Targeting Th2 transcription factors in experimental asthma

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Abstract

Antigen specific $CD4^+$ T cells adoptively transfer airway inflammation comprised mainly of lymphocytes and eosinophils. The ability of these transferred T cells to induce inflammation is dependent on the cytokines they express particularly Th2 cytokines. In order to better understand the mechanism by which adoptively transferred T cells induce airway inflammation, we chose to modulate the expression (GATA-3) and activity (STAT-6) of two key regulators of Th2 cytokine production.

To modify expression of GATA-3, we used a bicistronic retroviral vector encoding *GATA-3* and *enhanced green fluorescent protein* (EGFP). As a control, we used a retrovector encoding *EGFP* alone. By coupling *in vitro* antigen stimulation with retroviral transduction we generated antigen specific $CD4^+$ T cells expressing EGFP alone or GATA-3 and EGFP. When transferred into naïve recipients that were subsequently challenged, these transduced $CD4^+$ T cells induced lung inflammatory responses with an increase in both $CD4^+$ lymphocytes and eosinophils. This antigen specific inflammatory response was enhanced in animals receiving T cells overexpressing GATA-3. Analysis of the infiltrating cells also revealed that the $EGFP^+$ T cells were present in the lung following antigen challenge, comprising only a small fraction of the $CD4^+$ T cells recruited to the lung during the antigen response. Thus, GATA-3 amplifies antigen-specific inflammatory responses in the airways by augmenting the ability of antigen specific T cells to recruit inflammatory cells to the lung following antigen challenge.

To modify the activity of STAT-6 we used chimeric cell penetrating peptides containing a poly-arginine protein transduction domain (PTD) coupled to a sequence predicted to bind and inhibit STAT-6 activity (SIP-1). Using fluorescein-tagged SIP-1, we demonstrate that the poly-arginine PTD efficiently translocates to the cytoplasm within an hour. *In vitro*, antigen-induced IL-4 production was inhibited in SIP-1-treated splenocytes. *In vivo*, pretreatment of $CD4^+$ T cells with the SIP-1 peptide effectively inhibited the ability of these cells to induce eosinophilic airway inflammation and IL-4 production following

adoptive transfer and airway antigen challenge These data demonstrate that cell permeable peptides targeting STAT-6 inhibit antigen-induced cytokine production *in vitro* and $CD4^+$ T cell-dependent inflammatory responses *in vivo*

Resumé

Les cellules $CD4^+$ T à antigènes spécifiques transfèrent par adoption l'inflammation pulmonaire constituées principalement de lymphocytes et d'éosinophiles. L'habileté de celles-ci à transférer des cellules T pour induire l'inflammation est dépendante de leur expression de cytokines Th2. De manière à mieux comprendre le mécanisme par lequel les cellules T transmises par adoption induisent l'inflammation pulmonaire, nous avons choisi de moduler l'expression de GATA-3) ou l'activité de (STAT-6) des deux régulateurs-clés de production de cytokine Th2.

Afin de modifier l'expression de GATA-3 dans les cellules T destinées au transfert par adoption, nous avons utilisé un rétrovirus recombinant concentré avec une filtration par centrifugeuse. Ce procédé a dramatiquement augmenté leurs titres et ainsi leur habileté à transduire les cellules $CD4^+$ T en culture primaire. Nous avons utilisé un rétrovirus recombinant qui encode la GATA-3 et / ou *la protéine fluorescente verte* (EGFP). En couplant *in vitro* la stimulation d'antigènes avec la transduction par vecteur viral, nous avons généré des cellules $CD4^+$ T à antigènes spécifiques exprimant de l'EGFP seul ou bien de la GATA-3 et de l'EGFP. Lorsque transféré dans un rat qui avait subséquemment été provoqué avec des antigènes, ces cellules $CD4^+$ T induisent une réaction aux inflammations pulmonaires avec une augmentation des lymphocytes et éosinophiles. Cette réaction inflammatoire fut accrue chez les animaux recevant les cellules T surexprimant la GATA-3. L'analyse des cellules infiltrantes a aussi révélé que bien que les cellules $EGFP^+$ étaient présentes dans les poumons suivant la provocation par antigènes, elles étaient constituées seulement d'une petite fraction de cellules $CD4^+$ T recrutées dans les poumons. Ainsi, la GATA-3 amplifie la réaction inflammatoire des poumons induite par antigènes en augmentant l'habileté des cellules T à antigènes spécifiques à recruter des cellules inflammatoires au poumon en suivant la provocation par antigènes.

Afin de modifier l'activité de STAT-6 nous avons utilisé une cellule chimérique de peptide pénétrante contenant de la protéine poly-arginine (PTD) couplée à la séquence devant attacher et inhiber la STAT-6 (SIP-1). Nous avons

démontré que la SIP-1 marquée au fluorescent a rapidement transduit les cellules et s'est localisé dans le cytoplasme. L'antigène induit l'IL-4 dans la culture primaire de splénocytes qui est inhibée par la peptide SIP-1, également inhibée *in vitro*. Le traitement des cellules $CD4^+$ T *ex vivo* avec la peptide SIP-1 a efficacement inhibé l'habileté de ces cellules d'induire l'inflammation pulmonaire éosinophilique et la production d' IL-4 suivant la provocation pulmonaire par antigènes. Ces données démontrent que les cellules peptides perméables visant la STAT-6 inhibent la production IL-4 d'antigènes induites *in vitro* et que les cellules $CD4^+$ T dépendent de ces réactions inflammatoires *in vivo.*

.

Abbreviations

AHR- airway hyperresponsiveness

AMDCs - airway mucosal dendritic cells

CC10 - clara cell specific promoter 10

CCR-,CC - chemokine receptor

CD25- IL-2 receptor α chain, expressed on activated T cells B cells and monocytes

CD4 - co-receptor for MHC, expressed on T cells, monocytes, macrophages and NKT cells

CD62L - leukocyte adhesion molecule, expressed on monocytes, B cells, T cells and NK cells

CD86- ligand for CTLA-4, expressed on monocytes, B cells, and dendritic cells

CTLA-4 - cytotoxic T-lymphocyte associated gene

DLNs- draining lymph nodes

DSP-3,3′- dithiobis(succinimidylpropionate)

EGFP- enhanced green fluorescent protein

ERK- extracellular signal related kinase

FITC-SIP-1- fluorescein conjugated STAT-6 inhibitory peptide

HIV-TAT- human immunodeficiency virus transactivator of transcription

HS - heparan sulphate

HSCs - hematopoeitic stem cells

IFN- γ - interferon gamma

IL-interleukin

MAPK- mitogen activated protein kinase

MLV- murine leukemia virus

MoMLV- moloney murine leukemia virus

OVA- ovalbumin

OX-40 (also CD134)- adhesion molecule co-stimulator, expressed on activated T cells

PI3K - phosphoinositide-3-kinase

PIC- pre-integration complex

PTD- protein transduction domain

SCP-1- STAT-6 control peptide

SIP-1-STAT-6 inhibitory peptide

SSCH- side scatter height

STAT-6 - signal transducer and activator of transcription

TARC - thymus and activation regulated chemokine

TGF-β - transforming growth factor beta

VSV-G - vesicular stomatitis virus glycoprotein G

Chapter 1: Introduction

Targeting transcription factors in asthma

Introduction

Role of the T cell in asthma pathogenesis

Asthma is a complex, chronic inflammatory disease likely caused by dysregulated immune responses within the airways and characterized by several key features, namely i) reversible airway obstruction with recurrent wheezing and chest tightness; ii) airway hyperresponsiveness (AHR), defined as an increased sensitivity to bronchoconstrictors; iii) airway inflammation; iv) increased allergen-specific IgE; and v) airway remodeling (1). Though the origins for this aberrant immune response are multifactorial, atopy or allergy, which is the genetic predisposition to develop a Th2 response to environmental antigens, is a major factor.

The dysregulated immune response in the asthmatic lungs of both atopic and non-atopic individuals induces immune responses to common innocuous environmental proteins or antigens. This allergic response can be divided into an early phase that occurs within the first hour of allergen exposure, and which is induced by mast cell degranulation and the release of inflammatory mediators that increase vascular permeability and chemokines that increase cellular chemotaxis. Bronchoconstrictors such as prostaglandins and leukotrienes are also released during this early response. A late response occurs 4-24 hours later and is accompanied by the recruitment of inflammatory cells, particularly $CD4^+$ T lymphocytes (2) and eosinophils (3), to the airways.

Once recruited to the lung, activated $CD4^+$ T lymphocytes produce cytokines such as IL-4 which induces B cell maturation and production of

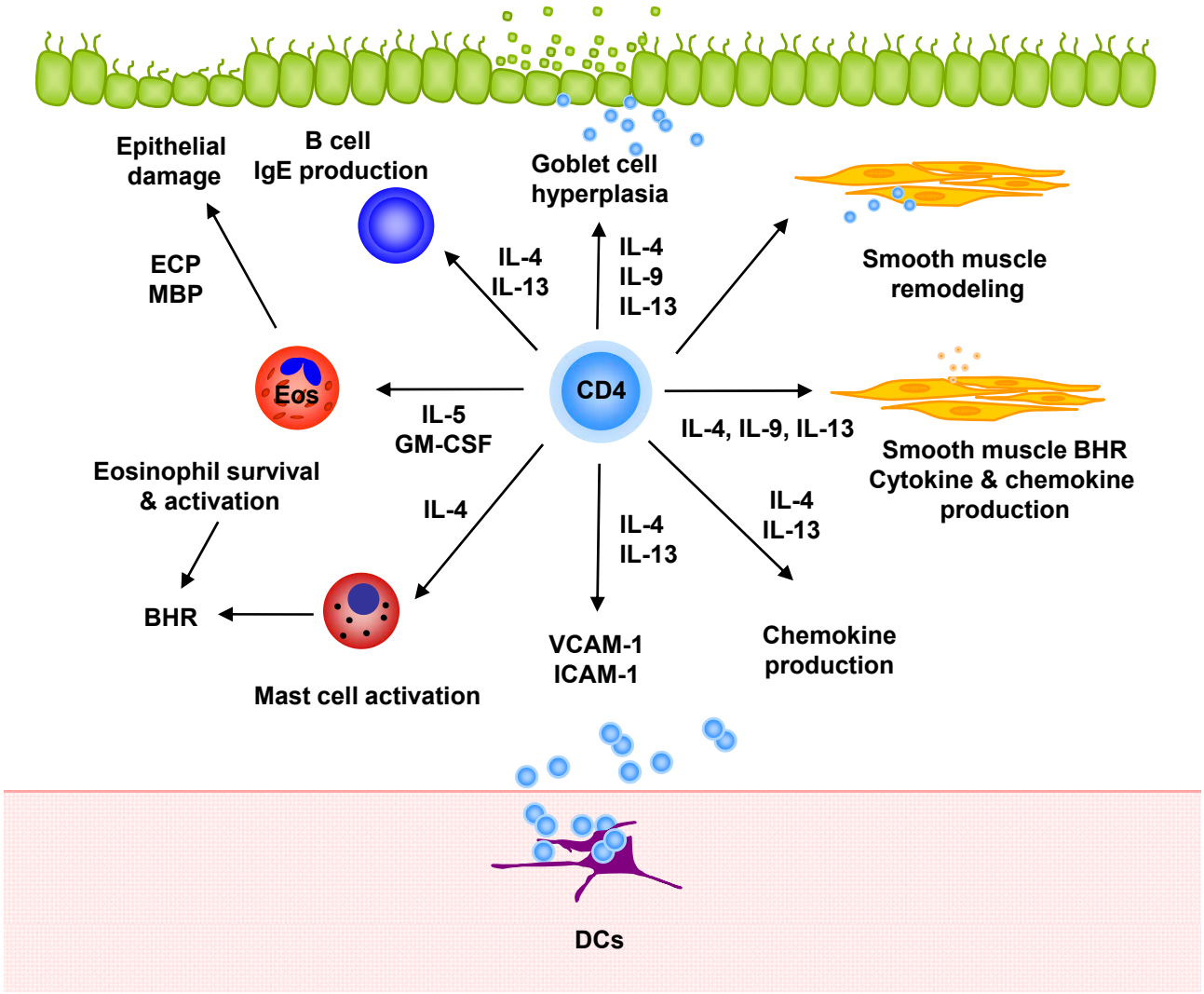
allergen-specific IgE (4). Bronchoconstriction is induced when allergen-specific IgE is captured on the cell surface receptor (FcεRI) on mast cells and eosinophils. Crosslinking of this receptor leads to the release of bronchoconstrictive mediators such as histamine, prostaglandins, leukotrienes and more cytokines (5-7). Chronic exposure to antigen eventually leads to structural changes within the lung such as goblet cell hyperplasia and mucus production, increases in smooth muscle mass, thickening of the subepithelium, and deposition of extracellular matrix proteins such as collagen and proteoglycans (8) (**Figure 1.1**).

Numerous studies show a positive correlation between the number of $CD4^+$ T lymphocytes in the lung and human asthma (9). In general, $CD4^+$ T cells traffic to the lung where they participate in immunosurveillance by producing inflammatory cytokines after antigen exposure (10). Hence, in the normal adult lung, $CD4^+$ memory T cells are found within the alveoli, airway lumen, intraepithelial layer, submucosa, and interstitium (10, 11). Due to an aberrant response to environmental antigens, asthmatic subjects have an increased accumulation of T cells in the lungs and airways (12-14). Furthermore, the number of $CD4^+$ T cells present in the lung correlates with both eosinophilia and disease severity (2). Larger numbers of $CD4^+$ T lymphocytes have also been detected using morphometric analyses of the airways in patients who died with severe asthma (15). Taken together, this data suggests that infiltrating $CD4^+$ T cells play an important role in the induction and maintenance of allergic asthma.

Figure 1.1

Functional role of the $CD4^+$ T cell in asthma.

The mechanism by which $CD4^+$ T cells induce airway hyperresponsiveness, mucus production, and airway eosinophilia is dependent upon the cytokines they produce. Activated T cells produce cytokines such as IL-4 which leads to B cell maturation and production of allergen-specific IgE. Production of IL-5 leads to eosinophil development, recruitment and activation. Activated eosinophils release inflammatory mediators such as major basic protein (MBP) and eosinophil cationic protein (ECP) which cause lung epithelial cell damage. In addition, activated eosinophils and mast cells produce bronchoconstrictive mediators such as prostaglandins and leukotrienes which then induce bronchial hyperresponsiveness (BHR). T cells also increase inflammatory cell chemotaxis by cytokine induced expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) and intracellular adhesion molecule (ICAM-1) on vascular endothelium. As well, T cell production of IL-4 and IL-13 increases production of chemokines which promote inflammatory cell extravasation into the lung. T cell derived cytokines also alter the contractility of smooth muscle cells by increasing their responses to bronchoconstrictors. Finally, chronic T cell inflammatory responses induce structural changes within the lung including goblet cell hyperplasia, mucus production and increases in smooth muscle mass.


Epithelial damage
B cell IgE production
Goblet cell hyperplasia
Smooth muscle remodeling
ECP MBP
IL-4 IL-13
IL-4 IL-9 IL-13
Eos
CD4
IL-5 GM-CSF
IL-4, IL-9, IL-13
Smooth muscle BHR Cytokine & chemokine production
Eosinophil survival & activation
IL-4
IL-4 IL-13
IL-4 IL-13
BHR
VCAM-1 ICAM-1
Chemokine production
Mast cell activation
DCs

Analysis of the infiltrating $CD4^+$ T cells from asthmatic patients also suggests that these cells react differently when compared to T cells from normal subjects. In an early study by Walker and colleagues, purified activated T lymphocytes from the airways of allergic and non-allergic asthmatic individuals were co-cultured with eosinophils. T cells from asthmatic donors secreted factors (cytokines) that extended the lifespan of eosinophils suggesting that T cells and the factors they secrete play an important regulatory function in asthma by promoting eosinophil survival (16). It is now well established that increased levels of Th2 cytokines are present in the bronchoalveolar lavage fluid (17, 18) and serum (19) of atopic and nonatopic (20) patients with asthma.

Definitive evidence that T cells are critical to the inflammatory responses and structural changes associated with asthma has come from experimental animal models. In these models, sensitized animals that are subsequently challenged with antigen develop AHR and eosinophilic airway inflammation along with an increase in airway $CD4^+$ T cells (21, 22). Depletion of $CD4^+$ T cells in these models prevents the induction of asthma pathogenesis (23), providing evidence that $CD4^+$ T cells are critical to the induction of allergic disease**.** Further evidence that $CD4^+$ T cells mediate pathogenesis in experimental asthma is provided by adoptive transfer models, in which T cells from antigen-sensitized animals are transferred into naïve recipients, which are subsequently antigen challenged. Results from these models have been particularly useful in elucidating the role of the T cell in asthma and demonstrate that antigen-specific $CD4^+$ T cells are sufficient to induce AHR, mucus production, and airway

eosinophilia in response to antigen challenge (24-27). Furthermore, the ability of these $CD4^+$ T lymphocytes to induce allergic inflammation is dependent on Th2 cytokine expression (28, 29).

The main objective of my thesis research was to evaluate the role of two Th2-specific transcription factors in the ability of adoptively-transferred, antigen-specific T cells to regulate airway inflammation. Although a number of studies have addressed the roles of Th2 cells in models of adoptive transfer, few of these studies have investigated the role of the transcription factors that modulate Th2 cytokine expression. To better understand the role of T cell-specific transcription factors in adoptively transferred experimental asthma we targeted two key transcription factors that regulate Th2 cytokine and chemokine expression: GATA-3 and signal transducer and activator of transcription factor 6 (STAT-6). Our goal was to modify the expression (GATA-3) or activity (STAT-6) of these two transcription factors in cells destined for adoptive transfer so as to evaluate how their modulation affects the ability of adoptively-transferred T cells to induce allergic inflammatory responses.

To modify the expression of GATA-3 in primary $CD4^+$ T cells we utilized ecotropic retroviruses encoding the rat *GATA-3* gene. The generation of retrovirally-transduced primary $CD4^+$ T lymphocytes has been limited by low titer retroviral preparations, which inefficiently transduce primary lymphocytes. To overcome this, we developed a simple method to prepare high-titer ecotropic retrovirus stocks by concentrating dilute retroviral preparations using centrifugal filtration (Chapter 2). I demonstrate that ecotropic retroviral preparations

concentrated by this method can be used to generate transduced antigen-specific T cells in sufficient numbers for *in vivo* studies in rodents. With this technology, I then generated retrovirally-transduced $CD4^+$ T cells that overexpressed GATA-3.

These gene-modified T cells were used in an established Brown Norway rat model of adoptively-transferred asthma to examine the role of GATA-3 in the ability of T cells to induce airway inflammation (Chapter 3). We hypothesized that following adoptive transfer, our antigen stimulated retrovirally-transduced T cells would retain the ability to induce airway inflammation in antigen challenged naïve recipients. We also expected that T cells transduced with the GATA-3 retrovector would express higher levels of Th2 cytokines compared to cells transduced with the control vector. We further hypothesized that overexpression of GATA-3 in these cells would enhance their ability to induce airway inflammation and eosinophilia. Thus by using these retroviruses we hoped to define the role that GATA-3 plays in the ability of adoptively transferred T cells to induce airway inflammation in experimental asthma.

We next investigated the contribution of the STAT-6 transcription factor in the ability of adoptively-transferred T cells to induced inflammation in the BN rat model of adoptively-transferred asthma (Chapter 4). To achieve this, we utilized a cell penetrating peptide targeting STAT-6. This STAT-6 inhibitory peptide (SIP-1) is comprised of a poly-arginine-based protein transduction domain fused to peptide sequences predicted to bind to and inhibit STAT-6. Our first goal was to test the hypothesis that STAT-6 regulates the production of IL-4 in antigen stimulated T cells in vitro. We also used the peptide to test the

hypothesis that the ability of adoptively transferred T cells to induce airway inflammation was STAT-6 dependent. Thus by using this peptide we hoped to determine if targeting STAT-6 could inhibit antigen-induced cytokine production *in vitro* and $CD4^+$ T cell-dependent inflammatory responses *in vivo*. These experiments will also provide evidence that treatment of T cells with cell penetrating peptides may be useful in identifying the signaling molecules that regulate the ability of these cells to induce adoptively transferred experimental asthma.

Below, I will summarize the current literature on the role of the T cell and the Th2 cytokines in asthma, focusing primarily on murine and rodent models of adoptive transfer. I will then summarize the current understanding of two key Th2 transcription factors (GATA-3 and STAT-6) involved in asthma. I will also discuss mechanisms by which these transcription factors regulate cytokine expression. Finally, I will present an overview of the experimental methodology I used to modify GATA-3 expression and STAT-6 activity.

T cell effector mechanisms: Th2 cytokines in allergic disease

$CD4^+$ helper T cells can be classified primarily into three functionally different polarized effector subsets: Th1 cells that produce Th1 cytokines, including interferon gamma (IFN-γ) and IL-12; Th2 cells that produce Th2 cytokines including IL-4, IL-5, and IL-13; and Th17 cells that produce IL-17A (IL-17), IL-17F and IL-22 (**Figure 1.2)** (30-33). Evidence from transgenic mice and murine models of experimental asthma have now shown that the Th2

cytokines: IL-4, IL-5, IL-6, IL-9, GM-CSF and IL-13 promote various indices of allergic airway inflammation (**Table 1.1**) (34-41). The most extensively studied of these cytokines are IL-4, IL-5 and IL-13, all of which are upregulated in response to antigen exposure in the lung (42).

Figure 1.2

$CD4^+$ effector T cell subsets.

Peripheral naive $CD4^+$ T cell precursor cells (Thp) can differentiate into three effector subsets (Th1, Th2 and Th-17). CD4 T cell differentiation is dependent on activation of specific transcriptions factors (bold) as well as specific cytokines. IL-4 induces development to Th2 phenotype; IL-12 and interferon gamma (IFN γ) favor a Th1 phenotype and IL-6/TGFβ lead to the development of Th17 cells.

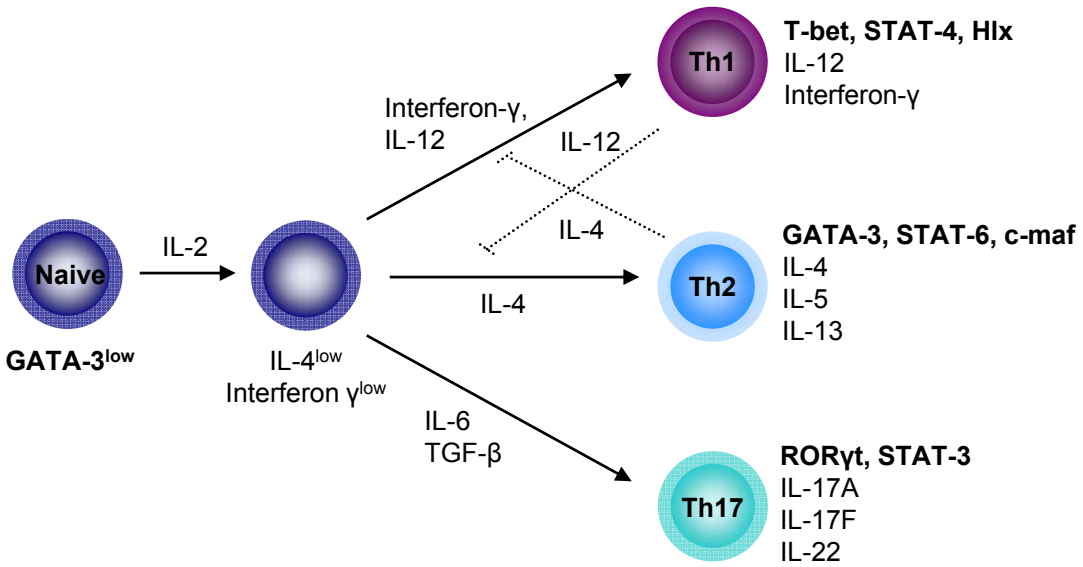

Naive
GATA-3low
IL-2
IL-4low
Interferon γlow
Interferon-γ,
IL-12
IL-12
IL-4
IL-4
IL-6
TGF-β
Th1
T-bet, STAT-4, Hlx
IL-12
Interferon-γ
Th2
GATA-3, STAT-6, c-maf
IL-4
IL-5
IL-13
Th17
RORγt, STAT-3
IL-17A
IL-17F
IL-22

Table 1.1 Cytokines that regulate allergic airway inflammation

	Function in allergic immune response	**Transcription factor**
IL-4	Induces mucus production (43) Induces airway hyperresponsiveness (44) Promotes expression of vascular cell adhesion molecule (VCAM-1) on vascular endothelium (45) Induces expression of T cell chemokine thymus- and activation-regulated chemokine (TARC) (46) Induces B-cell isotype switching and promotes and sustains expression of IgE (47)	GATA-3 (48) STAT-6 (49)
IL-5	Promotes development, differentiation, recruitment, activation and survival of the eosinophil (50)	STAT-5/1 (51) GATA-3 (52)
IL-9	Induces goblet cell metaplasia by inducing mucin gene expression (53) Induces eotaxin expression (54)	NF-Kb (55)
IL-13	Facilitates inflammation by upregulating VCAM expression on endothelial cells (56) Regulates MHC Class II and IgE expression on B cells (57) Inductes chemokines such as eotaxin (1&2), monocyte chemoattractant protein (MCP1,2,3,5), macrophage inhibitory protein 1α (MIP1α), TARC and macrophage-derived chemokine (MDC) (58, 59) Indirectly induces bronchial smooth muscle proliferation by increasing expression of CysLT1R (60), TGFβ (61) and PDGF (62)	GATA-3 (63) STAT-6 (49)

IL-17	Increases airway mucin gene expression (64)	NF-κB (65) STAT-3 (65)
IL-25	Promotes Th2 cytokine expression (66, 67)	?
GM-CSF	Enhances development of dendritic cells towards the Th2 phenotype (68-70)	STAT-1 (71)

IL-4

IL-4 was initially cloned and characterized as a B cell stimulatory factor (72-74) and eosinophil differentiation factor (75). It is highly pleiotropic, functioning in a number of target cells and regulating different biological responses. One of the major functions of IL-4 is the control of growth and differentiation in both lymphoid and myeloid cell populations (76). In T lymphocytes, IL-4 drives the differentiation of naïve T cells into Th2 cells that produce Th2 cytokines (77, 78). Apart from this role in the development of T cells, IL-4 induces B-cell isotype switching and promotes (79) and sustains expression of IgE (47). IL-4 can also induce bone marrow progenitor cells to differentiate into Th2 cytokine producing eosinophils(80). Finally, IL-4 directs maturation, proliferation and activation of immature dendritic cells, polarizing them to a Th2 phenotype (81).

IL-4 mediates its activity by binding to one of two IL-4 receptors: the type I IL-4 receptor and the type II IL-4 receptor. The type I IL-4 receptor is comprised of a heterodimer of the high affinity IL-4 receptor α (IL4Rα) chain and the common γ chain (82, 83). The type II IL-4 receptor consists of the IL4Rα chain and the IL-13 receptor α1 chain (84). Signaling through each of these receptors leads to the activation of STAT-6 (85), which mediates IL-4-induced transcriptional responses (discussed later). In addition, IL-4-induced activation of STAT-6 leads to the expression of GATA-3, which regulates the initiation of transcription of Th2 cytokines. This early induction of GATA-3 is particularly

crucial in the initial activation of naive T cells and drives the development of the Th2 effector cell (discussed later).

Consistent with its role in driving Th2 immune responses, IL-4 also participates in allergic airway responses in several ways. For instance, IL-4 regulates allergen-induced IgE production from B cells (47). By promoting expression of vascular cell adhesion molecule (VCAM-1) on vascular endothelium (45, 86) IL-4 enhances extravasation of inflammatory cells into lung tissue. Additionally, IL-4 enhances inflammation by regulating expression of chemokines such as eotaxin (CCL11), a chemokine that induces eosinophil chemotaxis; thymus- and activation-regulated chemokine (TARC or CCL17), a T cell chemoattractant; and RANTES, a chemoattractant for eosinophils, lymphocytes, and monocytes (59, 87-90) from airway smooth muscle and epithelial cells.

The involvement of IL-4 in other features of allergic disease is somewhat more complex. For instance, whereas some studies support the involvement of IL-4 in mucus secretion, others contradict it (27, 43, 91, 92). Likewise there is no strong link between IL-4 and antigen induced AHR in mice. Use of an IL-4 blocking antibody in an acute murine model of asthma inhibits allergen-induced AHR and goblet cell metaplasia (93) whereas lung-specific overexpression of IL-4 using a Clara cell specific promoter (CC10) in a transgenic model leads to spontaneous eosinophilic and lymphocytic inflammation in the lung without AHR (36). Studies using IL-4 knockout (IL-4 -/-) mice reflect this since they exhibit

reduced airway inflammation in response to antigen challenge (94) but AHR and mucus production remain unaffected (95).

In contrast to mice, high levels of IL-4 have been strongly linked to AHR in humans. Treatment of mild asthmatics with nebulized IL-4 increases eosinophilia and airway responsiveness to the bronchoconstrictor, methacholine (44). Segmental antigen challenge of bronchi (96) and *in vitro* antigen challenge of peripheral mononuclear cells from atopic patients also induces IL-4 expression that correlates with disease severity (97). Promising results from clinical trials using soluble IL-4 receptors to neutralize IL-4 function demonstrate a significant improvement in the lung function of treated asthmatic subjects (98, 99). However, studies using a larger cohort of asthmatic patients have yet to confirm these preliminary results (100).

IL-13

Human and mouse IL-13, which were initially cloned from T cells, are structurally and functionally similar to IL-4 (101-104). Like IL-4, IL-13 activates the IL-4 type II receptor, comprised of the IL-4R α chain and the IL-13R α1 chain (83). Binding of IL-13 to this receptor, which is found on several different cell types including B cells (83), airway epithelial cells (105, 106), macrophages (106) and lung fibroblasts (107), leads to the phosphorylation and activation of STAT-6 (108) (discussed later). IL-13 also binds to the IL-13Rα2 chain, a decoy receptor present in both membrane and soluble forms (109), that is thought to play a role in IL-13 sequestration (110-113).

