal cells (MSCs) lead to the paracrine conversion of CCL2 to an antagonist form capable of specifically inhibiting plasma cells and activated Th17 cells. This mechanistic insight informed the design of a second class of suppression fusokine. Namely, the fusing of antagonist CCL2 to GMCSF – aka GMME1. We tested its potential use in autoimmune diseases such as EAE and rheumatoid arthritis (RA). We demonstrated that GMME1 leads to asymmetrical signalling and inhibition of plasma cells as well as Th17 EAE/RA-reactive CD4 T-cells. The net outcome of these pharmacological effects is the selective depletion of CCR2-reactive T-cells as demonstrated both *in vitro* and *in vivo*.

Overall, our data support the use of our fusion proteins as part of a powerful and specific immunosuppressive strategy either as directly injectable protein biopharmaceuticals or through the ex vivo generation of autologous Bregs in the case of GIFT15.

RÉSUMÉ

L'augmentation rapide et progressive des maladies auto-immunes et rejet de greffes dans les pays développés a mis en garde la communauté scientifique du manque de médicaments efficaces pour la gestion de ces problèmes médicaux. L'objectif principal de ma thèse était de développer de nouvelles protéines de fusion ciblant les récepteurs directement liés à l'inflammation, une stratégie qui permettra la suppression des cellules autoreactives tout en épargnant les lymphocytes non-activés. L'interleukine (IL) 15 exerce ses fonctions majoritairement sur les cellules T activées. Paradoxalement, nous avons constaté que la fusion de ``granulocyte-macrophage colony stimulating factor`` (GMCSF) à IL15 conduit à des activités de répression sur les cellules immunitaires *in vitro*. En effet, GIFT15 conduit à une signalisation aberrante par le récepteur IL15 et bloque la sécrétion d''interféron (IFN)-gamma pendant une réaction lymphocytaire mixte (MLR). Pour cela, nous avons émis l'hypothèse que l'usage de cette fusokine - alias GIFT15 - pourrait bloquer le rejet de cellules allogéniques et xénogéniques chez des souris immunocompétentes; ce qui était le cas. De plus, nous avons constaté que l'ajout de GIFT15 sur des splenocytes non-fractionnées conduit à la génération de cellules B régulatrice (Bregs), exprimant des niveaux élevés de MHCII, IL10 et capable de bloquer la présentation d'antigène (Ag) *in vitro* ainsi que les pathologies *in vivo* tel que démontré chez le modèle murin de sclérose en plaque.

Nous avons récemment constaté que les cellules stromales mésenchymateuses (MSCs) conduisent à la génération paracrine de CCL2 antagonistique capable d'inhiber les cellules plasma ainsi que les cellules Th17 activées. Nous avons donc fusionné la forme clivée de CCL2 à GMCSF - alias GMME1- et démontré que la protéine de fusion conduit à une signalisation asymétrique chez les cellules de plasma et Th17. L'effet pharmacologique net est la destruction sélective des cellules exprimant CCR2 tel que démonstré chez les souris de sclérose en plaques et d'arthrite rhumatoïde.

L'ensemble de nos données soutient l'usage de nos protéines de fusion dans le cadre d'une puissante thérapie immunosuppressive soit par l'injection directe de protéines recombinantes ou par l'intermédiaire de la génération de Bregs comme dans le cas de GIFT15.

ACKNOWLEDGMENTS

I would like to express my sincere and deepest gratitude to several individuals who have heavily contributed in helping me achieve this endeavour. First and foremost, I would like to thank my supervisor, Dr. Jacques Galipeau, for graciously accepting me as part of the ``*Galipean team*``. When I first started my PhD, I considered myself scientifically trained and ready to bypass any technical issue. However, I quickly realized that there was a lot to learn. As of my first interaction with Dr. Galipeau, I noticed a great passion for science supplemented with great ideas. He was always full of absolute support and ceaseless encouragements, but most importantly, Dr. Galipeau always knew how to adapt any unanticipated observation; a solution looking for a problem he says! Nevertheless, I have never seen optimism, patience, and complete belief in his students capacities. Thank you, Thank you very much Jacques! I would like also to express my sincere gratitude to my PhD committee that helped in the elaboration and guidance of my PhD objectives. More specifically, I would like to thank Dr. Moulay (Alaoui-Jamali), Dr. Prem (Ponka), Dr. Terry (Spithill), and especially Dr. Claude (Perreault) who contributed as my scientific consultant. Many thanks to my collaborators: Dr. Vilceu (Bordignon), Dr. Borhane (Annabi), Dr. Jian Hui (Wu), Dr. Simone (Zehetner), Yamina (Berchiche), Dr. Nikolaus (Heveker), Simon (Fortier), Karen (Doody), Dr. Michel (Tremblay), Dr. Adriana (Aguilar-Mahecha), Marguerite (Buchanan), and Dr. Mark (Basik). In addition, it was a pleasure and a honour to work beside: Dr. Astrid (Reimann), Dr. Nicoletta (Eliopoulos), Dr. Manaf (Bouchentouf), Dr. Ian (Copland), Dr. Oleg (Loutochin), Shala (Yuan), Jeremy (Hsieh), Patrick (Williams), Janik (Jacmain), Dr. Philippe (Campeau), Dr. Elena (Birman), Kathy Ann (Forner), Claudia (Penafuerte), Dr. Norma (Bautista-Lopez), Dr. Sabrina (Perri), Marie-Noëlle – aka MERRY CHRISTMAS (Boivin), Laurence (Lejeune), Daniel (Legault), Jessica (Cuerquis), François (Mercier), Jing (Zhao), Christian (Young), Terry (Kucic), and Milena (Crosato). I also would like to thank some of my MCETC/LDI friends for their support such as Dr. Orlando (Angulo), Abdel (Hussein), Mike (Giovinatso), Dr. Krikor (Bijian), Stépahine (Fuoco), Amanda (Kasneci) and Tomoko (Sugahara). I also would like to thank FRSQ, CIHR, American Society of Hematology, the International Society of Cell Therapy, the MCETC, and the LDI for all funding, scholarship and granted awards.

"When it is obvious that the goals cannot be reached, don't adjust the goals, adjust the action steps."

- Confucius

PREFACE

The immune system can normally distinguish self from non-self to avoid the development of autoimmune diseases. The etiology of such diseases in not completely understood but environmental factors such as bacteria, viruses, toxins, and some drugs may play a role in triggering cumulative signals leading to the development of autoimmunity in someone who already has a genetic (inherited) predisposition to develop such disorders.

Today, about 80 autoimmune diseases have been identified with more than 50 million patients in the United State alone, representing 20% of the entire population with escalading numbers (www.Womenshealthresearch.org/site/PageServer?pagename-hs_facts_autoimmune). The drugs most commonly used in the clinic are corticosteroid drugs with the possibility of using additive immunosuppressant compounds depending on the condition's severity. All of these drugs however lead to side effects, some severe. Thus, the search for new potent immunosuppressive compounds with higher specificity and low toxicity is actively being pursued.

During the course of my doctoral studies, I have developed novel receptor-based immunosuppressive fusokine strategies for the treatment/alleviation of autoimmune diseases and/or transplant-related problems with major focus on experimental autoimmune encephalomyelitis (EAE) as well as inflammatory arthritis (RA) as examples of applications. I have elected to present my research in a manuscript-based format. My work has been published in 7 first-author original peer-reviewed papers presented in their integrality as distinct chapters of this thesis in accordance with the guidelines for thesis preparation from the Faculty of Graduate Studies and Research at McGill University *("Guidelines for Thesis Preparation"):*

- Chapter 2 : **Rafei M**, Wu JH, Annabi B, Lejeune L, François M, Galipeau J. A GMCSF and IL-15 fusokine leads to paradoxical immunosuppression in vivo via asymmetrical JAK/STAT signaling through the IL-15 receptor complex. Blood. 2007; 109(5):2234-42.
- Chapter 3: **Rafei M**, Hsieh J, Zehntner S, Li M, Forner K, Birman E, Perreault C, and Galipeau J. A GMCSF/Interleukin-15 Fusokine Leads to the Generation of a

Novel Type of Immune Regulatory B-Cell with Potent Immune Suppressive Properties as Demonstrated in the EAE Mouse Model of Multiple Sclerosis (*In revision in Nature Medicine 2009*)

- Chapter 4 : **Rafei M**, Hsieh J, Fortier S, Li M, Yuan S, Birman E, Forner K, Boivin MN, Doody K, Tremblay M, Annabi B, Galipeau J. Mesenchymal stromal cell derived CCL2 suppresses plasma cell immunoglobulin production via STAT3 inactivation and PAX5 induction. Blood; 2008. 112(13):4991-8
- Chapter 5: **Rafei M**, Campeau PM, Aguilar-Mahecha A, Buchanan M, Williams P, Birman E, Yuan S, Young C, Boivin M, Forner K, Basik M and Galipeau J. Mesenchymal Stromal Cells Ameliorate EAE by Inhibiting CD4 Th17 T-cells in a CCL2-dependent Manner. (*Accepted in Journal of Immunology 2009*)
- Chapter 6: **Rafei M**, Campeau PM, Wu JH, Birman E, Forner K, Boivin M, and Galipeau J. Selective Inhibition of CCR2 Expressing Lymphomyeloid Cells in Experimental Autoimmune Encephalomyelitis by a GMCSF-MCP1 Fusokine. 2009; 182(5):2620-7.
- Chapter 7: **Rafei M**, Berchiche Y, Birman E, Boivin M, Heveker N, and Galipeau J. The CCR2 Antagonist GMCSF-MCP1 Fusokine as a Novel Antiarthritic Compound Inhibiting Inflammation in Mice with Collagen-Induced Arthritis. (*In revision by the Journal of Immunology 2009*)
- Supplemental Chapter: **Rafei M**, Birman E, Forner K, and Galipeau J. Allogeneic Mesenchymal Stem Cells as Universal Donors for Treatment of Experimental Autoimmune Encephalomyelitis. (*in revision in Molecular Therapy 2009*)

In addition to the work presented in this thesis, I was involved in collaborative studies with other members of the laboratory, which led to the following publications:

- Campeau PM, **Rafei M**, François M, Birman E, Forner K, and Galipeau J. Bone Marrow Mesenchymal Stromal Cells Engineered to Express Erythropoietin Induce Autoimmune Anemia in Immune Competent Allorecipients (*Accepted in Molecular Therapy 2008*)

- Campeau PM, **Rafei M**, Boivin M, Sun Y, Grabowski GA, and Galipeau J. Characterization of Gaucher Disease Bone Marrow Mesenchymal Stromal Cells Reveals Over-expression of the Proinflammatory Cytokine CCL2 (*In revision by Blood 2009*)
- Wang N, Fallavollita L, Nguyen L, Burnier J, **Rafei M**, Galipeau J, Yakar S, Brodt P. Autologous Bone Marrow Stromal Cells Genetically Engineered to Secrete an IGF-I Receptor Decoy Prevent the Growth of Liver Metastases. Molecular Therapy. 2009. (Epub ahead of print)

Furthermore, provisional patents have been submitted by McGill University on the developed fusokines:

- New United States Patent Application
  Title: Fusion Proteins and Methods for Modulation of Immune Response
  Country of Issue: Canada
  Inventors: Jacques Galipeau and **Moutih Rafei**

- New United States Provisional Patent Application
  Title: GMCSF and Truncated CCL2 Conjugates And Methods and Uses Thereof
  Country of Issue: Canada
  Inventors: Jacques Galipeau and **Moutih Rafei**

CONTRIBUTION OF AUTHORS

The data presented in Chapters 2, 3, 4, 5, 6, and 7 of this thesis has been performed by the doctoral candidate under the supervision of Dr. J. Galipeau. Other individuals who have contributed to the work are listed below:

Chapter 2

► ***Dr. Jian Hui Wu*** is an Assistant Professor in the Department of Oncology of McGill University and provided his expertise in the generation of 3D structure of GIFT15.

► ***Dr. Borhane Annabi*** is a Canada Research Chair in Molecular Oncology at Université du Québec à Montréal. Dr. Annabi provided information and data related to MMPs analysis.

► ***Laurence Lejeune*** was a research assistant in Dr. Galipeau's laboratory and provided her expertise in flow cytometry.

► ***Moira François*** was a research assistant in Dr. Galipeau's laboratory at the time and provided her expertise in animal-related experiments.

Chapter 3

► ***Jeremy Hsieh*** is an MD-PhD candidate in Dr. Galipeau's laboratory and provided help in the generation of Bregs.

► ***Dr. Simone Zehntner*** is the director of the Brain Imaging Centre of the Montreal Neurological Institute and provided her expertise in the establishment of the EAE mouse model of MS in Dr. Galipeau's laboratory.

► ***MengYang Li*** is an MD candidate at the Université de Montréal and provided help in the generation of Bregs.

► ***Kathy Forner*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Dr. Elena Birman*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Dr. Claude Perreault*** is a professor at the Department of Medicine of Université de Montréal and provided his expertise in the generation of bone marrow chimera mice.

Chapter 4

► ***Jeremy Hsieh*** is an MD-PhD candidate in Dr. Galipeau's laboratory and provided his expertise to study the biology of MSCs.

► ***Dr. Simon Fortier*** is a Master candidate in Dr. Annabi's laboratory and provided expertise in detected MMPs.

► ***MengYang Li*** is an MD candidate at the Université de Montréal and provided help in the analysis of MSC-plasma cells interplay.

► ***Elena Birman*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Kathy Forner*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

►***Marie-Noëlle Boivin*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in RT-PCRs.

►***Karen Doody*** is a PhD candidate in Dr. Tremblay's laboratory and provided her expertise on TC-PTP KO mice.

► ***Dr. Michel Tremblay*** is Professor in the Department of Biochemistry of McGill University and provided his expertise on TC-PTP KO mice.

► ***Dr. Borhane Annabi*** is a Canada Research Chair in Molecular Oncology at Université du Québec à Montréal. Dr. Annabi provided information and data related to MMPs analysis.

Chapter 5

► ***Dr. Philippe Campeau*** is an MD resident in Dr. Galipeau's laboratory and provided his expertise on the qRT-PCR arrays to study inflammation.

► ***Dr. Adriana Aguilar-Mahecha*** is a Proteomics Scientist at the SELDI-TOF unit of the MCETC. She performed the SELDI-TOF experiment on MSC-derived CCL2.

► ***Marguerite Buchanan*** is a Proteomics research assistant at the SELDI-TOF unit of the MCETC. She assisted Adriana with the SELDI-TOF experiment on MSC-derived CCL2.

► ***Patrick Williams*** is an MD-PhD candidate in Dr. Galipeau's laboratory and provided his expertise on Th17 cells.

► ***Dr. Elena Birman*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Shala Yuan*** is the manager and research assistant in Dr. Galipeau's laboratory and provided her expertise in the analysis of MSC-derived CCL2 by Tricine gels.

► ***Christian Young*** is a research assistant in Dr. Galipeau's laboratory and provided his expertise in flow cytometry.

► ***Marie-Noëlle Boivin*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in RT-PCRs.

► ***Kathy Forner*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Dr. Mark Basik*** is an Assistant Professor at the Departments of Surgery and Oncology of McGill University and provided his expertise on the analysis of SELDI-TOF data.

Chapter 6

► ***Dr. Philippe Campeau*** is an MD resident in Dr. Galipeau's laboratory and provided his expertise on the qRT-PCR arrays to study inflammation.

► ***Dr. Jian Hui Wu*** is an assistant Professor in the Department of Oncology of McGill University and provided his expertise in the generation of 3D structure of GMME1.

► ***Dr. Elena Birman*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Kathy Forner*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Marie-Noëlle Boivin*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in RT-PCRs.

Chapter 7

► ***Yamnina Berchiche*** is a PhD candidate in Dr. Heveker's laboratory and provided her expertise on the BRET study.

► ***Dr. Elena Birman*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Marie-Noëlle Boivin*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in RT-PCRs.

► ***Dr. Nikolaus Heveker*** is an Associate Professor at the Department of Biochemistry of Université de Montréal and provided his expertise on the analysis of BRET data.

Supplemental Chapter

► ***Dr. Elena Birman*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

► ***Kathy Forner*** is a research assistant in Dr. Galipeau's laboratory and provided her expertise in animal-related experiments.

TABLE OF CONTENT

LIST OF ABREVIATIONS

Bp	base pair
g	gram
KDa	kilodalton
Kg	kilogram
mg	microgram
ml	millilter
mm	millimiter
mM	millimolar
ng	nanogram
U	units
Ag	antigen
APC	antigen presenting cells
BCR	B cell receptor
Bregs	regulatory B cells
BRET	bioluminescence resonance electron transfer
CCL	CC chemokine ligand
CCR	CC chemokine receptor
CD	cluster of differentiation
CFP	cyan fluorescent protein
CNS	central nervous system
CSF	cerebro-spinal fluid
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
DC	dendritic cell
EAE	experimental autoimmune encephalomyelitis

FDA	food and drug agency
FOXP3	forkhead box P3
GAG	glycosaminoglycans
GFP	green fluorescent protein
GCSF	granulocyte colony stimulating factor
GIFT15	GMCSF-IL Fusion Transgene 15
GMCSF	granulocyte-macrophage colony stimulating factor
GMME1	GMCSG-MCP Fusion Transgene 1
GPCR	G-protein coupled receptor
H&E	hematoxylin and eosin
HBSS	Hank's balanced salt solution
IL	interleukin
IFN	interferon
Ig	immunoglobulin
IP10	CXC chemokine ligand 10
IRES	internal ribosomal entry site
IV	intravenous
KC	keratinocyte chemoattractant
LIF	leukemia inhibitory factor
LIX	LPS CXC-chemokine ligand 5
LPS	lipopolysaccharide
Luc	luciferase
MCP	monocyte chemotactic protein
MCSF	macrophage colony stimulating factor

MDC	macrophage-derived chemokine
MHC	major histocompatibility complex
MIP	macrophage inflammatory protein
MLR	mixed lymphocyte reaction
MMP	matrix metalloproteinase
MS	multiple sclerosis
MSC	mesenchymal stromal cells
NK	natural killer
NOD-SCID	non-obese diabetic severe combined immunodeficient
nTregs	naturally occurring regulatory T cells
PAF	
PBMC	peripheral blood mononuclear cells
PBS	phosphate-buffered saline
PD	programmed death
PI	propidium iodine
R	Receptor
RA	rheumatoid arthritis
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RBC	red blood cell
rhFVIII	recombinant human factor VIII
SC	subcutaneous
SELDI-TOF	surface-enhanced laser desorption ionization-time of flight
TAM	tumor-associated macrophages
TCR	T cell receptor
TGF	transforming growth factor
TLR	toll-like receptor

TNF	tumor necrosis factor
Tr1	inducible Tregs
Tregs	regulatory T cells
Th	T helper cell
VEGF	vascular endothelial growth factor
vWF	von Willebrand factor
YFP	yellow fluorescent protein

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1: GENERAL INTRODUCTION

1.1 IMMUNOBIOLOGY

Immunology, a medically-branched science specialized in examining the structure and function of the immune system and its interplay with the surrounding environment as early as 430 BC during the plague of Athens [1]. It was until the end of the 18th century, with the talented work of Edward Jenner on smallpox that the value of immunology and its impact of human diseases revolutionized medicine [2]. Additional scientists further studied acquired immunity where the germ theory was introduced by Louis Pasteur [2]. Since then, great advancements and rapid knowledge of how the immune system operates were made [3]. It is now clear that immunity of higher vertebrates is divided into two main arms known for their continuous interplay in order to generate effective immune responses [3]. More specifically, the more primitive innate immune system, distinct by its rapid response to various molecular patterns present on the surface of foreign bodies or agents, is primordial for the initial containment of unwanted foreign organisms [3]. In case where these foreign agents persist due to a failure in their eradication, a more specific immune reaction is induced [3]. Such response, termed adaptive immunity, is also known to confer lifelong protective memory response following termination of the immune reaction to permit rapid and more specific protection upon re-infection with the same pathogen [3].

The immune system is tightly regulated in order to avoid autoreactivity [4]. Cells of the adaptive immune system should be capable of distinguishing between the self repertoires composed of peptides derived from host's tissues and the non-self peptides found on the surface of foreign agents. Failure to make their distinction would lead to the auto-activation of the immune system to the body's own cells leading to major organ damage and eventually fatal autoimmune diseases [5]. The following sections present a general overview of the immune system and the mechanisms in place to avoid autoimmunity as well as various approaches to circumvent such diseases.

1.1.1 Basic Concepts in Immunology

i) Innate Immunity

The innate arm of the immune system is usually triggered when infectious agents or danger signals released from damaged, injured or stressed cells, are identified by pattern recognition molecules such as the toll-like receptors (TLR), which will lead to their elimination either through direct attack using cytotoxic compounds or through engulfment [3]. One of the hallmarks of innate immunity is inflammation, a process defined by redness and swelling caused by increased blood flow into a tissue. Furthermore, a controlled production of inflammatory soluble mediators leads to coordinated recruitment of leukocytes to sites of inflammation [6]. A given innate response confers no long-lasting immunity against the target in question but is primordial for the initiation of adaptive immunity [7].

ii) Adaptive Immunity

In case of failure in the containment of the initial immune-triggering stimulus, adaptive immunity is induced specifically via a process called Ag-presentation mediated by professional Ag presenting cells (APCs) [8]. The ability to re-mount such a tailored response in a quick and efficient manner in case of re-infection with the same original agent is maintained by memory cells kept quiescent over years [8]. Thus, adaptive immunity could lead to the generation of a wide repertoire of lymphocytes by VDJ recombination to produce effector lymphocytes with great diversity. The immune system is thus capable of responding to any non-self antigenic determinant in order to protect the host from threats that could be detrimental to its survival [8].

1.1.2 Cellular Immune Effectors

Lymphocytes, major effectors of adaptive immunity, carry distinct receptors that recognize specific Ags [8]. Today, cells of the adaptive immune system are divided mainly into i) killer T-cells, ii) helper T-cells, and iii) B cells.

i) Killer T-cells

The Killer T-cells only recognize peptides coupled to major histocompatibility complex (MHC) I on APCs [9]. The function of killer T-cells is to mediate protection against intracellular pathogens or tumor cells by specifically inducing targeted cell death. The main molecules implicated in such process are i) perforin, a form of cytotoxin known to generate holes in the plasma membrane of target cells and ii) the secretion of granulysin, molecules known to induce apoptosis such as Granzyme B [9].

ii) Helper T-cells

On the other hand, helper T-cells bind to peptides presented by MHCII molecules on APCs [10]. The main function of helper lymphocytes is to assist and support the generation of immune responses against extracellular pathogens or in helping/reinforcing the initiation of a Th1 response [10]. Thus, the activation of helper T-cells causes the release of cytokines that influence the activity of lymphocytes to either enhance the microbicidal function of macrophages and killer T-cells or to assist the development/generation of plasma cells secreting antibodies specific for extracellular pathogens [10].

ii) B cells

In addition to cell-mediated immunity, the humoral branch of the adaptive immune response is based upon B cells, which recognize or identify pathogens via their B cell receptor (BCR) composed of membrane-bound immunoglobulin (Ig) M [11]. Upon

the binding of a soluble Ag by the BCR, such complex is taken up by B cells and processed by proteolysis into peptides displayed on MHCII molecules to allow direct interaction with helper T-cells [11]. As B cells start dividing, a series of maturation processes lead to the generation of plasma cells that are responsible for the secretion of antibodies specific to the detected Ag [11].

1.1.3 Cell Surface Molecules

Successful initiation and termination of immune responses is critically dependent on cell surface molecules known for their regulatory properties. Some of the most widely studied cells surface factors are i) B7-1/2 and their shared receptors CD28 and CD152 (CTLA-4), and ii) PD-1 and its ligands PD-L1/2.

i) B7-CD28-CD152

Upon TCR interaction with MHC molecules, co-stimulation is required to avoid anergy, a state of T-cells non-responsiveness. CD28, expressed on the surface of T-cells, binds to B7.1 (CD80) and B7.2 (CD86), which are found on APCs [12-14]. Such interaction promotes activation and usually occurs at the initial step of Ag presentation. As a result, up-regulation of survival genes, enhancement and stabilization of cytokine secretions as well as cell cycle progression are triggered [14].

As a counterbalance, CTLA-4 (CD152) is known to interfere with the B7-CD28 interaction as a way of controlling immune responses in order to avoid over exuberant inflammation [15]. As such, the capacity of the high affinity CTLA-4 in competing with CD28 for B7-1/2 molecules is known to downregulate cellular proliferation, cytokine secretion while antagonizing the TCR signal [15]. CLTA-4 is therefore known to play a pivotal role in T-cell tolerance avoiding autoreactivity to self especially. This theory is supported by the study of Waterhouse *et al.* who showed that CTLA-$4^{-/-}$ mice develop massive CD4 T-cell blast infiltration in liver, heart, lung and pancreas tissues in addition

to elevated serum immunoglobulin by 3-5 weeks of age. As a result, all deficient mice developed cell-mediated tissue damages that led to their death [16].

ii) PD-1 and Its Ligands

Of the same family as CD28/CTLA-4 T-cell regulators, PD-1 and its binding ligands PD-L1 and PD-L2 have been demonstrated repetitively to inhibit lymphocyte proliferation [17]. Some of the earliest studies have shown that PD-$1^{-/-}$ mice developed autoimmune diseases spontaneously. More specifically, while C57Bl/6 developed lupus-like glomerulonephritis and arthritis [18], BALB/c mice with PD-$1^{-/-}$ developed fatal dilated cardiomyopathy [19]. In agreement with this notion, the overexpression of PD-L1 on pancreatic ß-islet cells or tumor cells protected them from killer CD8 lymphocytes [20]. Such observations were followed up by a remarkable study in which dendritic cells (DCs) derived from embryonic stem (ES) cell gene-engineered to express PD-L1 and the human invariant chain with the peptide epitope of myelin oligodendrocyte glycoprotein (MOG) lead to the generation of anergic T-cells unresponsive to *ex vivo* stimulation with the MOG peptide [21].

Overall, cell surface costimulatory molecules/regulators balance is known to play a major role in inflammation by controlling initiation and termination of immune responses to avoid the development of autoimmune pathologies. Nevertheless, elaborate studies have suggested a developmental contribution of these cell surface molecules during the selection processes of lymphocytes [22-24]. For example, co-ligation of CD28, CTLA-4 or B7-1/2 has been demonstrated to affect TCR signalling and hence plays a role in the control of thymocyte fates [24]. However, knock-out studies for every one of these molecules failed in supporting such claims [25, 26]. PD-1 was the unique molecule among the CD28 family of T-cell regulators directly involved in normal thymocyte maturation [27]. In fact, PD-1 is expressed on the surface of double-negative (DN) thymocytes and was shown to be involved in changing TCR signalling thresholds affecting thymocytes survival [28]. Such results were confirmed using PD-$1^{-/-}$ mice in which a substantial increase in apoptosis of thymocytes was observed [29]. These notions

suggest that T-cell surface regulators are involved in thymocytes selection but might dependent on a variety of factors such as timing, strength of signal, Ag as well as the APCs presenting the epitopes [30].

1.1.4 Soluble Factors

Cytokines, originally from the Greek word *cytokinos* (*cyto* – cell and *kinos* – movement) are soluble factors secreted by cells of the immune system heavily implicated in shaping, activating, and recruiting cells to sites of inflammation. Due to their continual increase in number and function, cytokines have been subdivided into the following groups: i) Th1, ii) Th2, iii) Th3, and iv) Th17 cytokines (Figure 1) [31].

i) Th1 Cytokines

The major Th1 cytokines: IFNs, IL1, tumor necrosis factor (TNF)-α and IL12, are known to be secreted by various cells of the immune system with pleotropic functions such as cell activation and secretion of additional pro-inflammatory cytokines in addition to the up-regulation of MHCI and MHCII on APCs to enhance Ag-presentation [32]. More specifically, IFNs are of great interest during Th1 responses and are subdivided in type I IFNs including IFN-α and ß and type-II IFN for IFN-γ. Type-I IFNs are central for adaptive immunity since IFN-ß, for example, is known to: i) induce the production of cytokines implicated in the differentiation and development of NK and memory T-cells, ii) induce the production of chemokines, iii) enhance DC maturation and, iv) enhance Ag-presentation [32]. Paradoxically, IFN-ß have been largely used for the treatment of the autoimmune disease multiple sclerosis (MS) [33]. IL1 and TNF-α, on the other hand, are major products of macrophages known to: i) lead to local tissue destruction, ii) increase access of effector cells, Ig, complement molecules by activating and increasing the permeability of vascular endothelium [34]. IL12, on the other hand, is a cytokine known to: i) activate NK cells and, ii) induce the differentiation of CD4 T-cells into Th1 cells [35].

ii) Th2 Cytokines

The Th2 response usually refers to a subset of CD4 T-cells – aka T helper cells 2– characterized by the cytokines they produce which include but are not limited to IL4, IL5, IL10 and IL13 [36]. The main function of a Th2 response is to assist the development of B cells via the CD40-CD40L interaction and presentation of a specific epitope to become plasma cells with the capacity to produce antibodies [36]. IL10 is considered, by far, the most interesting molecule of this family due to its pleotropic effects (Figure 2) [37]. This molecule, usually secreted by monocytes and T-cells, is know to inhibit Th1 cytokines including IL1α, IL1ß, IL6, IL12, IL18, GMCSF, GCSF, MCSF, TNF, LIF and PAF as well as CC (MCP1, MCP5, MIP1α, MIP1ß, MIP3α, MIP3ß, RANTES, and MDC) and CXC chemokines (IL8, IP10, MIP2, and KC) [38-43]. Interestingly, IL10 does not only inhibit the production of these factors but can also enhance the expression of their naturally occurring antagonists such as IL1RA and soluble TNFR [44, 45]. Furthermore, IL10 has been shown to downregulate MHCII, B71/2, CD54 (ICAM1), and TLR4 on monocytes [38, 46, 47]. Downregulation of these factors impedes T-cell activation capacity and induces their anergy. In fact, the activation of T-cells in the presence of IL10 leads to anergy and cannot be reversed by the addition of IL2, CD3 and/or CD28 antibodies [48]. When added to DC, IL10 leads to MHCII reduction, inhibits IL12 production and leads to enhanced phagocytosis [49]. IL10 seems however to lead to different pharmacological effects depending on the cell target [50]. Although MHCII is downregulated on monocytes and DCs upon addition of IL10, it is enhanced on the surface of B cells [51]. In addition, IL10 enhances B cell survival, proliferation and antibody production [52].

Therapeutically speaking, IL10 has shown encouraging results in chronic autoimmune diseases [50]. Direct evidences for the involvement of IL10 in EAE recovery were provided by studies in which neutralization of endogenous IL10 or the use of $IL10^{-/-}$ mice increased disease severity [53]. Consistent with this notion, the direct injection of IL10 in rats or mice with pre-developed EAE inhibited disease progression [53]. However, the recovery was dependent on the continual delivery of IL10 to animals, as the pharmacological effects were rapidly lost when injections ceased [53].

Figure 1. CD4 T helper cell fates.

The products, characteristic transcription factors, and cytokines critical for CD4 T-cell fate determination (Jinfang Zhu *et al.*, Blood. 2008).

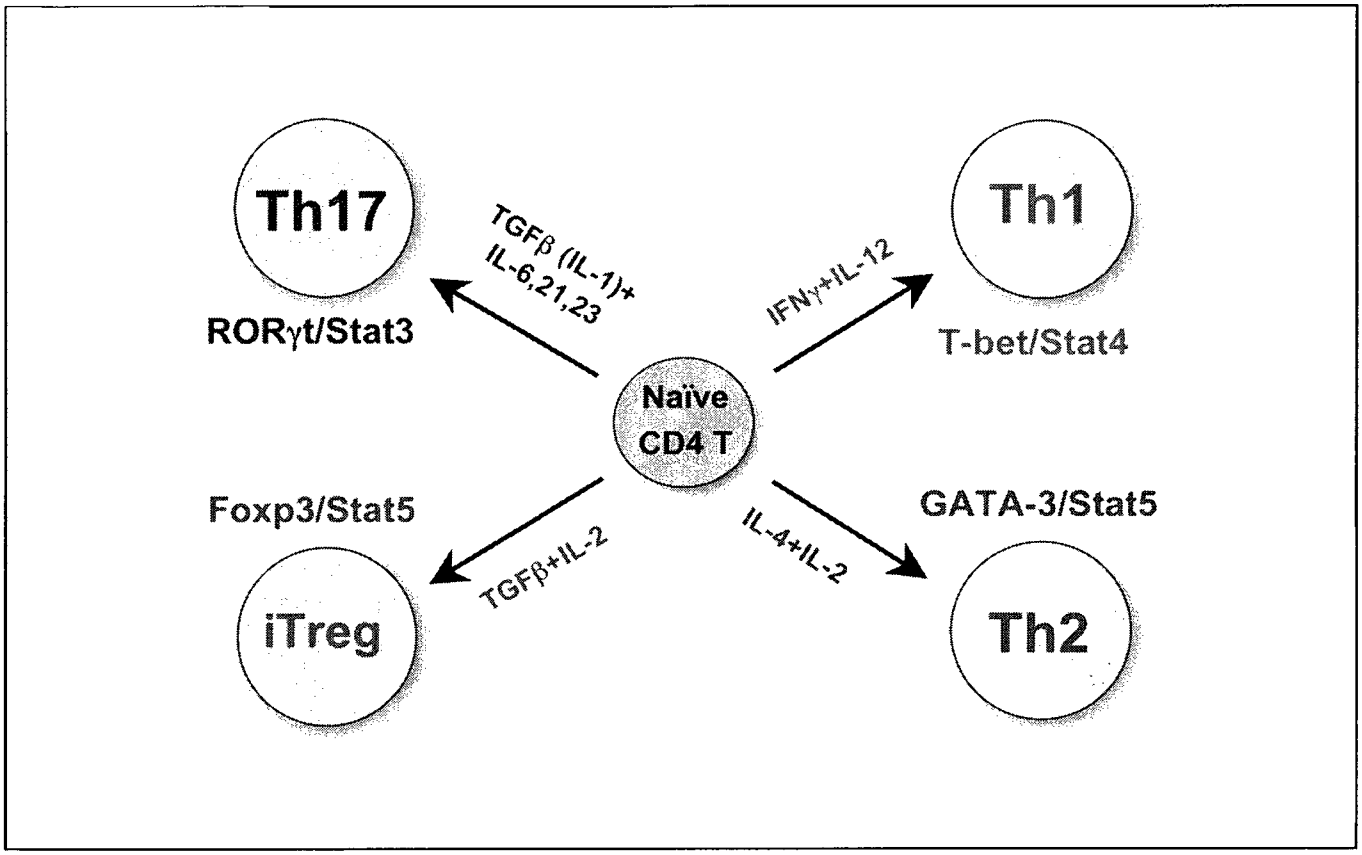
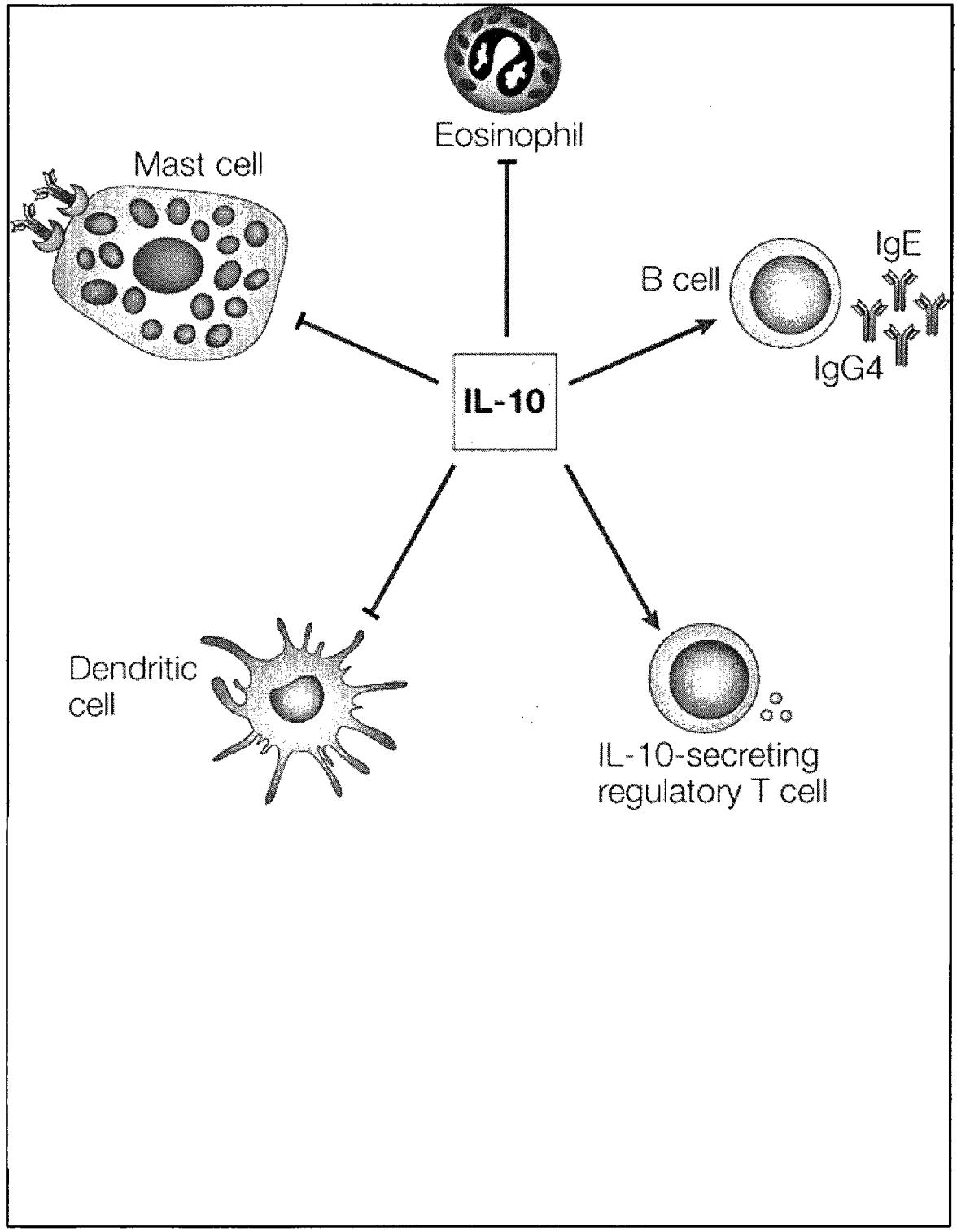

Th17
RORγt/Stat3
TGFβ (IL-1)+
IL-6,21,23
IFNγ+IL-12
Th1
T-bet/Stat4
Naïve
CD4 T
Foxp3/Stat5
TGFβ+IL-2
iTreg
IL-4+IL-2
GATA-3/Stat5
Th2

Figure 2: Pleotropic Functions of IL-10.

This cytokine has been shown to i) to inhibit mast cells, ii) eosinophils, iii) APC function, and iv) activation of Th2 cells. IL-10 has also been shown to enhance i) Ig isotype switching in B cells, and ii) to promote the induction of IL-10-secreting Tregs (Hawrylowicz CM *et al.* Nat Rev Immunol. 2005)


Eosinophil
Mast cell
B cell
IgE
IgG4
IL-10
Dendritic cell
IL-10-secreting regulatory T cell

cells [61]. TGF-ß is known to inhibit: i) growth of B cells and ii) activation of macrophages, T-cells and neutrophils [61].

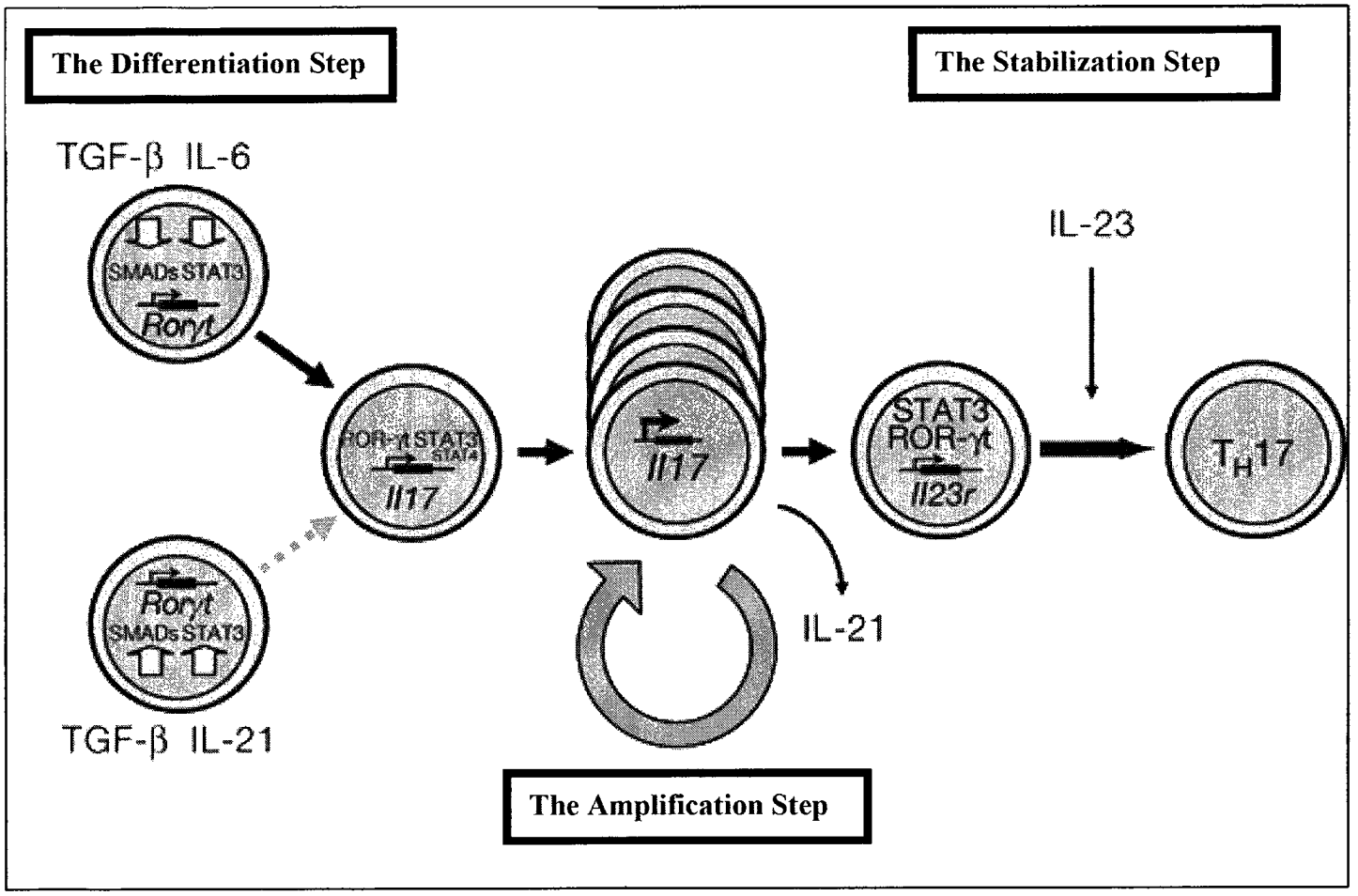
iv) Th17 Cytokines

Interestingly, a third subset of T-cells was discovered to exhibit distinct effector functions. Such cells were named Th17 due to their capacity to secrete IL17 [62]. This subset of lymphocytes is known to be generated from naïve T-cells under treatment with TGF-ß and IL6 [63]. interestingly, further analysis of Th17 cells lead to the identification of novel cytokines: i) IL21, known to amplify the frequency of Th17 cells, and ii) IL23 for its capacity in stabilizing previously generated Th17 cells (Figure 3) [63-66]. Th17 cells became of great interest in the field of immunology due to their wide involvement in immune-mediated pathologies. The concept by which Th1 cells or IFN-γ are solely responsible for exacerbated inflammation leading to autoimmunity has been lately challenged by various studies demonstrating the direct involvement of Th17 cells in pathology induction [55, 67-75]. This does not mean that IFN-γ is not involved but it suggests that additional compounds are also involved at certain stages of these ailments [63]. For instance, it was previously shown that deficiency in IFN-γ do lead to enhanced EAE suggesting an inhibitory role played by this cytokine [68]. Furthermore, deficiency of IL12, another powerful Th1-promoting cytokine, did not alter EAE pathologies as opposed to IL23 abolition, the main compound known for stabilizing Th17 cells [70]. In fact, IL23-expanded myelin-reactive Th17 cells *in vitro* were capable of adoptively transferring EAE to naïve mice [76]. It is therefore conceivable to mention that both Th1 and Th17 cells can induce autoimmunity perhaps via sequential involvement and synergistic effects. In addition to their ability to produce pro-inflammatory cytokines, Th17 cells can also produce chemokines and MMPs resulting in further recruitment of Th1 cells [62].

Th17 cell development relies on the action of lineage-specific transcription factor identified as the orphan nuclear receptor ROR-γt [77]. It has been demonstrated that the

Figure 3: Differentiation of TH17 Cells.

Both TGF-b and IL-6 are the differentiation factors for TH17 cells, whereas IL-21 acts in a positive feedback loop to increase the frequency of TH17 cells. STAT3 is essential for Th17 cell differentiation, which part of the signalling induced by IL6, IL21, IL23. Finally, IL23 plays major roles in expansion and stabilization of TH17 cells (Bettelli E *et al., Nature*, 2008)


The Differentiation Step
The Stabilization Step
TGF-β IL-6
SMADs STAT3
Rorγt
IL-23
RORγt STAT3
Il17
Il17
STAT3
RORγt
Il23r
TH17
Rorγt
SMADs STAT3
IL-21
TGF-β IL-21
The Amplification Step

transduction of naïve T-cells with a vector expressing ROR-γt induces the development of Th17 cells whereas its loss prevents the generation of these cells [77]. Interestingly however, a relationship between regulatory T-cells (Tregs) and Th17 cells has been suggested both on cytokines and transcription factors interplay. More specifically, TGF-ß is known to induce FOXP3, while the addition of IL6 to the system inhibits Tregs development by switching the entire cascade towards Th17 development [63]. In addition, FOXP3 and ROR-γt bind to each other antagonizing their distinct functions. This notion is further supported by the fact that FOXP3 conditional deletion in Tregs leads to ROR-γt up-regulation and IL17/IL21 expression [78, 79]. As a result, Th17 cells are generated.

The remarkable structure and effectiveness of the immune system is largely responsible for the adaptation of the host to its environment. However, like any other system, failures and dysregulation may occur leading to autoimmunity.

1.1.5 Autoimmunity

The development of lymphocytes is a tightly regulated process divided in 2 different steps known as positive and negative selections [80]. The main objective of such selections is to avoid the generation of auto-reactive lymphocytes deprived of their capacity to discriminate between self and non-self Ags [80]. As a result, the immune system starts reacting to specific or different parts of the host's tissues and organs leading to deleterious effect and eventually death. To avoid the generation of T-cells targeting self, reactive lymphocytes are usually deleted in the thymus by positive selection mediated by thymic cortical epithelial cells when they are found to bind tightly to presented T-cell epitopes [80]. However, surviving T-cells with low avidity to self-reactive epitopes are selected by bone marrow-derived DCs and macrophages to pursue their development in the periphery, a process known as negative selection [80]. If however, dysregulated selection or aberrant lympho-proliferation takes place, then the generation of autoreactive T-cells and eventually autoimmune diseases occur.

1.2 MODULATION OF IMMUNE RESPONSES

1.2.1 Existing Immunomodulatory Strategies

The importance of immunosuppression was first suggested by Gaspare Tagliacozzi, an Italian surgeon who worked mainly on skin transplants in the late 16^{th} century [81]. Until today, a continual struggle is pursued for the generation of efficacious immunosuppressive compounds to alleviate/block autoimmunity or transplant rejection. Therefore, further understanding of previously generated immunosuppressive compounds and their failure is required for the rational design of more effective drugs. The history of immunosuppressive agents is divided in 4 main stages as addressed in the following sections.

i) Inhibiting Effector Cell Proliferation

The first stage in the development of immunosuppressive compounds mainly focused on the generation of agents capable of inhibiting, non-specifically, rapidly diving cells. Such a concept was marked by the discovery of various chemicals such as benzene, nitrogen mustards, 6-mercaptopurine, and the imidazole derivative azathioprine, which were shown to prolong tissue transplant and lower antibody production [82-84]. Today, additional progress has been made in this field with the generation of more potent and specific anti-proliferative agents such as mycophenolic acid analogues, mycophenolate mofetil, and mycophenolate sodium [85, 86]. Unfortunately however, major inter-individual pharmacokinetic differences leading to inadequate immunosuppression were noticed in the clinic leaving these compounds behind in the list of immunosuppressive drugs [85, 86].

ii) Steroid Therapy

The second generation of immunosuppressive drugs was based on the development of agents capable of alleviating or reducing inflammatory processes [87].

Such compounds were created synthetically and are known as glucocorticoids. These steroids were mainly used by clinicians during renal transplants with one ultimate goal: the disruption of cellular reactions taken place downstream of a specific stimulus [88, 89]. Glucorticoids function intracellularly as modulators of protein biosynthesis [87]. Briefly, following their entrance in the cell's cytoplasm and binding to their appropriate ligands, the complex translocates to the nucleus where transcription of target genes is initiated with a net anti-inflammatory effect [87]. However, these drugs were soon found to be associated with a large range of toxicities such as sodium retention, hypertension, osteoporosis, reduced wound healing, hyperglycemia, and inability to fight infections. Fortunately, all the mentioned side effects were dose-dependent and thus the use of steroid therapy is feasible as part of a combination drug regimen [87].

iii) Lymphocyte Depletion

Since lymphocytes are known for their crucial role in autoimmunity and transplant rejection, the third stage in the development of immunosuppressive drugs focused mainly on lymphocyte depletion [87]. To achieve such objective, the use of monoclonal antibodies targeting a specific subset of cells was adopted [87]. One of the best examples was the development of OKT3, an antibody capable of binding the ε component of the CD3 signal-transduction complex on T-cells [87]. Such innovation was followed-up with the design of more specific antibodies targeting activated as opposed to resting T-cells [87]. The main target for this strategy was the α-chain of the IL2 receptor (R) (aka CD25), which is usually induced on activated T-cells following stimulation. Interestingly, the treatment of patients with CD25 neutralizing antibody showed encouraging data highlighted with high tolerance rates and very few side effects [87]. However, the α-chain of the IL2R confers specificity and selectivity of the receptor complex to IL2 only and thus does not confer potency for the blockade of other pathological cytokines that may have additive or independent inflammatory properties to IL2 [87]. In addition, interfering solely with the IL2R might lead to deleterious effects on apoptosis induced cell death (AICD) at the end of an immune reaction and can affect the

generation of Tregs. Therefore, targeting IL2R can abolish inflammation while disrupting physiological processes implicated in maintenance of peripheral tolerance [87].

In terms of clinical safety, antibody-based suppressive therapies are not without consequences due to major issues related to patient safety. For example, prolonged courses and unlimited doses have been shown to be involved in enhanced risks of viral infections and *de novo* malignancies, as well as the possibility of inducing allergic reactions [87].

iv) Cytokine Disruption and Neutralization

Cytokines are the main soluble factors involved in the activation, division, and differentiation of immune cells [88]. Therefore, they represent an appealing pharmaceutical target for the generation of novel drugs disrupting signal transductions that regulate lymphocyte activation during autoimmunity. The most popular agent of this category, cyclosporine A, is a cyclic endecapeptide that was originally isolated from *Fungi imperfecti* [90]. Additional studies led to the discovery of a similar agent, tacrolimus, with 50 times more potency than cyclosporine A [91]. Both drugs have similar mechanism of action antagonizing the activity of calcineurin, a serine-threonine phosphatase, implicated in the induction of gene transcription factors such as the nuclear factor of activated T-cells (NFAT), nuclear factor-κB (NF-κB) and JUN N-terminal kinase (JNK) [91]. However, as any other potent compounds, none of these drugs were completely capable of preventing transplant rejection due to off-target toxicity noticed with high dose administrations [90, 91]. In an attempt to improve existing therapies, disrupting signal transduction was further investigated by targeting transcription factors. Sirolimus, a macrolide derived from *Streptomyces hygroscopicus*, was found to share similar structure to tacrolimus [92, 93]. Its mechanism of action was however distinct due to it capacity to interfere with the multifunctional serine threonine kinase; also referred to as mammalian target of rapamycin (MTOR) [93]. The targeting of this specific kinase was found to be of extreme importance due to its implication in co-stimulation and cytokine induction [94]. Unfortunately however, targeting MTOR has limitations due to

its wide distribution in various tissues in addition to its crucial role in signal transduction pathways of almost every cytokine [94]. In addition, MTOR-mediated blockade can directly have negative effects on DC activation since it is implicated in Ag uptake and phosphorylation of signal transducer and activator of transcription 4 (STAT4) [94].

Although the main target in transplant rejection and autoimmune diseases is of T-cell nature, B cell-mediated antibody responses have also been implicated in immuno-pathologies and can participate in disease initiation or modify the phenotype and severity of pathologies [95]. For example, activated B cells and plasma cells secreting IgM and IgG directed against myelin are present in the cerebro-spinal fluid (CSF) of MS patients [96]. Furthermore, B cells can directly participate in Ag-presentation and cytokine secretion, which can lead to inflammatory perpetuation in the central nervous system (CNS) [96]. Therefore, deletion of these autoreactive B cells represents an attractive therapeutic strategy in MS. One of the most efficient B cell targeted therapy was the development of the chimeric antibody rituximab targeting CD20, a marker present on pre-B cells, mature B cells and to a lesser extent on memory B cells [97]. Rituximab has been approved by the food and drug agency (FDA) for the treatment of B cell non-Hodgkin's lymphoma as well as for RA [98]. Clinical studies have shown great efficacy of rituximab in reduction of MS lesions whereas its efficacy in delaying disease progression failed [99-102]. Although relatively safe, the use of rituximab as part of a combination approach such as chemotherapy and/or stem cell transplantation in hematological malignancies lead to the appearance of fatal progressive multifocal leukoencephalopathy (PML) [103]. However, the emergence of PML is these patients do not seem to be directly linked to the use of rituximab since rituximab monotherapy never induced PML [103]. Therefore, this side effect is most likely due to severe immunosuppression caused by the combination therapy [103].

All the above strategies have shown encouraging clinical outcome and helped refining the objectives for the development of novel therapies aiming at selectively targeting pathological cells/cytokines without widespread toxicities.

1.2.2 Cellular Therapy

As an alternative approach to solve toxicities related to the direct injection of drugs, researchers have focused for the last two decades on suppressive cellular therapy especially following the discovery that neutralizing CD25+ cells by monoclonal antibodies unveiled an antitumor response resulting in tumor rejection [104]. As such, the field progressed to a point where the concept of Tregs was accepted in the general community opening therefore the field of suppressive cellular therapy as a mean to treat autoimmunity and transplantation-related problems.

1.2.2.1 Tregs

Tregs are known to be naturally generated in the thymus using a diverse TCR repertoire [105]. During positive selection, some CD4+ T-cells recognizing auto-Ag are allowed to mature due to their high-avidity to self-Ag recognition, a process also dependent on CD28, CD80 and/or CD86 [106-108]. Once in the periphery, they survive as a long-lived population and replicate in response to self-Ag, IL2 and the engagement of CD28 [106-108]. Today, Tregs have been subdivided in 4 major groups: i) naturally occurring (nTreg), ii) inducible (Tr1), iii) anergic T-cells, and iv) Th3 Tregs [109]. Interestingly, nTregs mediate their suppressive effect either by membrane-bound TGF-β, CD223 (lymphocyte activation gene 3), or glucocorticoid-induced tumour-necrosis factor-receptor related protein (GITR) [110, 111] as opposed to Tr1 cells, which require IL10 for their generation *in vitro* as well as for inhibiting target lymphocytes [112]. The third group of Tregs requires the treatment of T-cells with Ag in the absence of co-stimulatory signals; a process known to induce anergic T-cells. These cells can suppress responding T-cells via cell-cell contact in addition to their ability to render APC tolerogenic [113, 114]. Finally, Th3 Tregs, mainly found in mucosal sites, depend on TGF-β for their development and suppression of target T-cells [115].

i) Phenotype

In terms of cell surface factors, Tregs are known to express a variety of molecules such as CD62L, CTLA-4 and GITR [116]. In addition, a hallmark of Tregs is the specific transcription factor, FOXP3, which controls both the development and function of these suppressor cells [116].

ii) Mechanism of Action

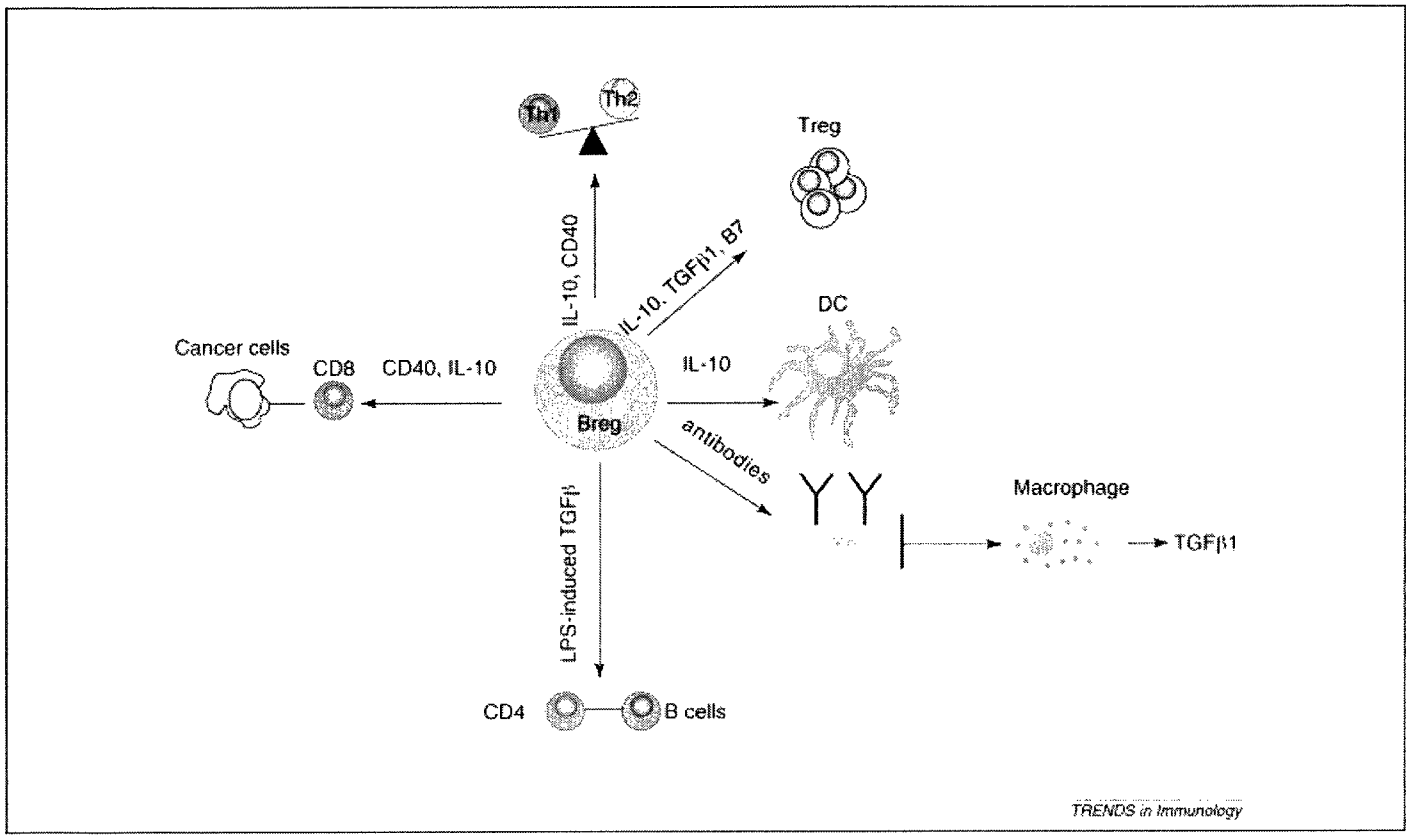
It has been demonstrated that the manipulation of conventional peripheral CD4 T-cells using Ag in the presence of a cytokine milieu can lead to the generation of Tregs *in vitro* [117-120]. However, there is controversy as to the main suppressive mechanism of action by which Tregs operate. Various studies have confirmed that Tregs use TGF-ß and IL10 to shut-down an alloimmune reaction whereas others believed it to be mediated via cell-cell contact [105, 110, 121]. However, the astonishing observation made recently by Ottenhoff's group showed that Tregs express labile membrane-bound active TGF-β capable of blocking T-cell proliferation. Neutralizing antibodies to TGF-β on the surface of these Tregs led to loss of suppressive efficacy. Such results suggest that the discrepancies in suggested hypotheses could just be explained by the inappropriate handling of Tregs, which might lead to the loss of surface TGF-ß hence both theories of soluble and cell surface-based mechanism of immunosuppression [110].

iii) Clinical Application

Although powerful in suppressing immune reactions, the potential use of Tregs in the clinic is still in doubt for various reasons. Tregs have the capacity to alleviate autoimmune-related diseases as well as transplantation problems, provide active and specific regulation, long-term tolerance and can be manipulated to a certain point *ex vivo* [121-125]. However, their relative low cell number and pan immunosuppression in addition to their potential uncontrolled expansion *in vivo* have raised some safety issues to be addresses before their translation into the clinic [126]. As potential solutions,

Figure 4: Bregs Suppressive Functions.

Regulatory B cells (Bregs) suppress activation and differentiation of T and NK T-cells via the release of IL-10 and/or TGF-ß. Bregs can also inhibit B and T-cell activation, Th1 differentiation, inhibition of DC, and can lead to the induction of Tregs (Mauri C. *et al.*, Trends in Immunology, 2008).


Th1
Th2
Treg
IL-10, CD40
IL-10, TGFβ1, B7
DC
Cancer cells
CD8
CD40, IL-10
Breg
IL-10
antibodies
Macrophage
TGFβ1
LPS-induced TGFβ
CD4
B cells
TRENDS in Immunology

various attempts are currently aiming at expanding Tregs *in vitro* using antibody-coated beads or artificial APCs [123, 124]. In addition, specificity and functional reliability was suggested to be solved by cytokines and *ex vivo* genetic alterations, solutions that might require years of further development [127].

1.2.2.2 Regulatory B Cells (Bregs)

Back in the 70s, B cells were suggested to play major roles in immune regulation especially after the discovery that spleen-derived B cells can suppress delayed-type hypersensitivity responses in guinea pigs [128, 129]. Today, B cells have been proven to modulate autoimmunity and inflammation in numerous mouse disease models. The term regulatory B cells (Bregs) was first coined by Mizoguchi and Bhan following their discovered role in EAE and colitis (Figure 4) [130, 131].

i) Phenotype

Bregs are also known as B10 cells due to their high capacity to secrete IL10 [132]. Phenotypically, B10 cells are $CD1d^{hi}CD5+$ [128]. Their development begins when immature B cells expressing a normal BCR exit the bone marrow and mature into transitional T1 B cells ($CD24^{hi}CD21$-B220+), to become eventually T2 B cells ($CD24^{hi}CD21$+B220+). T2 B cells will then branch into either mature B cells (CD24+CD21+B220+), known for their ability to differentiate into plasma cells, and secrete antibodies for the clearance of infectious agents or develop into marginal zone (MZ) B-cells ($CD1d^{hi}CD21^{hi}CD5+$), which will then be involved in suppressing pathological immune reactions [128].

Upon the recognition that IL10-producing B cells are important for the control of autoimmunity, various subsets of B cells possessing different phenotypes and origins where then identified and studied. For example, peritoneal CD5+ B-1 cells are known to produce IL10 whereas spleen B cells with a $CD21^{hi}CD23$- MZ phenotype produce IL10 in response to CpG or apoptotic cell stimulation [133]. During RA, a subset of spleen B

cells with a T2–MZ precursor phenotype ($CD21^{hi}CD23+IgM^{hi}$) has also been identified to produce IL10 [134]. Inflammatory bowel disease (IBD) generates $CD1d^{hi}CD21^{int}CD23+$ mesenteric lymph node B cells with IL10-producing capacities [135]. Bregs are so far known to share some phenotypic markers with CD5+ B-1 cells, $CD1d^{hi}CD23+$ T2–MZ precursor cells, and $CD1d^{hi}CD21^{hi}$ MZB cells.

ii) Mechanism of Action

The clearest evidence of the direct implication of Bregs in autoimmunity was first showed in the EAE mouse model of MS. Such studies were conducted in μMT mice, deficient in B cells, which were immunized using the MOG peptide. Interestingly, the absence of B cells did not lead to the prevention of EAE development but instead exacerbated the disease outcome [136, 137]. In agreement with this notion, the adoptive transfer of wild-type (WT) B cells in μMT mice with pre-established EAE ameliorated the disease severity whereas the transfer of $IL10^{-/-}$ B cells failed in leading to the same conclusion [136, 137]. It was then confirmed that the contribution played by these Bregs during EAE development was dependent on IL10 production [138]. However, one major question still remains: do Bregs affect immune responses centrally or locally? Various studies attempted to demonstrate a central mode of action for Bregs but unfortunately the results lead to more questions than answers. In a contact hypersensitivity (CHS) disease model, B cell infiltration was not observed in the challenged ears of mice. In addition, IL10 mRNA levels were not increased in B cells from draining lymph nodes following Ag challenge. However, B10 cells were found in the circulation during CHS responses suggesting the possibility of migration in small numbers to local sites of inflammation [139]. These contradictory observations suggest that perhaps the low frequency of migrating B10 driven by their potency in alleviating inflammation explains the failure in detecting Bregs at sites of inflammation. Bregs were also suggested to regulate inflammation by generating Tregs [140]. Such a concept was based on the observation that Bregs express B7.1 (CD80), which is believed to be implicated in Tregs generation hence through T-B interaction and further expansion/recruitment of Tregs [140]. In fact, Mann *et al.* found that EAE mice deficient in B cells resulted in a delay in the emergence

of FOXP3-expressing Tregs; an observation that was reversed upon the reconstitution with normal WT as opposed to B7-deficient B cells [140]. In agreement with this notion, the co-transfer of colitogenic CD4+ T-cells with mesenteric lymph nodes B cells in an IBD mouse model inhibited colitis progression [141].

iii) Clinical Application

The main technical difficulty with Bregs use in the clinic is limited, as in the case of Tregs, by their low frequency *in vivo* [142]. Unfortunately, there is no known method for the expansion of Bregs *ex vivo* due to the limited knowledge of their biology [142]. In addition, safety concerns were raised due to the ability of Bregs to secrete IL10, a cytokine that can favour tumor growth directly by affecting present tumor cells or indirectly by inhibiting immune effector cell function involved in immune surveillance [142, 143]. Solutions to these issues will require more mechanistic insights into the development, maturation as well as activation of Bregs.