Early studies assessing IL-13 function showed that it induced immunoglobulin production and proliferation in B cells as well as differentiation of cells of the monocytic lineage (102, 103, 114). The understanding of the effector function of IL-13 in asthma pathogenesis has dramatically expanded. IL-13 is now known to possess several overlapping activities with IL-4 including the promotion of B cell expression of MHC Class II molecules and the low affinity IgE receptor (57). Likewise, IL-13 also regulates inflammation by controlling expression of chemokines such as eotaxin (115) and MCP-1 (107). Like IL-4, IL-13 also induces expression of adhesion molecules such as VCAM on endothelial cells thereby facilitating recruitment of T cells into the lung.

Since IL-13 shares one receptor subunit with IL-4, it is not clear how IL-13, but not IL-4, induces some pathophysiological features of asthma, particularly mucus secretion (116, 117). Data also suggests that IL-13, but not IL-4, may indirectly participate in the induction of airway remodeling by inducing bronchial smooth muscle cell proliferation via increased expression of the leukotriene receptor $CysLT_1R$ (60) and vascular endothelial growth factor (VEGF) (118). This could be because the effect of Th2 cytokines in allergic disease is determined, at least in part by their levels of expression and protein stability. Not surprisingly, IL-13 is more stable and is expressed at higher levels (119) which may explain its ability to induce some aspects of airway remodeling even when IL-4 cannot.

There are numerous studies, both clinical and in animals, to support the role of IL-13 as the major effector cytokine in asthma. Increased levels of IL-13 are present in the BAL fluid from asthmatic patients (120). Intranasal

administration of recombinant IL-13 results in airway inflammation, AHR, and production of both mucus and eotaxin (39, 116, 117). Additionally, transgenic mice overexpressing IL-13 from the airway epithelial cell-specific CC10 promoter exhibit airway inflammation, AHR, mucus hypersecretion and subepithelial fibrosis (39). Consistently, the failure to develop airway inflammation and AHR in IL-13 deficient animals can be restored by administration of recombinant IL-13 (121). Likewise, *in vivo* inhibition of IL-13 using an IL-13Rα2-based decoy decreases antigen-driven AHR, airway inflammation, mucus production, and IgE production (9, 122). It is important to note that blockade of IL-13 in these models does not completely abolish allergen-induced eosinophilia (116). This is likely due to the continued production of IL-5 and eotaxin in these mice (123). Antigen-induced expression of IL-5 by T cells and eotaxin by bronchial epithelial cells is sufficient to drive airway eosinophilic infiltration. Furthermore, IL-5 and eotaxin may induce IL-13 production in T cells (124) further illustrating the interdependence and importance of several cytokine signaling systems in allergic disease.

IL-5

IL-5 was first identified in the 1980s (50) as a cytokine involved in the development (125), differentiation (126), recruitment (127), activation (128) and survival (129) of the eosinophil. IL-5 mediates these effects by binding to the IL-5 receptor, a heterodimer consisting of α- and β-subunits (130). The α-subunit is specific to IL-5, whereas the β-subunit is shared with the IL-3 and

granulocyte/macrophage colony-stimulating factor (GM-CSF) receptors. IL-5 binding and receptor activation on eosinophils leads to the propagation of signals through the Ras-MAPK and JAK-STAT pathways, which in turn promote IL-5 production and enhanced eosinophilic responses.

Allergen inhalation in humans leads to increased production of IL-5 in the bronchoalveolar lavage (131) and induced sputum (132). Allergen challenge also increases the number of $CD4^+$ lymphocytes and eosinophils producing IL-5 (133). Correspondingly, the characteristic eosinophilia seen in asthmatic airways is accompanied by a concomitant increase in IL-5 (134). As well, direct administration of recombinant human IL-5 to patients with allergic asthma causes AHR and sputum eosinophilia (26).

The increased recruitment of eosinophils to the upper and lower airways of asthmatic patients led to the hypothesis that blocking the activity or synthesis of IL-5 might be beneficial in the treatment of asthma. This was supported by data from IL-5 deficient mice which fail to develop eosinophilia following sensitization and challenge with antigen (135). Additionally, administration of a single intraperitoneal dose of anti-IL-5 antibody decreases the number of intraepithelial eosinophils and chronic inflammatory cells in the airway wall of mice in both acute (93, 136) and chronic models (137) of allergic airways disease, although AHR remains unaffected. Long-term treatment with the anti-IL-5 antibody nearly eliminates eosinophils from both the peripheral blood and the airways of antigen challenged animals (138).

Largely similar results were obtained in humans where airway eosinophilia is correlated with disease severity and may predict a decline in airway function (139). Surprisingly, clinical trials with a humanized anti-IL-5 monoclonal antibody showed mixed results. Whereas one study showed extensive decreases in eosinophils in the blood and sputum (140), another showed significant decreases in blood eosinophils and only modest decreases in bone marrow and bronchial mucosal eosinophils (141). There were no changes in airway function or disease symptoms in both these studies suggesting that AHR occurs independently of IL-5 and eosinophilia. However, results from this study should be taken with caution since the non treated asthmatic subjects did not exhibit AHR either (142). Interestingly, sustained treatment with the anti-IL-5 antibody in yet another study led to improvement in two indices of airway remodelling, smooth muscle mass and extracellular matrix deposition (143) indicating that further investigation into the mechanism underlying the role of IL-5 and eosinophils in airway function and remodelling is required.

To better understand the role of eosinophils in experimental asthma two independent groups generated mice that lacked eosinophils using two different methodologies (144). While the data are informative, they are not in complete agreement, likely because the background strains of the mice differed. Mice developed by Lee and coworkers expressed diphtheria toxin under the control of the eosinophil peroxidase promoter (*PHIL*) thereby selectively depleting eosinophils (145). Humbles *et al*. depleted eosinophils by generating mice with targeted mutations in the gene encoding GATA-1, a transcription factor essential

for differentiation of immature myeloid cells. Thus, deletion of the GATA-1 binding site in the GATA-1 promoter (Δdbl GATA) leads to selective ablation of the eosinophil lineage (146). Each strain of mice was tested in standard models of experimental asthma. *PHIL* mice did not develop AHR and showed partial reduction in airway mucus production. Importantly, Th2 cytokine production was also decreased in these mice. These data suggest that eosinophils participate in asthma pathogenesis at least in part by promoting production of Th2 cytokines at the site of disease. In contrast, mucus production and AHR were both intact in the Δdbl GATA mice. Airway remodeling was attenuated in these mice consistent with the effects seen in clinical trials with the IL-5 antibodies in humans. Although contradictory, these reports demonstrate that IL-5 plays an important role in asthma pathogenesis where it partially regulates both airway function and airway remodeling .

Th2 transcription factors in the allergic inflammatory response

The Th2 cytokines, IL-4, IL-5 and IL-13, are clustered at the Th2 locus in a region that spans 125 kb (147) on Chromosome 5q31 in humans and Chromosome 11 in mice. Genetic analyses have now linked this cluster (rev (148)) with total IgE serum concentration (149), eosinophil levels (150), bronchial hyperresponsiveness (151) and a predisposition to atopy. Abundant data from animal models and *in vitro* studies provide evidence that production of these cytokines as well as many of their effector functions are dependent on two key transcription factors, STAT-6 and GATA-3.

STAT-6

The signal transducer and activator of transcription (STAT) family of transcription factors mediate responses to multiple cytokines and growth factors (152, 153). STAT-6, which is activated by the Th2 cytokines, IL-4 and IL-13 (154), is essential for the development of Th2 cells from naïve T cells (49). Forced expression of STAT-6 is sufficient to drive Th2 cytokine production in differentiated or differentiating Th1 cells by inducing GATA-3 and c-maf expression whilst also down regulating IL-12 receptor chain (IL-12Rβ2) expression (155). However the ability of STAT-6 to induce this Th2 phenotype decreases with progressing Th1 cell commitment.

Activation of STAT-6 is dynamic and depends on repeated cycles of activation, nuclear transport, deactivation and subsequent reactivation (156). The initial activation occurs following receptor engagement and dimerization induced by IL-4 or IL-13. This leads to the activation of the receptor-associated JAK family of kinases which then phosphorylate Tyr residues on the IL-4Rα chain (**Figure 1.3**) (157) (rev by(158)). A STAT-6 monomer is recruited from the cytoplasm and binds to the Tyr phosphorylated IL4Rα chain via its SH2 domain. Once localized to the receptor, STAT-6 is phosphorylated on Tyr-641. This event allows two STAT-6 monomers to homodimerize via interactions between the SH2 domains and the phosphorylated Tyr641 residues on paired molecules. Following dimerization, STAT-6 translocates to the nucleus where it binds to a conserved sequence motif, TTC $(N)_{2-4}$ GAA, (where n represents any nucleotide) and

interacts with basal transcriptional machinery, other transcription factors, and/or transcriptional co-activators such as CBP and P300, to initiate transcription.

Protein interactions through the trans-activation domain contribute to transcriptional specificity from the promoter (159) where STAT-6 not only induces transcription of Th2 cytokines, but also expression of chemokines such as eotaxin and TARC, MHC class II molecules, CD23, IL-4Rα, and immunoglobulin class switching from IgG to IgE (46, 160). In addition to its ability to induce transcription, STAT-6 also regulates chromatin remodeling within the Th2 locus, a key regulatory region for cytokine production (161).

The importance of STAT-6 in Th2 cell development is well documented in studies using STAT-6 deficient animals. T cells in STAT-6 deficient animals have substantially reduced proliferative responses, fail to differentiate fully into Th2 cells (49) and produce high levels of IFN γ (162). Production of Th2 cytokines and IgE are thus lower (163, 164) and importantly, IL-13 signaling, which is regulated by IL-4-induced STAT-6 activation, is completely inhibited (108). STAT-6 deficient mice are thus protected from allergen-induced AHR and mucus production in acute asthma models. Chemokine expression and therefore lymphocyte (160) and eosinophil (115) trafficking to the lung are also diminished in STAT-6 -/- mice. The importance of STAT-6 in allergic airway inflammation is not only confined to T cells, since adoptive transfer of Th2 cells expressing STAT-6 into STAT-6 deficient animals is insufficient to induce experimental asthma (165). Interestingly, STAT-6 expression only in lung epithelial cells is

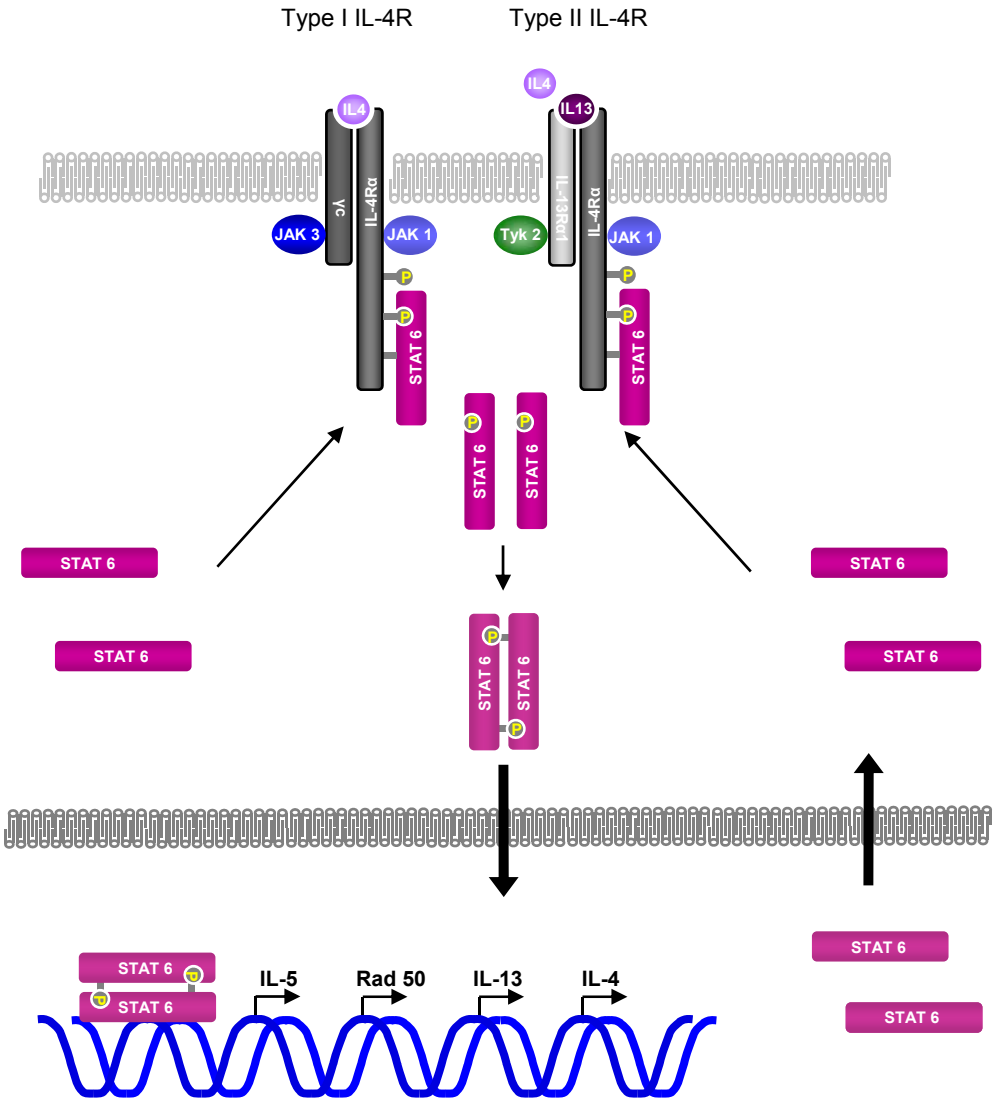
sufficient to induce IL-13 dependent AHR and mucus production, even in the absence of inflammation (166).

The role of STAT-6 in chronic asthma has yet to be fully elucidated. A study using STAT-6 deficient mice in a model of fungus-induced AHR and remodeling shows that the chronic development of airway disease during fungal-induced experimental asthma is STAT-6-independent. This raises the possibility that although STAT-6 is required for IL-13-dependent mucus production, other chronic features of asthma such as airway inflammation and AHR may develop in the absence of STAT-6 (167). Mechanistically this is supported by the fact that while Th2 cell differentiation is severely decreased in STAT-6 deficient $CD4^+$ T cells, STAT-6 independent Th2 cell differentiation is maintained in $CD4^+$ T cells (168) since STAT-5a may participate in Th2 cell differentiation in the absence of STAT-6 activation.

Figure 1.3

Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway.

Ligand binding on IL-4 or IL-13 receptors leads to a change in receptor conformation and the phosphorylation of tyrosine receptor associated JAKS. Activated JAKs then phosphorylate the cytokine receptors creating a binding site for STAT protein Src homology domains (SH2 domains). Recruited STAT-6 monomers are tyrosine phosphorylated by JAKS which then facilitates STAT-6 dimerization via interactions at the phosphorylated SH2 domains. Dimers then translocate to the nucleus where they initiate transcription. In the nucleus, STAT-6 dimers that are not bound to DNA are dephosphorylated and shuttle back to the cytoplasm as monomers.


Type I IL-4R
Type II IL-4R
IL4
IL13
γc
IL-4Rα
IL-13Rα1
JAK 3
JAK 1
Tyk 2
STAT 6
P
IL-5
Rad 50
IL-13
IL-4

GATA-3

The GATA family of zinc finger transcription factors is comprised of six members, each of which contains two conserved zinc finger DNA binding domains, which recognize and bind to a specific consensus nucleotide sequence (WGATAR where W is A/T and R is G/C)(169). The family of GATA proteins are divided into two subfamilies, GATA-1, -2, and -3 and GATA-4, -5, and -6. GATA-1, -2, and -3 are expressed in hematopoietic stem cells where they regulate differentiation and development of T-lymphocytes, erythroid cells, and megakaryocytes. GATA-4, -5, and -6 are expressed in mesoderm- and endoderm-derived tissues such as heart, liver, lung, gonad, and gut (170).

GATA-3, the third member of the GATA family is a lymphoid restricted transcription factor that regulates T cell development (52) by regulating expression of the T cell receptor (171, 172). GATA-3 protein expression is crucial for embryonic development. Consequently, deletion of GATA-3 is embryonic lethal (173, 174) even though *in vitro,* other GATA factors that are not normally expressed in T cells; GATA-1, GATA-2, and GATA-4, are capable of mimicking its effects (175, 176). More recent evidence also suggests that GATA-3 expression is important in the stage specific development of another $CD4^+$ cell, the invariant natural killer T (NKT) cell (177).

GATA-3 also plays a separate but crucial role in differentiation of naïve $CD4^+$ T cells into effector cells. In the current model of Th2 development, naïve T cells, which express low levels of GATA-3, are activated via their T cell receptor and increase both IL-4 and IFNγ expression in a STAT independent manner (178).

Subsequent responses to a polarizing IL-4 signal, leads to the optimal induction of GATA-3 though, interestingly, there is no evidence that STAT-6 activates the GATA-3 promoter directly. GATA-3 then induces expression of Th2 cytokines (52) as well as its own expression (52, 179). Thus, expression of GATA-3 is necessary for the development of the Th2 phenotype where its expression is a key checkpoint for Th2 lineage commitment (52). Acute expression of IL-4 in differentiated T cells is less dependent upon GATA-3 and is mediated by inducible, ubiquitous transcription factors such as c-maf after antigen encounter (180). Expression of GATA-3 is also sufficient for Th2 development since ectopic expression of GATA-3 fully reconstitutes Th2 development in STAT-6 deficient cells demonstrating that GATA-3 is a key regulator in both STAT-6-dependent and -independent Th2 development (181). GATA-3 also negatively regulates IFNγ expression (182) by inhibiting STAT-4 expression thereby preventing IL-12 dependent Th1 development (183). Hence, persistent expression of GATA-3 is required for established Th2 cells to maintain their Th2 cytokine production although in mature Th2 cells, expression of IL-4 becomes less dependent upon GATA-3 (184) while expression of both IL-5 and IL-13 remains highly dependent upon GATA-3 (185). Even conditional depletion of GATA-3 expression in T cells, *in vitro* and *in vivo*, severely inhibits Th2 cytokine production (186) as well as IL-4 independent and dependent Th2 differentiation (185). GATA-3 thus initiates the development and encourages the maintenance of Th2 cells by upregulating Th2 cytokine expression, inhibiting IFNγ expression, and promoting an outgrowth of Th2 cells via IL-4 production (187) (**Figure 1.4**).

At the molecular level, GATA-3 regulates the expression of three Th2 cytokines, IL-4, IL-5 and IL-13, in several ways. It directly induces IL-5 expression by binding to cis-acting elements in the IL-5 promoter (179, 188). There is also evidence to suggest that GATA-3 directly regulates transcription initiation from the IL-13 promoter (63, 189). Regulation of IL-4 production, however, is more complex. Initial studies of IL-4 expression show that GATA-3 only weakly transactivates the IL-4 promoter (190). Instead, it indirectly induces expression of IL-4 by providing access of the IL-4 locus to different transcription factors such as c-maf, a direct transactivator of the IL-4 promoter (48). Recent evidence also suggests that GATA-3 regulates expression of IL-10 since ectopic expression of GATA-3 in naive primary $CD4^+$ T cells enhances IL-10 (mRNA/protein) levels (191, 192).

GATA-3 also controls cytokine expression by regulating chromatin remodeling at the Th2 locus control region. In naïve T cells receiving normal developmental signals, epigenetic constraints such as the structure of chromatin or methylation of DNA limit the accessibility of transcription factors to their target genes. Upon T cell- or cytokine-receptor signaling and subsequent transcription factor activation, loci that have been repressed can become transcriptionally active following hyperacetylation of histones and demethylation of DNA. Cis-acting regulatory elements are more accessible to trans-acting factors at these open chromatin sites [which are experimentally more sensitive to DNase (DNase hypersensitive sites (HS)]. Such epigenetic control allows naive cells to "memorize" early signaling events and leads to the imprinting of Th2 phenotype

in subsequent progeny. In Th2 cells, histone hyperacetylation at the IL-4/IL-13 loci (193) and the IL-5 locus (194, 195) is enhanced by GATA-3. DNA demethylation at the IL-4 locus is also induced by GATA-3 expression (196). Conversely, in differentiated Th2 cells, ablation of GATA-3 expression using a Cre-lox recombinase system results in decreased histone hyperacetylation, increased DNA methylation and consequently decreased Th2 cytokine production (197).

Regulation of GATA-3 has been described at both the transcriptional and the protein level. GATA-3 expression is inhibited by fetal liver zinc finger protein (Fliz-1) which binds to the first intron of the GATA-3 gene (198). GATA proteins also bind to cofactors from the friend of GATA (FOG) family such as FOG1 which directly blocks GATA-3 activity and leads to suppressed Th2 cytokine expression in Th2 cells (199, 200). More recently, evidence has emerged that activation of the Ras-ERK MAPK cascade stabilizes GATA-3 protein in developing T cells thereby facilitating chromatin remodeling at the Th2 cytokine gene loci (201). This study suggests that phosphorylation of GATA-3 by activation of the ERK-MAPK cascade prevents its ubiquitin-mediated degradation through the proteosome pathway. In addition to the regulation of GATA-3 protein levels, activation of the MAPK cascade may have a direct effect on GATA-3 protein transcription and/or function (202).

Since GATA-3 regulates Th2 cytokine expression, it is not unexpected that AHR and airway remodeling are regulated by GATA-3. GATA-3 transgenic animals exhibit increased levels of AHR (203) and airway remodeling following

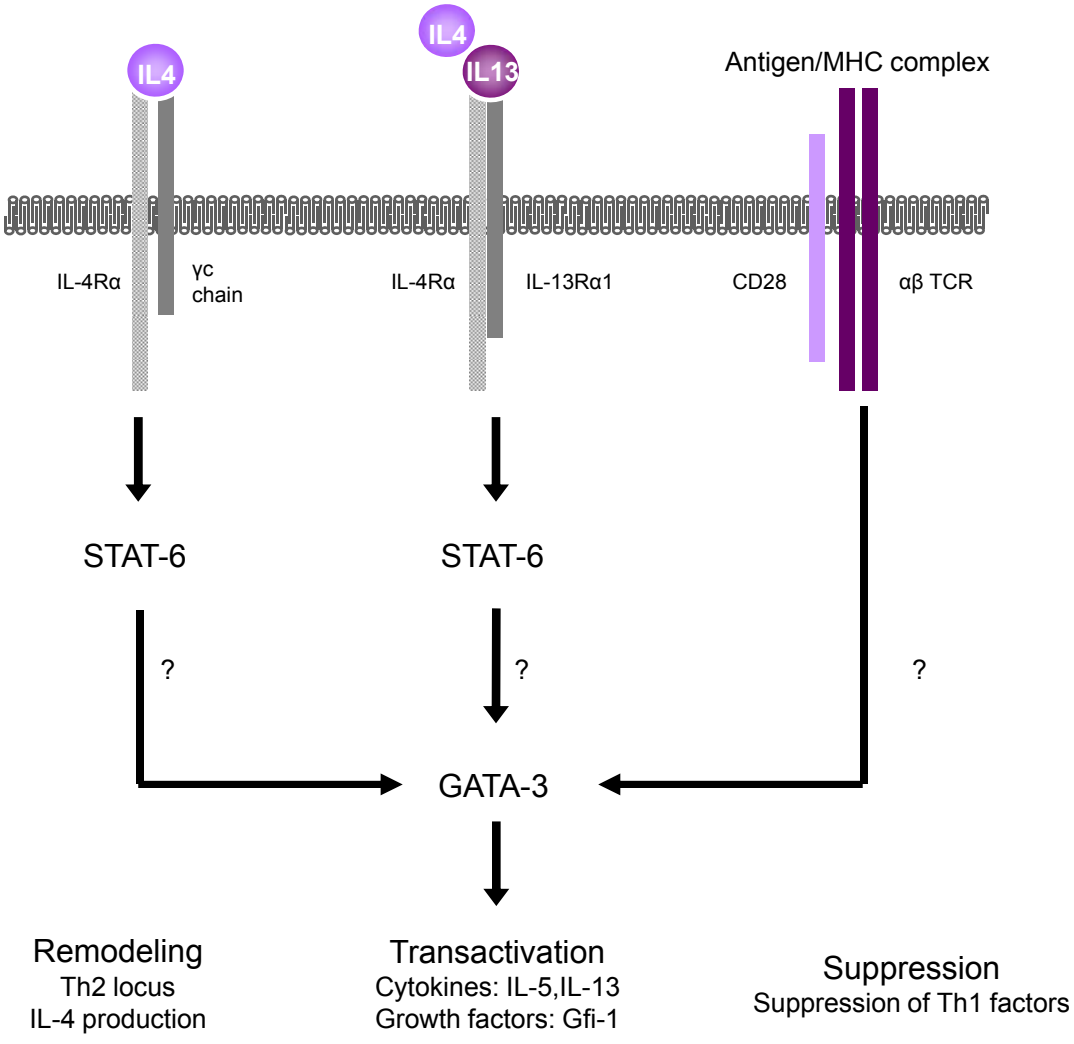
antigen challenge (204) when compared to wild type controls. Transgenic expression of a dominant negative GATA-3 mutant attenuates allergic inflammation with decreased mucus production, eosinophilia and IgE levels along with decreased IL-4, IL-5, and IL-13 expression (205). Allergic airway inflammation is also inhibited by suppressing GATA-3 expression using an intranasally administered antisense GATA-3 oligonucleotide (206). Interestingly, glucocorticoids which are the mainstay of asthma therapy, inhibit GATA-3 activity by direct binding of glucocorticoid receptors to the IL-5 and IL-13 promoters resulting in diminished cytokine expression (207). Recently, a study by Hasegawa et al. linked impaired GATA-3 dependent chromatin remodeling at the Th2 locus to attenuated allergic inflammation in aging mice. This attenuated airway response to antigen was due to lower expression of GATA-3 leading to inefficient remodeling at the Th2 locus (208). Although linkage between the Th2 locus and the asthma phenotype has already been established in humans, the role of aging has yet to be addressed (149, 209).

A link between GATA-3 and asthma has also been described in humans where higher numbers of cells that express GATA-3 protein have been found in bronchial biopsies and induced sputum of asthmatics (210). Expression of GATA-3 protein is also higher in individual cells (211) and is increased following antigen segmental challenge in asthmatic patients (96, 212). Not only is the level of GATA-3 protein higher, electrophoretic mobility shift assays (EMSAs) indicate that the number of GATA-3 DNA binding complexes in peripheral blood mononuclear cells (PBMCs) from asthmatic patients is higher as well (213).

Figure 1.4

GATA-3 regulation of Th2 cell development.

T cell– and cytokine-receptor signaling leads to the activation of GATA-3 which then initiates the development and encourages the maintenance of Th2 cells by upregulating Th2 cytokine expression, inhibiting IFN γ expression, and promoting remodeling at the Th2 locus.


IL4
IL4
IL13
Antigen/MHC complex
IL-4Rα
γc
chain
IL-4Rα
IL-13Rα1
CD28
αβ TCR
STAT-6
STAT-6
?
?
?
GATA-3
Remodeling
Th2 locus
IL-4 production
Transactivation
Cytokines: IL-5,IL-13
Growth factors: Gfi-1
Suppression
Suppression of Th1 factors

Exploring the role of $CD4^+$ T cells in asthma: Models of adoptive transfer

Most animal models of experimental asthma currently used involve an initial intraperitoneal (i.p) sensitization to antigen followed by multiple secondary exposures to the same antigen either intranasally (214) or by aerosol (215, 216). To promote the development of a Th2 response the initial sensitization is performed using antigen and a Th2 adjuvant such as aluminium hydroxide. Such active sensitization and challenge models lead to the development of: i) AHR (217-219); ii) airway infiltration by lymphocytes and eosinophils (215, 220-222); iii) increases in serum IgE (217, 222); and iv) induction of mucus production (218). Chronic exposure to antigen, usually over the course of weeks to months, also leads to airway remodeling in which increases in airway smooth muscle (204, 223, 224), collagen (225, 226)and extracellular matrix deposition (224, 227) have been demonstrated.

Overall, these active sensitization models have supported the participation of the T cell in the antigen-induced inflammatory process (9, 23) since activated T cells are consistently recruited to the lung following antigen challenge (228, 229). Furthermore, adoptively transferred T cells recruited to the lung following antigen challenge may drive airway smooth muscle remodeling (230). This effect may be dependent on direct T cell:smooth muscle cell contact suggesting that T cells must be present in the lung during chronic disease. There is some debate however as to whether or not T cell inflammation is necessary for the maintenance of certain aspects of airway dysfunction and airway remodeling since data from chronically

challenged mice suggests that inflammation may resolve in time even while structural changes do not (231).

Nevertheless, conclusive evidence supporting the importance of the $CD4^+$ T cells in the initiation of allergic inflammation has been demonstrated by murine models of adoptive transfer. In these models, $CD4^+$ T cells from sensitized donors are transferred into naïve recipients that are subsequently challenged with the antigen. These antigen-specific $CD4^+$ T cells are reactivated by antigen, release inflammatory mediators and induce AHR (232), mucus production (27) and airway inflammation (233). Adoptive transfer models have also been developed in the Brown Norway rat where $CD4^+$ T cells harvested from antigen-sensitized donors induce AHR and airway eosinophilia following adoptive transfer and antigen challenge in naïve recipients (24-26). Functional characterization of adoptively transferred T cells from sensitized donors shows that they express higher levels of CD4 and CD25, both markers of recent activation (234). In addition, these cells express low levels of CD62L, a marker required for entry into lymphoid tissue and expressed at high levels by naïve T cells (234). Prior activation of these cells in the presence of a Th2 adjuvant also predisposes them to express Th2 cytokines upon reactivation.

Although a majority of the data on adoptively transferred experimental asthma suggests that only Th2 cells can induce allergic inflammation (28, 233) some reports indicate that Th1 cells may also induce an allergic inflammatory response (29). In addition, Th1 cells may also exacerbate the effects of Th2 cells and enhance airway inflammation (235). Contrasting studies have also

demonstrated that when equal numbers of Th1 and Th2 cells are simultaneously adoptively transferred, Th1 cells may in fact counteract the effects of Th2 cells and prevent experimental asthma from developing (28). These conflicting results may be explained in part by the diverse experimental procedures used to generate the T cells destined for adoptive transfer.

Several methods have been used to obtain cells for adoptive transfer. In the BN rat, animals are first sensitized with antigen in the presence of a Th2 adjuvant. One to two weeks later, $CD4^+$ T cells from sites of inflammation are then purified and transferred into naïve recipients (25). The antigen-specific T cells that respond to antigen *in vivo*, are present at extremely low frequencies (236) and represent only a small fraction of the T cells that are transferred (234). In order to enrich for antigen-specific cells, $CD4^+$ T cells may be cultured in the presence of antigen where they proliferate in response to antigen presented on antigen presenting cells (234). Expansion of antigen specific T cells with a particular phenotype may also be performed by adding exogenous IL-4 (or IL-12) to generate antigen specific Th2 (or Th1) cell populations (28). In addition, transgenic mice carrying a T cell receptor specific for ovalbumin (OVA) have also been utilized as a source of antigenic T cells. Following *in vitro* stimulation, the majority of the effector $CD4^+$ cells (>80%) are OVA specific and can be tracked using an antibody against the transgenic TCR.

Tracking of adoptively-transferred, antigen-specific T cells shows that these cells have a complex migratory pattern following *in vivo* transfer. In an elegant study by Flugel and colleagues, encephalitogenic T cells expressing EGFP

were tracked in an adoptive transfer model of experimental autoimmune encephalomyelitis (EAE) (237). Within the first 36 hours following i.p. injection, these antigen-specific T cells migrate to the parathymic lymph nodes, which drain the peritoneal cavity. By 60 hours these antigen-specific T cells are found circulating in the blood, after which they migrate to the central nervous system (CNS), the site of inflammation. Within the CNS, adoptively transferred T cells become reactivated by antigen presenting cells consequently inducing the pathological features associated with EAE. Importantly, trafficking of antigen specific cells to the CNS occurs irrespective of local antigen presentation; however, cells that are not reactivated *in vivo* gradually decline in number. Thus, adoptively transferred antigen specific T cells preferentially home to sites of inflammation where their reactivation/survival is dependent upon antigen re-encounter.

Similar studies tracking antigen-specific T cells in experimental asthma have provided some information regarding mechanisms by which antigen-specific T cells regulate antigen-dependent inflammation. When OVA specific cells from TCR transgenic mice are tracked following i.v. transfer into histocompatible mice that are then antigen challenged, they enter the circulation, emigrate and accumulate at sites of inflammation and antigen deposition (238). These include the lymph nodes and non-lymphoid organs such as the lung (239). While in the lymph nodes, the T cells migrate to the T cell area where they are activated by antigen presenting airway DC that migrated from the lung following antigen challenge (**Figure 1.5**). Stable contacts between DCs and T cells induce T cell

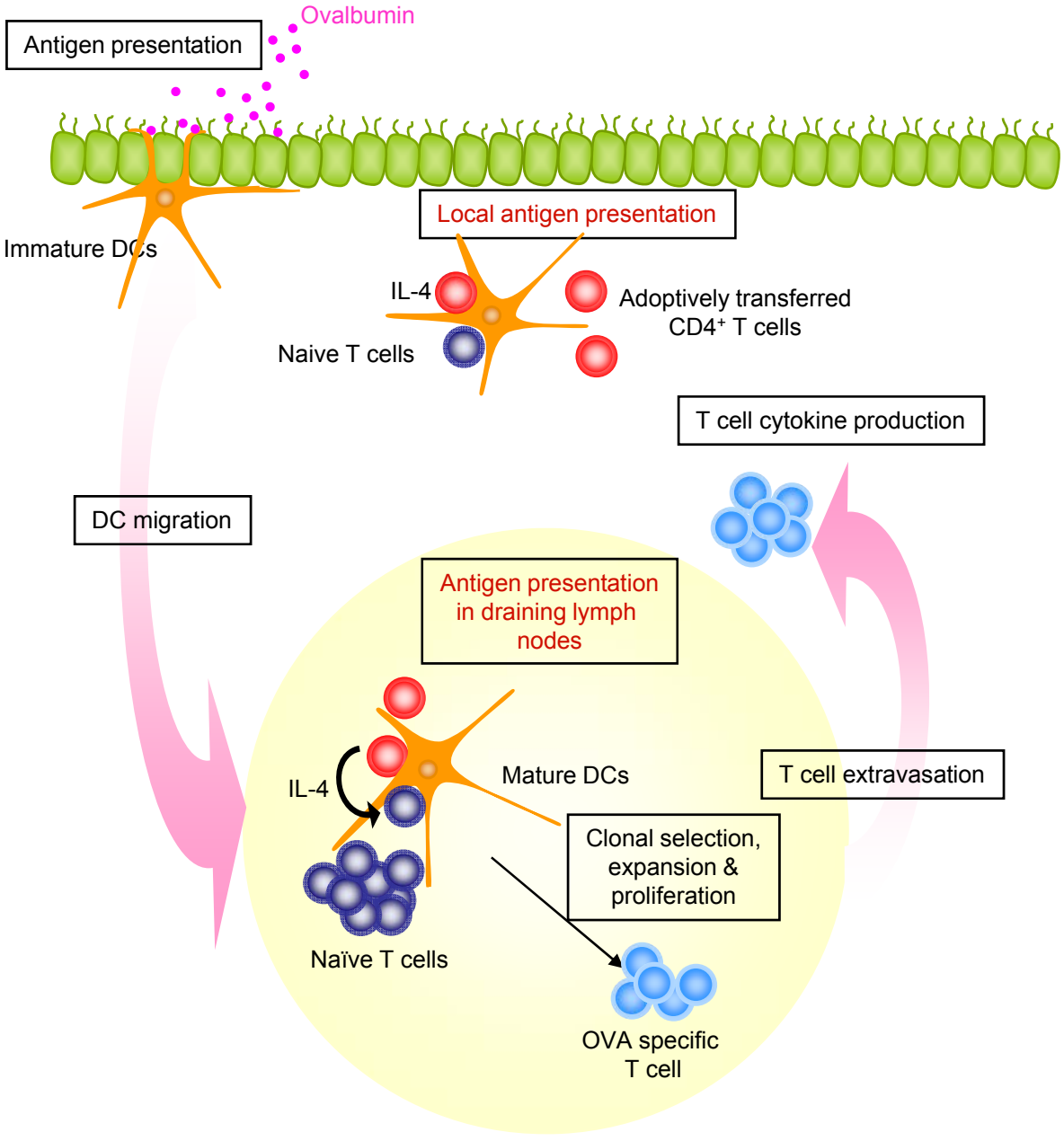
activation and cytokine production (240, 241). Production of IL-4 has a paracrine effect and enables the transgenic OVA-specific cells to induce expansion of endogenous $CD4^+$ T cell population as early as four days after antigen challenge (242). These newly activated cells migrate to the lung where they participate in allergic disease. Although less well defined, there is evidence that the adoptively transferred OVA-specific T cells may return to the lymph nodes via afferent lymph and participate in the maintenance of allergic immune responses (243).

Evidence suggests that local activation of antigen-specific T cells by resident airway mucosal DCs (AMDCs) may also occur (**Figure 1.5**). Interactions between AMDCs and adoptively transferred memory T cells leads to rapid maturation and cytokine production (244). During this process, resident lung AMDCs continuously interact with the memory T cells transiting through the airway mucosa. Once activated by aerosolized antigen, these immature AMDCs upregulate CD86, rapidly proliferate and influence local immune activation.

Figure 1.5

***In vivo* reactivation of adoptively transferred $CD4^+$ T cells.**

Following adoptive transfer, antigen specific cells enter the circulation, emigrate and accumulate in the lymph nodes and the lung, two sites of antigen reactivation (red). In the lymph nodes, the T cells migrate to the T cell area where they are activated by antigen presenting airway DC that migrated from the lung following antigen challenge. Stable contacts between DCs and T cells induce T cell activation, cytokine production and proliferation of endogenous effector T cells. These OVA-specific effector T cells return to the lung via efferent lymph where they participate in allergic immune responses. Alternatively adoptively transferred T cells accumulate in the lung where they are activated by resident DCs.


Antigen presentation
Ovalbumin
Immature DCs
Local antigen presentation
IL-4
Naive T cells
Adoptively transferred
CD4+ T cells
T cell cytokine production
DC migration
Antigen presentation
in draining lymph
nodes
IL-4
Mature DCs
T cell extravasation
Clonal selection,
expansion &
proliferation
Naïve T cells
OVA specific
T cell

Methods for targeting transcription factors in $CD4^+$ T cells

The retrovirus as a tool for targeted gene delivery

Retroviruses have been utilized for gene transfer since the 1980s when efficient retroviral gene delivery into a variety of cell types was first successfully performed (245). These early studies focused on retroviral transduction of stem cells and hematopoietic stem cells (HSCs) which could then proliferate and produce a large number of gene-modified progeny for therapeutic use. These gene-modified HSCs have now been utilized to successfully treat immunodeficiencies in clinical trials for beta thalassemia (246), adenosine deaminase deficiency (ADA) (247) and severe combined immunodeficiency (SCID) (248) where they repopulate lymphoid populations with gene-modified cells. Focus has also turned to retroviral gene modification of mature T lymphocytes as a therapeutic approach for treating diseases such as malignancies (249), HIV infection (250), and autoimmune diseases (251).

Retroviruses are lipid-enveloped spherical particles that contain two identical copies of a positive single stranded RNA genome. The retroviral life cycle is relatively complex **(Figure 1.6)** (rev by (252)). Briefly, in order for viral entry, retroviral particles bind to cell surface receptors after which viral proteins and RNA are released into the cytoplasm. Once in the cytoplasm, viral reverse transcriptase transcribes the RNA genome into double stranded DNA, which binds to cellular proteins and forms a nucleoprotein preintegration complex (PIC). PIC cannot cross the nuclear membrane and cells remain refractory to viral infection until they undergo mitosis, which disrupts the nuclear membrane

allowing integration of the provirus into the host chromosome (rev by (253)). The pre-requisite for cell division limits the efficacy of retroviruses as genetic vectors but is advantageous because it facilitates integration of the viral genes into the host chromosome, thus ensuring the subsequent transmission of the viral genome to all cellular progeny. Although it was once thought that integration into the host genome was random, there is new evidence to suggest that retroviruses like MLV (murine leukemia virus) integrate within or near gene promoters (254, 255). An increased understanding of the mechanisms regulating targeted retroviral integration would be an added advantage for the use of retroviruses in gene therapy.

Despite their limitations, retroviral vectors have become broadly used as tools for gene transfer (253). A number of technical complexities still remain in retrovirus-mediated gene therapy. Although retroviral titers from packaging cell systems can produce infectious particle titers between 10^6 and 10^7 cfus/ml, retroviral titers are often too low to efficiently transduce cells for gene therapy applications. High titer preparations are particularly necessary when the target cell population is difficult to transduce such as mature antigen specific lymphocytes destined for adoptive transfer. To date, several methods have been utilized to improve viral titers by concentrating viral particles. Increases in retroviral titers have been obtained by ultracentrifugation of virion particles pseudotyped with VSV-G protein, which confers resistance to ultracentrifugation (256). Although this process allows for the concentration of retroviruses to titers of 10^9 CFU/ml or greater, VSV-G pseudotyped viruses remain limited in their capacity to transduce

mature lymphocytes. Thus, even though concentration may lead to an increase in particle number, transduction efficiencies may not increase proportionally with the concentration factor (256). Likewise, attempts to purify retroviruses by other physical means such as high affinity chromatography (257) and precipitation (258) have had varied success (259).

In our experiments we needed to obtain sufficient numbers of antigen-specific T cells for *ex vivo* adoptive transfer experiments. Thus, we developed methodology to concentrate retroviral preparations by centrifugal filtration (Chapter 2). Using this technique, we demonstrated that ecotropic retroviral particles, which have a molecular mass of ~2.5 x 10^8 Da (260), can be concentrated using 100kDa nitrocellulose centrifugal filters (Centricons®). When centrifugal concentration is performed at 4°C, so as to minimize viral loss (261), we show that viral preparations can be concentrated at least 10 fold. More importantly, this increase in retroviral titer is proportional to the increase in transduction efficiency of primary rat splenocytes.

Our main goal was to obtain large volumes of high-titer retroviral preparations in order to generate enough gene-modified antigen-specific T cells for use in adoptive transfer experiments. To date, several studies have successfully utilized retroviral transduction to modulate the expression of cytokines in mature T lymphocytes destined for adoptive transfer (262, 263). Oh and colleagues inhibited AHR and pulmonary eosinophilic inflammation in antigen sensitized and challenged mice by adoptively transferring T cells engineered to produce IL-10, a cytokine with a regulatory role in the immune

response (262). Similar studies using T cells engineered to express another regulatory cytokine, TGF-β also demonstrated diminution of AHR and airway inflammation in comparison to control Th2 effector cells (263). In these studies, the majority of the gene modified cells were not antigen specific. To selectively enrich for antigen specific cells we and others have developed methodology whereby retroviral transduction is coupled to antigen stimulation (230, 264). Thus, since retroviruses infect only proliferating cells, antigen specific $CD4^+$ T cells cultured in the presence of antigen proliferate and are selectively transduced by retroviruses. Furthermore, since recruitment of antigen-specific T cells to the site of inflammation is dependent upon antigen challenge, selectively transducing antigen-specific T cells ensures their recruitment to the lung in an antigen-dependent manner thereby targeting these cells to the lung in experimental asthma. An additional advantage to this system is that once the provirus integrates into the host genome, antigen stimulation promotes retroviral LTR-driven gene expression (265, 266). Thus, following OVA challenge, *in vivo* LTR-driven expression of our genes of interest (EGFP and GATA-3) may actually be increased (Chapter 3).

Cell penetrating peptides targeting intracellular proteins

Despite their natural ability to deliver genes to target cells, retroviruses have several limitations. First, large volumes of retroviruses are necessary, particularly when gene modified cells are required for therapeutic use. Secondly, retroviral transduction requires time and manipulation of target cells *in vitro*. Finally, retroviral vectors integrate into the host chromosome, which may have

negative therapeutic consequences during clinical applications (267). Cell penetrating peptides (CPPs) also called protein transduction domains (PTDs) represent an alternative method for exogenous protein delivery and offer an advantage over viral based delivery systems since, unlike many retroviral transduction systems, relatively low concentrations of PTDs are able to transduce primary non-dividing cells with rapid kinetics and low toxicity.

The first reports demonstrating that proteins with specific functional domains had the ability to cross cell membranes were described using the transactivator of transcription factor (TAT) protein from human immunodeficiency virus 1 (HIV-TAT) (268, 269). The discovery of another PTD in *Drosophila* antennapedia led to the hypothesis that PTDs might act as efficient molecular transporters. Studies using chimeric molecules containing these PTDs proved this to be the case and demonstrated that these functional domains successfully facilitate cellular entry of associated cargo (270). Since these initial observations, naturally occurring PTDs from HIV-TAT (271, 272) and *Drosophila* antennapedia (273, 274) have been utilized for the intracellular delivery of a wide range of molecular cargo, including full length proteins, peptides, oligonucleotides, and small molecules (275-277).

Detailed analysis of naturally occurring PTDs has revealed that they consist of an abundance of highly basic amino acids (lysine and arginine) and has now led to the generation of a number of synthetic PTDs. Synthetic PTDs such as TAT-PTD (a derivative of HIV TAT), penetratin (a derivative of Antennapedia) (278), poly-lysine (279), and poly-arginine (280-282) have been utilized as

molecular transporters for biologically active peptides and proteins. One of the most efficacious of these synthetic PTDs is poly-arginine (280). Polymers of arginine with six or more amino acids enter cells more efficiently than other synthetic cationic polymers, the most favourable length being nona-arginine (283).

The mechanism by which PTDs traverse cellular membranes is still not clear, though several mechanisms for cellular uptake have been proposed. The entry of HIV-TAT is thought to be energy dependent involving either clathrin dependent endocytosis (284) or lipid raft dependent macropinocytosis, the latter being an actin driven mechanism in which the cell engulfs substantial amounts of extracellular fluid in macropinosomes (285, 286). In contrast, cellular internalization of synthetic poly-arginine may require heparan sulfate (HS) (281) or alternate unknown membrane-associated proteoglycans, which may serve as receptors during induction of macropinocytosis (287-289) (**Figure 1.6**). Alternate reports suggest that PTDs are internalized by energy independent plasma membrane translocation without the formation of any membrane vesicles (290, 291).

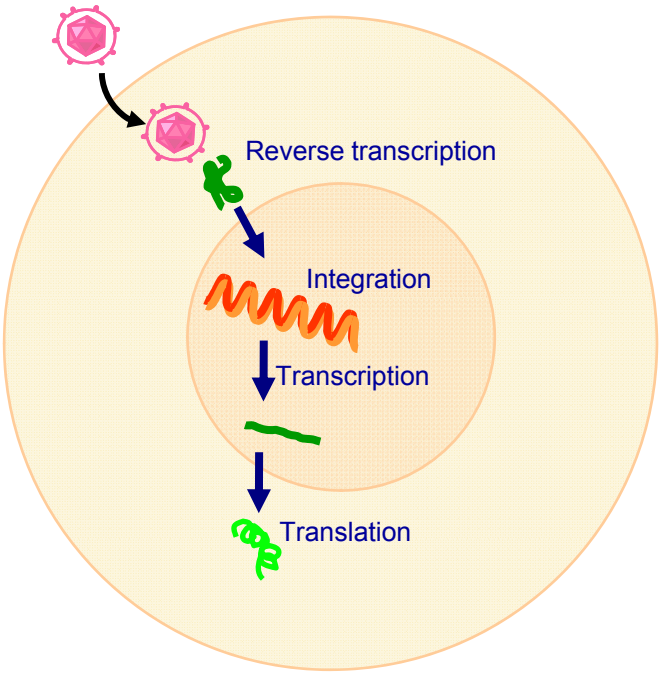
Cellular uptake of PTDs, poly-arginine in particular, is sensitive to temperature and peptide concentration (292). In fact, small increases in peptide concentration significantly enhance the concentration of peptide in the cytosol. When low levels of poly-arginine peptide are used (~2μM), small distinct intracellular vesicles are seen, suggestive of endocytic vesicle formation. Higher concentrations (~ 10μM) lead to a loss of vesicular labeling due to a more intense diffuse staining. These results are also highly dependent on the type of peptide

Figure 1.6

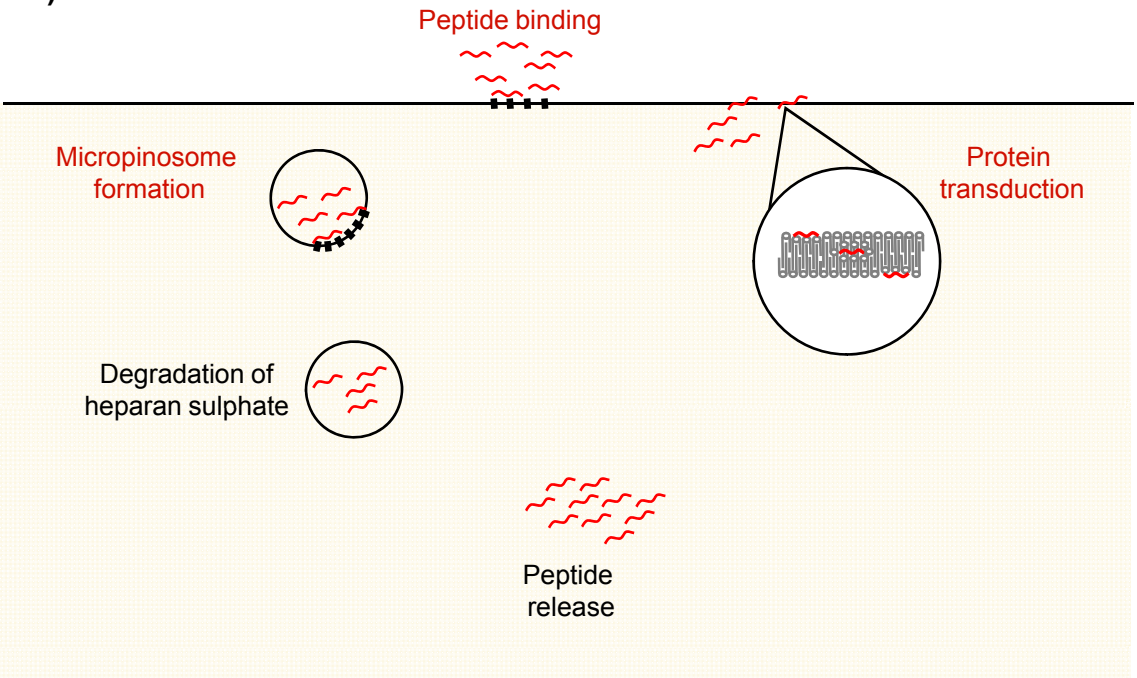
Methods for targeting transcription factors in $CD4^+$ T cells.

A) Long term gene modification using retroviral transduction. Following viral entry, the RNA genome is reverse transcribed into DNA. Proviral DNA integrates into the host chromosome following cell mitosis. Transcription initiation from viral long terminal repeats (LTRs) followed by translation leads to the expression of virally-encoded proteins. *B)* Mechanism(s) by which protein transduction domains cross cellular membranes. Peptides may traverse the cellular membrane by macropinocytosis (left) which involves the initial formation of macropinosomes that eventually degrade releasing the peptide into the cytoplasm. Other types of endocytosis may also be invovlved. Alternatively this process may simply involve direct protein transduction or translocation (right).

A)


Reverse transcription
Integration
Transcription
Translation

B)


Peptide binding
Micropinosome formation
Protein transduction
Degradation of heparan sulphate
Peptide release

used since enantiomers of poly-arginine behave differently (292). Similarly, the biological cargo in fluorescent conjugates could also influence mechanistic studies on uptake since they may interfere with peptide interactions with the cell membrane or influence translocation (293, 294).

While the mechanism(s) of uptake remain poorly defined, efficacy of these peptides has been clearly demonstrated in a wide range of disease models (295). Efficient delivery of therapeutic peptides and full-length proteins using PTDs has been demonstrated in several mouse models of antigen-induced allergic inflammation (296-298). In one study, phosphoinositide 3-kinase (PI3K), which is thought to contribute to the pathogenesis of asthma by modulating the recruitment, activation, and apoptosis of inflammatory cells, was targeted using a full-length, dominant-negative form conjugated to the HIV-TAT PTD (297). Intraperitoneal administration of this fusion protein led to transduction of cells within the lung and effectively inhibited antigen-induced Th2 cytokine secretion, airway inflammation, mucus production and AHR when administered during antigen challenge. Likewise intranasal delivery of the HIV-TAT PTD also led to the effective inhibition of antigen induced allergic disease. A similar study by Myou and colleagues also demonstrated efficient *in vivo* transduction by a full length TAT-dominant negative Ras fusion protein (dn-Ras) thereby preventing experimental asthma (296).

These studies were the first to show successful inhibition of antigen-induced experimental asthma by intranasal delivery of full-length cell penetrating proteins. An additional study demonstrated that intranasal delivery of a cell

penetrating peptide containing a sequence from the cytoplasmic domain of cytotoxic T-lymphocyte associated gene 4 (CTLA-4), a negative regulator of T cell function, also inhibits allergic inflammatory disease (298). More recently, McCusker and colleagues demonstrated that a PTD conjugated peptide containing a sequence predicted to inhibit the transcription factor STAT-6, inhibits STAT-6 dependent production of IL-4 from *in vitro* antigen stimulated splenocytes. Intranasal delivery of the cell penetrating peptide in a model of allergic disease decreased antigen dependent airway inflammation, mucus production and AHR to methacholine.

Despite efficient cargo delivery there are several limitations to the use of PTDs. Firstly, current understanding regarding the mechanism of intracellular uptake suggests that cellular uptake may be influenced by individual PTDs resulting in peptide localization to different cellular compartments (299). Secondly, *in vitro* studies suggest that high concentrations of some PTDs may be cytotoxic (300). Use of these peptides *in vivo* is also limited by to a lack of cellular specificity (277). For instance, intraperitoneal delivery of a HIV-TAT cell permeable fusion protein into mice leads to the transduction of all tissues including the brain (277, 301). Similarly, cell permeable fusion proteins, when delivered intranasally, are detected homogenously within the trachea and lungs (298). Nevertheless, the efficacy of protein transduction by PTDs conjugated to biologically active molecules demonstrates that this technology may be useful in dissecting cellular signaling pathways *in vivo*. To this end, we utilized a chimeric peptide in an *ex vivo* model of adoptively transferred allergic inflammation so as

to investigate the role of the transcription factor STAT-6 in the ability of adoptively transferred $CD4^+$ T cells to induce experimental asthma (Chapter 4).

Summary

Our current understanding of asthma pathology indicates that the asthmatic airway is inflamed with a number of cells that induce and exacerbate the disease. Central mediators in this inflammatory response are $CD4^+$ T cells which are recruited to the lung during antigen exposure where they produce Th2 cytokines that then contribute to pulmonary eosinophilic inflammation, airway obstruction, AHR and mucus overproduction.

To better define the mechanism by which these $CD4^+$ T cells initiate allergic disease investigators have utilized adoptive transfer models of allergic airway inflammation. These adoptive transfer models have demonstrated that $CD4^+$ T cells induce allergic inflammation in naïve recipients. This process is dependent upon Th2 cytokines derived from the adoptively transferred T cells themselves. Experimental models of allergic disease have further demonstrated that there is significant interdependence between cytokines possibly explaining why therapeutic targeting of individual Th2 cytokines has remained relatively ineffective in treating allergic disease.

Central to these cytokine effector pathways are transcription factors, which not only regulate the effector function of multiple cytokines but also cytokine production as well. The goal of this work is to build upon the current knowledge on two of these transcriptional activators, GATA-3 and STAT-6. By modulating the overexpression (GATA-3) and activity (STAT-6) of these factors in $CD4^+$ T cells we hope to not only improve upon the current understanding of

the way in which T cells induce and exacerbate allergic disease but also define the role of these molecules in this process.

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Chapter 2:

Enhanced transduction of antigen-stimulated T lymphocytes with recombinant retroviruses concentrated by centrifugal filtration

Abstract

Retroviral transduction is an efficient tool for the generation of gene modified T lymphocytes. The generation of retrovirally-transduced primary T lymphocytes however, is severely limited by low titer retroviral preparations which inefficiently transduce primary T lymphocytes. In order to generate large numbers of gene-modified antigen-specific T cells for use in *ex vivo* experiments, we developed a simple methodology to prepare high-titer ecotropic retrovirus stocks by concentrating dilute retroviral preparations using nitrocellulose centrifugal filters (Centricons®).

We compared the ability of nonconcentrated and concentrated preparations in transducing an immortalized fibroblast cell line by examining the transduction efficiencies as well as the mean fluorescence intensities of transduced cells. We demonstrated that ecotropic retroviral particles harvested from two independent packaging cell lines were efficiently concentrated with little or no loss of viral particles. Moreover, we showed that the concentrated preparations resulted in the generation of transduced populations with greater mean fluorescence intensities and therefore higher levels of transgene expression, compared to the nonconcentrated preparations. When we utilized the concentrated preparations to transduce antigen stimulated primary rat splenocytes, we found that compared to their nonconcentrated counterparts, the concentrated retroviral preparations, transduced antigen stimulated primary rat $CD4^+$ T cells with greater efficiency and successfully modified the expression of the proviral genes they encoded. Thus, concentrating retroviral preparations in this way could be a useful

tool for the generation of large numbers of gene-modified T cells for *ex vivo* studies or gene therapy.

Introduction

Gene transduction of mammalian cells with recombinant retroviruses has received much attention because of its potential for gene therapy. In fact, many gene therapy clinical trials, performed to date or currently ongoing, use retroviral vectors for gene transfer into cultured human cells that are then administered to patients (1). Retroviral transduction of hematopoietic stem cells has shown promise as a treatment for severe immunodeficiencies due to mutations in the genes encoding adenosine deaminase or the common gamma chain cytokine receptor subunit (2, 3). This genetic modification approach employs replication-defective retroviral vectors to deliver therapeutic genes into a host cell, where they are stably expressed due to the integration of the retroviral vector into the host genomic DNA (1). A number of technical complexities with retrovirus-mediated gene therapy still remain. One major factor determining the efficiency of retroviral transduction is the infectious particle titer of the retrovirus-containing medium that is delivered to the target cells. This becomes critical when the target is a difficult-to-transduce cell population such as mature lymphocytes. One approach to overcome this limitation has been the production of retroviruses pseudotyped with the vesicular stomatitis virus G (VSV-G) protein, which confers resistance to high *g* forces allowing the retrovirus-containing supernatants to be concentrated by ultracentrifugation (4, 5). Unexpectedly, pseudotype-dependent variability in transduction efficiency has been shown for different types of target cells, including mature B and T lymphocytes, which are relatively refractory to transduction with VSV-G pseudotyped retroviruses (6-9). On T cells, transduction efficiencies comparable to ecotropic or amphotropic retrovectors can be achieved

with VSV-G pseudotyped retroviruses provided that they are highly concentrated, therefore requiring production of large volumes of retrovirus. This limitation may become relevant for the objective of targeting rare antigen-specific T cell subpopulations.

Here, we describe the use of a simple method to prepare high-titer ecotropic retrovirus stocks by concentrating dilute retroviral preparations using low *g* force centrifugal filtration. This technique can be applied to retrovirus preparations harvested from virtually any packaging cell line without the need of modifying the retrovirus envelope protein. We demonstrate that ecotropic retroviral preparations concentrated by this method can be used to transduce antigen-stimulated T cells with high efficiency, resulting in the generation of gene-modified T cells in numbers sufficient for *in vivo* studies.

Materials and Methods

Antibodies, recombinant proteins, plasmid DNAs and cell lines. APC-conjugated mouse anti-rat CD4, mouse anti-rat CD25, APC-conjugated goat anti-mouse IgG secondary and IgG_1 isotype control as well as recombinant rat IL-4 were purchased from BD Biosciences (San Diego, CA). The pAP2 retrovector containing the EGFP open reading frame downstream from an internal ribosomal entry site (IRES) was generously provided by Dr. J. Galipeau (McGill University, Montreal, Quebec) (10). The rat GATA-3 cDNA had been previously RT-PCR amplified from the TIB236 cell line and cloned into pAP2 (by M. Kaufman in E. Fixman's lab). Phoenix-Ecotropic retrovirus packaging cells (Phoenix-Eco) were purchased from American Type Culture Collection (ATCC Manassas, VA) with permission from Dr. G. Nolan (Stanford University, CA) (11). Phoenix-Eco and NIH3T3 fibroblasts were cultured in DMEM (Invitrogen, Canada) containing 10% heat-inactivated fetal bovine serum (FBS) 2mM glutamate, 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen, Canada). Expression of the *gag*, *pol* and *env* gene products by the Phoenix-Eco packaging cells was maintained or boosted by selection in 300 ug/ml hygromycin B (Sigma-Aldrich, St. Louis, MO) and 1 ug/ml diphtheria toxin (Sigma Aldrich) for one week. All cells were maintained at 37°C and 5% CO_2.