1.2.2.3 Mesenchymal Stromal Cells (MSC)

The search for a novel naturally occurring primary cell population with high proliferative capacity and immunosuppressive effects led to the study of MSCs, a non-hematopoetic cell population structurally defined as a fibroblast-like plastic-adherent cell derived from bone marrow, supporting hematopoiesis and lymphopoeisis [144-146]. MSCs were discovered in the late 70s by Friedenstein *et al* as small fraction of adherent cells appearing following bone marrow aspirate with high proliferation capacities [147]. In addition, their multipotent ability to differentiate into cells of mesenchymal origin such as hepatocytes, myocytes, tenocytes, and endothelial cells were of great interest in the filed of regenerative medicine in addition to the great attention they generated due to their immunomodulatory properties [148]. Nevertheless, MSCs have also shown potency as a generic cellular vehicle for the delivery of pharmacologically relevant proteins [149-151].

i) Phenotype

In order to properly claim that the bone marrow-isolated cell population is composed of MSCs, various quality control tests must be conducted. First, MSCs must have the capacity to proliferate *in vitro* without the addition of complex growth factors [148]. Second, the isolated population must express a combination of cells surface factors such as: i) the TGF-ß receptor endoglin (CD105), ii) the membrane-bound ecto-5'-nucleotidase (CD73), and iii) the THYmocyte differentiation Ag 1 (CD90) and iv) the hyaluronate receptor CD44 while lacking the expression of i) CD45 (common leukocyte Ag), ii) CD31 (PECAM-1), iii) CD14 (co-receptor for TLR4), and iv) CD11b (Integrin alpha-M; aka MAC-1) to rule out any contamination by hematopoetic or endothelial cells. Some groups have also reported the constitutive expression of CD80/MHCI while MHCII was only shown to be induced upon treatment with IFN-γ [148]. Third, due to their high pluripotency, MSCs have to retain their capacity to differentiate under appropriate culture conditions into adipocytes, osteoblasts and chrondobalsts [148].

ii) Mechanism of Action

A broad variety of mechanisms by which MSCs exert their immunomodulatory effects have been suggested. Some groups believed that MSCs block alloresponses via cell surface molecules while others claim the unique involvement of soluble factors [152]. One of the early cell surface molecules believed to be involved was MHC [152]. However, this hypothesis was quickly ignored due to the fact that MSC lines with no MHCI expression could still inhibit lymphocyte proliferative responses [153]. Other reports even showed that MSCs with induced MHCII by IFN-γ still suppress alloreactive responses [154-156]. Thus, the role played by MHC molecules does not seem to be relevant for the suppressive properties of MSCs.

Using MLR assays, MSCs were shown to be capable of suppressing lymphocyte activation via cell-cell contact, whereas transwell cultures had some suppressive effects [157, 158]. It was then demonstrated that PD-1 and its ligand PD-L2 were mediating such

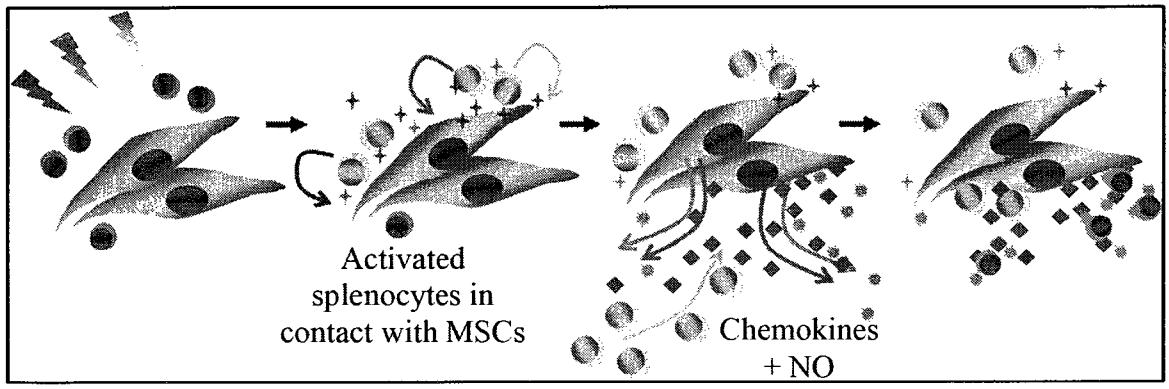
suppressive responses [159]. Further analysis of MSC biology and its interplay with cells of the adaptive immune system led the discovery that MSCs were capable of altering DC effector function [160-164]. More specifically, MSCs co-cultured with DCs inhibited the up-regulation of the costimulatory molecules CD80/86 as well as CD1a, CD40, CD14 and CD83. Furthermore, MSCs were capable of altering the cytokine secretion profile of DCs by secreting high levels of IL10 and blocking production of TNF-α and IFN-γ [165]. All these observation suggest a direct implication of soluble factors in suppression. Nevertheless, the supernatant isolated from an MSC/MLR co-culture had inhibitory effects on a secondary MLR [166]. In addition, MSC supernatant could inhibit the formation of cytotoxic lymphocytes in a transwell system, indicating a role for soluble factors. Some of the identified factors so far are i) IL10, ii) TGF-ß, iii) indoleamine 2,3-dioxygenase (IDO), iv) prostaglandin-(PGE)2, and v) hepatocyte growth factor (HGF) [152]. Interestingly however, it was lately demonstrated by *Sato et al.* that none of the above factors were secreted by MSCs or play a suppressive role [167]. In fact, this group demonstrated that a cell-cell contact is required between MSCs and T-cells leading to the induction of nitric oxide synthease and secretion of nitric oxide (NO), a molecule capable of blocking STAT5 phosphorylation and reduction in T-cells proliferation, an observation that was confirmed by Ren *et al.*(Figure 5) [167-169].

iii) Clinical Application

Culture expanded MSCs are indeed heterogeneous with intergroup variances in their analysis most likely due to laboratory handling techniques such as passage number, confluency, culture media...[170] As for MSCs efficacy *in vivo*, clinical trials have been initiated for the treatment of GVHD patients. The complementary properties of immunomodulation and wound healing would explain the synergistic potential of MSCs in treating human immune ailments. The previous completion of a trial demonstrating infusion of haplo-identical MSCs in a patient suffering from grade IV GVHD, resistant to conventional treatments, led to healing of damaged bowel epithelia [171]. This observation was followed by an additional trial on 40 patients with acute and chronic GVHD. Over 3 years after the therapy, more than half of the patients were alive [172].

Figure 5: Possible Mechanism of MSC-Mediated T-cell Suppression

Activated T-cells (light blue) release proinflammatory cytokines, which target MSCs leading to the release of chemokines (green) and NO (orange) from MSCs. The released NO suppresses nearby T-cells (purple) by inhibiting Stat5 phosphorylation, leading to cell-cycle arrest (Keating A. Cell Stem Cell, 2008).


Activated
splenocytes in
contact with MSCs
Chemokines
+ NO

Nevertheless, the recent success using MSCs to treat steroid-resistant GVHD as reported by LeBlanc K *et al*, predicts that the infusion of human MSCs in patient suffering from maladaptive immune ailments such as MS, RA, IBD or even pathological humoral responses is of great potential as well [171, 172]. However, the exact mechanism of action by which MSCs suppressed GVHD in these patients remains unknown. Although a recent study by Terwey *et al* has demonstrated that the chemokine receptor $CCR2^{+}$ is mandatory for a GVHD response [173]. Due to the ability of MSCs to secrete chemokines linked to the pathogenesis of various autoimmune diseases, it is conceivable to hypothesise the potential involvement of MSC-derived chemokines in the modulation of alloreactive T-cells [168].

1.3 NOVEL DRUG DESIGN STRATEGIES

Cytokines are the main mediators of immune-based biological effects [174]. They are known for their redundant ability to form a network connecting all immune cells during inflammation [174]. As a control mechanism, every immune response is followed by the secretion of various counterbalancing cytokines and inhibitors in order to terminate inflammation and avoid the establishment of pathologies that might lead to autoimmune disorders [174]. Understanding such physiological reactions can lead to the isolation and engineering of compounds that might interfere with unwanted immune reactions. Some of these strategies were covered previously and represent a group of non-peptidic molecules known to block cytokine synthesis, receptor binding or even signal transduction [175]. However, with the discovery of naturally occurring soluble receptors found in the bloodstream of patients with autoimmune ailments, further studies were run to mimic and optimize such approaches. As such, the generation of soluble receptors or antibodies to neutralize targeted cytokines became of great interest in the field of immunosuppressive drug-design some of which are discussed below (Figure 6) [174-176].

Table 1: Selected key cytokine activities implicated in the pathogenesis of rheumatoid arthritis

Cytokine	Advantages as a target	Disadvantages as a target	Development stage	Agent(s)
TNF	Plausible bioactivity *in vitro* and in models; validated clinical target; efficacy in approx 70% of recipients	Infection risk (such as tuberculosis); possible increased malignancy	Widespread clinical use	Infliximab*, adalimumab† (TNF-specific antibodies); etanercept§ (TNF receptor–Fc fusion protein)
IL-1	Plausible bioactivity *in vitro* and in models; particular role in matrix degradation	Limited efficacy in clinical trials; infection risk	Licensed for clinical use	Anakinra¶ (recombinant IL-1RA)
IL-6	Plausible bioactivity *in vitro* and in models; good efficacy so far in clinical trials	Essential role in host defence? Lipid and vascular modification?	Phase III clinical trials	Tocilizumab‡ (IL-6-receptor-specific antibody)
IL-12 or IL-23	Plausible bioactivity in models; role in T_H1- and/or T_H17-cell expansion; role in breach of tolerance?	Limited investigation in synovial biology; essential role in host defence?	Pre-clinical or proof of concept	Antibodies specific for p40, antibodies specific for p19
IL-15	Plausible bioactivity *in vitro* and in models; trends to efficacy in early clinical trials; role in breach of tolerance?	Essential role in host antiviral responses? Essential role in NK-cell biology?	Phase II clinical trials	AMG714**
GM-CSF	Plausible bioactivity *in vitro* and in models	Unclear hierarchical priority in rheumatoid arthritis	Pre-clinical or proof of concept	Antibody specific for cytokine or receptor
IL-17	Plausible bioactivity *in vitro* (synergy with TNF); key role in rodent models of autoimmunity	Human biology requires clarification; essential role in host defence?	Phase I clinical trials	Antibody specific for cytokine
IL-18	Plausible bioactivity *in vitro*	Ambiguous *in vivo* targeting; essential role in host defence?	Phase I clinical trials	Antibody specific for cytokine, IL-18-binding protein

*Remicade; Centocor. †Humira; Abbott Laboratories. §Enbrel; Amgen, Wyeth. ¶Kineret; Amgen. ‡Actemra; Chugai, Roche. **Amgen. GM-CSF, granulocyte/macrophage colony-stimulating factor; IL, interleukin; IL-1RA, IL-1 receptor antagonist; NK, natural killer; T_H, T helper; TNF, tumour-necrosis factor.

1.3.1 Current Molecular Targets

i) Interleukin-1

One of the widely studied inflammatory cytokines is IL1 due to its high hierarchy in the cytokine activation cascade. Its importance is due to its contribution to joint destruction during RA pathogenesis [177]. IL1 is know to mediate: i) activation of vascular endothelial cells, ii) increased expression of adhesion molecules, iii) stimulation of inflammatory prostanoids of connective tissue cells, and iv) bone and cartilage resorption [175]. For all these reasons, the search for drugs to neutralize IL1 received great attention. Interestingly, naturally occurring IL1 inhibitors have been reported in 1985, with the discovery of an IL1R antagonist in the urine of patients with monocytic leukemia, or febrile individuals [178, 179]. IL1R was also detected in the plasma of healthy volunteers injected with bacterial endotoxin or from human monocytes after GMCSF treatment [178, 179]. *In vitro* studies demonstrated that IL1R is capable of neutralizing all three forms of IL1. However, it must be bioavailable in excess – 10 to 500 fold – to inhibit 50% of IL1 activity *in vitro* and *in vivo*, hence explaining the poor to modest responses in RA patients during clinical trials testing IL1R efficacy [180, 181].

ii) Tumor Necrosis Factor (TNF)-α

TNF-α was shown to share similar properties to IL1 in addition to its pro-inflammatory effects on immune cells [182]. As a result, it was speculated that TNF-α neutralization would be of greater therapeutic interest than its homologue IL1. Similarly, TNF-α inhibitors were originally isolated from urine of healthy subjects or febrile patients in addition to supernatants from synovial fluid cells in culture. Two inhibitors were then found: i) TNFR1 (aka p55), which can be shed from macrophages, fibroblasts and epithelial cells and ii) TNFRII (aka p75), which is usually shed from T-cells [182-184]. Nevertheless, despite the detection of naturally occurring TNF-α soluble receptors in synovial fluids of RA patients, neutralization of TNF-α could not be achieved due to inadequate quantities naturally present in circulation [184].

The idea of TNF-α neutralization using therapeutic antibodies was first introduced in the early 90s and was eventually used for RA patients following its proven efficacy in animals [185]. As a follow up, a series of laboratory-engineered monoclonal antibodies and decoy receptor constructs were produced such as i) Infliximad (75% humanized and 25% of mouse origin – approved in Europe and USA for Crohn's disease and RA), ii) CDP571 (completely humanized), iii) Adalimumab D2E7 (completely humanized), iv) the humanized PEGylated Fab, v) Lenercept (containing the p55 component fused to human IgG Fc), vi) Entanercept (human TNFR p75 fused to human IgG Fc; approved in Europe and USA) and vii) the PEGylated truncated form of p55 [185].

Clinical studies using Infliximab (lately marketed by Centocor Inc. as Remicade®) have demonstrated the potency of this antibody in improving joint inflammation upon 8 to 26 weeks of treatment [186]. However, all patients relapsed indicating that the therapeutic effect was transient since it actively neutralizes circulating TNF-α right after injection. As such, concerns were raised about the long term efficacy of the treatment and the potential development of anti-idiotype antibodies especially due to the fact that Infliximab is of mouse origin. Furthermore, it must be noted that the response rate was only achieved in about 60% of treated patients [185]. Why do some patients not respond? Several hypotheses were made but the most probable ones have to do with i) genetic differences such as polymorphism with *TNF, TNFR* or other cytokine/receptor genes and ii) the involvement of additional disease-sustaining pro-inflammtory cytokines including IL2, IL15, IL17 and IL18 [185]. Further safety concerns were also raised concerning the long term regimen use of Infliximab, which might lead to increased infection risks such as tuberculosis, as well as malignancies [185].

iii) GM-CSF

GM-CSF was first described for its ability to generate *in vitro* colonies of mature myeloid cells derived from bone-marrow precursor cells [187]. As a follow up, GM-CSF was discovered to possess pro-inflammatory capacities since it can trigger the secretion of TNF-α, IL6, IL12p70 and IL23 [188, 189] in addition to its link to the Th17

inflammatory pathway [190]. Thus, this cytokine is known to form a crucial part of an inflammatory network allowing for communication between myeloid and adaptive immune cells.

GM-CSF is presently being used in the clinic for various reasons. First, it can increase the number of neutrophils after chemotherapy and the mobilization of myeloid stem cells, monocytes and other myeloid populations from the bone marrow to the blood [191]. In addition, many investigations have shown that monocyte treatment with GM-CSF helps priming an increased *in vitro* response to other stimuli, such as LPS [191]. As a result, studies are being conducted to study the adjuvant-like properties on macrophages and DCs [192]. In fact, GM-CSF treatment of APCs is known to upregulate the expression of the B7 costimulatory molecules, MHCI/II, in addition to increased Ag-presentation [193].

Today, various pre-clinical or proof-of-principle studies targeting GM-CSF are being pursued in an attempt to treat several autoimmune ailments and to clarify the relative importance of GM-CSF hierarchy in the inflammatory cascade [194]. Some of these strategies consist of the development of GM-CSF-specific antibodies – to neutralize the cytokine – or antibodies directed against the receptor to block signalling [194]. For example, neutralaizing GM-CSF in an arthritis mouse model lead to a decrease in both systemic and joint TNF and IL-1 [195]. In summary, GM-CSF is involved in the pathogenesis of autoimmune diseases and represent a target of choice to interfere with adaptive immunity.

iv) IL15

The pro-inflammatory cytokine IL15 was identified simultaneously by 2 independent groups based on its ability to stimulate proliferation of the IL2–dependent CTLL-2 T-cell line in the presence of neutralizing anti–IL2 antibodies [196, 197]. Computer-based analysis of the secondary structure of IL15 suggest high homology with IL2 and it was lately identified as part of the 4 α-helix bundle cytokine family including IL2, IL3, IL6, IL7, G-CSF, and GM-CSF [198, 199].

Monocytes/macrophages were the first immune cells identified for IL15 production [200, 201]. It was then demonstrated that APCs, such as blood-derived DCs were also capable of secreting this cytokine [202, 203]. Today, IL15 is known to be expressed and secreted by a variety of non-immune cells such as bone marrow stromal cells [204], thymic epithelium [205], fetal intestinal epithelium, epithelial and fibroblast cells from various tissues [206]. This semi-ubiquitous secretion profile of IL15 suggests that this cytokine is involved in a variety of biological processes independent of immunity.

The trimeric IL15 receptor is composed of the IL15Rα-chain which is known to bind IL15 with high affinity, and thus serves as the basis for specificity and selectivity of the entire receptor complex to IL15 [207]. The α-chain however, is not known to signal. Only the IL2/15Rβ and common-γ chains can lead to a biochemical response via the JAK-STAT pathways [207]. However, additional activated signalling pathways were identified following IL15 stimulation and include the *src*-related tyrosine kinases, and stimulation of the Ras/Raf/MAPK pathway that ultimately results in fos/jun activation [207].

Interestingly, IL15 is one of the most tightly regulated cytokine with a very short half-life (< 1 min) [208]. Such complex and tight control may indicate that IL15, if overproduced, may be deleterious to the host [207]. In support for this notion, transgenic mice overexpressing IL15 were shown to develop early expansion of NK and CD8+CD44^{hi} T-cells. At the age of 12-30 weeks, these mice were characterized as animals with low activity, rapid weight loss, and dyspnea which eventually led to their death. Also, IL15 overexpression leads to fatal NK/T lymphocytic leukemia with normal levels of CD4 T-cells [209].

IL15 is known to play major roles in the development of NK cells [207]. These *in vitro* generated CD56+ NK cells can effectively kill tumor targets cells and produce a normal cytokine profile in response to a stimulus. Furthermore, IL15 plays an important role in the development, homeostasis, and activation of dendritic epidermal T-cells,

intestinal intraepithelial lymphocytes, NKT-cells, as well as the maintenance of memory CD8 T-cells [207]. An important point to mention is the higher specificity of this cytokine for activated T-cells. It was observed that the addition of IL15 to resting primary T-cells leads to weak proliferation due to the absence of the IL15Rα-chain, whose expression is usually triggered by TCR ligation as demonstrated following the addition of anti-CD3 antibodies [207].

In terms of pathogenesis, IL15 has been detected in a variety of autoimmune diseases such as RA [210], sarcoidosis [211], IBD [212], MS [213-215] in addition to allograft rejection [216]. Analysis of immune infiltrates demonstrated that macrophages, T-cells as well as IL15 were always found in vicinity of inflammatory areas suggesting that the secretion of this cytokine is derived from macrophages leading to the activation of recruited T-cells [207]. Thus, IL15 is believed to be a major player implicated in exacerbating the development of pathologies. To further confirm the implication of IL15 in immunopathologies, various investigations demonstrated that the addition of anti–IL15 antibodies limited inflammation to a certain extent due to the complementary roles played by other pro-inflammatory cytokines. Such studies prompted the initiation of clinical trials using HuMAX-IL15 (Genmab Inc., Copenhagen); a fully humanized antibody against IL15 proven to be effective in interfering with inflammation during RA, psoriasis and Crohn's disease [217]. As a follow up step, the generation of IL15 mutants targeting the high affinity α-chain of the IL15R complex with higher half-life and antagonistic properties without any signalling capacity are currently being tested in pre-clinical models as proof-of-concept for further clinical trials initiation [218-220].

v) Chemokines and G-coupled Protein Receptors (GPCRs)

Immune reactions require the directed cell migration from one compartment to the other. Such reaction occurs via small polypeptide molecules secreted by immune cells called chemokines [221]. There is to date 50 identified chemokines divided in 4 major families and 20 receptors [221]. This means that a given receptor can bind more than one chemokine and thus a wide variety of redundancy occurs. Chemokine production is

usually triggered by inflammatory mediators such as cytokines. They are known to form dimers or higher oligomers upon their binding to glycosaminoglycans (GAGs), but can only interact with their cognate receptors as monomers [222]. Interestingly, cells exposed to uniform concentration of chemokines tend to move randomly in a given environment. However, the creation of gradient tends to guide the direct movement of cells towards their target [222]. This notion has been suggested to occur via GAGs binding, a process known to facilitate formation of gradient and perhaps other functions such as protein protection from proteolysis, storage or even signalling [222].

The N-terminal portion of chemokines has attracted a lot of attention especially since the N-terminus was identified a key player in GPCRs binding and signalling [222]. Deletion or modification of this chemokine's region leads to loss of signalling function, weakening binding, or even the generation of antagonist molecules [222]. Chemokines such as CCL2, CCL5, CCL9, CCL19 and CXCL10 all revealed to possess important functions in their N-terminus [222-224]. It was later found that the N-terminus is prone to natural proteolysis by many proteases such as CD26/dipeptidyl-peptidase IV and matrix metalloproteinases (MMPs) [222-224]. The role of these proteases was then suggested to be involved in the termination of cellular recruitment at the end of an immune reaction [222-224]. The discovery that biological effects of chemokines can be altered by proteases or modifications occurring at their N-terminus opened a great field of interest in the development of novel therapies targeting GPCRs.

GPCRs are composed of seven helical membrane spanning regions connected by extramembranous loops [222]. In terms of signalling, heterotrimeric G proteins bind to the intracellular loops. The Gα component is a GTPase responsible for the hydrolysis of GTP whereas the remaining Gβγ are involved in activating downstream effector pathways and thus mediate signalling [222]. The signal is controlled via the recruitment of arrestin molecules, which bind to phosphorylated GPCRs blocking further interactions with G proteins and mediating internalization of the receptor through clathrin coated pits and lipid rafts/caveolae [222]. In their resting state, GPCRs have been demonstrated to pre-form oligomers or even heterodimers before there interactions with chemokines

[222]. However, such interaction is not enough to lead to signalling and thus, chemokines are required to bring the 2 GPCRs in close proximity to induce receptor activation [222].

GPCRs and chemokines have been linked to inflammation and autoimmunity [221, 222]. Modifications on multiple portions of chemokines are presently in development in an attempt to generate powerful GPCRs antagonists. For example, the chemically modified forms of RANTES (aka CCL5), have been demonstrated to be extremely potent anti-HIV agent due to its capacity in blocking receptor cycling to the cell surface and thus viral entry [225-228]. Furthermore, a non-GAG-binding mutant of CCL5 has also shown potent inhibitory effects in cellular recruitment both *in vitro* and *in vivo* [229]. Today, active research targeting CCR1, CCR2 and CCR5 is being pursued for the rational design of antagonist receptor-based ligands [230].

1.4 SPECIFIC RESEARCH AIMS

The main objective of the research presented in this thesis was to develop novel approaches as a mean to improve upon cytokine-based therapeutics strategies for autoimmune/transplantation-related inflammation. The specific research aims were:

1. To test the hypothesis that an immunosuppressive fusokine generated from the fusion of two cytokines: GMCSF and IL15 (hereafter GIFT15), may induce powerful immunosuppression and thus lead to allogeneic and xenogeneic cell acceptance in immunocompetent mice.
2. To test the hypothesis that the fusokine GIFT15 can lead to the *ex vivo* generation of a novel suppressive cell population capable of alleviating/blocking EAE.
3. To test the hypothesis that primary MSCs could be exploited in an immunosuppressive cell therapy approach for the inhibition of humoral responses directed against therapeutically relevant proteins such as rhFVIII via the paracrine conversion of CCL2 to an antagonistic factor.
4. To test the hypothesis that MSCs-derived antagonist CCL2 could also be exploited in the alleviation of a Th1/Th17-mediated autoimmune disease such as EAE.
5. To test the hypothesis that the fusion of the two cytokines: GMCSF and antagonist CCL2 (hereafter GMME1), would lead to the generation of a powerful immunosuppressive compound capable of blocking inflammation during EAE pathogenesis.
6. To test the hypothesis that GMME1 would have powerful suppressive properties in a collagen-type II mouse model of RA.

CHAPTER 2

A GMCSF & IL15 Fusokine Leads to Paradoxical Immunosuppression *In Vivo* Via Asymmetrical JAK/STAT Signalling Through the IL15 Receptor Complex.

Reference: Rafei M, Wu JH, Annabi B, Lejeune L, François M, Galipeau J. A GMCSF and IL-15 fusokine leads to paradoxical immunosuppression in vivo via asymmetrical JAK/STAT signaling through the IL-15 receptor complex. Blood. 2007. 109(5):2234-42.

CHAPTER 2: A GMCSF & IL15 Fusokine Leads to Paradoxical Immunosuppression *In Vivo* Via Asymmetrical JAK/STAT Signalling Through the IL15 Receptor Complex.

2.1 ABSTRACT

We hypothesized that a granulocyte macrophage colony stimulating factor (GMCSF) and interleukin (IL)15 fusokine (GIFT15) would possess greater immune stimulatory properties than their combined use. Unexpectedly, tumor cells engineered to secrete GIFT15 protein led to suppression of natural killer (NK) and NKT-cell recruitment *in vivo*, suggesting an unanticipated immune suppressive effect. We found GIFT15 to have pleiotropic effects on an array of immune competent cells. Amongst these, macrophages treated with GIFT15 secrete *de novo* the tissue inhibitor of metalloproteinase-2 (TIMP-2); activated matrix metalloproteinase-2 (MMP-2); transforming growth factor-β (TGF-β) as well as vascular endothelial growth factor (VEGF). We show that the GIFT15 fusokine has increased affinity for the α chain component of the IL15R leading to aberrant signalling through the β chain manifested by the hyperphosphorylation of STAT3 both in macrophages and splenocytes. Suppression of common γ chain-mediated STAT5 phosphorylation and blockade of the IL15-dependent IFN-γ response in mouse splenocytes was also observed. We tested GIFT15 as an immunosuppressor and demonstrated that it allowed engraftment of allogeneic B16F0 and human xenograft U87MG glioma cells in immunocompetent mice. Thus, GIFT15 defines a new class of fusokine which mediates pro-angiogenic and immunosuppressive effects via aberrant signalling by the IL15R in lymphomyeloid cells.

2.2 INTRODUCTION

Immune stimulatory cytokines can be exploited to treat human ailments including cancer. Amongst the cytokines identified for such use, GMCSF has been under much scrutiny due to its direct action on the adaptive immune system through the enhancement of Ag presentation as well as costimulation [231, 232]. Furthermore, second generation

strategies linking innate and adaptive immunity using GMCSF delivered as a fusion cytokine (fusokine) with other immune stimulatory proteins such as IL2 are currently being developed [233, 234]. The utility of GMCSF containing fusokines having been established in animal models of cancer immunotherapy, begets the testing of novel combinatorial fusokines, especially since the biochemical behaviour of such chimeras can lead to unprecedented biopharmaceutical properties as we have previously reported[4]. IL15 possesses overlapping activities with IL2 such as the activation of T-cells and the stimulation of natural killing [235] as well as additive stimulatory effects on the immune system distinct from IL2 [236, 237]. These features make IL15 an attractive companion to GMCSF as part of an immunotherapeutic fusokine. In fact, it was previously reported that co-treatment of dendritic cells (DC) precursors with GMCSF and IL15 as separate entities can generate a powerful T-helper 1 (Th1) immune response both *in vitro* and *in vivo* [238]. Thus, we hypothesized that the generation of a GMCSF and IL15 Fusion Transgene (hereafter GIFT15) would lead to immunostimulatory synergy in the setting of cancer immunotherapy. Unexpectedly, we found that the GIFT15 fusokine behaved in a manner opposite to what was anticipated and possessed profoundly immune suppressive properties as well as robust pro-angiogenic features *in vivo*. These unanticipated features were found to arise from asymmetrical JAK/STAT signalling through the IL15 receptor complex in responsive lymphomyeloid cells. This novel pharmaceutical effect was further characterized in the setting of allogeneic and xenogeneic somatic cell transplantation. Here we describe our findings which support the potential use of GIFT15 as a novel immunosuppressive compound.

2.3 MATERIALS AND METHODS

2.3.1 Animals, Cell Lines, Recombinant Proteins, Antibodies, and ELISA kits.

All used female mice used were 6-8 weeks old (Jackson Laboratory, Bar Harbor, ME). The C57Bl/6-derived B16F0 and human U87GM cell lines were cultured in DMEM (Wisent Technologies, Rocklin, CA) supplemented with 10% FBS (Wisent Technologies) and 50U/ml of Pen/Strep (Wisent Technologies). The cell lines JAWSII

and CTLL2 (American Type Culture collections, Manassas, VA) were grown according to manufacturer's recommendations. Recombinant proteins (rIL15/rIL15Rα-Fc/rGMCSF/rTGF-β/rMMP2/rMMP9) and their antibodies were purchased form R&D systems (Minneapolis, MN). Anti-TIMP2 polyclonal antibodies and rTIMP2 were purchased from Chemicon (Single Oak Drive, Temecula, CA, USA). Antibodies against *vWF* (*Von Willebrand factor*) and α-tubulin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal anti-phosphorylated STAT3, phosphorylated STAT5, STAT3, STAT5 or Bcl-XL were purchased from Cell Signalling Technology (Danvers, MA). Anti-mouse Fcγ III/II, CD3, CD4, CD8, NK1.1, IFN-γ or isotype control antibodies for flow cytometry were purchased from BD Biosciences (San Diego, CA). The ELISA kits for mVEGF, mIL15 or mIFN-γ were purchased from R&D systems and BD Biosciences respectively. Angiogenic Protein arrays were purchased from Panomics (Fremont, CA). The Multiscreen-MIC plates were purchased from Millipore (Cambridge, Ontario, Canada).

2.3.2 Vector Construct and Protein Modelling.

The mIL15 cDNA (Invivogen, San-Diego, CA) was cloned into the bicistronic AP2 retrovector in frame with the cDNA encoding mGMCSF allowing the expression of both the chimeric transgene and GFP [234]. To build a structural model of GIFT15 by homology modeling, crystal structures of human GMCSF and human IL2 (D chain) were used as templates for mouse GMCSF and mouse IL15, respectively. The structural template for the region connecting GMCSF and IL15 was identified by fold recognition methods, using software PROSPECT v2 (Oak Ridge National Laboratory, Oak Ridge, TN). Based on the templates identified, 50 structural models of GIFT15 were generated using software MODELLER v6 (University of California at San Francisco). The structural model with lowest objective function was selected for further analysis. Next, a structural model of GIFT15 in complex with cytokine receptor was generated based on the crystal structure of the IL2 signaling complex, which is the trimeric assembly of IL2Rα, IL2Rβ and IL2Rγ in complex with IL2. Crystal structure of IL15Rα was used as an additional template.

2.3.3 Transgene Expression and Proliferation Assays

The AP2 retroviral plasmid was introduced into 293-GP2 packaging cell (Clontech, Mountain View, CA) and concentrated retroparticles were used to gene modify B16F0 melanoma cells. The supernatant from the polyclonal population was tested by western-blot. To test the bioactivity of GIFT15, the CTLL-2 or JAWSII cell lines were plated at a density of 10^5 cells/well in a 96-well plate and treated with increasing concentrations of cytokines for 72 hours. The reaction was read at 570 nm after adding 20 µL of 3-(4,5-dimethylhiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution for 4 hours at 37°C.

2.3.4 Murine B16F0 Tumor Implantation in Syngeneic C57Bl/6 Mice & Immune Infiltrate Analysis

One million cytokine-secreting B16F0 cells were injected subcutaneously in C57bl/6 mice, and tumor growth monitored over time. All implanted B16F0 polyclonal populations produced comparable molar quantities of cytokines (0.6 ± 0.1 pmol/10^6 cells/24 hours). For immune infiltrate analysis, one million cytokine-secreting B16F0 cells were mixed with 500 µL of Matrigel™ (BD Biosciences) at 4°C and injected subcutaneously in C57bl/6 mice. Implants were surgically removed two weeks post-transplantation and digested as reported previously [234]. After incubation with anti-Fcγ III/II mAb for 1 hour, cells were incubated for 1 hour at 4°C with appropriate antibodies and analyzed by flow cytometry using a Becton-Dickinson FACScan.

2.3.5 Murine B16F0 Tumor Implantation in NOD-SCID Mice

One million GIFT15-secreting or GFP-expressing B16F0 cells were injected subcutaneously in immunocompromised NOD-SCID mice, and tumor growth was monitored over time. For *vWF* immunostaining, animals were sacrificed and tumors retrieved for paraffin embedment before being cut and probed with anti-*vWF* as reported elsewhere [239]. Total blood vessels were counted and divided by the total surface area

calculated using Scion image software (Scion Corporation, MA, USA) to obtain blood vessel density.

2.3.6 Macrophage Migration Assays and Signalling

Murine peritoneal macrophages were isolated from C57bl/6 mice. Then, 10^5 cells per well were plated in the top chambers of a Transwell plate and the lower chambers were filled, in triplicates with 500 μL of serum-free RPMI with 0.1 or 1 nmol/L of GFP CM containing cytokines. After 18 hours of incubation at 37°C, the cells on the bottom filter of each well were counted at 10 high power fields (x400). For signalling analysis, GIFT15 was purified using an immunoaffinity column packed using CNBr-sepharose (Amersham, NJ, USA) according to manufacturer's instructions. To stimulate peritoneal macrophages, 30 pmols of cytokines were added to 10^6 cells for 15 minutes before being lysed and probed by western blot (WB) with rabbit anti-phosphorylated STAT3 or STAT5. Total STAT3 or STAT5 proteins were used as loading controls.

2.3.7 Angiogenic Protein Arrays and Secreted Factors

Murine peritoneal macrophages were cultured with 30 pmols of cytokines for 72 hrs at 37°C, and supernatants were then re-collected and screened using angiogenic protein arrays, according to manufacture's instructions. The detected protein (TIMP-2) was then confirmed by WB. Gelatin zymography was used to assess the extent of MMP-2 activity. Gelatinolytic activity was detected as unstained bands on a blue background. The same culture medium was used in a WB in order to confirm the presence of MMP2 and MMP9 at the protein level. Supernatants were also used to detect the presence of VEGF and TGF-β by ELISA according to manufacturer's instructions.

2.3.8 Surface Plasmon Resonance

The binding interaction between GIFT15 and rIL15Rα-Fc was examined in real-time using a BIACORE 3000 with research-grade CM5 sensor chips (Biacore AB, Uppsala,

Sweden) prepared by immobilizing rIL15Rα-Fc (10 μg/mL in 10 mM sodium acetate pH 5.0) using the Amine Coupling Kit (Biacore AB) and HBS-M running buffer. Corresponding reference surfaces were prepared in a similar manner in the absence of any ligand addition. As a positive control, rIL15 was injected at 50 μL/min (180 sec association + 180 sec dissociation) over the reference and amine-coupled rIL15Rα-Fc surfaces (1300 RU). Regeneration was achieved using two 30 second pulses of HBS-M containing 0.5 M NaCl, 50 mM EDTA, and 0.05% (v/v) TritonX-100 or Empigen. For the test sample, purified GIFT15 was injected over the same sensor chip surfaces and regenerated in an identical manner. All binding data presented were "double-referenced" and analyzed according to a 1:1 interaction model using BIAevaluation 4.1 (Biacore AB).

2.3.9 GIFT15-Mediated Biochemical Responses

Media containing 30 pmols of cytokines was used to stimulate 10^6 splenocytes for 15 minutes. The splenocytes were then probed with anti-phosphorylated STAT3 or STAT5. For apoptosis assays, 10^6 splenocytes were cultured using the same conditions for 36 hrs, then stained with Propidium Iodine (PI) and for annexin-V. The same experiment was repeated to analyse Bcl-XL protein expression by immunoblotting on cell lysate. For the splenocyte proliferation assay, 10^5 splenocytes were cultured with increasing concentrations of cytokines for 72 hrs at 37°C. The reaction was read at 570 nm after adding 20 μl of MTT reagent for 4 hours at 37°C.

2.3.10 Induction of IFN-γ secretion and 2-Way (Mixed Lymphocyte Reaction) MLR

The supernatant of 10^5 splenocytes stimulated for 36 hrs with equimolar concentrations of cytokines was centrifuged and used to detect IFN-γ secretion by ELISA. For the MLR assay, $1.5X10^5$ splenocytes of BALB/c and C57bl/6 mice were mixed or treated separately with GIFT15. All cells were incubated at 37°C for a period of 72 hrs then IFN-γ tested by ELISA. Intracellular IFN-γ staining on mouse splenocytes was performed using the BD Cytofix/Cytoperm™ Kit (BD Biosciences, 554714).

2.3.11 Allogeneic B16F0 and Xenogeneic U87GM Transplantations

Allogeneic transplantations were performed by injecting 10^7 live B16-GFP/GIFT15 in BALB/c mice and tumor growth followed over time. For spleen analysis, animals with GIFT15 tumors exceeding 1,000 mm^3 or with the largest B16-GFP tumors were sacrificed and their spleen removed and weighed. Paraffin-embedded slides were also prepared for Haematoxylin and Eosin (H&E) staining. For flow cytometry analysis, splenocytes were stained and analysed by FACS. For xenotransplantation, 10^7 live U87-GFP or U87-GIFT15 transduced as explained previously were injected subcutaneously to monitor tumor growth and graft survival over time in WT C57Bl/6 mice, $CD4^{-/-}$, $CD8^{-/-}$, or in *beige* mice (NK deficient).

2.3.12 Statistical Analysis

P values were calculated using the paired Student *t*-test.

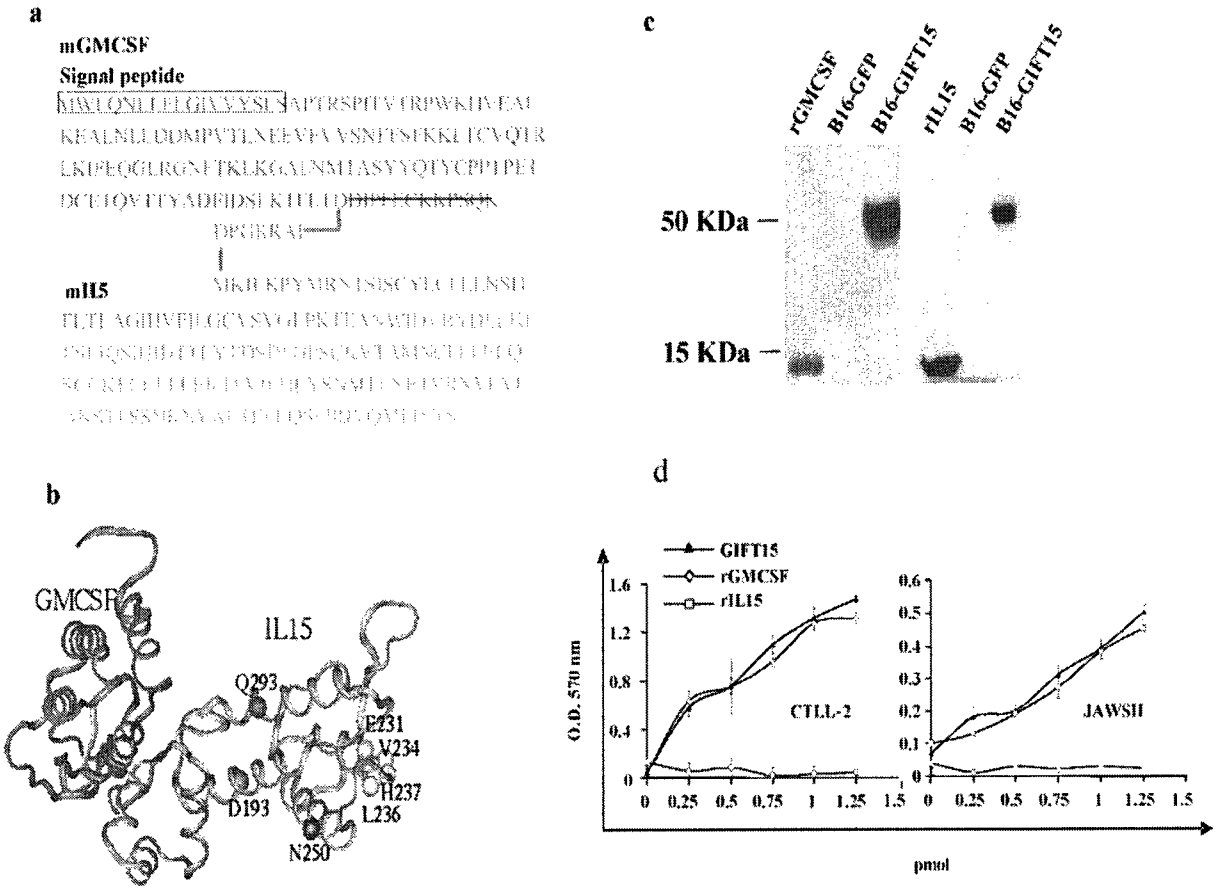
2.4 RESULTS

2.4.1 Design and Characterization of Murine GIFT15

The fusokine was created by cloning modified GMCSF cDNA, which was missing the nucleotides coding for the last 11 carboxy terminal aminoacids (aa), in frame with the 5' end of the mouse IL15 cDNA, including its long signal peptide [236, 237]. The final fusokine GIFT15 cDNA encodes for a single polypeptide chain of 299 aa (**Fig. 6a**). Computer-based analysis of the three-dimensional structure revealed that the 7 aa peptidic bridge and the uncleaved IL15 long signal peptide sequence forms an intercytokine bridge of 55 aa in length with a three alpha helixes configuration (**Fig. 6b**). Denaturing immunoblotting performed on the supernatant from B16F0 cells retrovirally transduced to express GIFT15 showed that the chimeric protein was efficiently secreted into the extracellular space and migrated at a molecular weight of 55 KDa (**Fig. 6c**). The bioactivity of both cytokine subunits within GIFT15 was confirmed by the proliferation

Figure 6: Construction and Expression of Bi-functional GIFT15.

(a) Schematic representation of the GIFT15 aa sequence. **(b)** The predicted structural model of GIFT15. GMCSF is shown in green, intercytokine bridge in grey, and IL15 in cyan ribbon. The IL15 residues experimentally identified to interact with IL15Rα, IL2Rβ and IL2Rγ are showed by yellow, purple and red balls, respectively. **(c)** Denaturing immunobloting using conditioned media (CM) from genetically-modified B16F0 expressing the green fluorescent protein (GFP) or GIFT15 probed with polyclonal goat anti-IL15 or anti-GMCSF antibodies. rIL15 and rGMCSF were used as positive controls. **(d)** Biological activity of GIFT15. To test the bioactivity of GIFT15, proliferation assays were performed by MTT incorporation using CTLL-2 and JAWSII cell lines concentrations (CTLL-2, $P>0.05$ between GIFT15 and IL15; JAWSII, $P>0.05$ between GIFT15 and GMCSF). Results are shown as mean of triplicates $\pm$ S.E.M of one representative experiment of three.


a
mGMCSF
Signal peptide
mIl5
b
GMCSF
IL15
Q293
E231
V234
H237
L236
D193
N250
c
rGMCSF
B16-GFP
B16-GIFT15
rIL15
B16-GFP
B16-GIFT15
50 KDa
15 KDa
d
GIFT15
rGMCSF
rIL15
O.D. 570 nm
CTLL-2
JAWSII
pmol

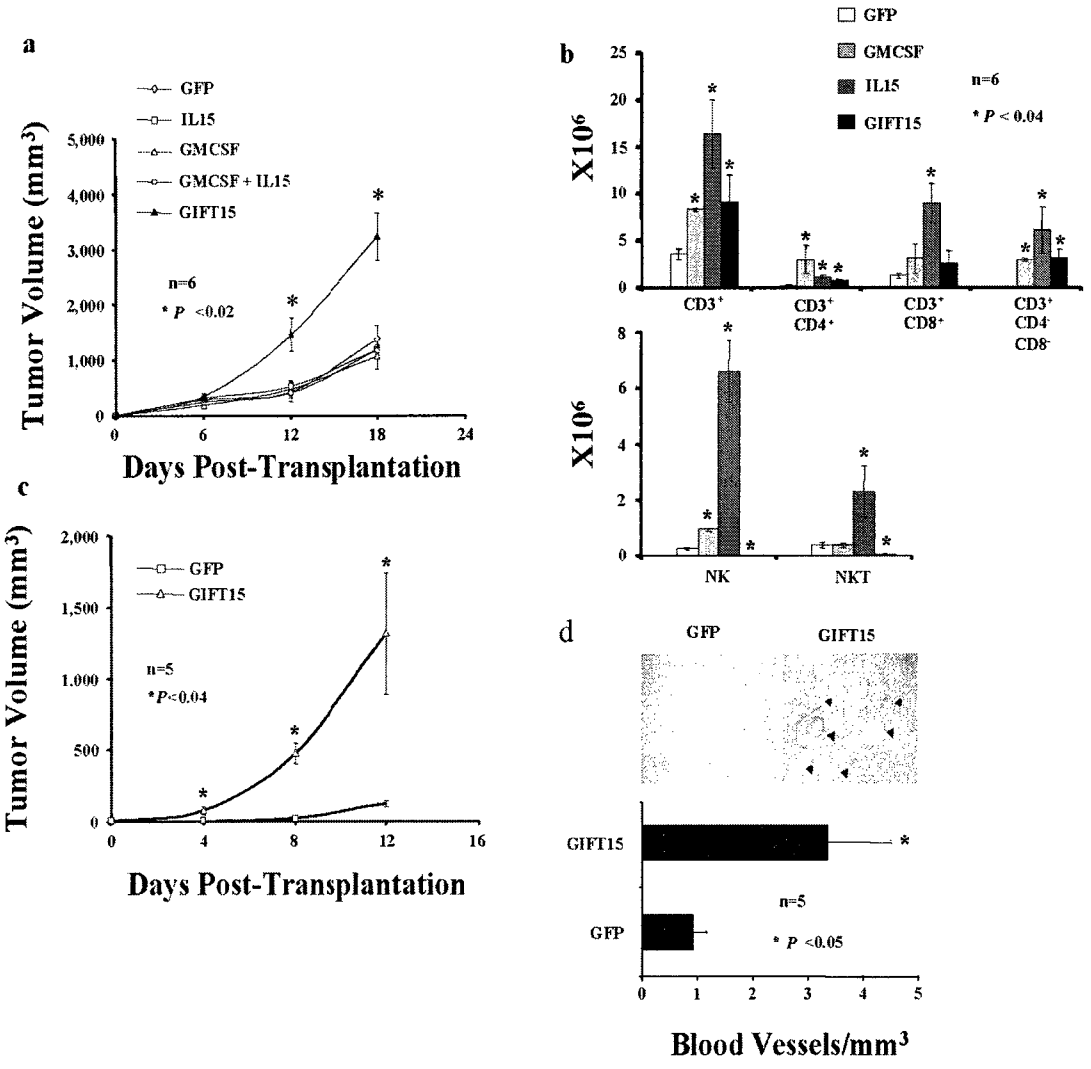
of the GMCSF-dependent JAWSII and IL15-dependent CTLL2 cell lines respectively (**Fig. 1d**).

2.4.2 GIFT15 Promotes Tumor Growth by Blocking the Recruitment of NK/NKT-cells and by Inducing Angiogenesis

To assess the ability of GIFT15 to induce an immune modulatory effect, polyclonal populations of B16F0 cells engineered to secrete equimolar levels of cytokines were injected subcutaneously in syngeneic immune competent C57Bl/6 mice. In contrast to our original working hypothesis, we observed that B16F0 cells secreting GIFT15 (hereafter B16-GIFT15) had acquired aggressive growth properties with an average tumor size three fold larger than that of control groups in the weeks following implantation (**Fig. 7a**). To determine whether this phenomenon was linked to an atypical immune response, we analyzed tumor infiltration by immune cells two weeks post-implantation of Matrigel^{TM} matrix embedded cells. We found that NK and NKT-cells were virtually absent in GIFT15-secreting tumors when compared to B16-GMCSF or B16-IL15 control groups, while the number of other CD3+ T-cell subsets were similar to controls (**Fig. 7b**). The observed absence in NK/NKT-cell recruitment by B16-GIFT15 cells contradicted what we predicted would occur *in vivo*, especially since IL15 has been shown by others to directly stimulate the development, expansion, recruitment and activation of NK and NKT-cells [204, 240-242]. B16-GIFT15 cells implanted in immunocompromised NOD-SCID mice also showed significantly enhanced tumorigenicity where we would have predicted a similar tumor growth rate to controls if immunosuppression was solely at play (**Fig. 7c**). Histological analysis of explanted tumors by immunostaining against *vWF* revealed a three fold increase in blood vessel density ($P<0.05$) in B16-GIFT15 tumors compared with the control (**Fig. 7d**) as well as a robust 4-fold increase of infiltration by tumour-associated macrophages (TAMs) (data not shown).

Figure 7: GIFT15 Possesses NK/NKT Depleting and Pro-angiogenic Properties

(a) GIFT15 effect on tumor growth in syngeneic immunocompetent C57Bl/6 mice. C57Bl/6 mice (n=6) were injected with 10^6 live cytokine-secreting B16F0 cells and tumor volume was monitored over time ($P<0.05$ between B16-GIFT15 and B16-GFP/IL15/GMCSF/IL15+GMCSF). Results are shown as mean tumor volume $\pm$ S.E.D. **(b)** Immune infiltrate analysis. Single cell suspension were generated from the enzymatic dissociation of matrix embedded B16F0-cells retrieved from sacrificed mice two weeks post-transplantation and analyzed by flow cytometry ($P<0.04$ between experimental groups and B16-GFP) Results are shown as mean of sixplicata $\pm$ S.E.D. **(c)** GIFT15 effect on tumor growth in NOD-SCID mice. NOD-SCID mice (n=5) were injected with 10^6 live B16-GFP or GIFT15 cells and tumor volume was assessed over time ($P<0.02$ between B16-GIFT15 and B16-GFP). Results are shown as mean tumor volume $\pm$ S.E.D. **(d)** Blood vessel density in tumors grown in NOD-SCID mice. Tumors removed from sacrificed NOD-SCID mice (n=5) were paraffin-embedded, prepared on slides then stained against *vWF*. Blood vessel density was calculated by dividing total amount of blood vessels by the tumor's surface area (mm^2) ($P<0.05$ between B16-GIFT15 and B16-GFP). Results are shown as mean blood vessel density $\pm$ S.E.D.

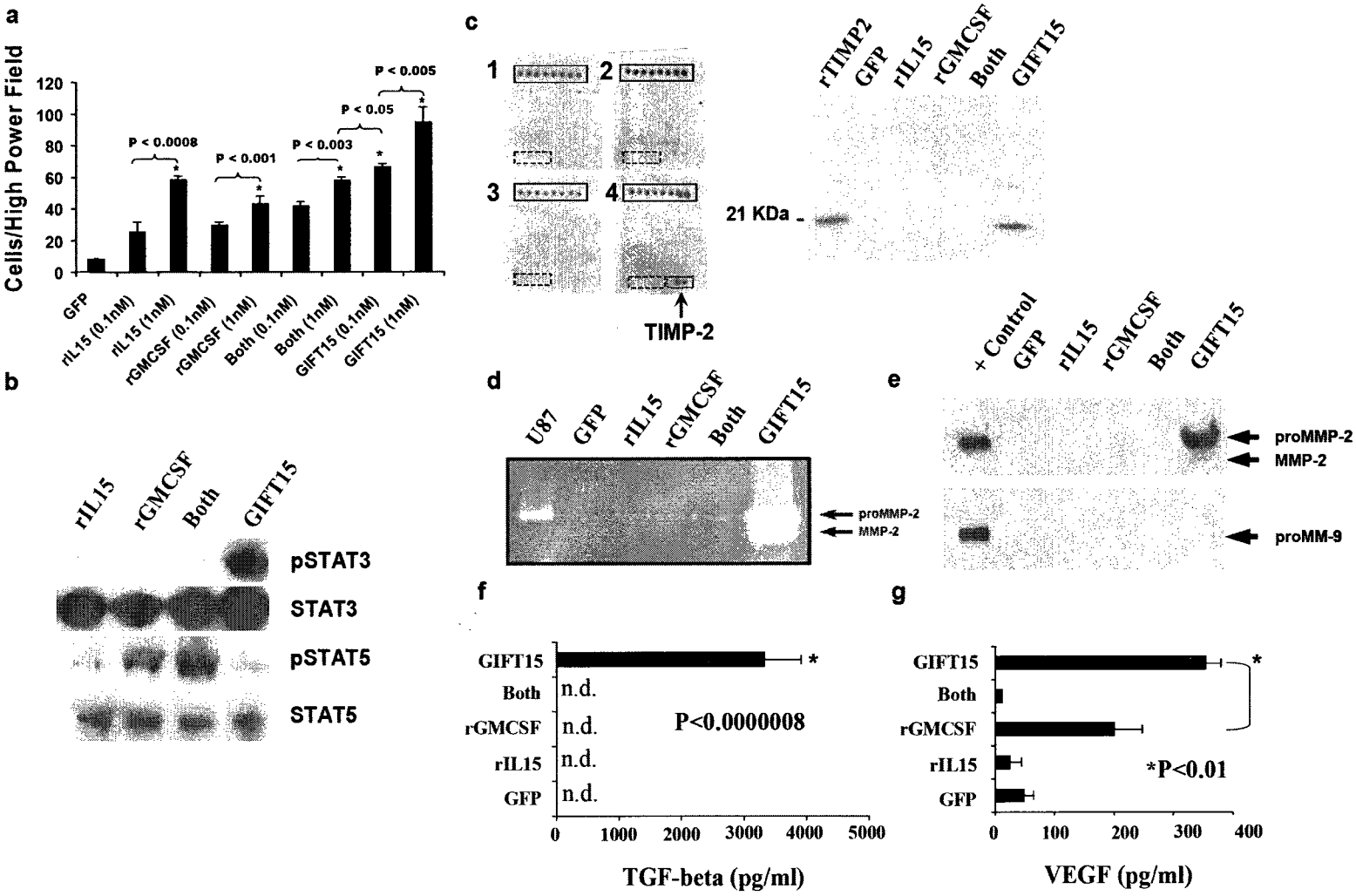

a
GFP
IL15
GMCSF
GMCSF + IL15
GIFT15
n=6
* P <0.02
Tumor Volume (mm3)
Days Post-Transplantation
b
GFP
GMCSF
IL15
GIFT15
n=6
* P < 0.04
X10^6
CD3+
CD3+ CD4+
CD3+ CD8+
CD3+ CD4- CD8-
NK
NKT
c
GFP
GIFT15
n=5
*P<0.04
Tumor Volume (mm3)
Days Post-Transplantation
d
GFP
GIFT15
n=5
* P <0.05
Blood Vessels/mm3

2.4.3 GIFT15 Effect on Macrophages

TAMs play an important role in immunosuppression and angiogenesis [243-245]. In the GIFT15-secreting tumors showed a 4-fold increase in macrophage content when compared with control B16 tumors (data not shown) suggesting a powerful macrophage chemotactic property for the fusokine *in vivo*. Macrophages are known to express the IL15R where intracellular signalling occurs through the β chain (JAK1/STAT3) and the γ chain (JAK3/STAT5). We therefore tested the chemotactic properties of GIFT15 on mouse peritoneal macrophages to further defined IL15R-mediated signalling pathways *in vitro*. The chemotactic effect of 0.1 nM GIFT15 on macrophages was significantly greater than that of control cytokines at equal or higher (1 nM) concentrations (**Fig. 8a)**, confirming the hypothesis that GIFT15 has the potential to enhance locoregional recruitment of TAMs as observed *in vivo*. On a molecular level, IL15R-mediated signalling analysis showed that STAT3 was hyperphosphorylated in response to GIFT15 when compared to control groups whereas STAT5 activation was similar to the response induced by rIL15 (**Fig. 8b**). Stimulation of macrophages using rIL15 and rGMCSF led to an additive effect on STAT5 phosphorylation whereas the fusokine containing both moieties failed in generating the same level of phosphorylation (**Fig. 8b**). To better understand the cell physiological consequences of asymmetrical signalling on macrophages at a cellular level, conditioned-media (CM) from cytokine treated macrophages was probed using a commercial angiogenic protein array revealing the presence of TIMP-2 following GIFT15 exposure, which was then confirmed by WB (**Fig. 8c**). Since TIMP-2 is implicated in the activation of MMP-2 [246], CM from treated macrophages was assayed by gelatin zymography. As shown in **Fig. 8d**, the GIFT15-treated group lead to MMP-2 activation as determined by gelatin degradation. MMP-2 was also detected at the protein level by WB confirming the data obtained by zymography whereas MMP-9 was totally absent (**Fig. 8e**). Since peritoneal macrophages stimulated with GIFT15 led to the activation of MMP-2 and the latter is known to play a role in angiogenesis [246-248], we were interested in identifying additional macrophage-

Figure 8: Peritoneal Macrophage Responses Following GIFT15 Treatment.

(a) *In vitro* Macrophage Migration Assay. Peritoneal macrophages were plated for 18 hours in transwell plates with lower chambers filled in triplicate with cytokines at increasing molarities. The cells on the bottom filters of 10 high power fields (x400) were counted for each well, and the results are depicted as mean cell number per high power field ± SED. **(b)** STAT3/STAT5 Phosphorylation in Peritoneal Macrophages. 10^6 macrophages were stimulated for 15 minutes with 30 pmols of rIL15, rGMCSF, or both cytokines inoculated in B16-GFP CM or with B16-GIFT15 CM, and cell lysate probed for phosphorylated STAT3/STAT5. Total STAT3 or STAT5 protein was used as loading control. GIFT15-induced phosphorylation of STAT5 protein. **(c)** TIMP-2 Secretion From GIFT15-Treated Macrophages. Peritoneal macrophages treated with 30 pmols of cytokines in serum-free media were incubated for 72 hrs and media tested by angiogenic protein arrays (U87 supernatant was used as positive control). TIMP-2 detection was further confirmed by WB (+ control is either rMMP2 or rMMP9). **(d)** GIFT15 Treatment Induces The Secretion and Activation of MMP2. 10^6 macrophages were deprived of serum and cultured with 30 pmols of cytokines. Gelatin zymography was performed and the hydrolytic activity of MMP-2 was assessed. **(e)** A confirmation by WB was performed to verify that MMP-2 but not MMP-9 was indeed induced by GIFT15. **(f)** Macrophage Treatments with GIFT15 Induces Active TGF-β. Serum-free supernatant collected from macrophages treated with 30 pmols cytokines was assessed for the presence of active TGF-β and only the GIFT15 group lead to its detection. **(g)** GIFT15-Treated Macrophages lead to VEGF Secretion. Stimulated peritoneal macrophages treated as previously mentioned were assessed by ELISA for the secretion of VEGF. Even though GMCSF lead to modest secretion of VEGF, GIFT15 treated group had a higher concentration than the remaining groups.


a
Cells/High Power Field
120
100
80
60
40
20
0
P < 0.0008
P < 0.001
P < 0.003
P < 0.05
P < 0.005
GFP
rIL15 (0.1nM)
rIL15 (1nM)
rGMCSF (0.1nM)
rGMCSF (1nM)
Both (0.1nM)
Both (1nM)
GIFT15 (0.1nM)
GIFT15 (1nM)
b
rIL15
rGMCSF
Both
GIFT15
pSTAT3
STAT3
pSTAT5
STAT5
c
1
2
3
4
TIMP-2
rTIMP2
GFP
rIL15
rGMCSF
Both
GIFT15
21 KDa
d
U87
GFP
rIL15
rGMCSF
Both
GIFT15
proMMP-2
MMP-2
e
+ Control
GFP
rIL15
rGMCSF
Both
GIFT15
proMMP-2
MMP-2
proMM-9
f
GIFT15
Both
rGMCSF
rIL15
GFP
n.d.
n.d.
n.d.
P<0.0000008
0
1000
2000
3000
4000
5000
TGF-beta (pg/ml)
g
GIFT15
Both
rGMCSF
rIL15
GFP
*P<0.01
0
100
200
300
400
VEGF (pg/ml)

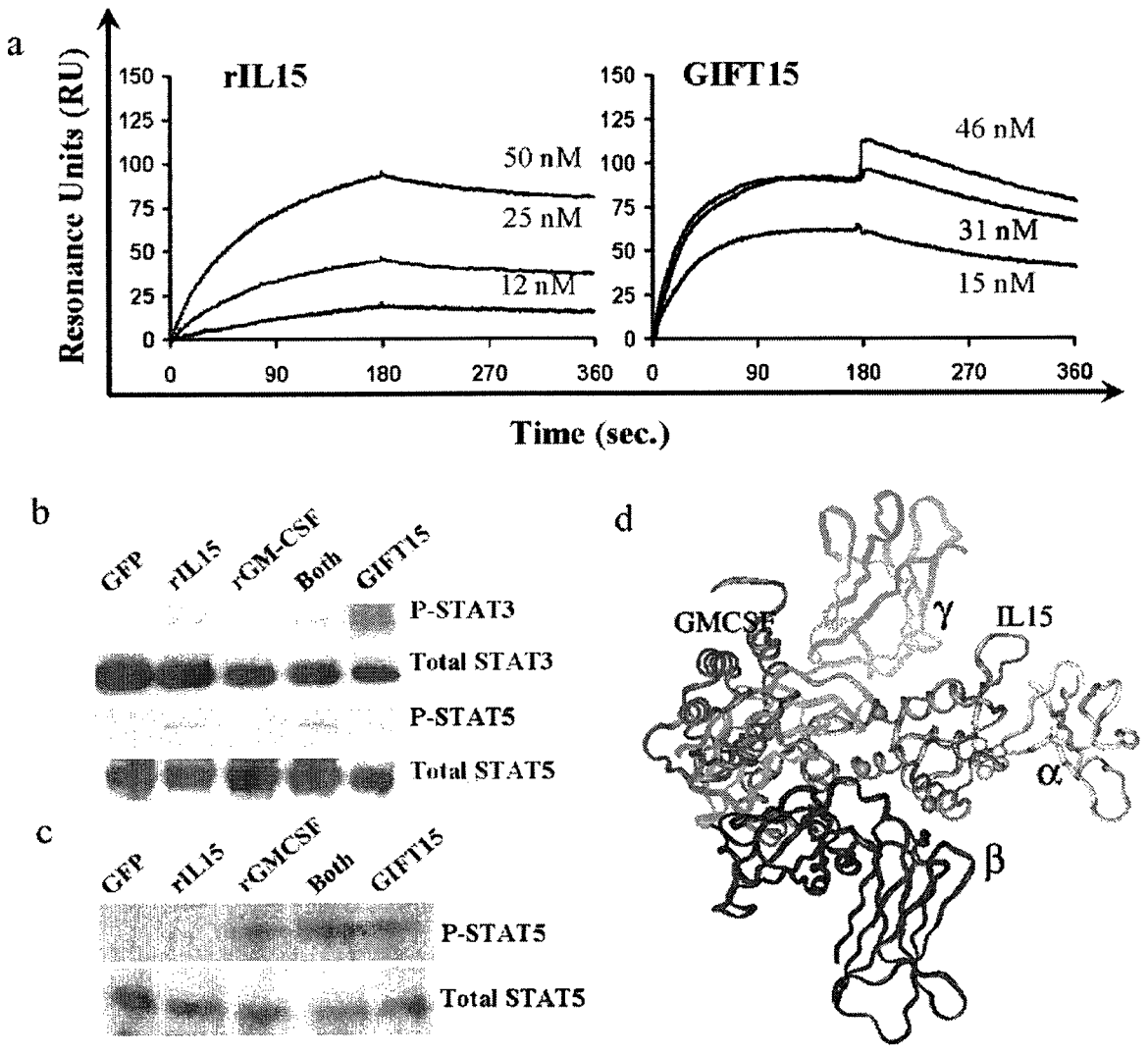
derived molecules that might help explain the increased blood vessel density observed *in vivo*. In comparison to control groups, GIFT15 treatment of macrophages leads to significant secretion/activation of TGF-β (**Fig. 8f**) and VEGF (**Fig. 8g**). Taken together, these data suggest that GIFT15-stimulated macrophages likely contribute to the observed *in vivo* immunosuppression via TGF-β production in addition to angiogenesis through MMP-2 and VEGF secretion and activation.

2.4.4 GIFT15 leads to Hyperphosphorylation of STAT3 and Blockade of STAT5 Activation in Lymphoid Cells

CD3+ and NK/NKT lymphoid cells express the full IL15R composed of the α, β, and common γ chains [235-237]. In order to further characterize the molecular mechanism by which GIFT15 exerts its suppressive effects on lymphoid cells, we first assessed the interaction of GIFT15 with individual components of the IL15R [236, 237]. Though the β and γ chains of the IL15R are components shared by the IL2R complex, the high affinity IL15Rα chain provides specificity. Its binding affinity to GIFT15 was assessed by BIAcore analysis. We found that the average dissociation equilibrium (KD) of rIL15 was of 3 nM whereas purified GIFT15 interacted with a higher affinity with an average KD of 1.4 nM (**Fig. 9a**). Since IL15R-dependent intracellular signalling in immune competent cells occurs through JAK/STAT downstream of both the β chain and the γ chain, we investigated the effect of GIFT15 on these pathways in primary mouse splenocytes. After a 15 minute stimulation with GIFT15 or controls in equimolar concentrations, we found that the fusion protein substantially increased the β chain-dependent phosphorylation of STAT3 and suppressed the γ chain-dependent phosphorylation of STAT5 (**Fig. 9b**). To determine the effect of GIFT15 on GMCSF receptor complex (GMCSFR) mediated signalling, we examined STAT5 phosphorylation following stimulation of JAWS-II cells, a GMCSF-dependent cell line devoid of the IL15R. We did not observe any difference between GMCSF and GIFT15 mediated activation of STAT5 in this cell line suggesting that GIFT15 binds to and activates the GMCSFR in a manner indistinguishable to that of monomeric GMCSF (**Fig. 9c**). This observation suggests that the function of the GMCSF moiety of GIFT15 remains

Figure 9: GIFT15 and Receptor-Mediated Interactions Signaling.

(a) BIAcore analysis of IL15Rα chain interaction with rIL15 and purified GIFT15. Representative sensorgrams for the binding of (A) 12, 25, or 50 nM rIL15 or (B) 15, 31, 46 nM purified GIFT15 to 1300 RU rIL15Rα-Fc as detected by SPR. **(b)** GIFT15-induced phosphorylation of STAT3/5 protein. 10^6 splenocytes were stimulated for 15 minutes with 30 pmols of rIL15, rGMCSF, or both cytokines inoculated in B16-GFP CM or with B16-GIFT15 CM, and cell lysate probed for phosphorylated STAT3/STAT5. Total STAT3 or STAT5 protein was used as loading control. GIFT15-induced phosphorylation of STAT5 protein. **(c)** GMCSFR-Mediated Signaling. 10^6 JAWS-II were stimulated as performed previously with primary splenocytes, and cell lysate probed for phosphorylated STAT5. Total STAT5 protein was used as loading control. **(d)** Structural model of GIFT15 (green, grey and cyan ribbon) complexed with IL15Rα (yellow ribbon), IL2β (purple ribbon) and IL2Rγ (red ribbon).


a
Resonance Units (RU)
rIL15
GIFT15
50 nM
25 nM
12 nM
46 nM
31 nM
15 nM
150
125
100
75
50
25
0
0
90
180
270
360
Time (sec.)
b
GFP
rIL15
rGM-CSF
Both
GIFT15
P-STAT3
Total STAT3
P-STAT5
Total STAT5
c
GFP
rIL15
rGMCSF
Both
GIFT15
P-STAT5
Total STAT5
d
GMCSF
γ
IL15
α
β

unchanged despite the tethering of IL15 at its carboxyterminus. The asymmetrical signalling through the IL15R mediated by GIFT15 suggests that an altered ligand/receptor interaction is at play. As a hypothesis generating experiment, we utilized molecular modelling to predict GIFT15 and IL15R interaction at the structural level. Based on the known molecular structure of IL15 interaction with the IL15Rα chain [249, 250] and on the predicted homologous interaction of IL15 with the IL15Rβ and γ chains to that of IL2 [251], we modelled the best fit for GIFT15 with the IL15R (**Fig. 9d**). This virtual interaction suggests that the GMCSF domain component of the GIFT15 fusokine may hinder the interaction of the IL15 domain component with the IL15Rγ chain, explaining in part the observed down regulation of signalling through the JAK3/STAT5 pathway.

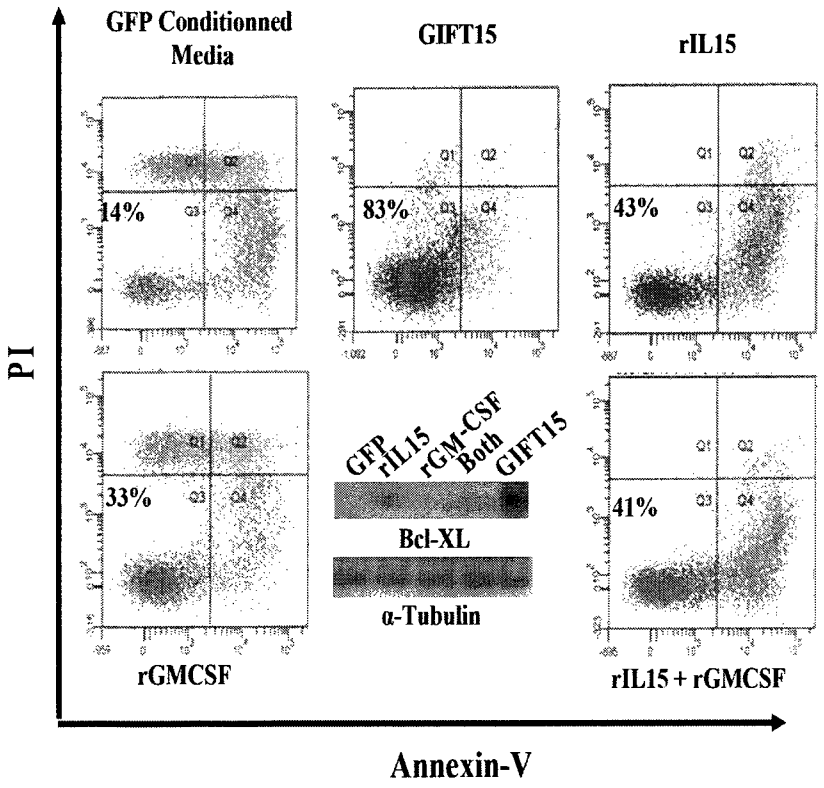
2.4.5 GIFT15 Protects Splenocytes from Apoptosis, Stimulates Proliferation and Blocks IFN-γ Secretion

STAT3 activation has been linked to a variety of biochemical and cellular events such as survival, proliferation, angiogenesis and immunosuppression [252-255]. Since we have shown that splenocytes hyperphosphorylate STAT3 in response to GIFT15, we tested whether GIFT15 enhances survival of splenocytes *in vitro* using serum-free media as a pro-apoptotic stimulus. Splenocytes stained for PI and annexin-V revealed that 83% of cells treated with GIFT15 survived as compared to 33% with rGMCSF, 43% using rIL15 or 41% with both molecules (**Fig. 10a**). In addition, cell lysate immunoblotting against the anti-apoptotic molecule Bcl-XL (**Fig. 10a**) provided evidence that GIFT15 rescues splenocytes from cell death through an increase in Bcl-XL level, a process known to occur when STAT3 is dominantly activated [252, 253]. Interestingly, splenocyte proliferation does not seem to be affected by the relative decrease in STAT5 phosphorylation (**Fig. 10b**; $P<0.05$) despite the fact that the latter is associated with mitogenic activities [256-258]. Based on the asymmetrical signalling mediated by GIFT15 through the IL15R, we tested for expression levels of known target genes of STAT5 in immune competent cells – such as IFNγ [237] - which may help to explain the

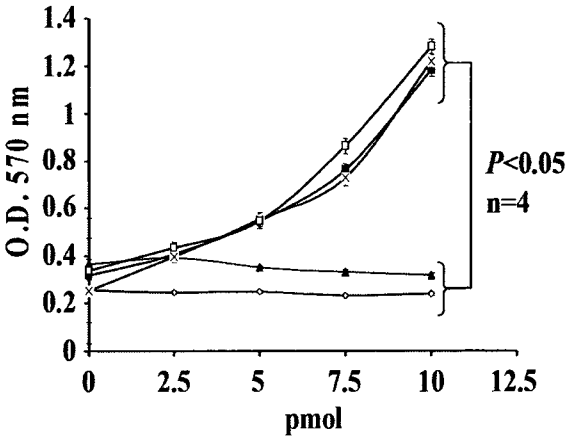
Figure 10. GIFT15 and Biochemical Responses

(a) GIFT15 and splenocyte apoptosis. Splenocytes cultured for 36 hrs in the presence of equimolar concentrations of cytokines were stained for PI and Annexin-V. The same conditions were applied for Bcl-XL immunobloting. α-Tubulin was used as loading control for this experiment. **(b)** Splenocyte proliferation. 10^5 splenocytes were cultured with increasing concentrations of cytokines for 72 hrs before MTT incorporation. Results are shown as mean of triplicates ± S.E.M of one representative experiment of two independent assays ($P<0.05$ between rIL15 (□), rIL15 + rGMCSF (X), GIFT15 (■) and rGMCSF (Δ) or GFP CM (O)). **(c)** Splenocyte activation and secretion of IFNγ. The supernatant of 10^5 splenocytes cultured for 36 hrs in the presence of GFP CM inoculated with cytokines or GIFT15 CM was used to detect the presence of IFNγ by ELISA. Results are shown as mean of quadruplets ± S.E.D of one representative experiment of five (n.d. = not detected; $P<0.005$ between rIL15 or rIL15 + rGMCSF and GFP control group).

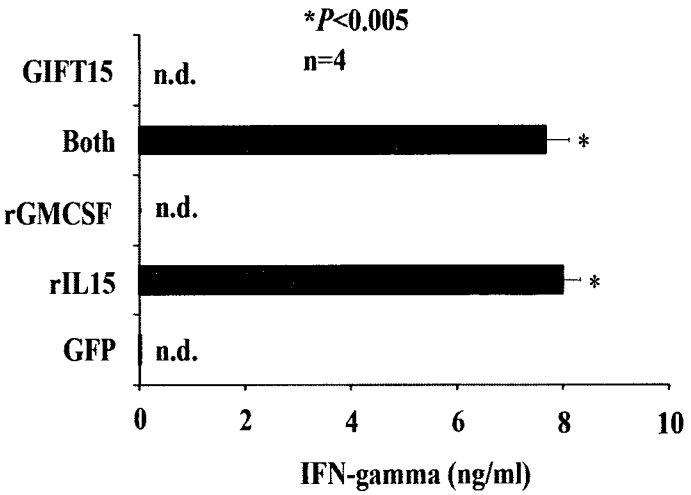
a


GFP Conditionned Media
GIFT15
rIL15
14%
83%
43%
33%
41%
GFP
rIL15
rGM-CSF
Both
GIFT15
Bcl-XL
α-Tubulin
rGMCSF
rIL15 + rGMCSF
PI
Annexin-V

b


O.D. 570 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2.5
5
7.5
10
12.5
pmol
P<0.05
n=4

c


*P<0.005
n=4
GIFT15
n.d.
Both
*
rGMCSF
n.d.
rIL15
*
GFP
n.d.
0
2
4
6
8
10
IFN-gamma (ng/ml)

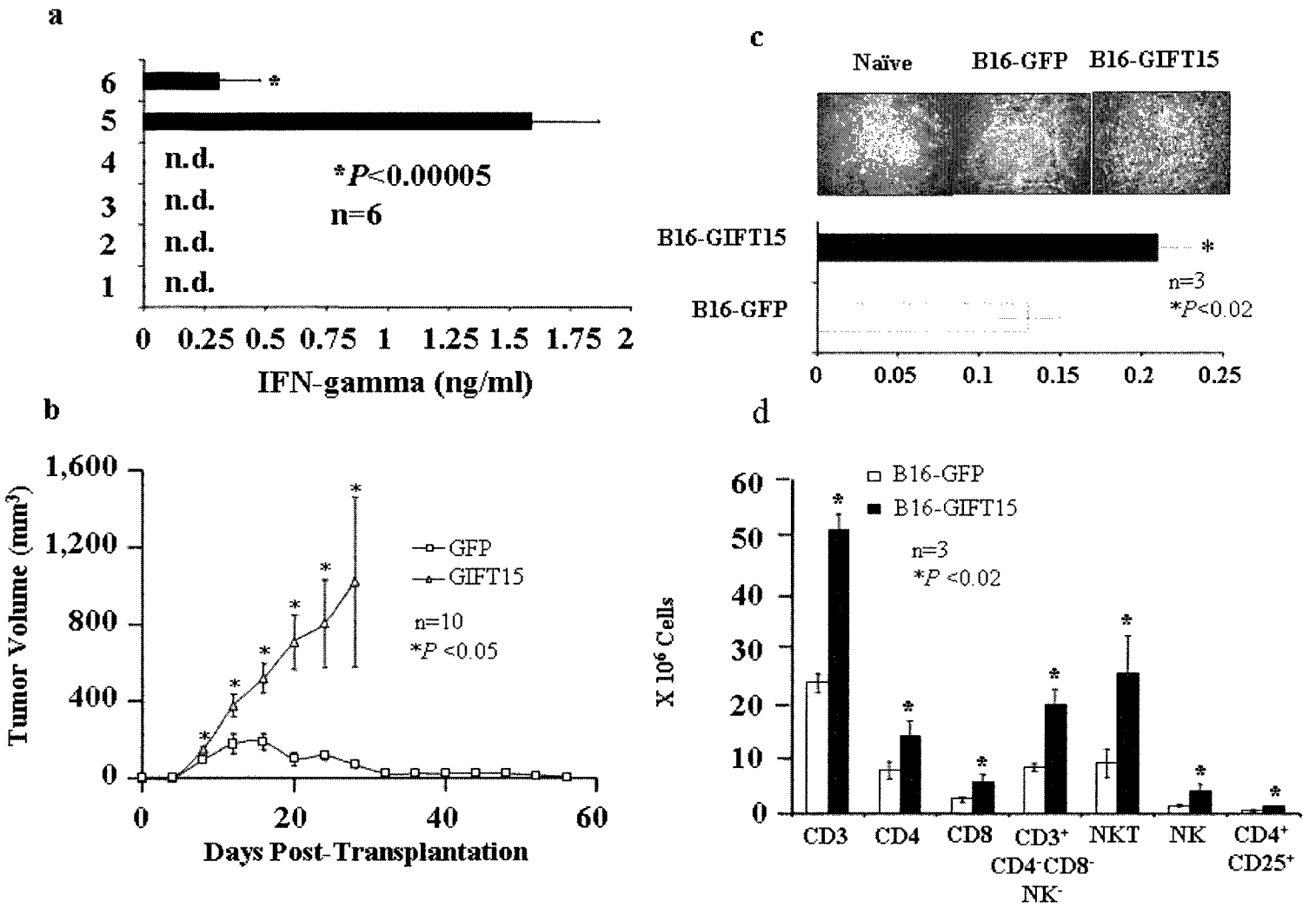
observed immunosuppressive effects *in vivo*. Although GIFT15-treated splenocytes proliferate normally when compared to rIL15, the IFN-γ response was completely abrogated when the fusokine was added to the system as opposed to a strong induction with rIL15 alone or in the presence of rGMCSF (**Fig. 10c,** $P<0.0005$).

2.4.6 GIFT15 Can Block Lymphocyte Activation Arising From a Mixed Lymphocyte Reaction and Allows Engraftment of GIFT15-expressing Allogeneic Somatic Cells in Immune Competent Recipients

Since GIFT15 can signal aberrantly through the IL15R by hyperphosphorylating STAT3 without leading to IFN-γ secretion in lymphocytes, we wanted to test its ability to inhibit an MLR between MHC-mismatched C57Bl/6 and BALB/c splenocytes. Interestingly, 0.18 pmols of purified GIFT15 was enough to suppress by 6 fold the levels of IFN-γ secretion (**Fig. 11a**). We examined interferon-gamma production by intracellular staining in T-cell subsets part of the 2-way MLR and found that GIFT15 abolished interferon-gamma production by CD3/CD4 and CD3/CD8 T-cells (**Supplemental figure 1**). This observation implies that the fusokine can be exploited for tolerance induction of allogeneic cells in immune competent recipients. To test this hypothesis, 10^7 B16-GFP or GIFT15 cells were injected subcutaneously in MHC-mismatched BALB/c mice, and tumor growth was assessed as a measure of graft tolerance. Even though the B16-GFP graft was rejected completely two months post-transplantation, the B16-GIFT15 cells lead to continuous tumor growth up to a volume of 1000 mm^3 (**Fig. 11b**). Mice with established B16-GIFT15 allogeneic tumors were sacrificed and their spleens weighed and analyzed by histochemistry. The spleens of naïve or B16-GFP mice had similar white pulp structures, which they disappeared in the GIFT15 group as the white and red pulps merged and showed no apparent organized structures (**Fig. 11c**). In addition, spleens from the GIFT15 group had a significant increase in weight suggesting that splenocytes did indeed proliferate *in vivo* probably due to the large amount of GIFT15 secreted from the B16-GIFT15 tumors (**Fig. 11c**). There was no selective proliferation of a specific immune cell population as all lymphocyte

Figure 11. GIFT15 and Allogeneic Tumor Transplantation.

(a) GIFT15 direct effect on the 2-way MLR. 0.18 pmols of purified GIFT15 was added directly to $1.5X10^5$ BALB/c + $1.5X10^5$ C57Bl/6 splenocytes for 72 hrs. The supernatant was tested by ELISA for IFN-γ. Every condition was performed in sixplicata ± S.E.D ($P<0.00005$ between MLR condition containing GIFT15 and BALB/c + C57BL/6 only). **(b)** Effect of GIFT15 on B16F0 tumor growth in allogeneic BALB/c mice. 10^7 live B16-GFP or GIFT15 were transplanted subcutaneously in immunocompetent BALB/c mice (n=10) and tumor volume monitored over time. The GIFT15 experiment had to be stopped by day 28-post-transplantation due to large tumors developed by these mice ($P<0.05$ between B16-GIFT15 and GFP group). Results are shown as mean tumor volume ± S.E.D. **(c)** GIFT15 BALB/c mice developed splenomegaly. Splenomegaly was observed in mice transplanted with GIFT15 tumors based on the spleen's weight characterized by white pulp structural loss as shown by H & E staining. Spleens from naïve or B16-GFP mice were used for comparison. **(d)** Increased T and NK cell number within the GIFT15 group. Flow cytometry analysis performed on splenocytes from GIFT15 or GFP mice revealed a significant increase in the absolute number of T and NK cells (n=3; $P<0.02$ between the GIFT15 and GFP group). Results are shown as mean average of triplicates ± S.E.D.


a
6
5
4
3
2
1
n.d.
n.d.
n.d.
n.d.
*P<0.00005
n=6
0 0.25 0.5 0.75 1 1.25 1.5 1.75 2
IFN-gamma (ng/ml)
c
Naïve
B16-GFP
B16-GIFT15
B16-GIFT15
B16-GFP
n=3
*P<0.02
0 0.05 0.1 0.15 0.2 0.25
b
Tumor Volume (mm3)
1,600
1,200
800
400
0
GFP
GIFT15
n=10
*P <0.05
0 20 40 60
Days Post-Transplantation
d
X 10^6 Cells
60
50
40
30
20
10
0
B16-GFP
B16-GIFT15
n=3
*P <0.02
CD3
CD4
CD8
CD3+ CD4- CD8- NK-
NKT
NK
CD4+ CD25+

levels increased significantly in the GIFT15 group when compared to the GFP control group (**Fig. 11d**).

2.4.7 GIFT15 Allows for Xenotransplantation of the Human Glioma U87GM in Immunocompetent Mice

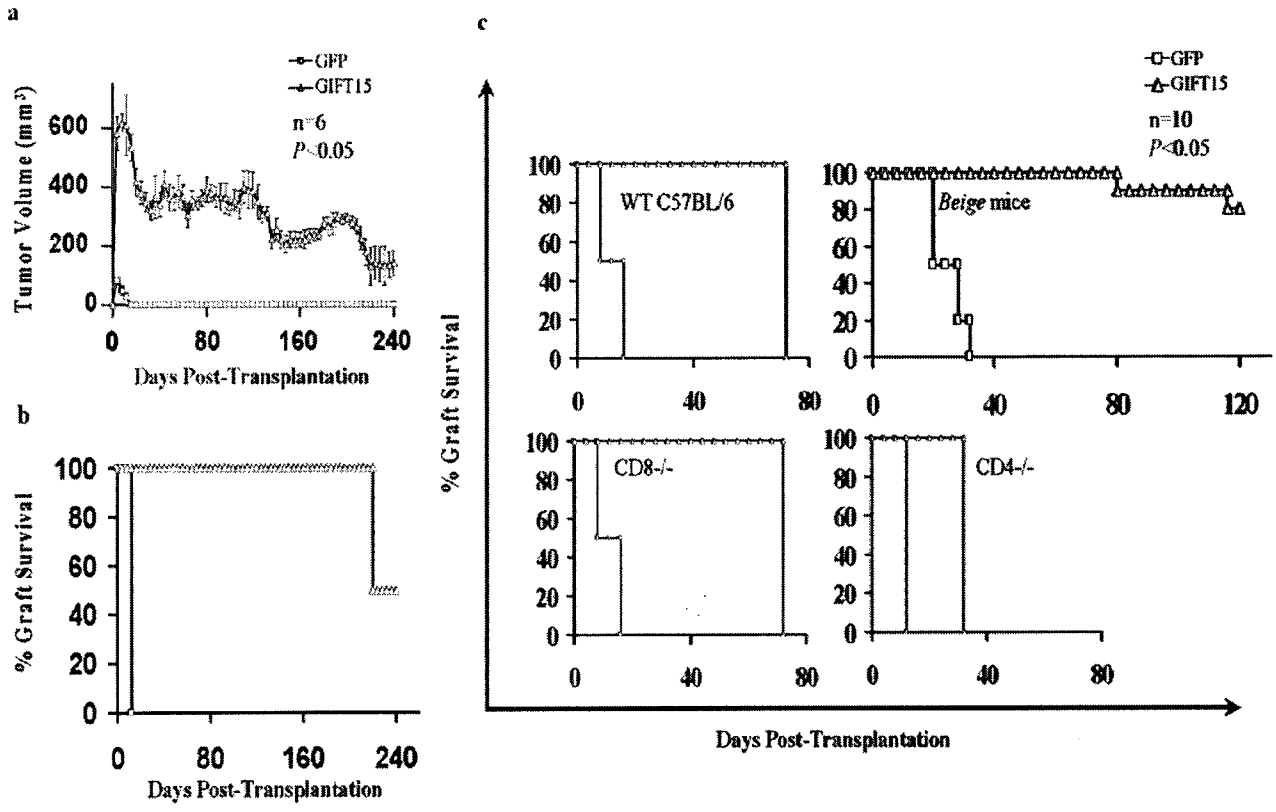
The ability of GIFT15 to allow the engraftment of allogeneic cells in immunocompetent mice demonstrates that the fusokine possess powerful immunosuppressive abilities. Thus, we tested the limits of GIFT15-mediated tolerance in the context of xenotransplantation using the human glioma cell line U87GM transduced to secrete the fusokine (10^7 U87-GFP or GIFT15 cells were injected subcutaneously in BALB/c mice). GIFT15 secreting tumors were accepted in all mice until day 224 where half the group rejected the graft. All control mice had rejected the U87-GFP graft 12 days post-transplantation (**Fig. 12a-b**). However, WT C57Bl/6 mice allowed the tumor GIFT15-secreting tumor graft to survive for up to two months starting from the day where the GFP tumors were rejected (**Fig. 12c**). Interestingly, implanted $CD8^{-/-}$ mice reproduced the same graft survival curve obtained by the WT C57Bl/6 strain indicating that CD8 T-cells are not implicated in the rejection of xenogeneic cells. An NK deficiency, on the other hand, seemed to promote the survival of the graft since 80% of mice accepted the transplant for a period longer than 100 days post-transplantation (**Fig. 12c**). In addition, the rejection profile was faster in $CD4^{-/-}$ mice when compared to WT or $CD8^{-/-}$ mice implying that CD4 lymphocytes are necessary for GIFT15-mediated graft acceptance (**Fig. 12c**). In sum, GIFT15 interacts with a subset of CD4 T-cells to prevent NK-mediated rejection of xenografts.

2.5 DISCUSSION

We have previously demonstrated that the merging of two distinct immune stimulatory cytokines – in occurrence GMCSF and IL2 – can lead to a fusokine with synergistic proinflammatory properties in the setting of cancer immunotherapy [234]. Operating within the same developmental mind frame, we sought to improve this

Figure 12: GIFT15 and Tumor Xenotransplantation.

(a-b) GIFT15 effect on U87GM xenotransplantation. Immunocompetent BALB/c mice (n=6) were grafted with 10^7 live U87-GFP (□) or GIFT15 (Δ) subcutaneously and tumor volume as well as percentage survival were monitored over 8 months ($P<0.05$ between the GIFT15 and GFP group). **(c)** Xenotransplantation of U87-GFP/GIFT15 in WT C57BL/6 or KO mice. 10^7 live U87-GFP or GIFT15 were transplanted subcutaneously in WT C57BL/6 (n=6) mice, $CD8^{-/-}$ (n=10), $CD4^{-/-}$ (n=10), or *beige* mice (n=10), and graft survival monitored over time ($P<0.05$ between U87-GIFT15 and GFP). Results are shown as mean tumor volume ± S.E.D.


a
GFP
GIFT15
n=6
P<0.05
Tumor Volume (mm³)
600
400
200
0
0
80
160
240
Days Post-Transplantation
b
% Graft Survival
100
80
60
40
20
0
0
80
160
240
Days Post-Transplantation
c
GFP
GIFT15
n=10
P<0.05
% Graft Survival
WT C57BL/6
Beige mice
CD8-/-
CD4-/-
100
80
60
40
20
0
0
40
80
120
Days Post-Transplantation

antitumor biopharmaceutical by generating a GM-CSF and IL-15 fusokine, aka. GIFT15. We observed that B16 melanoma cells expressing GIFT15 displayed markedly enhanced tumor growth properties *in vivo*. This observation was contradictory to our working hypothesis. In an attempt to decipher this totally unexpected result we first observed that tumor-associated angiogenesis was markedly enhanced in association with a robust recruitment of TAMs and we further noted that tumor-associated lymphoid cells, in particular NK/NKT-cells were significantly reduced in number. These two seminal observations led us to revise our hypothesis on the putative biochemical effect of GIFT15: GIFT15 behaves as a proangiogenic and immunosuppressive compound. These interesting and unanticipated properties suggest that GIFT15 may be useful as a compound for treatment of those medical ailments where pharmaceutical immunosuppression and/or angiogenesis are desirable. To best understand the cellular physiology and biochemistry of GIFT15, we chose to tackle the investigation of angiogenesis and immunosuppression separtly.

Our initial observation that B16-GIFT15 cells led to massive recruitment of TAMs, gave us the insight that host-derived macrophages may be playing a significant role in the observed enhanced angiogenesis. It has been previously shown that GM-CSF and IL-15 can induce migration of macrophages both *in vitro* and *in vivo* [234-237]. Since macrophages express both the GM-CSFR and the IL-15R, it follows that GIFT15 would lead to selective recruitment of these cells. Not surprisingly, we found GIFT15 to be a potent chemotactic agent for macrophages *in vitro*. There is much precedent in the field of cancer angiogenesis of TAMs play an important role in releasing a wide array of proangiogenic factors as part of a maladaptive injury repair response to a "wound that does not heal" [243-245]. We demonstrate that primary peritoneal macrophages stimulated with GIFT15 protein preferentially phosphorylate STAT3 via the IL2/IL15 β chain and subsequently adopt an unprecedented phenotype where they secrete TIMP-2 *de novo*. Indeed, there is virtually no published precedent describing TIMP-2 production by monocyte/macrophages, suggesting a completely novel property of macrophages. TIMP-2 is known to act either as an inhibitor of MMP-2 if a soluble complex is formed or as an activator if it is bound to MT1-MMP on cell surfaces, which can lead to matrix

remodelling and angiogenesis [246, 247]. Moreover, STAT3 phosphorylation has been shown to control the level of MMP-2 secretion by directly binding to its promoter and activating gene expression [259] suggesting that both TIMP-2 and STAT3 hyperphosphorylation can lead to MMP2 secretion and activation. Indeed, enzymaticaly active MMP-2 was found to be produced by GIFT15 treated macrophages as shown by gelatin zymography. On the other hand, MMP-9 was absent and thus cannot be accounted to play a role in angiogenesis. MMP-2 possesses a variety of functions such as anti-inflammation, increasing the levels of active TGF-β as well as the activation of VEGF and a variety of other growth factors [223, 260-265]. In fact, significantly increased levels of active TGF-β and VEGF levels were documented in GIFT15-treated macrophages. Though the qualitative interaction of GIFT15 with the GMCSFR appears identical to that of monomeric GMCSF, it must be noted that GMCSF's half-life *in vivo* is more than 240 minutes [266, 267], whereas IL15 has a much shorter plasma half-life of less than 1 minute [208]. Therefore, it is possible that the *in vivo* half-life of GIFT15 is closer to that of native GMCSF and may lead to prolonged interaction with the IL15R. In sum, GIFT15 appears to act as a macrophage chemotactic agent *in vivo* and also modulates their phenotype in a manner rendering them profoundly proangiogenic.

A second seminal observation is the extensive cell-mediated immune suppression by GIFT15 which tolerizes to both allogeneic and xenogeneic somatic cell implantation in otherwise immunologically intact recipient mice. This phenomenon – at least in regards to xenotolerance - is dependent *in vivo* upon modulation of NK cells via CD4 cells. On a molecular basis, we have found that GIFT15 has increased affinity for the IL15R α chain and also leads to asymmetrical signalling through the IL15R complex manifest as STAT3 hyperphosphorylation and reciprocal STAT5 underphosphorylation. Downstream effects of STAT3/STAT5 asymmetrical signalling include induction of Bcl-XL and suppression of IFNγ production leading to protection from apoptosis and anergy respectively. The latter observation is of particular interest since it suggests that GIFT15 can quite literally shut down IFNγ production in "activated" lymphoid cells. Indeed, we found that GIFT15 could significantly antagonize IFNγ secretion arising from a 2-way MLR. Since STAT3 activation has recently been reported to mediate

immunosuppression by inhibiting the expression of pro-inflammatory cytokines [254, 255], we propose that the combined effect of enhanced STAT3 activation coupled to a relative decrease in STAT5 phosphorylation may explain the potent blunting of the IFNγ response following GIFT15 exposure.

In light of the remarkable immunosuppressive effects of GIFT15, we tested whether its expression could protect allogeneic tumor cells from rejection in immune competent MHC-mismatched recipient animals. As proof of concept, 10 million B16-GFP or B16-GIFT15 (H-2K^b) were grafted in BALB/c (H-2K^d) mice and we observed that tumors secreting the fusion protein were accepted in all recipient mice. As GIFT15 tumors grew, we noted that splenomegaly evolved with effacement of the spleen's white pulp structures and an increase in T and NK cell number, likely reflecting the systemic effect of high dose GIFT15 on host immune system. We further investigated the utility of GIFT15 immunosuppression in the context of xenotransplantation. In this case, a transduced polyclonal population of the human glioma cell line U87GM was transplanted subcutaneously in BALB/c mice. All mice accepted the GIFT15 xenograft for up to 7 months whereas the control U87-GFP xenograft was rejected 12 days post-injection. Due to the fact that different mouse strains generate variable immune responses [268], we pursued our studies by xenotransplanting C57Bl/6 mice, which are known to possess a biased Th1 immune response [269]. Even though both GFP and GIFT15 xenografts were rejected in these mice, there was a two month delay for the complete regression of the U87-GIFT15 transplants in comparison to the U87-GFP group. Experiments performed in knock-out mice revealed that the immunosuppressive property of the fusion protein was impaired once U87-GIFT15 were injected in CD4$^{-/-}$ model of C57Bl/6 mice. The B16 melanoma cell line used in Fig. 2 is syngeneic to C57Bl/6 mice, and will lead to tumor growth when implanted subcutaneously in immune competent C57Bl/6 mice. B16 tumors fail to induce an effective immune response in immunologically naïve mice, though it is possible to elicit a specific anti-B16 curative CD8-mediated cellular immune response when an appropriate pharmacological immune stimulus is applied, such as interleukin-2. In our system, we observed that B16 tumors cells are associated with a CD3+ lymphoid cell infiltrate and that the composition of this infiltrate is characterized

by depletion of NK/NKT-cells in the presence of GIFT15. Since tumor growth is accelerated in this setting, it suggests that NK/NKT-cells may play a role in delaying syngeneic tumor growth in immunologically naïve mice, especially if these tumors express low levels of MHCI. In contrast, xenogeneic human cells cannot lead to a physiological immune synapse in murine hosts due to the interspecies discrepancy between human MHC and murine TCR, thereby limiting cellular rejection to MHC-independent cytotoxic cells such as NK. Indeed, in the well studied porcine/simian xenotransplant system, NK cells appear to be important effectors in xenotissue rejection. Our model system behaves similarly, since we observed that NK-deficient mice are permissive to human xenograft tumor growth and that CD4 and CD8 deficient mice robustly reject xenografts. The observation that GIFT15 allows human xenograft tumor growth in normal mice strongly supports the hypothesis that its suppressive effect – in the xenogeneic setting - is targeted towards NK cells. Furthermore, this anti-NK effect of GIFT15 is dependent upon interplay with CD4 cells. Hence, we do not propose that CD4 cells are directly cytotoxic in xenorejection. Rather, GIFT15 modulates a subset of CD4 cells which may interact with NK directly. Therefore, in the absence of a species paired immunological synapse between effector and target cell, GIFT15 likely tolerizes to xenografts by promoting a suppressive cross talk between host derived CD4 and NK cells [270].

Taken together, our observations and experiments support the hypothesis that GIFT15 possesses novel biochemical properties leading to altered affinities to components of the IL15R and asymmetrical downstream signalling via its two dependent STAT/JAK pathways in lymphomyeloid cells. As a result, cellular proliferation, reduced apoptosis and blunting of the IFNγ response following activation can be achieved in lymphoid cells as well as a proangiogenic response by macrophages. The sum of these effects mediates a profound immunosuppressive state permissive to allo/xenotransplantation which is CD4 dependent. We speculate that the GIFT15 fusion protein can be exploited using two different approaches: tissue transplantation and autoimmune disease. First, genetic modification of allogeneic or xenogenic somatic cells or tissue to express GIFT15 can be readily achieved using currently available gene

transfer technology and *in vivo* tolerance achieved as we have here demonstrated. As a specific and achievable translational example, we could envisage GIFT15 engineering of islet cells from allogeneic human or xenogeneic non-simian source and use these cells for the treatment of type I diabetes without need of systemic immunosuppressive regimen in the recipient. Indeed, a wide array of cells/tissues/organs from allogeneic or xenogeneic source could be similarly engineered for treatment of a variety of human ailments. Another therapeutic avenue for this fusokine could be its use as a purified recombinant protein biopharmaceutical that can be administered parenterally for the treatment of severe and life-threatening autoimmune diseases such as graft versus host disease or multiple sclerosis where immunosuppression is clinically desirable. In conclusion, we have generated a novel biopharmaceutical providing a potential therapeutic platform for tolerization to immunologically mismatched tissue and treatment of ailments responsive to pharmacological immunosuppression.

2.6 ACCESSION CODES

PBD entries for the crystal structures of GMCSF (2gmf); human IL2 (1erj); region connecting GMCSF and IL15 (1orc); IL2 signalling complex (1erj); and IL15Rα (2ers)

2.7 ACKNOWLEDGMENTS

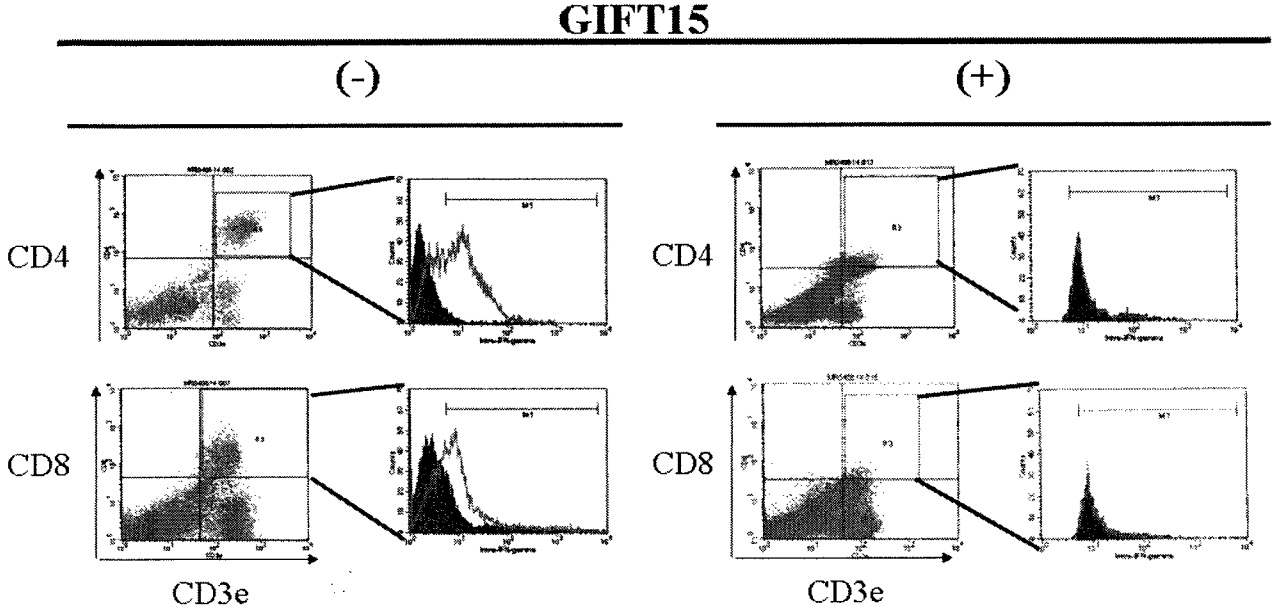
Moutih Rafei is a recipient of a *Fonds de recherches en Santé du Québec* Schorlaship and Jacques Galipeau is a CIHR Physician-Scientist Scholar. We thank Dr. M. Hancock (Sheldon Biotechnology Center) for the BIAcore experiments. Sheldon Biotechnology Centre at McGill University is supported by a Multi-User Maintenance Grant from CIHR. We Thank Drs N. Eliopoulos, I. Copland, and J. Stagg for technical advice and materials. We thank Mrs Milena Crosato for the critical reviewing of the manuscript.

2.8 COMPETING FINANCIAL STATEMENT

The authors declare that they have no competing financial interests.

Supplemental Figure 1:

Effect of GIFT15 on IFN-γ production in T-cell subsets part of a 2-way MLR. To induce IFN-γ, allogeneic splenocytes ($1.5X10^5$ BALB/c + $1.5X10^5$ C57Bl/6) were admixed for 72 hrs in the absence or presence of 0.18 pmols of GIFT15. The cells were then washed, and analyzed for intracellular IFN-γ expression in CD3/CD4 and CD3/CD8 subsets.


GIFT15
(-)
(+)
CD4
CD8
CD3e
CD4
CD8
CD3e

CHAPTER 3

A GMCSF/Interleukin-15 Fusokine Leads to the Generation of a Novel Type of Immune Regulatory B-Cell with Potent Immune Suppressive Properties as Demonstrated in the EAE Mouse Model of Multiple Sclerosis

Reference: Moutih Rafei, Jeremy Hsieh, Simone Zehntner, MengYang Li, Kathy Forner, Elena Birman, Claude Perreault, and Jacques Galipeau. A GMCSF/Interleukin-15 Fusokine Leads to the Generation of a Novel Type of Immune Regulatory B-Cell with Potent Immune Suppressive Properties as Demonstrated in the EAE Mouse Model of Multiple Sclerosis (*In revision in Nature Medicine 2009).*

Preface to Chapter 3:

We found in Chapter 2 a link between CD4-deficiency and GIFT15 using our xenotransplantation model suggesting that the fusokine can lead to the generation of a population of regulatory T-cells. In an attempt to clarify this point, we characterized the emerging population following GIFT15 *ex vivo* treatment of splenocytes and tested the hypothesis of whether these cells can lead to immunosuppression in an autoimmune disease model as part of a cell therapy approach. To our surprise, we discovered that GIFT15 converts naïve B-cells to a novel B-regulatory (Breg) phenotype with potent immune suppressive properties.

CHAPTER 3: A GMCSF/Interleukin-15 Fusokine Leads to the Generation of a Novel Type of Immune Regulatory B-Cell with Potent Immune Suppressive Properties as Demonstrated in the EAE Mouse Model of Multiple Sclerosis.

3.1 ABSTRACT

We have previously shown that a GMCSF and IL15 fusokine (GIFT15) exerts potent immune suppression via aberrant signalling through the IL15 receptor on lymphomyeloid cells. We here demonstrate that *ex-vivo* GIFT15 treatment of murine splenocytes generates a novel type of suppressive regulatory cell of B-cell ontogeny (GIFT15 Bregs). Arising from $CD19^+$ B-cells, GIFT15 Bregs shed CD19 yet otherwise express known Breg markers as well as high levels of MHCI/II. GIFT15 Bregs are capable of suppressing *in-vitro* T-cell activation and this activity is dependent upon MHCII expression and IL10 production. Mice with experimental autoimmune encephalomyelitis went in complete remission following intravenous GIFT15 Breg administration with a robust decrease in lymphomyeloid cell migration to the central nervous system. The clinical effect was abolished when GIFT15 Bregs were derived from $MHCII^{-/-}$, $STAT6^{-/-}$, $IL10^{-/-}$, or allogeneic splenocytes. We propose that cell therapy with autologous GIFT15 Bregs may serve as a novel treatment for autoimmune ailments.

3.2 INTRODUCTION

A well established model of Multiple Sclerosis (MS), Experimental Allergic Encephalitis (EAE), is a chronic inflammatory disease of the central nervous system (CNS) characterized by the activation of the immune system against myelin components entailing irreversible damages in axons and their myelin sheaths [271]. According to the dominant paradigm, induction of MS comprises multiple factors that culminate in the activation of peripheral autoreactive T-cells. After receiving a trigger to leave the periphery and cross the blood-brain barrier, T-cells recognize CNS antigens presented by microglial antigen presenting cells (APCs), get re-activated, and secrete pro-inflammatory cytokines resulting in tissue damage [271-273]. So far, four disease-modifying treatments

have been approved for relapsing-remitting MS. Interferon (IFN)-β has been approved due to its capacity to inhibit T-cell proliferation, and block their migration to the CNS [274, 275]. The second medication is glatiramer acetate (GA), a synthetic aminoacid polymer thought to promote the development of a specific Th2 cell population that migrate to the CNS and exert anti-inflammatory effects [276]. Finally, Mitoxantrone has anti-proliferative and pro-apoptotic properties [277], while natalizumad is a monoclonal antibody directed against the adhesive molecule alpha4 integrin [278]. However, despite exciting results in human trials, these therapies have only succeeded in modestly alleviating MS progression [278-280]. Therefore, there is an urgent need for the development of novel therapies that can be both safe and potent in inhibiting MS pathological progression.

An alternative approach under investigation in pre-clinical models involves the use of cell therapy, in particular the use of suppressive regulatory cells best exemplified by Tregs [281-283]. *Ex vivo* manipulated cells could exert a persisting suppressive effect by provoking the secretion of the host's own suppressive factors without inducing side effects observed with injectable drugs [284]. We have lately created a GMCSF and IL15 Fusion Transgene (GIFT15) and discovered that the fusokine is very potent in suppressing immune cells [285]. In fact, GIFT15 was originally engineered for the stimulation of the immune system in an attempt to induce tumor rejection in mice. However, we found through various in vitro cellular assays and in vivo proof-of-concepts that GIFT15 behaves as a powerful immunosuppressive compound. Its mechanism of action relies on the fact that the GMCSF moiety creates a physical steric hindrance to the IL15 domain of the protein and thus leads to aberrant signalling taking place downstream of the IL15 receptor (R) [285]. The end effect is potent immune suppression. This property of GIFT15 was unheralded and quite a surprise. Indeed, we further discovered the ability of GIFT15 of converting naïve CD19+ B-cells to a novel type of suppressive regulatory cell of B-cell ontogeny. We demonstrate herein that GIFT15-driven B-regulatory cells (GIFT15 Bregs) can completely reverse EAE symptoms and neuropathology. The sum of these data suggest that GIFT15 can be used as a method to generate *ex vivo* regulatory cells derived from naïve autologous B-cells that can be exploited for the treatment of autoimmune disease such as MS.

3.3 RESULTS

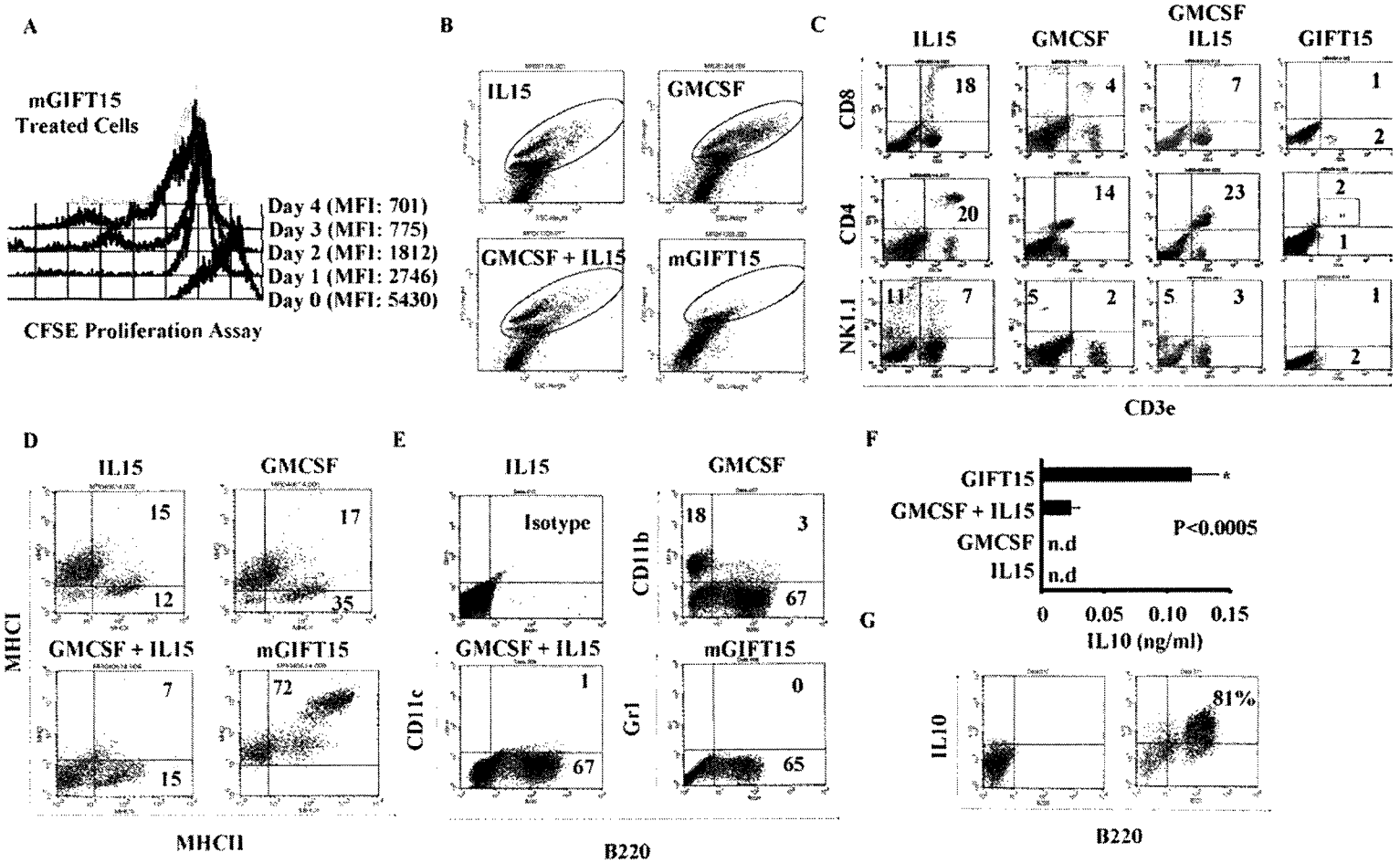
3.3.1 GIFT15 treatment of unfractionated murine splenocytes leads to enrichment of an IL10-secreting B-cell derived population expressing high levels of MHCI/II.

To study the mitogenic effect of GIFT15 on unfractionated splenocytes, CFSE-labelled splenocytes were assessed daily by flow cytometry for their proliferation. We observed that on day 4, the ≈ 8-fold decrease in CFSE MFI indicates that the dominant lymphoid cellular subset had undergone three cell divisions ($2^3 = 8$) **(Figure 13A)**. These cells define a unique population of cells with a distinct FSC/SSC signature as compared to the output of splenocytes treated with IL15, GMCSF or both cytokines at equimolar concentrations (**Figure 13B**). These observations led us to investigate the phenotype of the generated population. Surprisingly, none of the GIFT15 treated cells expressed T or NK cell markers (**Figure 13C**). However, splenocytes treated with GIFT15 were MHCI and MHCII double-positive (72% vs 15%, 17% or 7% for IL15, GMCSF or both cytokines, respectively; **Figure 13D**). In an attempt to define their lineage, GIFT15 treated splenocytes were analyzed for expression of surface markers associated with myeloid suppressor cells (MSC) or regulatory dendritic cell (DC) phenotype. We found that B220 expressing GIFT15 Bregs did not express CD11b, CD11c or Gr1 (**Figure 13E**). Screening the secretome of GIFT15 treated splenocytes revealed the presence of IL10 (**Figure 13F**), an observation that was further confirmed by intracellular staining of IL10 in B220+ cells (**Figure 13G**).

3.3.2GIFT15 Regs are of a B-cell lineage.

We have demonstrated that GIFT15 treatment of unfractionated splenocytes enriches for a population of cells with high MHCI/II and IL10 expression. To identify the lineage ontogeny of the generated GIFT15-derived cells, purified T, B, or NK cells collected from the spleens of C57BL/6 (B6) mice were subsequently cultured for 6 days in the presence of GIFT15 as performed previously, then analyzed for expression of MHCII (**Figure 14A**). Only cells of B lineage expressed high levels of MHCII while

Figure 13: GIFT15 Regs Phenotype. (A) B6 splenocytes proliferation in response to GIFT15. Following CFSE labelling, B6 splenocytes were cultured with 10 pmols of GIFT15 and proliferation assessed over 4 days. The shift of the peak to the left is reflective of cellular division. Every peak represents CFSE labelling at a given day. (**B**) Flow cytometry gating. For phenotypic analysis, all cells were gated after 6-8 days of culture with the different cytokine conditions. (**C**) GIFT15 Regs do not express T or NK markers. Following 6-8 days culture, splenocytes were stained for CD3e vs CD8, CD4, or NK1.1. None of these markers appeared following GIFT15 treatment as opposed to the other cytokines. (**D**) GIFT15 Regs express high levels of MHCI and MHCII. Same culture conditions as previously shown, GIFT15 Regs express high levels of MHCI and II vs the other test conditions (72% vs 15% for IL15, 17% for GMCSF, and 7% for both cytokines at equimolar conditions). (**E**) GIFT15 Regs are not MSCs or regulatory DC. To verify whether our generated cells are of an MSC or regulatory DC lineage, cells were stained with CD11b, CD11c and Gr1 versus B220. (**F**) GIFT15 treatment induces IL10 secretion. High levels of IL10 are secreted from B6 splenocytes cultured with GIFT15 (n=4/group; data are shown as mean ± SD). (G) Intracellular staining for IL10 (n.d. non-detectable).


A
mGIFT15
Treated Cells
Day 4 (MFI: 701)
Day 3 (MFI: 775)
Day 2 (MFI: 1812)
Day 1 (MFI: 2746)
Day 0 (MFI: 5430)
CFSE Proliferation Assay
B
IL15
GMCSF
GMCSF + IL15
mGIFT15
C
IL15
GMCSF
GMCSF IL15
GIFT15
CD8
CD4
NK1.1
18
4
7
1
2
20
14
23
2
1
11
7
5
2
5
3
1
2
CD3e
D
IL15
GMCSF
GMCSF + IL15
mGIFT15
15
12
17
35
7
15
72
MHCI
MHCII
E
IL15
GMCSF
Isotype
18
3
67
CD11b
GMCSF + IL15
mGIFT15
1
67
0
65
CD11c
Gr1
B220
F
GIFT15
GMCSF + IL15
GMCSF
IL15
n.d
n.d
*
P<0.0005
0
0.05
0.10
0.15
IL10 (ng/ml)
G
81%
IL10
B220

losing their CD19 expression. Interestingly, the removal of GIFT15 following the generation of Bregs did not lead to the re-expression of CD19 either 2 or 4 days post-removal (**Figure 14B**). To further analyze the phenotype of these cells, purified B-cells were analyzed by flow cytometry for their purity by staining for CD11b, CD11c or Gr1 to avoid any myeloid contaminant. B-cells of 93-94% purity (**Figure 14C; top panel**) were then treated with GIFT15 and analyzed for expression other surface B-cell markers. We found that GIFT15-treated B-cells are positive for CD21, CD22, CD23, CD24, CD43, CD79b, CD1d, IgD, IgM as well as B220. On the other hand, CD27, a plasma cell marker and AA4.1 were absent. As such, GIFT15 treatment converts naïve B-cells to a CD19 null cell population otherwise expressing B-cell markers, IL10 and high levels of MHCI/MHCII.

3.3.3 Biochemical responses of GIFT15 treated splenocytes to LPS and suppressor function.

LPS is known to trigger a cytokine response profile in lymphoid cells via toll-like receptor 4 [286]. We therefore tested the cytokine activation response of both naïve and GIFT15-treated splenocytes following LPS stimulation. When comparing GIFT15-treated to naive splenocytes, only the TNF-α response was comparable upon LPS stimulation whereas IFNγ, IL2 and IL12 responses were substantially impaired (**Figure 15A**). However, IL10, which was detected upon GIFT15 treatment at time 0, was completely abrogated following LPS stimulation suggesting that LPS can suppress IL10 secretion by GIFT15-treated splenocytes (**Figure 15A**). The fact that GIFT15-treated splenocytes secreted IL10 and had impaired responses to LPS suggested that they might possess suppressive properties. To test this possibility, GIFT15-treated splenocytes derived from B6 mice were co-cultured with naïve MHC-mismatched BALB/c splenocytes and we measured IFNγ produced following 2-way mixed lymphocyte reaction (MLR). Compared to the positive controls, IFNγ was virtually undetectable in the supernatant of the MLR containing GIFT15 pre-treated B6 splenocytes (**Figure 15B**). To clarify whether MHCII or IL10 are responsible for the observed IFNγ inhibition during the MLR, splenocytes derived from MHCII$^{-/-}$ or STAT6$^{-/-}$ mice (which cannot produce IL10) were treated with

Figure 14: GIFT15 Leads to the Development of Bregs. (A) GIFT15 Regs are of B-cell lineage. CD3e T-cells, CD19 B-cells or NK cells were purified from the spleens of normal B6 mice, and then cultured for 6-8 days in the presence of GIFT15, and MHCII expression was assessed by flow cytometry. Only cells of B-cell origin lost their CD19 expression while expressing high levels of MHCII. **(B)** GIFT15 Bregs do not Re-express CD19 *in vitro*. Four days following the removal of GIFT15 from B-cells, CD19 re-expression did not occur. **(C)** GIFT15 Bregs express B-cell markers. The purity of B-cells was verified by double staining CD19 with CD11b, CD11c, and Gr1 to avoid any myeloid contaminant. Our starting B-cell population was 93-94% pure. The screening of other cell surface markers, reveals that GIFT15 Bregs are positive for CD21, CD22, CD23, CD24, CD27, CD79b CD1d, IgM, IgD, as well as 45B/B220, but negative for AA4.1.

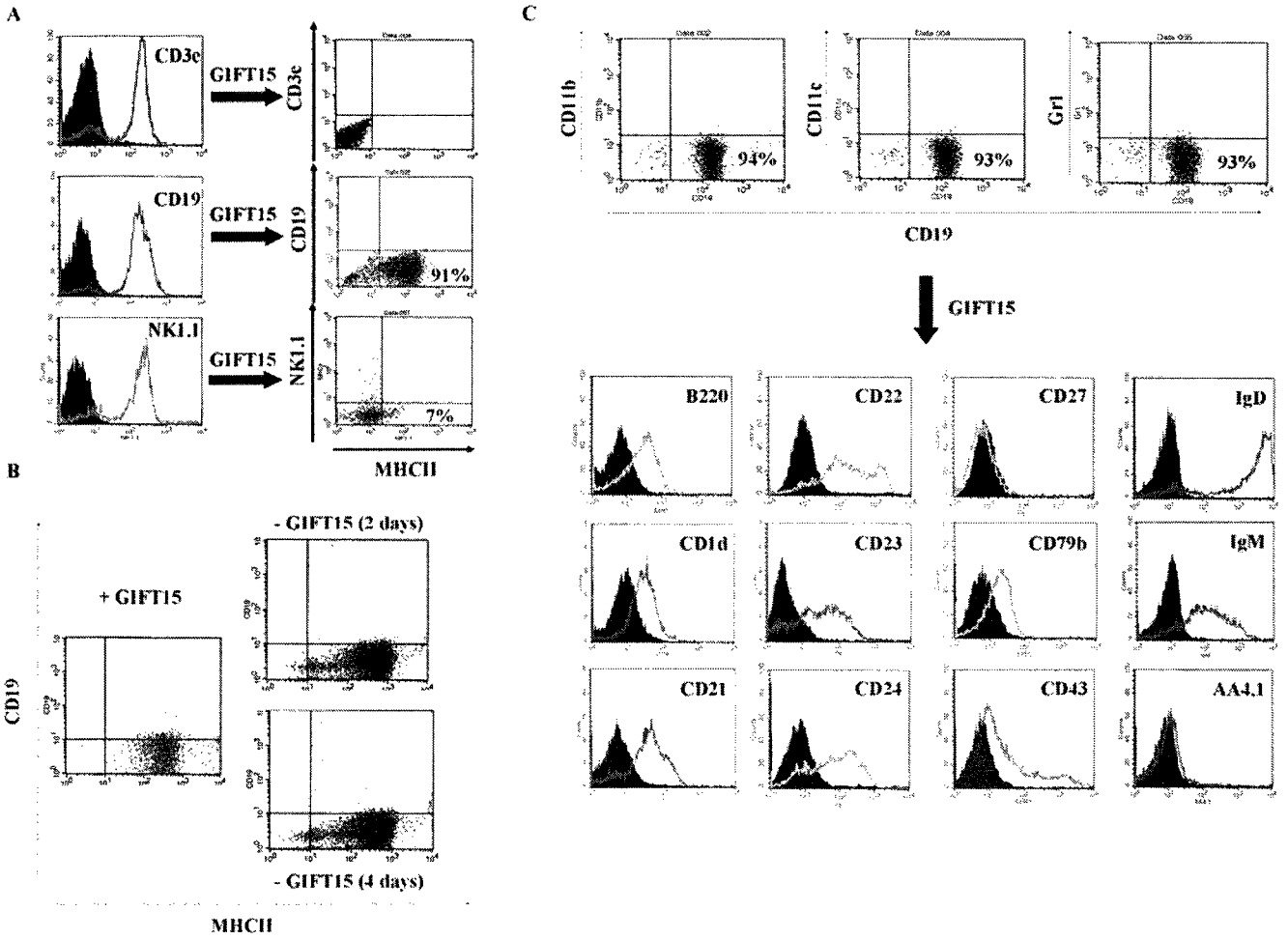
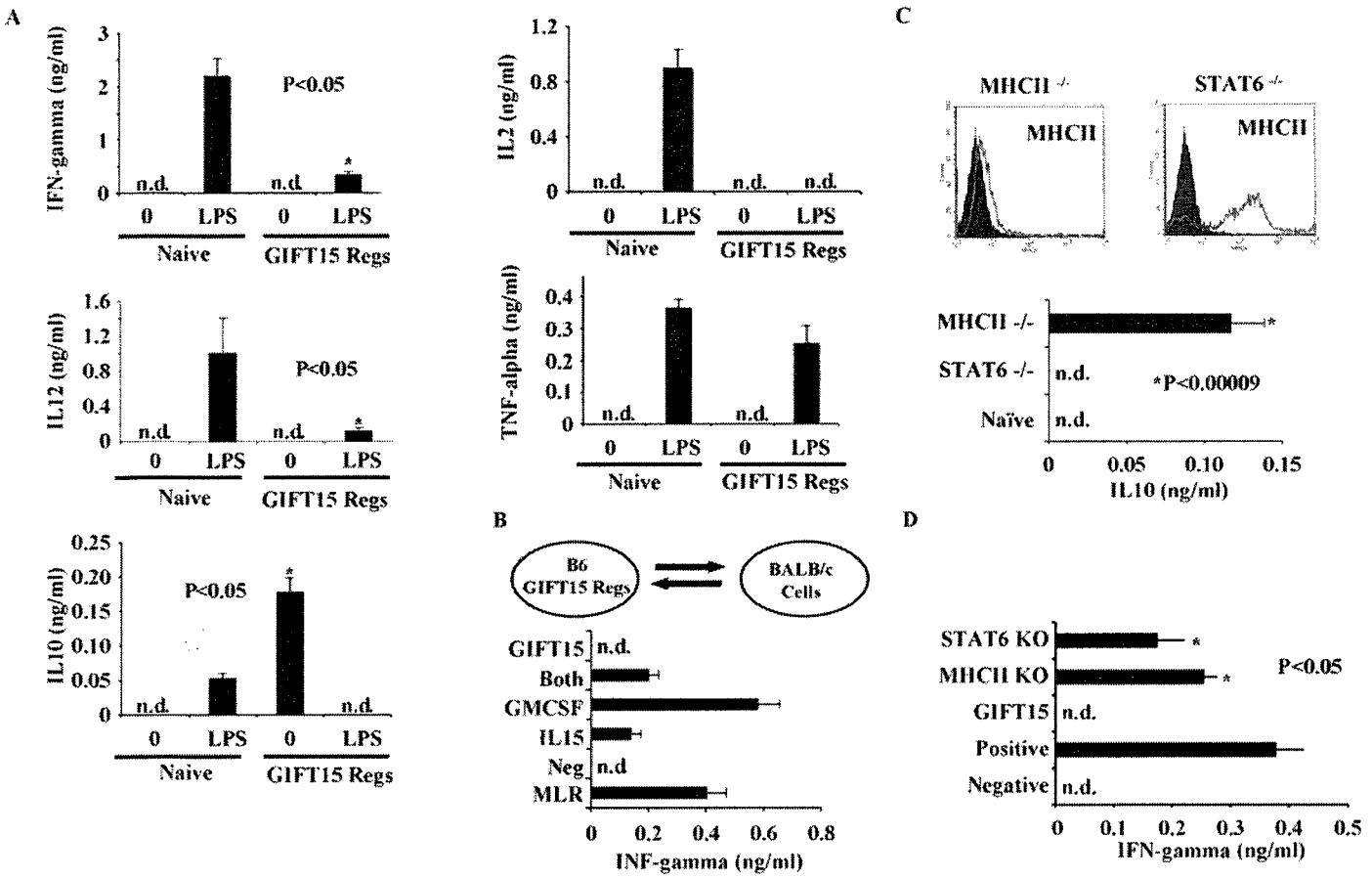

A
CD3e
GIFT15
CD3e
CD19
GIFT15
CD19
91%
NK1.1
GIFT15
NK1.1
7%
MHCII
B
+ GIFT15
- GIFT15 (2 days)
- GIFT15 (4 days)
CD19
MHCII
C
CD11b
94%
CD11c
93%
Gr1
93%
CD19
GIFT15
B220
CD22
CD27
IgD
CD1d
CD23
CD79b
IgM
CD21
CD24
CD43
AA4.1

Figure 15: GIFT15 Regs Biochemical Responses. (A) Response of GIFT15 Regs and control splenocytes to LPS. Naïve vs GIFT15 Regs were assessed for their secretion levels of IFNγ, IL2, IL12, TNFα, and IL10 (n=5/group; data are shown as mean ± SD). **(B)** GIFT15 Regs block IFNγ secretion during a 2-way MLR. BALB/c splenocytes and GIFT15 Regs derived from B6 mice were co-cultured for 72 hrs (n = 5/group; data are shown as mean ± SD). **(C)** MHC and STAT6 deficient mice response to GIFT15. Following GIFT15 treatment, $MHC^{-/-}$ splenocytes do not upregulate MHCII while $STAT6^{-/-}$ splenocytes failed in secreting IL10. **(D)** IFNγ blockade requires MHCII and IL10. If GIFT15 treated splenocytes derived from $MHC^{-/-}$ or $STAT6^{-/-}$ are used, they fail in blocking IFNγ secretion during a 2-way MLR (n=4 perData are shown as mean ± SD).

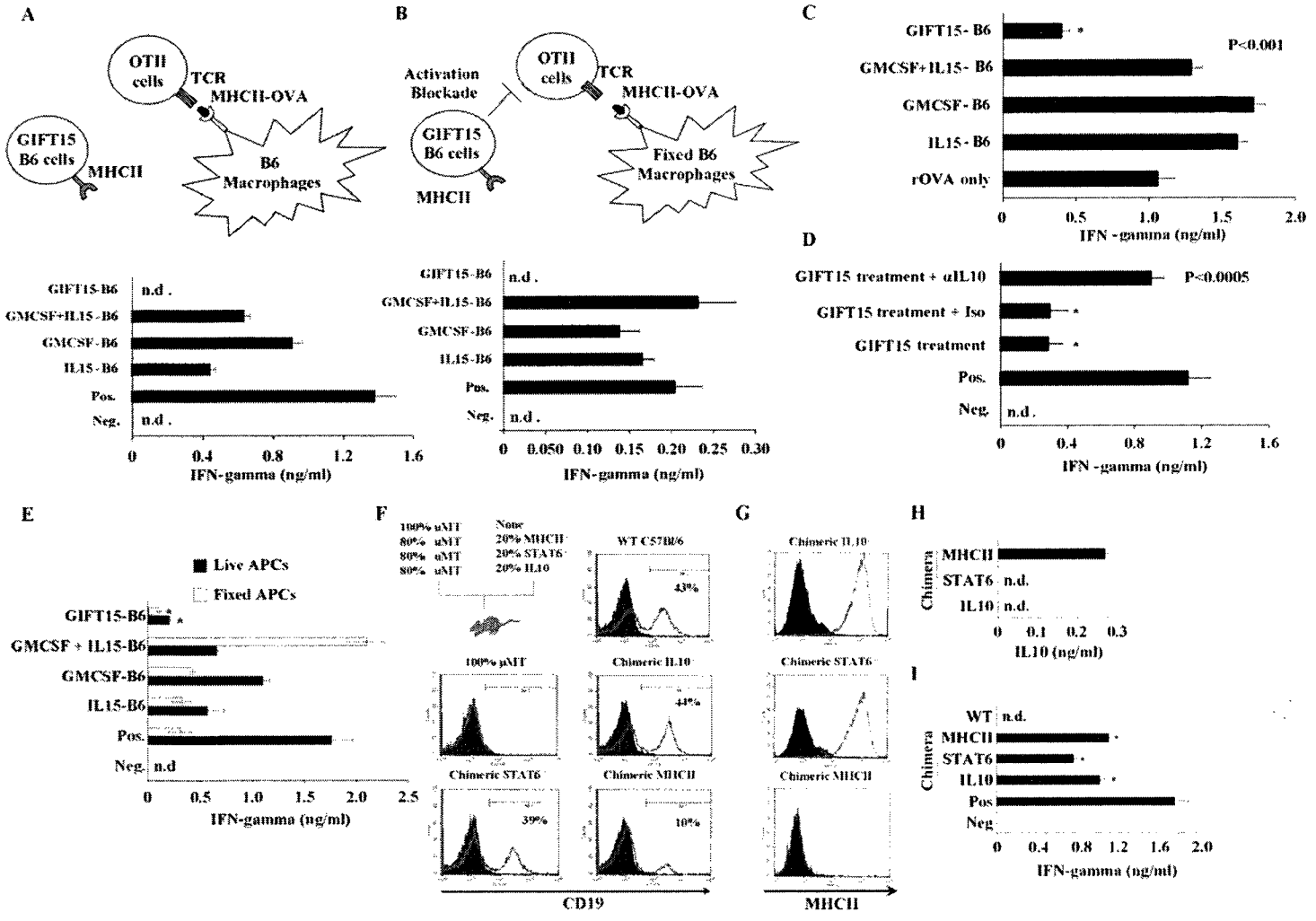

A
IFN-gamma (ng/ml)
P<0.05
n.d.
0
LPS
Naive
GIFT15 Regs
IL12 (ng/ml)
IL10 (ng/ml)
IL2 (ng/ml)
TNF-alpha (ng/ml)
B
B6 GIFT15 Regs
BALB/c Cells
GIFT15
Both
GMCSF
IL15
Neg
MLR
INF-gamma (ng/ml)
C
MHCII -/-
STAT6 -/-
MHCII
*P<0.00009
Naïve
IL10 (ng/ml)
D
STAT6 KO
MHCII KO
GIFT15
Positive
Negative
IFN-gamma (ng/ml)

GIFT15 (**Figure 15C**) and were tested using the same experimental design as in panel (B). While GIFT15-treated wild-type (WT) splenocytes completely abrogated IFNγ production, GIFT15-treated splenocytes derived from $MHCII^{-/-}$ or $STAT6^{-/-}$ mice were permissive for partial alloantigen reactivity suggesting that both elements – MHCII & IL10 - are required by GIFT15-treated splenocytes to block IFNγ secretion from alloreactive lymphoid cells (**Figure 15D**).