Phoenix-Eco cells were transfected with FspI linearized pAP2 (5 μg) or GATA-3/AP2 (5μg) and pIRESPUR (0.5μg) as the selectable marker using lipofectamine. The next day cells were washed twice with PBS and placed into DMEM/10% FBS. When cells were approximately 70% confluent, puromycin

(2µg/ml) was added. Following two weeks of drug selection, the puromycin-resistant Phoenix-Eco cell population was sorted by multiparameter fluorescence activated cell sorting (FACS) to select cells expressing high levels of $EGFP^+$.

Recombinant retrovirus production and generation of concentrated stocks by centrifugal filtration. Puromycin resistant Phoenix-Eco cells producing retrovirus were plated in 150-mm cell culture dishes and grown to confluence. The cell culture medium was then replaced by fresh medium and retrovirus-containing medium was harvested 24h and 48h later (twice from each confluent dish). Medium was filtered (0.45µm) and then loaded into the upper chamber of 100 kDa molecular weight cutoff Centricon Plus® filters (Millipore, Canada). The assembled filters containing the media were then centrifuged at 1000 x g and 4°C until the desired volume reduction was achieved. The concentrated retrovirus-containing supernatants were then recovered through an inverted spin as per manufacturer's instructions, aliquoted and stored at -80°C for later use. Sample aliquots were used for virus titration on NIH3T3 cells. For systematic production of large stocks, the used filters were washed twice after each use by running 80 ml PBS through a spin followed by inverted spin, and the filters used up to three times.

Retrovirus titrations. Immortalized NIH3T3 fibroblasts were used to titer retroviral supernatant. Fibroblasts were cultured on 6-well plates at a seeding density of 2-4 x 10^4 cells/well. Forty-eight hours later, two wells were trypsinized, washed twice with PBS and counted. Increasing volumes of

retrovirus-containing supernatants were added in a 0.5, 5, 50, 500 µl progression for unconcentrated and concentrated virus stocks. Cells were incubated with the retroviral supernatant in the presence of 6 µg/ml polybrene for 24 h after which the cell culture media was changed. Three days after adding the viruses, the cells were trypsinized and analyzed for EGFP expression by flow cytometry. The percentage of $EGFP^+$ cells was calculated by fluorescence intensity histogram subtraction referenced to control culture wells. Virus titers were estimated from titration volumes of 5µl for the unconcentrated virus preparations and 0.5µl for the concentrated virus preparations. With these volumes of retroviral supernatant the transduction efficiencies ranged from 2 to 13.4 percent and the average mean fluorescence intensity (MFI) of the transduced cells did not differ (**see Figures 2.4 and 2.5**). With larger volumes the MFI increased, consistent with cells being transduced with multiple retroviral particles and thus these values were not used to calculate titers. Titers were calculated using the following formula:

$$\text{Titer} = \text{Cell No} \times EGFP^+/100 \times 1/T_V$$

"Cell No" is the average number of NIH3T3 cells per well the day the retroviruses were added; $EGFP^+/100$ is the percentage of $EGFP^+$ cells; and T_V is the volume of retrovirus added to the test well (10, 12).

Animals, antigen sensitization, T cell stimulation and retroviral transduction. Inbred BN rats (Harlan-U.K. Bicester, Oxon, England) were sensitized intraperitoneally by injection of 200 µg OVA and 100 mg aluminum hydroxide in 1 ml PBS. Fourteen days after sensitization, animals were sacrificed and the spleens were removed. The splenocyte cell suspensions were then washed in PBS

and filtered, and most of the red blood cells were eliminated using red blood cell lysis buffer.

Helper T cells were stimulated with antigen *in vitro* by culturing the total splenocyte populations in 6-well cluster dishes at a seeding density of 10 x 10^6 cells/ml in 4ml of complete DMEM supplemented with 50 μM 2-mercaptoethanol, 200 μg/ml OVA and 10 U/ml of rat rIL-2. Following 48 h of stimulation, the cells were infected with retroviruses at an MOI of 1 by centrifugal spin infection. Briefly, the cell culture medium was gently withdrawn from the wells by pipetting, pooled and centrifuged to recover the cells. The cells were then resuspended in concentrated retrovirus-containing medium supplemented with 6 μg/ml of polybrene and 10 U/ml of rat IL-2. This mixture was then returned to the original cell culture plates, where the cells had been left behind in a thin layer of culture medium to avoid dehydration. The plates were then spun at 455 x g for 45 min, and returned to the cell culture incubator overnight. The spin infection cycles were performed four times over the course of 48 hours and the retrovirus-containing media was replaced with fresh medium 24h following the last spin. The transduced cells were analyzed by flow cytometry for EGFP expression five days after the last spin. All experimental protocols involving animals were approved by the McGill University Animal Care Committee and complied with the guidelines of the Canadian Council on Animal Care.

Flow cytometry. Following transduction, splenocytes were cultured for an additional five days after which expression of EGFP, CD4 and CD25 was

evaluated. Briefly, cells were labeled with anti-CD4 APC conjugated antibody or anti-CD25 and goat anti-mouse APC secondary as per manufacturer's instructions. Labeled cells were then analyzed using a FACSCalibur flow cytometer (BD Biosciences) and expression of EGFP, CD4 and CD25 quantified in live cells using Cell Quest Software (BD Biosciences). Density plots shown are plotted on a four-decade log scale and are representative of three separate experiments using cells from a total of six animals.

Data analysis. Data are presented as the mean ± SEM. Each experiment was repeated three times. For figures 2.2, 2.3, 2.4 and 2.5 a representative flow cytometry histogram or dot plot are presented and the means ± SEM are presented in the text.

Results

Generation of stable retrovirus packaging cell lines.

To obtain a permanent source of high-titer retrovirus-containing supernatants for transduction of peripheral T cells, we generated puromycin-resistant Phoenix-Eco packaging cells (11) stably transfected with the pAP2 retrovector, which contains the EGFP open reading frame downstream of an internal ribosome entry site (IRES)(10). In addition, we generated cell lines stably transfected with a derivative of the pAP2 retrovector containing the full-length rat GATA-3 cDNA cloned upstream of the IRES **(Figure 2.1)**. To increase retroviral titers the puromycin-selected Phoenix-Eco cells were FACS-sorted to select $EGFP^+$ cells.

Recombinant retroviral preparations are efficiently concentrated by centrifugal filtration.

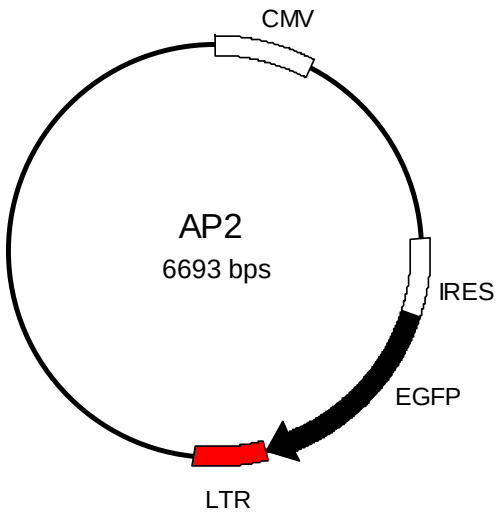
To meet the requirements for *in vivo* experiments employing adoptive transfer of retrovirally transduced $CD4^+$ T cells, we tested a method to concentrate regular retrovirus-containing supernatants by centrifugal filtration. We used filters with a 100 kDa molecular weight cutoff that retain virion particles with an average diameter of 80-120 nm. To concentrate the retrovirus-containing supernatants we employed 80-ml capacity filters Centricon Plus® that were washed and recycled up to three times. Following centrifugal filtration, the numbers of infectious virus particles present in the filtrate averaged 1.2 ± 0.3% of the loaded viruses, indicating nearly 99% retention efficiency by the filters (**Figure 2.2**) and there was no significant loss in titer after reuse of the centrifugal

Figure 2.1

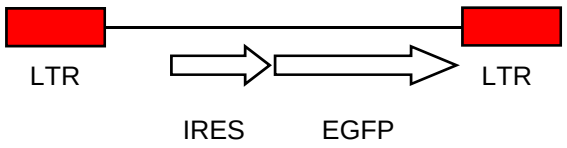
Schematic representation of pAP2 and GATA-3 retrovectors

(A) The pAP2 retrovector containing the EGFP open reading frame downstream from an internal ribosomal entry site (IRES) was used to generate retroviral particles (10). *(B)* During transduction, the pAP2-derived retroviral genome integrates into the host chromosome where subsequent viral gene expression is driven by a promoter in the the 5' long terminal repeat (LTR). *(C)* A derivative retrovector encoding the transcription factor GATA-3 upstream of the IRES was also generated.

A)


CMV
AP2
6693 bps
IRES
EGFP
LTR

B)


LTR
LTR
IRES
EGFP

C)

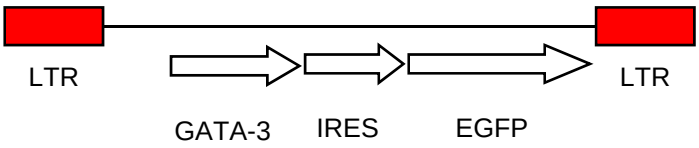
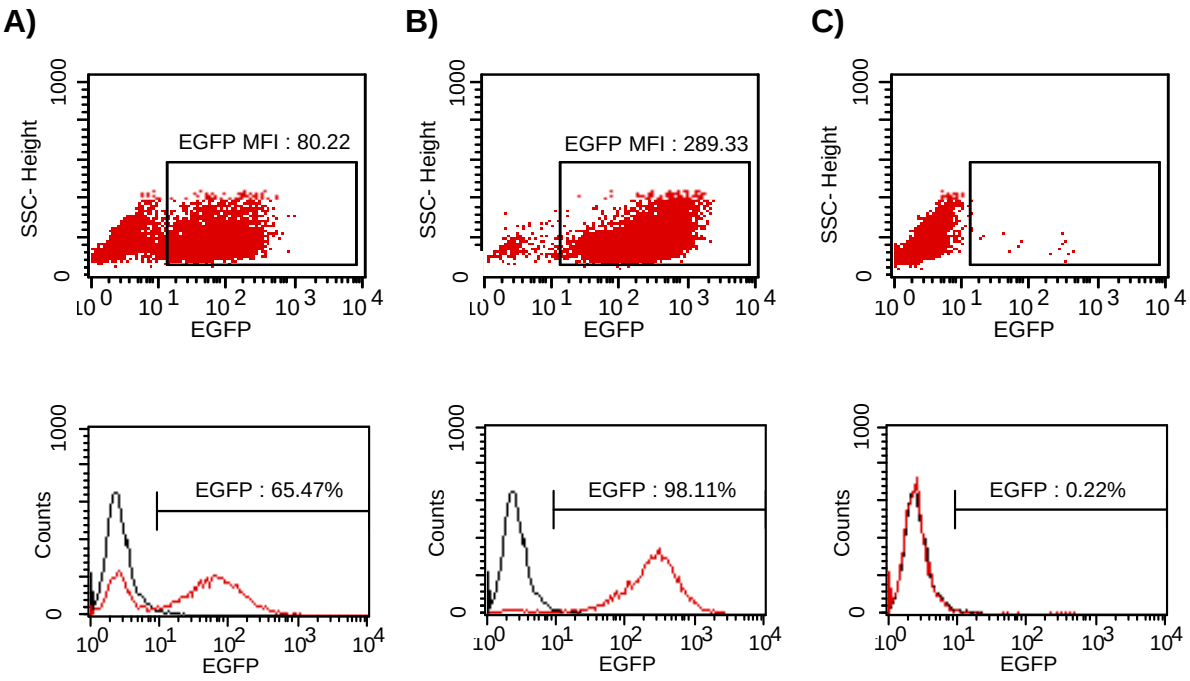

LTR
LTR
GATA-3
IRES
EGFP

Figure 2.2

Recombinant retroviruses are efficiently concentrated by centrifugal filtration.

Cell culture supernatants from Phoenix-Eco cells producing retrovirus derived from pAP2 were harvested and concentrated by means of centrifugal filtration. Retroviral preparations, both before and after centrifugal concentration, as well as the filtrate, were then titered on NIH3T3 fibroblasts. (*A, B*). Flow cytometry density plots and histograms of EGFP expression in cells infected with 50 µl of the retrovirus-containing supernatant, before (*A*) and after (*B*) concentration by centrifugal filtration. (*C*) Flow cytometry density plots and histograms of EGFP expression in cells infected with 500-µl of filtrate. The mean fluorescence intensity (MFI) and percentage of cells transduced are indicated in the density plots and histograms, respectively. Results are representative of three experiments.


A)
B)
C)
SSC- Height
EGFP MFI : 80.22
EGFP MFI : 289.33
EGFP
Counts
EGFP : 65.47%
EGFP : 98.11%
EGFP : 0.22%

filters (**Figure 2.3**). Both concentrated and unconcentrated virus preparations efficiently transduced NIH3T3 cells (**Figure 2.4**). For pAP2-derived retroviral preparations, a 10-fold reduction of supernatant volume resulted in a 9.4 ± 0.7-fold increase in retroviral titer. As expected, increasing the volume of pAP2-derived retrovirus, either unconcentrated or concentrated, applied to the NIH3T3 cells resulted in an increase in the number of cells transduced, with nearly 100% of the cells being transduced with 500µl of the unconcentrated retrovirus and with 50µl (and 500µl) of the concentrated retrovirus. In addition to more cells being transduced, the mean fluorescence intensity of EGFP expression in the cells also shifted to the right with increasing volume of retrovirus applied, consistent with cells expressing EGFP from multiple sites of integration (**Figures 2.4 and 2.5**). In fact, though nearly all of the NIH3T3 cells were transduced with either the 50µl and 500µl volumes of concentrated retrovirus, the mean fluorescence intensity of EGFP expression still doubled following transduction with the larger volume. These data are summarized in **Table 2.1**.

Similar results were obtained with the GATA-3 expressing retroviral preparations where a 10-fold reduction of supernatant volume resulted in a 6.3 ± 1.7-fold increase in retroviral titer (**Table 2.2**). Although transduction efficiencies did not reach 100% with the unconcentrated retroviral preparations, the mean fluorescence intensity of EGFP expression also shifted to the right with increasing volume of retrovirus applied (**Figure 2.6**). Retroviral transduction using 500µl volumes of concentrated GATA-3 virus resulted in a significant increase in mean fluorescence intensity albeit dramatically lower than the concentrated pAP2 viral supernatant (**Figure 2.7**). Altogether, these data demonstrate that centrifugal

Figure 2.3

Changes in retroviral particle recovery following centrifugal concentration with

recycled filters.

Retroviral supernatants were filtered to remove cellular debris and then loaded into the upper chamber of Centricon Plus® filters (Millipore). The assembled filters containing the media were then centrifuged at 1000 x g and 4°C until the desired volume reduction was achieved. Filters were washed twice after each use and re-used up to three times. Viral titers of the concentrated retrovirus-containing supernatants from each of the three spins were then compared. Titrations were performed on NIH3T3 cells.

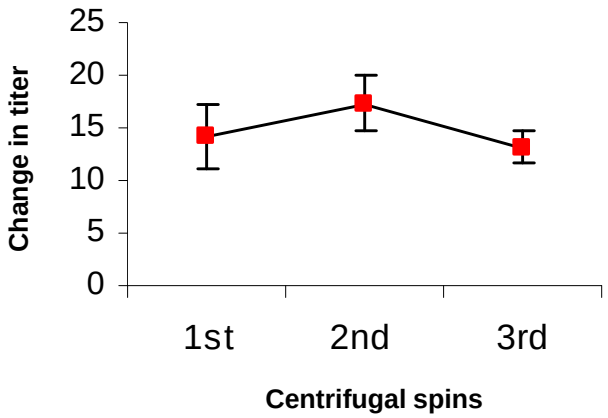

25
20
15
10
5
0
Change in titer
1st
2nd
3rd
Centrifugal spins

Figure 2.4

Efficiency of retrovirus supernatant concentration by centrifugal filtration.

Cell culture supernatants from Phoenix-Eco cells producing retrovirus derived from pAP2 were subjected to a 10-fold volume reduction. Retroviral preparations, both before and after centrifugal concentration were then titered on NIH3T3 cells. Volumes of 0.5µl, 5µl, 50µl, and 500µl were used and EGFP expression monitored by flow cytometry. Density plots show EGFP expression in transduced NIH3T3 cells following incubation with the indicated volumes of retrovirus-containing supernatant, before (left column) and after (right column) concentration. The percentage of cells transduced and the mean fluorescence intensity of the EGFP expressing cells are indicated. Results are representative of three experiments.

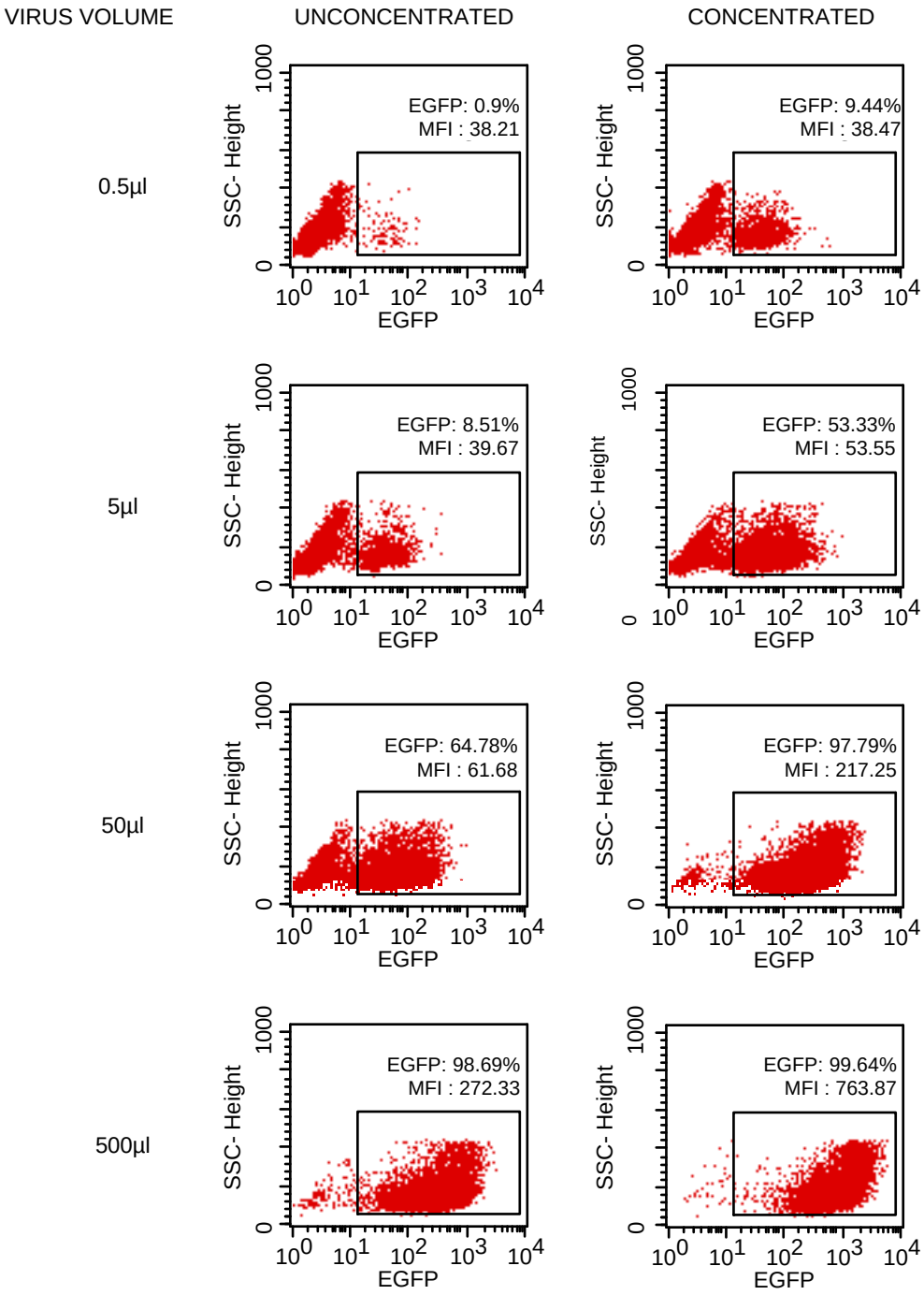

VIRUS VOLUME
UNCONCENTRATED
CONCENTRATED
0.5µl
5µl
50µl
500µl
EGFP: 0.9%
MFI : 38.21
EGFP: 9.44%
MFI : 38.47
EGFP: 8.51%
MFI : 39.67
EGFP: 53.33%
MFI : 53.55
EGFP: 64.78%
MFI : 61.68
EGFP: 97.79%
MFI : 217.25
EGFP: 98.69%
MFI : 272.33
EGFP: 99.64%
MFI : 763.87
SSC- Height
EGFP
0
1000
10^0 10^1 10^2 10^3 10^4

Figure 2.5

Mean fluorescence intensity values of transduced NIH3T3 fibroblasts.

Cell culture supernatants from Phoenix-Eco cells producing retrovirus derived from pAP2 were subjected to a 10-fold volume reduction. Retroviral preparations, both before and after centrifugal concentration, were then titered on NIH3T3 cells using the indicated volumes. The average mean fluorescence intensity of EGFP expression ± SEM determined by flow cytometry for three experiments is presented.

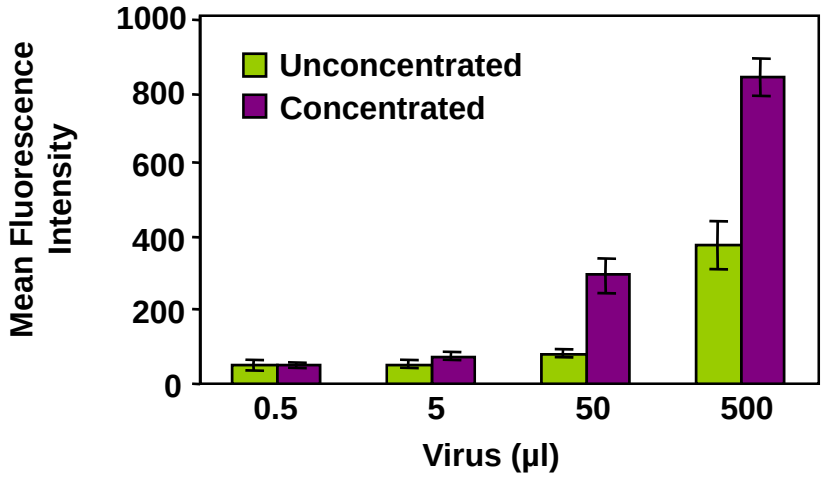

Mean Fluorescence Intensity
1000
800
600
400
200
0
Unconcentrated
Concentrated
0.5
5
50
500
Virus (µl)

Table 2.1

Average retroviral titer and mean fluorescence intensity empty vector concentrated and unconcentrated retroviral preparations

	Viral Titer (x 10^6 cfu/mls)	Mean Fluorescence Intensity[a]	Transduction efficiency (splenocytes)[b]
Unconcentrated virus	1.1 ± 0.14	351.5 ± 34.5	6.8 ± 2.0
Concentrated virus	10.4 ± 1.1	796.4 ± 28.5	30.2 ± 4.5
Centricon filtrate	0.01 ± 0.002	3.1 ± 1.03	ND

ND: not determined.

[a]Mean fluorescence intensity following transduction of NIH3T3 fibroblasts with 0.5 ml viral supernatant.

[b]Transduction efficiency determined by EGFP expression of transduced splenocytes.

Table 2.2

Average retroviral titer and mean fluorescence intensity of GATA-3 concentrated and unconcentrated retroviral preparations

	Viral Titer (x 10^6 cfu/mls)	Mean Fluorescence Intensity[a]
Unconcentrated virus	1.2 ± 0.07	15.14 ± 2.7
Concentrated virus	8.0 ± 1.0	47.1 ± 4.5
Centricon filtrate	0.01 ± 0.002	2.0 ± 1.01

ND: not determined.

[a]Mean fluorescence intensity following transduction of NIH3T3 fibroblasts with 0.5 ml viral supernatant.

Figure 2.6

Efficiency of GATA-3 encoding retrovirus concentration by centrifugal filtration.

Cell culture supernatants from Phoenix-Eco cells producing retrovirus derived from GATA-3/AP2 were subjected to a 10-fold volume reduction. Retroviral preparations, both before and after centrifugal concentration were then titered on NIH3T3 cells. Volumes of 0.5µl, 5µl, 50µl, and 500µl were used and EGFP expression monitored by flow cytometry. Density plots show EGFP expression in transduced NIH3T3 cells following incubation with the indicated volumes of retrovirus-containing supernatant, before (left column) and after (right column) concentration.

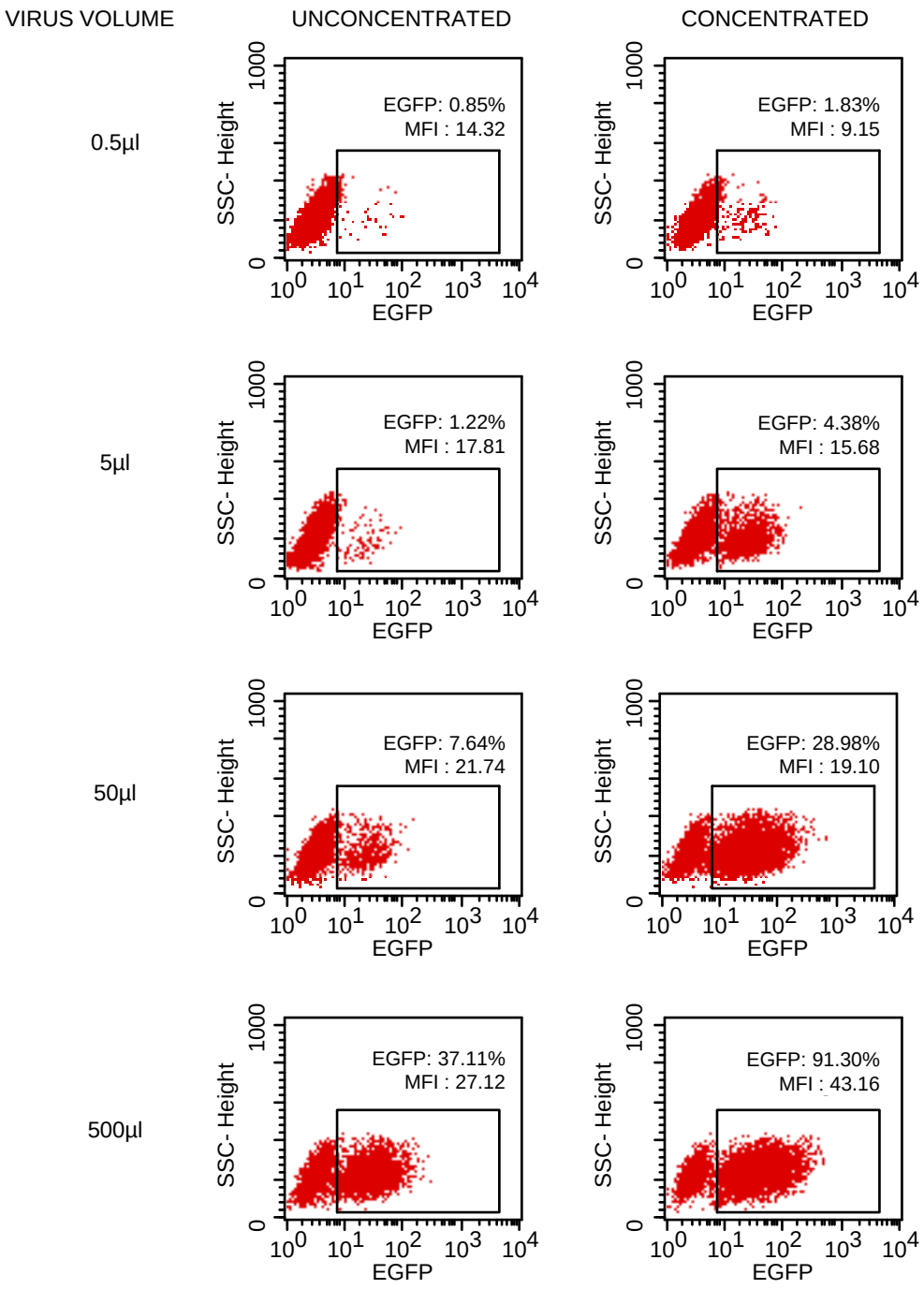
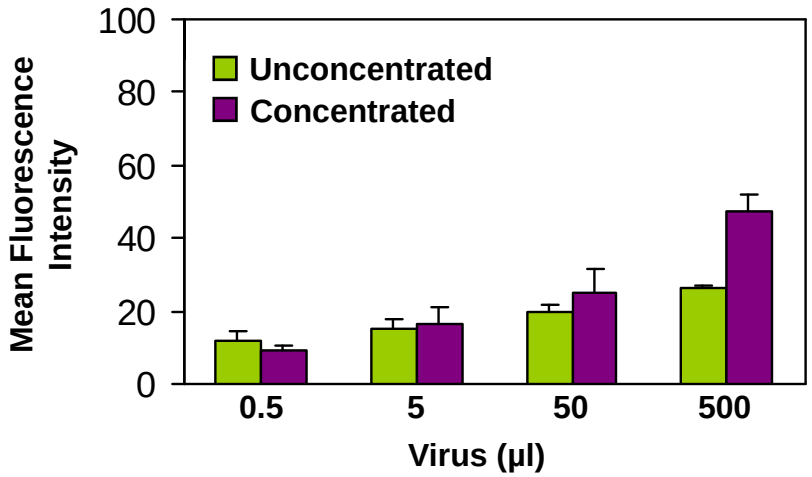

VIRUS VOLUME
UNCONCENTRATED
CONCENTRATED
0.5µl
5µl
50µl
500µl
EGFP: 0.85%
MFI : 14.32
EGFP: 1.83%
MFI : 9.15
EGFP: 1.22%
MFI : 17.81
EGFP: 4.38%
MFI : 15.68
EGFP: 7.64%
MFI : 21.74
EGFP: 28.98%
MFI : 19.10
EGFP: 37.11%
MFI : 27.12
EGFP: 91.30%
MFI : 43.16
SSC- Height
EGFP
0
1000
10^0 10^1 10^2 10^3 10^4

Figure 2.7

Mean fluorescence intensity values of transduced NIH3T3 fibroblasts.