3.3.4 GIFT15-treated splenocytes suppress antigen-driven immune responses.

To test whether GIFT15-treated splenocytes can suppress a syngeneic antigen-driven immune response, they were added as third party cells to an *in vitro* antigen stimulation assay. B6 peritoneal macrophages pulsed with recombinant chicken ovalbumin (rOVA) present OVA-derived peptides in the context of MHCII and served here as APCs. As responder cells, we added naïve T-cells from TCR-transgenic OTII mice. When primed with APCs, OTII CD4 T-cells secrete IFNγ in an OVA/MHCII-specific manner. Naïve GIFT15-treated splenocytes derived from normal B6 mice were added as third party cells (**Schema Figure 16A**). Compared to controls, we found that GIFT15-treated splenocytes completely blocked IFNγ production by responder OTII splenocytes (**Figure 16A**). More specifically, this blockade occurred at the level of responding OTII T-cells since assays using OVA-pulsed fixed macrophages as stimulators (dead cells with no capacity to metabolically respond to cellular interactions yet preserving stimulator function *in vitro*) generated identical results (**Figure 16B**).
We have shown in Figure 2 that GIFT15 treated splenocytes produced substantial amounts of IL10 which in turn can suppress a MLR. We queried whether IL10 in this system can also modulate an antigen-driven syngeneic immune response. To test this hypothesis, B6 splenocytes were treated with GIFT15 for 96 hrs, removed, and conditioned media (CM) collected in the subsequent 48-72 hrs from the previously GIFT15 treated cells. The CM was substituted for third party cells in the culture system depicted in Figure 4A. A partial but significant inhibition of IFNγ production was noted (**Figure 16C**). Adding an IL10 neutralizing antibody restored IFNγ production by responder cells (**Figure 16D**). These data confirm that GIFT15-treated splenocytes can

Figure 16: GIFT15 Regs Bystander Inhibition. (**A**) GIFT15 Regs block IFNγ production by antigen stimulated OTII cells. rOVA-induced production of IFNγ by responding OTII T-cells was completely blocked when GIFT15 regs were added as third-party cells. (**B**) Same set-up as in (A) but with fixed APCs. (**C**) Soluble factor(s) contribute to IFNγ inhibition. Using CM 4 days following washed cytokine treated cells on the antigen presentation assay, a significant decrease in IFNγ was obtained using the GIFT15 regs. (**D**) IL10 is the only soluble inhibitory factor induced by GIFT15 regs. IL10 neutralizing antibodies were added to the CM using the antigen presentation assay. (**E**) $IL10^{-/-}$-derived splenocytes lose most of their suppressive capacities. $IL10^{-/-}$ were used as in panel (A) with live or fixed macrophages. Complete IFNγ inhibition was not achieved using these deficient cells clearly demonstrating its necessity. (**F**) Generation of bone marrow chimeric mice with specific targeting in B-cells. Generated chimeric mice generate normal B-cells except in the $MHCII^{-/-}$ group. (**G**) MHCII up-regulation in chimeric mice following GIFT15 treatment. Only $MHCII^{-/-}$ mice were not able to up-regulate MHCII upon GIFT15 treatment as opposed to $IL10^{-/-}$, $STAT6^{-/-}$-purified B-cells. (**H**) IL10 up-regulation in chimeric mice following GIFT15 treatment. Both $IL10^{-/-}$, $STAT6^{-/-}$-purified B-cells were incapable of secreting IL10 upon GIFT15 treatment. (**I**) Chimeric Bregs partially block IFNγ during antigen presentation as a bystander cell. When compared to WT Bregs, chimeric GIFT15-treated B-cells were incapable of leading to complete suppression of IFNγ (n=5/group; data are shown as mean ± SD).


A
OTII cells
TCR
MHCII-OVA
GIFT15 B6 cells
MHCII
B6 Macrophages
B
Activation Blockade
OTII cells
TCR
MHCII-OVA
GIFT15 B6 cells
MHCII
Fixed B6 Macrophages
C
GIFT15- B6
GMCSF+IL15- B6
GMCSF- B6
IL15- B6
rOVA only
P<0.001
IFN -gamma (ng/ml)
GIFT15-B6 n.d.
GMCSF+IL15-B6
GMCSF-B6
IL15-B6
Pos.
Neg. n.d.
IFN-gamma (ng/ml)
GIFT15-B6 n.d.
GMCSF+IL15-B6
GMCSF-B6
IL15-B6
Pos.
Neg. n.d.
IFN-gamma (ng/ml)
D
GIFT15 treatment + αIL10
GIFT15 treatment + Iso
GIFT15 treatment
Pos.
Neg. n.d.
P<0.0005
IFN -gamma (ng/ml)
E
Live APCs
Fixed APCs
GIFT15-B6
GMCSF + IL15-B6
GMCSF-B6
IL15-B6
Pos.
Neg. n.d
IFN-gamma (ng/ml)
F
100% μMT
80% μMT 20% MHCII
80% μMT 20% STAT6
80% μMT 20% IL10
WT C57Bl/6
43%
100% μMT
Chimeric IL10
44%
Chimeric STAT6
39%
Chimeric MHCII
10%
CD19
G
Chimeric IL10
Chimeric STAT6
Chimeric MHCII
MHCII
H
Chimera
MHCII
STAT6 n.d.
IL10 n.d.
IL10 (ng/ml)
I
Chimera
WT n.d.
MHCII
STAT6
IL10
Pos
Neg
IFN-gamma (ng/ml)

suppress an Ag-specific T-cell response in a paracrine IL10-dependent manner independently of co-existent contact suppression. To further confirm the importance of IL10 in the inhibition of antigen-driven immune responses, splenocytes derived from $IL10^{-/-}$ mice were treated with GIFT15, then stimulated as previously described with live or fixed macrophages. We noticed that $IL10^{-/-}$ splenocytes provided partial suppression as opposed to WT GIFT15-treated cells (**Figure 16E**). Thus, we can conclude that both IL10 and MHCII are required to achieve full immunosuppression. We then asked whether lack of IL10, STAT6 and MHCII affected Bregs in a cell-autonomous manner or by altering B-cell environment. To ensure that MHCII or STAT6 is defective exclusively in B cells while overcoming the possibility that B-cells isolated from STAT6 or MHC II are defective from start, due to "generational" problems, we created mixed hematopoietic chimeras by injecting to irradiated WT B6 mice a cell inoculum containing 80% μMT bone marrow cells (B-cell deficient) and 20% bone marrow cells from $MHCII^{-/-}$, $STAT6^{-/-}$, or $IL10^{-/-}$ mice. As expected, the frequency of B-cells obtained was comparable to that of WT B6 mice expect for recipients of $MHCII^{-/-}$ cells where the percentage of generated B-cells was decreased (10% vs 39-43% in other groups) (**Figure 16F**). In contrast to $MHCII^{-/-}$ B-cells, purified $IL10^{-/-}$ and $STAT6^{-/-}$ B-cells up-regulated MHCII upon GIFT15 treatment (**Figure 16G**). Furthermore, IL10 was present in the supernatant of $MHCII^{-/-}$ B-cells but could not be detected in the supernatant of $IL10^{-/-}$and $STAT6^{-/-}$ B-cells (**Figure 16H**). To further confirm our antigen presentation assays, B-cells from mixed chimeras were treated with GIFT15 and added at a 1:1 ratio to responding T-cells. Only partial IFNγ inhibition was obtained (**Figure 16I**) confirming that MHCII, STAT6 and IL10 have a direct and B-cell autonomous role in the suppressor function of GIFT15-induced Bregs.

3.3.5 GIFT15-treated splenocytes suppression of EAE is dependent upon STAT6 and MHCII.

Considering the potent inhibitory effects of GIFT15-treated splenocytes *in vitro*, we investigated the therapeutic effects of these cells *in vivo* in the context of EAE. *In vitro* antigen presentation assays using myelin-oligodendrocyte-glycoprotein (MOG)-pulsed

B6-derived macrophages as APCs and as responders, T-cells collected from mice with symptomatic EAE, demonstrated that third party GIFT15-treated B6 splenocytes were capable of robustly decreasing T-cell reactivation (**Figure 17A**). Such effects correlated very well with *in vivo* studies in which B6 mice suffering from EAE completely recovered following intravenous (IV) injection of 2 million GIFT15-treated splenocytes (**Figure 17B**). In a separate set of experiments, spinal cords and brains derived from EAE following IV administration of GIFT15-treated splenocytes were analyzed for content of pathogenic myeloid and CD4 T-cells. Both cell types were significantly reduced in number compared with untreated EAE controls (**Figure 17C**). To analyse the effect of GIFT15-treated splenocytes on lymphoid cells causative of EAE, we harvested splenocytes of EAE mice that had undergone cell therapy. Following *in vitro* stimulation with MOG_{35-55} peptide, these splenocytes proliferated weakly and showed decreased IFNγ response and enhanced production of IL10 relative to controls (**Figure 17D**). GIFT15-treated splenocytes derived from MHC-mismatched BALB/c mice failed to reverse EAE in B6 mice (**Figure 17E**) even though they decreased by about 50% *in vitro* T-cell proliferation induced by MOG_{35-55} (**Figure 17D**). Since we have previously demonstrated that the *in vitro* suppressor function of GIFT15-treated splenocytes depends upon MHCII and STAT6, we assessed the *in vivo* contribution of these effector pathways by treating EAE B6 mice with GIFT15-treated splenocytes derived from $MHCII^{-/-}$ (**Figure 17F**) or $STAT6^{-/-}$ (**Figure 17G**). In both cases, the intravenous injection of 2 million GIFT15-treated splenocytes had no effect on the clinical progression of symptomatic EAE suggesting that both MHCII and STAT6 (and downstream IL10 production) were required to suppress EAE *in vivo*.

3.3.6 Purified GIFT15 Bregs are highly efficient in reversing EAE.

While using unfractionated splenocytes for the generation of GIFT15 Regs, IL10 was demonstrated as being the only detectable secreted soluble factor with known suppressor function. Therefore, IL10 secretion was monitored following the treatment of purified splenic naïve B-cells with GIFT15. We found that all secreted IL10 was of a B-cell origin (**Figure 18A**) and only GIFT15 Bregs were capable of completely blocking

Figure 17: Therapeutic Effects of GIFT15 Regs on EAE. (**A**) GIFT15 Regs can block IFNγ from pathogenic EAE T-cells. MOG_{35-55}-specific T-cells were enriched from EAE mice then stimulated in the presence of APCs presenting the MOG_{35-55} peptide and GIFT15 Regs. A robust decrease of IFNγ was obtained (Data are shown as mean ± SD). (**B**) GIFT15 Regs can completely reverse EAE pathology. B6 mice immunized with the MOG_{35-55} peptide developed EAE but their disease score was quickly reversed back to 0 upon IV administration of $2X10^6$ GIFT15 Regs. (**C**) GIFT15 Regs Block the Infiltration of macrophages and T-cells in the CNS. Spinal cords and brains of EAE mice treated with GIFT15 Regs were isolated and used to generate single-cell suspension for FACS analysis. GIFT15 Regs treated EAE mice had less infiltrating CD4CD45 or CD11bCD45 positive cells when compared to the PBS control group (n=5/group; data are shown as mean ± SD). (**D**) GIFT15 Regs treated cells and *in vitro* MOG re-stimulation (Left panel). Splenocytes isolated from treated EAE mice were stimulated *in vitro* with 5 ug of MOG_{35-55} then analyzed by MTT for their proliferative ability. Splenocytes-derived from GIFT15 Regs lost most of their ability to proliferate in response to MOG_{35-55} re-stimulation. ELISA analysis of IFNγ and IL10 (right panel). Supernatant collected from the same experiment were analyzed by ELISA for IFNγ and IL10 secretion. A significant reduction in IFNγ levels was detected in the GIFT15 group along with higher IL10 induction when compared to control mice (data are shown as mean ± SD). (**E**) Allogeneic GIFT15 Regs failed in reversing EAE. Repeating the same treatment of EAE mice but using MHC mismatched BALB/c-derived splenocytes failed in leading to a therapeutic effect in B6 EAE mice despite the substantial inhibition observed *in vitro* during the antigen presentation assay (n=5/group; data are shown as mean ± SD). (**F**) Reversing EAE pathology is MHCII-dependent. $MHCII^{-/-}$ GIFT15 Regs did not show any therapeutic potential when delivered to EAE mice, whereas an *in vitro* inhibition of IFNγ during a MOG APC assay was observed. (n=5/group; data are shown as mean ± SD). (**G**) STAT6 is required along with MHCII to treat EAE. The same outcome as in (F) occurred using the $STAT6^{-/-}$ GIFT15 Regs. However no detectable inhibition was obtained *in vitro* during the APC assay (n=5/group; data are shown as mean ± SD).

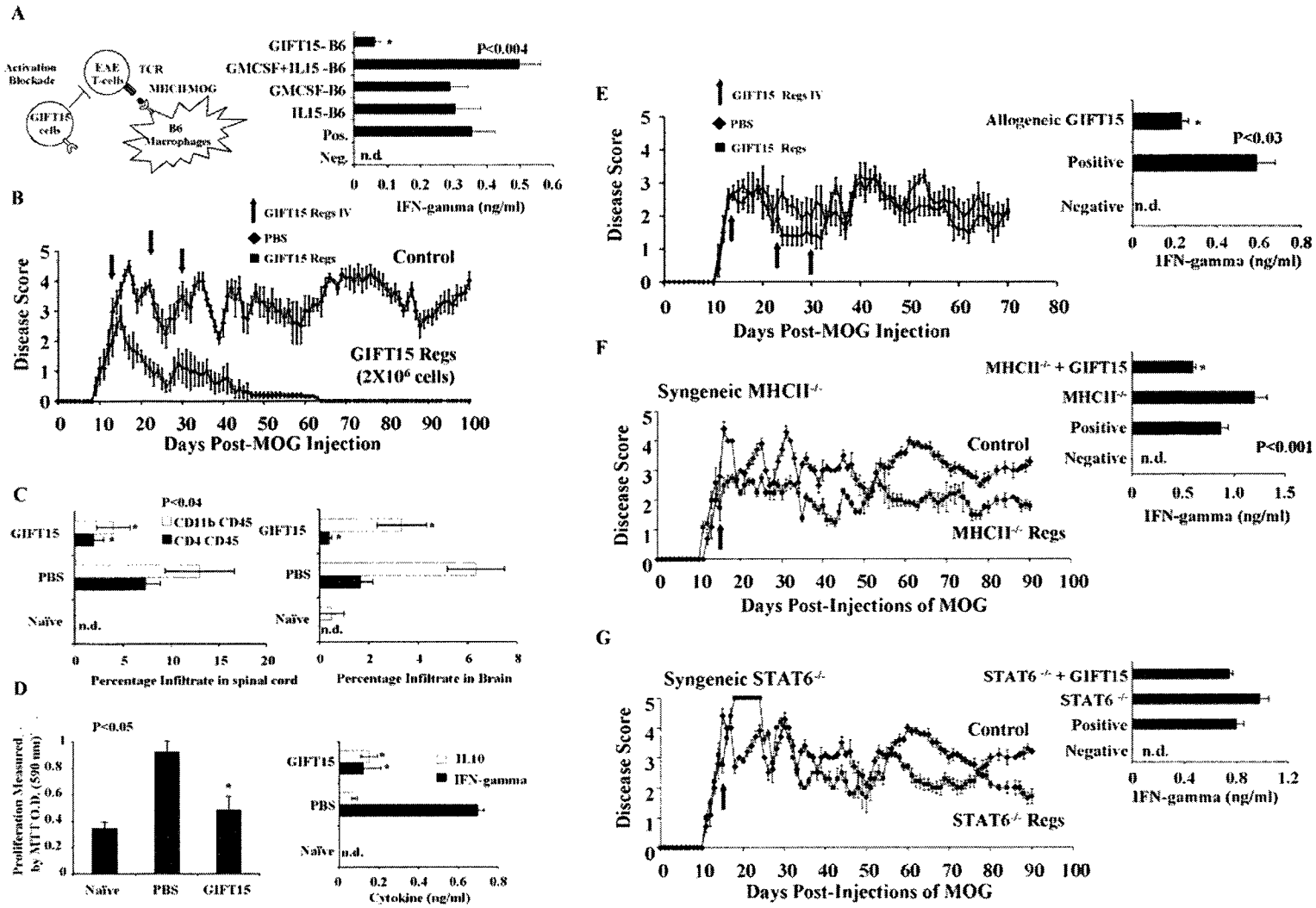
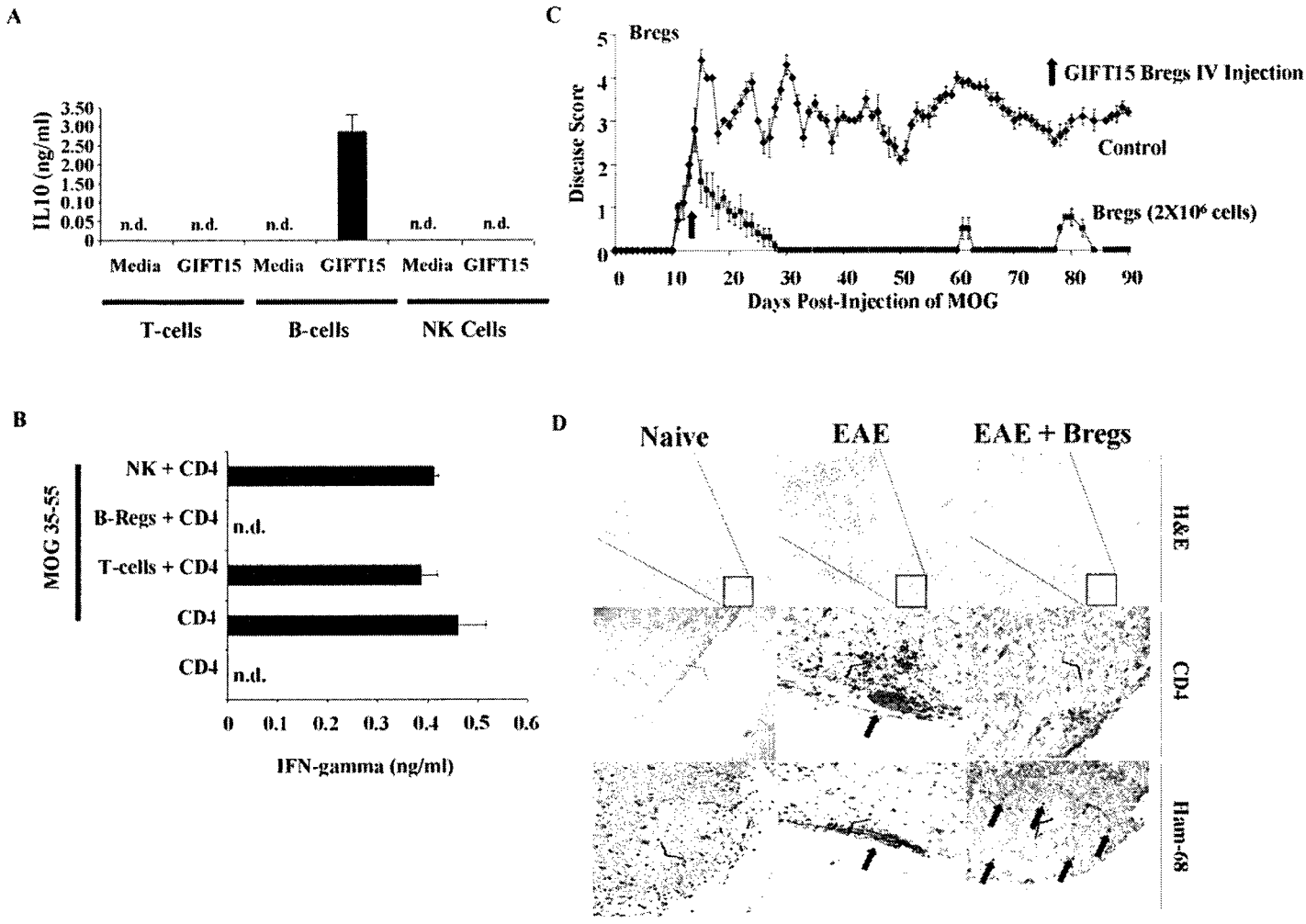

A
Activation Blockade
EAE T-cells
TCR
MHCIIMOG
GIFT15 cells
B6 Macrophages
GIFT15- B6
GMCSF+IL15 -B6
GMCSF-B6
IL15-B6
Pos.
Neg.
P<0.004
n.d.
IFN-gamma (ng/ml)
B
GIFT15 Regs IV
PBS
GIFT15 Regs
Control
GIFT15 Regs (2X10⁶ cells)
Disease Score
Days Post-MOG Injection
C
P<0.04
CD11b CD45
CD4 CD45
GIFT15
PBS
Naïve
n.d.
Percentage Infiltrate in spinal cord
Percentage Infiltrate in Brain
D
P<0.05
Proliferation Measured by MTT O.D. (590 nm)
Naïve
PBS
GIFT15
IL10
IFN-gamma
Cytokine (ng/ml)
E
GIFT15 Regs IV
PBS
GIFT15 Regs
Disease Score
Days Post-MOG Injection
Allogeneic GIFT15
Positive
Negative
P<0.03
n.d.
IFN-gamma (ng/ml)
F
Syngeneic MHCII-/-
Control
MHCII-/- Regs
Disease Score
Days Post-Injections of MOG
MHCII-/- + GIFT15
MHCII-/-
Positive
Negative
P<0.001
n.d.
IFN-gamma (ng/ml)
G
Syngeneic STAT6-/-
Control
STAT6-/- Regs
Disease Score
Days Post-Injections of MOG
STAT6-/- + GIFT15
STAT6-/-
Positive
Negative
n.d.
IFN-gamma (ng/ml)

Figure 18: Bregs are Highly Efficient In Reversing EAE.

(**A**) Only purified B-cells treated with GIFT15 secrete IL10. IL10 ELISA performed using the CM of T, B or NK cells treated with GIFT15 demonstrate that only Bregs produce IL10 (n = 5/group; data are shown as mean ± SD). (**B**) Bregs completely block IFNγ during MOG_{35-55} APC assay. Using peritoneal macrophages presenting the MOG_{35-55} peptide, T, B or NK cells treated with GIFT15 were co-cultured in a 1:1 ratio with EAE derived T-cells and IFNγ assessed by ELISA. Only Bregs were capable of completely blocking IFNγ secretion (n=5/group; data are shown as mean ± SD). (**C**) Bregs completely reverse EAE pathology. GIFT15 induced Bregs or PBS was injected in EAE mice and their disease score was monitored over time. A faster recovery was observed as compared to previously generated data (Figure 4B) using GIFT15 Regs. (**D**) Spinal cord histopathology. Spinal cords of naïve, untreated or treated EAE mice were stained for H&E, CD4 or Ham-69 (macrophage marker). Less CD4 and almost no detectable macrophages could be detected in GIFT15 Bregs treated EAE mice.


A
IL10 (ng/ml)
3.50
3.00
2.50
2.00
1.50
0.10
0.05
0
n.d.
n.d.
n.d.
n.d.
n.d.
Media
GIFT15
Media
GIFT15
Media
GIFT15
T-cells
B-cells
NK Cells
C
Bregs
GIFT15 Bregs IV Injection
Control
Bregs (2X10⁶ cells)
Disease Score
Days Post-Injection of MOG
B
MOG 35-55
NK + CD4
B-Regs + CD4
T-cells + CD4
CD4
CD4
n.d.
n.d.
IFN-gamma (ng/ml)
D
Naive
EAE
EAE + Bregs
H&E
CD4
Ham-68

IFNγ secretion during an antigen presentation assay using the MOG peptide (**Figure 18B**). To verify whether the entire therapeutic effect seen with the use of unfractionated GIFT15-treated splenocytes was due to the activity of cells of B-cell ontogeny, EAE mice were injected with 2 million GIFT15 Bregs derived from preselected naïve B-cells and disease score was followed over time. The kinetics of clinical remission was significantly accelerated compared to the use of unfractionated GIFT15-treated splenocytes (**Figure 18C**) and this correlated with improved neuro-pathological findings as well (**Figure 18D**).

3.4 DISCUSSION

We have demonstrated that treatment of murine splenocytes with the immunosuppressive fusokine GIFT15 leads to the generation of a novel regulatory population of B-cell lineage capable of completely blocking and reversing EAE pathology on a long-term basis in mice. This therapeutic property of GIFT15 Bregs is lost if both MHCII and STAT6 (and secondarily IL10) are absent.

Recent data suggest that naturally occurring Bregs exist and are capable of suppressing inflammation while enhancing tolerance [287-289]. Breg-mediated suppression appears to be independent from the quintessential B-cell function, antibody production [290]. More specifically, IL10-producing B-cells were previously shown to suppress the pathology seen in arthritis as well as during chronic intestinal inflammation [291, 292]. Furthermore, preliminary evidence demonstrated that in humans suffering from MS, a certain subset of B-cells secrete low levels of IL10, which is known to limit the production of pro-inflammatory cytokines such as IFNγ thereby restraining Th1 differentiation and disease exacerbation [293]. In agreement with these findings, we were able to show in this study using antigen presentation assays with both OVA and MOG peptide that GIFT15 Bregs are capable of robustly inhibiting IFNγ secretion as bystander third party cells. Notably, GIFT15-induced Bregs from $STAT6^{-/-}$ mice, which cannot produce Th2 cytokines, displayed reduced albeit significant suppressive ability. Thus, the suppressive ability of GIFT15-induced Bregs depends partly but not entirely on STAT6 and IL10.

Based on our *in vitro* data using macrophages as APCs, we have demonstrated that MHCII expression by GIFT15 Bregs is required in part to mediate suppression on responding T-cells. Our *in vivo* data using MHCII$^{-/-}$ GIFT15 Bregs also demonstrate the pivotal role of MHCII expression for suppressor function. It has been shown by Lau *et al* that IL10 stimulated dendritic cells recruit and activate suppressor Tregs via MHCII-mediated antigen presentation [294]. However, our *in vitro* experimental work demonstrates that GIFT15 Bregs can suppress T-cell response in an antigen independent manner, which suggests a distinct role for MHCII is operative for GIFT15 Bregs. We cannot rule out the possibility that GIFT15 Bregs administered *in vivo* preserve the capacity to sample and present exogenous antigen as part of their suppressor function akin to that described for IL10 DCs. Though we unambiguously show the importance of MHCII in GIFT15 Breg function, understanding the mechanistic underpinnings of this observation cannot be answered in full by published art and will require further investigation. As an aggregate, our work strongly suggests that GIFT15 Bregs inhibit T-cell responses via both paracrine IL10 and cell contact mechanism dependent on MHCII.

Based on our observation that GIFT15 Regs are of B-cell lineage, close attention was given to their phenotype. In general, B-cells can be subdivided into two categories depending on their distribution pattern in the host. B1 cells are known to be localized in the peritoneal and pleural cavities, whereas B2 cells are mainly found in secondary lymphoid organs such as the spleen [295, 296]. GIFT15 Bregs were induced *in vitro* using spleen-derived naïve B-cells, and their acquired phenotype (CD21+, CD23+, IgM+, CD1d+, IgD+) is consistent with a transitional 2 marginal zone B-cell[20]. In addition, B-cells respond to numerous stimuli that regulate their response to antigen or stimulation leading either to the generation of a humoral response or the maintenance of tolerance [297]. Amongst all cell surface markers, CD19, CD21 and CD45 have been identified as positive regulators that augment signals through the B-cell receptor (BCR) [298]. Interestingly, CD19 overexpression on B-cells show augmented proliferation with higher capacity to produce autoantibodies while CD19$^{-/-}$ mice exhibit reduced proliferation compared to WT B-cells [298]. Thus, the capacity of GIFT15 to completely downregulate cell surface CD19 expression, while expressing MHCII and secreting IL10, dovetails well with phenotypic and functional features of Bregs in other models. On the

other hand, CD22 is considered a negative regulator of BCR signaling [298]. That our GIFT15 Bregs are $CD19^{-}$ but $CD22^{hi}$ suggests that these cells are incapable of responding to an antigen in addition to their remarkable ability to suppress T-cell activation. An additional marker has been closely associated with Bregs phenotype: CD1d [299]. This MHCI-like protein, usually presenting lipids to NKT-cells, is considered to play an important role in the control of autoimmune diseases such as colitis, as emphasized by the development of severe intestinal inflammation in $CD1d^{-/-}$ mice [299]. However, CD1d does not seem to be of great importance in our Breg model for 2 main reasons. First, it was previously reported that CD1d absence does not exacerbate EAE [299], suggesting that it might be a disease-specific marker, and second, CD1d ligation triggers IL10 secretion, a process induced in our case only upon GIFT15 treatment. Nevertheless, additional markers have been claimed to distinguish four major Breg subsets [140, 300]. For example, the expression of B7 costimulatory molecules such as CD80/CD86 [140] or the direct interaction between CD40 and CD154 [301, 302] was shown to be crucial for the development of IL10-secreting Bregs. Our Breg population is distinct from the previously reported groups due to the absence of CD80/86, CD19, and CD40 ligand since GIFT15-induced Bregs were generated without the use of anti-CD40 antibodies or expressing CD154. In addition, the notion of CD154 being implicated in alleviating autoimmunity is difficult to conciliate with the previously well established principle that tolerogenic effects are induced with the use of blocking anti-CD154 monoclonal antibody in arthritis and EAE [301, 302]. We must recognize that the phenotype of our GIFT15 Bregs is indeed quite similar to that of Bregs described by Mizoguchi *et al.* [292]. One of the main distinguishing features is that our B-suppressor cells lose expression of CD19. We feel this distinctive feature is meaningful enough that we cannot claim to have derived the same cells as those described by Mizoguchi *et al.* as are naturally found in a normal host [292]. Indeed, it has been demonstrated that B-cells from CD19-deficient mice exhibited markedly decreased proliferative responses to mitogens, and serum immunoglobulin levels were also significantly decreased [298]. Considering its important role in B-cell immunobiology, we feel that we cannot trivialize CD19 expression loss by GIFT15 Bregs. It is apparent that the suppressor effect of our GIFT15 Bregs does not require CD19 function or activity, but the same statement cannot be

unilaterally made of Bregs described by other groups [288, 289, 292]. Hence, we feel it more cautious to claim that GIFT15 Bregs are a distinct type of B-suppressor cells than those previously described, though they may be very closely related otherwise, in particular to transitional 2-marginal zone Bregs.

In summary, B-cell deficiency was previously reported to enhance EAE pathology, whereas IL10-producing B-cells expressing MHCII have been suggested to represent a powerful regulatory cellular population capable of inhibiting autoimmune pathologies [303, 304]. However, this naturally occurring B-cell subset is found in very limited amounts *in vivo*, restricting therefore the possibility of their isolation and use in clinic. We have demonstrated in this study that GIFT15 treatment of naïve B-cells can lead to the acquisition of a regulatory phenotype capable of robustly inhibiting EAE progression and to induce complete recovery from EAE signs in mice. Such an approach could be directly validated using the human homologue of GIFT15 for the adaptation of a cellular therapy protocol using autologous GIFT15-treated blood B-cells in humans suffering from MS or other autoimmune ailments.

3.5 METHODS

3.5.1 Animals, proteins, antibodies, and cytokine ELISAs.

WT, MHCII$^{-/-}$, STAT6$^{-/-}$, IL10$^{-/-}$, μMT, and retired breeder C57Bl/6 (B6) mice were all age-matched and purchased from Jackson laboratories (Bar Harbor, ME). Reagents such as rGMCSF, rIL15 or their appropriate antibodies, as well as cytokine ELISAs were purchased from R&D System (**Minneapolis, MN**) and used according to manufacturer's instructions. Pure chicken rOVA and pertussis toxin were purchased from Sigma-Aldrich (Oakville, ON, Canada). All antibodies used in flow cytometry were purchased from Pharmingen (San Diego, CA) and used according to manufacture's instructions. CD3e, CD19 or NK1.1 cellular enrichment kits were purchased from StemCell Technologies (Vancouver, BC, Canada). The MOG_{35-55} peptide was obtained from the Sheldon Biotech Centre (McGill University, Montreal, Canada). Complete Freund's Adjuvant from Cedarlane (Montreal, Qc, Canada) and Mycobacterium

tuberculosis H35RA from Difco Laboratories (Detroit, Michigan, USA) were used according to manufacturer's instructions. All flow cytometry and histology antibodies were obtained from Pharmingen (Mississauga, ON, Canada).

3.5.2 Generation and characterization of GIFT15 regulatory cells.

Whole splenocytes population of C57Bl/6 mice were harvested and placed in culture in complete splenocytes media (RPMI, 10% FBS, 5mM L-glutamine, 10 mM sodium pyruvate, and 25 mM Beta-mercaptoethanol) supplemented with the different cytokine conditions (10 pmols for every treatment). Six to eight days later, cells were stained for markers CD3e, CD8, CD4, NK1.1, MHCI, MHCII, CD11b, CD11c, and Gr1. CM collected from treated splenocytes derived from WT, $MHCII^{-/-}$, or $STAT6^{-/-}$ was also assessed for IL10 by ELISA according to manufacturer's instructions. For *in vitro* proliferation assays, splenocytes were labelled with 5ug/ml CFSE for 8-10 min at 37°C and then washed once with complete medium and three times with PBS. To assess the proliferation induces by the fusokine over time, cells were treated with GIFT15 and analysed daily by flow cytometry.

3.5.3 GIFT15 Bregs lineage identification.

To identify the population responding to GIFT15, CD3e, CD19 or NK1.1 positive cells were purified using the StemCell purification kits then treated with GIFT15 for 6-8 days before assessing their cellular markers versus MHCII. The expression of CD19 was assessed by flow cytometry 2 or 4 days following the removal of GIFT15 from the Bregs population. Further phenotypic analysis of the Bregs was performed on purified B-cells free of CD11b, CD11c, or Gr1 positive cells and analyzed by flow cytometry for surface expression of CD21, CD22, CD23, CD24, CD27, CD43, CD79b, IgD, IgM, CD1d, AA4.1 and CD45b/B220.

3.5.4 Biochemical response of GIFT15 regulatory cells.

Naïve or GIFT15 regulatory cells were probed for IFNγ, IL2, IL12, TNF-alpha, and IL10 by ELISA before and after LPS stimulation (10 ng/ml). For the 2 way MLR, 10^5 of different cytokine treated cells derived from WT, MHCII$^{-/-}$, or STAT6$^{-/-}$ B6 mice were co-cultured with BALB/c splenocytes and IFNγ probed by ELISA.

3.5.5 Generation of mixed bone marrow-chimera mice.

Naïve recipient B6 mice received 6.5 Gray of γ-irradiation via a cesium source followed by a bone marrow transplant 24 hrs later. In order to specifically target the MHCII, STAT6 and IL10 deficiency to B-cells, the $3X10^6$ bone marrow cell inoculum consisted of 80% μMT bone marrow (deficient in B-cells) and 20% bone marrow from MHCII$^{-/-}$, STAT6$^{-/-}$, or IL10$^{-/-}$ mice. As control for irradiation and re-constitution, 100% μMT bone marrow was transplanted in irradiated B6 mice. Seven weeks post-transplantation, mice were sacrificed and CD19 staining was performed by flow cytometry on splenocytes. All mice, except for the MHCII$^{-/-}$, showed similar percentage of CD19 positive cells relative to control naïve B6 mouse.

3.5.6 Antigen presentation assays.

APC assays were performed using peritoneal macrophages collected from retired B6 breeder mice. Adherent macrophages were treated with rOVA or MOG_{35-55} peptide for 24 hrs, then washed before the addition of i) responder T-cells: OVA-specific OTII CD4 T-cells at a 1:1 ratio, and ii) regulatory cells: WT, MHCII$^{-/-}$, STAT6$^{-/-}$ or IL10$^{-/-}$ B6 splenocytes previously treated with different cytokines. Supernatants were collected and centrifuged 72 hrs later for IFNγ analysis by ELISA. The same assay was repeated using paraformaldehyde-fixed macrophages to identify the cell targeted by the GIFT15-induced regulatory cells. Additional APC assays were performed where regulatory cells were replaced by culture supernatant collected from cytokine treated cells, with or without IL10 neutralizing antibody. Splenocytes from mixed hematopoietic chimeras were also

tested to discriminate cell-intrinsic from bystander effects. Secretion of IL10 and IFNγ-following antigen presentation were also assessed using purified Bregs.

3.5.7 EAE induction and analysis.

Purified synthetic MOG_{35-55} peptide at 1mg/ml was emulsified (1:1 volume ratio) in Complete Freund's Adjuvant containing 4mg/ml Mycobacterium tuberculosis H35RA and injected subcutaneously (sc) at the base of the tail (50ul/side). Animals also received pertussis toxin immediately after the sc injection (300 ng in 0.2 ml saline for a 20g mouse, eg. 0.015 mg/kg) by IP injections, repeated two days later. Mice were clinically scored every 2 days as follows: 0, no disease; 1, floppy tail; 2, hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund stage. Following the appearance of EAE symptoms, treatment was administered when the mean EAE grade reached a score of 2. Then, mice received IV injections of PBS or GIFT15 treated splenocytes derived from WT, $MHCII^{-/-}$, or $STAT6^{-/-}$ mice or Bregs from WT B6 mice ($2X10^6$cells per injection).

3.5.8 Immune infiltrate analysis and *in vitro* responses.

For EAE analysis, spinal cords and brains of EAE mice were isolated and mechanically disrupted in splenocyte culture medium in order to generate single cell suspension. After washing and blocking Fc receptors, cells were stained for CD11b or CD4 versus CD45 for flow cytometry analysis. In addition, splenocytes were isolated from the same mice, disrupted mechanically to generate a single cells suspension for *in vitro* re-stimulated with MOG (10ug/ml) to assess proliferation by MTT and IFNγ and IL10 secretion by ELISA.

3.5.9 Spinal cord histology.

EAE mice were perfused with 20 ml PBS before the removal of their spinal cord. For histological analysis, spinal cords were fixed in 10% formalin before H&E staining or Ham-68 (for macrophages) and CD4 immunohistology.

3.5.10 Statistical analysis.

P values were calculated by paired Student *t*-test.

3.6 AKNOWLEDGMENTS

Moutih Rafei is a recipient of a *Fonds de Recherches en Santé du Québec (FRSQ)* Scholarship and Jacques Galipeau is a *FRSQ chercheur-boursier sénior*. Claude Perreault holds a Canada Research Chair in Immunobiology. This work was supported by the Canadian Institute of Health Research grant MOP-15017.

CHAPTER 4

Mesenchymal Stromal Cell Derived CCL2 Suppresses Plasma Cell Immunoglobulin Production via STAT3 Inactivation and PAX5 Induction.

Reference : Rafei M, Hsieh J, Fortier S, Li M, Yuan S, Birman E, Forner K, Boivin MN, Doody K, Tremblay M, Annabi B, Galipeau J. Mesenchymal stromal cell derived CCL2 suppresses plasma cell immunoglobulin production via STAT3 inactivation and PAX5 induction. Blood 2008 112(13):4991-4998.

Preface to Chapter 4:

We showed in Chapter 2 and 3 that the bio-engineering of a chimeric fusion protein between GMCSF and IL15 leads to drastic immunosuppression as demonstrated in blocking rejection of allogeneic/xenogeneic cells or inflammation during autoimmunity. Based on these observations, we wished to study the mechanism by which other potential soluble factors, which could be used as part of a novel fusion protein, behave when naturally delivered from bone marrow-derived MSCs. In the next chapter, we tested the hypothesis that primary MSCs could modulate antibody production via paracrine conversion of secreted CCL2 to an antagonist form.

CHAPTER 4: Mesenchymal Stromal Cell Derived CCL2 Suppresses Plasma Cell Immunoglobulin Production via STAT3 Inactivation and PAX5 Induction.

4.1 ABSTRACT

We demonstrate that the secretome of mesenchymal stromal cells (MSCs) suppresses plasma cell (PC) immunoglobulin production, induces plasmablast proliferation, and leads to IL10–mediated blockade *in vitro*. We found that these effects are due to MSC-derived CC-chemokines CCL2 and CCL7. More specifically, we found that MSCs further processed these CC chemokines by the activity of matrix metalloproteinases (MMPs) leading to the generation of proteolytically processed antagonistic CCL2 variant. Neutralizing CCL2 or inhibiting MMP enzymatic activity abolished the PC suppressive effect of MSCs. We also observed that MMP-processed CCL2 suppresses STAT3 activation in PC. As a result, the transcription factor PAX5 is induced thus explaining the inhibition of immunoglobulin synthesis. The absence of inhibitory effects by MSC on the humoral response of $CCR2^{-/-}$ mice to xenoAg suggests that MMP-cleaved CCL2/CCR2 interaction as well as downstream phosphatase activity is necessary for antagonistic effect. We tested syngeneic MSCs in hemophilic B6 mice with pre-developed anti-human Factor VIII (hFVIII) antibodies and demonstrated a robust decrease in hFVIII-specific IgG levels. Thus, marrow resident MSCs may play a physiological role in modulating Ig production by PCs via MMP processing of paracrine CCL2 secretion and may represent an appealing cell therapy approach for pathological humoral responses.

4.2 INTRODUCTION

Mesenchymal stromal cells (MSCs) normally reside in marrow where they lay as pericytes and interstitial cells. MSCs have long been felt to play a supportive role in hematopoiesis and lymphopoiesis as part of the marrow microenvironmental niche and have been the object of more than quarter century of research as bone mesenchymal precursors [305]. The precise interaction between MSCs and hematopoietic cells remains

to be determined but appears to involve complex contact and paracrine factors which can be recapitulated *in vitro* by the stroma-dependent long term bone marrow culture hematopoietic assay [306]. More recently, it was discovered that MSCs possessed potent immune modulatory properties – both suppressive and stimulatory – which could be exploited clinically [165, 307, 308]. Indeed, MSCs can be harvested from a simple low volume aspirate, *ex vivo* expanded to large numbers with routine tissue culture techniques and subsequently readministered to either autologous or allogeneic recipients with clinical effects[5]. The use of *ex vivo* expanded MSCs in suppressing T-cell reactivity via secreted soluble factors opened a new field of interest in cellular immunosuppression [165, 309], well exemplified by the remarkable effects of allogeneic MSCs in attenuating the symptoms of steroid-resistant Graft versus Host Disease [310]. The immune suppressive effects of MSCs also may extend to pathological autoreactive lymphocytes, at least in animal models of autoimmune disease. Indeed, experimental autoimmune encephalomyelitis (EAE) was strikingly ameliorated in mice following injection of MSCs [311], and such pre-clinical results buttress the use of MSCs as a cell-based pharmaceutical in more than two dozen clinical trials worldwide for this and other autoimmune conditions and regenerative medicine applications (www.clinicaltrials.gov). However, the molecular mechanisms by which MSCs mediate their suppressive effect *in vivo* remains controversial. Most data generated to date with human MSCs was inferred from *in vitro* experimentation which have identified multiple, at time conflicting, candidate pathways [308].

MSCs have also been shown to suppress B cell dependent immunoglobulin production *in vitro* as well by unknown mechanisms [312]. Though it is believed that marrow resident MSCs are linked to lymphoid development, the underpinning molecular mechanism by which this relationship and modulatory effects are unknown. Recent *in vivo* studies performed in mice with experimental GVHD, identified a key role to MSC interferon gamma responsiveness and their ability to generate nitric oxide (NO) and speculated that supplementary components of the MSC secretome, including chemokines, were at play [313]. Studies performed on MSCs secretome revealed the presence of a wide range of cytokines, and chemokines including CCL2 [313]. Furthermore, MSCs are

also capable of producing an array of MMPs with the capacity of cleaving a variety of target molecules, including chemokines [223, 314, 315]. Such MMP processing, at least on CC chemokines, was demonstrated to convert the biochemical property of the targeted molecules from an agonist to an antagonist form [223]. Knowing that B cell development is dependent in part on close interaction of B cell progenitors with MSCs and that unidentified soluble factors produced by MSC can also suppress B cell activation, proliferation and differentiation to immunoglobulin (Ig)-producing cells *in vitro* [312], we hypothesize that MSCs may modulate Ig production by PCs as part of a physiological response to xenoAg or in the setting of pathological humoral responses. More specifically, we investigated the capacity of MSCs in modulating Ig production and PC biology both *in vitro* and *in vivo* by specifically studying CCL2 and its MMP-processed derivative that can target CCR2-expressing PCs. The observations here made could give insights on the physiological role of MSCs in marrow PC homeostais as well as in their properties when administered as an anti-inflammatory biopharmaceutical.

4.3 MATERIALS AND METHODS

4.3.1 Mice and Immunizations

WT and $CCR2^{-/-}$ B6 mice were all age-matched and purchased from Jackson laboratories (Bar Harbor, ME). Hemophilic B6 mice were kindly provided by Dr. Mark Blostein (Lady Davis Institute, Montreal, QC, Canada). TC-$PTP^{-/-}$ mice were kindly provided by Dr. Michel Tremblay (McGill University, Montreal, Qc, Canada). rOVA immunizations were performed IP at a concentration of 10 ug per animal twice at an interval of 2 weeks. For rhFVIII experiments, 0.02U/g of weight was delivered 4 times every 2 days for the first week followed by a single injection one week later. Two millions syngeneic or allogeneic MSC are injected IP at the same sites of rOVA immunizations once high IgG levels are reached. Approval of animal studies was obtained from the Lady Davis Institute Animal Care Committee.

4.3.2 Proteins, Chemicals, Antibodies, and Cytokine ELISA

Reagents such as rOVA, rhMMP1, ascorbic acid-2 phosphate, insulin, Alizarin Red S, Oil Red O and actinonin were purchased from Sigma-Aldrich (Oakville, ON, Canada). Anti-mouse IgG secondary antibodies used in ELISPOT assays were purchased from Amersham (NJ, USA). Anti-PAX5 and anti-pAKT/STAT3 were purchased from Pharmingen and Cell Signalling (Danvers, MA) respectively. Anti-CCL2 antibody and all cytokine ELISAs were purchased from R&D System (Minneapolis, MN) and used according to manufacturer's instructions. Anti-MMPs antibodies were purchased from Chemicon (Cambridge, Ontario, Canada).

4.3.3 Generation and differentiation of WT and CCL2$^{-/-}$ MSC

Whole bone marrow from femurs and tibias of a female B6 or BALB/c mouse was harvested and placed in culture in complete media. Five days later, nonadherent cells were washed and adherent cells were kept in culture for a period of 5-6 weeks before the appearance of a homogeneous polyclonal population. For osteogenic differentiation, MSC (70-80% confluent) were cultured in media supplemented with ß-glycerol phosphate (10 nM), dexamethasone (10^{-8} M), and ascorbic acid-2 phosphate (5ug/ml) for 4 weeks with media changes every 2-3 days. Alizarin Red S was then used to stain calcium in the mineralized extracellular matrix as show previously (Eliopoulos *et al.*, 2005). To induce adipogenic differentiation, MSC (50-60% confluent) were cultured in complete media supplemented with indomethacin (46 uM), 3-isobutyl-methylxanthine (0.5 nM), dexamethasone (1 uM), and insulin (10 ug/ml) for 7 days with continual media changes every 2 days. Oil Red O was used for lipid droplet staining as reported previously [316].

4.3.4 Flow Cytometry

All antibodies used in flow Cytometry were purchased from Pharmingen (San Diego, CA). Antibody staining was performed according to manufacture's instructions.

4.3.5 ELISPOT Assays, Protein Arrays, and RT-PCR

ELISPOTs were performed according to manufacturer's instructions. Briefly, rOVA-coated ELISPOT plates **(Millipore, Cambridge, Ontario, Canada)** were washed 3X with PBS, blocked with 1% BSA before the addition of test conditions. Following 3 washes with PBS, secondary anti-mouse alkaline phosphatise-labelled antibodies were added for 4 hrs at 4°C before development. Protein arrays (RayBiotech, Norcross, GA, USA) was used to screen the secretome of MSC according to manufactures instructions. For CCR2 expression on MSC, RT-PCR was performed on RNA extracted using the AllPrep DNA/RNA Mini Kit (Qiagen, Huntsville, Alabama, USA). The used primers were purchased from R&D Systems (Minneapolis, MN, USA).

4.3.6 Generation of Antagonist CCL2 *in Vitro*

To generate antagonist CCL2 *in vitro*, 10 ng of pure rhMMP1 was added directly to 50ug pure rCCL2 for a period of 4 hrs at 37°C. mpCCL2 was directly used in assays without further modifications.

4.3.7 Western Analysis

For phosphorylated AKT, STAT3 and PAX5 analysis by western blot, whole-cell lysate from sorted CD138+ cells were separated on 4-20% gradient SDS-PAGE (Invitrogen, Burlington, ON, Canada) and blotted with appropriate antibodies according to manufacturer's instructions. For the detection of CCL2, CM from MSC or from $CCL2^{-/-}$ MSC was collected and concentrated 20-folds using Amicon's (Millipore, Cambridge, Ontario, Canada) then run on tricine SDS-PAGE gel prepared as reported previously [317] and probed with anti-CCL2 antibody. The same concentrated supernatants was run on 12% SDS-PAGE and probed with anti-MMP-1, MMP-3, and MMP-8.

4.3.8 Carboxyfluorescein diacetate Succinimidyl Ester (CFSE)-Labelling and *in vitro* Proliferation

For *in vitro* proliferation assays, splenocytes were labeled with 5ug/ml CFSE for 8-10 min at 37°C and then washed once with complete medium and three times with PBS. To assess the proliferation over time, cells were stained with anti-CD138 daily and analysed by flow cytometry.

4.3.9 Statistical Analysis

P values were calculated by paired Student *t*-test.

4.4 RESULTS

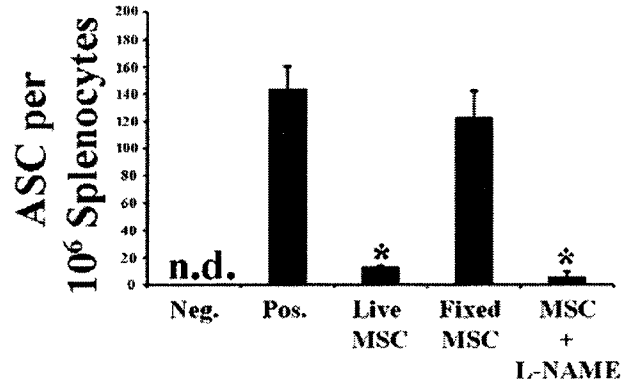
4.4.1 MSC Conditioned Media Inhibits Immunoglobulin Production by PCs

Splenocytes collected from B6 mice immunized with recombinant ovalbumin (rOVA) were treated *in vitro* with live or fixed MSC and the number of antibody secreting cells (ASCs) assessed by an OVA-specific ELISPOT assay **(Fig. 19A)**. The observed decrease in OVA-specific Ig-producing ASCs in the presence of MSC is dependent on MSC-derived soluble factors since only metabolically active MSC or MSC-derived conditioned media (CM) **(Fig. 19B)** was inhibitory. This suggests the absence of a contact-dependent inhibitory mechanism on ASC-dependent Ig production. In addition, nitric oxide (NO), a previously described MSC-produced T-cell inhibitor [318], does not seem to affect ASCs, since inhibition of NO synthesis by the addition of the inhibitor L-NAME to MSC failed in rescuing Ig secretion **(Fig. 19A)**. Several studies have suggested that leukocyte infiltration to inflammatory sites following CC chemokine secretion is regulated by enzymatic chemokine processing [223, 264]. Such regulation occurs through the proteolytic processing of CCL2/CCL7 at their N-termini by MMPs converting these

Figure 19: MSC CM Can Block IgG Secretion from Plasma Cells

(A) Live MSC Can Block IgG Secretion from splenocytes. Splenocytes collected from OVA-immunized mice were either cultured *in vitro* in splenocytes media or with MSC 24 hrs prior to ELISPOT read-outs. For the L-NAME test group, MSC were treated for 24 hrs with the inhibitor prior to the addition of splenocytes. 4% PFA was used to fix MSC. Only metabolically active MSC even in the presence of L-NAME had IgG inhibitory properties. Fixing MSC led to lost in inhibition (n=3/group; $P<0.00009$) **(B)** MSC Secrete a Soluble Inhibitory Factor(s). Based on the observation made previously, MSC CM was used alone in ELISPOT to demonstrate that it contains soluble factor(s) mediating IgG inhibition (n=3/group; $P<0.0005$). **(C)** WT MSC Lead to MMP-cleaved CCL2. CM-derived from WT MSC or $CCL2^{-/-}$ MSC was run on a Tricine SDS-PAGE to detect CCL2. **(D)** MSC Do Not Express CCR2. RNA extracted from MSC was used in an RT-PCR to demonstrate the absence of CCR2 expression on MSC suggesting no autocrine loop induced by CCL2/CCR2 interaction.

A


ASC per
10^6 Splenocytes
200
180
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Live MSC
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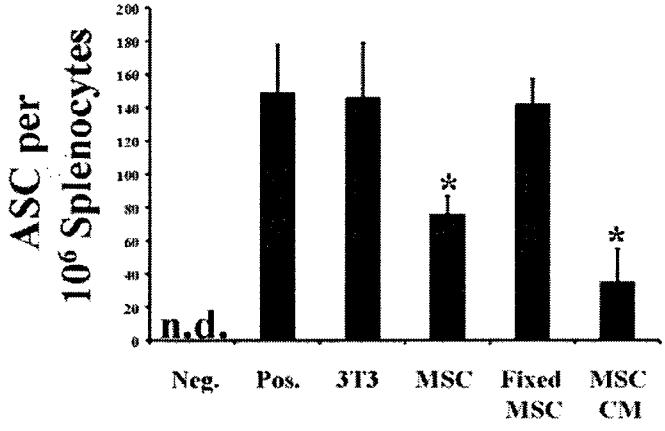
MSC + L-NAME

B


ASC per
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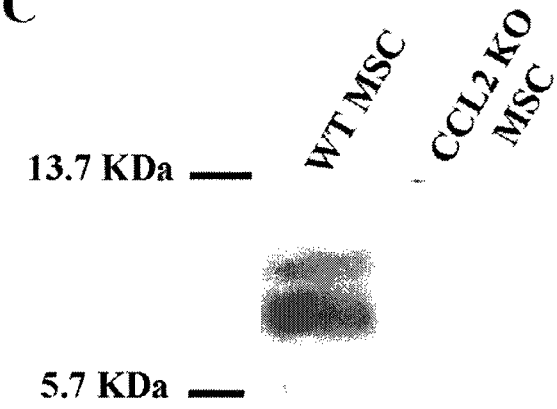
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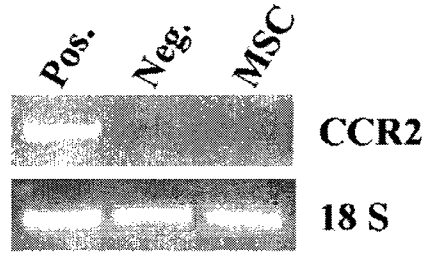
CCL2 KO MSC
13.7 KDa
5.7 KDa

D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Neg.
MSC
CCR2
18 S

chemokines from agonists to antagonists of chemotaxis for CCR2-expressing cells. Using protein arrays to identify candidate secreted molecules with potential inhibitory properties on Ig production, we identified the CC chemokine CCL2 whose secretion was confirmed by western blot (**Fig. 19C**). We also found that cultured MSCs do not express the mRNA encoding for the CCL2 receptor (CCR2) **(Fig. 19D)**.

4.4.2 Paracrine MSC Processing of CCL2/CCL7 by MMPs

Ig production by ASCs was restored following CCL2 neutralization, whereas CCL7 inactivation had partial effects **(Fig. 20A)**. Taken together, our data suggest an additive inhibitory effect of mostly CCL2 and partly CCL7 on spleen-derived ASCs since their simultaneous neutralization completely rescued Ig secretion **(Fig. 20A)**. To determine the mechanism by which CCL2 inhibits Ig secretion, spleen-derived PC were cultured in media with full-length recombinant CCL2 (rCCL2). The level of ASCs in that group was comparable to non-treated control **(Fig. 20B)** whereas the addition of *in vitro* MMP1-processed rCCL2 (antagonist CCL2 as defined by others [223] – hereafter mpCCL2) led to almost 90% Ig inhibition, but could be reversed upon the addition of CCL2 neutralizing antibody **(Fig. 20B)**. MSC are known to secrete multiple MMPs and western-blot analysis on MSC CM demonstrates the presence of MMP1, 3 as well as 8, all of which are implicated in CC chemokine processing and conversion to an antagonistic variant [223] **(Fig. 20C)**. To further confirm the direct implication of MMPs in our model, CM derived from MSC treated with actinonin, a broad MMP inhibitor, failed in suppressing ASCs **(Fig. 20D)**. These data suggest that MSC secrete CCL2 that becomes targeted by MSC-derived MMPs generating an antagonist compound capable of blocking Ig production from ASCs.

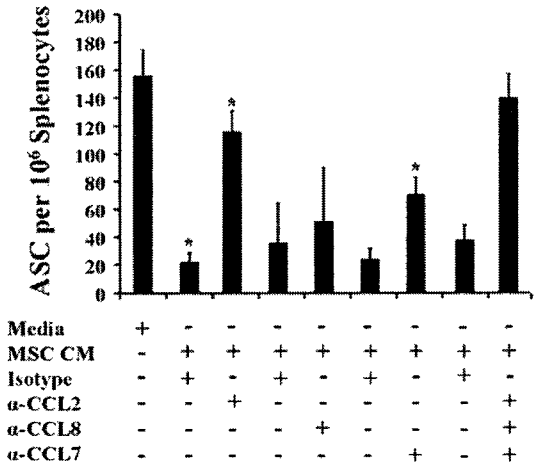
4.4.3 MSC-Derived CCL2 Leads to Plasmablast Proliferation and IL10-mediated Blockade

In order to explain the observed decrease number of ASCs following treatment with mpCCL2, we tested whether suppression of Ig production was due to induced PC

Figure 20: MSC-Derived MMP-Processed CCL2/CCL7 Block IgG Secretion.

(A) MSC Block IgG Secretion Via CCL2/7. An ELISPOT assay was performed in the presence of CCL2/8 and 7 neutralizing antibodies. IgG Secretion is partially restored upon CCL2 and CCL7 neutralization but a complete rescue is achieved following simultaneous inhibition of both CCLs (n=3/group; $P<0.001$). **(B)** MSC Secrete Antagonist CCL2. To prove that a truncated form of CCL2 with antagonistic activities is responsible for IgG blockade, *in vitro* MMP1-mpCCL2 was added on OVA-derived splenocytes and led to 90% IgG inhibition (n=3/group; $P<0.0004$). **(C)** MSC Secrete MMP-1, MMP-3 and MMP-8. To generate the cleaved form of CCL2, an MMP digestion is required. MMPs were indeed detected in MSC CM by WB. **(D)** MMP Inhibition Rescues IgG Blockade. Since MMPs enzymatic cleavage of CCL2 as well as CCL7 is responsible for the IgG secretion inhibition, we added actinonin (a generic MMP inhibitor) and demonstrate that plasma cells can secrete IgG following MSC CM treatment in a comparable way to the non-treated splenocytes (n=3/group; $P<0.05$). The negative control consists of splenocytes from naïve mice. All experiments were repeated three times. All error bars show $\pm$ S.D.

A


ASC per 10^6 Splenocytes
200
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Media + - - - - - - - -
MSC CM - + + + + + + + +
Isotype - + - + - + - + -
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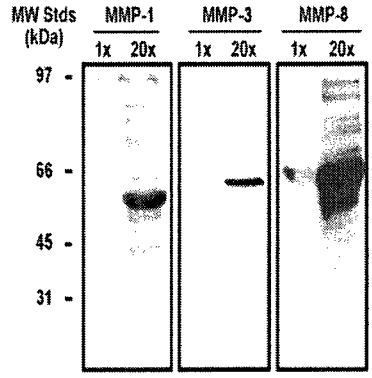
α-CCL7 - - - - - - + - +

C


MW Stds (kDa)
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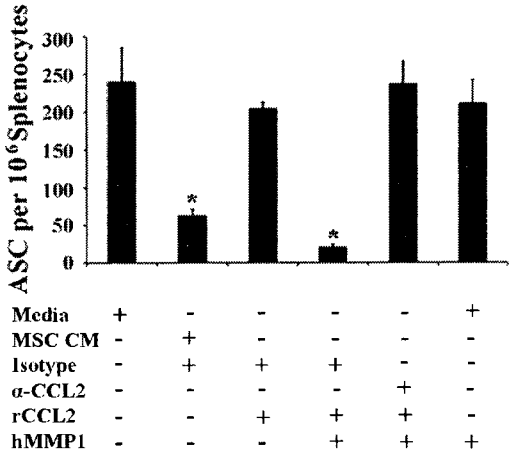
MMP-3
MMP-8
1x 20x
1x 20x
1x 20x
97
66
45
31

B


ASC per 10^6 Splenocytes
300
250
200
150
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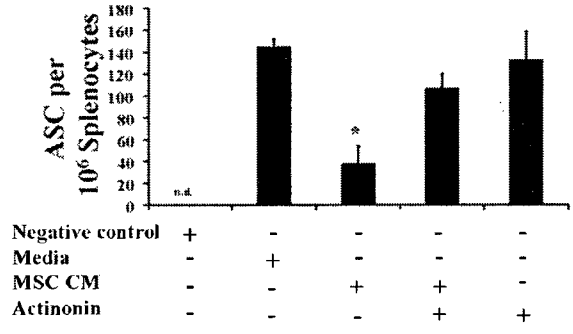
Media + - - - - +
MSC CM - + - - - -
Isotype - + + + - -
α-CCL2 - - - - + -
rCCL2 - - + + + -
hMMP1 - - - + + +

D


ASC per 10^6 Splenocytes
180
160
140
120
100
80
60
40
20
0
n.d.
Negative control + - - - -
Media - + - - -
MSC CM - - + + -
Actinonin - - - + +

cell death. We found that mpCCL2 treatment of purified spleen-derived $CD138^+$ cells did not lead to enhanced apoptosis as compared to an untreated control group (data not shown). Indeed, in experimental work with unfractionated splenocytes, we observed an apparent mitogenic effect of mpCCL2 (data not shown). Therefore, we tested whether spleen-derived CFSE-labeled $CD138^+$ PC behaved similarly by measuring proliferative response to mpCCL2. Interestingly, MSC CM induced the proliferation of $CD138^+$ cells as noticed by the loss of CFSE intensity over time, which could be reversed by CCL2 neutralizing antibody **(Fig. 21A)**. A significantly higher percentage of CD138/CD19 double positive cells was also noticed at day 3 following MSC CM treatment in the absence of CCL2 neutralization as opposed to control groups suggesting a specific induction of plasmablast proliferation by mpCCL2 **(Fig. 21B)**. Furthermore, interleukin (IL)-6 secretion was detected in MSC CM independent of CCL2 neutralization whereas significantly higher levels of IL10 were noticed following CCL2 blockade **(Fig. 21C)**. These findings are consistent with previous studies showing that CCL2 neutralization or CCR2 signaling blockade augmented IL10 concentrations in serum during inflammation [319-323]. Taken together, our data suggest that mpCCL2 present in MSC CM interacts with CCR2 leading to suppression of IL10 production, PC dedifferentiation and plasmablastic proliferation.

4.4.4 mpCCL2 Inhibits AKT and STAT3 Phosphorylation While Inducing PAX5 in PCs

Our data supports the hypothesis that mpCCL2 signals through the CCR2 receptor in a manner distinct to that of agonist, full-length CCL2. Examining signal transduction downstream of CCR2, we observed lower pAKT levels on sorted PCs ($CD19^-CD138^+$) following mpCCL2 treatment, or during the addition of both full-length and mpCCL2 in 1:1 ratio **(Fig. 22A)**. A striking observation was the inhibition of STAT3 phosphorylation in $CD19^-CD138^+$ PC following mpCCL2 or MSC CM treatments **(Fig. 22B)**. With the use of T-cell protein-tyrosine phosphatase $(TC\text{-}PTP)^{-/-}$ $CD19^-CD138^+$ PC, we show this inhibition is dependent on the phosphatase TC-PTP, known to directly interact with

Figure 21: Biological Responses of Plasma Cells to mpCCL2.

(A) MSC CM Leads to CD19$^-$CD138+ Cells Proliferation. Following treatment of splenocytes with MSC CM, CD138$^+$ gating demonstrates that the cells proliferate as of day 1, whereas the addition of CCL2 neutralizing antibody blocks this proliferative activity. The same profile was noticed for days 2 and 3. **(B)** MSC CM Leads to Plasmablast Proliferation. No apparent CD138/CD19 double positive cells could be found following 3 days culture with splenocytes media (control group). However, a high percentage of plasmablast is detectable (17%) when cultured with MSC CM with a decrease to 10% following CCL2 neutralization. **(C)** CCL2 Neutralization Leads to IL10 Secretion. From the same experiments performed above, IL6 and IL10 ELISAs were performed. IL6 does not seem to be altered if CCL2 is neutralized whereas IL10 is up-regulated (n=3/group; $P<0.005$).

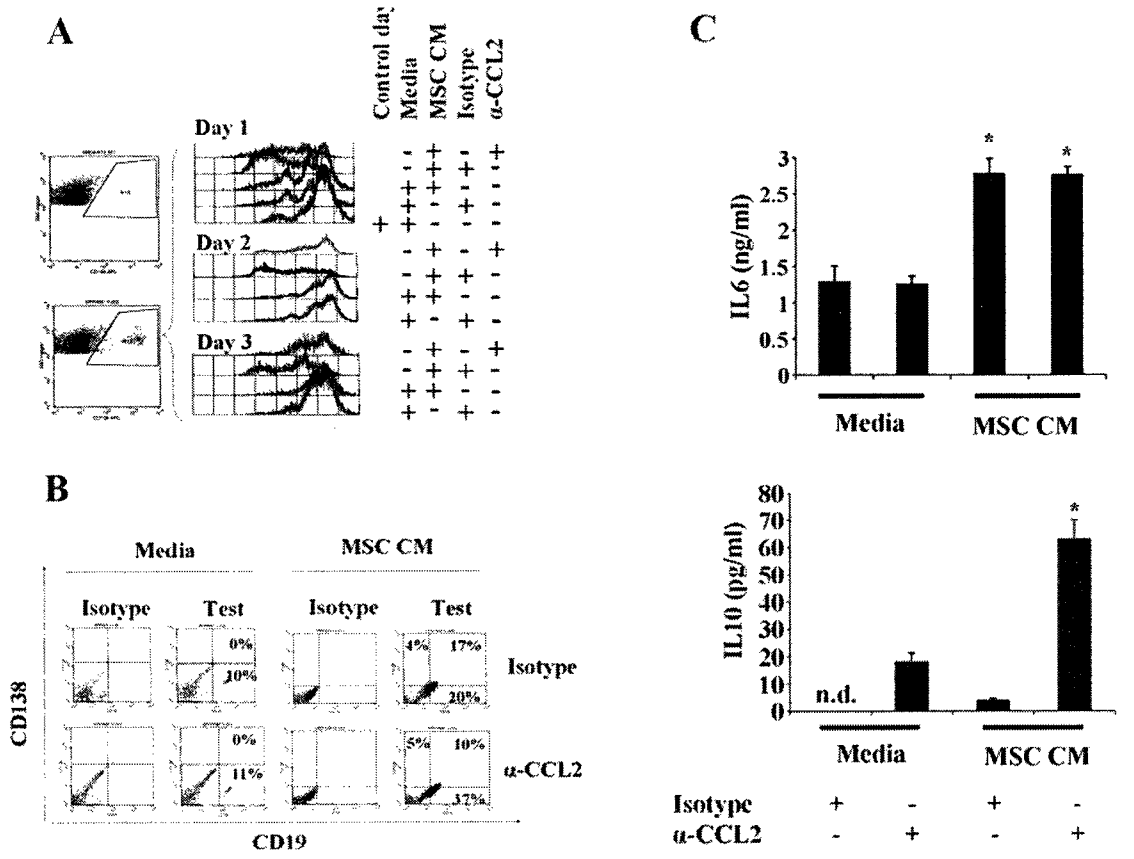

A
Control day
Media
MSC CM
Isotype
α-CCL2
Day 1
Day 2
Day 3
B
Media
MSC CM
Isotype
Test
Isotype
Test
CD138
CD19
0%
10%
4%
17%
20%
0%
11%
5%
10%
32%
Isotype
α-CCL2
C
IL6 (ng/ml)
3
2.5
2
1.5
1
0.5
0
*
*
Media
MSC CM
IL10 (pg/ml)
80
70
60
50
40
30
20
10
0
n.d.
*
Media
MSC CM
Isotype + - + -
α-CCL2 - + - +

Figure 22. Biochemical Responses of Plasma Cells to mpCCL2.

(A) Antagonist CCL2 Acts as a Dominant Negative Molecule for AKT Phosphorylation (pAKT). 1- Unstimulated, 2- rCCL2, 3- rhMMP1. 4-mpCCL2, 5- rCCL2 + mpCCL2 (1:1). rCCL2 stimulation of CD138+ plasma cells leads to pAKT, whereas mpCCL2 alone or in combination with full length CCL2 leads to a reduced AKT activation demonstrating antagonistic activities occurring at the level of CCR2. **(B)** Antagonist CCL2 Blocks STAT3 Activation. Whole cell lysate of $CD138^{+}$ sorted cells was tested for pSTAT3 as follow: unstimulated control (1), rCCL2 (2), cCCL2 (3), MSC CM + isotypes (4), or MSC CM in the presence of CCL2 neutralizing antibody (5). pSTAT3 decreased following mpCCL2 stimulation, but was completely absent upon the addition of MSC CM. The pSTAT3 was induced following the addition of CCL2 neutralizing antibody. In addition, PC-derived from TC-$PTP^{-/-}$ splenocytes were refractory to STAT3 dephosphorylation by mpCCL2 as no changes were noticed following treatment. **(C)** mpCCL2 induces PAX5 expression in PC.). 1- Unstimulated, 2- MSC CM + iso, 3- MSC CM + α-CCL2, 4- rhMMP1, 5- rCCL2 + rhMMP1 + iso, 6- rCCL2 + rhMMP1 + α-CCL2, 7-A2B5 (positive control for PAX5). PAX5 was mainly detected in the lysate of sorted PCs following cCCL2 and MSC CM treatments whereas CCL2 neutralization decreases significantly PAX5 levels.

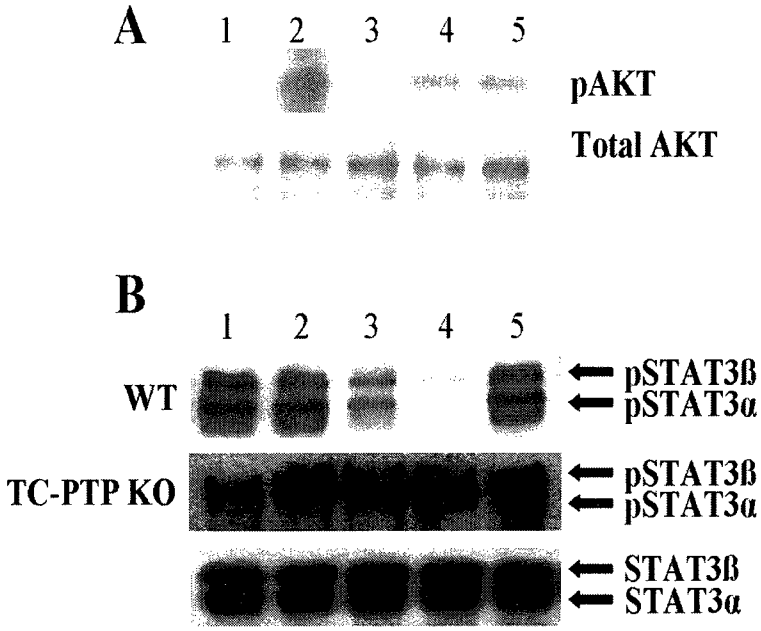
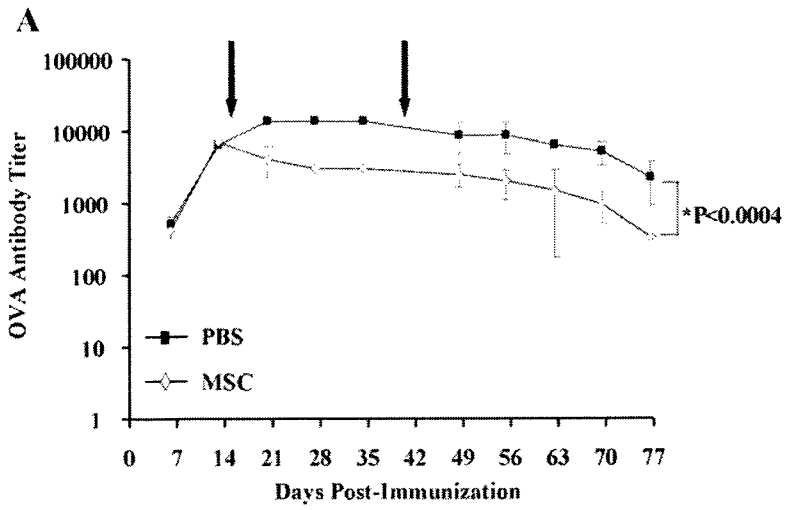
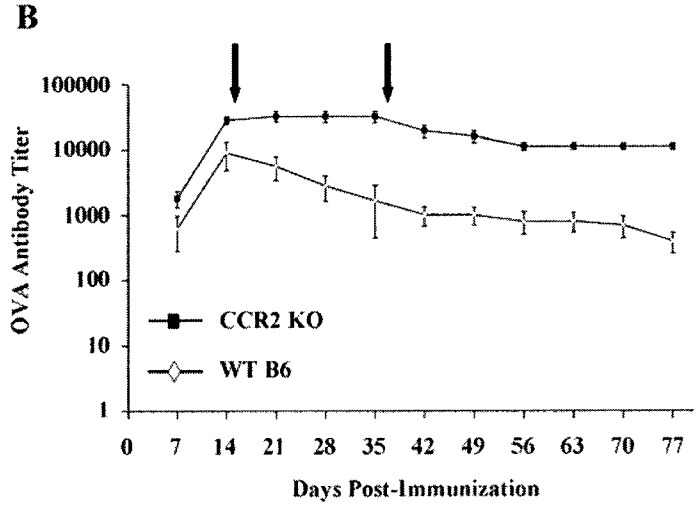
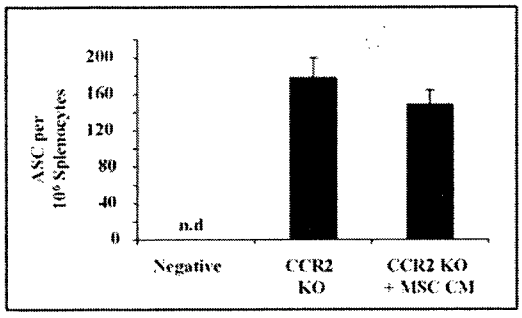

A
1 2 3 4 5
pAKT
Total AKT
B
1 2 3 4 5
WT
pSTAT3β
pSTAT3α
TC-PTP KO
pSTAT3β
pSTAT3α
STAT3β
STAT3α
C
1 2 3 4 5 6 7
PAX5
Tubulin

Figure 23: MSC Injection *in vivo* Decreases IgG Titers.

(A) MSC Injection in OVA-Immunized Mice Can Lead to Faster Clearance of IgG Titers. Following immunization of B6 mice with rOVA, MSC were injected IP at 4 weeks intervals and bled weekly for anti-OVA IgG titer by ELISA. MSC lead to a faster decrease in IgG levels as opposed to control group. **(B)** ELISPOT Assay Using $CCR2^{-/-}$ Cells. $CCR2^{-/-}$ Mice Are Not Affected by MSC. $CCR2^{-/-}$ mice were immunized with rOVA along with WT B6 mice and injected with MSC as explained in (A). High IgG titers were developed in $CCR2^{-/-}$ with no significant decrease following MSC injection. **(C)** Total IgG Titers. *In vitro* ELISPOT performed with $CCR2^{-/-}$ and WT B6 splenocytes demonstrate that $CCR2^{-/-}$ cells are refractory to antagonist CCL2 (n=3/group). **(D)**Total IgG Titers. Total IgG levels screened by ELISA shown that the overall circulating IgGs were not affected by MSC injection.


A
OVA Antibody Titer
100000
10000
1000
100
10
1
0 7 14 21 28 35 42 49 56 63 70 77
Days Post-Immunization
PBS
MSC
*P<0.0004


B
OVA Antibody Titer
100000
10000
1000
100
10
1
0 7 14 21 28 35 42 49 56 63 70 77
Days Post-Immunization
CCR2 KO
WT B6


C
ASC per
10⁶ Splenocytes
200
160
120
80
40
0
n.d
Negative
CCR2
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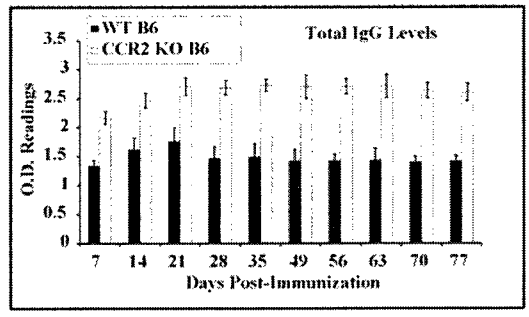
CCR2 KO
+ MSC CM


D
WT B6
CCR2 KO B6
Total IgG Levels
O.D. Readings
3.5
3
2.5
2
1.5
1
0.5
0
7 14 21 28 35 49 56 63 70 77
Days Post-Immunization

phosphorylated STAT3 [324], suggesting that mpCCL2 promotes TC-PTP-dependent STAT3 dephosphorylation once it interacts with CCR2. STAT3 activation in PC is crucial for antibody production [325] since it leads to the expression of BLIMP-1, an important repressor of the transcription factor PAX5 to allow Ig synthesis [326-329]. Concordantly with the STAT3 data, PAX5 protein levels was increased in the presence of MSC CM or mpCCL2, but was repressed following CCL2 neutralization **(Fig. 22C)**.

4.4.5 MSC Administration Lowers OVA-specific IgG Titer in Vaccinated Mice

In light of the inhibitory effects of MSC on activated PC production of Ig, we examined the effect of administering exogenous syngeneic MSC in xenoAg immunized mice. We found that rOVA-immunized mice responded to MSC administration with significantly decreased anti-OVA IgG titers **(Fig. 23A)**. Since mpCCL2 interacts with CCR2, we presumed that CCR2 receptor expression by target PCs is required to inhibit IgG secretion. Therefore, rOVA-immunized $CCR2^{-/-}$ mice were given MSC. Not only did MSC injection fail in modulating IgG titers *in vivo*, but these mice developed a stronger anti-OVA humoral response than the wild-type (WT) control group **(Fig. 23B)**. Furthermore, spleen-derived PCs from $CCR2^{-/-}$ mice were refractory to MSC CM effect *in vitro* **(Fig. 23C)**. However, the fact that overall IgG levels in both WT and $CCR2^{-/-}$ mice were not altered following MSC delivery suggests that the cellular therapy is specific for CCR2-expressing activated PC only and not all Ig producing PCs **(Fig. 23D)**.

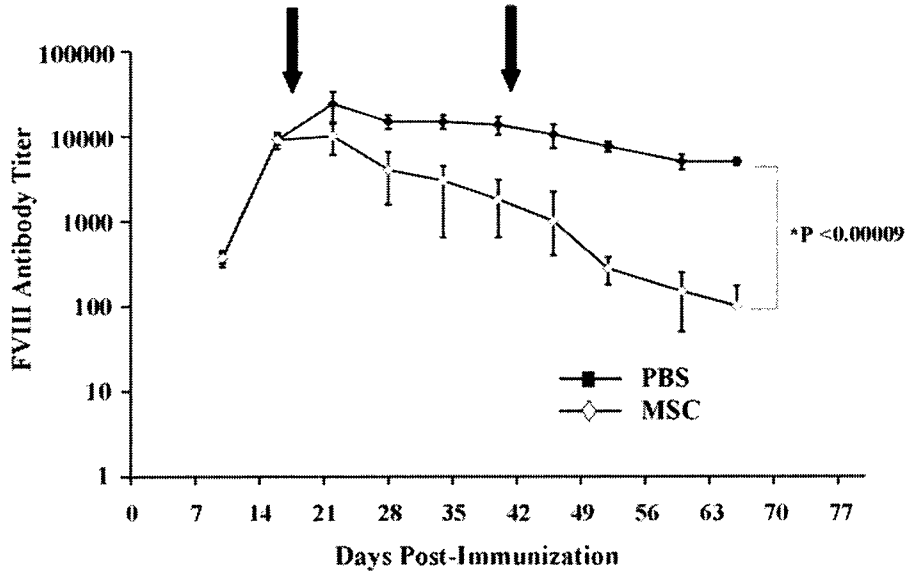
4.4.6 MSC Administration to Haemophilic Mice Lowers hFVIII-Specific IgG Titer

Acquired alloantibodies directed against therapeutic proteins, such as recombinant human FVIII used in the treatment of hemophilia A, can be a serious complication to therapy [330]. To test the hypothesis that MSC can help decrease the level of neutralizing alloantibodies, we induced anti-hFVIII antibodies in B6 hemophilic mice and subsequently administered syngeneic MSC, and observed a near two log decrease in anti-

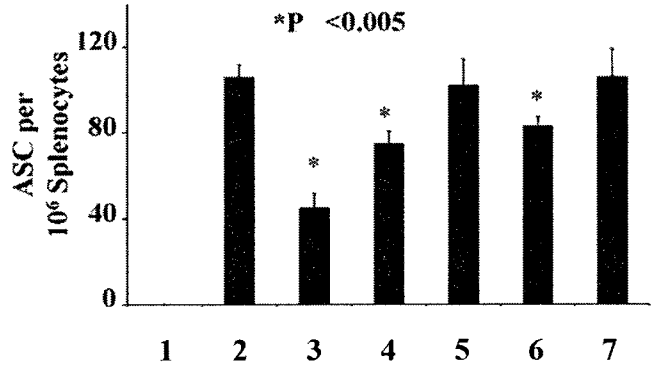
Figure 24: MSC Effect on Pathological FVIII Response.

(A) Faster Clearance of hFVIII IgG upon MSC Injection in hemophilic Mice. hFVIII immunized hemophilic B6 mice were injected with MSC (IP) following the same scheme as in (A). The IgG profile shows high IgG clearance compared to non-treated group. **(B)** ELISPOT Assay Using FVIII-derives splenocytes. *In vitro* ELISPOT performed with immunized hemophilic B6 mice splenocytes demonstrates that CCL2 neutralization rescue about 75% of IgG secretion ability by PC whereas simultaneous inhibition of CCL2/7 completely restored Ig secretion (1: Negative; 2: Positive; 3: MSC CM + isotype; 4: MSC CM + CCL2 neutralizing antibody; 5: MSC CM + neutralizing CCL2/7 antibodies; 6: $CCL2^{-/-}$ MSC CM + isotype; 7: $CCL2^{-/-}$ MSC CM + CCL7 neutralizing antibody).

A


100000
10000
1000
100
10
1
FVIII Antibody Titer
*P <0.00009
PBS
MSC
0
7
14
21
28
35
42
49
56
63
70
77
Days Post-Immunization

B


*P <0.005
ASC per
10^6 Splenocytes
120
80
40
0
1
2
3
4
5
6
7

hFVIII IgG titer **(Fig. 24A)**. ELISPOT assays performed on spleen-derived ASCs from hemophilic mice showed decreased number of anti-hFVIII Ig-secreting PC following treatment with WT MSC CM and to a lesser extent with $CCL2^{-/-}$ MSC. The weak inhibition seen by $CCL2^{-/-}$ MSC CM was due to CCL7 secretion since its neutralization blocked the observed anti-hFVIII Ig-secreting PC inhibition **(Fig. 24B)**.

4.5 DISCUSSION

We have demonstrated that the MSC secretome has the capacity to decrease the number of spleen-derived Ag-specific antibody secreting cells (ASCs) from rOVA immunized mice. However, this inhibitory property was lost upon MSC fixation prior to splenocyte treatment implying that metabolically active MSCs are required for the generation of inhibitory soluble factors. Though NO was identified as an inhibitor of activated T-cells following a direct MSC/T-cell contact [264, 313], we found that it played no role in modulating ASCs *in vitro*. However, we identified at least two CC chemokines – CCL2 & CCL7 – produced by MSCs and that represent the sum total of the suppressor activity contained in MSC secretome. More specifically, the MMP processed variant of CCL2 and possibly CCL7 are the key B-cell regulatory effector molecules. This discovery gives some insight into how MSCs – as part of the marrow microenvironment – may play a role in humoral immune response homeostasis.

B cell maturation is a process that occurs both in spleen and bone marrow to form fully mature naïve B cells [331]. Upon Ag stimulation, germinal centers appear in the spleen leading to the generation of plasmablast and ultimately PCs, which do not divide but secrete antibodies [332]. These developmental stages are controlled by a variety of transcription factors such as E2A, and EBF, which coordinately induce the expression of B-cell lymphoid genes at least at the early progenitor stage [331]. However, this early progenitor commitment to the B-lymphoid lineage is dependent on PAX5, which was identified as the key regulator of B-cell identity [332]. More specifically, a *Pax5* mutation is capable of arresting B cell development while its overexpression in $Pax5^{-/-}$ pro-B cells leads to the formation of mature B cells [333]. In addition, PAX5 must be

repressed to allow PC differentiation since it directly represses the X-box-binding protein 1 (XBP1), IgH, IgL and the immunoglobulin (J) chain [334-337] in addition to more than 100 repressed genes [338]. One of the most important reactivated gene upon PAX5 repression is CCR2 (the sole receptor for CCL2), which was proven to be important for PC differentiation and function [338]. Thus, a dynamic machinery of regulation is involved in the generation of fully functional antibody secreting PCs. Successful immune reactions lead to the accumulation of PCs in inflamed tissues, also known as survival niches until the Ag of interest is fully neutralized. At that stage, the elimination of survival signals in these niches leads to PC death and eventually to a decrease of circulating antibody titers [339]. In parallel, a proportion of plasmablasts migrates to the bone marrow and eventually become immobile long-lived plasma cells for further secondary responses upon Ag re-stimulation [340]. MSCs in the bone marrow are known to be involved at the early stages of B-cell development and PC homeostasis. We here demonstrate that MSC production of MMP-processed CCL2 may represent a key molecular event in the paracrine modulation of PCs production of Igs. Indeed, during PCs terminal differentiation, PAX5 is repressed allowing therefore the reactivation and expression of *ccr2* [338], which could eventually represent a receptor of choice for modulation by MSC-derived CCL2 and derivatives.

The biological activity of CCL2 can be modulated via various mechanisms [341]. For example, the glycosylation pattern leading to chemokines with a molecular weight of 12 or 13.5 KDa can decrease by 2-3 fold the chemotactic activity of CCL2 on primary monocytes while enhancing their half-life [341]. In addition, it was also found that N-terminal processing of CCL2 by MMPs leading to variants 5-76 or 6-76 were practically devoid of agonist bioactivity when tested for their ability to trigger Ca^{2+} influx in CCR2-expressing cells [341]. Furthermore, C-terminally processed CCL2 (1-69), on the other hand, fully retained its chemotactic and Ca^{2+}-inducing capacity when compared to variant 5-76 or 6-76 suggesting very complex regulation mechanisms on CCL2 activity [341]. When compared for their antagonistic capacities, only variant 5-76 could compete with full length CCL2 for binding to the receptor as opposed to the 6-76 truncated form [341]. Since MSC CM contains an antagonist variant capable of inhibiting pAKT or

STAT3, we predict that the variant present in MSC CM is a N-terminal truncated form, acting as dominant negative signalling molecule on CCR2.

Several signals alone or in combination such as IL2, IL4, IL5, IL6 and IL10 are know to support the survival of PCs [342]. Amongst many, IL6 was believed to be the most important survival factor due to its capacity to support PC survival *ex vivo* [342]. However, it was then discovered that IL6-deficient mice had no defects in the maintenance of humoral memory responses [342, 343]. Our finding that MMP-processed CCL2 triggers the proliferation of plasmablasts *in vitro* in an IL6-independent manner correlates perfectly with this model. Since plasmablasts are substantially less efficient than fully differentiated plasma cells at producing Igs, we speculate that this explains the robust reduction of alloAg IgG titers we observed *in vivo*. We also noted that PCs treated with MSC CM or cleaved mpCCL2 *in vitro* do not undergo increased apoptosis relative to controls (data not shown), which supports the notion that Ig suppression is not achieved through PC killing but rather by their conversion to a low Ig secretor phenotype. To address mechanistically this observation, we performed a series of *in vitro* and *in vivo* experiments. Firstly, we found that $CCR2^{-/-}$ mice are utterly unresponsive to the suppressive effects of MSCs on Ag-specific Ig production, suggesting that all downstream effects of MMP-processed CCL2 on Ig-producing cells occur through receptor interaction with CCR2 only. Focusing on the biochemical responses of PCs following MSC CM or *in vitro* MMP1-cleaved CCL2 treatment, we obtained a robust dephosphorylation of STAT3 in a TC-PTP-dependent manner. It was previously reported that conditional $STAT3^{-/-}$ in PCs blocks antibody synthesis of the IgG isotype since it directly affects the expression of BLIMP-1 allowing an additional de-repression mechanism on PAX5 expression [325]. Furthermore, we observed that presence of antagonist CCL2 in MSC CM suppressed IL10 production by splenocytes *in vitro*. Since IL10 normally induces BLIMP-1 and plays an important role in the humoral immune response [344], its blocked production can also account in part for the observed IgG inhibition. As a result, the synergizing effects of IL10 production blockade and STAT3 dephosphorylation leads to *de novo* expression of PAX5 in PCs, de-differentiation to plasmablasts and suppressed Ig production.

Since PCs express CCR2 and can be involved in maladapted immune responses exemplified by autoimmune diseases [330, 344], we hypothesized that the inhibition of PCs via CCL2 antagonism could allow a robust blockade of Ig secretion and thus alleviate clinically-relevant humoral pathologies. To test this idea, we alloimmunized hemophilic mice to human recombinant factor VIII. These mice develop neutralizing anti-FVIII antibodies akin to what is seen in more than 30% of hemophilia A sufferers treated with recombinant FVIII replacement therapy [330]. We tested whether autologous MSCs could reduce the anti-FVIII titers following alloimmunization and observed a significant and stable decrease in anti-FVIII IgG titers, yet total IgG levels remained normal and unaffected. This inhibition was lost if $CCR2^{-/-}$ mice were used in addition to the fact that a higher IgG titer was obtained in $CCR2^{-/-}$ as compared to WT mice.

How can we reconcile the suppression of alloantibodies without a substantial effect on total IgG levels? It is well known that helper CD4 T-cells are implicated in providing T-cell help for the initiation of a humoral response [345]. Interestingly, CCR2 has been reported to be absent on naïve lymphocytes and upregulated on activated T-cells (both CD4 and CD8) as reported by Rabin RL *et al.* [345]. Thus, we propose that only activated CD4 T-cells implicated in the ongoing Th2 help will be targeted by mpCCL2 and inhibited. In addition, plasma cells were reported by Delogu *et al* to up-regulated CCR2 as well. As such, the MSC-derived antagonist CCL2 would target both Th2 CD4 T-cells providing the T-cell help as well as $CCR2^{+}$ plasma cells secreting anti-FVIII antibodies without affecting quiescent memory B or T-cells. Therefore, it is possible that there is a measure of selectivity in Ig suppression, based on CCR2 expression by "activated" in contrast to steady-state or quiescent lymphoid targets.

In summary, the development of Ag-specific Ig-producing PC is fundamental to humoral response. Our observations demonstrate that MSC secrete and process CCL2 generating a molecule with profound inhibitory effects on PCs ability to secrete Ig due to TC-PTP-dependent STAT3 inhibition and PAX5 induction. The induction of PAX5 is likely transcriptional in origin [346, 347]. In addition, the specific proliferation of plasmablast is favored to the detriment of PC while IL10 levels are low in the presence of

mpCCL2. As a result, plasmablast may compete for the bone marrow or survival niche affecting overall Ig production as demonstrated by the study of Odenhal *et al.* [338]. Thus, through its MMP-dependent extracellular proteolytic processing of CC chemokine CCL2 and possibly to a lesser extent CCL7, marrow-resident MSCs may play an important physiological role in Ig response and Th2 homeostasis. It must be noted that culture expanded polyclonal MSCs are heterogeneous and there are intergroup variances in their analysis most likely due to various reasons such as number of passages, cell confluency, handling, culture media, etc... In regards to CCL2 expression, we and other groups have identified CCL2 as a chemokine produced by MSCs, and this from an array of mouse strains (C57Bl/6 and BALB/c) and from human MSCs [348]. This reinforces the notion that CCL2 is of a common ground between MSC populations derived from multiple mouse strains and from humans as well and does not represent a idiosyncratic artefact of the MSCs used by our group. As for MSCs efficacy *in vivo*, the recent success in the use of MSCs to treat steroid-resistant GVHD as reported by LeBlanc K *et al*, allows one to predict that the infusion of human MSCs in patient suffering from a maladaptive humoral ailment is of great potential as well [310]. The exact mechanism of action by which MSCs suppress GVHD remains unknown. Interestingly, it has been demonstrated by Terwey *et al* that donor $CCR2^{+}$ CD8 effector lymphocytes are mandatory for a GVHD response [173]. It is conceivable that the MSC CCR2-dependent mechanism of action we have here uncovered may be operative in suppression of alloreactive T-cells as well. We can therefore speculate that autoimmune and alloimmune humoral and cell-mediated ailments driven by the CCL2/CCR2 axis such as acute & chronic GVHD, diseases driven by pathological autoantibodies and the like would be responsive to MSC-based cell therapies. These observations and our data regarding suppression of Ag-specific alloimmune response to ovalbumin and FVIII open up the possibility of exploiting MSCs as part of a cell therapy approach to interfere with the development of pathogenic allo or autoantibodies as well.

4.6 AKNOWLEDGMENTS

Moutih Rafei is a recipient of a *Fonds de Recherches en Santé du Québec (FRSQ)* Schorlaship and Jacques Galipeau is a *FRSQ chercheur-boursier sénior*. We thank Dr. G.E. Rivard for kindly providing rhFVIII for our studies. This work was supported by the Canadian Institute of Health Research grant MOP-15017.

4.7 COMPETING INTERESTS STATMENT

The authors declare that they have no competing financial interests.

CHAPTER 5

Mesenchymal Stromal Cells Ameliorate EAE by Inhibiting CD4 Th17 T-cells in a CCL2-dependent Manner

Reference : Moutih Rafei, Philippe M. Campeau, Adriana Aguilar-Mahecha, Marguerite Buchanan, Patrick Williams, Elena Birman, Shala Yuan, Christian Young, Marie-Noelle Boivin, Kathy Forner, Mark Basik and Jacques Galipeau (*Accepted in Journal of Immunology 2009).*

Preface to Chapter 5:

We demonstrated in Chapter 4 that the paracrine conversion of CCL2 to an antagonist form by MSCs inhibits plasma cells ability to secrete antibodies by targeting CCR2. As a follow-up on this subject, we wished to test the suppressive capacity of MSC-derived CCL2 on EAE, a Th1-driven autoimmune disease dependent upon CCR2. As such, WT or $CCL2^{-/-}$ MSCs were compared to decipher the exact mechanism by which the antagonist form of CCL2 functions in alleviating EAE pathologies.

CHAPTER 5: Mesenchymal Stromal Cells Ameliorate EAE by Inhibiting CD4 Th17 T-cells in a CCL2-dependent Manner

5.1 ABSTRACT

The administration of *ex vivo* culture expanded mesenchymal stromal cells (MSCs) has been shown to reverse symptomatic neuroinflammation observed in experimental autoimmune encephalomyelitis (EAE). The mechanism by which this therapeutic effect occurs remains unknown. In an effort to decipher MSC mode of action, we found that MSC conditioned media (CM) inhibits EAE-derived CD4 T-cell activation by suppressing STAT3 phosphorylation (pSTAT3) via MSC-derived CCL2. Further analysis demonstrates that the effect is dependent on MSC-driven matrix metalloproteinase (MMP) proteolytic processing of CCL2 to an antagonistic derivative. We also show that antagonistic CCL2 suppresses phosphorylation of AKT and leads to a reciprocal increased phosphorylation of ERK associated with an upregulation of B7.H1 in CD4 T-cells derived from EAE mice. CD4 T-cell infiltration of the spinal cord of MSC-treated group was robustly decreased along with reduced plasma levels of IL17 and TNF-alpha levels and *in vitro* from re-stimulated splenocytes. The key role of MSC-derived CCL2 was confirmed by the observed loss of function of $CCL2^{-/-}$ MSCs in EAE mice. In summary, this is the first report of MSCs modulating EAE biology via the paracrine conversion of CCL2 from agonist to antagonist of CD4 Th17 cell function.

5.2 INTRODUCTION

Multiple sclerosis (MS), a chronic inflammatory condition characterized by the infiltration of peripheral immune cells to the central nervous system (CNS), can lead to acquired disabilities due to major damages caused to neuronal axons [349]. It was long believed that cells of the T-helper type 1 (Th1) lineage were solely responsible through interferon (IFN)-gamma production for the initiation and progression of MS and its murine equivalent: EAE [350]. Today, there is a general consensus that interleukin-17 (IL17), a pro-inflammatory cytokine produced by CD4 T-cells of the Th17 lineage, is

implicated in EAE onset and plays a major role in the induction of the demyelination process [73]. Nevertheless, EAE lesions do not rely only on pro-inflammatory cytokines but rather at the dynamic migration of inflammatory cells from the periphery to the CNS. More specifically, the critical importance of chemokines during EAE was defined through various neutralization experiments [351]. As a result, CCL2 was identified as one of the major chemokines orchestrating the dynamic migration of inflammatory cells to the CNS [351, 352]. In line with this observation, the absence of CCL2 receptor (CCR2) rendered mice resistant to EAE development [353]. Taken together, these data strongly suggest the necessity of CCL2/CCR2 interaction for EAE development and progression in addition to the importance of IL17 in promoting inflammation. Therapeutic interventions which modulate the CCL2/CCR2/Th17 maladaptive pathways would be of interest for patients suffering of MS. Along this line of thought, much effort has been invested in the use of cellular products as a pharmaceutical for immunomodulation and suppression of a pathological autoimmune response. This is best exemplified by the development of protocols for use of *ex vivo* expanded autologous T-regulatory cells (Tregs) for treatment of autoimmune ailments, including EAE [354]. Mining a similar vein, MSCs are the focus of much pre-clinical and clinical research from regenerative medicine to immunomodulation [149, 307]. Their ability to inhibit allogeneic T-cell activation has been exemplified by various murine studies in which MSC-based cell therapy proved its clinical utility for immunosuppression [157, 311]. In particular, the induction of T-cell anergy following MSC administration to EAE mice improved their disease score suggesting profound suppressive properties believed to be achieved via unidentified secreted soluble factors [355]. MSCs are known to produce an array of cytokines and chemokines, including CCL2 [356]. Furthermore, MSCs secrete a variety of MMPs [314, 357, 358] known to cleave a wide spectrum of target molecules, including CC chemokines [223, 264]. Indeed, MMP-mediated cleavage of the 4 N-terminal aminoacids of CCL2 converts CCL2 from an agonist to an antagonist of T-cell chemotaxis and activation [223]. We have recently reported that MSC-derived CCL2 subsequently undergoes paracrine cleavage by MSC-derived MMPs leading to the generation of the antagonistic form of CCL2 (hereafter mpCCL2) with potent suppression of immunoglobulin production by Ag-activated CCR2-expressing B

lymphocytes [358]. The sum of these observations leads to the proposal that MSCs may mediate their *in vivo* EAE suppressive effects by modulating the biology of CCL2-driven pro-inflammatory lymphoid cells. Indeed, Th17 CD4 T-cells causative of MS/EAE are known to express CCR2 both in humans and mice during autoimmune diseases [359-361]. We here validate the previous reports that autologous MSCs can serve as a cellular pharmaceutical to attenuate the clinical and pathological manifestation of EAE and further demonstrate that this effect is mediated via MSC-derived CCL2 secretion, its subsequent processing by MMPs to an antagonistic form and its profoundly suppressive effect on pro-inflammatory Th17 T-cells causative of EAE.

5.3 MATERIALS AND METHODS

5.3.1 Reagents.

Female WT BALB/c, C57Bl/6, $CCL2^{-/-}$, and retired breeder mice were purchased from Jackson laboratories (Bar Harbor, ME, USA). Recombinant human MMP1 (rhMMP1), and actinonin were purchased from Sigma-Aldrich (Oakville, ON, Canada). STAT3 and AKT antibodies were purchased from Cell Signalling (Danvers, MA, USA). ERK antibodies were purchased from Santa-Cruz (Santa Cruz, CA, USA). Anti-CCL2 neutralizing antibody and its isotype, rCCL2, CCR2/5 primers as well as IFN-gamma, TNF-alpha, IL-6 and IL17 ELISAs were purchased from R&D System (Minneapolis, MN, USA). CD4 T-cell enrichment kit was purchased from StemCell Technologies (Vancouver, BC, Canada). All FACS antibodies and IL6 neutralizing antibody were purchased from Pharmingen (San Diego, CA, USA). The ProteinChip antibody capture kit was purchased from Bio-Rad Labs (Mississauga, ON, Canada). The 5-76 antagonist CCL2 was synthesized by Genecust (Dudelange, Luxembourg).

5.3.2 MSC Characterization.

Whole bone marrow from femurs and tibias of WT female C57Bl/6 or $CCL2^{-/-}$ C57BL/6 mice was harvested and placed in culture in complete media. After a period of

5-6 weeks a homogeneous polyclonal population of MSCs appeared then was phenotyped by FACS Calibur cytometer (BD) using R-phycoerythrin (PE)-conjugated anti-CD31, CD44, CD45, CD73, CD90, CD105, MHCI and MHCII. MSCs plasticity was tested by the induction of differentiation into parenchymal lineages using adipocytic or osteogenic differentiation media as show previously [149].

5.3.3 Two-way MLR.

BALB/c and C57Bl/6 splenocytes were co-cultured with or without MSC supernatant concentrated 20X using Amicon's (Millipore, Cambridge, Ontario, Canada) treated with CCL2 neutralizing antibody or isotype. Three days later, the co-culture was centrifuged and 100 ul was used for IFN-gamma measurement by ELISA.

5.3.4 SELDI-TOF Analysis

PG20 arrays (ProteinChip antibody capture kit, Bio-Rad, Mississauga, ON, Canada) were placed in a bioprocessor (Ciphergen Biosystems) and CCL2 antibody was applied to each spot. Antibodies were cross-linked to the array surface by applying 30µl of crosslinking reagent (ProteinChip antibody capture kit, Bio-Rad). Ag capture from CM was performed by applying 20µl of sample per spot or rCCL2 at a final concentration of 1ng/ul was applied per spot. Following incubation overnight at 4°C on an agitating plate, arrays were washed once with wash buffer, rinsed twice with PBS and rinsed twice with 1mM HEPES. Chips were analysed on the SELDI Protein Chip System Series 4000 Enterprise Edition (Ciphergen Biosystems) and a protocol created using CiphergenExpress™ version 3.0.6. Data was acquired with laser intensity set at 3500nj and focus mass to 8 kDa.

5.3.5 EAE Induction and MSC Treatments.

Purified synthetic peptides of MOG_{35-55} 1mg/ml (Sheldon Biotech Center, McGill University, Montreal, Qc, Canada) was emulsified (1:1 volume ratio) in Complete

Freund's Adjuvant (Cedarlane, Montreal, Qc, Canada) containing 4mg/ml Mycobacterium tuberculosis H35RA (Difco Laboratories, Detroit, Michigan, USA) and injected subcutaneously (sc) at the base of the tail. Animals also received pertussis toxin (Sigma Aldrich, Oakville, ON, Canada) immediately after the sc injection by IP injections, repeated two days later. Mice were clinically scored every 2 days as follow: 0, no disease; 1, floppy tail; 2, hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund stage. Following the appearance of EAE symptoms, all groups were normalized to possess a grade 2 average before receiving IP injections of WT or $CCL2^{-/-}$ MSC ($2X10^6$cells per injection). All mice were bled a month later for cytokine analysis and MOG-specific antibody titer.

5.3.6 Generation of mpCCL2 and *Ex-Vivo* Re-stimulation of Splenocytes with MOG_{35-55}.

To generate mpCCL2 *in vitro*, 10 ng of pure rhMMP1 was added directly to 50ug pure rCCL2 in PBS for a period of 4 hrs at 37°C. mpCCL2 or 5-76 were detected by western-blot (WB) using tricine gels as reported previously [362]. Both forms were directly used (at the chosen concentration) in assays without further modifications. Spleens from MOG_{35-55}-immunized C57Bl/6 mice were collected and mechanically disrupted in complete RPMI 1640 medium. Viable splenocytes were cultured for 3 days with or without MOG_{35-55} (1ug/ml) in the presence of rCCL2, mpCCL2, concentrated WT MSC CM (alone or actinonin), or $CCL2^{-/-}$ MSC CM. Supernatants were analyzed for IL17 by ELISA according to manufacturer's instructions. Splenocytes derived from control or MSC treated groups were stimulated with MOG_{35-55} then their supernatant was analyzed by ELISA for IL17 and TNF-alpha. In addition, B7H-1 and CD86 cell surface expression of MOG_{35-55} stimulated splenocytes was analyzed by flow cytometry. For IL17 intracellular staining, cells were first labelled with CD4 before fixation for 20 min. After the wash, stained cells were incubated for 30 min with permeabilization buffer containing anti-IL17A antibody then analyzed by flow cytometry after the last wash.

5.3.7 EAE-derived CD4 T-cells Purification and Western Blotting.

Splenocytes from MOG_{35-55}-immunized C57Bl/6 mice were used to purify CD4 T-cells according to manufacturer's instructions. Purity of CD4 T-cells was confirmed by flow cytometry. For CCR2/CCR5 expression, RT-PCR was performed on RNA extracted using the AllPrep DNA/RNA Mini Kit (Qiagen, Huntsville, Alabama, USA). For pSTAT3 analysis by western blot (WB), whole-cell lysate from enriched CD4 T-cells treated with CM derived from WT or $CCL2^{-/-}$ MSCs was separated on 4-20% gradient SDS-PAGE (Invitrogen, Burlington, ON, Canada) and blotted with pSTAT3 or total STAT3 antibodies according to manufacturer's instructions

5.3.8 APC Assays.

APC assays were performed using peritoneal macrophages collected from retired breeder C57Bl/6 mice. Upon binding and washing from non-adherent cells, macrophages were treated with MOG_{35-55} for 24 hrs, then washed and fixed using paraformaldehyde before the addition of MOG_{35-55}-specific enriched CD4 T-cells under different treatment conditions. Supernatants were collected and centrifuged 72 hrs later for IL17 analysis by ELISA. The assay was repeated with the use of anti-IL6 neutralizing antibody.

5.3.9 Spinal cord Histology and Immune Infiltrate Analysis.

EAE mice were perfused with 20 ml PBS before the removal of their spinal cord. For histological analysis, spinal cords were fixed in 10% formalin before luxol fast blue/crystal violet co-staining or CD4 immunohistology. Flow cytometry analysis of CD4 T-cells was performed as follow. Spinal cords were mechanically disrupted to generate single cell suspension. Cells were then stained with CD4-PE and analyzed by FACS Calibur cytometer (BD). Results are shown as absolute cell numbers.

5.3.10 B7H.1 Induction and *In-Vitro* Response Analysis

EAE-derived CD4 T-cells were cultured for 24 hrs in the presence of media only, rCCL2, mpCCL2, WT MSC CM, or $CCL2^{-/-}$ CM. B7H.1 expression was then assessed by flow cytometry using B7H.1-PE antibody. CD4 T-cells stimulated with media only, rCCL2 or mpCCL2 for 15 min where then lysed, and run on a 4-20% gradient gel for pAKT or pERK analysis. Total AKT, or total ERK was used as loading control for these WBs. *In vitro* stimulation of EAE-derived splenocytes with MOG_{35-55} was performed as explained earlier but in the presence of B7H.1 neutralizing antibody or its appropriate isotype. 3 days later, the culture media was collected and analyzed for IL17 by ELISA.

5.3.11 Statistical Analysis

P values were calculated by paired Student *t*-test.

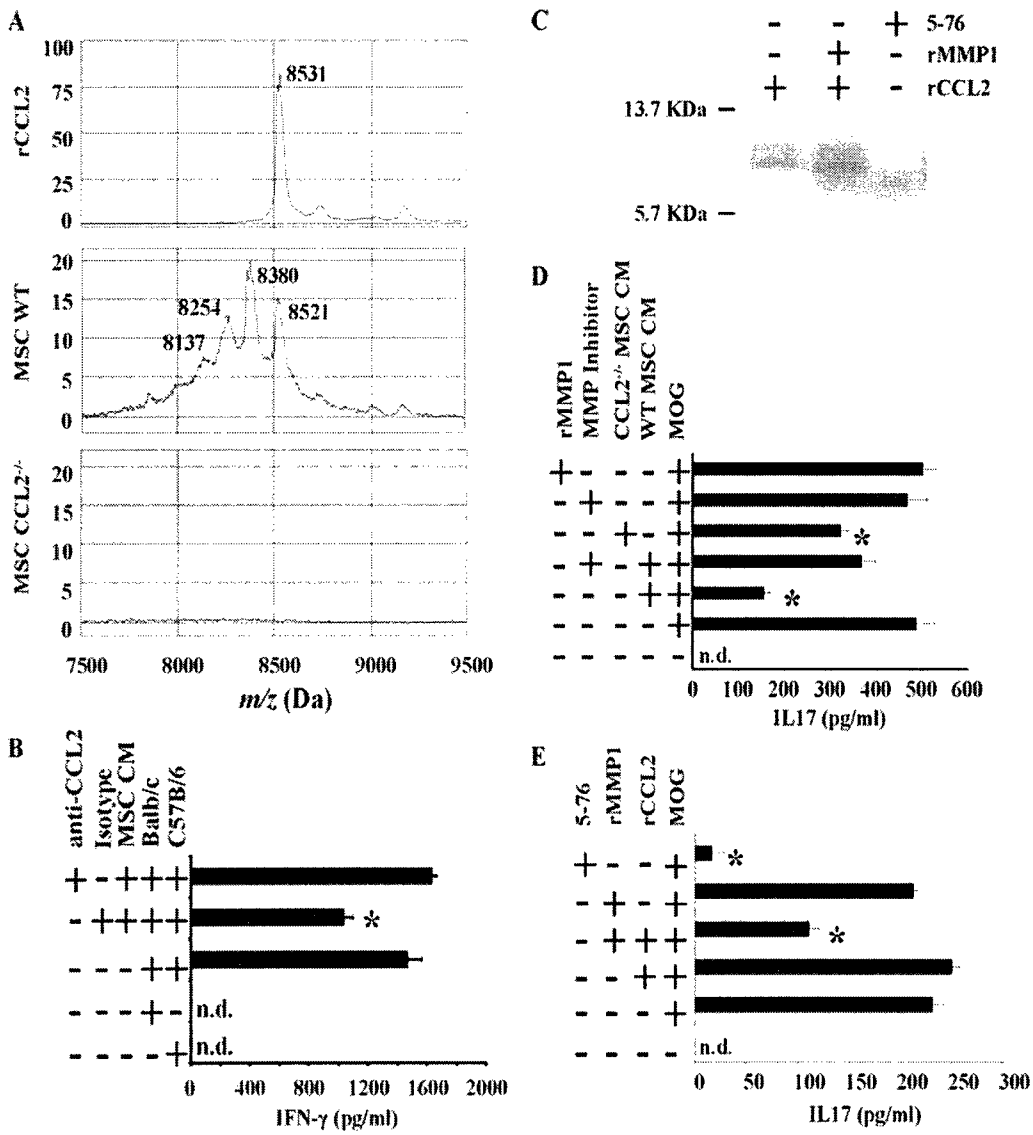
5.4 RESULTS

5.4.1 MSC Characterization.

A homogeneous MSC population was obtained from C57Bl/6 mice after 5-6 passages *in vitro*. Their phenotypic analysis by flow cytometry confirmed their expression of CD44, CD73, and CD105, whereas CD31, CD45, CD90, MHCI, and MHCII could not be detected **(data not shown)**. The same phenotype was obtained for MSCs derived from $CCL2^{-/-}$ mice **(data not shown)**. When cultured in appropriate differentiation media, MSCs gave rise to adipocytes and osteoblasts **(data not shown)**. To confirm the presence of CCL2 and derivatives in MSC secretome, a surface enhanced laser desorption ionisation-time of flight (SELDI-TOF) analysis was performed on WT or $CCL2^{-/-}$ MSC CM (**Figure 25A**). As predicted, we were unable to detect CCL2 in the secretome of $CCL2^{-/-}$ MSC CM (**Figure 25A, lower panel**). As a positive control (**Fig 25A, top panel**), we analyzed pure bacteria-derived bioactive recombinant mouse CCL2.

Figure 25: Characterization of MSC-Derived CCL2.

(A) SELDI-TO Analysis on MSC CM. To investigate the presence of CCL2 and its truncated form, rCCL2 was analyzed by SELDI-TOF and compared to WT MSC CM or $CCL2^{-/-}$ MSC CM. Interestingly, 4 major peaks appeared in WT MSC CM suggesting the presence of 4 distinct variants of CCL2 **(B)** Suppression of a 2-way MLR. C57Bl/6 and BALB/c splenocytes were co-cultured with C57Bl/6-derived MSCs CM with or without neutralizing CCL2 antibody, and IFNγ production was assessed by ELISA after 3 days (n = 3 per group and $P<0.005$). **(C)** WB analysis of cleaved rCCL2. To demonstrate that MMP1 addition on rCCL2 leads to a variant similar in size to CCL2 5-76, a WB was performed on rCCL2, rCCL2 incubated with rMMP1 or on 5-76. As expected rMMP1 addition to rCCL2 leads to the generation of a truncated form that migrates at the same MW as 5-76. **(D)** MOG_{35-55} re-stimulation under different conditions. Splenocytes from EAE mice were recovered 15 days following MOG_{35-55} immunization and stimulated *in vitro* for 72 hours with the MOG peptide in the presence of WT or $CCL2^{-/-}$ MSC CM. Supernatants were tested for mouse IL17 by ELISA (n = 3 per group and $P<0.03$). No IL17 release was detected when splenocytes where cultured in the absence of MOG_{35-55} whereas a remarkable inhibition was seen following the addition of WT MSC CM. The addition of the generic MMP-inhibitor actinonin lead to almost complete rescue of IL17. Data are shown as average ± SD **(D)** mpCCL2 and *in vitro* MOG_{35-55} re-stimulation. The same experiment as in (D) was repeated but using *in vitro* cleaved CCL2 or variant 5-76. A stronger inhibition of IL17 was noticed with 5-76 due to the partial cleavage of rCCL2 by rMMP1 as shown in Fig. 2A. (n = 3 per group and $P<0.01$). Data are shown as average ± SD.


A
rCCL2
MSC WT
MSC CCL2-/-
8531
8380
8254
8521
8137
m/z (Da)
B
anti-CCL2
Isotype
MSC CM
Balb/c
C57B/6
n.d.
IFN-γ (pg/ml)
C
5-76
rMMP1
rCCL2
13.7 KDa
5.7 KDa
D
rMMP1
MMP Inhibitor
CCL2-/- MSC CM
WT MSC CM
MOG
n.d.
IL17 (pg/ml)
E
5-76
rMMP1
rCCL2
MOG
n.d.
IL17 (pg/ml)

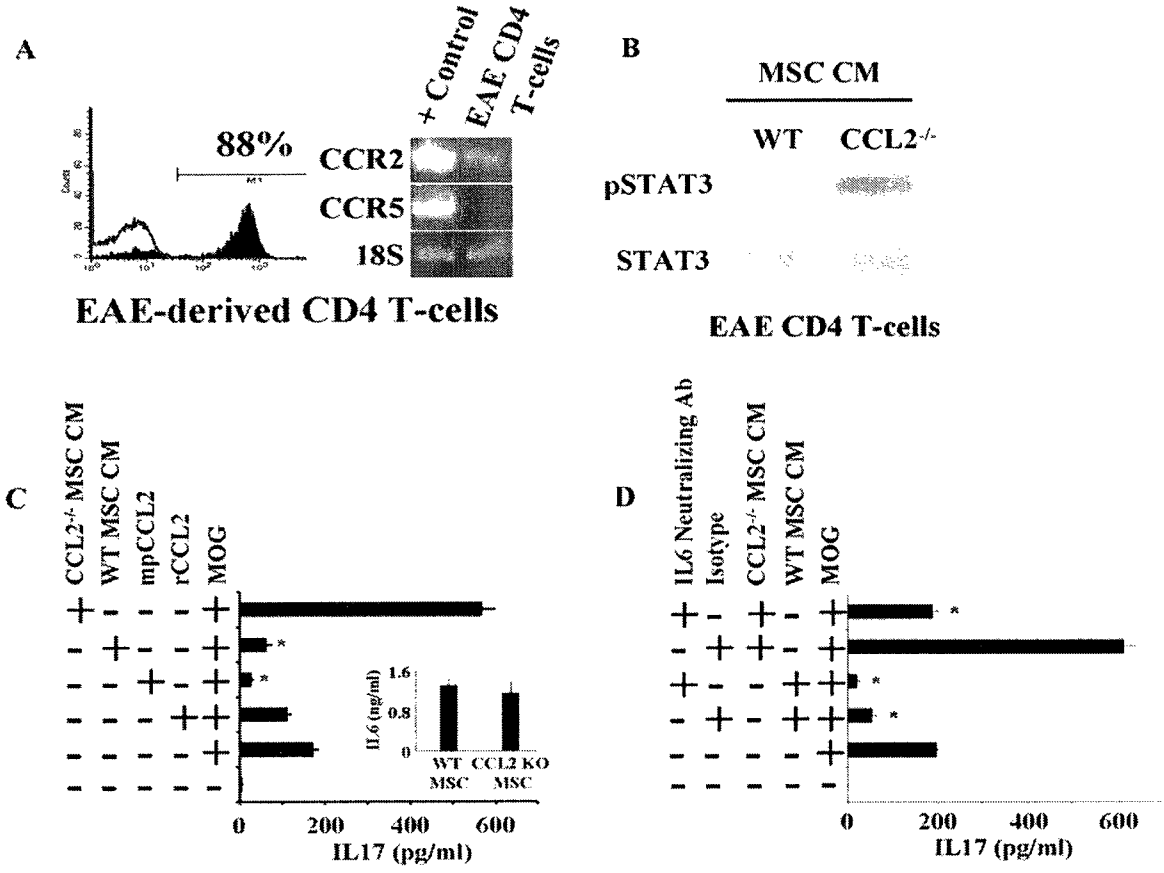
As per manufacturer specification, this recombinant CCL2 with a MW of 8525 has an intact QPDA N-terminus and also bears a spontaneous C-terminal cleavage. This polypeptide's MW is consistent with CCL2 1-73, as predicted by best fit with aminoacid sequence. Our analysis of this protein gave a MW of 8531 in good concordance with manufacturer specifications. Analyzing MSC CM (middle panel), we found that MSCs produce at least 4 CCL2 variants (MWs of 8521, 8380, 8254, and 8137 respectively). To demonstrate the immunosuppressive property of the secretome of wild-type (WT) MSCs, 20X concentrated MSC CM was added to a 2 way mixed lymphocyte reaction (MLR) using live C57Bl/6 and BALB/c splenocytes. A significant decrease in IFN-gamma **(Figure 25B)** was obtained with CM derived from WT MSCs whereas the addition of CCL2 neutralizing antibody partially abolished the suppressive effect. These data identify MSC-derived CCL2 (or one of its derivatives) as a direct inhibitor of splenocyte activation as assayed by MLR.

5.4.2 Effect of MMP-cleaved CCL2 on MOG_{35-55} Stimulated Splenocytes.

Since lymphocyte-derived IL17 is believed to be a major component of EAE physiopathology, we tested whether full-length recombinant CCL2 (rCCL2) and its MMP-processed derivative – mpCCL2 – modulated IL17 production by lymphocytes. First, we confirmed the presence of the cleaved form of rCCL2 following rMMP1 processing **(Figure 26C)**. Splenocytes harvested from mice symptomatic with MOG_{35-55}-induced EAE were re-stimulated *ex-vivo* with MOG_{35-55} in the presence of rCCL2, mpCCL2 as well as CM from WT and $CCL2^{-/-}$ MSCs. A potent reduction in IL17 was noticed using WT MSC CM along with a slight but significant inhibition with the $CCL2^{-/-}$ MSC CM **(Figure 26D)**. The addition of actinonin (a broad-action MMP inhibitor) to WT MSC CM completely restored IL17 secretion to comparable levels as the MOG_{35-55} stimulation only, suggesting that MMP-processing of CCL2 is essential for its suppressive effects **(Figure 26D)**. Working with pure rCCL2 protein, we demonstrate that the full-length rCCL2 has no effect on IL17 production by MOG_{35-55} stimulated EAE splenocytes, whilst CCL2 *in vitro* processed by human MMPs (akin to the extracellular

Figure 26: Effect of mpCCL2 on WT MOG-specific CD4 T-cells.

(A) Analysis of MOG_{35-55}-specific CD4 T-cells. Flow analysis of purified CD4 T-cells from EAE mice was performed to ensure that the cell population is homogeneous and CCR2 expression was confirmed by RT-PCR whereas CCR5 was absent. The positive (+) control is supplied with the CCR2 primers kit. **(B)** Assessment of pSTAT3 protein. 10^6 splenocytes were stimulated for 15 minutes with WT MSC CM or $CCL2^{-/-}$ MSC CM and cell lysate probed for pSTAT3. Total STAT3 protein was used as loading control. **(C)** IL17 assessment in an APC assay. Fixed C57Bl/6 peritoneal macrophages presenting MOG_{35-55} were co-cultured with purified EAE-derived CD4 T-cells under the different test conditions. Only WT MSC CM or mpCCL2 were capable of reducing the secretion levels of IL17 ($n = 3$ per group and $P<0.0003$). IL6 was detected in the CM of WT MSC and $CCL2^{-/-}$ MSC by ELISA ($n = 3$ per group). **(D)** IL6 is responsible for the hyper-induction of IL17 in $CCL2^{-/-}$ MSC CM. An APC assay was performed as in Fig. 3D but using anti-IL6 neutralizing antibody.. Data are shown as average ± SD. Data are shown as average ± SD.


A
+ Control
EAE CD4 T-cells
88%
CCR2
CCR5
18S
EAE-derived CD4 T-cells
B
MSC CM
WT
CCL2-/-
pSTAT3
STAT3
EAE CD4 T-cells
C
CCL2-/- MSC CM
WT MSC CM
mpCCL2
rCCL2
MOG
IL6 (ng/ml)
WT MSC
CCL2 KO MSC
0
200
400
600
IL17 (pg/ml)
D
IL6 Neutralizing Ab
Isotype
CCL2-/- MSC CM
WT MSC CM
MOG
0
200
400
600
IL17 (pg/ml)

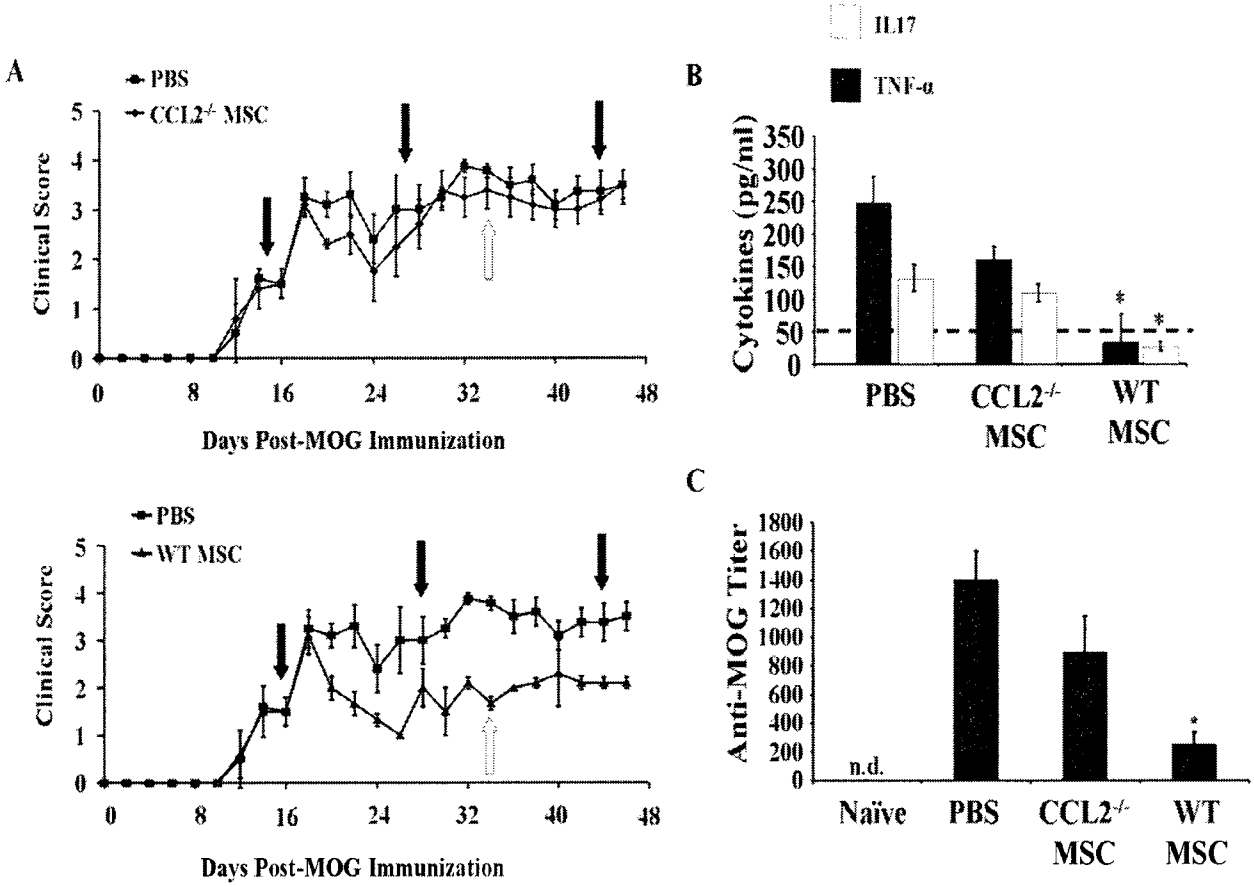
processing of CCL2 by MSC-derived MMPs) leads to significant suppression of IL17 secretion (**Figure 26E**). Interestingly, the use of the synthesized CCL2 5-76 antagonist at equimolar concentrations with rCCL2 + rMMP1 leads to a more robust inhibition of IL17 from EAE-derived CD4 T-cells **(Figure 26E)**

5.4.3 Biochemical Effect of mpCCL2 on MOG_{35-55}-Specific Th17 CD4 T-cells.

As detailed above, we show that MMP-processed CCL2 either in its recombinant form or secreted by MSCs can suppress IL17 production by Ag-specific restimulated lymphoid cells. However, the CD4 T-cell subset of lymphocytes is critical for development of EAE. Therefore, we here examine the biochemical effect of mpCCL2 directly on purified MOG_{35-55}-specific IL17-secreting CD4 T-cells. It is important to note that Th17 CD4 T-cells were characterized in humans to be $CCR2^{+}CCR5^{-}$ whereas $CCR2^{+}CCR5^{+}$ are known to be part of the Th1 lineage and thus secrete IFN-gamma [360]. Our purified CD4 T-cells derived from EAE mice expressed CCR2 but were negative for CCR5 as confirmed by RT-PCR **(Figure 2A)**. Since pSTAT3 is required for IL17 secretion and CCR2 engagement by CCL2 is known to lead to STAT3 activation, purified EAE CD4 T-cells were probed for pSTAT3 following the different stimuli. WT MSC CM completely suppressed pSTAT3 as opposed to $CCL2^{-/-}$ MSC CM **(Figure 2B)**. To understand the significance of these findings on IL17 secretion, purified CD4 T-cells were cultured in the presence of fixed syngeneic peritoneal macrophages presenting MOG_{35-55} under the different test conditions. As such, macrophages act as stimulators without the capacity of secreting soluble factors that might interfere with the assay. As expected, both mpCCL2 and WT MSC CM significantly inhibited IL17 secretion from responder CD4 T-cells, whereas $CCL2^{-/-}$ MSC CM promoted it **(Figure 2C)**. We propose that the IL17 induction by $CCL2^{-/-}$ MSC may unmask the effect of MSC-derived IL6 (**Figure 2C**) since IL6 neutralization blocked IL17 production to a comparable level to MOG stimulation only (**Figure 2D**). Taken together, these data demonstrate that MOG_{35-55}-specific CD4 T-cells exposed to mpCCL2 respond by suppressing pSTAT3 and IL17 production.

Figure 27: IP injection of WT MSCs Ameliorate EAE.

(A) $CCL2^{-/-}$ MSC versus WT MSC administration *in vivo*. WT MSCs injected at days 15, 27, and 45 after MOG_{35-55} immunization improved disease severity compared to PBS control mice. No differences were observed between controls and mice treated with $CCL2^{-/-}$ MSCs. Black arrows indicate days of MSC injection and white arrows indicate the bleeding time (n = 10 per group). **(B)** IL17 and TNF-alpha assessment by ELISA. Administration of WT MSCs lead to a robust decrease in circulating IL17 and TNF-alpha as shown by the ELISA performed on serum retrieved at day 34 from treated mice when compared to remaining groups (n = 10 per group and $P<0.002$). **(C)** Anti-MOG_{35-55} titer. The same serum sample was probed by ELISA for MOG_{35-55}-specific antibodies. The data demonstrate a 4 fold decrease in anti-MOG antibody titer in WT MSC treated mice whereas no major differences are noticed between the $CCL2^{-/-}$ MSC and the PBS control group (n = 10 per group and $P<0.0008$). Data are shown as average ± SD.


A
PBS
CCL2-/- MSC
Clinical Score
Days Post-MOG Immunization
PBS
WT MSC
Clinical Score
Days Post-MOG Immunization
B
IL17
TNF-α
Cytokines (pg/ml)
PBS
CCL2-/- MSC
WT MSC
C
Anti-MOG Titer
n.d.
Naïve
PBS
CCL2-/- MSC
WT MSC

5.4.4 MSCs Ameliorate EAE in a CCL2-dependent Manner *In Vivo*

To address whether MSC-derived CCL2 is operative in suppressing EAE symptoms in mice, we compared the therapeutic utility of WT MSCs and $CCL2^{-/-}$ MSCs in EAE mice. We found that intraperitoneal (IP) administration of WT MSCs in symptomatic mice significantly reduced disease score over time, whereas $CCL2^{-/-}$ MSCs had no significant impact akin to the PBS control group **(Figure 27A)**. Analysis of mouse serum at day 34 revealed elevated levels of circulating IL17 and TNF-alpha in mice given PBS or $CCL2^{-/-}$ MSCs, whereas a significant reduction for both cytokines was observed following the administration of WT MSCs **(Figure 27B)**. In addition, a 4 fold decrease in anti-MOG_{35-55} antibody titer was achieved with WT MSCs administration as compared to PBS or $CCL2^{-/-}$ MSC groups **(Figure 27C)**.

5.4.5 MSCs Block CD4 T-cell Infiltration to the Spinal Cord.

Based on the evidence that MSCs alleviate EAE symptoms, we examined whether there was a neuropathological correlate. The spinal cords of all treated mice were analyzed for histological evidence of inflammation. We found immune infiltrates in the spinal cords of PBS or $CCL2^{-/-}$ MSC-treated EAE mice with clear demyelination as shown by the luxol fast blue/crystal violet co-staining **(Figure 28A)**. Interestingly, immune cells were found surrounding the spinal cord derived from the WT MSC treated group, more specifically in the dura sheath, with no evident spinal cord infiltration **(Figure 28A)**. CD4 T-cell immunohistology revealed a similar pattern **(Figure 28A)** and their increased number infiltrating the neuraxis was confirmed by flow cytometry of dissociated neural tissue **(Figure 28B)**.

5.4.6 In Vitro Recall Response to MOG_{35-55} in MSC-treated Mice.

We further tested the response of splenocytes derived from MSC treated mice to MOG_{35-55} and noticed a strong reduction in the proliferative ability of cells **(Figure 29A)**

Figure 28: Pathologic Findings in the Spinal cord of Treated EAE Mice.

(A) Histological analysis of immune infiltrates. Staining of the spinal cord shows demyelination in control mice at the areas of immune infiltrates of the spinal cord as shown with luxol blue fast/crystal violet co-staining. Compared to MSC-treated mice, more immune cells where found within the spinal cord as opposed to an alignment of lymphocytes in the dura sheath surround it. CD4 immunohistology confirmed the previous observation. Arrows indicate representative histological abnormalities. **(B)** CD4 T-cell quantification by flow cytometry. Flow cytometry analysis of single cell suspension prepared from isolated spinal cords demonstrate a potent reduction in the levels of CD4 T-cell infiltration in WT MSC treated EAE mice (4-6 fold decrease compared to $CCL2^{-/-}$ MSC or PBS respectively; n = 3 per group and $P<0.001$). Data are shown as average ± SD.