Cell culture supernatants from Phoenix-Eco cells producing retrovirus derived from GATA-3/AP2 were subjected to a 10-fold volume reduction. Retroviral preparations, both before and after centrifugal concentration, were then titered on NIH3T3 cells using the indicated volumes. The average mean fluorescence intensity of EGFP expression ± SEM determined by flow cytometry for three experiments is presented


Mean Fluorescence Intensity
100
80
60
40
20
0
Unconcentrated
Concentrated
0.5
5
50
500
Virus (µl)

filtration can be used to concentrate supernatants at least 6-10 fold in order to generate ecotropic retrovirus preparations with titers of 7-10 x 10^6 CFU/ml.

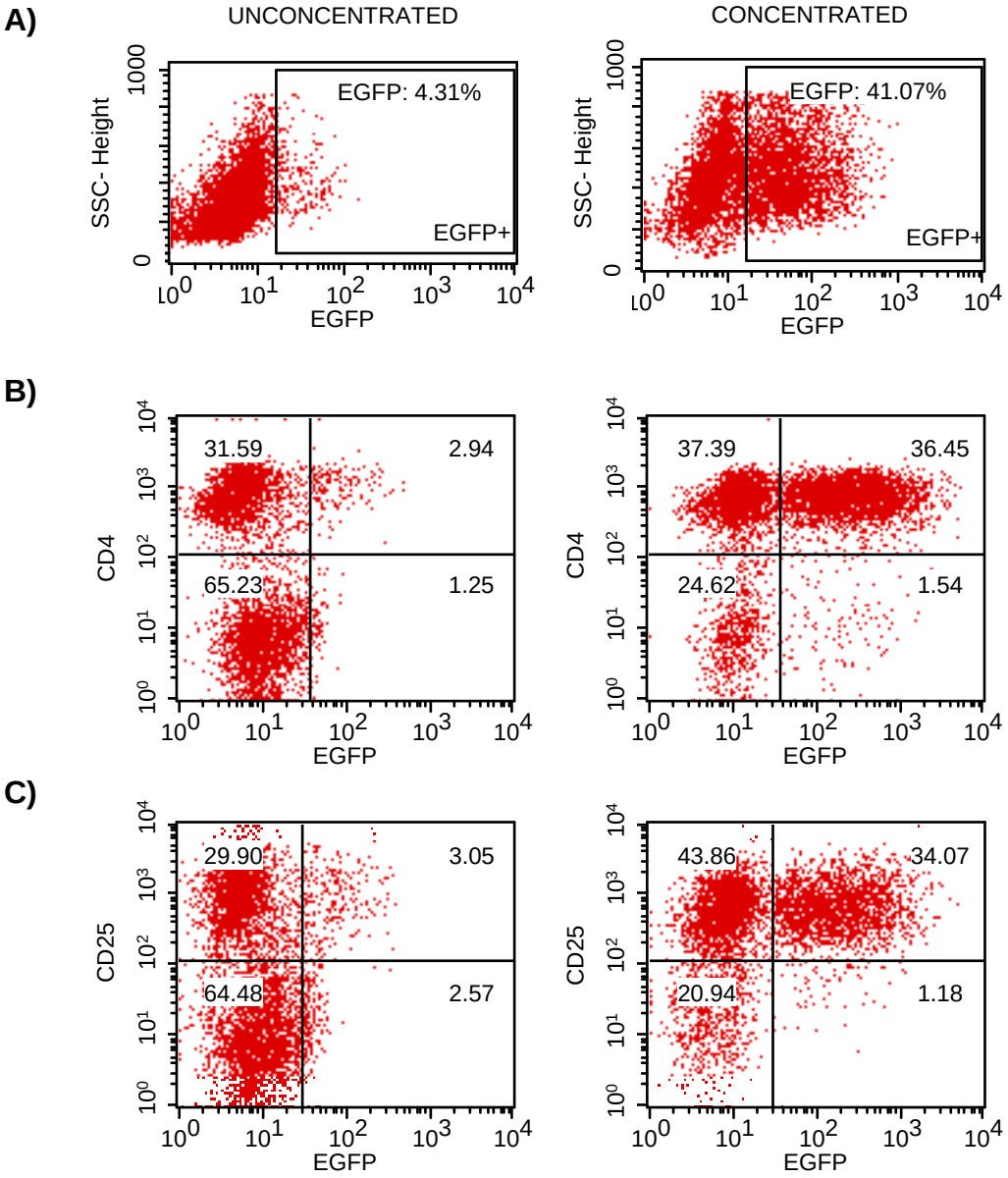
Primary rat $CD4^+$ T cells are efficiently transduced by concentrated retroviral preparations.

Immortalized NIH3T3 fibroblasts are efficiently transduced by recombinant retroviruses. However, the target cells of interest for our studies are primary, antigen-specific $CD4^+$ T lymphocytes. Thus, we investigated the ability of the concentrated and unconcentrated retroviral preparations derived from pAP2 to transduce rat splenocyte T cells stimulated with antigen in vitro. Firstly, splenocytes were harvested from ovalbumin (OVA) sensitized rats and cultured with OVA for two days prior to retroviral transduction. Both unconcentrated and concentrated retroviral preparations, with titers of approximately 10^6 CFU/ml and 10^7 CFU/ml, respectively, transduced $CD4^+$ T cells stimulated by antigen *in vitro*, without altering the levels of cell death present in control non-transduced cells. When concentrated retroviral stocks were used, 30.2 ± 4.5% of the splenocytes were transduced compared to 6.8 ± 2.0% when unconcentrated retroviral supernatants were used (**Figure 2.8A**). Moreover, in addition to transducing a larger number of cells, the mean fluorescence intensity of EGFP expression in splenocytes transduced with the concentrated retrovirus was higher due to the larger MOI and therefore greater rate of infection per cell. Significantly, although total splenocyte populations were incubated with retroviruses, $CD4^+$ T cells were selectively transduced (**Figure 2.8B**). In addition, the transduced $CD4^+$ T cells expressed T cell markers of activation, CD25 (**Figure 2.8C**) and OX-40 (data not

Figure 2.8

Primary $CD4^+$ T cells are efficiently transduced by concentrated retroviral preparations.

Splenocytes harvested from OVA-sensitized Brown Norway rats were cultured with OVA for 48h prior to being spin-infected with either unconcentrated (left panels) or concentrated (right panels) retroviral preparations. Two days after the final spin, expression of CD4, CD25 and EGFP in live cell populations was assessed by flow cytometry. *(A)* Flow cytometry density plots of EGFP expression in splenocyte populations. *(B)* Flow cytometry density plots of EGFP expression in $CD4^+$ T cell populations. *(C)* Flow cytometry density plots of EGFP expression in $CD25^+$ T cell populations. Results are representative of three experiments.


A)
UNCONCENTRATED
CONCENTRATED
SSC- Height
EGFP: 4.31%
EGFP+
EGFP: 41.07%
EGFP+
EGFP
B)
CD4
31.59
2.94
65.23
1.25
37.39
36.45
24.62
1.54
EGFP
C)
CD25
29.90
3.05
64.48
2.57
43.86
34.07
20.94
1.18
EGFP

shown). Similar results were obtained using the concentrated retroviruses expressing GATA-3 (see Chapter 3), though experiments comparing unconcentrated vs concentrated preparations were not done.

Though splenocytes were cultured with OVA, the increase in transduction efficiency may have been in part a response to the increase in retroviral particles present in the concentrated retroviral preparations. Thus, to confirm that addition of OVA increased retroviral transduction efficiency, we investigated the ability of pAP2-derived retroviral preparations to transduce splenocyte populations, harvested from OVA-sensitized donors, and cultured in the presence or absence of OVA(11, 13). The addition of OVA increased the overall viability of the cultured splenocytes and induced a 7.2 ± 2.3 fold increase in transduced cell numbers for unconcentrated retroviral preparations and a 3.0 ± 1.3 fold increase for concentrated retroviral preparations. These data provide evidence that addition of OVA to the cultures increased proliferation of antigen-specific T cells that were then transduced. Though these data do not rule out a nonspecific T cell response to the retroviral preparations themselves, they are consistent with OVA-specific T cells comprising at least a subset of the transduced cell population. Moreover, following FACS-sorting (of $EGFP^+CD4^+$ T cells), adoptive transfer, and airway OVA challenge, these transduced $CD4^+$ T cells induce OVA-specific lung inflammatory responses in naïve Brown Norway rats (13) and Chapter 3). Altogether, these data demonstrate that the transduction efficiency of difficult-to-transduce cells, such as primary T cells activated with antigen, can be increased at least four-fold by using retroviral preparations concentrated by centrifugal filtration.

Discussion

Replication-defective recombinant retroviruses are ideal vectors for gene transfer into proliferating cell populations because, following infection, virion internalization and reverse transcription of the retroviral RNA, the proviral DNA integrates into the host cell chromosomal DNA (1). This allows long-term expression of the retrovirally encoded gene product(s) in the target cell as well as its progeny. This is particularly relevant when the target is an antigen specific T cell, which will undergo proliferation and clonal expansion upon activation. Indeed, the ability of retroviruses to transduce only dividing cells (14) has been exploited to select rare subpopulations of antigen-specific helper (11, 13) or cytotoxic (15) T cells, by coupling antigen-specific T cell activation with retrovirus infection. Since antigen-specific T cells enter the cell cycle upon stimulation by antigen, these cells can be selectively transduced *in vitro* following activation by antigen-presenting cells. Therefore, recombinant retroviruses are a promising tool for therapeutic applications based on genetically modified, antigen-specific T cells. Pre-clinical studies using T cell hybridomas, TCR transgenic mice or T cell clones have already demonstrated the feasibility of using genetically modified, antigen-specific $CD4^+$ T cells to release immunomodulatory molecules at disease specific sites *in vivo* (16-21).

However, the advantages of retroviruses and their potential for gene therapy applications targeting peripheral T cells, are still hindered by technical limitations making it difficult to obtain transduced, antigen-specific T cells in clinically significant numbers, especially if a physiological immune response is the source of such cells. This is the case of wild-type animal strains sensitized to

an antigen or, for potential therapeutic purposes, human subjects. A central problem is that various mammalian cell types respond differently to retroviral gene transduction. While clinical trials have succeeded in correcting severe combined immunodeficiencies by transducing hematopoietic stem cells (2, 3), mature T lymphocytes are a particularly difficult-to-transduce population and require the use of high titer retroviral preparations. One strategy employed to increase retrovirus titers is ultracentrifugation of virion particles pseudotyped with VSV-G protein. VSV-G pseudotyped retroviruses have a very broad host range because the VSV-G protein interacts with a membrane phospholipid component and viral entry does not appear to depend on a specific protein receptor (22). Importantly, VSV-G pseudotyping confers resistance to ultracentrifugation and allows the concentration of retroviruses to titers of 10^9 CFU/ml or greater (23, 24). Surprisingly, despite a lack of tropism and high titers, these retroviral preparations are still limited in their ability to infect mature T and B lymphocytes (12).

In the absence of VSV-G pseudotyping, attempts to concentrate retroviruses by centrifugation or other physical means have generally resulted in loss of infectious particles and little gain in titers (24). Here we demonstrate that centrifugal filtration can be used to concentrate ecotropic retroviruses at least ten fold. The leak of infectious particles through the filters was negligible and the virus degradation throughout the process was limited. We tested this strategy with the goal of transducing $CD4^+$ T cells in spleen cell populations in order to target rare populations of antigen-specific $CD4^+$ T cells (11, 13). For this purpose, following OVA sensitization of wild-type Brown Norway rats, spleen

cell populations were cultured with OVA and subsequently incubated with retroviral preparations at an MOI of 1. To achieve this MOI, retroviral preparations were first concentrated by centrifugal filtration. The concentrated retrovirus-containing supernatants were incubated with total splenocytes, yet selectively transduced activated $CD4^+$ T cells. Compared to unconcentrated retroviruses, cells transduced with concentrated retroviral preparations did not show any obvious toxic effects; were transduced in greater numbers; and expressed higher levels of the transgene product, EGFP. Likewise, $CD4^+$ T cells were successfully transduced with retrovirus encoding the transcription factor GATA-3 (See also Chapter 3). However, $EGFP^+$ cells transduced with the GATA-3 retrovirus consistently had lower overall MFIs compared to cells transduced with retrovirus derived from pAP2. This is likely due in part to the slightly lower viral titers obtained from concentrated GATA-3 retroviral preparations. The lower viral titers from the GATA-3 transfected Phoenix packaging cell line may be due to decreased viral particle production since this cell line exhibits slower growth rates compared to the pAP2 control cells (unpublished observations). Slower growth rates have also been observed due to enforced expression of GATA-3 in HEK293 cells, from which phoenix retroviral packaging cell lines are derived (25). An additional explanation for the lower MFI's obtained in GATA-3 transduced cells may be that the cells with high MFIs, representing high levels of expression of both GATA-3 and EGFP, succumb to cell death due to the toxicity high levels of GATA-3 expression.

Interestingly, nearly all of the retrovirally transduced cells expressed the T cell activation marker, CD25. Moreover, when splenocytes were transduced with

the concentrated retroviral preparations (as opposed to unconcentrated retroviruses), a larger percentage of cells expressed CD25, whether transduced or not. Thus, CD25 may have been upregulated in response to both the retrovirus and the OVA. Nevertheless, our data indicate that cell viability and transduction efficiency were increased in cells cultured with OVA. Moreover, following FACS-sorting and adoptive transfer into naïve BN rats, transduced $CD4^{+}EGFP^{+}$ T cells efficiently induced OVA-specific airway inflammatory responses *in vivo* (See Chapter 3).

In summary, our data demonstrate that centrifugal filtration is a simple method that can be used to concentrate retroviruses to titers sufficient to transduce antigen-specific primary T cells. This method can be used to effectively increase the titer of retroviruses at least ten fold. The concentrated retroviral stocks do not have altered physical or chemical properties that adversely affect transduction of primary T cells. Compared to unconcentrated retroviral preparations these concentrated retroviruses transduced larger numbers of T cells and induced higher levels of transgene expression. Concentrating retroviral preparations in this way may increase the efficiency with which retroviral transduction coupled with T cell stimulation with antigen can be used to generate gene-modified, antigen-specific T cells for gene therapy studies in animal models of autoimmune or allergic disease (11, 13). This methodology may also be applied to increase the efficiency of gene transfer into other difficult to transduce cells.

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Chapter 3:

Th2-dependent early recruitment of antigen non-specific $CD4^+$ T cells in experimental asthma

Abstract

Following antigen challenge, adoptively-transferred antigen-specific $CD4^+$ T cells induce allergic airway inflammation, comprised primarily of an increase in lymphocytes and eosinophils. To better understand the contribution of the GATA-3 transcription factor to the ability of adoptively transferred T cells to induce airway inflammation we have used the Brown Norway rat model of adoptively-transferred asthma. We transduced antigen-stimulated $CD4^+$ T cells with recombinant retroviruses encoding EGFP only or EGFP and the GATA-3 transcription factor. Our data indicate that T cells transduced with retroviruses encoding GATA-3 expressed high levels of GATA-3 protein as well as Th2 cytokines. Following adoptive transfer and airway antigen challenge, these gene-modified T cells induced robust inflammatory responses in the lungs and draining lymph nodes. Increased numbers of total inflammatory cells and eosinophils were recovered in the bronchoalveolar lavage fluid. In addition, the number of $CD4^+$ T cells recovered in the bronchoalveolar lavage fluid as well as the lung and draining lymph nodes was enhanced in recipients of GATA-3-overexpressing T cells following antigen challenge. Nevertheless, the transduced $CD4^+$ T cells comprised only a small percentage of the population of $CD4^+$ T cells infiltrating the lung and were not detectable at all in the draining lymph nodes. These data provide evidence that GATA-3 plays a significant role in the ability of antigen-specific T cells to amplify allergic inflammatory responses *in vivo* by promoting the recruitment of endogenous T cells to the lung.

Keywords: asthma, inflammation, GATA-3, T cell, retroviral transduction, adoptive transfer

Introduction

$CD4^+$ T cells producing the Th2 cytokines, IL-4, IL-5, IL-9 and IL-13, play a central role in allergic asthma, a disease characterized by eosinophilic airway inflammation and mucus production accompanied by airway hyperresponsiveness (1, 2). Adoptive transfer of antigen-specific $CD4^+$ T cells is sufficient to induce allergic airway inflammation following antigen challenge. The magnitude of inflammation following T cell adoptive transfer is similar to that produced in actively sensitized animals providing evidence that $CD4^+$ T cells are sufficient for the induction and maintenance of inflammation in experimental asthma (3). Phenotypically, the cells responsible for transfer of allergic responses are activated, antigen-specific T cells that express high levels of CD4 and produce Th2 cytokines (3-7). Following culture with antigen, adoptive transfer of remarkably few $CD4^+$ T cells is sufficient to induce inflammation following antigen challenge in the Brown Norway (BN) rat (8). While several million T cells expressing a transgenic TCR that recognizes OVA (from DO11.10 mice) are typically transferred in murine models (9, 10) fewer than 1200 Ag-specific $CD4^+$ T cells may be sufficient to induce a robust airway eosinophilic inflammatory response in experimental asthma in the mouse (3).

The ability of adoptively-transferred $CD4^+$ T cells to induce asthma pathogenesis is due in part to the production of Th2 cytokines that then regulate chemokine and cytokine production and the recruitment of other effector cells to the lung (10-12). Production of IL-4, IL-5 and IL-13 is regulated by the transcription factor GATA-3 (13-17). In addition to inducing Th2 cytokine

expression, GATA-3 autoactivates its own expression by a positive feedback mechanism (18). Significantly, expression of GATA-3 is increased in atopic asthmatics (19-21). Moreover, in experimental asthma, disruption of GATA-3 expression or activity inhibits airway eosinophilia, Th2 cytokine expression and airway hyperresponsiveness (22, 23).

We have used an adoptive transfer model in the BN rat to investigate the role of GATA-3 in the ability of adoptively transferred T cells to induce airway inflammation in experimental allergic asthma. Our data show that adoptively transferred T cells overexpressing GATA-3, though present in small numbers in the airways, induce robust lymphocytic and eosinophilic inflammatory responses within days of antigen challenge. These data suggest that antigen-specific T cells overexpressing GATA-3 and Th2 cytokines have the ability to amplify allergen-specific inflammatory responses in the airways by enhancing the recruitment of endogenous T lymphocytes.

Materials and Methods

Animals. Inbred Brown Norway rats aged 10-12 weeks were obtained from Harlan Laboratories (Harlan-U.K., Bicester, Oxon, England). All animal protocols complied with Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care Committee.

Antibodies and cytokines. Allophycocyanin- (APC-) conjugated mouse anti-rat CD4, mouse anti-rat CD25, mouse anti-rat CD62L, mouse anti-rat IL-4, mouse anti-rat IFN-γ, APC-conjugated anti-mouse IgG, phycoerythrin (PE)-conjugated anti-mouse IgG, and recombinant rat IL-2 were obtained from BD Biosciences (Canada). Murine anti-rat CD134 (OX40) and murine anti-rat IL-5 were purchased from Cedarlane (Canada). Fluorescein isothiocyanate-(FITC-) conjugated goat polyclonal anti-green fluorescent protein (GFP) antibody was purchased from Abcam (Cambridge, MA). Mouse anti-rat GATA-3 antibody (HG3-31) was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA).

Production of recombinant retroviruses. Retroviruses were harvested from Phoenix-Eco packaging cells stably transfected with the pAP2 retroviral vector, which encodes enhanced green fluorescent protein (EGFP) only (24), or its derivative containing the full-length GATA-3 cDNA isolated from rat TIB-236 cells and cloned upstream of the IRES-EGFP cassette in pAP2. Recombinant retroviruses, concentrated 10-fold by centrifugal filtration, had titers on NIH3T3 fibroblasts in the range of 5-10 x 10^6 CFU/ml (25). Stimulated splenocytes were

transduced with the control retrovector encoding EGFP only (control) or the GATA-3 retrovector encoding both GATA-3 and EGFP (GATA-3).

Antigen sensitization, splenocyte isolation and retroviral transduction. BN rats (ages 10-12 weeks) were sensitized intraperitoneally (i.p.) with a suspension of 200μg of OVA (Sigma-Aldrich, St Louis, MO) and 1 mg of aluminum hydroxide (EM Industries, Gibbstown, NJ). Animals were sacrificed 14 days later and spleens harvested and passed through a cell strainer to obtain single cell suspensions. Red blood cells were removed using red blood cell lysis buffer (Sigma-Aldrich). To enhance proliferation and thus retroviral transduction of OVA-specific T cells (8, 26), total splenocyte populations were cultured in DMEM (Invitrogen, Canada) containing 200 μg/ml OVA, 100U/ml IL-2, 100U/ml penicillin, 100ug/ml streptomycin, and 2mM L-glutamine (Invitrogen, Canada) at 37^0C and 5% CO_2. Following 48 hours of culture with OVA, cells were resuspended in retroviral supernatants containing 6μg/ml of hexadimethrine bromide (polybrene) (Sigma-Aldrich) and IL-2 (100U/ml). Cells and retroviruses were then centrifuged at 1600 rpm for 1 hour. A total of four spin infections were carried out over the course of 48 hours and 72 hours after the last spin, cells were assessed for EGFP expression using a FACS Calibur flow cytometer (BD Biosciences) and CELLQUEST Software (BD Biosciences). Five days following the last spin, $CD4^+$ cells were purified using magnetic cell sorting (MACS; Miltenyi Biotec GMBH, Germany) by negative selection and live, $EGFP^+$ cells were sorted for adoptive transfer on a MoFlo flow cytometer (Cytomation, Fort

Collins, CO). Alternatively, cells were maintained in culture for two weeks in order to assess cytokine expression.

Analysis of GATA-3 and cytokine expression in transduced cells. To detect GATA-3 expression following retroviral transduction, splenocytes were fixed in 1% paraformaldehyde, permeabilized with 0.1% Triton X-100 (Sigma), and incubated with primary GATA-3 antibody followed by APC-conjugated anti-mouse IgG. GATA-3 protein expression was subsequently analyzed using a FACS Calibur flow cytometer. As a control, cells were stained with APC-conjugated anti-mouse IgG only. Expression of GATA-3 mRNA was assessed by RT-PCR using RNA isolated from transduced splenocyte populations and primers derived from the rat GATA-3 sequence. (Forward primer: 5'-CGA AAG ACG TCT CCC CAG AC-3' and Reverse primer: 5'-AGC GTT TGC AAA GGT AGT GC-3'). Briefly, total RNA was extracted from splenocytes, and 1μg was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Canada) which was then PCR amplified (2 min at 95ºC followed by 30 sec at 94ºC, 30 sec at 56ºC, 1 min 30 sec at 72ºC for 35 cycles, then 10 min at 72ºC). PCR products were resolved by gel electrophoresis and visualized on a FluorChem 8000 Imaging System. Expression of IL-4, IL-5 and the housekeeping gene cyclophilin A were also assessed by RT-PCR using RNA isolated from transduced splenocyte populations as previously described (27).

Cytokine expression in transduced splenocytes was also examined by intracellular cytokine staining using the BD Cytofix/Cytoperm intracellular

cytokine staining kit (BD Biosciences) as per the manufacturer's instructions. Briefly, cells were stimulated with 1ug/ml of recombinant rat CD3 in the presence of Golgi stop for 8 hours. Cells were then fixed, permeabilized and stained for IL-4, IL-5 and IFN-γ. Cytokine expression was then determined following analysis by flow cytometry.

Adoptive transfer and antigen challenge (Figure 3.3A). In order to increase homing of OVA-specific $CD4^+$ T cells to the lungs, naïve recipient rats were intubated and intratracheally challenged with an aerosol of 5% OVA or PBS for five minutes 24h prior to adoptive transfer. The next day, 10^6 FACS-sorted $CD4^+$ $EGFP^+$ T cells, obtained from sensitized donor rats, were injected i.p. into anaesthetized BN recipient rats. Animals were challenged immediately after receiving T cells as above with 5% OVA or PBS for five minutes. Rats were sacrificed 72 hours following challenge with a lethal dose of sodium pentobarbital and bronchoalveolar lavage (BAL) was performed. Subsequently, the right lung was inflated and fixed in 10% formalin for 24 hours after which it was embedded in paraffin for immunofluorescence. The left lung was homogenized into a cell suspension for flow cytometry analysis. Spleens and draining (mediastinal) lymph nodes were also harvested and homogenized to form single cell suspensions for flow cytometry. Draining lymph nodes from recipient animals were then cultured in the presence of OVA for 72 hours, after which cell supernatants were collected and analyzed for cytokine production.

Cytospin and flow cytometry analysis. For analysis of inflammatory infiltrates, cells in BAL fluid were cytospun onto microscope slides and differential cell counts were performed using Diff Quick (Dade Behring Inc, Newark, DE). Cells from the BAL fluid were also stained with APC-conjugated anti-rat CD4 or murine anti-CD134 (OX40) and APC-conjugated goat anti-mouse IgG, respectively, and surface expression of CD4 and OX-40 quantified on a FACS Calibur flow cytometer (BD Biosciences) using CELLQUEST software (BD Biosciences).

Enzyme-Linked Immunosorbent Assays. Rat IL-4, IFN-γ, and IL-13 were quantified using ELISA kits from BD Biosciences and Biosource (Medicorp, Canada), respectively, and performed as per manufacturer's instructions.

Immunofluorescence and histology of lung tissue. Lung tissues were inflated, fixed in 10% buffered formalin and embedded into paraffin blocks from which five-micron thick sections were made. To assess airway inflammation, lung sections were stained with hematoxylin and eosin (H&E) and examined using light microscopy. To detect EGFP, tissue sections were permeabilized using PBS containing 0.5% Triton X-100 for 20 mins and then stained using goat polyclonal FITC-conjugated green fluorescent protein (Abcam) overnight at 4 °C. Nuclei were counterstained using Hoechst 33342 (Molecular Probes, Invitrogen). Slides were mounted using PermaFluor mounting medium (Shandon, Pittsburg, PA) and examined using a fluorescence microscope (Olympus BX 51 Fluorescence

Microscope System [Olympus America, Melville, NY]). Images were then analyzed using Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Silver Spring, MD).

Statistical analysis. Data are presented as the mean ± S.E.M. Data were analyzed using Student's T test (two sample assuming unequal or equal variances). $p < 0.05$ was considered significant. Data is from a minimum of three experiments using a total of five to seven animals per group.

Results

Retroviral transduction of antigen stimulated splenocytes

To overexpress GATA-3 in $CD4^+$ T cells destined for adoptive transfer we established methodology to produce high titer retroviral preparations that efficiently transduce BN rat splenocyte $CD4^+$ T cells cultured with the antigen, OVA (8, 25). OVA-stimulated splenocytes were transduced efficiently by the control retrovirus as well as that encoding GATA-3 (**Figure 3.1A**). As we have shown previously (8, 25), when total splenocytes are cultured with antigen, $CD4^+$ cells are preferentially transduced (**Figure 3.1B**). The majority of the transduced cells (>80%) expressed high levels of CD4 (**Figure 3.1B**) as well as the activation markers, CD25 (**Figure 3.1C**) and OX-40 (data not shown) and low levels of CD62L ($CD62L^{low}$), a marker commonly expressed by naïve T cells (**Figure 3.1D**). We first assessed expression of GATA-3 in splenocyte populations by flow cytometry 48h after retroviral transduction. As predicted, compared to cells transduced with control retrovector, cells transduced with the GATA-3 retrovector expressed higher levels of GATA-3 protein (**Figure 3.2A**). Expression levels correlated with the levels of EGFP expression, consistent with translation of each protein from a single bicistronic mRNA. Similarly, levels of mRNA encoding GATA-3 were also increased (**Figure 3.2B**). GATA-3 overexpression was maintained in transduced cell populations for at least two weeks (**Figure 3.2C**). Significantly, overexpression of GATA-3 did not alter the number of $EGFP^+$ cells recovered following culture for 6 days (**Figure 3.2D**), providing evidence that

Figure 3.1

Recombinant retroviruses selectively transduce CD4$^+$ T cells in splenocyte cultures harvested from OVA-sensitized donors.

(A) OVA-stimulated splenocytes were retrovirally transduced with a control retrovirus or a retrovirus encoding GATA-3. EGFP expression was monitored using FACS analysis. (*B*) Total splenocyte populations were stained with APC-conjugated anti-CD4 to identify CD4$^+$ T cells. (*C, D*) Transduced cells were also stained with anti-CD25 (*C*) or anti-CD62L (*D*) to determine the level of activation 48 hours after the last transduction. All data obtained from FACS were analyzed by gating on live cells and are representative of at least three independent experiments.