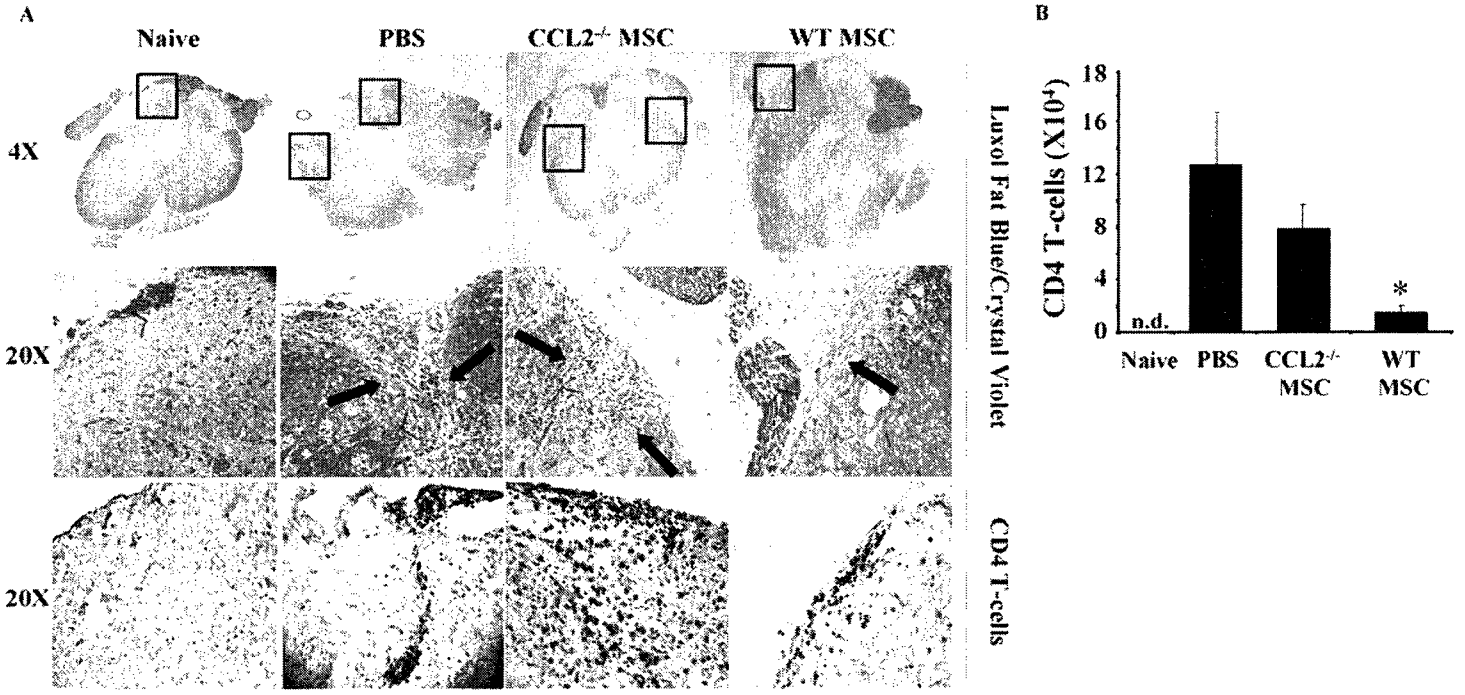
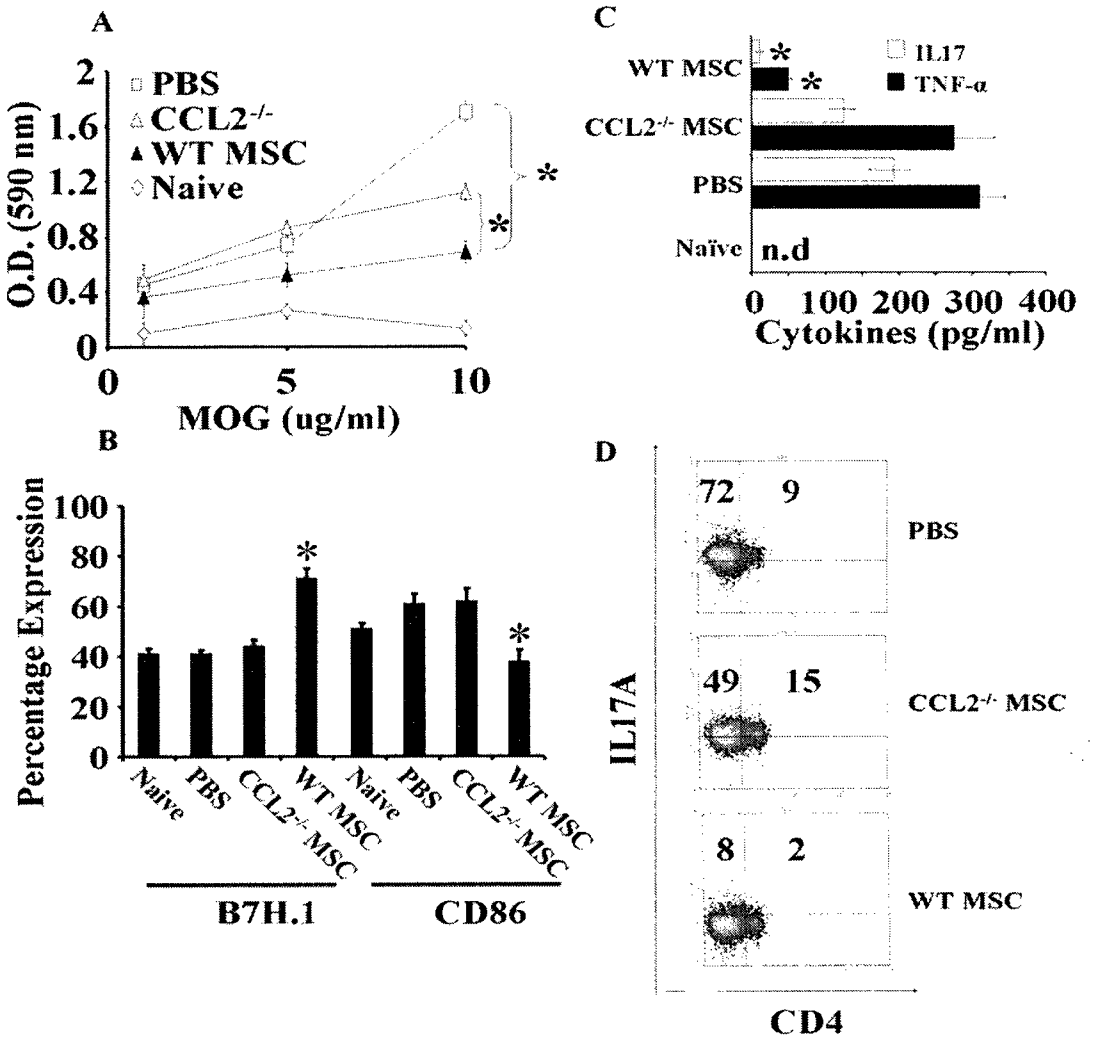

A
Naive
PBS
CCL2-/- MSC
WT MSC
4X
20X
20X
Luxol Fat Blue/Crystal Violet
CD4 T-cells
B
CD4 T-cells (X10⁴)
18
16
12
8
4
0
n.d.
*
Naive
PBS
CCL2-/- MSC
WT MSC

Figure 29: *In-Vitro* Recall Response.

(A) WT MSC administration leads to weaker T-cell proliferation. An MTT assay using splenocytes derived from EAE treated mice performed using MOG_{35-55} showed a reduced proliferation of WT MSC-derived cells (▲) in a dose-dependent manner compared to PBS (□) or $CCL2^{-/-}$ MSC (Δ) groups. **(B)** Flow cytometry analysis of immune regulatory molecules. Following MOG_{35-55} stimulation, B7H.1 levels were increased on splenocytes of WT MSC treated mice while a reduction was noticed with respect to the costimulatory molecule CD86. The remaining test groups were of similar intensity (n = 10 per group and $P<0.03$). **(C)** IL17 and TNF-alpha secretion from stimulated splenocytes. Naïve splenocytes (◊) did not respond as expected, whereas the PBS or $CCL2^{-/-}$ MSC-treated mice showed high secretion of both cytokines at comparable levels. A strong decrease was noticed however, in the WT MSC-treated group. (n = 10 per group and $P<0.00002$). Data are shown as average ± SD. **(D)** IL17 intracellular staining. As a verification step, CD4 T-cells from unfractionated splenocytes derived from EAE mice were stained for intracellular IL17 to confirm the ELISA data. About 2% of CD4 T-cells in the WT MSC group were positive for IL17 (top right quadrant) as opposed to 9 or 15% following PBS or $CCL2^{-/-}$ MSC treatment *in vivo*.

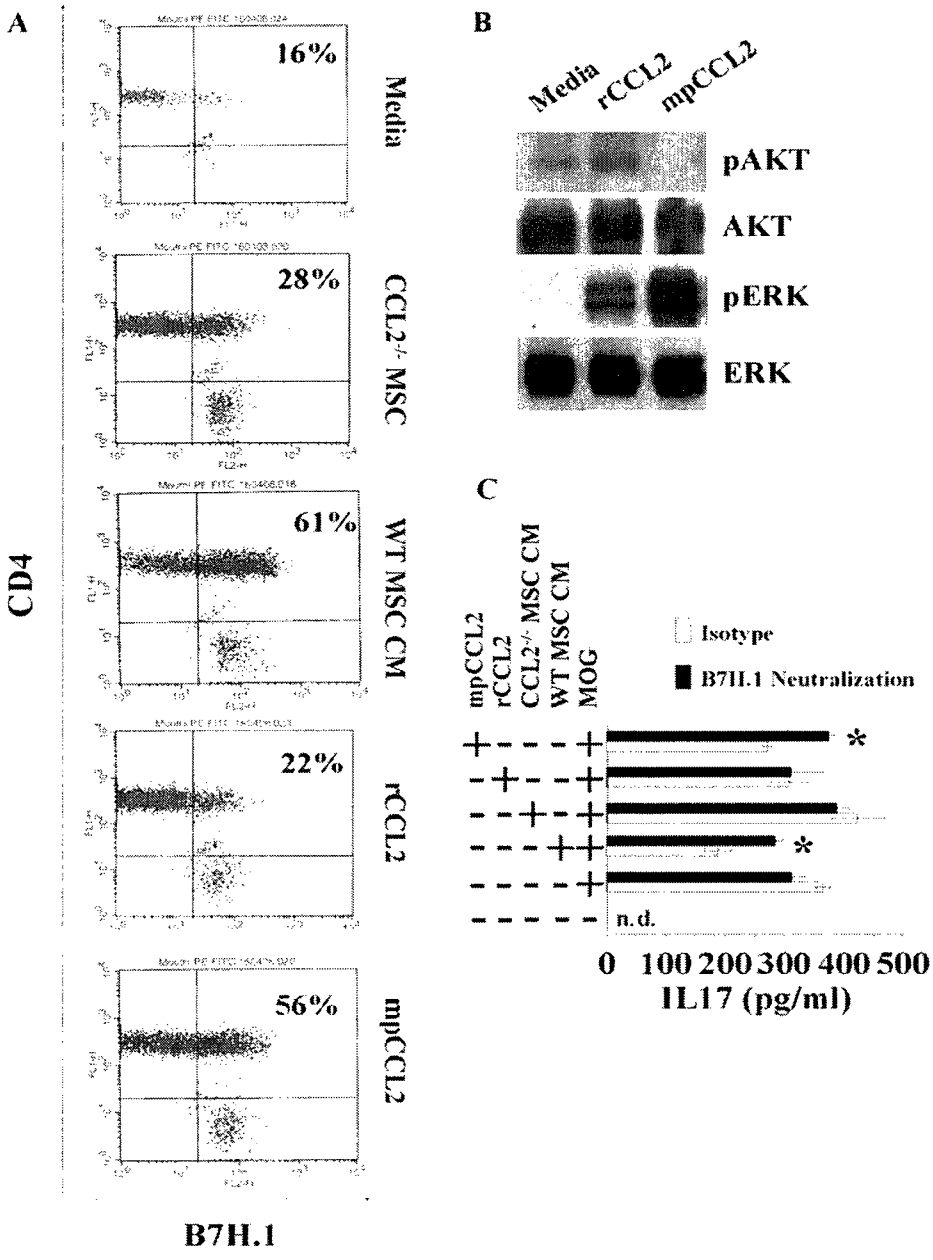

A
O.D. (590 nm)
PBS
CCL2-/-
WT MSC
Naive
MOG (ug/ml)
B
Percentage Expression
Naive
PBS
CCL2-/- MSC
WT MSC
B7H.1
CD86
C
WT MSC
CCL2-/- MSC
PBS
Naïve
IL17
TNF-α
n.d
Cytokines (pg/ml)
D
IL17A
CD4
72
9
PBS
49
15
CCL2-/- MSC
8
2
WT MSC

while expressing higher levels of the negative regulatory molecule B7H-1 (**Figure 29B**). A significant decrease of CD86 levels were also observed in splenocytes-derived from MSC-treated group **(Figure 29B).** In addition, a reduction of both IL17 and TNF-alpha from MSC-treated EAE mice was observed as opposed to the remaining groups **(Figure 29C)**. To confirm the decrease in IL17 secretion, splenocytes derived from EAE treated mice were stimulated with MOG_{35-55} then stained for intracellular IL17A. About 9% of CD4 T-cells were IL17 positive in the PBS treated group as opposed to 15% following $CCL2^{-/-}$ MSC administration. In the WT MSC group, approximately 2% of CD4 T-cells produced IL17 **(Figure 5D)**.

5.4.7 EAE-derived CD4 T-cells Up-regulate B7H.1 Following Exposure to mpCCL2.

Due to the increase B7H.1 expression following re-stimulation of MSC-treated mice with MOG_{35-55}, we assessed the level of B7H.1 expression following culture of purified CD4 T-cells under the different test conditions. We observed a robust up-regulation of B7H.1 expression **(Figure 30A)** upon the addition of WT MSC CM or mpCCL2 (61% and 56%) when compared to $CCL2^{-/-}$ MSC CM or rCCL2 (22% and 28%). This mpCCL2-dependent increase of B7H.1 prompted us to analyze the activation status of AKT and ERK that are known to play a role in B7H.1 regulation and expression. Using purified EAE-derived CD4 T-cells, pAKT or pERK (**Figure 30B**) were assessed following media, rCCL2 or mpCCL2 treatments. Interestingly, we noticed a strong inhibition of pAKT and increased pERK with mpCCL2. To test the potential inhibitory effects of B7H.1 on IL17 secretion, splenocytes derived from EAE mice were stimulated with MOG_{35-55} in the presence or absence of B7H.1 neutralizing antibodies. We found an up-regulation of IL17 secretion with mpCCL2, $CCL2^{-/-}$ MSC CM supplemented with mpCCL2, or WT MSC CM upon the addition of B7H.1 neutralizing antibodies as compared to isotype control (**Figure 30C**). Taken together these data demonstrate that mpCCL2 suppresses pAKT and activates pERK which correlates with B7H.1 upregulation in CD4 T-cells. Furthermore, we find that B7H.1 is materially important in suppressing IL17 production in bystander cells.

Figure 30: mpCCL2 Increases B7H.1 Expression in an ERK-dependent Manner. **(A)** B7H.1 assessment of CD4 T-cells upon different treatments. EAE-derived CD4 T-cells cultured with mpCCL2, or WT MSC CM all show increased expression of B7H.1 on their cell surface. rCCL2 or $CCL2^{-/-}$ MSC CM slightly up-regulated B7H.1 as compared to the remaining groups. **(B)** pAKT/ERK Assessment. Using purified EAE-derived CD4 T-cells, an inhibition of pAKT was obtained following stimulation with mpCCL2 as opposed to rCCL2 or media only. Total AKT was used as loading control. pERK was induced by rCCL2 but to higher extent upon stimulation with mpCCL2. Total ERK was used as loading control for that WB (**C**) B7H.1 neutralization rescues IL17 production. The addition of B7H.1 neutralizing antibodies to EAE-derived splenocytes stimulated with MOG_{35-55} in the presence of mpCCL2 or WT MSC CM leads to an increase in IL17 secretion as opposed to isotype control. ($n = 3$ per group and $P<0.002$). Data are shown as average ± SD.


A
16%
Media
28%
CCL2-/- MSC
61%
WT MSC CM
22%
rCCL2
56%
mpCCL2
CD4
B7H.1
B
Media
rCCL2
mpCCL2
pAKT
AKT
pERK
ERK
C
mpCCL2
rCCL2
CCL2-/- MSC CM
WT MSC CM
MOG
Isotype
B7H.1 Neutralization
n.d.
0 100 200 300 400 500
IL17 (pg/ml)

5.5 DISCUSSION

Naïve CD4 T-cells can acquire a Th17 phenotype, characterized by the production of IL17, upon their T-cell receptor engagement in the presence of transforming growth factor (TGF)-beta and IL6 [77]. Their expansion could be further enhanced by IL-1β, tumor necrosis factor (TNF)α and IL23 all capable of triggering inflammation and, under certain circumstances, buttress a maladapted autoimmune response [363]. Interestingly, IL17 neutralization by antibodies [364], soluble receptor constructs [365], the use of IL17-deficient mice [366] and auto-vaccination [367] was beneficial in inhibiting autoimmune pathology such as joint destruction in rheumatoid arthritis, diminishing symptoms in inflammatory bowel as well as improve EAE. In addition to IL17, specific chemokines modulation strategies have also demonstrated encouraging results in blocking the migration of inflammatory cells to inflamed sites therefore limiting disease progression. Such observations were supported by various studies in which MIP1α [368] CCL2 [369] or RANTES [370] neutralization attenuated the manifestations of autoimmunity. Since Th17 CD4 T-cells secrete IL17 and are responsive to CCL2 [371, 372], we hypothesized that the suppression of these lymphocytes via CCL2 antagonism could allow a robust blockade of IL17-driven inflammation and cellular infiltration of the CNS. In this regard, MSCs were of great interest for two reasons. The study by Zappia *et al.* demonstrated a significant improvement of EAE disease score following IV injection of syngeneic, *ex vivo* expanded MSCs, via the induction of T-cell anergy by an unknown mechanism [311]. Second, the remarkable capacity of MSCs in converting some factors of its own secretome into antagonist molecules via MMP paracrine cleavage – a processed coined "degradomics" [373]- as demonstrated with CCL2, suggests a novel mechanism by which MSCs can modulate inflammation [358]. In an attempt to determine whether MSC processing of its own chemokine secretome could be linked to their therapeutic benefit in EAE, we find that a causal link indeed exists. In addition to their mesenchymal plasticity, we have demonstrated that CM collected from MSCs has the capacity to partially block allogeneic *in vitro* T-cell activation as initiated in a 2 way MLR. A property that was lost upon the addition of CCL2 neutralizing antibodies. We further demonstrate that the MMP-processed form of CCL2 is required for suppression of

MLR, in keeping with our prior observation made under similar experimental condition on B-cells [358]. Interestingly, we find that the secretome of mouse MSCs contains at least 4 CCL2 variants consistent with unglycosylated forms with MW spanning 8137 to 8521. Taking in to consideration that the predicted MW of fully unglycosylated mouse CCL2 1-73 is 8525, these derivatives are all consistent with N-terminal cleaved variants of unglycosylated CCL2 and possibly CCL2 1-69. These data are reminiscent of what was observed in the secretome of human mononuclear cells, where a substantial fraction of CCL2 1-76 was unglycosylated and accompanied by unglycosylated N-terminal truncated variants 5-76, 6-76 and 1-69. Only the 1-76 and 1-69 variants maintain agonist properties, the N-terminal truncated variants, 5-76 and 6-76, are antagonists [341].

This observation of CCL2 effect does not rule-out the absence of the other reported soluble suppressive factors [166]; however it re-enforces the notion that a direct MSC/T-cell contact is not absolutely required for the subsequent induction of suppressive factors as shown with the nitric oxide (NO) case [318]. Indeed, we observed that $CCL2^{-/-}$ MSC CM led to a measurable decrease in IL17 secretion by CD4 T-cells *in vitro* (yet less than WT MSCs).

From a cell biochemistry perspective, we focused on the interplay of CCL2 and its MMP-processed derivative on CD4 Th17 T-cells causative of the immune pathology of EAE. In terms of intracellular T-cell signaling, a link between IL17 production and response to CCL2 is the activation of STAT3, which is known to selectively mediate Th17 differentiation by directly binding to IL17 promoter [374]. We found that WT MSC CM completely abrogated pSTAT3 once added on Th17 CD4 T-cells as opposed to $CCL2^{-/-}$ MSC CM, suggesting the active recruitment of a cellular phosphatase as part of the response to mpCCL2. Ag presentation (APC) assays using fixed peritoneal macrophages presenting MOG_{35-55} cultured in the presence of EAE-derived CD4 T-cells led to a reduction in IL17 production under WT MSC CM or mpCCL2 conditions while enhancement was obtained if $CCL2^{-/-}$ MSC CM is used. The latter observation was unexpected and could be explained by MSC secretion of IL6 that could directly affect responding CD4 T-cells. This piece of data suggest that the presence of the truncated

form of CCL2 binds to Th17 CD4 T-cells leading to a dominant negative effect despite the presence of the pro-inflammatory signal delivered by IL6 and possibly others.

The control of Ag-specific T-cell activation is regulated through various mechanisms including co-stimulation via CD80/CD86, soluble factors such as cytokines, activation-induced cell death, anergy, or through negative signals delivered by regulatory costimulatory pathways [375, 376]. In particular, the importance of the negative regulatory molecule B7H.1 and its interaction with the programmed death-1 (PD-1) receptor has been recently highlighted. This B7H.1/PD-1 engagement blocks proliferation and IL2 secretion, in addition to a robust induction of CD4/CD8 cell cycle arrest [377]. Critical roles have been attributed to B7H.1 especially in the EAE model where its neutralization or inhibition resulted in more severe disease score and increased secretion of pro-inflammatory cytokines [377]. Our MOG_{35-55} stimulated splenocytes derived from WT MSC-treated EAE mice proliferated weakly *in vitro* compared to $CCL2^{-/-}$ MSC or PBS treated EAE mice and showed increased expression of B7H.1. This prompted us to study the expression levels of other immune regulatory molecules such as CD80 and CD86 where the latter was down-regulated in the WT MSC group. This unbalance in B7H.1/CD86 expression could explain the poor responsive levels of re-stimulated splenocytes and their reduced ability to secrete both IL17 and TNFα. Such observations correlate well with the study of Zappia *et al.* since B7H.1 is known to block T-cell proliferation and IL2 secretion, consistent with the notion of anergy especially that the up-regulation of B7H.1 during Th1-driven inflammation was proven to serve as a negative feedback mechanism for controlling pathogenic T-cell responses [377]. Furthermore, an increase of MOG_{35-55}-specific CD4 T-cell responses derived from $B7H.1^{-/-}$ mice was observed reflecting therefore the multiple negative regulatory functions of B7H.1 in limiting the expansion and differentiation of naïve CD4 T-cells as well as regulating the reactivation of effector CD4 T-cells during autoimmunity [377]. In support of this claim, we demonstrate that mpCCL2 has the capacity to induce the expression of B7H.1 on CD4 T-cells via the inhibition of pAKT and activation of ERK as reported in multiple myeloma cells [378]. As such, we believe that the poor responsiveness of T-cell proliferation that we observe in our model is due to the

inhibitory effects exerted by B7H.1. An additional element of proof was the increased IL17 secretion of MOG_{35-55} re-stimulated splenocytes cultured with WT MSC CM or mpCCL2 following the addition of B7H.1 neutralizing antibody. It is also important to note that both B7H.1 and PD-1 are co-expressed on T and B-cells and might therefore allow negative regulation of T/T or T/B-cell interactions hence creating a blockade at the level of T-cell help during the stimulation/differentiation of Ag-activated B-cells [379]. Our observation of a 4-fold decrease of MOG titer in the serum of WT MSC-treated EAE confirms this concept in addition to the possibility that a direct interaction of WT MSC-derived CCL2 with CCR2-expressing $CD138^{+}$ plasmablasts or plasma cells could lead to their subsequent inhibition [358]. Nevertheless, the substantial reduction in CD86 levels could also amplify the poor responses obtained at the cellular and humoral levels as previously reported [376].

We investigated the therapeutic outcome following IP injection of WT versus $CCL2^{-/-}$ MSCs in EAE mice with pre-established disease pathology. We observed a significant and stable improvement of EAE score following WT MSC administration with decreased CD4 T-cell infiltration to the CNS. In contrast, the use of $CCL2^{-/-}$ MSCs did not alleviate EAE symptoms and was associated with inflammatory demyelination. This observation complements a recent report finding that MSC suppression effect in a mouse model of GVHD is dependent upon their response to interferon IFNγ, production of NO and secondary enhancement of chemokine secretome [380]. Taken together, we can speculate that MSC's response to IFNγ, as reported, leads to enhanced CCL2 production *in vivo*, and presumably its antagonistic derivative, and accounts for the near totality of anti-EAE suppressive effect. If there were supplementary suppressor molecules generated by MSCs such as CCL7, NO or any of the myriad factors suggested to date [381], these failed to provide any meaningful clinical reversal of EAE disease in mice treated with $CCL2^{-/-}$ MSCs. Reduced plasma levels of both TNFα and IL17 correlated well with the scores since minimal amounts were detected in the serum of WT MSC-treated mice and upon MOG_{35-55} re-stimulation in *vitro* of their splenocytes. The study by Zappia *et al.* claimed that intravenous (IV) administration of MSCs lead to T-cell anergy since EAE-derived splenocytes failed in responding to MOG_{35-55} unless IL2 was added to

the system[311]. Our experiments did not reveal any sign of T-cell anergy since MOG_{35-55} stimulation of splenocytes from WT MSC-treated mice proliferated weakly, but in a dose-dependent manner. This discrepancy could be due to the different MSC delivery routes and doses administered. Whereas IV MSC injection lead to their accumulation in the spleen, draining lymph nodes as well as in the subarachnoid space of the spinal cord [311], it is difficult to administer more than 500,000 cells in an IV bolus to a mouse since these tend to suffer a high rate of sudden death at even modestly higher doses. In contrast, IP administration of MSCs allows delivery of doses up to 10,000,000 cells without any noticeable side effect to experimental mice. The effectiveness observed following IP administration of MSCs reinforces the model by which they exert their effect via paracrine delivery of suppressive CCL2 acting upon distant disease sites such as CNS. It has been shown that chemokine receptor expression profile is linked to the stage of maturation of CD4 cells. Naïve CD4 T-cells are null for CCR2, yet fully differentiated CD4 cells will express CCR2 [382]. We may therefore speculate that MMP-processed CCL2 will inhibit mainly memory/effector CD4 T-cells rather than their naïve CCR2-null progenitors. Our work and that of Zappia *et al.* validate the use of syngeneic MSCs to treat EAE mice [311]. We and others have shown that MHC-mismatched allogeneic MSCs are immune rejected by immune competent murine recipients [149]. Therefore, it remains to be seen whether the pharmaceutical use of MHC-mismatched MSCs – as opposed to autologous cells - will be of optimal benefit in patients suffering from MS. In conclusion, we demonstrated the specific capacity of MSCs in regulating Th17 CD4 T-cell activation and migration in EAE mice via the production of MMP-processed CCL2. We can speculate that MSCs may serve well for treatment of autoimmune ailments driven by a pathological CD4 Th17 mechanism.

5.6 ACKNOWLEDGMENTS

We thank Denis Rodrigue, Julie Hinsinger, and Micheline Fortin from the histopathology facility at the Institute for Research in Cancer Immunotherapy (Montreal University) for tissue processing and staining. Moutih Rafei is a recipient of a *Fonds de Recherches en Santé du Québec (FRSQ)* Schorlaship and Jacques Galipeau is a *FRSQ chercheur-*

boursier sénior. This work was supported by the Canadian Institute of Health Research grant MOP-15017.

5.7 DISCLOSURES

The authors declare that they have no relevant competing interest.

CHAPTER 6

Selective Inhibition of CCR2 Expressing Lymphomyeloid Cells in Experimental Autoimmune Encephalomyelitis by a GMCSF-MCP1 Fusokine

Reference: Moutih Rafei, Philippe M. Campeau, Jian Hui Wu, Elena Birman, Kathy Forner, Marie-Noelle Boivin, and Jacques Galipeau (*In Press in Journal of Immunology 2009).*

Preface to Chapter 7:

Chapters 4, and 5 clearly suggest that MSC-derived antagonist CCL2 has powerful suppressive abilities on immune cells expressing CCR2. This mechanistic insight on the pharmacological properties of MSCs prompted us to examine the utility of a fusokine incorporating N-terminal truncated CCL2 as a means to replicate the effect of MSCs with a classic protein pharmaceutical. Therefore, in an attempt to enhance the pharmacological property of truncated CCL2, we tested the hypothesis that a GMCSF-CCL2 (6-76) could lead to a novel fusokine with robust immunosuppressive action in the context of EAE as an autoimmune disease model.

CHAPTER 6: Selective Inhibition of CCR2 Expressing Lymphomyeloid Cells in Experimental Autoimmune Encephalomyelitis by a GMCSF-MCP1 Fusokine

6.1 ABSTRACT

We describe the generation of a fusion cytokine consisting of granulocyte macrophage colony stimulating factor (GMCSF) in tandem with N-terminal truncated Monocyte Chemotactic Protein-1 (MCP1 6-76), hereafter GMME1. Treatment of activated T-cells with recombinant GMME1 protein leads to pro-inflammatory cytokine reduction and apoptosis via a CCR2-restricted pathway. Similarly, cell death is triggered in macrophages cultured with GMME1 while an inhibition of antibody production from plasma cells is observed. Treatment of CD4 T-cells derived from experimental autoimmune encephalomyelitis (EAE) mice with GMME1 leads to p38 hyperphosphorylation, inhibition of p44/42, AKT and STAT3 phosphorylation and caspase-3 activation. GMME1 administration to EAE mice suppresses symptomatic disease and correlates with decreased levels of inflammatory cytokines including interleukin-17, MOG-specific antibody titers and blockade of CD4 and CD8 T-cell infiltration in spinal cords. We propose that GMME1 defines a new class of agents for the treatment of autoimmune ailments by selectively targeting lymphomyeloid cells expressing CCR2.

6.2 INTRODUCTION

INTRODUCTION

Chemokines are implicated in inflammatory responses especially at the co-ordination and control of immune cell trafficking [383]. These proteins usually bind to their cognate G protein-coupled receptors (GPCR) initiating a pro-inflammatory and chemotactic response in CC receptor-expressing lymphomyeloid cells [384-388]. Intriguingly, CC chemokines, MCP-1 in particular (hereafter referred to as CCL2), have also been shown to be amenable to MMP processing, converting full length CCL2 to a N-terminal

truncated derivative with potent antagonistic and anti-chemotactic properties relative to the parental polypeptide [223]. Indeed, we have previously demonstrated that MMP-processed CCL2 (mpCCL2) is profoundly inhibitory on immunoglobulin production by CCR2-expressing plasma cells [358]. In addition, it was previously demonstrated that the antagonist variants 5-76 as well as 6-76 of CCL2 (missing the first 4 or 5 N-terminal aminoacids respectively) were devoid of chemotactic activity [341]. However, only the 5-76 truncated CCL2 is considered as a potent antagonist, as opposed to the 6-76, since it can desensitize intact CCL2 by competing for its receptor CCR2 [341]. The CCL2 (6-76) loss of function derivative prompted us to exploit and optimize the use of this variant using the combinatorial fusion cytokine (fusokine) technology as part of a novel approach for GPCR modulation. A fusokine represents the fusion of 2 distinct and functionally unrelated cytokine domains as one single polypeptide that might lead to unanticipated pharmacological properties as we have previously reported [285]. Along this line of thought, we have shown that fusion of GMCSF to interleukin-2 (IL2) to generate the GIFT2 fusokine led to a potent proinflammatory cytokine with unanticipated pro-survival effects on NK cells, which co-express the GMCSF and the IL2 receptor [389]. Our laboratory has previously demonstrated that as the N-terminal domain of a fusokine, that GMCSF is permissive for high fusokine secretion efficiency by transfected cells [285, 389]. Furthermore, GMCSF has a fairly long plasma half-life reported to be about 6 hours [390, 391] which may markedly extend the *in vivo* bioavailability of C-terminal domains such as truncated CCL2. In addition, from our previous experience with another fusion protein: GIFT15, we found that the GMCSF N-terminal domain can markedly alter the receptor binding properties of the C-terminal partner leading to unheralded cell signalling features and novel pharmaceutical properties [285]. Therefore, GMCSF-based fusokines may lead to pro-inflammatory synergy or profoundly antagonistic properties to the C-terminal companion domain in manner dictated by the influence of GMCSF on fusokine half-life, synergy with GMCSFR-mediated signalling and steric influence on the C-terminal fusokine partner signalling pathway. These features would allow an unanticipated gain of function for truncated CCL2 (6-76) as part of a fusion protein with GMCSF and thus interesting pairing for the generation of a novel fusokine targeting cells expressing CCR2. We here show that such a fusokine leads to profoundly altered

signalling through the CCR2 GPCR pathway and initiates a potent pro-apoptotic response in CCR2 expressing lymphomyeloid cells. Although multiples mouse strains, including $CCR2^{-/-}$ mice, are susceptible to EAE [392], the major consensus remains that selective depletion of CCR2-expressing pathogenic cells involved in autoimmune ailments such as multiple sclerosis (MS) [353] would theoretically offer selectivity and minimize off-target toxicity as can be seen with broad action immune suppressive drugs. We here demonstrate that in the EAE mouse model of MS, delivery of GMME1 is a potent suppressor of CCR2-expressing cells causative of MS/EAE, thereby validating the use of a novel class of chimeric fusokines in the selective depletion of CC-expressing cells involved in a maladapted immune response.

6.3 MATERIAL AND METHODS

6.3.1 Reagents

All used female mice were 6-8 weeks old (Jackson Laboratory, Bar Harbor, ME). Recombinant proteins (rGMCSF/rCCL2) and their antibodies were purchased form R&D systems (Minneapolis, MN). Antibody for α-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved caspase 3, p44/42, p38, p38, AKT, and STAT3 antibodies were purchased from Cell Signalling Technology (Danvers, MA). rhMMP1 was purchased from Sigma-Aldrich (Oakville, ON, Canada). All flow cytometry antibodies were purchased from BD Biosciences (San Diego, CA). CCR2 primers, apoptosis detection kits, ELISAs for mouse CCL2, mouse IFN-γ, mouse IL2/IL4/IL13/IL17, mouse CXCL5 and mouse TNF-α were purchased from R&D systems. RNA extraction kit was purchased from Qiagen (Mississauga, ON, CANADA). CD3/CD28 beads and CFSE dye were purchased from Invitrogen (Burlington, ON, CANADA). Contigen was purchased from Bard Urological Division (Covington, GA, USA). The MOG_{35-55} was synthesized by Sheldon Biotech Center (McGill University, Montreal, Qc, Canada) and Complete Freund's Adjuvant was obtained from Cedarlane (Montreal, Qc, Canada). The pertussis toxin was purchased from Sigma Aldrich (Oakville, ON, Canada). CD4 and CD8 T-cell enrichment kits were purchased from

StemCell Technologies (Vancouver, BC, Canada). Inflammatory qRT-PCR arrays were purchased from SABiosciences (Frederick MD, USA) and used according to manufacturer's instructions.

6.3.2 Fusokine Design and Expression

Mouse CCL2 was amplified by PCR in order to generate a 5 aa truncation at the N-terminus then cloned into the mammalian expression vector pCMV in frame with the cDNA encoding mouse GMCSF. HeLa cells were transiently transfected using Polyfect (Qiagen, Mississauga, ON, Canada) and supernatant tested by western-blot. HeLa cells were also transfected using pGMME1 and selected with G418 for a total period of 3 weeks to generate stable expression of the fusokine.

6.3.3 Prediction of Fusokine 3-Dimentional Model

The structural models of CCR2 and GMME1 were obtained by homology modeling using MODELLER 9v3 (University of California at San Francisco). The crystal structure of human β_2 adrenergic G-protein-coupled receptor (PDB entry: 2R4R) is used as a template for CCR2 (32). For the fusion protein GMME1, crystal structures of human GM-CSF (PDB entry: 2gmf) and CCL2 (PDB entry: 2nz1) are used as the templates for residues 21-190 in GMME1. Fold recognition method as implemented in PROSPECT 2.0 (Oak Ridge National Laboratory, Oak Ridge, TN) was subsequently employed. The crystal structure of photosystem I protein (PDB entry: 1pse) was identified as a template. Based on templates 2gmf, 2nz1 and 1pse, 100 structure models of GMME1 were generated and the one with lowest objective function was selected for further analysis.

6.3.4 Induction of CCR2 Expression and GMME1 Effects

For CCR2 induction, CD4 T-cells were purified using the Spinsep kit and cultured in 1:1 ratio with anti-CD3/CD28 beads in 96 well plates for 72 hrs. Cells were then washed and analyzed for CCR2 expression by RT-PCR and flow cytometry. For *in vitro*

proliferation assays, CD4 T-cells were labelled with 5ug/ml CFSE for 8-10 min at 37°C and then washed once with complete medium and three times with PBS. To assess the proliferation induces by the CD3/CD28 beads in the presence of the fusokine over time, cells were treated with GMME1 and analysed daily by flow cytometry. Supernatants were collected daily and assessed by ELISA for IL2 levels.

6.3.5 Mixed Lymphocyte Reaction (MLR) and ELISPOTS

The supernatant of co-cultured 10^5 splenocytes-derived from BALB/c or C57Bl/6 mice for 72 hrs with equimolar concentrations of chemokines was centrifuged and used to detect IFN-γ secretion by ELISA. For the MLR assay using $CCR2^{-/-}$ C57Bl/6 splenocytes, 10^5 splenocytes of BALB/c were fixed with paraformaldehyde prior to their addition to C57Bl/6 splenocytes. After 3 days of culture with the test conditions, IFN-γ levels were assessed by ELISA. ELISPOTS were performed as follow: rOVA-coated ELISPOT plates (Millipore, Cambridge, Ontario, Canada) were washed 3X with PBS, blocked with 1% BSA before the addition of cells under different test conditions. Following 3 washes with PBS, secondary anti-mouse alkaline phosphatise-labelled antibodies were added for 4 hrs at 4°C before development.

6.3.6 Apoptosis Analysis

Peritoneal macrophages cultured for 24 hrs with equimolar concentrations of rGMCSF, rCCL2, mpCCL2, rGMCSG/rCCL2, rGMCSF/mpCCL2, or GMME1 were lysed and whole cell lysate was loaded on a 4-20% gradient gel and probed with anti-cleaved caspase-3 according to manufacturer's instructions. The result obtained with GMME1 was confirmed by PI and annexin-V staining. Staining for purified CD4 and CD8 T-cells was performed similarly. To generate antagonist CCL2 *in vitro*, 10 ng of pure rhMMP1 was added directly to 50ug pure rCCL2 in PBS for a period of 4 hrs at 37°C. MMP-processed CCL2 was directly used in assays without further modifications.

6.3.7 qRT-PCR on Stimulated Splenocytes

RNA was extracted from mouse cells using Qiagen (Mississauga, ON) RNeasy minikit and Qiashredder columns according to the manufacturer's instructions. 1 μg of RNA was reverse transcribed using RT² First Strand Kit and applied to PCR array plates, both from SABiosciences (Frederick, MD). Plates were processed in an Applied Biosystems 7500 Fast Real-Time PCR System, using automated baseline and threshold cycle detection. Data was interpreted by using SABiosciences' web-based PCR array data analysis tool.

6.3.8 Biochemical Responses and Signalling Analysis

For signalling analysis, EAE-derived CD4 T-cells were purified and treated for 5 min with 1 pmol of rCCL2 or GMME1 then lysed using ice-cold cell lysis buffer before their sonication. The activation of p38, p44/42, pAKT, and pSTAT3 were probed by WB using 4-20% gradient SDS-PAGE gels and appropriate antibodies.

6.3.9 APC Assays

APC assays were performed using peritoneal macrophages collected from retired breeder C57Bl/6 mice. Upon binding and washing from non-adherent cells, macrophages were treated with MOG_{35-55} for 24 hrs, then washed and fixed using paraformaldehyde before the addition of MOG_{35-55}-specific enriched CD4 T-cells under different treatment conditions. Supernatants were collected and centrifuged 72 hrs later for IFN-γ and IL17 analysis by ELISA.

6.3.10 Gene Engineering MSC to Express GMME1

Whole bone marrow from femurs and tibias of $CCL2^{-/-}$ C57BL/6 mice was harvested and placed in culture in complete media until the appearance of a homogeneous MSC polyclonal population, which was later on phenotyped by flow cytometry while

their plasticity tested by inducing them to differentiate into osteoblasts and adipocytes **(data not shown)**. The GMME1 cDNA was cloned in the AP2 retroviral plasmid and retropracticles generated as shown previously [285]. Concentrated retroparticles were then used to gene modify $CCL2^{-/-}$ C57Bl/6 MSCs. Secretion and expression levels of GMME1 by MSCs were analyzed by WB and CCL2 ELISA respectively.

6.3.11 EAE Induction and $CCL2^{-/-}$ MSC-GMME1 Contigen Implantation

The synthetic MOG_{35-55} peptide was emulsified in Complete Freund's Adjuvant and injected subcutaneously (sc) at the base of the tail. Animals also received pertussis toxin immediately after the sc injection by IP injections, repeated two days later. Mice were clinically scored every 2 days as follow: 0, no disease; 1, floppy tail; 2, hind limb weakness; 3, partial hind limb paralysis; 4, complete hind limb paralysis; 5, moribund stage. Following the appearance of EAE symptoms, all groups were normalized to possess a grade 2 average before receiving a subcutaneous implant of contigen-embedded MSCs as a neo-organoid as previously described by our group [393]. Contigen implants contained $CCL2^{-/-}$ MSCs gene-engineered to express GMME1 or null $CCL2^{-/-}$ MSC ($2X10^6$cells per implant).

6.3.12 Hematological and Histological Analysis

To investigate the levels of circulating pro-inflammatory cytokines, mice sera were collected weekly from treated EAE-mice 1 week following MSC implantation and tested by ELISA. For antibody titer, ELISA plates were coated with 1mg/ml of MOG peptide then screened using diluted sera to calculate final titers. White blood cell (WBC) count was performed using the Z2 coulter Particle Count and Size analyzer (BD Biosciences). For histological analysis, mice were perfused with PBS and their spinal cords removed. For H&E staining, spinal cords were fixed, embedded and cut. For CD4 and CD8 immunohistology, sections were frozen before cutting and staining.

6.3.13 Statistical Analysis

P values were calculated by paired Student *t*-test.

6.4 RESULTS

6.4.1 Engineering the GMME1 Fusokine

The GMME1 fusokine was created by cloning a modified cDNA of mouse GMCSF missing the nucleotides coding for the last 11 carboxy terminal aminoacids (aa) in frame to the 5' end of a mouse CCL2 cDNA missing the nucleotides coding for the first 5 N-terminal aa (corresponding to aa 6-76 of the signal peptide-free form of CCL2). An interdomain single aa asparagine linker was introduced. The final fusokine GMME1 cDNA encodes for a single polypeptide chain of 250 aa (**Fig. 33A**). Computer-based analysis of the theoretical three-dimensional protein structure of GMME1 predicts for a direct and stable interaction of the CCL2 domain of GMME1 with CCR2 (**Fig. 33B**). Denaturing immunoblotting performed on the supernatant from HeLa cells transiently transfected to express GMME1 showed that the chimeric protein is efficiently secreted in the extracellular space and has a molecular weight of 50 KDa (**Fig. 33C**).

6.4.2 Biological Effects of GMME1 on Lymphomyeloid Cells

Following stimulation, activated T-cells are known to induce the expression of CCR2 *de novo* [345]. To confirm that notion, naïve CD4 T-cells were purified and cultured with media alone or supplemented with beads containing anti-CD3/CD28 antibodies to induce CCR2 expression as shown by RT-PCR and flow cytometry analysis (**Fig. 34A**). To determine the functional impact of GMME1 on CD3/CD28 activated CD4 T-cells, CFSE-labelled CD4 lymphocytes were cultured in the presence of splenocyte conditioned media only or supplemented with GMME1. Interestingly, a strong proliferation of lymphocytes occurred under CD3/CD28 stimulation whereas the addition of GMME1 robustly inhibited it as seen with the CFSE peak profiles (**Fig. 34B**). Since

IL2 secretion accompanies lymphocyte activation, we analyzed its levels in the supernatant of CFSE-labelled cells and noticed a strong reduction following GMME1 treatment even after CD3/CD28 stimulation (**Fig. 34C**). To confirm our observations, we analyzed the effect of GMME1 on physiologically activated lymphocytes. To do so, a mixed lymphocyte reaction (MLR) was performed using splenocytes derived from wild-type (WT) C57BL/6 mice or $CCR2^{-/-}$ C57BL/6 mice as responders and paraformaldehyde fixed BALB/c splenocytes as stimulators. The up-regulation of interferon (IFN)-γ production was measured as a surrogate marker of allo-activation of the C57BL/6 responders. We observed a significant inhibition in IFN-γ production by WT cells when exposed to GMME1, whereas $CCR2^{-/-}$ responder lymphocytes were unaffected by GMME1 treatment (**Fig. 34D**). GMME1 was similarly assessed on plasma cells. Following WT or $CCR2^{-/-}$ C57Bl/6 mice immunization using recombinant ovalbumin (rOVA) to induce an OVA-specific humoral response, their splenocytes were cultured in the presence of GMME1 before performing an OVA-specific ELISPOT assay. A robust inhibition of antibody production was observed using both mpCCL2 (rCCL2 + hMMP1) and GMME1 on WT antibody-secreting cells (ASCs) only suggesting that the fusokine is as potent as the antagonistic form of CCL2 in suppressing plasma cells expressing CCR2 (**Fig. 34E**). In addition to B and T lymphocytes, macrophages are also known to express CCR2 [394], so we assessed the effect of GMME1 treatment on these cells as well. Peritoneal macrophages harvested from C57BL/6 mice and exposed to GMME1 died by apoptosis 24 hrs later as shown by activation of caspase-3 (**Fig. 34F**) as well as annexin-V/PI co-staining (**Fig. 34G**).

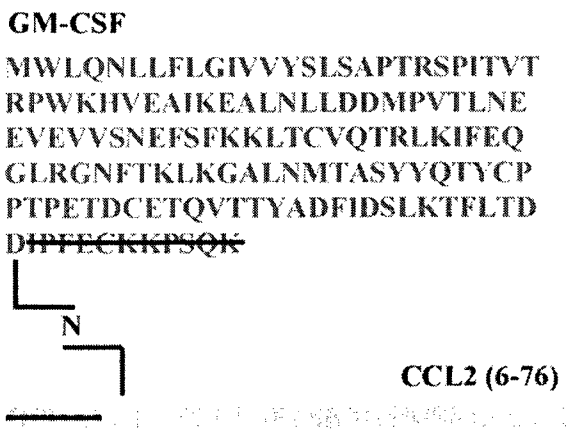
6.4.3 Biochemical Effects of GMME1 on Pathogenic Lymphoid Cells

To understand the molecular mechanism of GMME1 on lymphocytes involved in the pathogenesis of EAE, splenocytes collected from EAE mice were stimulated *in vitro* with MOG alone, in the presence of rCCL2 or a combination of rCCL2 and GMME1 to examine the effect of the fusokine under competitive conditions. A quantitave RT-PCR (qRT-PCR) array was then performed on isolated RNA to identify modulated

Figure 33: Design and Expression of GMME1.

(A) Schematic Representation of the GMME1 aminoacid sequence. **(B)** GMME1-CCR2 Interaction. Structural models of CCR2 and GMME1 predicted by homology modeling and placed according to the putative interacting surfaces. CCR2 is coloured according to the secondary structure and its seven trans-membrane helices are labelled as TM1-TM7. The solvent accessible surface of CCR2 is in grey transparent. GMME1 is in yellow and blue ribbons. Residues Y138, R160, P162, K163 and R174 in GMME1 (greyish spheres) are the CCL2 residues implicated in the interaction with CCR2. (**C**) Denaturing immunobloting using CM from transiently transfected HeLa cells with GMCSF, CCL2 or GMME1 probed with polyclonal goat anti-CCL2. Recombinant CCL2 was used as positive controls.

A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EVEVVSNEFSFKKLTCVQTRLKIFEQ
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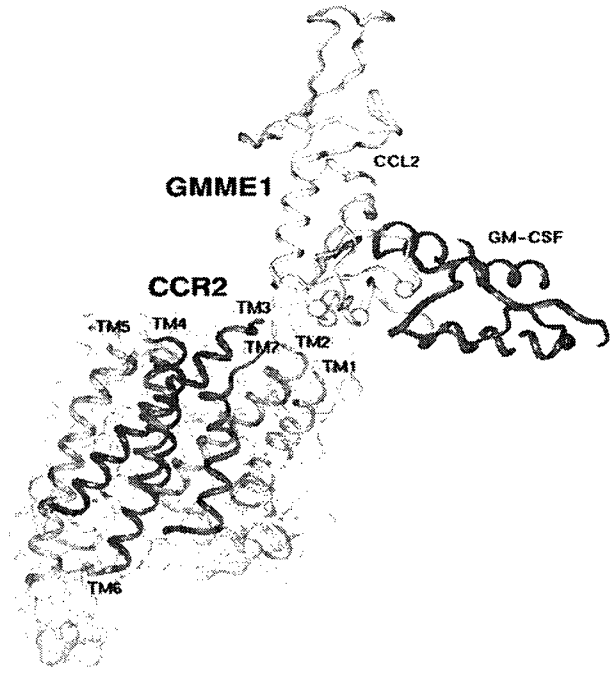
N
CCL2 (6-76)

B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GMME1
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C

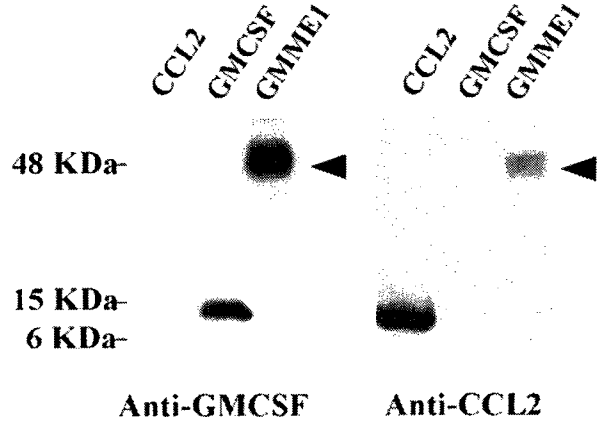
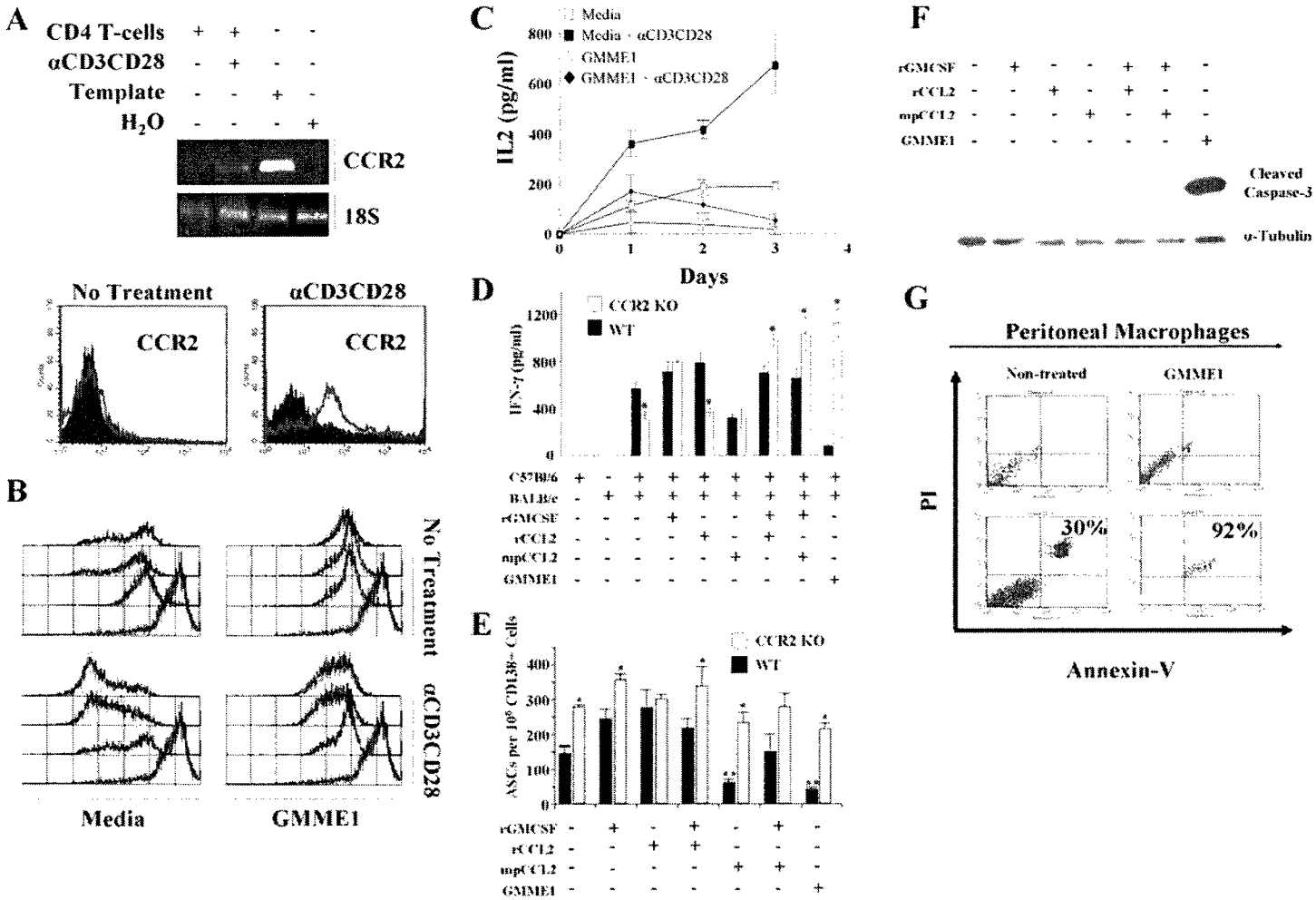

CCL2
GMCSF
GMME1
CCL2
GMCSF
GMME1
48 KDa-
15 KDa-
6 KDa-
Anti-GMCSF
Anti-CCL2

Figure 34: Pharmacological Effects of GMME1 on Activated Lymphocytes.

(**A**) RT-PCR and flow cytometry analysis demonstrate induction of CCR2 expression following CD3/CD28 stimulation. (**B-C**) CD3/CD28 stimulated CFSE-labeled CD4 T-cells were cultured with GMME1 or media. GMME1 can robustly block CD4 proliferation and IL2 secretion. (n=3 per group with a $P<0.05$). Results are shown as mean $\pm$ SD (**D**) Using a 1-way MLR with fixed BALB/c but live C57Bl/6 splenocytes for the $CCR2^{-/-}$ MLR and a 2-way MLR with live splenocytes (n=5 per group with a $P<0.05$). Results are shown as mean $\pm$ SD (**E**) Same outcome occurred in the ELISPOT assay demonstrating that GMME1 can block antibody production from plasma cells. (n=5 per group with a $P<0.05$). Results are shown as mean $\pm$ SD (**F-G**) Peritoneal macrophages cultured for 24 hrs with test conditions then cell lysate probed for cleaved caspase-3. GMME1-induced apoptosis was confirmed by PI/Annexin-V staining.


A
CD4 T-cells + + - -
αCD3CD28 - + - -
Template - - + -
H2O - - - +
CCR2
18S
No Treatment
αCD3CD28
CCR2
CCR2
B
No Treatment
αCD3CD28
Media
GMME1
C
Media
Media + αCD3CD28
GMME1
GMME1 + αCD3CD28
IL2 (pg/ml)
Days
D
CCR2 KO
WT
IFN-γ (pg/ml)
C57Bl/6
BALB/c
rGMCSF
rCCL2
mpCCL2
GMME1
E
CCR2 KO
WT
ASCs per 10^6 CD138+ Cells
rGMCSF
rCCL2
mpCCL2
GMME1
F
rGMCSF
rCCL2
mpCCL2
GMME1
Cleaved Caspase-3
α-Tubulin
G
Peritoneal Macrophages
Non-treated
GMME1
PI
30%
92%
Annexin-V

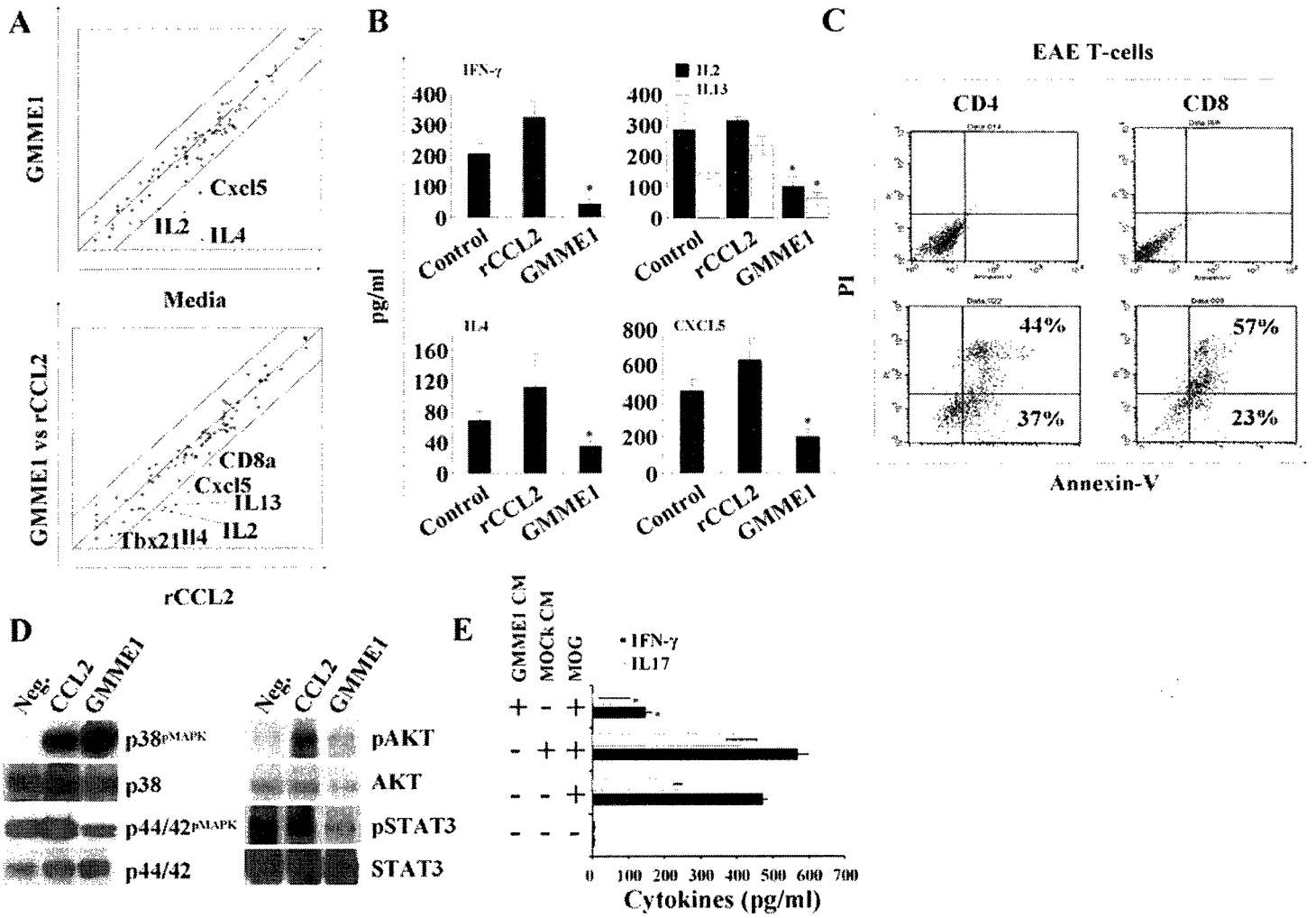
inflammatory genes. As shown in **Fig. 35A,** GMME1 lead to the downregulation of interleukin (IL)2, IL4, IL13, CXCL5 as well as the transcription factor T-box 21 involved in IFN-γ induction even in the presence of rCCL2 in a 1:1 molar ratio to GMME1. All identified factors were then confirmed by ELISA (**Fig. 35B**). Interestingly, following CD4 and CD8 T-cells purification from EAE mice and GMME1 treatment for 48 hrs, almost 80% of both cell populations were seen undergoing apoptosis (**Fig. 35C**). To understand GMME1's effect on molecular pathways, purified CD4 T-cells stimulated for 5 min with GMME1 or rCCL2 demonstrate an asymmetrical activation of the MAPK pathway by hyper-activating p38 while inhibiting p44/42 phosphorylation (**Fig. 35D**). Furthermore, GMME1 was capable of completely blocking both AKT and STAT3 activation as opposed to rCCL2 clearly showing antagonizing properties on the AKT and JAK-STAT pathways (**Fig. 35D**). To examine the significance of these findings on EAE CD4 lymphocytes implicated in pathology induction, we cultured purified EAE CD4 T-cells in the presence of fixed syngeneic peritoneal macrophages presenting MOG_{35-55} under different test conditions. As such, macrophages act as stimulators without the capacity of secreting soluble factors that might interfere with the assay. As expected, $CCL2^{-/-}$ MSC conditioned-media (CM) significantly induced both IL17 and IFN-γ, pro-inflammatory cytokines implicated in promoting EAE. However, GMME1 significantly inhibited their secretion from responder CD4 T-cells **(Figure 35E)**.

6.4.4 GMME1 Delivery to EAE Mice Ameliorates Pathology

As an experimental method to test delivery GMME1 in a continuous manner to EAE mice, we chose the use of gene-engineered Mesenchymal Stromal Cells (MSCs) as an *in vivo* delivery platform. Our group has previously demonstrated that MSCs engineered to produce plasma proteins, embedded in biomatrix and implanted subcutaneously form a neo-organoid – in essence a synthetic ectopic endocrine tissue – which allows for long term delivery of proteins such as erythropoietin [393].

Figure 35: GMME1 Pharmacological and Biochemical Responses.

(**A-B**) Splenocytes derived from EAE mice were re-stimulated with MOG with or without GMME1 and qRT-PCR analysis was performed. Relative changes in RNA levels as compared between the 2 treatments were then confirmed by ELISA to detect the modulated factors at the protein level. ($n=5$ per group with a $P<0.005$). Results are shown as mean $\pm$ SD (**C**) Following 48 hrs of splenocytes culture with media (top panel) versus GMME1 (lower panel), CD4 and CD8 T-cells were analyzed by flow cytometry following Annexin-V/PI co-staining. (**D**) Purified CD4 T-cells were stimulated for 5 min with rCCL2 or GMME1. GMME1 leads to asymmetrical signalling in the MAPK pathway (p38 and p44/42) whereas the AKT and JAK-STAT pathways are inhibited. (**E**) GMME1 addition reduced the secretion levels of IL17 and IFN-γ ($n = 5$ per group and $P<0.0003$).


A
GMME1
Cxcl5
IL2
IL4
Media
GMME1 vs rCCL2
CD8a
Cxcl5
IL13
Tbx21
Il4
IL2
rCCL2
B
IFN-γ
IL2
IL13
IL4
CXCL5
pg/ml
400
300
200
100
0
160
120
80
40
800
600
Control
rCCL2
GMME1
C
EAE T-cells
CD4
CD8
PI
44%
37%
57%
23%
Annexin-V
D
Neg.
CCL2
GMME1
p38pMAPK
p38
p44/42pMAPK
p44/42
pAKT
AKT
pSTAT3
STAT3
E
GMME1 CM
MOCK CM
MOG
IFN-γ
IL17
0
100
200
300
400
500
600
700
Cytokines (pg/ml)

Since MSCs are known to secrete CCL2 and its cleaved variant [358], we utilized MSCs derived from $CCL2^{-/-}$ mice as a cell-based platform to deliver GMME1 *in vivo*, thus avoiding any bias arising from contemporaneous CCL2 secretion. Therefore, to evaluate the *in vivo* efficacy of GMME1 under pathological conditions, C57Bl/6 mice with pre-established EAE were implanted with a neo-organoid contigen implant containing $CCL2^{-/-}$ MSCs gene-engineered to secrete GMME1. The continual delivery of the fusokine led to a progressive and stable recovery of EAE disease score up to 2 months with no apparent relapse. One advantage of using neo-organoid implants is the possibility to surgically remove the implant with full reversal of *in vivo* protein delivery as we have previously shown [393]. Therefore, we tested whether GMME1's removal from EAE remitted mice affected outcome. To verify whether GMME1 levels are absent from circulation upon implant removal, an ELISA was performed on mice sera at different time points. GMME1's level was detected as of the first week post-implantation and persisted up to the fifth week in mice bearing the contigen implant (**Fig. 36B**), whereas no detectable levels of the fusokine was observed 1 week post-removal (**Fig. 36C**). Mice whose GMME1 implants were removed remained in EAE remission for duration of observation. Spleens from control or GMME1 implanted mice were analyzed at week 2 and 5. Splenic atrophy was observed in the GMME1 group early during inflammation (week 2) but resolved to full size at week 5 (**Fig. 36D**). Furthermore, the spleen of mice whose GMME1 implant was removed looked similar to the remaining control groups (**Fig. 36D**). Flow cytometric analysis of lymphoid cells contained in the smaller spleens of GMME1-treated mice revealed a lympho-depletion of $CD3^+$ and $CD19^+$ cells occurring at week 2 with normal levels at week 5 once the pathology was resolved (**Fig. 36E**). However, peripheral blood leukocyte counts over the same period were not significantly affected (**Fig. 36F**). *In vitro* re-stimulation of splenocytes derived from treated or control EAE mice demonstrate a weak proliferation in the GMME1 or GMME1 removed implant group with a robust decrease in levels of IFN-γ (**Fig. 36G**).

6.4.5 Hematological and Pathological Analysis of EAE Mice Treated with GMME1

Plasma cytokine analysis show that the presence of the fusokine correlates well with the decrease in levels of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL17 in circulation (**Fig. 37A**). A noticeable improvement was also obtained at the humoral level since MOG antibody titer decreased significantly in GMME1-treated mice (**Fig. 37B**). One of the most important hallmarks of EAE is spinal cord infiltration by MOG-specific CD4 T-cells. Once they have crossed the blood-brain barrier, CD4 T-cells get reactivated by microglia cells presenting the MOG peptide leading to the induction of pro-inflammatory cytokines and chemokines, which in turn will amplify inflammation, neuronal damage and recruitment of further effector immune cells [345]. Analysis of CD4 and CD8 T-cell absolute cell number in the spinal cord demonstrate a large infiltration of immune cells in the EAE control group as opposed to the GMME1 treated group or in mice where the implant was removed (**Fig. 37C**). The same outcome was obtained by H&E staining of spinal cords or through immunohistology for CD4 and CD8 lymphocytes (**Fig. 37B**) demonstrating the potency of GMME1 in robustly blocking inflammatory cell migration to the CNS most likely due to their cell death in the periphery.

6.5 DISCUSSION

Chemokines and their receptors are involved in normal physiological immune responses but can, under certain circumstances, participate in maladaptive pathological immune reactions [383]. For example, CCR2 is largely implicated in the pathophysiology of graft-versus host disease, EAE, inflammatory bowel disease and other inflammatory diseases [173, 351, 395-397]. As a result, CCR2 as part of the family of GPCRs, has generated considerable interest as a potential target for treatment of autoimmune and alloimmune ailments. Various difficulties, however, were encountered in generating compounds that can specifically target CCRs. For instance, the development of intrakine [398] or degrakine molecules [399], which are specific

Figure 36: GMME1 Inhibits EAE.

(A) Implants secreting GMME1 inhibit EAE progression and its removal did not lead to relapse. (n = 10 per group). **(B-C)** The fusokine was detected in circulation up to the 5^{th} week whereas it disappeared upon implant removal. (n=5 per group and $P<0.0008$). Data are shown as average ± SD. **(D)** Noticeable atrophy was observed at week 2 post-implantation whereas spleen size recovered at week 5 once mice have recovered from EAE. (**E**) A significant decrease in $CD3^+$ and $CD19^+$ cells were noticed only at the 2^{nd} week in the GMME1 group (**F**) GMME1 does not lead to systemic lymphomyeloid depletion in circulation as observed in the spleen (**G**) Splenocytes derived from mice were re-stimulated with MOG. Both GMME1 groups had less proliferative activity consistent with the decrease in IFN-γ secretion. (n=5 per group and $P<0.005$). Data are shown as average ± SD.