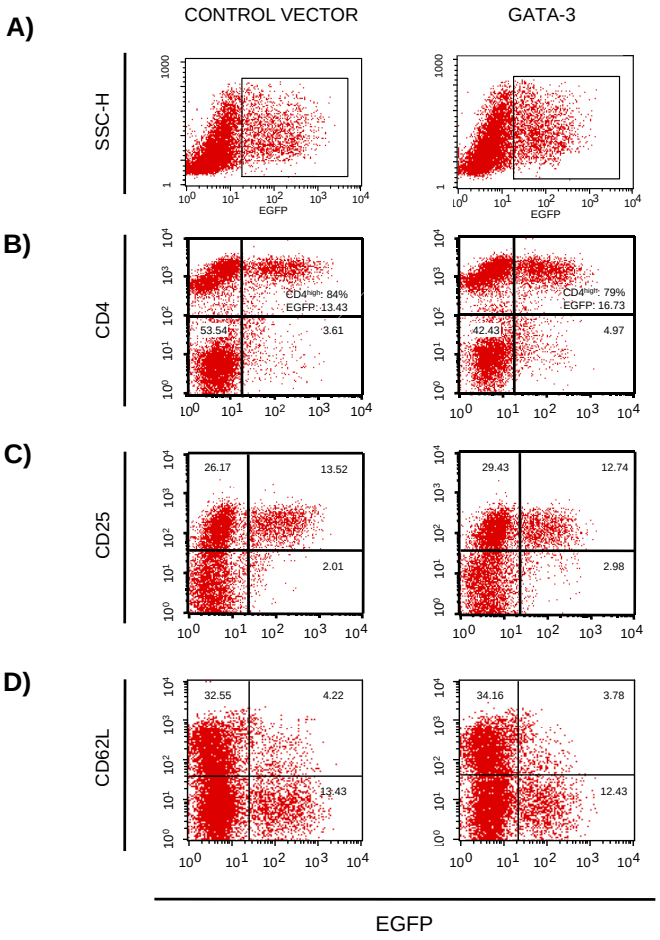
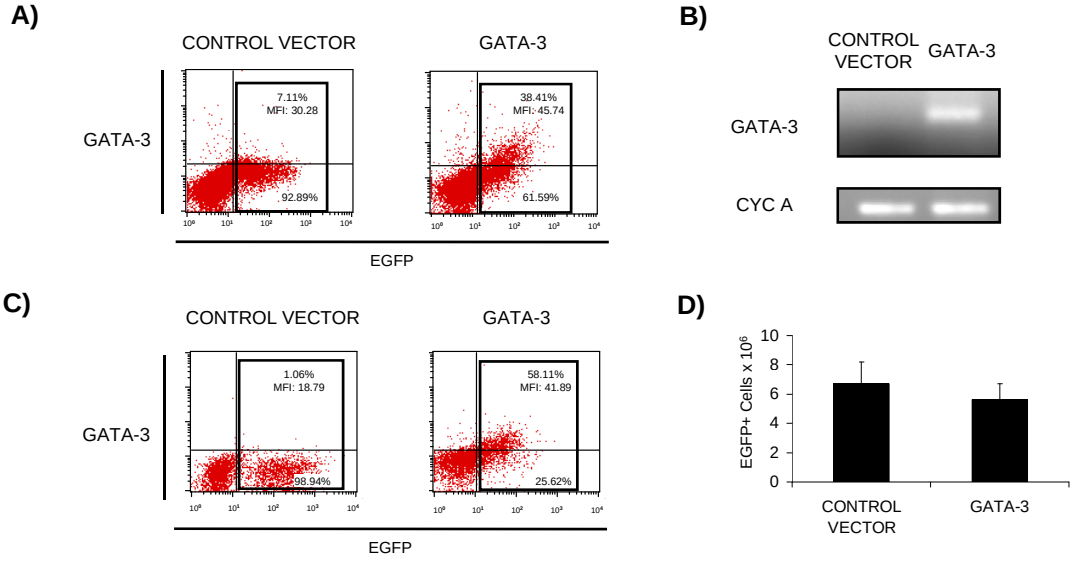

A)
CONTROL VECTOR
GATA-3
SSC-H
EGFP
B)
CD4
CD4high: 84%
EGFP: 13.43
53.54
3.61
CD4high: 79%
EGFP: 16.73
42.43
4.97
C)
CD25
26.17
13.52
2.01
29.43
12.74
2.98
D)
CD62L
32.55
4.22
13.43
34.16
3.78
12.43
EGFP

Figure 3.2

Retroviral vector encoding GATA-3 increases expression of GATA-3 in transduced splenocytes.

(*A*) Two days after transduction splenocytes were fixed, permeabilized, and stained with a primary anti-GATA-3 antibody and a secondary APC-conjugated antibody. GATA-3 and EGFP protein expression were then assessed by flow cytometry. (*B*) RT-PCR was performed to assess expression of GATA-3 and cyclophilin mRNA in transduced splenocytes. (*C*) Transduced splenocytes were cultured for two weeks, fixed, permeabilized, and stained with GATA-3 antibody as in (*A*). GATA-3 and EGFP expression were then assessed by flow cytometry. (*D*) $EGFP^+$ $CD4^+$ T cells were quantified prior to adoptive transfer of splenocyte populations transduced with a control retrovirus or a retrovirus encoding GATA-3.


A)
CONTROL VECTOR
GATA-3
GATA-3
7.11%
MFI: 30.28
92.89%
38.41%
MFI: 45.74
61.59%
EGFP
B)
CONTROL VECTOR
GATA-3
GATA-3
CYC A
C)
CONTROL VECTOR
GATA-3
GATA-3
1.06%
MFI: 18.79
98.94%
58.11%
MFI: 41.89
25.62%
EGFP
D)
EGFP+ Cells x 10^6
10
8
6
4
2
0
CONTROL VECTOR
GATA-3

GATA-3 overexpression did not modify proliferation and/or survival of transduced cells in the splenocyte cultures.

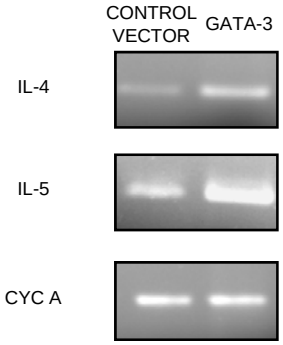
Since GATA-3 positively regulates Th2 cytokine expression, we assessed cytokine production in transduced splenocyte populations. Compared to cells transduced with control virus, cells overexpressing GATA-3 expressed higher levels of mRNA encoding IL-4 and IL-5 (**Figure 3.3A**). Forty eight hours after retroviral transduction, intracellular cytokine staining showed that the $EGFP^+$ T cells upregulated expression of both IL-4 and IFN-γ (Figure 3.3B left panel). When these cells were rested for two weeks in culture, 68.8% of the $EGFP^+$ cells transduced with retroviruses encoding GATA-3 expressed IL-4 whereas, only 49.1% of the $EGFP^+$ cells transduced with control retroviruses were IL-4 positive (**Figure 3.3B,** right panel). As well, the mean fluorescence intensity of IL-4 expression in GATA-3 overexpressing cells was also increased. In contrast to IL-4 expression, IFN-γ was not detected in these cells (**Figure 3.3B,** lower right panel). Additionally, intracellular cytokine staining for IL-5 forty eight hours following transduction showed greater levels of IL-5 expression in cells transduced with the GATA-3 retrovirus compared to those transduced with control virus (**Figure 3.3C**). Altogether, these data demonstrate that recombinant retroviruses can be used to generate primary rat $CD4^+$ T cells overexpressing GATA-3 and Th2 cytokines.

Figure 3.3

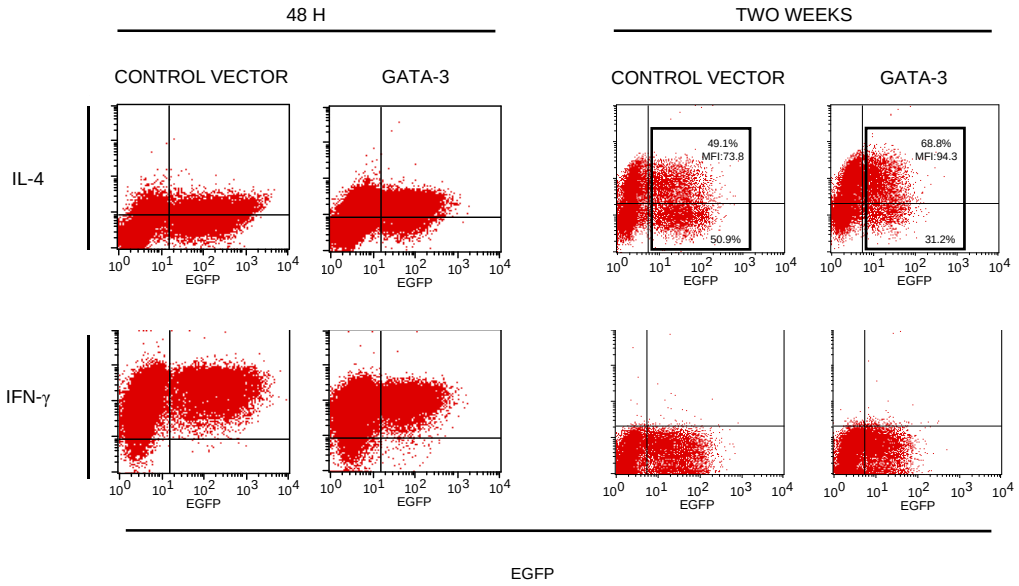
Retroviral vector encoding GATA-3 increases expression of Th2 cytokines in transduced splenocytes.

(*A*) RT-PCR was performed to detect expression of IL-4 and IL-5 mRNA in transduced splenocytes (mRNA was the same as in Figure 3.2 and the cyclophilin image is the same as that in 3.2B). (*B*) Splenocytes transduced with the control retrovirus or retrovirus encoding GATA-3 were activated with anti-CD3 in the presence of Golgistop both 48h (left panel) and two weeks (right panel) following transduction. Cells were then stained for IL-4 (upper panels) and IFN-γ production (lower panels). *(C)* Transduced cells were also stained for IL-5 so as to determine IL-5 expression 48h after transduction. Analysis of cell populations in B and C was performed on gated, live cells. Data shown is representative of at least two independent experiments.

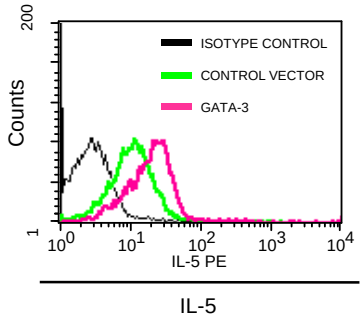
A)


CONTROL VECTOR
GATA-3
IL-4
IL-5
CYC A

B)


48 H
TWO WEEKS
CONTROL VECTOR
GATA-3
CONTROL VECTOR
GATA-3
IL-4
IFN-γ
49.1%
MFI:73.8
50.9%
68.8%
MFI:94.3
31.2%
EGFP
EGFP

C)


ISOTYPE CONTROL
CONTROL VECTOR
GATA-3
Counts
200
1
IL-5 PE
IL-5

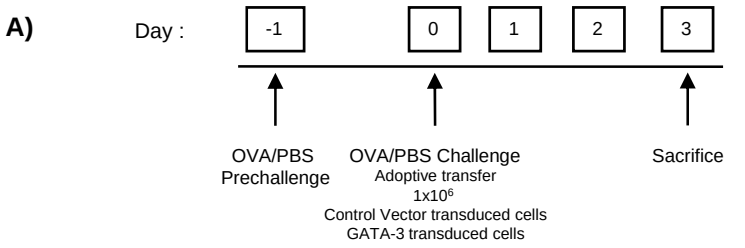
Adoptively transferred T cells overexpressing GATA-3 induce enhanced airway inflammation and eosinophilia

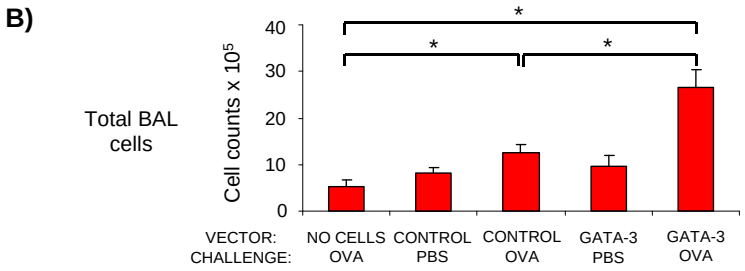
To investigate the ability of these cells to regulate allergic responses *in vivo*, gene-modified $CD4^+EGFP^+$ cells were purified and delivered by i.p injection into naïve recipient rats using the protocol presented in **Figure 3.4A**. To promote recruitment of $CD4^+$ T cells to the lung, animals were prechallenged 24h prior to adoptive transfer (8, 28). Immediately following delivery of T cells, animals were challenged with OVA or vehicle and 72 hours post challenge animals were sacrificed and airway inflammation in BAL fluid and lungs assessed. Though the $CD4^+$ T cells had been activated and retrovirally transduced *in vitro*, they did not induce airway inflammation in recipient rats in the absence of OVA challenge (**Figure 3.4B**). Moreover, compared to animals that received $CD4^+$ T cells transduced with control vector, a larger number of inflammatory cells were recovered in the BAL fluid from animals that received $CD4^+$ T cells overexpressing GATA-3 (**Figure 3.4B**). Similarly, following OVA challenge, the number of eosinophils recovered in the BAL fluid from recipients of T cells overexpressing GATA-3 was enhanced 2.3 ± 0.4 fold (**Figure 3.4C**), consistent with the ability of T cells overexpressing GATA-3 to produce higher levels of IL-5, a potent chemoattractant, activator, and differentiation factor for eosinophils (16, 29).

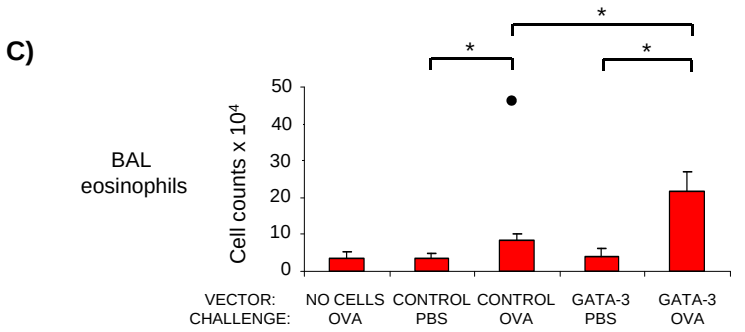
Figure 3.4

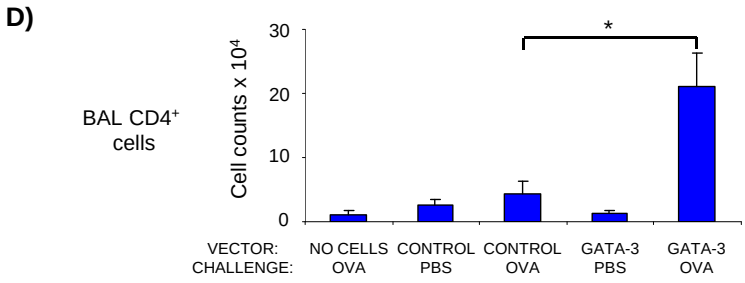
Adoptively transferred T cells overexpressing GATA-3 induce enhanced airway inflammation and eosinophilia following antigen challenge.

(*A*) Protocol for adoptive transfer of transduced splenocytes and antigen challenge. Bronchoalveolar lavage (BAL) was performed following sacrifice of challenged recipients. (*B*) Total BAL fluid cells and (*C*) BAL fluid eosinophils recovered from challenged recipients are shown (*$p < 0.05$). The dot in (*C*) represents an animal that was excluded because the number of BAL fluid eosinophils was greater than three standard deviations from the mean. (*D*) BAL fluid cells were stained with anti-CD4 antibodies and analyzed by flow cytometry (*$p < 0.01$). Pooled data from four independent experiments are presented as mean ± S.E.M. (n = 3-7 rats per group).


A)
Day :
-1
0
1
2
3
OVA/PBS
Prechallenge
OVA/PBS Challenge
Adoptive transfer
1x10^6
Control Vector transduced cells
GATA-3 transduced cells
Sacrifice


B)
Total BAL cells
Cell counts x 10^5
40
30
20
10
0
*
*
*
VECTOR:
CHALLENGE:
NO CELLS
OVA
CONTROL
PBS
CONTROL
OVA
GATA-3
PBS
GATA-3
OVA


C)
BAL eosinophils
Cell counts x 10^4
50
40
30
20
10
0
*
*
*
VECTOR:
CHALLENGE:
NO CELLS
OVA
CONTROL
PBS
CONTROL
OVA
GATA-3
PBS
GATA-3
OVA


D)
BAL CD4+ cells
Cell counts x 10^4
30
20
10
0
*
VECTOR:
CHALLENGE:
NO CELLS
OVA
CONTROL
PBS
CONTROL
OVA
GATA-3
PBS
GATA-3
OVA

We also quantified $CD4^+$ T cells recovered in the BAL fluid and the lungs. Compared to animals in all other groups, large numbers of infiltrating $CD4^+$ T cells were recovered in the BAL fluid of animals that received $CD4^+$ T cells overexpressing GATA-3 (**Figure 3.4D, 3.5A and 3.5B**). Following OVA challenge, the number of $CD4^+$ T cells recovered from the lungs was also enhanced in recipients of T cells overexpressing GATA-3 (**Figure 3.6A**). Histologic analyses revealed increased inflammation in recipients of T cells overexpressing GATA-3 compared to recipients of T cells transduced with control vector (**Figure 3.6B**). Cytokine levels in the BAL fluid were assessed following antigen challenge. Both IL-4 and IL-13 were increased in the BAL fluid of animals receiving $CD4^+$ T cells overexpressing GATA-3 while levels of IFN-γ did not differ significantly in any group (**Table 3.1**). These data provide evidence that adoptively transferred, gene-modified $CD4^+$ T cells induced a Th2-biased airway eosinophilic inflammatory response that was dependent upon antigen challenge and enhanced by T cell overexpression of GATA-3. Nevertheless, remarkably few $EGFP^+$ T cells were recovered in the BAL fluid and lung (see below).

Recruitment of adoptively transferred $CD4^+$ $EGFP^+$ cells to airways

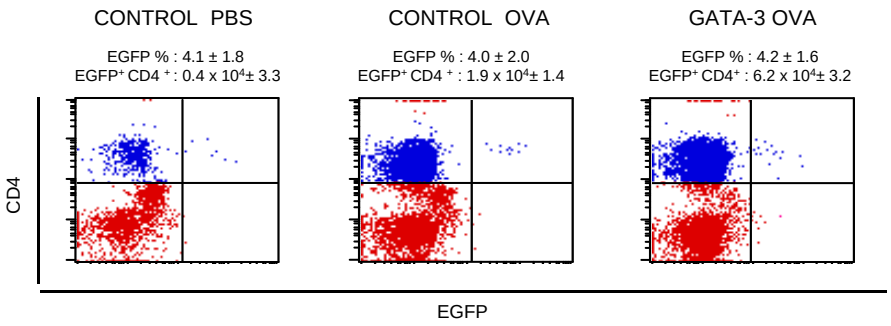
In order to induce airway inflammation, antigen-specific T cells are recruited to the lung and draining lymph nodes. We therefore quantified $EGFP^+$ cells recovered from BAL fluid, lung homogenates, and draining lymph nodes and assessed EGFP expression in lung sections by immunofluorescence. $EGFP^+$ cells were detected in the BAL fluid and lung homogenates of recipient animals,

Figure 3.5

Adoptively transferred EGFP$^+$ CD4$^+$ T cells comprise only a small proportion of total CD4$^+$ T cells recruited to the airway.

(*A*) BAL fluid cells from T cell recipients were stained with anti-CD4 antibodies and analyzed by flow cytometry to detect CD4$^+$ and EGFP$^+$ cells. (*B*) Quantification of total CD4$^+$EGFP$^+$ and CD4$^+$EGFP$^-$ cells recovered in BAL fluid of transduced T cell recipients. (* $p < 0.05$). The red bar represents CD4$^+$EGFP$^+$ T cells and the blue bar represents CD4$^+$EGFP$^-$ T cells. Pooled data from at least three independent experiments are presented as mean ± S.E.M. (n = 3-7 rats per group).

A)


CONTROL PBS
EGFP % : 4.1 ± 1.8
EGFP+ CD4 + : 0.4 x 10⁴± 3.3
CONTROL OVA
EGFP % : 4.0 ± 2.0
EGFP+ CD4 + : 1.9 x 10⁴± 1.4
GATA-3 OVA
EGFP % : 4.2 ± 1.6
EGFP+ CD4+ : 6.2 x 10⁴± 3.2
CD4
EGFP

B)

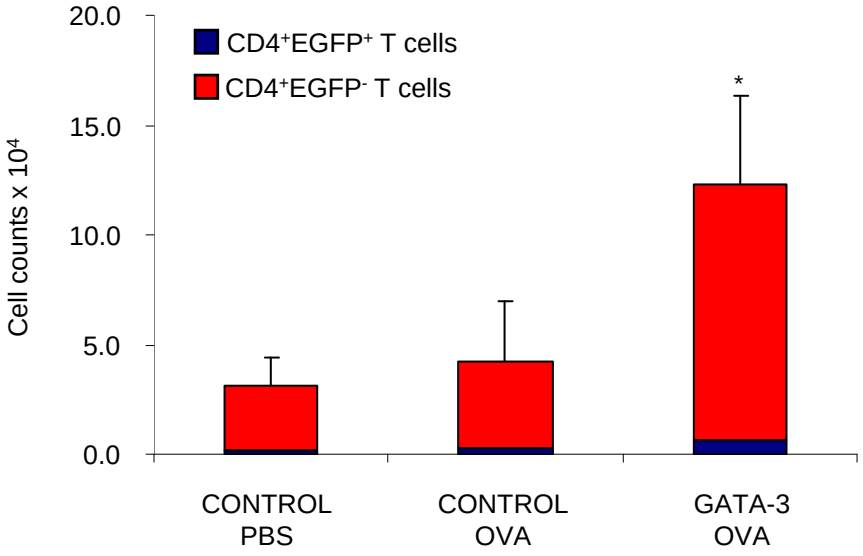

20.0
15.0
10.0
5.0
0.0
Cell counts x 10⁴
CD4+EGFP+ T cells
CD4+EGFP- T cells
*
CONTROL PBS
CONTROL OVA
GATA-3 OVA

Table 3.1

Cytokine production in bronchoalveolar lavage of challenged recipients of transduced cells.

	NO CELLS	CONTROL PBS	CONTROL OVA	GATA-3 PBS	GATA-3 OVA
IL-4	0	3.43 ± 1.57	3.27 ± 1.38	4.88 ± 2.64	5.92 ± 1.89*
IL-13	40.83 ± 12.01	48.11± 12.75	28.21± 11.22	32.08 ± 9.83	72.25 ± 14.79*
IFNγ	93.17 ± 2.12	107.16 ± 0.01	92.67 ± 6.72	99.16 ± 2.12	86.17 ± 0.71

One experiment representative of three independent experiments is shown.
Values shown are in pg/mls
* $p < 0.05$ compared to CONTROL OVA.

although they comprised only a minor fraction of the total $CD4^+$ cells present (**Figure 3.5A, B and 3.6A**). Consistent with the flow cytometry data, $EGFP^+$ cells were also identified in lung sections from recipient animals following OVA challenge where they comprised only a minor fraction of the inflammatory cells present (**Figure 3.7**). Interestingly, these $EGFP^+$ cells were still present in the lung following multiple OVA challenges over the course of nine days suggesting that they were retained in the airway upon repeated exposure to OVA. (**Figure 3.8**).

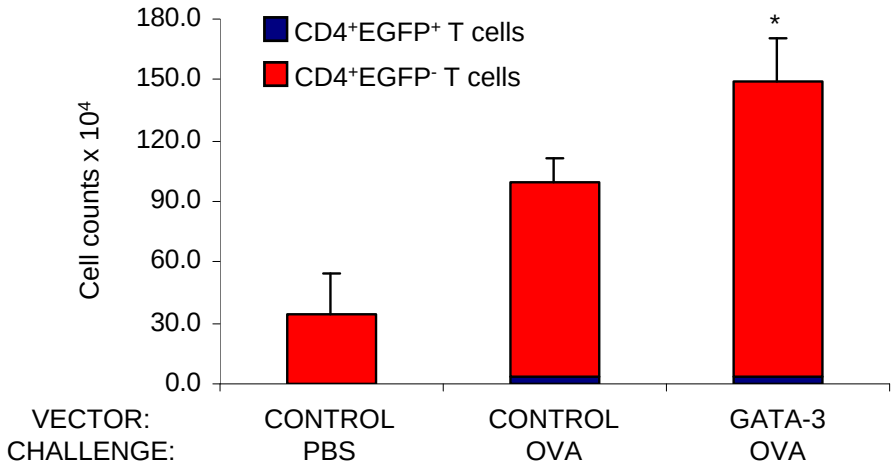
We quantified the total number of $CD4^+EGFP^+$ positive cells isolated from the BAL fluid and lung. Of the one million $CD4^+EGFP^+$ cells administered by i.p. injection, we recovered $4.1 \pm 2.1 \times 10^4$ and $3.3 \pm 1.6 \times 10^5$ respectively, in saline and OVA challenged recipients of cells transduced with control virus and $4.0 \pm 0.6 \times 10^5$ from OVA challenged recipients of cells overexpressing GATA-3 (**Figure 3.9**) demonstrating that the OVA challenge enhanced recruitment of antigen-specific $EGFP^+$ cells to the lung. However, the number of $EGFP^+$ cells recovered in the lung did not differ between OVA-challenged groups, suggesting that the number of antigen-specific cells trafficking to the lung is not as important as the cytokines they produce in the ability of adoptively transferred T cells to induce airway inflammation.

Figure 3.6

Adoptively transferred T cells overexpressing GATA-3 induce enhanced antigen non-specific $CD4^+$ T cell recruitment to the lung following antigen challenge.

(*A*) Lung cell suspensions were stained with APC-conjugated anti-rat CD4 antibody and analyzed by flow cytometry to quantify total $CD4^+EGFP^+$ and $CD4^+EGFP^-$ cells recovered. (* $p < 0.05$). Pooled data from three independent experiments are presented as mean ± S.E.M. (n = 3-4 rats per group). (*B*) Lungs from challenged recipients were fixed in 10% formalin and paraffin embedded. Tissue sections were then stained with H&E and examined by light microscopy. Representative sections are shown.

A)


180.0
150.0
120.0
90.0
60.0
30.0
0.0
Cell counts x 10⁴
CD4⁺EGFP⁺ T cells
CD4⁺EGFP⁻ T cells
*
VECTOR:
CHALLENGE:
CONTROL
PBS
CONTROL
OVA
GATA-3
OVA

B)

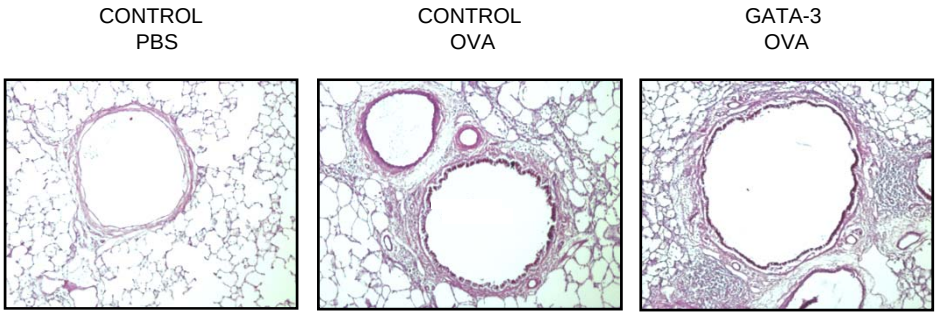

CONTROL
PBS
CONTROL
OVA
GATA-3
OVA

Figure 3.7

Adoptively transferred $CD4^+EGFP^+$ T cells traffic to the lung.

Lungs from antigen challenged recipients of transduced cells were fixed in 10% formalin and paraffin embedded. Prepared tissue sections were then permeabilized with 0.1 % Triton X-100 and stained with goat polyclonal GFP antibody to detect $CD4^+EGFP^+$ cells in the lung (*A, C, E*). Nuclei were counterstained using Hoechst 33342 (*B, D, F*). Data are representative of at least two independent experiments. Arrows indicate EGFP positive cells.

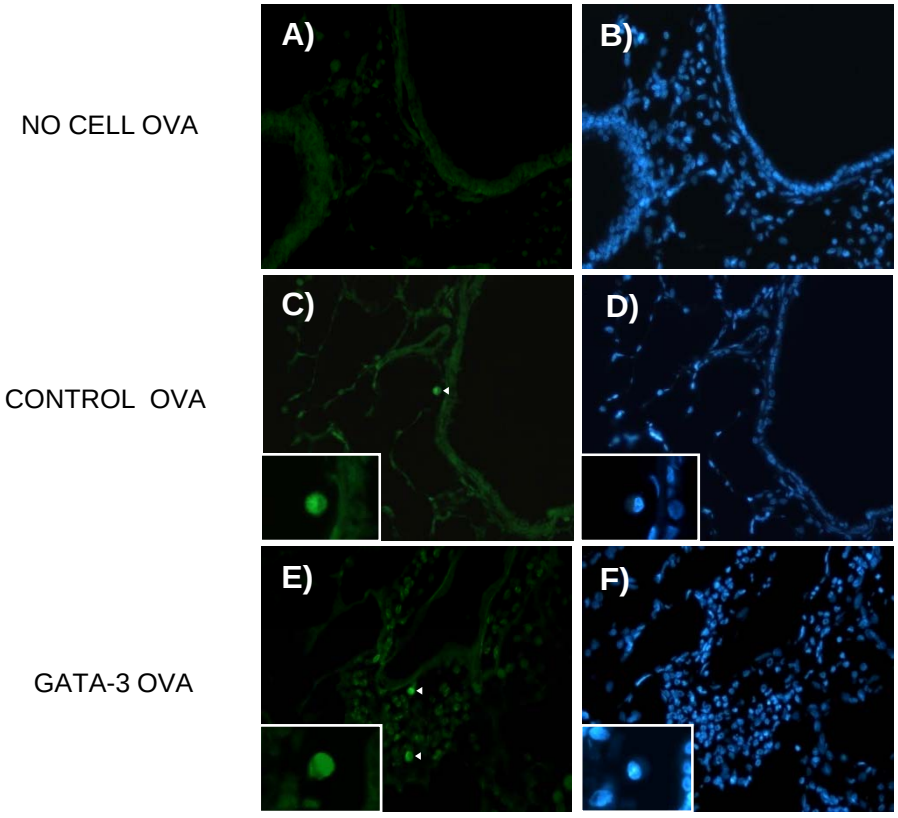

NO CELL OVA
CONTROL OVA
GATA-3 OVA
A)
B)
C)
D)
E)
F)

Figure 3.8

Adoptively transferred EGFP$^+$ CD4$^+$ T are still detectable in the lung following multiple OVA challenges.

Retrovirally transduced T cells were adoptively transferred into animals that were then challenged once (left panel) or four times over the course of nine days (right panel). Cells recovered in the BAL fluid were then stained with anti-CD4 antibodies and analyzed by flow cytometry to detect CD4$^+$ and EGFP$^+$ cells.