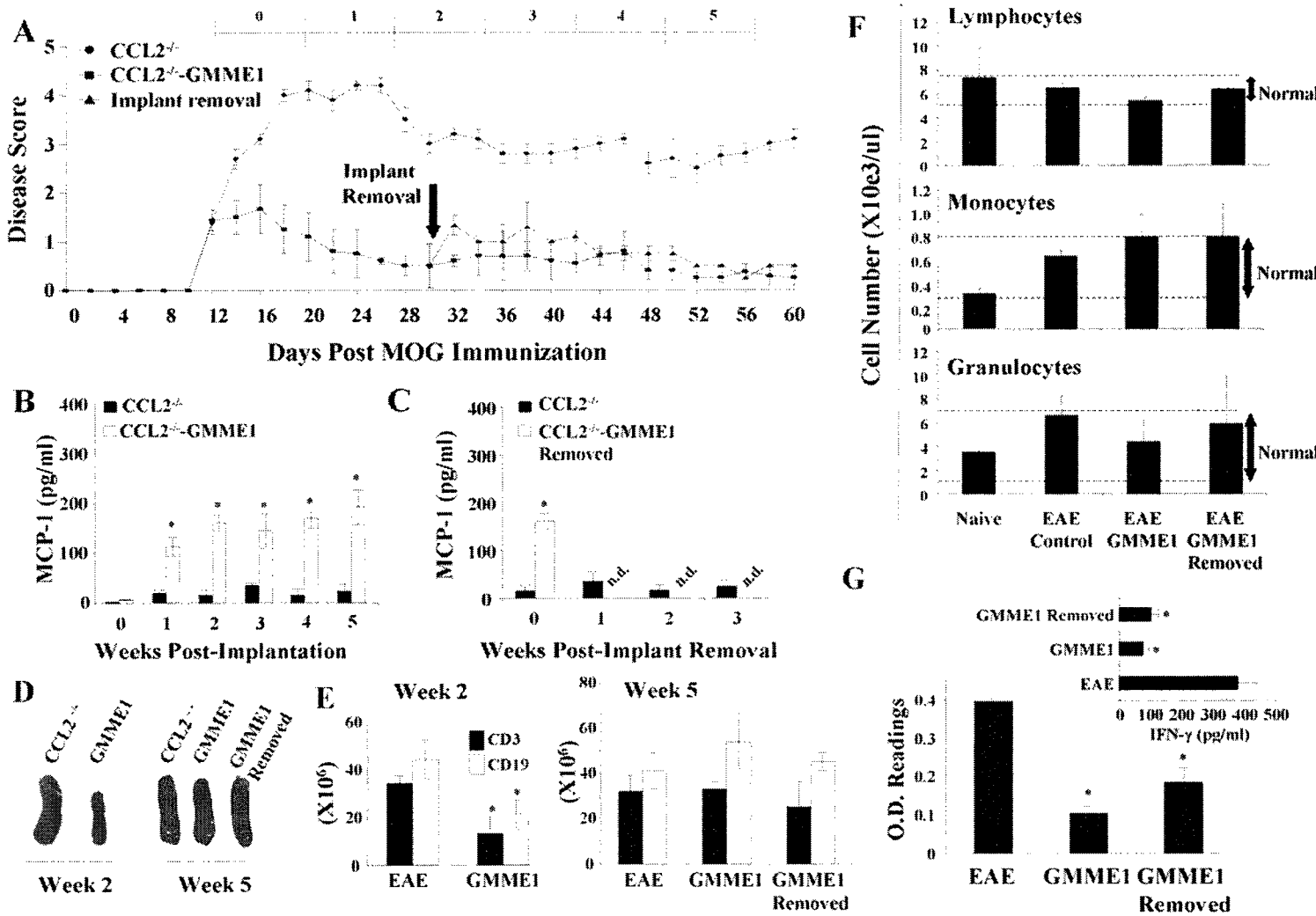
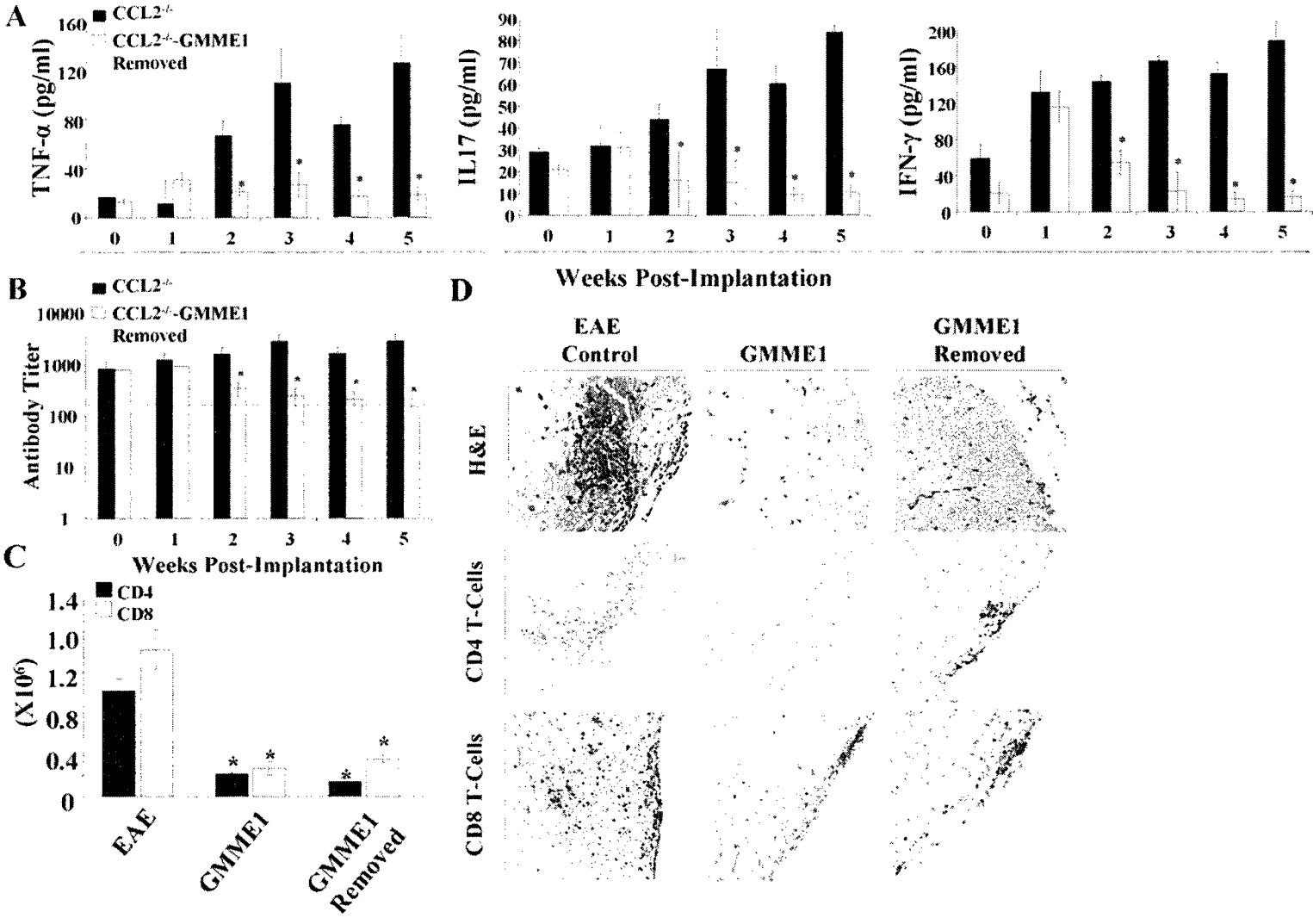

A
Disease Score
CCL2-/-
CCL2-/--GMME1
Implant removal
Implant Removal
Days Post MOG Immunization
B
MCP-1 (pg/ml)
CCL2-/-
CCL2-/--GMME1
Weeks Post-Implantation
C
MCP-1 (pg/ml)
CCL2-/-
CCL2-/--GMME1 Removed
n.d.
Weeks Post-Implant Removal
D
CCL2-/-
GMME1
GMME1 Removed
Week 2
Week 5
E
Week 2
Week 5
(X10^6)
CD3
CD19
EAE
GMME1
GMME1 Removed
F
Lymphocytes
Monocytes
Granulocytes
Cell Number (X10e3/ul)
Normal
Naive
EAE Control
EAE GMME1
EAE GMME1 Removed
G
GMME1 Removed
GMME1
EAE
IFN-γ (pg/ml)
O.D. Readings
EAE
GMME1
GMME1 Removed

Figure 37: Hematological and Pathological Analysis.

(**A**) Levels of TNF-α, IL17 and IFN-γ in mice sera were assessed by ELISA and demonstrate a decrease in the GMME1 group. (n=5 per group and $P<0.008$). Data are shown as average ± SD. (**B**) MOG-specific antibody titers were analyzed by ELISA and demonstrate a robust decrease in antibody levels in mice with the GMME1-implant. (n=5 per group and $P<0.005$). Data are shown as average ± SD. (**C**) CD4 and CD8 lymphocytes infiltrating the spinal cord were analyzed by flow cytometry. Both cell populations decreased in absolute numbers in the GMME1 and GMME1 sensitized group. (n=5 per group and $P<0.0008$). Data are shown as average ± SD. (**D**) H&E staining demonstrates a robust infiltration of immune cells as opposed to the GMME1 groups. The same outcome was obtained for CD4 and CD8 immunostaining.


A
CCL2-/-
CCL2-/--GMME1 Removed
TNF-α (pg/ml)
IL17 (pg/ml)
IFN-γ (pg/ml)
Weeks Post-Implantation
B
Antibody Titer
C
CD4
CD8
(X10⁶)
EAE
GMME1
GMME1 Removed
D
EAE Control
GMME1
GMME1 Removed
H&E
CD4 T-Cells
CD8 T-Cells

chemokines linked to an endoplasmic reticulum (ER) retention signal sequence (KDEL) on their carboxy termini, have been shown to sequester target GPCRs in the ER to prevent their transport to the cell surface or induce their degradation. Even though efficient in preventing or reducing chemokine stimulation, these molecules were linked to intracellular toxicity not to mention their passive diffusion outside of the cell [400]. Nevertheless, transducing target cells is required for the success of this strategy, an approach that is unfeasible in the context of ubiquitous expression of target GPCR [400]. We are proposing in this study, a strategy enabling the direct targeting of GPCR. We chose CCR2 due to its wide involvement in various immune pathologies and expression profile on target effector cells. The developed novel fusokine, GMME1, is capable of specifically targeting CCR2 on activated T-cells or macrophages leading to their cell death or inhibition of antibody production from plasma cells. In addition, GMME1 can actively compete for CCR2 in the presence of its ligand CCL2 demonstrating a high capacity for competitive antagonism leading to a blockade in inflammatory cytokine secretion.

The MAPK, AKT and JAK-STAT pathways are important for the induction of EAE pathologies [401-403]. One specific example is the recruitment of tyrosine kinases to CCR2 upon rCCL2 binding to CCR2 and phosphorylation of the tyrosine residue 139 promoting CCR2 homodimerization for consequent downstream signalling [404]. The sum of the unheralded biochemical effects of GMME1 demonstrate an asymmetrical signalling taking place in the MAPK pathway in addition to the complete inhibition of AKT and JAK-STAT activation leading to cellular apoptosis in a caspase-3 dependent manner. We can also conclude that GMME1 tropism seems to be CCR2 specific since C57Bl/6 $CCR2^{-/-}$ splenocytes were completely refractory to the biochemical effects of GMME1. The fact that GMME1 pharmacological effects are lost on $CCR2^{-/-}$ cells suggest high target selectivity and we cannot invoke binding to and signalling through other CCR receptors.

In light of the potent depletion of CCR2-expressing lymphomyeloid cells induced by GMME1 *in vitro*, we tested whether its expression directly *in vivo* could lead to EAE

recovery in C57BL/6 mice. We found that within a fortnight, GMME1 lead to near full recovery from symptomatic pre-established EAE. In addition, spleen atrophy observed at week 2 post GMME1 administration is consistent with lymphomyeloid depletion and is also completely reversible as demonstrated in the spleen of mice on long-term GMME1 following implant removal.

In comparison to the previously reported antagonist derivatives of CCL2, our study demonstrates that GMME1 mechanism of action is distinct. Rather than behaving as a passive dominant negative competitive inhibitor of native CCL2 binding to CCR2, GMME1 displays novel CCR2-driven signalling which qualifies it as a distinct, *bona fide*, chimeric chemokine with properties diametrically opposite to that of native CCL2. Indeed, GMME1's activity usurps CCR2 signal transduction machinery and leads to subsequent apoptotic cascade. GMME1's cell biochemistry is also distinct from that of CCL2 post translational derivatives or mutants MCP-1 (9-76) [405] or 7ND [406]. These latter polypeptides are thought of as non-biologically active decoys capable of preventing cells from responding to chemokines, blocking induction of adhesion molecules, or changes in cytoskeleton. Furthermore, P8A-MCP-1, a mutated form of CCL2, was shown to possess antagonist activity due to its ability in downregulating the MAPK pathway, which could also be due to its binding capacity to CCR2 without triggering any further biochemical response [397]. Taken together, our experiments demonstrate that GMME1 possesses novel biochemical properties that are distinct from CCL2 and its MMP-derived antagonist derivatives via its specific effect on CCR2-expressing lymphoid cells.

In conclusion, GMME1 represents the first member of a new class of chimeric cytokines which co-opts CCR2 signal transduction machinery and leads to selective and potent apoptosis of CCR2-expressing lymphomyeloid cells and consequent immune suppression. We propose that the GMME1 fusokine could be therapeutically exploited in the treatment of CCR2-driven autoimmune and alloimmune ailments as a classic protein biopharmaceutical.

6.6 ACKNOWLEDGMENTS

Moutih Rafei is a recipient of a *Fonds de Recherches en Santé du Québec (FRSQ)* Scholarship, and Jacques Galipeau is a *FRSQ chercheur-boursier sénior.*

6.7 DISCLOSURES

The authors declare that they have no competing financial interests.

CHAPTER 7

An Engineered GMCSF-MCP1 Fusokine is a Potent Inhibitor of CCR2-driven Inflammation as Demonstrated in a Murine Model of Inflammatory Arthritis

Reference: Moutih Rafei, Yamina A. Berchiche, Elena Birman, Marie-Noëlle Boivin, Nikolaus Heveker, and Jacques Galipeau *(Submitted 2009).*

Preface to Chapter 7:

We successfully demonstrated in Chapter 6 that the fusokine GMME1 could selectively deplete CCR2-expressing cells in a Th1-driven autoimmune disease. As an extension of the previous study, we tested the suppressive properties of GMME1 in an autoimmune disease implicating both cellular (Th1)/(Th17) and humoral (Th2) pathogenic processes. For such, we chose the collagen-induced arthritis mouse model which parallels the human Rheumatoid Arthritis equivalent.

CHAPTER 7: An Engineered GMCSF-MCP1 Fusokine is a Potent Inhibitor of CCR2-driven Inflammation as Demonstrated in a Murine Model of Inflammatory Arthritis

7.1 ABSTRACT

Objective. CCR2 is widely expressed by lymphomyeloid cells involved in maladaptive autoimmune ailments. Therefore CCR2 is of great interest as a biological target for immune suppression due to its direct implication in autoimmune diseases such as rheumatoid arthritis (RA).

Methods. We have generated a novel fusion protein using GMCSF and a N-terminal truncated version of MCP1 (6-76) (hereafter GMME1) and investigated its utility as a CCR2-specific immune suppressor.

Results. Using BRET studies, we found that, unlike CCL2, GMME1 did not induce conformational changes in the CCR2 homodimer, and did not induce the recruitment of ß arrestin 2 to the receptor. However, CCR2-dependent calcium influx, BAX induction and caspase-3 activation followed by cell death was observed. Using Th17 cells harvested from mice ill with bovine collagen-induced arthritis, we demonstrate that GMME1 is indeed capable of blocking the secretion of IL17 *in vitro*. Upon its delivery to mice symptomatic with inflammatory arthritis, a robust clinical recovery occurred with decreased paw thickness to normal levels, significant reduction in: anti-collagen antibody titer, Rheumatoid Factor titer as well as reduction of pro-inflammatory cytokines levels both intra-articularily and systemically.

Conclusion. Our data demonstrate that GMME1 is a powerful synthetic suppressor cytokine which co-opts CCR2 dependent cellular signaling to deplete lymphomyeloid cells causative of autoimmune arthritis.

7.2 INTRODUCTION

Rheumatoid arthritis (RA) is an autoimmune ailment with unmet medical needs characterized by joint inflammation leading to their erosion and destruction [407]. This

polyarthritis is shown to be mediated, in part, by chemokines such as CC ligand (CCL) 2 and 5; known chemotactic factors for monocytes and T-cells [408, 409]. Furthermore, the chemokine receptors CCR1, CCR2, and CCR5 have been previously reported in synovial fluids during RA pathology [408, 409]. The aim of present and future RA therapies would be to interfere with inflammation early on during disease onset to block further damage to joints. Two major types of treatments are available: anti-inflammatory drugs such as steroids, which are used to suppress symptoms, and disease-modifying antirheumatic drugs, for reversal of disease process [410]. Unfortunately, these treatments do not cure RA but rather alleviate the symptoms and/or modify disease progress. For example, various antibody constructs targeting TNF-α such as Infliximab [411], Adalmumab [412] or Etanercept [413] are highly effective for treatment of RA but remain less-than-perfect for sustained clinical remission. Furthermore, increased susceptiblity to infections is of concern [414]. Additional anti-inflammatroy strategies, including: soluble cytokine receptors, receptor antagonists, anti-inflammatory cytokines, or regulatory T-cells (Tregs) are promising yet perfectible [176]. The aggregate observations culled from these strategies suggest that blockade of cytokines or their receptors alone may not be sufficient due to the redundancy and orchestrated cross-talk among cytokines/chemokines.

We have engineered a novel pro-apoptotic fusokine consisting of granulocyte-macrophage colony stimulating factor (GMCSF) and truncated MCP-1 (aka CCL2); referred to as GMME1. The pharmacological effects of this fusokine consist of selectively depleting CCR2-expressing lymphomyeloid cells as we have previously demonstrated in the experimental autoimmune encephalomyelitis mouse model of multiple sclerosis (Rafei *et al.* Journal of Immunology *In Press* 2009). GMME1's mechanism of action depends upon binding to CCR2 and subsequently leading to aberrant signalling leading to MAPK over-activation and caspase-3 induced cell death. Since lymphocytes and monocytes implicated in RA initiation and progression express CCR2, we speculate that GMME1 could be used in this context as a CCR2-depleting agent capable of blocking articular inflammation and clinical seropositive arthritis. To investigate the therapeutic effect of GMME1, mice with pre-established bovine collagen-

induced inflammatory arthritis were implanted with a neo-organoid implant composed of $CCL2^{-/-}$ mesenchymal stromal cells (MSCs) gene-engineered to continually express and deliver GMME1 to the host, akin to what we have previously described [393, 415-417]. We analyzed the impact on clinical disease score as well as levels of systemic and articular inflammatory cytokines and found strong reduction both locally and systemically. In addition, pathological $CCR2^{+}$ lymphomyeloid cells were found to be depleted in both joints and circulation clearly demonstrating the powerful pharmaco-therapeutic effects of GMME1 in the collagen type II-induced mouse model of RA.

7.3 MATERIALS AND METHODS

7.3.1 Mice and Reagents

All used DBA/1 female mice were 6-8 weeks old (Jackson Laboratory, Bar Harbor, ME). Recombinant proteins (rGMCSF/rCCL2), their antibodies, MMP9 reagents, apoptosis detection kits, RT-PCR primers, ELISAs and ELISPOTS were purchased form R&D systems (Minneapolis, MN). Antibody for α-tubulin was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Cleaved caspase-3, p38, Ikbα and BAX antibodies were purchased from Cell Signalling Technology (Danvers, MA). rhMMP1 was purchased from Sigma-Aldrich (Oakville, ON, Canada). All flow cytometry antibodies and bovine collagen type-II (CII) were purchased from BD Biosciences (San Diego, CA). The Premo™ Cameleon Calcium Sensor was purchased from Invitrogen (Burlington, ON, Canada). CD4 purification kit was purchased from StemCell Technologies (Vancouver, Canada) and migration kit was obtained from Chemicon (Ontario, Canada). Rheumatoid factor (RF) ELISA was purchased from Cedarlane (Montreal, Qc, Canada). AllPrep DNA/RNA Mini Kit was purchased from Qiagen (Alabama, USA).

7.3.2 GMME1 Engineering and Expression

A 5 aa truncated form of mouse CCL2 (6-76) was amplified by PCR and cloned in frame with the cDNA encoding a 3'-truncated version of mouse GMCSF. The used plasmid

backbone is pCMV, a generic mammalian expression vector. HeLa cells were transiently transfected using Polyfect (Qiagen, Mississauga, ON, Canada) and supernatant tested by western blot for the detection of GMME1 using both GMCSF and CCL2 specific antibodies. HeLa cells were also transfected using pGMME1 and selected with G418 for a total period of 3 weeks to generate stable expression of the fusokine. The structural models GMME1 was obtained as reported previously (Rafei *et al.* Journal of Immunology *In Press* 2009)

7.3.3 Transfection and Resonance Electron Transfer Assays

HEK293T-cells were transiently transfected using the polyethylenimine method [418]. For the CCR2 homodimer Bioluminescence Resonance Energy Transfer (BRET), cells were transfected with CCR2-RLuc, alone or with YFP-tagged CCR2. For the β-arrestin recruitment assay, Rluc-tagged β-arrestin 2 was transfected, alone or with of YFP-tagged CCR2. Readings were then collected using a multidetector plate reader MITHRAS LB940 (Berthold Technologies, Bad Wildbad, Germany) allowing the sequential integration of the signals detected in the 480 ± 20 nm and 530 ± 20 nm windows for luciferase and YFP light emissions respectively. The BRET signal is determined by calculating the ratio of the light intensity emitted by the YFP-tagged receptor over the light intensity emitted by the RLuc-tagged protein. For Ca^{2+} mobilization, the mix of Premo™ cameleon calcium sensor reagent was used according to manufacture's instructions. The stimulations were performed over 2 min.

7.3.4 Apoptosis Analysis

10^6 HEK293T-cells expressing CCR2 cultured for 24 hrs with equimolar concentrations of rGMCSF, rCCL2, mpCCL2, rGMCSG/rCCL2, rGMCSF/mpCCL2, or GMME1 were stained for PI and annexin-V then analyzed by flow cytometry. To generate antagonist mpCCL2 *in vitro*, 10 ng of pure rhMMP1 was added directly to 50ug pure rCCL2 in PBS for a period of 4 hrs at 37°C. mpCCL2 was directly used in assays without further modifications. For cleaved caspase-3 analysis by western blot, CCR2-transiently

transfected HEK293T-cells were sorted, stimulated with the cytokine conditions then whole-cell lysates blotted with caspase-3 antibodies.

7.3.5 Induction of Mouse Arthritis

CII was emulsified in Complete Freund's Adjuvant (Cedarlane, Montreal, Qc, Canada) containing Mycobacterium tuberculosis H35RA (Difco Laboratories, Detroit, Michigan, USA) and injected subcutaneously (sc) at the base of the tail. The same injection was repeated 3 weeks later. Mice were clinically scored every week. Following the appearance of RA symptoms, all groups were normalized to possess a total average grade of 4 before receiving $CCL2^{-/-}$ MSCs expressing GMME1 as part of a contigen neo-organoid implanted subcutaneously ($5X10^6$cells per implant). Grading consisted of giving a score of 0-4 for each paw and adding all obtained scores for each mouse for a maximum of 16 per mouse. A paw score of 0: no signs; 1: slight swelling; 2: moderate swelling; 3: pronounced edema with limited joint usage; and 4: excess edema with joint rigidity. Mice were also bled for systemic cytokine analysis and assessment of circulating lymphomyeloid cells.

7.3.6 GMME1 Pharmacological Effects on RA-derived CD4 T-cells

After the purification of RA CD4 T-cells by SpinSep, lymphocytes were used in a migration assay using rCCL2 as the chemoattractant with a consistent dose of 1 pmol at the bottom chamber. To investigate the anti-chemotactic ability of the fusokine, an increasing concentration of GMME1 was added on the cells at the top chamber starting at 0.01 pmol and increasing by 2 fold. The migration was set-up over 12 hrs and cells at the bottom chamber were lysed, stained and read using a fluorescence reader. To investigate the effect of GMME1 at the molecular level, RA-derived CD4 T-cells were stimulated with rCCL2 or GMME1 for 10 min and both NF-κb and p38 were analyzed. For BAX analysis, the same purified population was cultured for a period of 48 hrs in the presence of equimolar concentration of rCCL2 or GMME1. Cells were then lysed and run on a 4-

20% gradient gel. As a confirmation of cell death induction, a PI/Annexin-V co-staining was performed.

7.3.7 APC Assays and IL17 Analysis

To analyse the effect of GMME1 on CD4 T-cells during antigen presentation, an assay was performed using peritoneal macrophages collected from retired breeder C57Bl/6 mice. Upon binding and washing of non-adherent cells, macrophages were treated with CII for 24 hrs, washed then fixed using 2% paraformaldehyde before the addition of RA-derived enriched CD4 T-cells under different treatment conditions. Supernatants were collected and centrifuged 72 hrs later for IL17 analysis by ELISA. For IL17 intracellular staining, CD4 T-cells were collected from the assay, washed, labelled with CD4 before fixation. After the wash, stained cells were permeabilized, stained with anti-IL17A antibody then analyzed by flow cytometry.

7.3.8 Inflammation Analysis

After removal of fat and muscle, joints were minced into small pieces with scissors, and resuspended in 0.5% trypsin solution for about 1 hour at 37°C. Tissues were then washed and incubated with a collagenese solution (2ug/ml) for another hour. The obtained cell suspension was centrifuged and resuspended in splenocytes media for further analysis. To induce an *in vitro* proliferation response, collected cells were cultured in the presence of increasing concentration of bovine CII and proliferation assessed by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma-Aldrich, Oakville, ON, Canada). For the analysis of lipolysaccharide CXC-chemokine ligand 5 (LIX), collected cells were plated for 48 hrs in splenocytes media and supernatant analyzed by ELISA. All remaining cytokines were analyzed by RT-PCR using RNA extracted from collected cells. The remaining cells were stained and analyzed by flow cytometry for the assessment of immune infiltrates. To analyse the systemic response, spleens of sick or treated mice were mechanically disrupted and cultured for 3 days with CII to assess proliferation. On the side, cells were also used in ELISPOT assays to detect the presence

of IFN-γ, TNF-α, IL17 and IL6. Supernatants were analyzed for MMP9 by ELISA and western blot. To analyse the humoral response induced in RA mice, ELISA plates were coated with 1mg/ml of CII then screened using diluted sera to calculate final titers. On the side, the sera were also used to detect levels of RF according to manufacturer's instructions.

7.3.9 Histological Analysis

Paws of sick or treated mice were removed, fixed in 4% formalin and decalcified for a week before performing Hematoxylin and Eosin (H&E) or masson's trichrome stainings. These sections were then used to analyze synovial inflammation and cartilage erosion using the following score: 0 for no change; 1 for partial change; and 3 for massive change.

7.3.10 Statistical Analysis

P values were calculated by paired Student *t*-test.

7.4 RESULTS

7.4.1 Engineering the GMME1 Fusokine

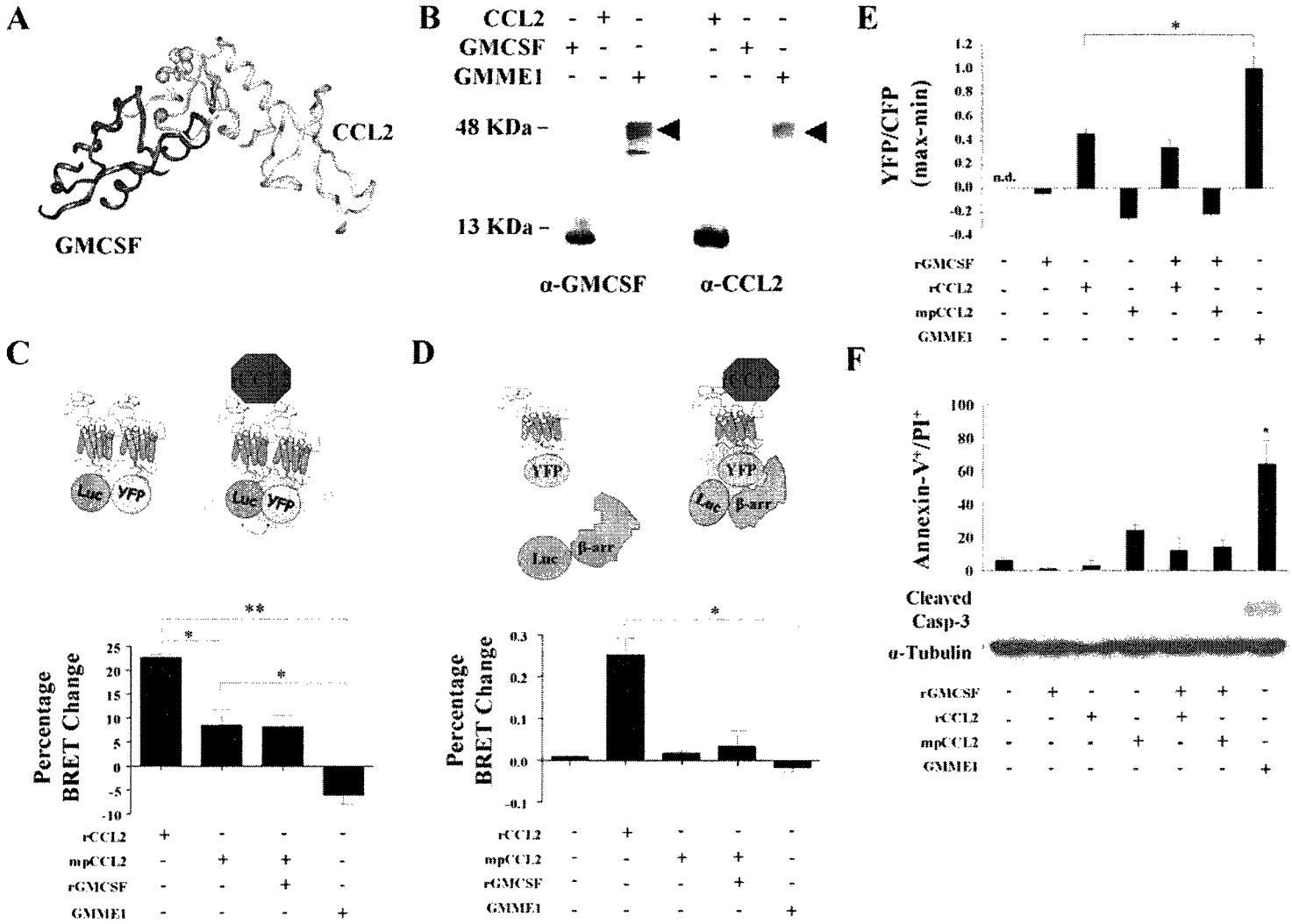
The GMME1 fusokine was created using a C-terminus truncated cDNA of mouse GMCSF in frame to the truncated 5' end of a mouse CCL2 cDNA as reported previously (Rafei *et al.* Journal of Immunology *In Press* 2009). The single aminoacid introduced (Asparagine) represents the linker. Computer-based analysis of the theoretical three-dimensional structure of GMME1 was predicted based on known GMCSF and CCL2 crystal structures (**Fig. 38A**). Denaturing immunoblotting performed on the supernatant from HeLa cells transiently transfected to express GMME1 showed that the chimeric protein is efficiently secreted in the extracellular space and has a molecular weight of 50 KDa (**Fig. 38B**).

7.4.2 Ligand-induced Modulation of CCR2 Behaviour and Biochemical Response

To detect potential differences in the conformational rearrangements of homodimeric CCR2 following GMME1 and CCL2 binding to the receptor, HEK293T-cells were cotransfected with CCR2 constructs fused to BRET energy donor (Renilla luaciferase, RLuc) and acceptor (the yellow fluorescence protein YFP). Using these fusion proteins, previous BRET studies have demonstrated that CCR2 forms constitutive homodimers in the absence of ligand, and that CCL2-binding induces conformational changes within the receptor dimer that can be monitored as BRET changes [419]. In addition, such conformational sensors show distinct BRET changes in response to different receptor ligands, suggestive of distinct functional consequences of ligand binding [419]. As shown in **Figure 38C**, incubation of CCR2-RLuc/CCR2-YFP expressing HEK293 cells with CCL2 induced a robust BRET increase. Since we have previously demonstrated that MMP-processed CCL2 (mpCCL2) is profoundly inhibitory on immunoglobulin production by CCR2-expressing plasma cells [358], we compared the effect of this truncated form of CCL2 vs GMME1. This effect is significantly attenuated by mpCCL2, due to changes in the receptor that are different from those induced by full-length chemokine. Incubation with GMME1 induced a slight BRET decrease, significantly distinct from the signals induced by both full length CCL2 and mpCCL2, suggestive of distinct conformational effects of GMME1 upon CCR2 binding. To determine signalling downstream of CCR2 induced by GMME1, CCL2 and mpCCL2, respectively, we investigated the recruitment of β-arrestin 2 to CCR2 using BRET. β-arrestin 2 plays a primordial role in receptor desensitization [420]. As opposed to CCL2 which robustly recruits β-arrestin 2 to CCR2, GMME1 and MMP-processed mpCCL2 failed to produce such a response (**Fig. 38D**). To study the consequence of GMME1 distinct signalling on intracellular Ca^{2+} mobilization, CCR2-expressing HEK-293T-cells were further modified using the Premo™ calcium sensor reagent to allow the expression of cameleon, a calmodulin molecule fused to cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). If Ca^{2+} molecules are mobilized, they will bind to cytoplasmic calmodulin and allow a change in the conformation of the entire protein leading to changes in the

Figure 38. Construction and Expression of GMME1.

(A) Schematic representation of the predicted 3D structure of GMME1. (**B**) Denaturing immunobloting of GMME1 CM from transiently transfected HeLa cells probed with polyclonal goat anti-GMCSF or CCL2. **(C)** HEK293T-cells coexpressing CCR2-RLuc and CCR2-YFP were stimulated with 45nM CCL2, mpCCL2, or GMME1 and CCR2 homodimer and basal BRET was monitored. (n=3 per group with * = $P<0.05$, ** = $P<0.001$) (**D**) Using a BRET readout for β-arrestin 2 recruitment to CCR2, addition of 45 nM GMME1 or mpCCL2 on HEK293T-cells expressing RLuc-β-arrestin 2 and CCR2-YFP fusion proteins does not induce the recruitment of β-arrestin 2, as opposed to 45 nM full length CCL2 (n=3 per group with * = $P<0.05$). (**E**) Treatment of CCR2-expressing HEK293T-cells with GMME1 triggers a robust induction of Ca^{2+} release as opposed to the remaining control groups. Results are the average of three independent experiments performed in triplicate (n=3 per group with * = $P<0.05$). (**F**) A robust increase in apoptosis was noticed using GMME1 as opposed to the other conditions. To confirm apoptosis by caspase-3 activation, 10^6 HEK293T-cells expressing CCR2 were cultured for 48 hrs with 1 pmol of rGMCSF, rCCL2, mpCCL2, rGMCSF/rCCL2, rGMCSF/mpCCL2 or with GMME1, and cell lysate probed for cleaved caspase-3. α-tubulin protein was used as loading control. Results are shown as mean ± S.E.D (* = $P<0.05$).


A
CCL2
GMCSF
B
CCL2 - + - + - -
GMCSF + - - - + -
GMME1 - - + - - +
48 KDa –
13 KDa –
α-GMCSF
α-CCL2
E
YFP/CFP (max-min)
n.d.
rGMCSF - + - - + + -
rCCL2 - - + - + - -
mpCCL2 - - - + - + -
GMME1 - - - - - - +
C
Luc
YFP
Percentage BRET Change
rCCL2 + - - -
mpCCL2 - + + -
rGMCSF - - + -
GMME1 - - - +
D
YFP
Luc
β-arr
Percentage BRET Change
rCCL2 - + - - -
mpCCL2 - - + + -
rGMCSF - - - + -
GMME1 - - - - +
F
Annexin-V+/PI+
Cleaved Casp-3
α-Tubulin
rGMCSF - + - - + + -
rCCL2 - - + - + - -
mpCCL2 - - - + - + -
GMME1 - - - - - - +

emitted wavelengths. Upon the addition of the different chemokine test conditions, we observed Ca^{2+} mobilization as the BRET ratio increased following stimulation with rCCL2 or the combination of rGMCSF/rCCL2 (**Fig. 38E**). The use of mpCCL2 or its combination with rGMCSF led to a negative BRET signal suggesting inhibition of basal Ca^{2+} activity within the cell as opposed to GMME1, which induced robust Ca^{2+} mobilization (**Fig. 38E**). We analyzed apoptosis by flow cytometry looking at annevin-V/propidium iodine (PI) co-staining and found a substantial and significant proportion of GMME1 treated cells dead (55% annexin-V/PI positive) as opposed to controls (1% for rGMCSF; 11% rCCL2, 17% mpCCL2; 12% for rGMCSF/rCCL2; and 14% rGMCSF/mpCCL2) (**Fig. 38F**). Ca^{2+} influx is known to trigger cytochrome C release from mitochondria and pro-caspase-3 activation [421]. To test whether GMME1 induces caspase-3 activation, CCR2-expressing HEK293T-cells were cultured with the same chemokine test conditions as previous for 48 hrs and whole cell lysates were probed for cleaved caspase-3. A modest increase was noticed with the use of mpCCL2 as compared to the robustly significant activation seen with GMME1 (**Fig. 38F**).

7.4.3 Biochemical Effects of GMME1 on RA-derived CD4 T-cells

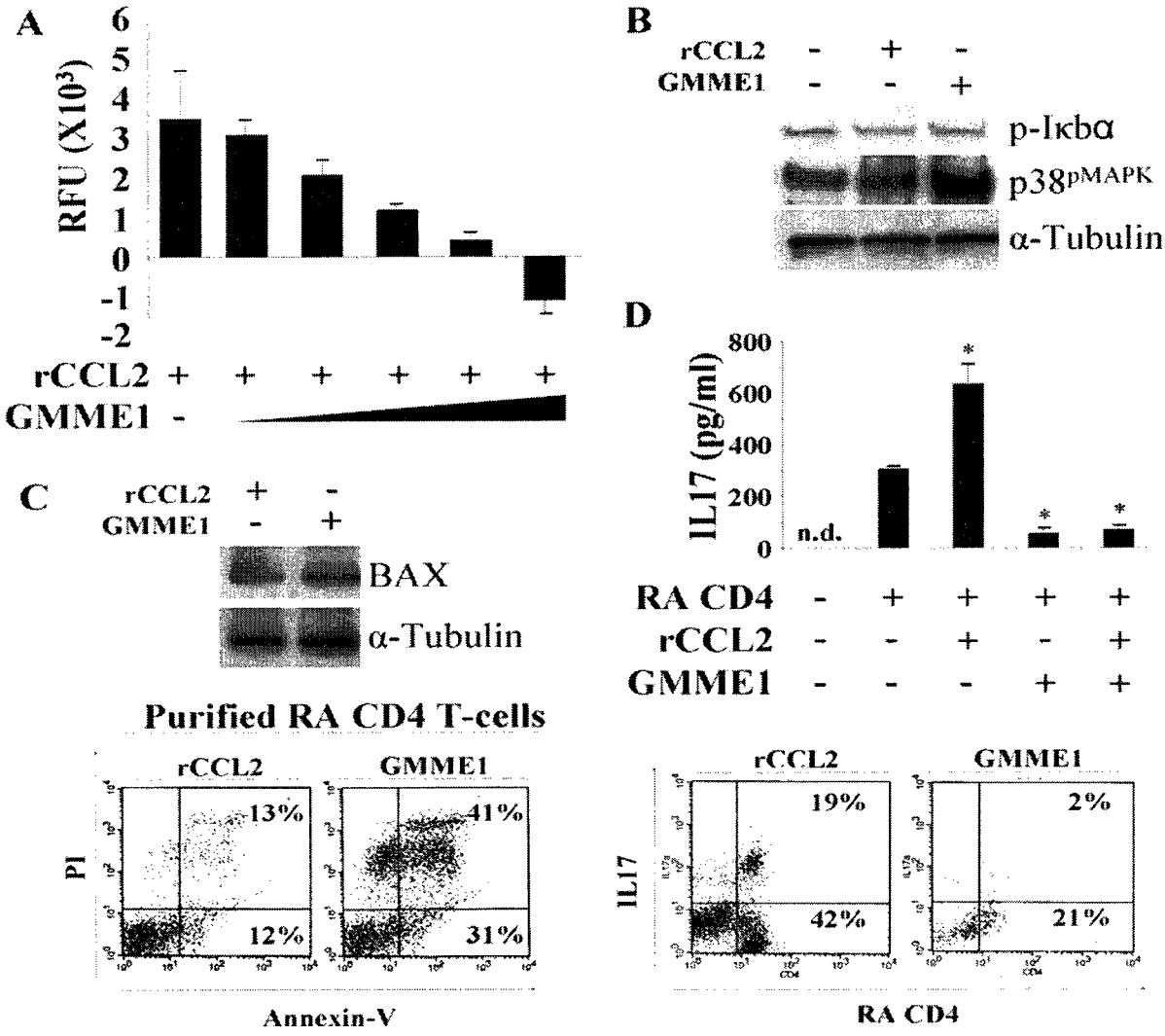
The major biochemical effect of CCL2 on $CCR2^{+}$ target cells is a chemotactic response [345]. Since GMME1 C-terminus moiety is composed of a truncated variant of CCL2 missing 5 aminoacid at its N-terminus, we investigated the chemotactic effect of the fusokine in the setting of a competitive assay using rCCL2 on purified RA-derived CD4 T-cells. We found that rCCL2 can trigger CD4 T-cell migration, whereas the addition of increased concentrations of GMME1 on the upper chamber inhibited such reaction until a complete migration blockade was observed (**Fig. 39A**). Since the NF-κB and MAPK pathways are implicated in chemotaxis in addition to RA pathogenesis [422], we wished to decipher the effect of the fusokine on these pathways. Interestingly, GMME1 does not lead to any noticeable effect on the phosphorylation of IκB-α, representative of NF-κB activation, whereas p38 was hyperphosphorylated, demonstrating a direct modulatory effect on the MAPK signalling pathway (**Fig. 39B**). Due to our previous observation that GMME1 induces apoptosis, we screened all pro-apoptotic proteins (Bcl-2 pro-apoptotic

family) by western blotting using CD4 T-cells cultured with rCCL2 or GMME1 for 48 hrs and found an increased expression of the BAX protein (**Fig. 39C**). This observation was confirmed by an increased cell death percentage as shown by PI/Annexin-V co-staining (80% cell death in the GMME1 group as opposed to 15% in the rCCL2 control group; **Fig. 39C**). To understand the significance of these findings on IL17 secretion, a pro-inflammatory cytokine involved in RA exacerbation, purified CD4 T-cells were cultured in the presence of fixed syngeneic peritoneal macrophages presenting CII under competitive GMME1 condition **(Fig. 39D)**. In this context, macrophages act only as stimulators without secreting soluble factors interfering with the assay. As expected, the addition of rCCL2 exacerbated IL17 from responder CD4 T-cells, whereas the addition of GMME1 in equimolar concentration to rCCL2 robustly prompted it **(Fig. 39D)**. Intracellular staining of CD4 T-cells for IL17 demonstrate that about 19% of responding T-cells are indeed secreting IL17 as opposed to about 2% under GMME1 treatment (**Fig. 39D**).

7.4.4 GMME1 Lead to RA Recovery and Depletion of Pathological Lymphomyeloid Cells

We chose RA as a mouse model to test the efficacy of GMME1 since $CCR2^{+}$ lymphocytes, granulocytes and macrophages are implicated simultaneously in the physiopathology of this ailment [423]. As an experimental method to test delivery GMME1 in a continuous manner to RA mice, we chose the use of gene-engineered Mesenchymal Stromal Cells (MSCs) as an *in vivo* delivery platform. Our group has previously demonstrated that MSCs engineered to produce plasma proteins, embedded in biomatrix and implanted subcutaneously form a neo-organoid – in essence a synthetic ectopic endocrine tissue – which allows for long term delivery of proteins such as erythropoietin [393]. Since MSCs are known to secrete CCL2 and its cleaved variant [358], we utilized MSCs derived from $CCL2^{-/-}$ mice as a cell-based platform to deliver GMME1 *in vivo*, thus avoiding any bias arising from contemporaneous CCL2 secretion, as previously described (Rafei *et al.* Journal of Immunology *In Press* 2009). Therefore, to evaluate the *in vivo* efficacy of GMME1 under

Figure 39. Pharmacological Properties of GMME1 on RA-Derived CD4 T-cells.
(**A**) CD4 migration assay towards CCL2 with increasing concentrations of GMME1. (**B**) GMME1 does not affect the NF-κB pathway but leads to p38 hyperphosphorylation when compared to CCL2 stimulation. (**C**) CD4 T-cells cultured with GMME1 for 48 hrs have higher levels of BAX protein as opposed to rCCL2. In addition, PI/Annexin-V co-staining demonstrates that GMME1 leads to apoptosis induction. (**D**) Fixed peritoneal macrophages presenting CII to RA-derived CD4 T-cells induce the secretion of IL17 and is up-regulated following the addition of CCL2. If GMME1 is added alone or in equimolrar concentration to rCCL2, a robust decrease is noticed. Intracellular staining of IL17 RA-derived CD4 T-cells demonstrates that 19% of responding T-cells are positive for this cytokine whereas about 2% are still found in the GMME1 treatment group. Results are shown as mean $\pm$ S.E.D (* = $P<0.05$).


A
RFU (X10³)
6
5
4
3
2
1
0
-1
-2
rCCL2 + + + + + +
GMME1 -
B
rCCL2 - + -
GMME1 - - +
p-Iκbα
p38pMAPK
α-Tubulin
C
rCCL2 + -
GMME1 - +
BAX
α-Tubulin
D
IL17 (pg/ml)
800
600
400
200
0
n.d.
*
RA CD4 - + + + +
rCCL2 - - + - +
GMME1 - - - + +
Purified RA CD4 T-cells
rCCL2
GMME1
PI
13%
12%
41%
31%
Annexin-V
rCCL2
GMME1
IL17
19%
42%
2%
21%
RA CD4

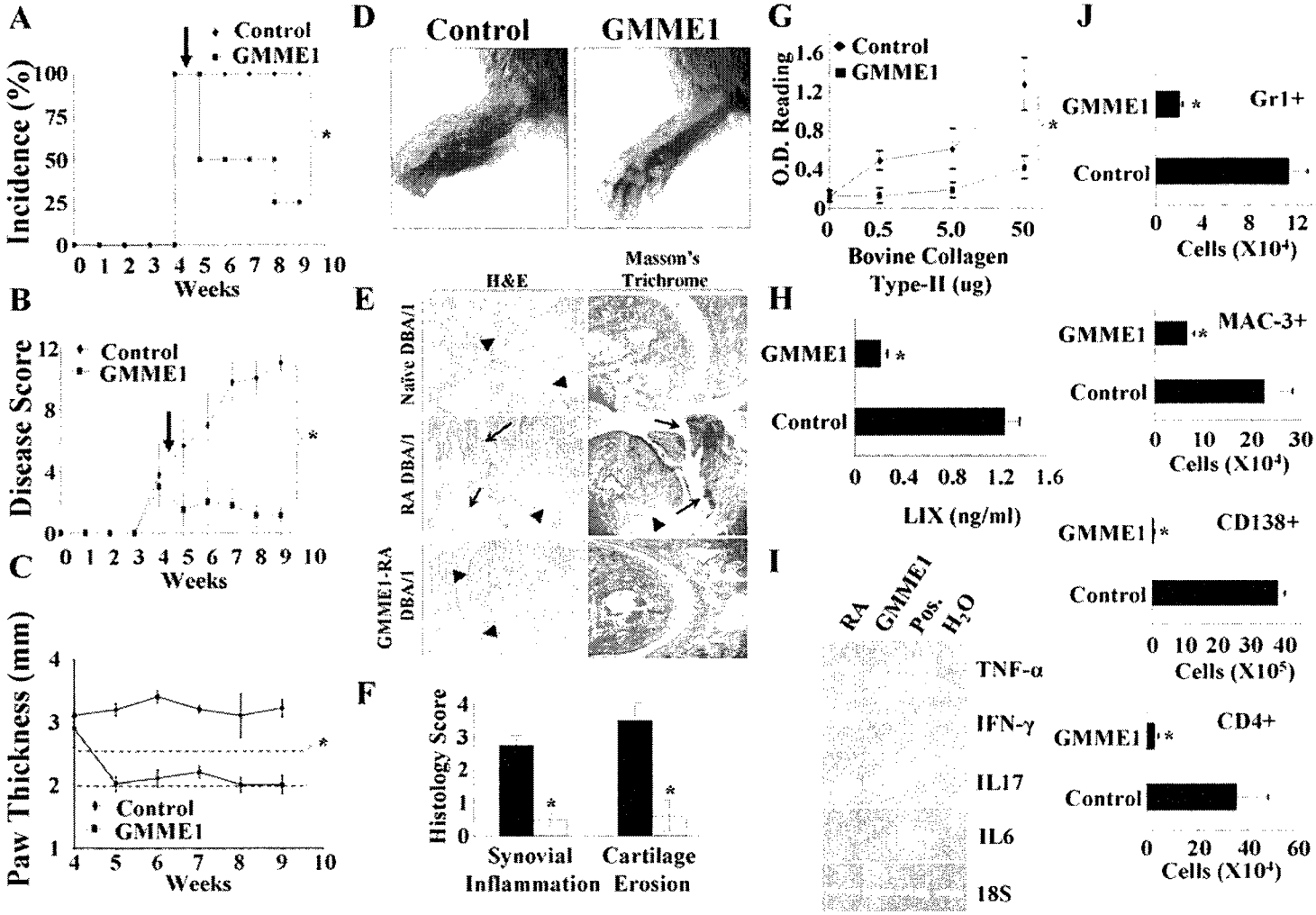
pathological conditions, CII-treated BDA/1 mice with pre-established RA were implanted with neo-organoids containing $CCL2^{-/-}$ MSC expressing GMME1. Following the neo-organoid implantation 4 weeks post immunization, we observed a decrease in RA incidence (**Fig. 40A**) in addition to a robust recovery of disease score in the GMME1 group (**Fig. 40B**). We analyzed paw thickness of both sick and treated mice and noticed a substantial and significant decrease in size to normal range (**Fig. 40C and D**). Histological and quantitative analysis of the whole joints demonstrate great similarities between naïve and GMME1-treated DBA/1 mice, whereas a robust infiltration of immune cells and bone erosion were observed in sick control mice. Similar results were obtained with the masson's trichrome staining (**Fig. 40F**). The obtained *in vivo* date correlates with the weak splenocyte recall response in the GMME1 group (**Fig. 40G**) as well as the secretion of LIX (**Fig. 40H**). Analysis of various other cytokine in the joints demonstrate a blockade in TNF-α, IFN-γ, IL17, and IL6 upon GMME1 treatment as opposed to the presence of these cytokines in the sick control mice (**Fig. 40I**). To further confirm these observations, analysis of joint infiltrates demonstrates a decrease in absolute cell number of granulocytes, macrophages, CD4 T-cell as well plasma cells (**Fig. 40J**).

7.4.5 Effect of GMME1 on Systemic Cytokines, Immune Cells, and Humoral Response.

RA mice with reversed clinical manifestation of articular disease showed a systemic increase of plasma detectable GMME1 as measured with the CCL2 ELISA as opposed to control sick mice (**Fig. 41A**). GMME1 increase correlates negatively with levels of TNF-α and IL17 in circulation (**Fig. 41A**). In addition, we observed that absolute numbers of circulating lymphocytes, monocytes as well as granulocytes decreased significantly (**Fig. 41B**). Since an important component of RA pathogenesis is humoral mediated, we analyzed CII-specific antibody titer and found a robust decrease in the GMME1 treated group (**Fig. 41C**). The levels of plasma RF were also significantly diminished (**Fig. 41D**).

Figure 40. GMME1 Leads to RA Recovery *In Vivo*.

(**A**) Delivery of GMME1 to RA mice leads to pathology reversal as only 25% of GMME1 treated mice still show pathological signs as opposed to 100% in the sick control group. (**B**) Similarly, a strong decrease in RA pathology score was noticed over 5 weeks of GMME1 treatment. (**C**) Paw thickness in GMME1 treated mice fall in the normal range of 2.4 $\pm$ 0.2 mm (shown in grey background) whereas sick mice have an average paw thickness of at least 3.2 mm. (**D**) Representative pictures of GMME1 versus sick mice paw. (**E**) H&E and Masson's trichrome histological analysis of mice joints demonstrate that both naïve and GMME1 treated DBA/1 mice have normal bone structures whereas sick mice show a powerful infiltration by immune-derived cells and bone destruction. Black triangles show bone structures whereas black arrows point at immune infiltrates. (**F**) Histological scores performed on stained sections demonstrate huge synovial infiltration and cartilage erosion in sick mice as opposed to GMME1-treated mice. (**G**) *In vitro* re-call response performed using joint immune infiltrates-derived from sick mice demonstrate a strong proliferative response to CII as opposed to GMME1-derived cells. (**H**) LIX analysis by ELISA and RT-PCR. (**I**) Screening of joints show a decrease in pro-inflammatory cytokines in the GMME1 group whereas strong induction of these cytokines is detected in control mice. (**J**) Concordantly, granulocytes, macrophages, plasma cells and T-cells were all found in diminished amounts in the GMME1 group as opposed to sick control mice. All Results are shown as mean $\pm$ S.E.D (* = $P<0.05$).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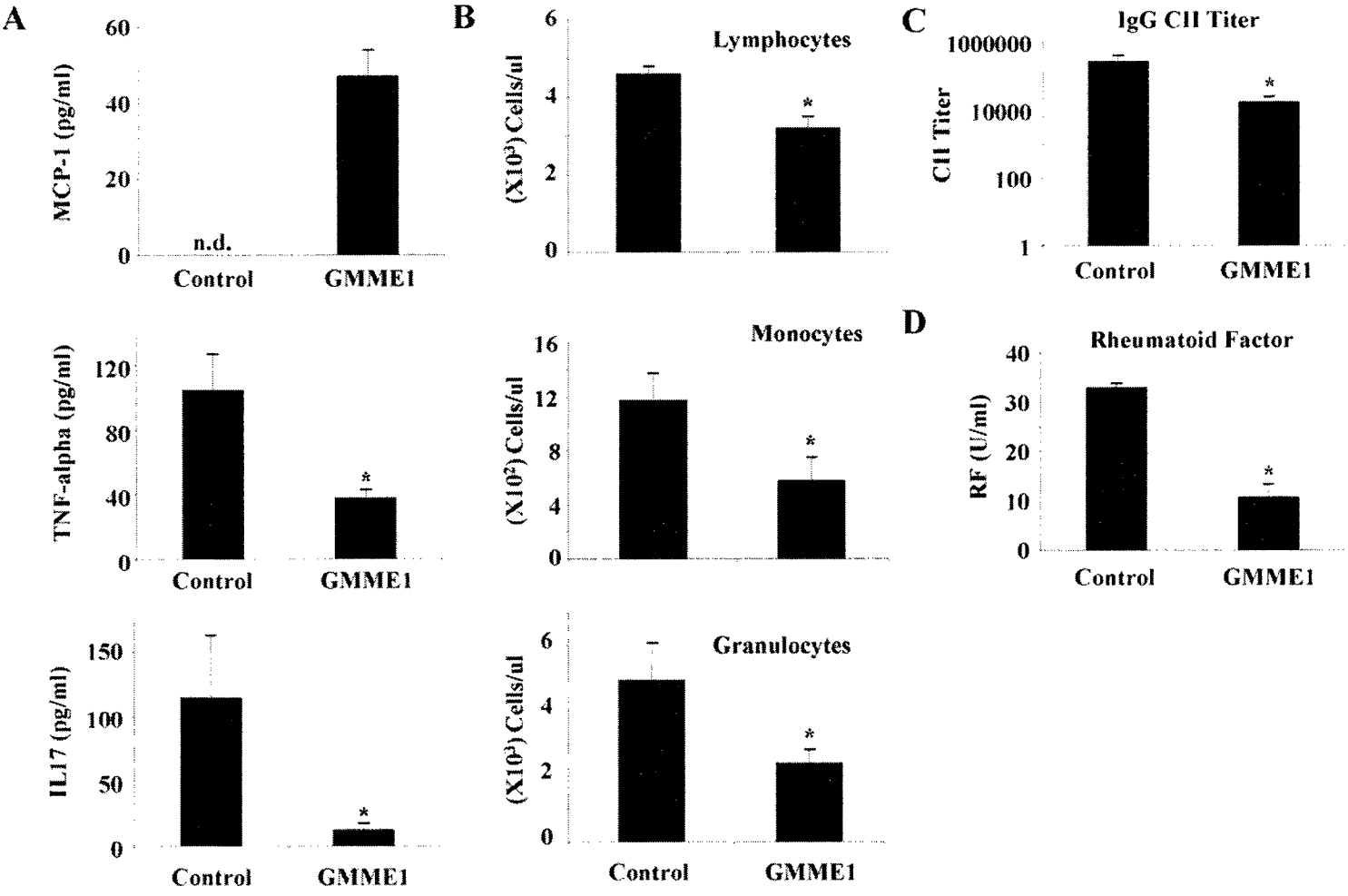
Control
GMME1
Incidence (%)
100
75
50
25
0
0 1 2 3 4 5 6 7 8 9 10
Weeks
*
B
Control
GMME1
Disease Score
12
8
4
0
0 1 2 3 4 5 6 7 8 9 10
Weeks
*
C
Paw Thickness (mm)
4
3
2
1
Control
GMME1
4 5 6 7 8 9 10
Weeks
*
D
Control
GMME1
E
H&E
Masson's Trichrome
Naïve DBA/1
RA DBA/1
GMME1-RA DBA/1
F
Histology Score
4
3
2
1
0
*
*
Synovial Inflammation
Cartilage Erosion
G
Control
GMME1
O.D. Reading
1.6
1.2
0.8
0.4
0
0 0.5 5.0 50
Bovine Collagen Type-II (ug)
*
H
GMME1
Control
*
0 0.4 0.8 1.2 1.6
LIX (ng/ml)
I
RA
GMME1
Pos.
H2O
TNF-α
IFN-γ
IL17
IL6
18S
J
GMME1
Control
*
Gr1+
0 4 8 12
Cells (X10⁴)
GMME1
Control
*
MAC-3+
0 10 20 30
Cells (X10⁴)
GMME1
Control
*
CD138+
0 10 20 30 40
Cells (X10⁵)
GMME1
Control
*
CD4+
0 20 40 60
Cells (X10⁴)

Figure 41. GMME1 Effects on Systemic Cytokines, Lymphocytes and Humoral Responses.

(**A**) To demonstrate the therapeutic efficacy of systemic circulation of GMME1, ELISA analysis on mice sera demonstrate an increase in GMME1 in circulation whereas both TNF-α and IL17 levels were decreased. The opposite outcome occurred in the sick control group. (**B**) All immune cells known to express CCR2 were found decreased in absolute number in the GMME1 group. (**C**) Anti-CII titer was also diminished in the GMME1 group along with RF units. (**D**) Analysis of RF in the bloodstream of GMME1 mice demonstrate a robust decrease as compared to sick control mice. All Results are shown as mean $\pm$ S.E.D (* = $P<0.05$).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MCP-1 (pg/ml)
60
40
20
0
n.d.
Control
GMME1
TNF-alpha (pg/ml)
120
80
40
0
*
Control
GMME1
IL17 (pg/ml)
150
100
50
0
*
Control
GMME1
B
Lymphocytes
(X10³) Cells/ul
6
4
2
0
*
Monocytes
(X10²) Cells/ul
16
12
8
4
0
*
Granulocytes
(X10³) Cells/ul
6
4
2
0
*
Control
GMME1
C
IgG CII Titer
CII Titer
1000000
10000
100
1
*
Control
GMME1
D
Rheumatoid Factor
RF (U/ml)
40
30
20
10
0
*
Control
GMME1

7.4.6 *In Vitro* Re-call Responses

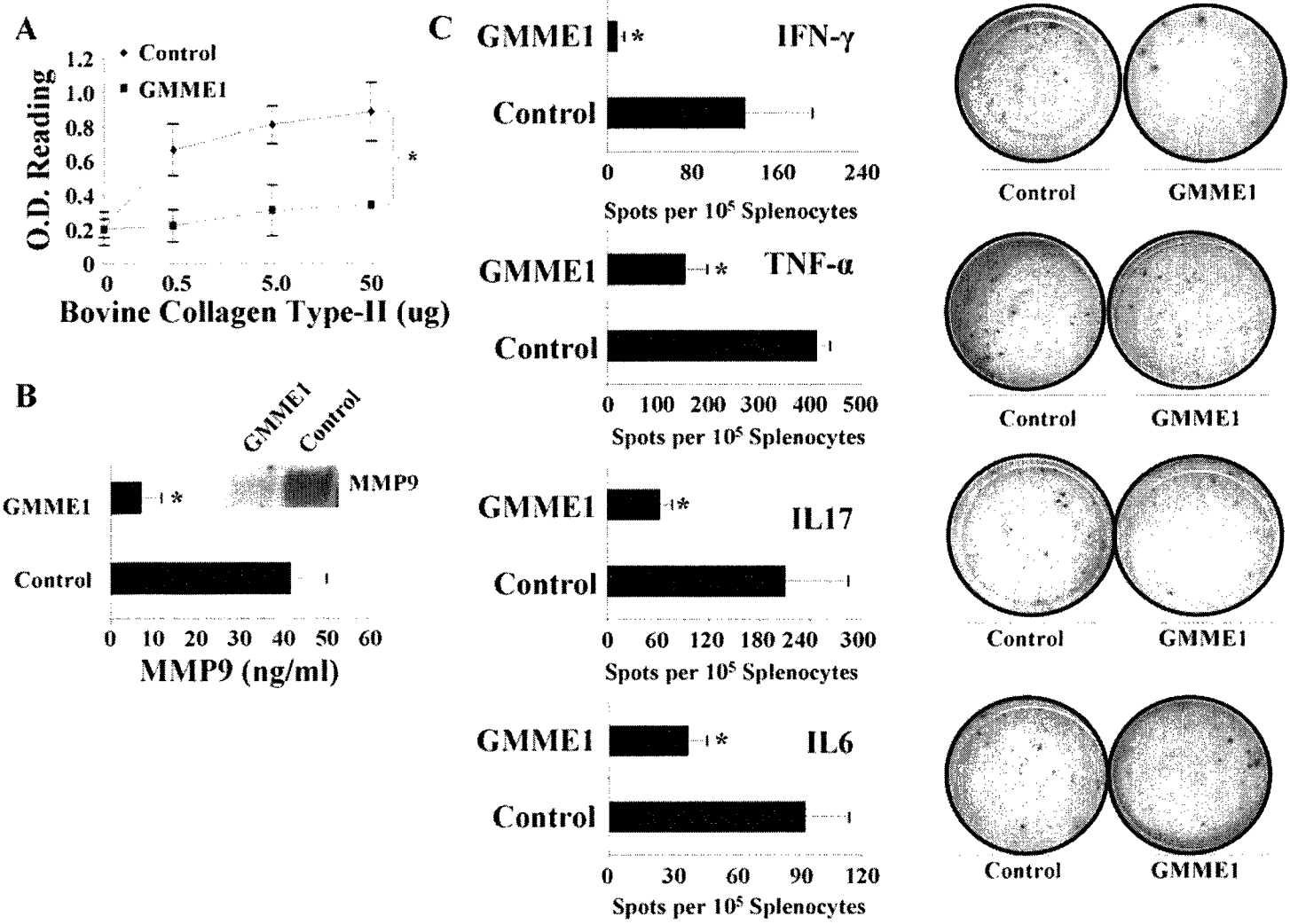
As a measure for the intensity of immune responses in RA mice, splenocytes were collected and re-stimulated *in vitro* with increasing concentration of CII. As expected, splenocytes-derived from sick mice proliferated in a CII dose-dependent manner as opposed to GMME1-derived cells (**Fig. 42A**). Amongst the secreted factors implicated in the pathophysiology of RA, tissue-degrading enzymes, such as MMPs are induced and participate in cartilage digestion and bone deformation (23). We chose MMP9 as its role in RA pathogenesis is heavily underlined (23). We found that MMP9 is highly induced by CII restimulation both by ELISA and western blotting (**Fig. 42B**). In addition, all screened pro-inflammatory cytokines such as IFN-γ, TNF-α, IL17 and IL6 were significantly reduced relative to control mice with RA (**Fig. 42C**).

7.5 DISCUSSION

The maladaptive interplay of immune cells involved in RA pathophysiology is a complex process and is known to implicate a cascade of reactions [424]. It is believed that, following activation of B-cells – by an unknown mechanism - , immunoglobulins and RFs are induced and deposited in synovial tissues. This subsequently leads to complement activation and recruitment of phagocytic cells, which further exacerbates synovial inflammation leading to edema, vasodilatation and infiltration of activated CD4 T-cells. Upon entry, CD4 T-cells are reactivated by resident APCs leading to the secretion of pro-inflammatory factors including IFN-γ, TNF-α, IL6, and IL17. As a result, granulation occur at the edges of the synovial lining (pannus) with extensive angiogenesis and production of MMPs that cause tissue damage. The synovium thickens and the cartilage underlying the bone begins to disintegrate leading to deformation [424]. It is therefore very unlikely that targeting of any one inflammatory cytokine or cell population involved in disease ontogeny will bear meaningful clinical results. In this context, CCR2 – as a therapeutic target - is of great interest since it is widely expressed on immune effector cells and predominantly on monocytes/macrophages [425]. In fact, inflammatory monocytes in circulation are characterized as $CCR2^{hi}CX3CR1^{low}$, whereas

Figure 42. *In Vitro* Re-call Responses.

(**A**) Stimulation with increased CII amounts lead to dose-dependent proliferation of splenocytes-derived from sick mice with a slight proliferative response in the GMME1 group. (**B**) MMP9 analysis shows a robust inhibition in the GMME1 group by both ELISA and western blot. (**C**) ELISPOTS performed on the same cells lead to significant decrease in IFN-γ, TNF-α, IL17 and IL6 levels.