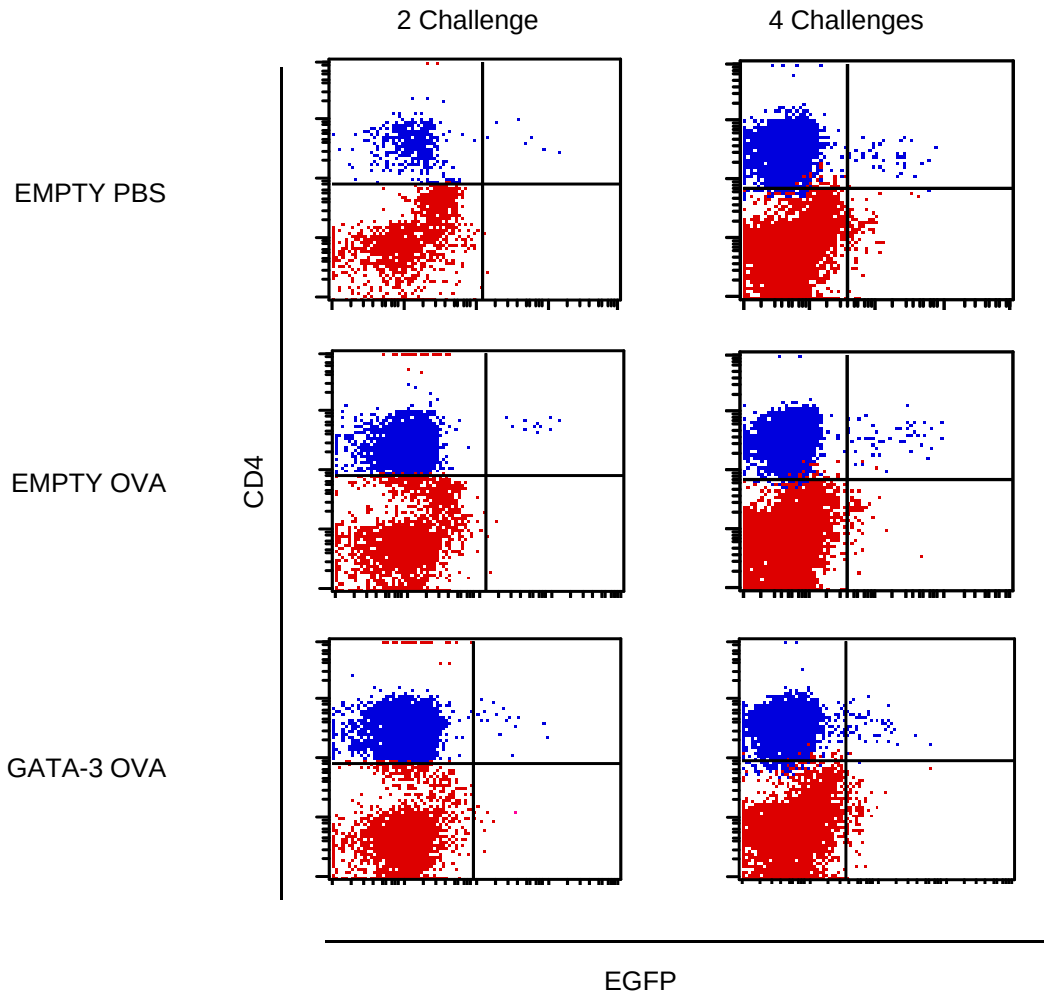
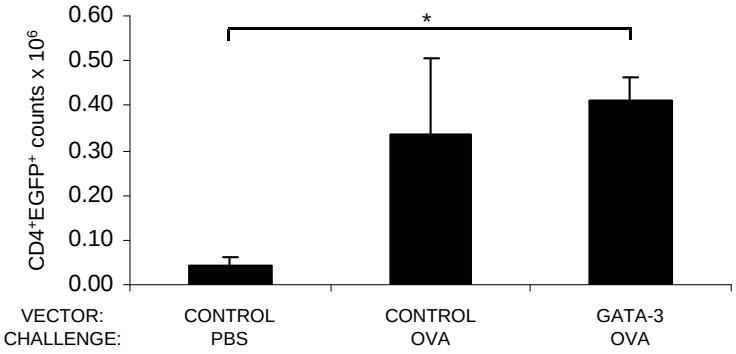

2 Challenge
4 Challenges
EMPTY PBS
EMPTY OVA
GATA-3 OVA
CD4
EGFP

Figure 3.9

Quantification of adoptively-transferred $CD4^+EGFP^+$ T cells recovered from the BAL fluid and the lung.

(*A*) Total numbers of $CD4^+EGFP^+$ cells recovered from the BAL fluid and lung were quantified (* $p < 0.05$). Pooled data from at least three independent experiments are presented as mean ± S.E.M. (n = 3-7 rats per group).


0.60
0.50
0.40
0.30
0.20
0.10
0.00
CD4+EGFP+ counts x 10^6
*
VECTOR:
CHALLENGE:
CONTROL
PBS
CONTROL
OVA
GATA-3
OVA

In vivo expansion of lymph nodes in recipients of T cells overexpressing GATA-3

Finally, inflammatory responses in the lung draining (mediastinal) lymph nodes and spleens of recipient animals were assessed. Adoptively transferred $CD4^+$ T cells did not induce inflammation in the spleen, providing evidence that these cells did not induce inflammation at sites distinct from that of antigen challenge (**Table 3.2** and data not shown). However, increased numbers of $CD4^+$ T cells were recovered from the draining lymph nodes in T cell recipients challenged with OVA (**Figure 3.10A** and data not shown). As in the lung, $CD4^+$ T cell numbers were further enhanced in recipients of T cells overexpressing GATA-3. In addition to the increase in $CD4^+$ T cells in the lymph nodes, increased numbers of total cells were also recovered from draining lymph nodes of animals receiving T cells overexpressing GATA-3 following OVA challenge (**Figure 3.10B**). Nevertheless, unlike in the lung, $EGFP^+$ cells were not detected in the lymph nodes. Interestingly, our preliminary data show that when cells were harvested from the draining lymph nodes of OVA-challenged animals and cultured in the presence of OVA, significantly greater levels of IL-13 were produced in cultures from animals receiving T cells overexpressing GATA-3 (**Figure 3.10C**). This increase in IL-13 was concomitant with a decrease in IFN-γ production (**Figure 3.10D**). Taken together, these data suggest that T cells overexpressing GATA-3 may indirectly induce proliferation or recruitment of cells to the draining lymph node and modify their cytokine production.

Table 3.2

Percentage of $CD4^+$ T lymphocytes recovered from recipients of transduced cells

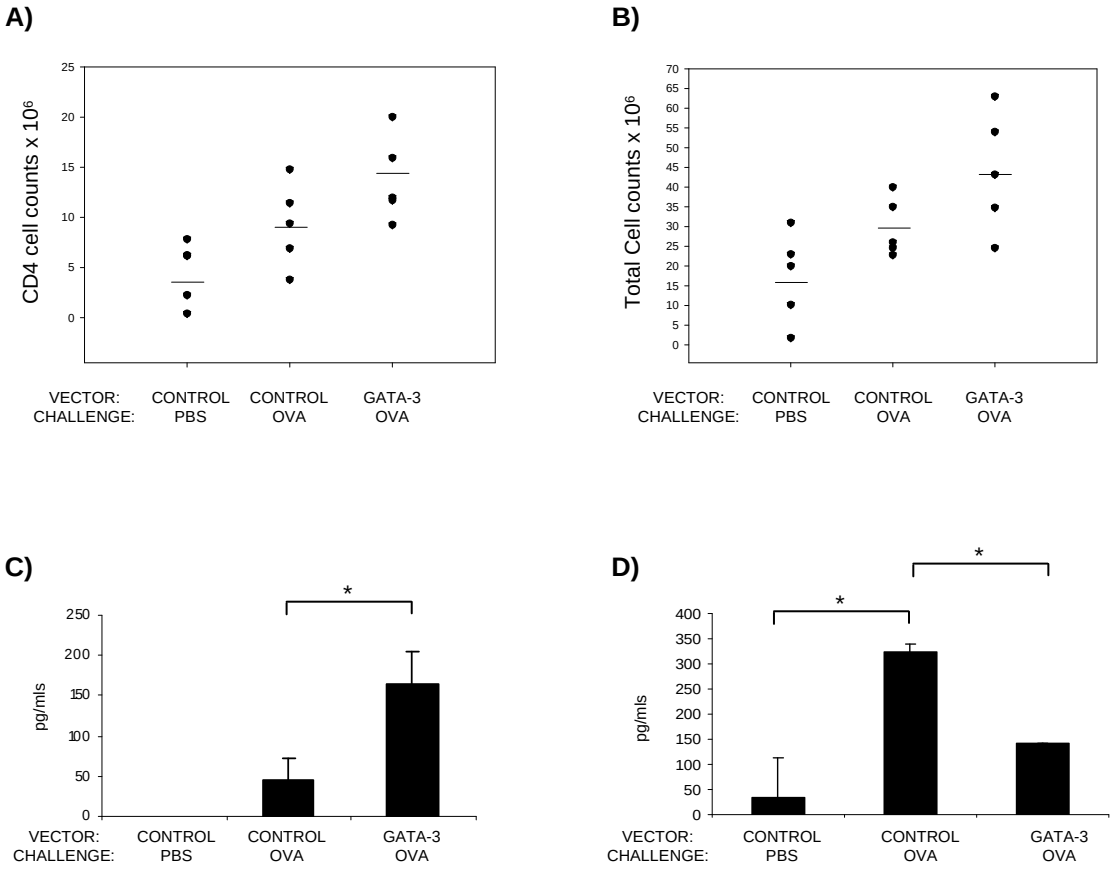
	Spleen	Draining Lymph nodes
CONTROL PBS	31 ± 1.8	24.65 ± 1.8
CONTROL OVA	29.16 ± 2.9	14.48 ± 7.2
GATA-3 OVA	31.99 ± 3.7	28.83 ± 2.1 *

Data from two independent experiments is shown, n=6
* $p < 0.05$ compared to CONTROL OVA

Figure 3.10

Antigen-induced expansion of lymphoid cell populations in recipients of T cells overexpressing GATA-3.

(*A*) Total DLNs were recovered 72 hours following challenge and were stained with anti-CD4 antibodies to quantify the lymphoid $CD4^+$ T cell population by flow cytometry. (*B*) DLNs from antigen challenged recipients were recovered and total cells were counted. Control PBS vs. Control OVA, $p < 0.01$; Control OVA vs. GATA-3 OVA, $p < 0.05$. Pooled data from two independent experiments are presented as mean ± S.E.M. (n = 5 rats per group). DLNs from antigen challenged recipients were recovered and cultured with OVA for seventy two hours. Levels of *(C)* IL-13 and *(D)* IFN-γ in cell supernatants were determined using ELISA. Pooled data from three animals are presented as mean ± S.E.M. (* $p < 0.05$)


A)
CD4 cell counts x 10^6
25
20
15
10
5
0
VECTOR: CONTROL CONTROL GATA-3
CHALLENGE: PBS OVA OVA
B)
Total Cell counts x 10^6
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
VECTOR: CONTROL CONTROL GATA-3
CHALLENGE: PBS OVA OVA
C)
pg/mls
250
200
150
100
50
0
*
VECTOR: CONTROL CONTROL GATA-3
CHALLENGE: PBS OVA OVA
D)
pg/mls
400
350
300
250
200
150
100
50
0
*
*
VECTOR: CONTROL CONTROL GATA-3
CHALLENGE: PBS OVA OVA

Discussion

Models of adoptive transfer have helped to define the role of T cells and their cytokines in orchestrating allergic asthma. In experimental asthma, adoptively-transferred, antigen-specific $CD4^+$ T cells induce airway inflammation in part by activating other effector cells that participate in airway inflammatory responses (7, 9, 30). We have previously shown that small numbers of T cells, harvested from antigen-sensitized donor rats and retrovirally transduced *in vitro*, drive robust inflammatory responses 48h following adoptive transfer and antigen challenge (8). By adoptively transferring $CD4^+$ T cells expressing EGFP, we have now shown that the majority of $CD4^+$ T cells that infiltrated the lung within days of antigen challenge were not the adoptively transferred T cells themselves. Moreover, these $CD4^+$ T cell-dependent allergen-driven inflammatory responses in the lung and draining lymph nodes were augmented in recipients of $CD4^+$ T cells overexpressing the Th2-specific GATA-3 transcription factor.

By stimulating splenocytes from OVA-sensitized Brown Norway rat donors with antigen *in vitro* we are able to obtain a population of T cells that can be efficiently retrovirally transduced in numbers sufficient for adoptive transfer experiments (8, 25). Following culture with antigen, the T cells that originally encountered antigen *in vivo* during sensitization become reactivated and undergo proliferation, allowing them to be transduced with recombinant retroviruses (25, 26). As we and others have shown previously, a subset of the transduced cells are OVA-specific (8, 25, 26). Consistent with this, upon sequential *in vitro* exposure to OVA and recombinant retroviruses, $CD4^+$ T cells are selectively transduced [Fig 1 and (8)]. For the studies described here we used

retroviruses derived from a control retroviral vector encoding EGFP only as well as retroviruses encoding the rat GATA-3 transcription factor. Cells transduced with retroviruses encoding GATA-3 expressed higher levels of both GATA-3 mRNA and protein as well as the Th2 cytokines, IL-4 and IL-5. We hypothesized that cells transduced under these conditions and subsequently adoptively transferred to naïve recipients would induce airway inflammation and eosinophilia following OVA challenge. Since the effector role of Th2 cells in asthma is dependent upon Th2 cytokine release, we expected that animals receiving T cells overexpressing GATA-3 would have an augmented response to OVA challenge. Following adoptive transfer and OVA challenge, we found that recipients of T cells retrovirally transduced with the control vector exhibited a small but significant increase in airway inflammation particularly eosinophilia. The inflammatory response was OVA dependent, eosinophilic in nature, and was greatly enhanced by T cells overexpressing GATA-3. We found larger numbers of $CD4^+$ T cells in recipients of T cells overexpressing GATA-3. However, the vast majority of these $CD4^+$ T cells did not express EGFP. These data suggest that the GATA-3^+ $EGFP^+$ cells, upon OVA challenge, recruited large numbers of endogenous $CD4^+$ T cells to the lung. This increased recruitment was also reflected in the number of $CD4^+$ T cells recovered in the draining lymph nodes of recipients post OVA challenge.

Our *in vitro* data indicate that IL-4 production was increased in both EGFP positive and negative cells 14 days following transduction of $CD4^+$ T cells with retroviruses encoding GATA-3. The increased IL-4 expression in $EGFP^+$ GATA-3^+ cells is likely due to the direct binding of retrovirally-encoded GATA-3 to cis-acting elements in the IL-4 gene locus. Because GATA-3 autoregulates its own expression, there may

also be a contribution from host cell GATA-3 induced by the virally-encoded protein (18). Subsequently, increased IL-4 production by the transduced cells likely promoted the Th2-biased differentiation and IL-4 production of nontransduced EGFP negative cells in the same culture.

IL-4 production by antigen-specific T cells has also been shown to promote differentiation of naïve T cells to Th2 type effector cells with specificity toward the same or other antigens *in vivo* in adoptive transfer models of experimental asthma (9, 31, 32). The ability of adoptively transferred, antigen-specific T cells to promote differentiation of naïve T cells into antigen specific effector cells is due to the colocalization of both naïve and effector T cells with dendritic cells in the lymph node and the subsequent IL-4-dependent differentiation of the naïve T cells (31, 32). Several other studies have investigated the localization of adoptively transferred T cells *in vivo* (9, 10, 31-34). Data from Bradley and colleagues indicate that memory T cells migrate and localize to the site of antigen challenge (33) consistent with our ability to identify $EGFP^+$ cells in the lung. Data from other groups has also demonstrated preferential trafficking of effector $CD4^+$ T cells expressing low levels of CD62L to the lung (34, 35). These cells are unable to proliferate yet maintain their ability to execute effector functions, such as cytokine production (34). The $CD4^+$ T cells that we adoptively transferred expressed CD25 and OX40 and were $CD62L^{low}$ and thus likely consisted of a mixture of effector and memory-effector T cells. These cells would be predicted to traffic primarily to nonlymphoid tissues, including the lung, following adoptive transfer. Indeed, small numbers of $EGFP^+$ T cells were detected in the lungs, both by flow cytometry and immunofluorescence. These $EGFP^+$ cells were still detectable in the lung after three OVA challenges over a

nine-day time course. Moreover, GATA-3 overexpressing $CD4^+$ T cells retained the ability to enhance recruitment of host $CD4^+$ T cells to the airway. It is likely that the transduced $CD4^+$ T cells we adoptively transferred migrated to the lungs and, following antigen challenge, produced abundant Th2 cytokines, which led to the recruitment of host cells that participated in the inflammatory response. We believe it is unlikely that the host $CD4^+$ T cells recruited to the lung in this model are antigen specific due to the short time frame of our experiments. Animals were sacrificed 3 days following adoptive transfer and challenge and it is unlikely that this would be sufficient time for IL-4 produced by the transduced cells to promote differentiation of naive host cells. However, a small percentage of $CD4^+$ T cells recruited to the lung by retrovirally transduced GATA-3 cells expressed OX-40, a T cell marker of activation, suggesting that a small number of the infiltrating cells could be antigen specific. Alternatively, the infiltrating $CD4^+$ cells could have been NKT cells which also express CD4. To clarify this we used RT-PCR to amplify the NKT cell invariant T cell receptor(38, 39). Though expression of this receptor was detected in the lungs of rats, it did not change following OVA challenge, suggesting that although NKT cells are present, their levels did not increase following OVA challenge in this experimental system.

In an elegant study by Huh et al, primed T cells located in the lung were shown to induce maturation of resident mucosal dendritic cells, which subsequently migrated to the regional lymph nodes, where they would be expected to have potent antigen presentation capacity (40). These data raise the possibility that, in our study, following allergen challenge, the adoptively transferred $CD4^+EGFP^+$ T cells present in the lung induced rapid maturation of mucosal dendritic cells, which subsequently migrated to and induced

robust T cell activation in the draining lymph node. Moreover, these data suggest that T cells overexpressing GATA-3 and Th2 cytokines more efficiently induced maturation of mucosal dendritic cells and thus their ability to activate host T cells that then participated in Th2-biased, allergen-induced inflammatory responses.

Interestingly, when draining lymph node cells, harvested from antigen-challenged T cell recipients, were cultured with OVA, they produced IL-13 and IFN-γ. In the cultures containing draining lymph node cells harvested from recipients of GATA-3 overexpressing $CD4^+$ T cells, IL-13 production was enhanced whereas IFN-γ production was decreased. Since we were unable to detect $EGFP^+$ T cells in the draining lymph nodes, this antigen-dependent increase in IL-13 production was likely mediated by endogenous T cells. The enhanced IL-13 and decreased IFN- production could be explained by the fact that migration of dendritic cells from the lung to the draining lymph nodes is dependent on the inflammatory conditions in the lung (36). Additionally, only mature dendritic cells expressing high levels of MHC II would migrate to the draining lymph nodes (37). The presence of antigen-specific T cells overexpressing GATA-3 may have lead to the rapid maturation of dendritic cells within the lung with consequently higher numbers of dendritic cells migrating to lymph nodes to activate T cells. Therefore, in animals receiving GATA-3-transduced T cells, a greater number of endogenous lymph node T cells may have been activated in response to antigen challenge. Indeed, in our study we recovered greater numbers of cells (both $CD4^+$ and $CD4^-$) from the draining lymph nodes of animals receiving GATA-3 transduced T cells. When restimulated with antigen *in vitro*, these cells readily expressed IL-13 which, as it has been previously

demonstrated (37), enhances the capacity of dendritic cells to suppress T cell secretion of IFN-γ.

There are several implications for the enhanced recruitment of host T cells in the allergic inflammatory response. Firstly, recruitment of antigen non-specific $CD4^+$ T cells to the lung provides a mechanism by which Th2 responses to neo-allergens may occur. It has been proposed that the lymph node is the site of collateral priming and phenotype spread. Nevertheless, because dendritic cell activity localized to the lung is required to maintain allergic airway responses (40), we propose that the early recruitment of antigen non-specific $CD4^+$ T cells to the lung may also contribute to phenotype spread. In addition, antigen non-specific T cells recruited to the lung may also contribute to airway remodeling. We have recently shown that small numbers of adoptively-transferred antigen specific $CD4^+$ T cells drive smooth muscle remodeling. $CD4^+EGFP^+$ cells can also be localized in apparent contact with airway smooth muscle bundles following adoptive transfer and antigen challenge. These data suggest that the increase in smooth muscle mass may depend in part upon direct T cell:smooth muscle cell contact. Our data presented here provide the possibility that antigen non-specific host T cells recruited to the lung may also participate in this process.

In conclusion, we have presented evidence that adoptively transferred $CD4^+EGFP^+$ T cells mobilized to the lung where they induced antigen-dependent eosinophilic inflammation. In recipients of T cells overexpressing GATA-3 influx of both eosinophils and $CD4^+$ T cells was augmented, likely due to increased production of Th2 cytokines resulting in enhanced recruitment of endogenous T cells. This process underscores the importance of the phenotype of T lymphocytes recruited at the initial

stages of allergen challenge and suggests that highly polarized T cells producing abundant Th2 cytokines may accelerate the development and severity of allergy by inducing the recruitment of a larger number of T lymphocytes that may then participate in airway inflammation and asthma pathogenesis.

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Chapter 4:

T cell specific inhibition of STAT-6 attenuates experimental asthma in the Brown Norway rat

Abstract

Cell permeable peptides are chimeric peptides comprised of a protein transduction domain coupled to sequences targeting intracellular proteins. We designed a cell permeable inhibitory peptide targeting the STAT-6 transcription factor, which drives Th2 inflammatory processes associated with asthma. The STAT-6 inhibitory peptide (SIP-1) is comprised of a polyarginine-based protein transduction domain fused to peptide sequences predicted to bind to and inhibit STAT-6. Our data demonstrate that fluorescein-tagged SIP-1 efficiently transduced $CD4^+$ T cells and remained detectable for at least twenty four hours. *In vitro*, antigen-induced IL-4 production was inhibited in SIP-1-treated splenocytes. *In vivo*, pretreatment of $CD4^+$ T cells with the SIP-1 peptide effectively inhibited the ability of these cells to induce eosinophilic airway inflammation and IL-4 production following adoptive transfer and airway antigen challenge. These data indicate that cell permeable peptides targeting STAT-6 have the ability to inhibit antigen-induced cytokine production *in vitro* and $CD4^+$ T cell-dependent inflammatory responses *in vivo*. They also provide evidence that treatment of T cells destined for adoptive transfer with cell permeable inhibitory peptides can be used to identify signaling molecules that regulate the ability of these cells to induce pathogenesis in experimental asthma.

Keywords: STAT-6, inflammation, asthma, peptide, cytokine, $CD4^+$ T cell

Introduction

Protein transduction domains (PTDs) effectively deliver molecular cargo such as proteins, oligonucleotides, and small molecules across cellular membranes (reviewed in (1-3)). PTDs were first identified in HIV TAT and *Drosophila* Antennapedia, two transcription factors with the ability to enter cells in a receptor-independent fashion (4-6). Several peptides, both naturally occurring and synthetic are now known to possess this activity. One of the most effective synthetic PTDs is the nona-arginine peptide, a highly cationic 9-mer of L-arginine (7, 8).

In addition to transducing cells *in vitro*, several studies have documented efficient uptake of cargo by PTDs *in vivo*. In one of the first studies, recombinant, chimeric HIV-TAT PTD-beta-galactosidase was injected intraperitoneally into mice and beta-galactosidase activity was subsequently localized to all tissues, including the brain (9, 10). While distribution throughout the body may be advantageous, in many cases it would be preferable to deliver fusion proteins/peptides to specific target tissues. This has recently been demonstrated through the use of tissue-specific PTDs or by using chimeric cell permeable peptides fused to cell-type specific ligands (11). Cell permeable proteins or peptides have also been delivered directly to the tissue of interest (12-14).

We are interested in exploiting protein transduction technology to better understand mechanisms regulating pathogenesis in experimental asthma and thus are interested in targeting cell permeable proteins/peptides directly to the lung. While PTD-containing proteins delivered intraperitoneally can traffic to the lung

(15), we and others have shown that intranasal application to anaesthetized mice is an efficient delivery route for PTD-containing proteins/peptides to the lung (13-16). Because asthma pathogenesis, which is orchestrated by Th2 cells, can be induced upon adoptive transfer of antigen-specific $CD4^+$ T cells, direct exposure of T cells destined for adoptive transfer with cell permeable peptides/proteins could also be used to better understand mechanisms by which these cells regulate pathogenesis in experimental asthma.

The Brown Norway rat model of adoptively transferred experimental asthma has been widely used to examine the role of the T cell in asthma pathogenesis. In this model, adoptive transfer of small numbers of antigen-primed $CD4^+$ T cells is sufficient to induce allergic airway inflammation in naïve recipients following antigen challenge (17-19). This airway inflammation is similar in magnitude and timing to the inflammation induced by active antigen sensitization protocols making it an elegant tool to investigate the role of the T cell in allergic inflammation and asthma pathogenesis. We hypothesized that treatment of $CD4^+$ T cells with cell permeable inhibitory peptides would allow us to investigate the role of signaling molecules, many of which cannot be targeted with pharmacological inhibitors, in the ability of adoptively transferred $CD4^+$ T cells to induce experimental asthma. The first protein we targeted is the signal transducer and activator of transcription 6 (STAT-6), which regulates Th2 cell differentiation and cytokine production. Significantly, gene-modified mice lacking STAT-6 are protected from allergen-induced inflammation and airway hyperresponsiveness in acute models of experimental asthma. To inhibit STAT-6

activity in T cells destined for adoptive transfer, we designed a chimeric cell permeable peptide comprised of the nona-arginine $(Arg)_9$ PTD in association with a phosphotyrosine (*Y) containing peptide predicted to bind with high affinity to the STAT-6 SH2 domain. The phosphoTyr containing sequence we chose is $*Y_{641}$VST from murine and rat STAT-6, which, when phosphorylated, mediates SH2-domain dependent STAT-6 homodimerization in cells stimulated with IL-4 or IL-13. Thus, the STAT-6 binding peptide [$(Arg)_9$-*YVST], SIP-1, is predicted to enter cells and then bind to and disrupt homodimerization of wild-type STAT-6.

Here we show that the SIP-1 peptide was efficiently internalized into rat splenocyte $CD4^+$ T cells where it inhibited STAT-6-dependent IL-4 production. Following adoptive transfer into naïve recipients, SIP-1-treated $CD4^+$ T cells failed to promote antigen-induced IL-4 production as well as an influx of eosinophils and lymphocytes into the lungs. These data provide evidence that the SIP-1 peptide inhibited STAT-6 function in T cells, thereby inhibiting the ability of adoptively transferred T cells to induce airway inflammatory responses following antigen challenge in the BN rat. They also provide evidence that treatment of T cells with cell permeable inhibitory peptides can be used to identify signaling molecules that regulate the ability of adoptively transferred T cells to induce pathogenesis in experimental asthma.

Methods

Animals. Inbred Brown Norway rats aged 9-12 weeks were obtained from Harlan Laboratories (Harlan-U.K., England). All animal protocols complied with Canadian Council on Animal Care guidelines and were approved by the McGill University Animal Care Committee.

Antibodies and cytokines. Mouse anti-rat myeloid differentiation antigen (CD172a (ED9)), mouse anti-rat CD8, and mouse anti-rat B cell CD45RA/B were purchased from Cedarlane (Canada). Mouse anti-rat CD3, mouse anti-rat CD4, phycoerythrin-(PE-) conjugated mouse anti-rat interleukin (IL)-4 and allophycocyanin-(APC-) conjugated mouse anti-rat CD4 were both purchased from BD Biosciences (Canada). Rabbit anti-phospho-STAT-6 (Tyr641) antibody was obtained from Cell Signaling (Boston, MA) and horseradish peroxidase-conjugated goat anti-rabbit IgG was obtained from Santa Cruz (Santa Cruz, CA). Alexa-555 conjugated goat anti-mouse was obtained from Molecular Probes, Invitrogen (Canada).

Antigen sensitization and splenocyte isolation. Brown Norway rats (9-12 weeks old) were sensitized intraperitoneally (i.p.) with an emulsion containing 200μg of OVA (Sigma-Aldrich, St Louis, MO) and 1 mg of aluminum hydroxide (EM Industries, Gibbstown, NJ) (16). Animals were sacrificed 14 days later and spleens harvested and passed through a cell strainer to obtain single cell suspensions. Cell suspensions were then treated with red blood cell lysing buffer

(Sigma-Aldrich) to remove erythrocytes and either placed in culture with 10% fetal bovine serum or further purified for adoptive transfer as described below.

Peptide synthesis and uptake. Peptides were purchased from Biomer Technology (Hayward, CA). The sequence of the SIP-1 peptide is $(Arg)_9$-*YVST, where *Y represents a phosphotyrosine residue. A similar negative control peptide was also synthesized $(Arg)_9$-FVST (SCP-1). To examine the efficiency of uptake into splenocytes in culture, we used a fluorescein-conjugated derivative of the SIP-1 peptide (FITC-SIP-1).

Splenocytes from BN rats were incubated with 0.1, 1, 10, or 100μM of the FITC-SIP-1 peptide for 4 hours. Cells were washed once with PBS and trypsinized for 5 minutes at 37°C after which cells were washed again and then labeled with a mouse anti-rat CD4 primary antibody and fluorescent cells detected using a FACS Calibur flow cytometer (BD Biosciences) and analyzed using CELLQUEST software (BD Biosciences). Splenocytes were also incubated with 100 μM FITC-SIP-1 for 5mins, 15mins, 30mins, 1h, 4h, or 24h, washed and trypsinized to remove surface associated peptides, and subsequently analyzed by FACS.

For intracellular localization of cell permeable peptides, splenocytes were incubated with the FITC-SIP-1 for 4h after which cells were washed with PBS and trypsinized for 5 minutes at 37°C. Cells were then stained with mouse anti-rat CD4 followed by Alexa 555 conjugated goat anti-mouse antibodies and nuclei counterstained with Hoechst 33342 (Molecular Probes, Invitrogen, Canada). Cells

were then cytospun onto microscope slides and peptide uptake monitored by fluorescence microscopy using an Olympus BX 51 Fluorescence Microscope System [Olympus America, Melville, NY]. Fluorescent images were analyzed using Image-Pro Plus 4.5 software (Media Cybernetics Inc., Silver Springs, MD).