A
Control
GMME1
O.D. Reading
1.2
1.0
0.8
0.6
0.4
0.2
0
0
0.5
5.0
50
*
Bovine Collagen Type-II (ug)
B
GMME1
Control
MMP9
GMME1
*
Control
0 10 20 30 40 50 60
MMP9 (ng/ml)
C
GMME1
*
IFN-γ
Control
0 80 160 240
Spots per 10^5 Splenocytes
GMME1
*
TNF-α
Control
0 100 200 300 400 500
Spots per 10^5 Splenocytes
GMME1
*
IL17
Control
0 60 120 180 240 300
Spots per 10^5 Splenocytes
GMME1
*
IL6
Control
0 30 60 90 120
Spots per 10^5 Splenocytes
Control
GMME1
Control
GMME1
Control
GMME1
Control
GMME1

$CCR2^{low}CX3CR1^{hi}$ cells are defined as resident monocytes [425]. This notion further confirms the importance of CCR2 in inflammation due to the ubiquitous presence of monocytes and their focused migration within the host in response to CCL2 and other chemokines. Surprisingly, previous clinical studies using inhibitors or neutralizing antibodies for CCR2 in RA showed limited amelioration for various reasons. For example, the administration of the therapeutic antibody MLN102 failed due to its inefficiency in fully covering the entire CCR2 repertoire [426]. Another small molecule inhibitor of CCR2, MK0812, failed due to poorly tolerated off-target effects [427]. More specifically, this inhibitor targeted CCR2 but also possessed a higher affinity for CCR5, a CCR2 homologous receptor. This miss-specificity is detrimental to RA since CCR5 is also expressed on Tregs and may therefore lead to their depletion explaining the lack of efficacy observed during phase II arthritis trial [427]. As an alternative strategy, we here build upon the observation that CCR2-specific pro-apoptotic effect of GMME1 could be exploited to deplete pathogenic lymphomyeloid cells implicated in RA (Rafei *et al.* Journal of Immunology *In Press* 2009). We showed that the fusion of the CCL2 fragment 6-76 to GMCSF resulted in a fusion protein that induced different changes in the CCR2 conformation than CCL2 or mpCCL2, suggesting that GMME1 induced different downstream signalling. In addition, GMME1 did not lead to ß-arrestin 2 recruitment, implicated in receptor down-regulation/recycling. As a result, the cells received various abnormal signalling such as strong Ca^{2+} influx, hyperphosphorylation of p38, BAX induction and caspase-3 activation. In addition, the fusokine acquired the capacity to compete for CCR2 in the presence of the native ligand CCL2 as opposed to the parental 6-76 variant form of the chemokine.

As proof of its therapeutic potency, $CCL2^{-/-}$ MSCs expressing the fusokine were implanted as a neo-organoid delivering the fusokine as a strategy to increase the bioavailibity and the pharmacokinetic properties of GMME1 in an RA mouse model. This technology also allows the surgical removal of the implant at any time point especially if the ailment is resolved [393, 417]. We were indeed capable of detecting GMME1 systemically in recovered mice in addition to a substantial decrease in levels of lymphomyeloid cells in circulation. Moreover, almost no immune cells were detected in

the joints of GMME1-treated mice clearly demonstrating the potency of the fusokine in eradicating pathological immune cells. LIX is believed to play substantial roles in mediating polymorphonuclear cells joint infiltration and exacerbation of the inflammatory process [428]. The minimal amount of LIX from host-derived cells found within joints of treated mice additionally confirms the therapeutic efficacy of GMME1.

Chemokine redundancy represents a major barrier in the development or rational design of therapies directed towards a specific G-coupled receptor [424, 429]. We have shown in a previous study the specificity of GMME1 on $CCR2^{+}$ cells since $CCR2^{-/-}$ lymphocytes failed in responding to the treatment (Rafei *et al.* Journal of Immunology *In Press* 2009). This observation demonstrates that GMME1 has a great advantage over many other experimental therapies since we have not observed any off-target toxicities. In summary, by targeting and selectively depleting CCR2-expressing lymphomyeloid cells, we were able to completely inhibit disease progression and markedly repressed the production of most, if not all, pro-inflammatory factors identified so far in RA pathogenesis. Thus, GMME1 is the lead member of a new class of drugs which may be of substantial clinical interest for RA and related ailments.

7.6 ACKNOWLEDGMENTS

The authors are grateful to Michel Bouvier for the generous gift of plasmids coding for RLuc-arrestin 2 and Gγ2-YFP. Moutih Rafei is a recipient of a *Fonds de Recherches en Santé du Québec (FRSQ)* Scholarship, YAB holds a Canadian Institutes of Health Research (CIHR) scholarship, NH is a CIHR New Investigator and Jacques Galipeau is a *FRSQ chercheur-boursier sénior*. This work was supported by the Canadian Institute of Health Research grants MOP-15017 to JG and HOP-79210 to NH.

CHAPTER 8

CONCLUSION

CHAPTER 8: Conclusion

Even after almost a century of investigation for the development of novel strategies to treat/block autoimmune disease progression or transplantation rejection, a considerable number of issues remain to be addressed in order to obtain clinical efficacy. An impressive array of immunossuppressive agents capable of blocking proliferation, inducing anti-inflammatory effects, leading to lymphoid depletion, or interfering with cytokine activation have been developed and tested experimentally in various animal models [430]. Unfortunately, every category of such immunosuppressive agents is still confronted to the following limitations:

i) *Anti-proliferative drugs*: various gastro- and hepato-toxicities in addition to myelosuppression
ii) *Steroids*: salt retention, hypertension, bone changes, diabetes mellitus
iii) *Lymphocyte depletion agents* : allergic reactions and immune-based problems related to cytokine inhibition
iv) *Cytokine disruption*: nephro- and neurotoxicities, hypertension, diabetes mellitus, myelodepression, diarrhoea, and defects in wound healing

The main reason for all these adverse side effects is related to the wide distribution of most of the compound-specific targets in human tissues [431]. In addition, autoimmunity usually involves many cell types simultaneously, which confers a higher level of difficulty to target all implicated pathological subsets while sparing "normal" cells [431]. Today, redesign of new immunosuppressive compounds aims at three major objectives:

i) Specific depletion of pathological lymphocytes
ii) Blocking autoreactive cell trafficking
iii) Interruption of pathological APCs and lymphocyte activation

As such, the development of novel suppressive strategies to solve autoimmunity is directed towards selective destruction of pathological lymphocytes while minimizing side

effects and off-target toxicities. In view of these challenges, the main objective of my thesis was to develop novel means targeting the disruption of pro-inflammatory cascades via specific receptors. Such a strategy will allow the interference with major signal transduction pathways usually involved in the activation of pathological lymphocytes without affecting normal resting cells. The specific research aims of my thesis were to test the hypothesis that:

1. The immunosuppressive fusokine GIFT15 generated from the fusion of two cytokines (GM-CSF and IL-15), may induce powerful immunosuppression and thus lead to allogeneic and xenogeneic cell acceptance in immunocompetent mice.
2. The fusokine GIFT15 can lead to the *ex vivo* generation of a novel suppressive cell population capable of alleviating/blocking EAE.
3. Primary MSCs could be exploited in an immunosuppressive cell therapy approach for the inhibition of humoral responses directed against therapeutically relevant proteins such as rhFVIII via the paracrine conversion of CCL2 to an antagonistic factor.
4. MSCs-derived antagonist CCL2 could also be exploited in the alleviation of Th1-mediated autoimmune disease such as EAE.
5. MSCs can be used as universal donor cells for the suppression of EAE.
6. The fusion of the two cytokines: GMCSF and CCL2 (6-76), would lead to the generation of an immunosuppressive compound – GMME1 - capable of blocking inflammation during EAE pathogenesis.
7. GMME1 would have suppressive properties in a collagen-type II mouse model of RA.

Hypothesis 1

The field of transplant therapeutics has been extremely disappointing at least for the first decade of the millennium [430]. Several compounds such as anti-CD154, CD28 or IL15 selected for clinical trials in organ transplantation have failed with only six new agents being tested currently in the pipeline [430]. It was lately proposed that established drugs in autoimmunity used to target specific cellular subsets could be exploited in transplantation, while others still believe the importance of targeting cytokine pathways to achieve targeted immunosuppression [430]. Due to the major role played by IL2 and its receptor in T-cell activation and proliferation, various studies aimed at targeting this cytokine/receptor interplay in an attempt to halt unwanted immune reactions [431]. However, it was lately observed that such a strategy might not be beneficial especially since IL2 is implicated in the induction of Tregs [431]. As an alternative approach, IL15 was suggested for various reasons. First, both IL2 and IL15 share similar biological activities such as the activation of NK and T-cells [207]. Second, IL15 is mainly effective on activated T-cells expressing the IL15R-α chain - possessing a relatively high affinity for IL15 [207]. As such, cells expressing only the IL2/15Rßγ chains are not or weakly responsive to IL15 but can however proliferate upon IL2 addition [207]. Third, IL15 does not only play major roles in protecting CD8 T-cells from apoptosis, as opposed to IL2, but is also involved in the maintenance of memory CD8 T-cells, which implies that IL15 can confer long term persistence of pathological memory [207]. Therefore, IL15 or IL15R represent already a more convenient strategy for targeting autoreactive T-cells. In agreement with this notion, studies have demonstrated a relative prolonged allograft survival following IL15 blockade using mutant structural antagonists or fusion protein [432, 433].

We originally believed that GIFT15 would lead to synergistic stimulation of all IL15R-expressing cells, but quickly realized that the fusokine delivers aberrant signalling and eventually immunosuppression. We thus decided to use this bi-functional fusokine in the context of allo/xenogeneic cell transplantation in immunocompetent mice. Such study would serve as a proof-of-concept for the development of GIFT15 as a small

biopharmaceutical to block transplant rejection as well as autoimmunity problems. My studies demonstrated that:

i) The GIFT15 protein is bi-functional since both the GMCSF and IL15 moieties retained their biological effects on GMCSFR and IL15R-expressing cells respectively.

ii) When expressed by live B16 tumor cells and implanted in syngeneic mice, the GIFT15-expressing tumor grew faster than control tumor cells due to NK and NKT-depleting capacity of the fusokine in the tumor vicinity.

iii) The fusokine possesses powerful angiogenic properties as demonstrated by the increased blood vessel density in GIFT15-expressing tumors and through the induction of MMP2 and VEGF secretion by macrophages.

iv) The fusokine induces immunosuppression via asymmetrical signalling downstream of the IL15R leading to hyperphosphorylation of STAT3 and blockade of STAT5 activation.

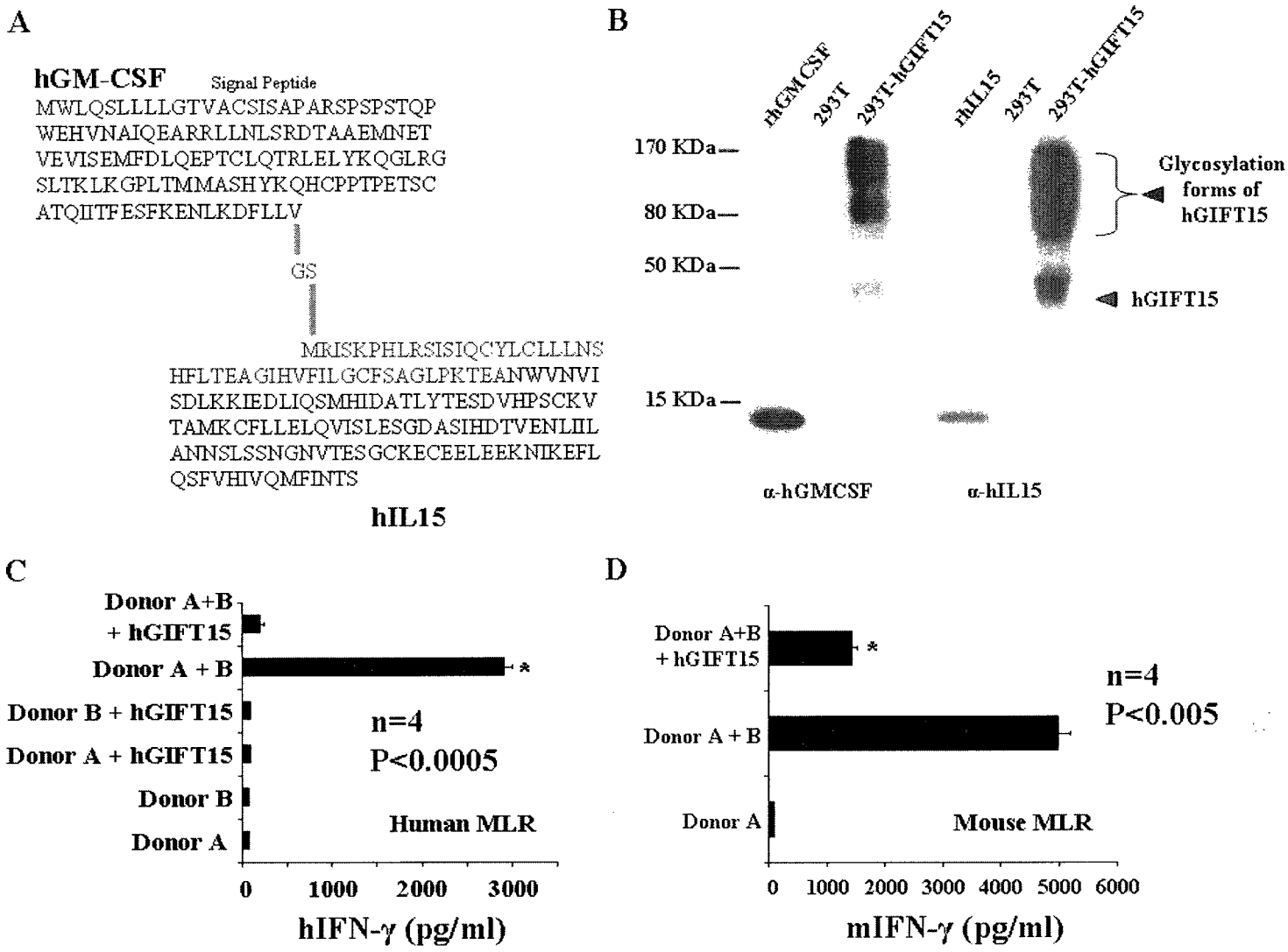
v) The fusokine inhibits IFN-gamma secretion during a mouse MLR while protecting IL15R-expressing lymphocytes from apoptosis.

vi) Expression of GIFT15 by allogeneic or xenogeneic cells protects them from rejection in immuncompetent mice over a long period of time in a CD4-depedent manner

This proof-of-principle study supports the use of GIFT15 as a major immunosuppressant without further use of suppressive compounds. Interestingly, its effect seems to be strictly dependent on the IL15 moiety since the GMCSF portion of the fusokine does not lead to any aberrant signalling on cells expressing the GMCSFR. In addition, we have engineered the human homolog of mouse GIFT15 and demonstrated inhibition of IFN-gamma secretion during both mouse and human MLR (Figure 43). This observation is of great importance since GMCSF, as opposed to IL15, is species-specific [434]. In other words, mouse GMCSFR only responds to mouse GMCSF whereas human IL15 can activate both human and mouse cells [434]. As such, GIFT15's suppressive capacity is directly linked to the IL15 moiety hindered by the GMCSF part of the

Figure 43. Cloning The Human Homolog of mGIFT15.

(a) Schematic representation of the human GIFT15 aa sequence. **(b)** Denaturing immunobloting using conditioned media (CM) from transiently transfected CHO cells expressing GFP or human GIFT15 probed with polyclonal goat anti-IL15 or anti-GMCSF antibodies. rIL15 and rGMCSF were used as positive controls. **(c)** Human GIFT15 direct effect on human and mouse 2-way MLR. Same CM as in (**b**) was added directly to co-cultured PBMCs or mosue splenocytes for 72 hrs. The supernatant was tested by ELISA for IFN-γ. Every condition was performed in sixplicata ± S.E.D ($P<0.0005$ between MLR condition containing GIFT15 PBMCs and $P<0.005$ between MLR condition containing GIFT15 and splenocytes).


A
hGM-CSF
Signal Peptide
MWLQSLLLLGTVACSISAPARSPSPSTQP
WEHVNAIQEARRLLNLSRDTAAEMNET
VEVISEMFDLQEPTCLQTRLELYKQGLRG
SLTKLKGPLTMMASHYKQHCPPTPETSC
ATQIITFESFKENLKDFLLV
GS
MRISKPHLRSISIQCYLCLLLNS
HFLTEAGIHVFILGCFSAGLPKTEANWVNVI
SDLKKIEDLIQSMHIDATLYTESDVHPSCKV
TAMKCFLLELQVISLESGDASIHDTVENLIIL
ANNSLSSNGNVTESGCKECEELEEKNIKEFL
QSFVHIVQMFINTS
hIL15
B
rhGMCSF
293T
293T-hGIFT15
rhIL15
293T
293T-hGIFT15
170 KDa
80 KDa
50 KDa
15 KDa
Glycosylation forms of hGIFT15
hGIFT15
α-hGMCSF
α-hIL15
C
Donor A+B + hGIFT15
Donor A + B
Donor B + hGIFT15
Donor A + hGIFT15
Donor B
Donor A
*
n=4
P<0.0005
Human MLR
0
1000
2000
3000
hIFN-γ (pg/ml)
D
Donor A+B + hGIFT15
Donor A + B
Donor A
*
n=4
P<0.005
Mouse MLR
0
1000
2000
3000
4000
5000
6000
mIFN-γ (pg/ml)

fusokine. An interesting observation however, was the GIFT15 partial loss of function in CD4-deficient mice suggesting a role for Tregs in the maintenance of suppression. Given this observation, we formulated a new hypothesis consisting of the *ex vivo* generation of Tregs using GIFT15; a strategy that can be exploited as part of a immunosuppressive cell therapy approach to modulate pathological immune reactions.

Hypothesis 2:

In Chapter 2, we have demonstrated the capacity of GIFT15 in leading to aberrant signalling and allowing the survival of both allogeneic and xenogeneic cells in immunocompetent mice. The latter effect was confirmed to be mediated in a CD4-dependent manner and thus, we speculated that GIFT15 would lead to the generation of Tregs directly *in vivo*. In an attempt to prove our claim, we *ex-vivo* cultured unfractionated splenocytes with GIFT15 and analyzed their phenotype by flow cytometry. In addition, we tested the generated cells in various *in vitro* cell-based assays before their use therapeutically in the EAE mouse model of MS. Our findings are summarized as follow:

i) *Ex vivo* culturing of unfractionated splenocytes leads to a homogeneous population expression high levels of MHCI/II as well as IL10.
ii) These cells are not of NK, T, DC or myeloid origin as they do not express any of the generic markers
iii) The suppressive population is of B cell ontogeny as only purified B cells lead to the previously reported phenotype and shed CD19; thus we referred to our cells as Bregs.
iv) The suppressive ability of GIFT15 Bregs is through cell contact, MHCII and IL10 secretion.
v) GIFT15 generated Bregs can lead to complete and durable remission of EAE in mice following a single iv injection

The finding that GIFT15 can lead to the generation of Bregs was unanticipated based on observations in Chapter 2. Various studies have demonstrated the existence of naturally occurring Bregs in mice with autoimmune diseases and B cell deficiency in EAE, for example, exacerbates inflammation due to the absence of IL10 [134, 137, 139, 142]. We are, to our knowledge, the first to demonstrate an *ex-vivo* method of artificially generating a novel suppressive Bregs population characterized phenotypically as $CD19^{-}$ $MHCII^{+}IL10^{+}$. The major difference observed in our GIFT15-Bregs is the complete absence of CD19 expression as opposed to the previously reported naturally occurring Bregs. Interestingly, CD19 is considered as a signal-transducing element during B cell activation [435]. $CD19^{-/-}$ mice are immunodeficient with reduced B cell proliferation, while CD19-transgenic mice are autoimmune-prone with augmented B cell proliferation [435]. CD19 is also known to be regulated by BCR response regulators such as CD22 [435]. This trans-membrane molecule regulates CD19 activation by acting as a phosphatase through is intracellular domain [435]. Our phenotypic analysis of the surface of GIFT15 Bregs revealed the complete absence of CD19 and high expression levels of CD22. Interestingly, Arruffo *et al*, previously demonstrated that the addition of soluble CD22 on CD3-triggered T-cells inhibit their function by binding to CD45 and blocking intracellular calcium increase while inhibiting tyrosine phosphorylation of phospholipase Cγ1 [435]. This notion is consistent with our *in vitro* observation that GIFT15 Bregs are capable of directly inhibiting responding T-cells during an APC assay. This observation suggests a possible role for CD22 on the surface of these GIFT15 Bregs while MHCII might be helping in the binding process to T-cells. It must be noted however, that the possiblity of directly generating Tregs *in vivo* is plausible following GIFT15 Bregs injection. Mann *et al.* have reported that Bregs can lead to the generation of Tregs directly *in vivo* via cell contact [140], an observtaion that might help explain the powerful suppressive effects seen following one single injection of Bregs in EAE mice as well as the partial loss of GIFT15 suppression in the xenogeneic transplantation model using CD4-deficient mice. Furthermore, Breg-derived IL10 could enhance Treg generation [130]. All these points remain speculative at this time point but could be major investigative hypotheses in the future. In addition, the unlimited *ex-vivo* generation of Bregs from naïve B cells using GIFT15 could be used as a molecular tool to help

understanding more in depth the biology of Bregs in order to improve future cell therapies using this technology platform.

Hypothesis 3:

MSCs possess powerful immunosuppressive properties as demonstrated in various disease models [149, 151, 152, 157, 169]. So far, a wide array of soluble factors such as HGF, TGF-β, and IL10 has been suggested to play a role in inhibiting lymphocytes [152]. Interestingly however, it was lately demonstrated that MSCs do not constitutively secrete such factors and are not likely detected in all MSC preparations perhaps due to methods related to culturing/handling of MSCs [169]. An alternative mechanism of action was proposed by two different groups in which there is a need for a direct T-cell-MSC contact to trigger the secretion by MSCs of NO; a molecule capable of inhibiting T-cell proliferation and STAT5 activation [318, 380]. In addition, Ren *et al.* suggested the direct implication of additional factors such as CC chemokines [380]. In an attempt to identify the implication of soluble factors in the inhibition of lymphocytes, we have analyzed the secretome of MSCs using protein arrays and identified CCL2/CCL7 as potential candidates. We focused our efforts on CCL2 and hypothesized that MSC-derived CCL2 would directly affect plasma cells by targeting CCR2 and blocking their ability to secrete antibodies. My study demonstrated that:

i) MSC CM inhibits IgG production by PCs *in vitro*.
ii) The presence of a paracrine MSC processing mechanism of CCL2/CCL7 by MMPs.
iii) MSC-derived CCL2 leads to plasmablast proliferation and IL10-mediated Blockade.
iv) Antagonist CCL2 inhibits AKT and STAT3 phosphorylation while inducing PAX5 in PCs.
v) MSC administration lowers pathogenic autoreactive IgG titer in immunized mice.

MSCs are known to play a physiological role in the development of pre-B cells in the bone marrow [331]. Therefore, it is not surprising to observe a direct link between MSC-B cell development and the capacity of MSCs to control B cell proliferation, migration, maturation as well as differentiation into plasma cells [312]. One of the striking observations in our study was the ability of MSCs to produce a variety of soluble factors that can interact with one another. Of interest was the simultaneous secretion of active MMP1, 3 and 8 known to directly bind and cleave CCL2 at its N-terminus [223]. Such reaction occurs readily *in vitro*, but might be under direct control *in vivo*. Our study suggest that this paracrine conversion of CC and/or various other chemokines may represent one of the various physiological mechanisms by which MSCs control plasma cell homeostasis at the end of a humoral reaction. This concept might shed some light on the potential mechanism responsible for the decrease in antibody titer over time upon clearance of pathogens. All these results demonstrate that MSCs could modulate CCR2-dependent pathologies via truncated CCL2 and/or CCL7 and thus, would be of great interest to inquire whether it can interfere with autoreactive T-cells as well.

Hypothesis 4:

As a disease model for autoreactive T-cells, we chose the EAE mouse model of MS for 2 main reasons. First, CCL2 as well as its receptor CCR2 are both implicated in EAE onset and progression [352, 353, 368]. In fact, it is believed that $CCR2^{-/-}$ mice are resistant to the onset of EAE due to a major defect in leukocyte migration to the CNS [353]. Since we have demonstrated in our previous chapter that MSCs can convert CCL2, in a paracrine fashion, to an antagonist molecule with inhibitory capacities, we hypothesized that MSC-derived CCL2 can play an important role in the alleviation of EAE. In fact, Zappia *et al.* previously demonstrated that MSCs can ameliorate the outcome of EAE but the group did not investigate nor propose the mechanism by which MSCs operated in this disease model [311]. Thus, the major aim of our study was to test MSCs suppressive ability in EAE via antagonist CCL2. We have demonstrated that:

i) SELDI-TOF analysis of MSC CM reveals the presence of various truncated forms of CCL2.

ii) MSC-derived CCL2 can inhibit IFN-gamma and IL17 secretion from MOG_{35-55} stimulated splenocytes.

iii) The addition of MSC-derived CCL2 on CD4 T-cells derived from EAE mice blocks STAT3 phosphorylation and IL17 secretion during an APC assay.

iv) The injection of WT MSCs leads to EAE score amelioration with significant decrease in pro-inflammatory cytokines as well as anti-MOG titer.

v) A significant decrease in CD4 T-cells was also observed in the spinal cord of WT MSC-treated mice as demonstrate by both histology and flow cytometry analysis.

vi) Splenocytes-derived from WT as opposed to $CCL2^{-/-}$ MSCs proliferate weakly *in vitro* in response to MOG_{35-55} peptide and secrete less pro-inflammatory cytokines while expressing high levels of B7H.1.

vii) B7H.1 neutralization rescued IL17 secretion *in vitro*

viii) Cleaved CCL2 can modulate signaling in Th17 cells by inhibiting AKT phosphorylation and activation of ERK.

Taken together, the observations in Chapters 3 and 4 clearly demonstrate that MSCs can modulate both T and B pathological lymphocytes by interfering with the MAPK kinase signaling pathway via the paracrine generation of antagonist CCL2. In addition, our *in vitro* data suggest an indirect effect of truncated CCL2 on the induction of B7H.1, a cell surface molecule shown to play important suppressive roles [377, 379]. Due to the importance played by CCR2 in the system and the selective expression of this receptor on the surface of Th17 cells [360], our data clearly show that MSCs can lead to specific targeting of Th17 cells under pathological conditions. We therefore propose a new immunosuppressive mechanism of action by MSCs that could explain the previously reported results by *Zappia et al.* group in which MSCs were shown to play beneficial effects [311].

Hypothesis (Supplemental Article)

Even though our previous studies demonstrated that the use of MSCs as a suppressive cell therapy for devastating autoimmune diseases such as EAE is of potential, there is still a major barrier in the translation of such therapy to the clinic. Harvesting autologous MSCs from every patient still poses logistic, time consuming and economical issues which might not be in favor of patients suffering from acute disorders. Furthermore, it must be noted that most patients who would benefit from such cellular therapy are elderly, and MSCs obtained from these patients have reduced capacity to proliferate, differentiate, neovascularize, and are prone to apoptosis both *in vivo* and *in vitro* [436]. As a possible solution, the idea of universal donor cell therapy was introduced in the early 90s by which one single MSC preparation is generated and readily available for any patient; an elegant strategy for chronic patients where the MSC suppressive role is only required for a short laps of time [436]. Thus, we hypothesized that allogeneic MSCs would be capable of alleviating EAE in a similar fashion as in the syngeneic model via the paracrine conversion of antagonist CCL2. Indeed, we have shown in our supplemental study that:

i) Allogeneic MSCs can block EAE similarly to syngeneic preparations.
ii) The stimulation of allogeneic MSCs with IFN-gamma – to increase the level of CCL2 expression – led to complete loss of therapeutic capacity, likely due to accelerated immune rejection of allogeneic MSCs .
iii) The injection of non-IFN-gamma treated MSCs in EAE mice lead to a decrease in IL17 and IFN-gamma systemically.
iv) Less CD4 T-cell infiltrates are found in the spinal cord as shown with syngeneic MSCs.

Almost all groups working with MSCs are consistent with the fact that these bone marrow-derived cells are MHCI positive with no detectable levels of MHCII [436]. This means that MSCs could evade, to a certain point, adaptive CD4 T-cell responses due to the deficiency in presenting alloantigens to responding T-cells [436]. The presence of

MHCI, on the other hand, can still lead to their rejection by CD8 T-cells. As for the escape from NK-mediated killing via MHCI mismatch, MSCs were suggested to secrete TGF-ß and PGE-2; compounds know to decrease the proliferation and activation of NK cells [436]. Interestingly, using allogeneic MSCs would lead to their rejection after a few weeks with the acquisition of anti-MSC cellular response as we have previously demonstrated [437]. As such, the use of allogeneic MSCs in the context of EAE could still lead to a useful therapeutic effect in a short "window" of activity following a first injection. IFN-gamma could be the main cause since it is known to lead to cellular activation and enhancement of the transcription machinery [438]. We have demonstrated in our laboratory, that MSCs treatment with IFN-gamma leads to the up-regulation by 2-3 fold the levels of CCL2 secretion, a biological process, which we thought could be exploited for the enhancement of the MSC suppressive capacity in the universal donor approach. However, we found that the treatment of MSCs with IFN-gamma leads to the loss of therapeutic efficacy most likely due to their fast rejection mediated by the up-regulation of MHCI and II. Thus, we can conclude that the use of allogeneic MSCs as universal donors is a feasible approach and the presence of high endogenous doses of IFN-gamma in the host can impede their immunomodulatory capacities.

Hypothesis 6

Our investigations of MSC immunobiology led us to the discovery of a novel mechanism by which MSCs can inhibit lymphocytes. The intriguing finding of a paracrine conversion of CCL2 to an antagonist in the secretome of MSCs and its capacity in targeting CCR2 is of great interest not only in experimental animal models but in the clinic as well [351, 353, 385, 387, 439]. Unfortunately, most of the clinical trials targeting CCR2 have shown modest responses due to the inefficacy of the used drugs to cover the entire CCR2 repertoire or due to off-target cytotoxicity [439]. Thus, there is still a great room of improvement for the development of novel strategies targeting CCR2. Since we have demonstrated in the past that the use of GMCSF as part of a fusion transgene may lead to unanticipated pharmacological properties either of immuno-stimulatory or suppressive nature [285], we sought of fusing GMCSF to the cleaved form

of CCL2 (6-76) as a single polypeptide (GMME1) and its use in autoimmune diseases. We hypothesized that GMME1 could be used to inhibit CCR2-expressing cells in the context of EAE. We found the following observations:

i) The GMME1 fusokine can block activated CD4 T-cell proliferation and IL2 secretion.

ii) GMME1 addition to an MLR leads to IFN-gamma inhibition in a CCR2-dependent manner.

iii) GMME1 addition to an ELISPOT leads to inhibition of antibody production from plasma cells in a CCR2-dependent manner.

iv) Treatment of macrophages with GMME1 induces caspase-3 activation and apoptosis.

v) GMME1 addition on EAE-derived CD4 T-cells can block to secretion of IFN-gamma, IL2, IL4, IL13 and CXCL5.

vi) We have also demonstrated that GMME1 leads to apoptosis in both CD4 and CD8 T-cells derived from EAE mice.

vii) GMME1 leads to asymmetrical signaling in CD4 T-cells as shown by the hyperphosphorylation of p38 and the inhibition of p44/42, as well as the complete blockade of AKT and STAT3 activation.

viii) An inhibition of both IFN-gamma and IL17 was observed *in vitro* during the APC assay.

ix) The implantation of $CCL2^{-/-}$ MSCs expressing GMME1 in EAE mice lead to a robust therapeutic recovery and the removal of the implant did not lead to relapse.

x) All analyzed immunological and histological parameters demonstrate an inhibition of inflammation as shown by antibody titer and pro-inflammatory cytokines decrease in addition to a blockade in both CD4 and CD8 migration to spinal cords.

Our main objective was the design of a compound capable of inhibiting inflammation via the modulation of signaling pathways as previously described using the

fusokine GIFT15 [285]. However, novel pharmacological properties were discovered as GMME1 is capable of inducing apoptosis in CCR2-expressing cells. In fact, apoptosis has been suggested to serve as a therapeutic tool to deplete macrophages, synovial fibroblasts, osteoclasts, and even T-cells [440]. However, the main difficulty encountered would be the targeted depletion of autoreactive cells in a receptor-specific approach. This is the most striking characteristic of GMME1 as it specifically targets activated CCR2-expressing T-cells since naïve CD4 T-cells do not express CCR2 unless they are induced by CD3/CD28. We have also demonstrated that $CCR2^{-/-}$ cells do not respond to the pharmacological effects of GMME1. As a proof-of-concept, GMME1 was tested *in vivo* during EAE onset once the disease has been initiated. The use of a contigen-based neo-organoid offers the possibility of removing the implant in case of ailment recovery or major side effects [393]. We wished originally to demonstrate that the removal of the implant would lead to relapse to prove that the detected improvement depended upon the direct presence of the fusokine. However, we were surprised to notice no relapses in EAE mice following the removal of the implant due to the depletion of activated EAE-specific cells. In addition, the lympho-depletion noticed 2 weeks following treatment with GMME1 was lost 3 weeks later (total of 5 weeks under treatment) even though GMME1 was still detected in circulation. The results clearly demonstrate that GMME1 continual presence in the system does not lead to any toxicity due to the absence of CCR2 expression on lymphocytes. As such, we could conclude from our EAE study that GMME1 represents a novel therapeutic strategy with long lasting effects that could lead to the depletion of pathological cells implicated in autoimmune ailments.

Hypothesis 7

RA is a devastating disease leading to major disabilities. Interestingly, almost all classes of immune cells play a role in the initiation, induction and exacerbation of RA pathologies [440]. For instance, plasma cells are heavily implicated due to their ability to produce RF, T-cells are major secretors of pro-inflammatory cytokines such as IFN-γ, TNF-α and IL17, and granulocytes generate an autocrine secretion loop of additional pro-inflammatory cytokines such as TNF-α and LIX [410]. Targeting pro-inflammatory

cytokines was believed to be the best strategy for the alleviation of RA [410]. However, one cannot expect to eradicate all associated immunopathologies based on the inhibition of one or two secreted factors. A more radical approach targeting the source itself is required to interfere with the entire pro-inflammatory cascade. GMME1 was shown to target specifically CCR2-expressing lymphocytes leading to cell death without affecting naïve/resting lymphocytes. Therefore, we tested GMME1 for the depletion of RA-pathological cells akin to what we have performed in the EAE mouse model of MS. Our studies show that:

i) GMME1 could antagonize CCR2 homodimerization and block recruitment of β-arrestin 2 for receptor recycling in HEK29T-cells.

ii) An abnormally high Ca^{2+} influx signal is delivered to the cell following GMME1 treatment leading to caspase-3 activation and apoptosis.

iii) GMME1 is a powerful antagonist as it can block the migration of RA-derived CD4 T-cells towards CCL2.

iv) GMME1 leads to hyperphosphorylation of p38 in the MAPK pathway with no major effects on the NF-Kb pathway.

v) GMME1 addition to RA-derived CD4 T-cells lead to BAX induction and apoptosis.

vi) During an APC assay, GMME1 antagonizes the CCL2 signal and inhibits IL17 secretion.

vii) The delivery of GMME1 to RA mice as part of a neo-organoid MSC-based implant leads to impressive recovery from RA sings with robust decrease of immune cell infiltration to the joints as well as a decrease in pro-inflammatory cytokines levels both locally in the joints and systemically.

The effects of current therapies used in RA might be mediated to a certain point by apoptosis [440]. For example, methotrexate and sulfasalazine, two drugs commonly used as part of a combined therapy for RA, have been shown to exert some of the pharmacological effects via monocytes/macrophages cell death in a non-specific manner [440]. These observations support the potential importance of immune cell apoptosis as a

therapeutic tool. The data obtained in our study were similar to the EAE outcome in the sense that we have demonstrated the capacity of GMME1 in depleting pathological RA lymphocytes and sustained recovery from autoimmune pathologies. Histological analysis of joints demonstrated almost no immune infiltrates in mice as opposed to RA sick mice with a substantial decrease in levels of pro-inflammatory cytokines such as TNF-α, IFN-γ, and IL17; considered as major players in RA immuno-pahtogenesis. Our therapeutic approach has shown better outcome than the remaining available strategies due to the direct targeting of immune effector cells implicated in the secretion of inflammatory cytokines as opposed to targeting one or two cytokines. Thus, the use of GMME1 ensures the removal of all sources responsible for inflammation thus interfering with all autoreactive immune cells-based pathologies.

Final Conclusion and Future Directions

In conclusion, my research has demonstrated that the fusion between 2 functionally unrelated cytokines can lead to unanticipated pharmacological properties that might not be observed with the use of either cytokines alone or in combination. GIFT15 has shown great potential for the blockade of cellular rejection and thus would be greatly useful for cell or organ transplantation. In fact, our laboratory has initiated collaboration for the generation of transgenic pigs expressing GIFT15 endogenously for the purpose of xenotransplanting ß-islet cells in immunocompetent mice. The success of such project would then allow further creation of a transgenic pig expressing the human homolog for validation of beta-islet xenotransplantation in non-human primates. As for the ability of GIFT15 to generate Bregs, a follow-up investigation would be the use of these autologous regulatory cells in a mouse allogeneic heart, kidney and islet cell transplant project to study their full capacity in blocking organ rejection. Thus, GIFT15 will not only be useful in autoimmune ailments, but could furthermore have a great impact on allogeneic & xenogeneic cell and organ transplantation.

The study of MSC biology allowed us to discover novel suppressive mechanisms mediated by soluble CCL2 alone or in combinantion with CCL7. As such, secreted

factors like CC or CXC chemokines, and their processing or modification by MMPs suggest that various other secreted factors might be modulated by proteases leading to immune cells modulation. The CCL2 study was a model for additional CC and CXC chemokines, which could be regulated in a similar fashion as well. The use of MSCs to control pathological humoral responses in mice encouraged us to pursue the use of MSCs in larger mammals. For example, a study will be initiated soon in which hemophilic dogs with pre-developed anti-FVIII antibodies will be injected with MSCs. Such study could serve as a basis for the preparation of clinical trials especially since MSCs have been lately used with success in human suffering from GVHD [310].

A second branch for the importance of MSCs would be the screening of its secretome for various factors that could be exploited as part of novel fusion proteins. The successful use of truncated CCL2 as part of a fusokine with pro-apoptotic capacities is a powerful example of application. Our studies demonstrated that it is very difficult to predict the outcome of a fusokine based on the biology of isolated entities. I propose to pursue investigating the generation of other cytokine chimeric gene products between GMCSF and other suppressive cytokines such as IL10, TGF-ß as well as full or truncated chemokines. This technology platform could then serve as the basis to enhance the pipeline of protein-based immunosuppressive strategies.

CONTRIBUTION TO ORIGINAL KNOWLEDGE

Contribution to Original Knowledge

The data presented in this thesis has provided several original contributions to the existing body of scientific knowledge:

i) I generated and characterized the immunosuppressive effects of a chimeric bi-functional mouse GMCSF/IL15 fusion transgene (GIFT15)

ii) I demonstrated that GIFT15 leads to aberrant signaling in immune cells and leads to a pronounced angiogenic response

iii) I demonstrated that GIFT15 could protect allogeneic tumor cells from rejection in immunocompetent mice by depleting NK and NKT-cells in the tumor vicinity

iv) I demonstrated that GIFT15 allows the survival of human tumor cells in immuncompetent mice in a CD4-dependent manner

v) I have demonstrated that GIFT15 *ex vivo* treatment of splenocytes leads to a homogeneous $CD19^{-/-}$ Breg population expressing high levels of MHCII and IL10

vi) I demonstrated that this Breg population could block Ag-dependent immune stimulation as a third party bystander cells *in vitro*

vii) I demonstrated that syngeneic Bregs could reverse inflammation in EAE mice in a MHCII and STAT6-dependent manner

viii) I demonstrated that allogeneic Bregs could not lead to the same therapeutic effect *in vivo* as seen with syngeneic cells

ix) I demonstrated that MSCs could convert in a paracrine manner CCL2 to an antagonist form

x) I demonstrated that MSC-derived CCL2 could lead to the expression of PAX5 in plasma cells, a blockade in IL10 production as well as the non-specific proliferation of plasmablasts

xi) I demonstrated that MSCs injection in mice with pre-developed antibodies leads to a strong decrease in antibody titer

xii) I demonstrated that MSC injection in EAE leads to pathology amelioration as the effect was lost upon the use of $CCL2^{-/-}$MSCs

xiii) I demonstrated that MSC-derived CCL2 leads to the up-regulation of B7H.1 on immune cells, a decrease in pro-inflammatory cytokines and MOG-specific proliferation

xiv) I demonstrated that cleaved CCL2 derived from MSCs leads to active signaling in CCR2 expressing cells

xv) I demonstrated that the neutralization of B7H.1 on lymphocytes-derived from MSCs-treated mice re-gain their ability to secrete pro-inflammatory cytokines *in vitro*

xvi) I generated and characterized the immunosuppressive effects of a chimeric bi-functional mouse GMCSF/CCL2 (6-76) fusion transgene (GMME1)

xvii) I demonstrated that GMME1 could lead to aberrant signaling in CCR2-expressing CD4 T-cells and block IFN-gamma and IL17 secretion

xviii) I demonstrated that GMME1 leads to cell death in lymphocytes and macrophages

xix) I demonstrated that GMME1 could lead to the recovery of EAE pathologies by depleting the pool of autoreactive lymphocytes

xx) I demonstrated that GMME1 inhibits the production of inhibitory cytokines directly *in vivo* as well as *in vitro*

xxi) I demonstrated that GMME1 could antagonize CCR2 homodimerization and block ß-arrestin 2 recruitment

xxii) I demonstrated that GMME1 is a competitive antagonist as it can override the chemotactic effects of full length CCL2

xxiii) I demonstrated that GMME1 leads to a strong recovery of RA mice by blocking recruitment of immune infiltrates to joints

xxiv) I demonstrated that GMME1 inhibits all tested inflammatory cytokines both locally and systemically

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APPENDIX

SUPPLEMENTAL CHAPTER

Allogeneic Mesenchymal Stem Cells as Universal Donors for Treatment of Experimental Autoimmune Encephalomyelitis

Reference: Moutih Rafei, Elena Birman, Kathy Forner, and Jacques Galipeau

Preface to supplemental chapter:

We have demonstrated in Chapter 5 that MSCs could alleviate EAE via paracrine conversion of CCL2 to an antagonistic form. As a follow up to this study, we wished to investigate the potential use of allogeneic MSCs as universal donor cells for EAE therapy. We found that allogeneic MSCs could decrease EAE pathologies as much as syngeneic cells and thus could be exploited as part of a universal cell therapy approach for autoimmune diseases.

Supplemental Chapter: Allogeneic Mesenchymal Stem Cells as Universal Donors for Treatment of Experimental Autoimmune Encephalomyelitis

ABSTRACT

We hypothesized that the preparation of an allogeneic mesenchymal stem cell (MSC) population as universal donor cells for the treatment of experimental autoimmune encephalomyelitis (EAE) can lead to comparable improvement as seen with syngeneic cells and found that it was indeed the case. A decrease in interferon (IFN)-gamma during a mixed lymphocyte reaction (MLR); mediated by MSC-derived CC chemokine ligand (CCL)2 secretion, immune cell infiltration to the spinal cord and circulating levels of IFN-gamma and interleukin (IL)-17 were noticed. However, the pre-treatment of MSCs with IFN-gamma to increase the expression levels of antagonist CCL2 prior to their injection did not lead to any significant improvement. Such observation could be explained by the rapid rejection of IFN-gamma-stimulated allogeneic MSC due to up-regulation of major histocompatibility complex (MHC) molecules I and II. Therefore, only the direct use of allogeneic MSCs is as efficient as syngeneic cell preparation to improve EAE pathologies.

INTRODUCTION

Mesenchymal stem cells (MSCs) possess clinical potential for the treatment of many ailments[1,2,3,4,5,6]. Although, found in very low amount in the bone marrow, MSCs can be easily isolated and expanded *in vitro* without loosing their capacity to differentiate into multiple parenchymal lineages, hence their great potential in regenerative medicine[7]. Furthermore, one of the most remarkable and perhaps least understood ability of MSCs is their immunomodulatory capacity. More specifically, MSCs have been shown to prolong skin allograft survival in baboons[8], prevent the rejection of allogeneic tumor cells in immunocompetent mice[9] in addition to their capacity to alleviate EAE[10] and GVHD[11]. We have recently demonstrated that syngeneic MSCs can inhibit plasma cells[12] *in vitro* and *in vivo* and lead to therapeutic improvement in EAE mice[13] via paracrine conversion

of CCL2 to an antagonist form by matrix metalloproteinase (MMPs) processing. However, the efficient use of MSCs in the clinic is most likely readily achieved using a single allogeneic cellular product that could be used in multiple recipients as universal donor cells. As such, MSCs were shown to be rejected by the recipient immune system if used as part of a cell delivery platform for therapeutically relevant proteins[14]. This does not completely rule out the possibility of exploiting allogeneic MSCs in other ailments that may dependent on MSCs therapeutic effects for short laps of time. Thus, we hypothesized that allogeneic MSCs can improve the outcome of the neurological autoimmune disease EAE. We demonstrate in the following study that both syngeneic and allogeneic MSCs can alleviate EAE pathology with almost the same capacity. More specifically, allogeneic MSCs can lead to a decrease in immune cell infiltrate to spinal cords and secretion of pro-inflammatory cytokines. Interestingly however, pre-treating allogeneic MSCs with IFN-gamma showed no therapeutic improvement over the PBS control group.

MATERIALS AND METHODES

REAGENTS

Female BALB/c and C57Bl/6 mice were purchased from Jackson laboratories (Bar Harbor, ME, USA). Ascorbic acid-2 phosphate, insulin, Alizarin Red S, and Oil Red O were purchased from Sigma-Aldrich (Oakville, ON, Canada). IFN-gamma, CCL2, IL17 ELISAs and CCL2 neutralizing antibody were purchased from R&D System (Minneapolis, MN, USA). All FACS antibodies were purchased from BD Pharmingen (San Diego, CA, USA).

MSCs PREPARATION

Whole bone marrow from femurs and tibias of a female B6 or BALB/c mouse was harvested and placed in culture in complete media as reported previously[14]. Their phenotype was analyzed by flow cytometry using anti-CD44, CD45, CD90, CD73 and

CD105 antibodies. For differentiation, MSC were cultured in osteogenic or adipogenic media for 4 weeks. Alizarin Red S was then used to stain calcium in the mineralized extracellular matrix whereas oil Red O was used for lipid droplet staining. MSC conditioned-media (CM) was used to quantify CCL2 levels before and after IFN-gamma stimulation by ELISA according to manufacturer's instructions. To test the immunosuppressive property of syngeneic and allogeneic MSC CM, 100X concentrated CM was serially diluted by two fold and added to a 2 way MLR. The supernatants were then collected and tested by ELISA for IFN-gamma. The same experiment was repeated but using CCL2 neutralizing antibody.

EAE INDUCTION AND MSC INJECTION

Purified synthetic peptides of MOG_{35-55} 1mg/ml (Sheldon Biotech Center, McGill University, Montreal, Qc, Canada) was emulsified (1:1 volume ratio) in Complete Freund's Adjuvant (Cedarlane, Montreal, Qc, Canada) containing 4mg/ml Mycobacterium tuberculosis H35RA (Difco Laboratories, Detroit, Michigan, USA) and injected subcutaneously (sc) at the base of the tail. Animals also received pertussis toxin (Sigma Aldrich, Oakville, ON, Canada) immediately after the sc injection by IP injections, repeated two days later and mice were clinically scored every 2 days. Following the appearance of EAE symptoms, all groups were normalized to possess a grade 2 average before receiving IP injections of allogeneic or syngeneic MSC ($2X10^6$cells per injection). The same procedure was performed for IFN-gamma treated allogeneic MSCs.

IMMUNE AND HISTOLOGICAL ANALYSIS

All mice were bled a month later to analyse circulating levels of IFN-gamma and IL17. For histology purposes, EAE mice were perfused with 20 ml PBS before the removal of their spinal cord. Sections were then stained for Hemoxylin and Eosin. For Flow cytometry analysis of CD4 T-cells, spinal cords were mechanically disrupted to generate

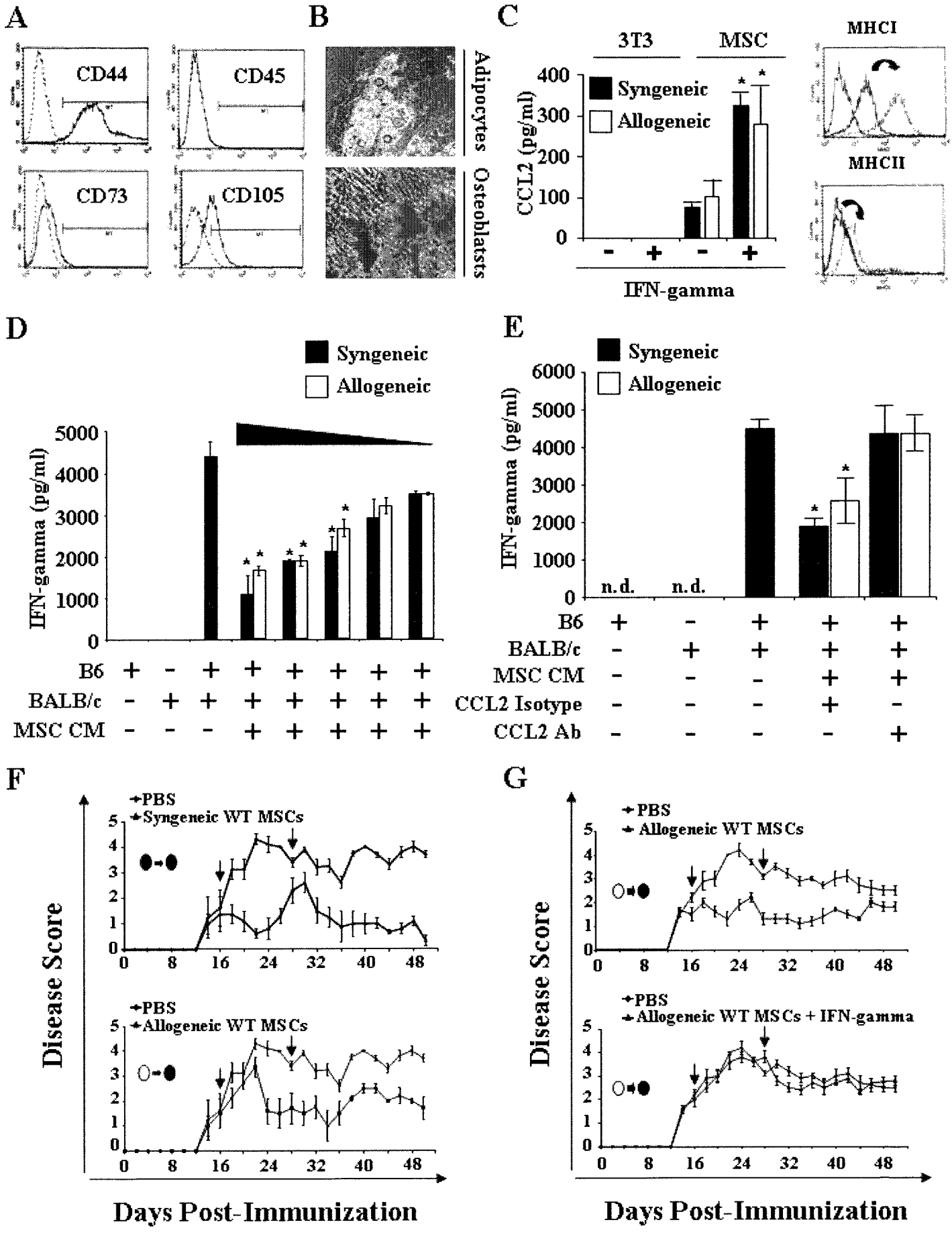
single cell suspension. Cells were then stained with CD4-PE and analyzed by FACS Calibur cytometer (BD).

RESULTS AND DISCUSSION

Syngeneic MSCs have great potency in reducing inflammation in EAE mice[10,13]. However, the translation of this technology platform to the clinic could be cumbersome due to customized preparation of MSCs from every patient; a time consuming procedure that might not be suitable for patients in active phase of pathology progression. Therefore, we wished to compare the therapeutic efficacy of allogeneic and syngeneic MSCs in alleviating EAE. Both cell populations had similar phenotypes characterized by the expression of CD44, CD73 and CD105 while being negative for CD45 (**Fig. 1A**). Their expansion *in vitro* did not alter their capacity to differentiate into adipocytes and osteoblasts confirming therefore that the expanded cells are of MSC origin (**Fig. 1B**). The presence of CCL2 in the CM of both allogeneic and syngeneic MSCs was confirmed by ELISA and shown to be modulated by IFN-gamma (**Fig. 1C**) and capable of blocking a 2-way MLR in a dose-dependent manner (**Fig. 1D**). The addition of CCL2 neutralizing antibody to the MLR completely abolished the observed suppression demonstrating the direct involvement of MSC-derived CCL2 inhibiting IFN-gamma secretion (**Fig. 1E**). Once delivered *in vivo*, both syngeneic and allogeneic MSCs populations were capable in reducing EAE severity by almost 50% over 2 months (**Fig. 1F**). Interestingly however, the prior treatment of allogeneic MSCs with IFN-gamma, as a mean to up-regulate the expression and convertion of CCL2 to an antagonist form did not lead to any improvement when compared to the PBS group (**Fig. 1G**). This loss of immunosuppressive property could be explained by the up-regulation of MHCI and MHCII molecules as we have shown *in vitro* following MSC treatment with IFN-gamma hence their rejection by the recipient's immune system (**Fig. 1C**). Consistent with the latter claim, the levels of circulating pro-inflammatory cytokine IFN-gamma and IL17 were comparable between the PBS control group and the allogeneic MSCs-treated with IFN-gamma (**Fig. 2A**), an observation that was further confirmed by the *in vitro* restimulation of splenocytes with MSCs (**Fig. 2B**). As for pathological findings, immune

Figure 1: Therapeutic Effects of Syngeneic and Allogeneic MSCs in EAE.

(A) MSC Phenotypic Analysis. Cultured MSCs were stained for various cell surface markers and shown to express high levels of CD44, low levels of CD73, medium levels of CD105 and no apparent CD45. **(B)** MSC Plasticity. As shown in this panel, MSCs culture under adipogenic or osteogenic conditions lead to their differentiation. **(C)** MSCs Secrete CCL2. To prove that MSCs are capable of secreting the CCL2 chemokine, CM collected from NIH-3T3 or MSCs with or without IFN-gamma (10ng/ml) treatment were collected and tested by ELISA. CCL2 was only detected in MSC CM and was up-regulated following IFN-gamma stimulation ($n=5$ and $P<0.0004$). MHCI and II were assessed by flow cytometry as indicative of successful IFN-gamma treatment **(D)** MSC CM Blocks 2-Way MLR in a Dose-Dependent Manner. CM was collected from resting syngeneic or allogeneic MSCs and concentrated up to a 100X. A 2 fold serial dilution was then prepared and added to the MLR reaction. IFN-gamma inhibition could only be achieved using a concentration $\geq$ 25X ($n=5$ and $P<0.0003$). **(E)** MSC-Derived CCL2 Blocks IFN-gamma During MLR. To demonstrate the direct involvement of CCL2 in IFN-gamma inhibition, the addition of CCL2 neutralizing antibody to the MLR reaction as reversed the IFN-gamma inhibition effect to a comparable level with the positive control ($n=5$ and $P<0.00002$). **(F)** Syngeneic and Allogeneic MSCs Effects on EAE. Mice with developed EAE symptoms were injected IP at 2 different time points as shown by arrows. Both syngeneic and allogeneic MSCs can lead to EAE improvement. **(G)** IFN-gamma Pre-treatment of Allogeneic MSCs Leads to no Therapeutic Effects. The same *in vivo* EAE experiment as in **(F)** was repeated but comparing allogeneic MSCs with or without IFN-gamma pre-treatment *in vitro*. No therapeutic improvements were noticed in the IFN-gamma pre-treated allogeneic MSCs compared to the PBS control group.


A
CD44
CD45
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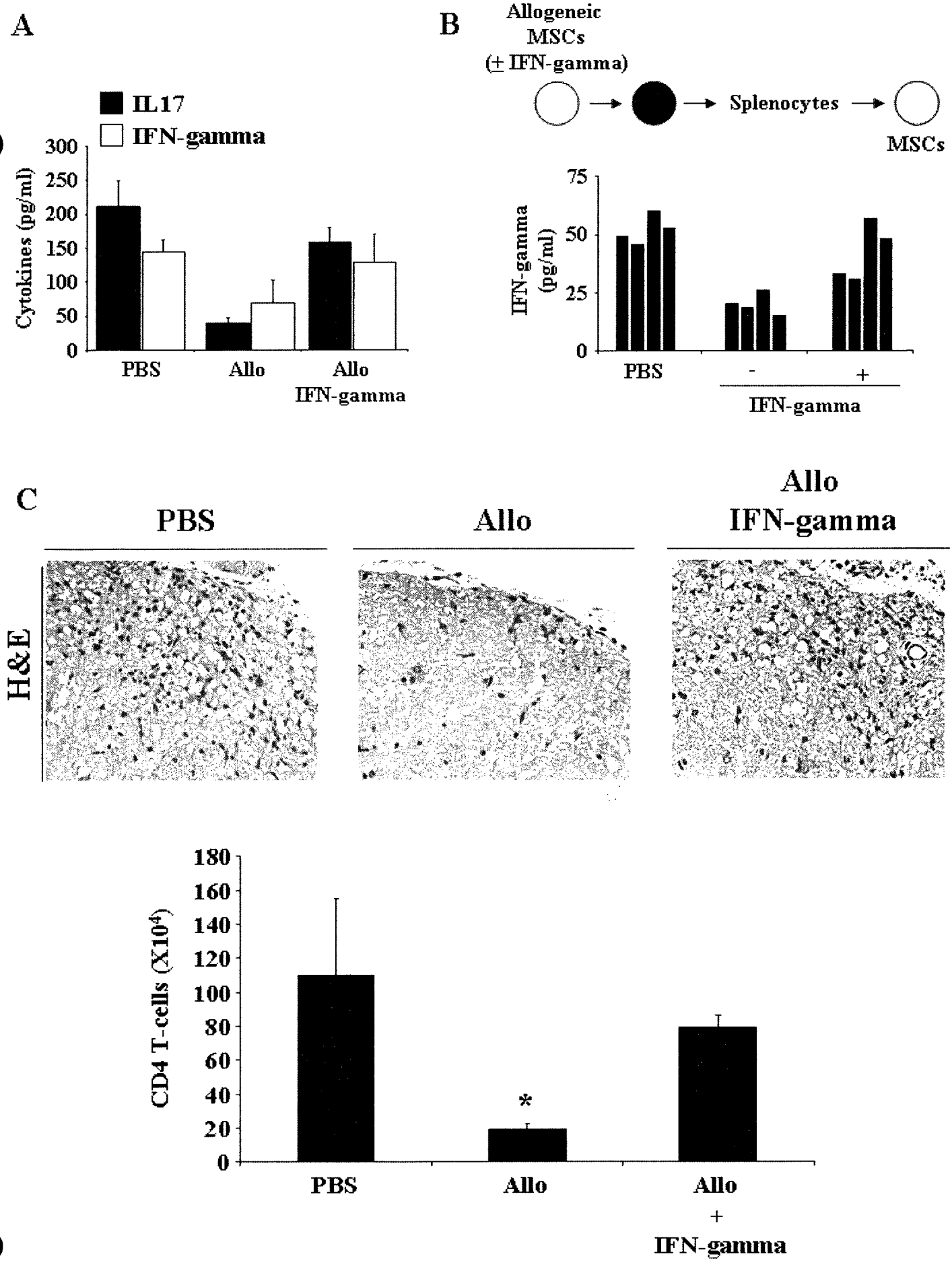
CD105
B
Adipocytes
Osteoblatsts
C
3T3
MSC
Syngeneic
Allogeneic
CCL2 (pg/ml)
400
300
200
100
0
- + - +
IFN-gamma
MHCI
MHCII
D
Syngeneic
Allogeneic
IFN-gamma (pg/ml)
5000
4000
3000
2000
1000
0
B6 + - + + + + + +
BALB/c - + + + + + + +
MSC CM - - - + + + + +
E
Syngeneic
Allogeneic
IFN-gamma (pg/ml)
6000
5000
4000
3000
2000
1000
0
n.d.
n.d.
B6 + - + + +
BALB/c - + + + +
MSC CM - - - + +
CCL2 Isotype - - - + -
CCL2 Ab - - - - +
F
PBS
Syngeneic WT MSCs
PBS
Allogeneic WT MSCs
Disease Score
0 8 16 24 32 40 48
Days Post-Immunization
G
PBS
Allogeneic WT MSCs
PBS
Allogeneic WT MSCs + IFN-gamma
Disease Score
0 8 16 24 32 40 48
Days Post-Immunization

Figure 2: Allogeneic MSCs Decreases the Levels of Pro-inflammatory Cytokines and CD4 T-cell Infiltration of the Spinal Cord.

(A) IFN-gamma and IL17 Levels in Systemic Circulation. Only allogeneic MSCs were capable of decreasing the circulating levels of IFN-gamma and IL17 in EAE mice whereas IFN-gamma pre-treatment of allogeneic MSCs lead to no difference as compared to the PBS control group ($n=5$ and $P<0.00002$). **(B)** MSCs Tolerogenic Effects. To demonstrate the tolerogenic effects of MSCs towards allogeneic cells, splenocytes collected from EAE mice injected with allogeneic MSCs were stimulated *in vitro* with the same MSCs they have been injected with. A robust decrease in IFN-gamma secretion was obtained with mice treated with allogeneic MSCs, whereas MSC pre-treatment with IFN-gamma prior to their injection leads to no differences in comparison to the PBS control group. **(C)** Immune Infiltrate Analysis. Spinal cords of EAE mice were stained with H&E to demonstrate the levels of immune cells. As demonstrated in this panel, no differences could be seen between the PBS EAE mice or EAE mice injected with IFN-gamma pre-treated MSC. The same outcome was obtained when CD4 T-cells infiltrates were assessed by flow cytometry.


A
IL17
IFN-gamma
Cytokines (pg/ml)
300
250
200
150
100
50
0
PBS
Allo
Allo
IFN-gamma
B
Allogeneic
MSCs
(± IFN-gamma)
Splenocytes
MSCs
IFN-gamma
(pg/ml)
75
50
25
0
PBS
-
+
IFN-gamma
C
PBS
Allo
Allo
IFN-gamma
H&E
CD4 T-cells (X10⁴)
180
160
140
120
100
80
60
40
20
0
*
PBS
Allo
Allo
+
IFN-gamma

infiltration in the spinal cord of EAE treated mice was robustly diminished following allogeneic MSCs injection whereas IFN-gamma pre-treatment leads to comparable immune infiltrate as seen in the PBS control group (**Fig. 2C**). Due to the important of MOG-specific CD4 T-cell in EAE pathologies, a flow cytometry analysis was performed on the spinal cord of treated EAE mice demonstrating a potent decrease in the total number of CD4 T-cells only in the allogeneic MSCs treated mice (**Fig. 2C**).

Long-term persistence of MSCs in immunocompetent recipient might not be necessary to achieve therapeutic treatment as long as the cells could persist long enough to suppress the ongoing inflammation. Such strategy could be possible as we have demonstrated in our study using allogeneic MSC for the treatment of EAE. However, our results suggest that in diseases highly driven by IFN-gamma, a fast rejection of injected allogeneic cells could impede the therapeutic effects. Thus, more studies are required to elucidate the appropriate cell therapy protocol for targeted ailments.

ACKNOWLEDGMENT

Moutih Rafei is a recipient of a *Fonds de Recherches en Santé du Québec (FRSQ)* Schorlaship and Jacques Galipeau is a *FRSQ chercheur-boursier sénior*. This work was supported by the Canadian Institute of Health Research grant MOP-15017. We thank Denis Rodrigue, Julie Hinsinger, and Micheline Fortin from the histopathology facility at the Institute for Research in Cancer Immunotherapy (Université de Montréal) for tissue processing and staining.

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MARK A. WAINBERG, O.C., PH.D., F.R.S.C.
DIRECTOR OF RESEARCH

PROFESSOR OF MEDICINE
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MCGILL UNIVERSITY AIDS CENTRE

January 14, 2009

RE: Moutih Rafei
Thesis submission to McGill University

To Whom It May Concern:

The following will confirm the safety and ethical conditions with regard to all laboratories at the Lady Davis Institute for Medical Research of the Sir Mortimer B. Davis Jewish General Hospital.

The Lady Davis Institute for Medical Research is a free-standing biomedical research institute attached to the Sir Mortimer B. Davis Jewish General Hospital, a major adult teaching hospital affiliated with McGill University. There is approximately 110,000 sq. ft. of laboratory space occupied by over 75 independent investigators all of whom hold McGill University appointments in appropriate departments. At the present time we have approximately 100 graduate students and post-doctoral fellows registered with McGill University. Total external funding is in the range of $18 million (exclusive of overhead), which is large by Canadian standards.

Professor Andrew Mouland functions as Chair of our Health and Safety Committee, while Professor John Hiscott, a virologist, heads the subcommittee of the Health and Safety Committee on Biohazards.

Dr. Mouland affirms the safety program which addresses the question of radioactive material licence and Dr. Hiscott affirms the question of the use of recombinant DNA in the experiments. Dr. Lorraine Chalifour affirms the ethical use of animals.

As a constituent part of McGill University the Institute follows and meets all of the requirements of the *McGill Laboratory Biosafety Manual*. For those rare circumstances not covered by the *McGill Laboratory Biosafety Manual* we use the following as reference guidelines:

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3755, CH. DE LA CÔTE-STE-CATHERINE ROAD, MONTRÉAL (QUÉBEC) H3T 1E2
TÉL / TEL: 514-340-8307 • FAX: 514-340-7537
E-MAIL: mark.wainberg@mcgill.ca • WEB: www.jgh.ca

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Regular inspection of safety procedures are conducted by the Canadian Nuclear Safety Commission (CNSC) (annual), Contex Environment (laboratory health and safety consultants – several times per year), the Sir Mortimer B. Davis Jewish General Hospital Chairman of Infection Control – Dr. Mark Miller (monthly), the Canadian Council on Animal Care (bi-annually), the McGill animal Care Committee (monthly), as well as by the Hospital Fire Marshal – Mr. Thomas Prokos, and the City of Montreal Fire Department (semi-annually).

The Lady Davis Institute (LDI) laboratories in which the research will be conducted are specially designed and equipped for scientific research and are used solely for that purpose. Specialized facilities are provided for the safe conduct of scientific research, including but not limited to, waste disposal facilities for biohazardous, radioactive and chemical wastes, a level 3 containment facility and state-of-the-art animal facilities. An automatic sprinkler system (water) is installed throughout the building. Fire extinguishers are located in several locations on each of six floors, as are fire alarms. Fire doors and alarm pulls are marked. In general, the laboratories and animal rooms are designed to have pressure differentials negative to adjacent areas. Fume hoods are monitored on a semi-annual basis. Fume hoods exhaust to the outside. Biological safety cabinets are certified semi-annually.

The use of chemicals is governed by McGill University regulations which are distributed to each laboratory. Standing operative procedures have been formulated to address actions to be taken after spills or accidents involving potentially hazardous materials under the supervision of the LDI Health and Safety Committee. Training which meets the requirements of various inspection teams is undertaken twice annually and the Health and Safety Committee meets monthly. Radiation worker training is offered on a regularly scheduled basis consistent with the requirements of the CNSC. Dr. Richard Latt, Director of the McGill Animal Resources Centre, is employed as a part-time veterinary consultant. All personnel working with animals hold a certificate as an Animal Health Technician as provided under the laws of the Province of Quebec. The Institute provides hepatitis immunization to all employees potentially exposed to human blood or blood products.