In vitro cytokine expression in antigen stimulated splenocytes. Splenocytes from sensitized BN rat donors were harvested and cultured in DMEM (Invitrogen, Canada) containing 200 μg/ml OVA, 100U/ml IL-2, 100U/ml penicillin, 100ug/ml streptomycin, and 2mM L-glutamine (Invitrogen, Canada) at 37^0C and 5% CO_2. SIP-1 (100uM) or negative control SCP-1 (100uM) were added daily for five days. Cells were then activated by addition of recombinant anti-rat CD3 in the presence of GolgiStop™ (BD Biosciences). After 8h, cells were harvested and stained with PE-conjugated mouse anti-rat IL-4 and APC-conjugated mouse anti-rat CD4 as per manufacturer's instructions. Expression of IL-4 and CD4 was assessed by FACS analysis and quantified using CELLQUEST software.

$CD4^+$ T lymphocyte adoptive transfer and challenge. $CD4^+$ T cells from OVA-sensitized BN rat donors were isolated by magnetic cell sorting (MACS) (20). Briefly, cells were incubated with an antibody cocktail consisting of mouse anti-rat myeloid differentiation antigen (CD172a (ED9)), mouse anti-rat CD8, and mouse anti-rat B cell CD45RA/B. Cells were then labeled with goat anti-mouse magnetic microbeads and applied to a magnetic selection column (Miltenyi Biotec, Auburn, CA). Following negative selection, greater than 95% $CD4^+$ T cell

enrichment was obtained. $CD4^+$ T cells were then incubated with 100μM SIP-1 or the negative control SCP-1 for 4 hours. Afterward, $5x10^6$ $CD4^+$ cells were injected i.p. into naive recipient rats, which were immediately challenged with OVA or PBS. Animals were sacrificed forty eight hours following antigen challenge at which time bronchoalveolar lavage (BAL) fluid was collected. For analysis of inflammatory infiltrates, cells obtained from BAL fluid were cytospun onto microscope slides and differential cell counts performed using Diff Quick (Dade Behring Inc, Newark, DE).

Histological analysis of lung tissue following antigen challenge. Lung tissues were inflated, fixed in 10% buffered formalin and embedded into paraffin blocks from which five micron thick sections were made. To assess airway inflammation, lung sections were stained with hematoxylin and eosin (H&E) and examined using light microscopy. Images were analyzed using Image-Pro Plus 4.5 software (Media Cybernetics Inc., Silver Spring, MD).

Measurement of cytokines in BAL fluid. Rat IL-4 and IFN-γ were quantified using ELISA kits from BD Biosciences and Biosource (Medicorp, Canada), respectively, and performed as per manufacturer's instructions.

Analysis of STAT-6 dimerization by western blot analysis. Splenocytes from non-OVA-sensitized donors were harvested and cultured in complete DMEM with 100μM SIP-1 or SCP-1 for 4 hours. Cultured splenocytes were then

stimulated with IL-4 (1ng/ml) for 30mins or 1hr. Cells were then lysed in lysis buffer (200 μM sodium vanadate, 5 μg/ml leupeptin, 10 μg/ml pepstatin A, 1mM PMSF, 20 μg/ml aprotinin, 0.5% NP-40, 40mM HEPES, 120mM NaCl, 1mM EDTA, 20mM sodium fluoride, 2mM sodium pyrophosphate, 2.5mM sodium metabisulfite, 5mM benzimidine, 50mM β-glycerol phosphate) containing 2.5mM of the crosslinker 3,3′-dithiobis(succinimidylpropionate) (DSP) (Pierce Biotechnologies, Rockford, IL) (21). Lysates were incubated for 30m at room temperature during the crosslinking reaction, which was then stopped by addition of 100mM TrisCl. Afterward, lysates were clarified by centrifugation and supernatants collected for western immunoblotting. Briefly, equal quantities of protein (40μg) were boiled for 5 mins in SDS sample buffer in the absence of reducing agent to maintain cross-linked proteins and subsequently resolved by 8% SDS polyacrylamide gel electrophoresis (PAGE). Alternatively, DSP-dependent cross-links were reduced by boiling samples in the presence of β-mercaptoethanol. Following electrophoresis, proteins were transferred overnight at 40V onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). For immunoblotting, membranes were blocked in Tris buffered saline (10 mM Tris-Cl pH 7.4, 2.5 mM EDTA, 150 mM NaCl) containing 0.1% Tween-20 (TBST) and 5% (wt/vol) dry milk for 1 h. Membranes were then probed with anti-phospho-STAT-6 antibody (1:1000) in TBST for 1 h. Following extensive washing, blots were incubated in TBST containing bovine anti-rabbit IgG-HRP (1:5000) for 1 h. Proteins were then detected using the ECL Plus chemiluminescence detection system (GE Healthcare, Canada) and visualized on

a FluorChem8000 Imaging System using AlphaEase software (Alpha Innotech, San Leandro, CA).

Statistical analysis. Data are presented as the mean ± S.E.M. Data were analyzed using Student's T test (two sample assuming unequal or equal variances). $p < 0.05$ was considered significant. *In vivo* data is from a minimum of three experiments using 1-2 animals per group.

Results

In vitro uptake and inhibitory activity of the SIP-1 peptide

The sequences of the peptides used are shown in **Figure 4.1A**. To examine the efficiency of uptake we used the fluorescein-conjugated SIP-1 peptide (FITC-SIP-1) and examined uptake into rat splenocytes by flow cytometry. Cells exhibited a concentration-dependent increase in mean fluorescence intensity (MFI) (**Figure 4.1B**), with the largest increases in MFI at 10μM or 100μM. The FITC-SIP-1 peptide transduced nearly all cells after a 30-minute incubation and remained detectable for at least 24h (**Figure 4.1C**). In addition, both CD4 positive and CD4 negative cells were transduced (**Figure 4.1D**). Intracellular uptake of the FITC-SIP-1 was confirmed by fluorescence microscopy. As shown in **Figure 4.2** the FITC-SIP-1 peptide was localized to the cytoplasm and/or the nucleus of transduced splenocytes. Together, these data indicate that the $(Arg)_9$ PTD entered cells and facilitated uptake of the SIP-1 peptide.

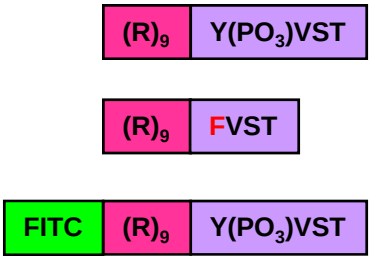
We next assessed the ability of the SIP-1 peptide to inhibit IL-4 production from splenocytes cultured with OVA. As expected, the number of IL-4 producing cells was increased in OVA-stimulated splenocytes (**Figure 4.3A**). While addition of the negative control SCP-1 peptide had no significant effect on the number of IL-4 expressing cells, culture of splenocytes in the presence of the SIP-1 peptide inhibited OVA-induced IL-4 production (**Figure 4.3B**).

Figure 4.1

Efficient transduction of $CD4^+$ T cells using a nona-arginine conjugated chimeric STAT-6 peptide (FITC-SIP-1).

(A) Sequence of the cell permeable peptides used in this study. (B-D) To examine the efficiency of $(Arg)_9$ as a protein transduction domain, we assessed uptake of the FITC-SIP-1 peptide into BN rat splenocyte $CD4^+$ cells. *(A)* Cells were incubated for 4h with increasing doses of the FITC-SIP-1 peptide (0.1-100μM). Uptake into $CD4^+$ T cells was then assessed by FACS analysis. *(B)* Cells were incubated with 100μM FITC-SIP-1 for 0.5-24h. Uptake into $CD4^+$ T cells was then assessed by FACS analysis. (D) One representative experiment of three is shown.

A)

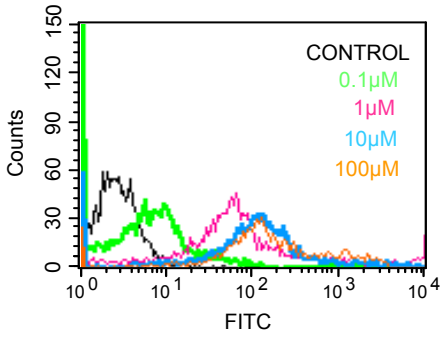


SIP-1 (STAT-6 INHIBITORY PEPTIDE)

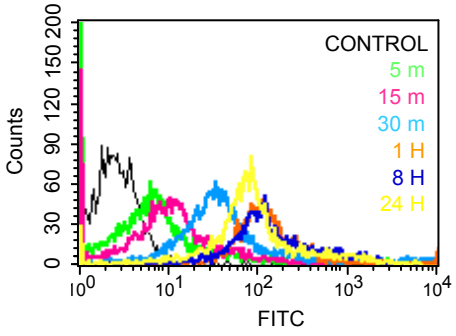
SCP-1 (STAT-6 CONTROL PEPTIDE)

FITC-SIP-1

B)



C)



D)

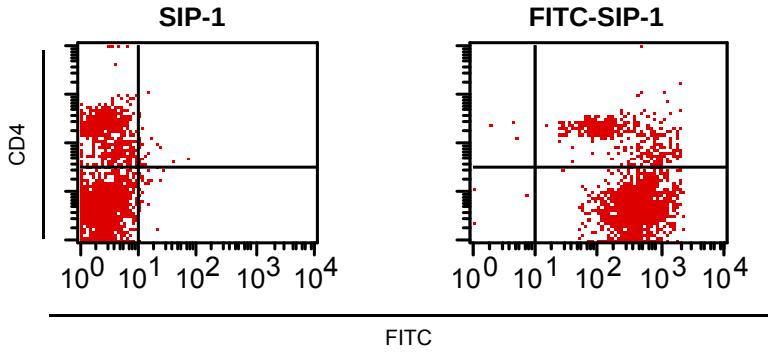
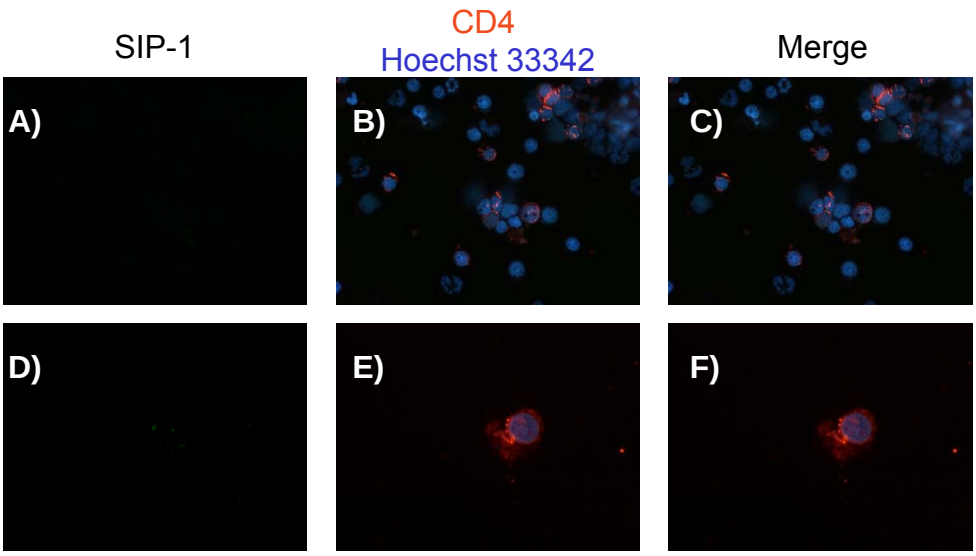
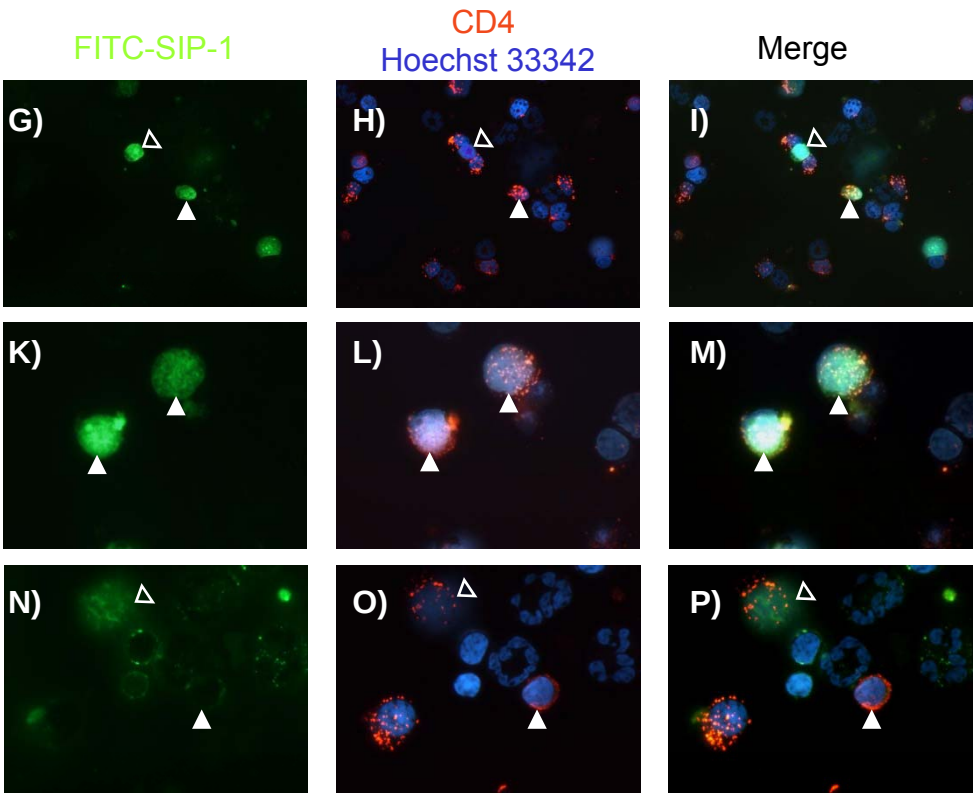


Figure 4.2

The $(Arg)_9$ chimeric peptide effectively delivers molecular cargo across cell membranes.

To examine the uptake of the FITC-SIP-1, rat splenocytes were treated with 100μM of FITC-SIP-1 or unlabelled SIP-1 for 4h. Transduced cells were washed and trypsinized to remove extracellular peptide. Splenocytes were then stained with anti-CD4 antibodies and counterstained with Hoechst 33342 before being cytospun onto microscope slides. Peptide uptake into cells was then examined using fluorescence microscopy for FITC (left panel), Hoechst 33342 (blue) and CD4 (red) (middle panel), and overlay (right panel). Representative slides from SIP-1 (*A-F*) and FITC-SIP-1 (*G-P*) are shown. Images were taken at 40x magnification (*A-C, G-I*) and 100x magnification (*D-F, K-P*).


SIP-1
CD4
Hoechst 33342
Merge
A)
B)
C)
D)
E)
F)


FITC-SIP-1
CD4
Hoechst 33342
Merge
G)
H)
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K)
L)
M)
N)
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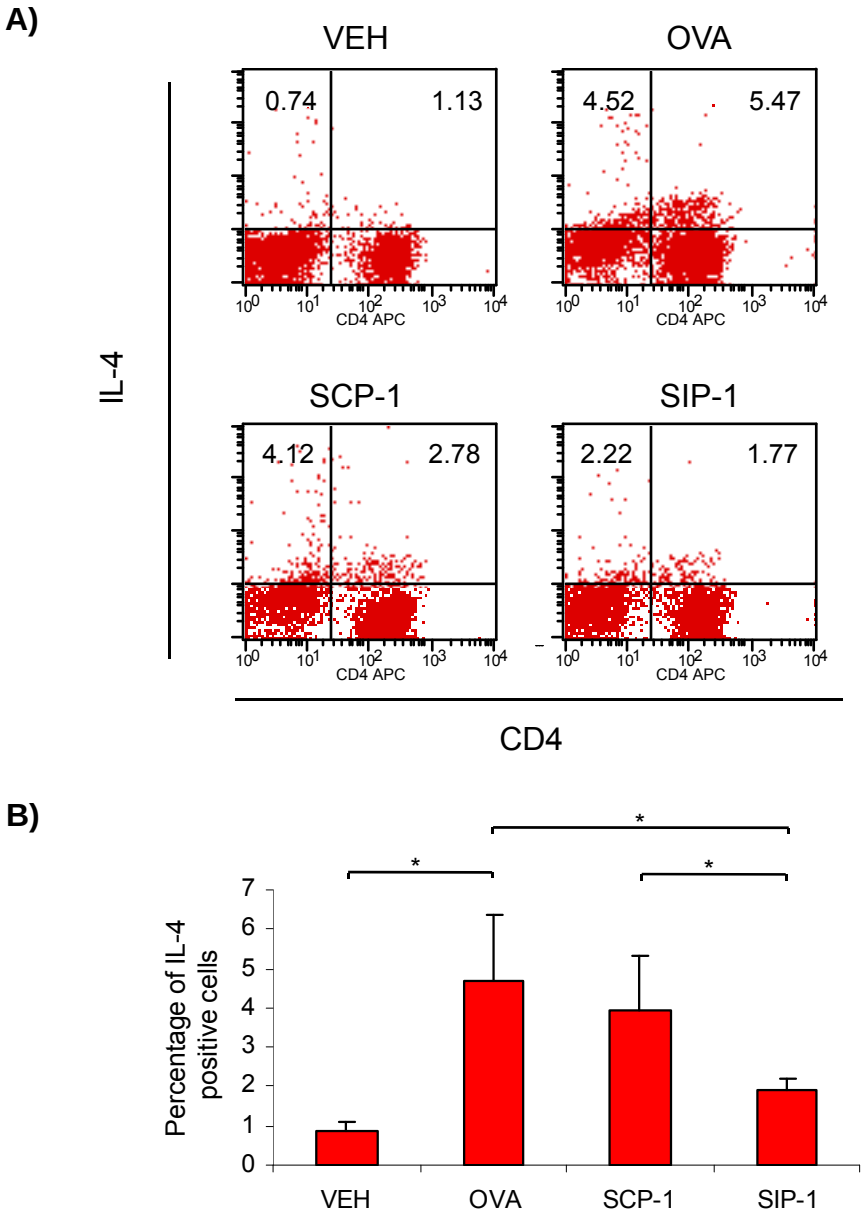
Figure 4.3

SIP-1 inhibits OVA-induced IL-4 production in cultured BN rat splenocytes. *(A)* Splenocytes from OVA-sensitized BN rat donors were stimulated with OVA and cultured for five days. During this period, the SIP-1 (100μM) or SCP-1 (100μM) was added daily. Cytokine production was assessed using FACS analysis following anti-CD3 stimulation. One experiment, representative of four independent experiments, is shown. *(B)* Mean percentage of IL-$4^{+}CD4^{+}$ cells (± SEM) from four independent experiments is shown. *$p < 0.05$


A)
VEH
OVA
SCP-1
SIP-1
0.74
1.13
4.52
5.47
4.12
2.78
2.22
1.77
CD4 APC
IL-4
CD4
B)
Percentage of IL-4 positive cells
0
1
2
3
4
5
6
7
VEH
OVA
SCP-1
SIP-1
*
*
*

SIP-1 treatment reduces the ability of adoptively transferred $CD4^+$ T cells to induce inflammation in experimental allergic asthma.

To determine if the SIP-1 peptide regulated the ability of $CD4^+$ T cells to induce airway inflammation, we pretreated purified cells with SIP-1 prior to adoptive transfer into naïve recipients (**Figure 4.4**). As shown previously, following OVA challenge, the total number of inflammatory cells recovered in the BAL fluid was increased in animals that received $CD4^+$ T cells (**Figure 4.5A**). This inflammation was eosinophilic in nature (**Figure 4.5A**) and was accompanied by an increase in the number of lymphocytes (**Figure 4.5C**) as well as an increase in IL-4 recovered in the BAL fluid (**Figure 4.6A**) (17, 20). Pretreatment of $CD4^+$ T cells with the negative control SCP-1 peptide did not inhibit their ability to induce OVA-specific airway inflammatory responses (Figures 4.5 and 4.6). In contrast, treatment of cells with the SIP-1 peptide attenuated airway inflammation, with decreases in IL-4 levels as well as decreases in total cells, eosinophils and lymphocytes recovered in the BAL infiltrates (Figures 4.5, 4.6, and 4.7). Surprisingly pre-treatment with either SIP-1 and SCP-1 lead to a decrease in IFNγ levels recovered in the BAL fluid (**Figure 4.6B**). Consistent with the changes in the BAL fluid, histology of lungs revealed that OVA-induced inflammation remained abundant in recipients of control, SCP-1 treated $CD4^+$ T cells, and was reduced in recipients of SIP-1-treated $CD4^+$ cells (**Figure 4.7**).

Figure 4.4

Brown Norway rat model of adoptively transferred experimental asthma.

$CD4^+$ splenocyte T cells from sensitized BN rats were incubated with 100μM SIP-1 or the negative control SCP-1 for 4 hours. Afterward, $5x10^6$ $CD4^+$ cells were injected i.p. into naive recipient rats, which were immediately challenged with OVA or PBS (VEH). Animals were sacrificed forty eight hours following antigen challenge at which time bronchoalveolar lavage (BAL) fluid was collected.

Brown Norway rat model of adoptively transferred experimental asthma

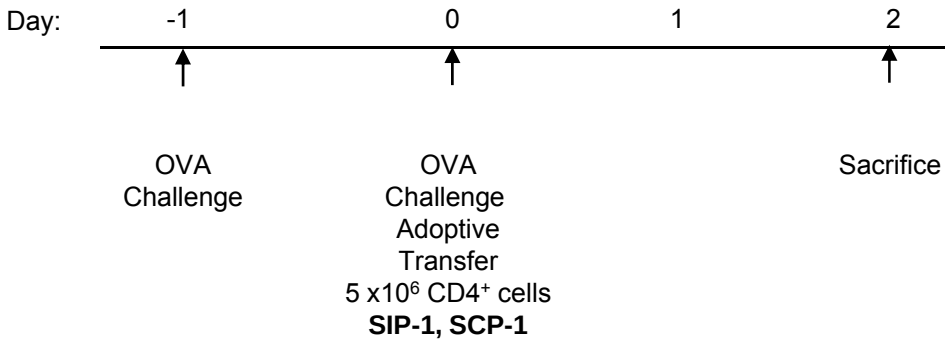
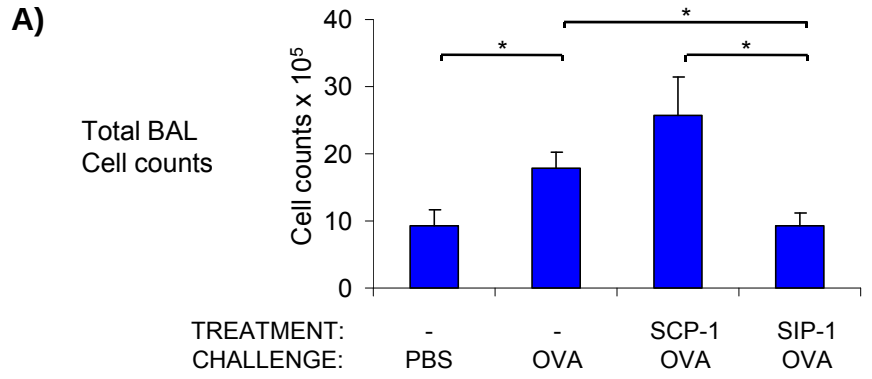


Figure 4.5

SIP-1 attenuates OVA-induced airway inflammation.

(A) Total BAL fluid cells recovered from recipients of adoptively-transferred $CD4^+$ T cells following OVA challenge. *(B)* BAL fluid eosinophils *(C)* BAL fluid lymphocytes. Mean cell counts ± SEM from three independent experiments are shown. *$p < 0.05$


A)
Total BAL
Cell counts
Cell counts x 10^5
40
30
20
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TREATMENT:
CHALLENGE:
-
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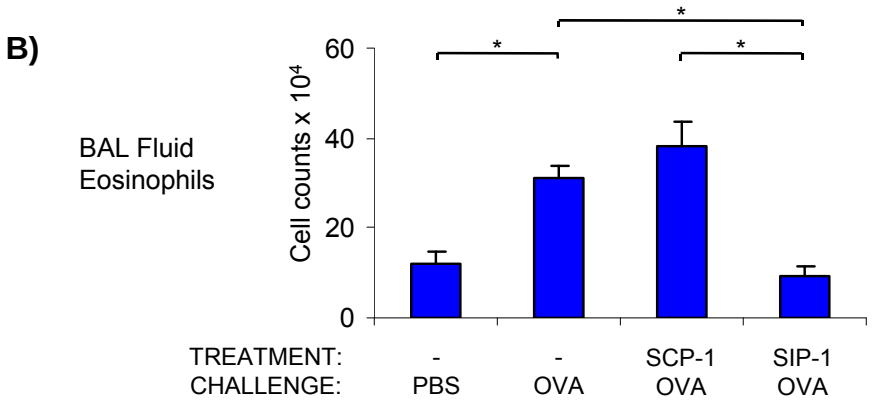
B)
BAL Fluid
Eosinophils
Cell counts x 10^4
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TREATMENT:
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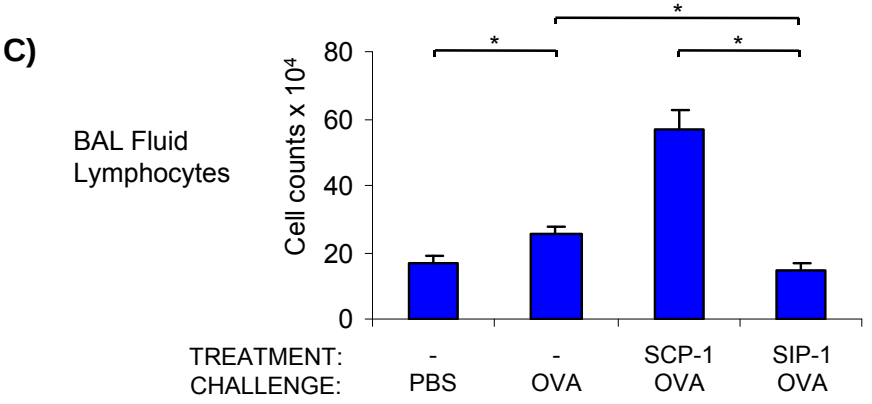
C)
BAL Fluid
Lymphocytes
Cell counts x 10^4
80
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TREATMENT:
CHALLENGE:
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PBS
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SCP-1
OVA
SIP-1
OVA

Figure 4.6

SIP-1 inhibits airway inflammation induced by adoptively transferred T cells.

Following OVA challenge, lungs were harvested and inflammation assessed in tissue sections stained with H&E. In *A)* and *B)* animals received control, untreated T cells and were challenged with PBS or OVA, respectively. In *C)* and *D)* prior to adoptive transfer $CD4^+$ T cells were treated with SCP-1 *(C)* or SIP-1 *(D)*. Data are representative of three independent experiments.

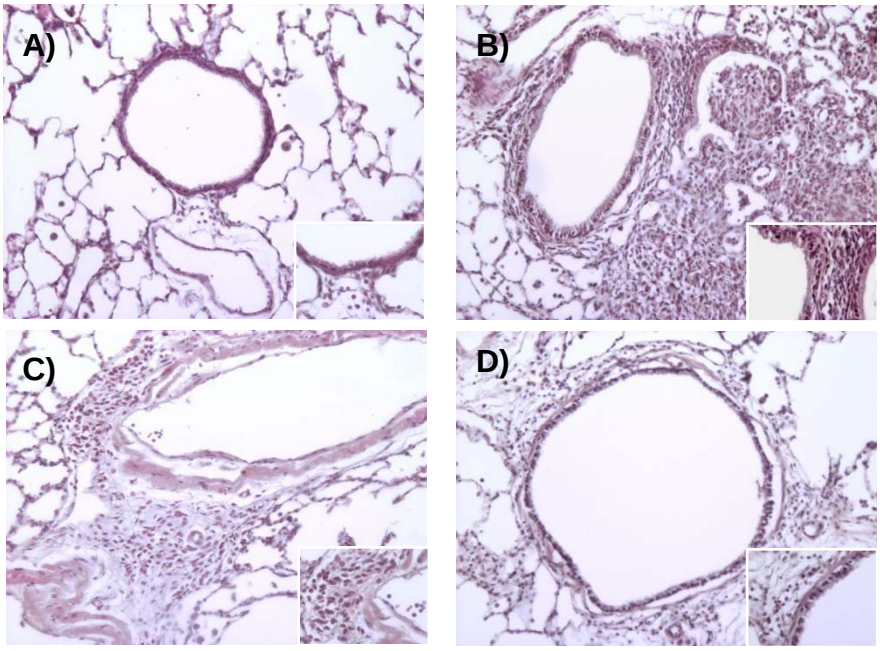
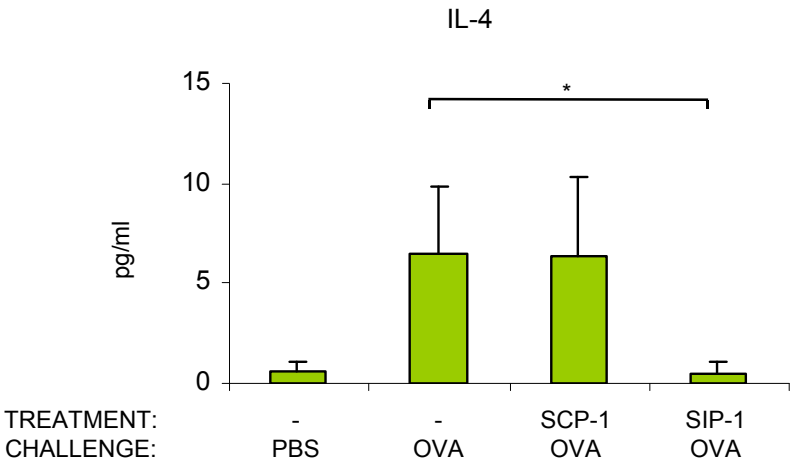

A)
B)
C)
D)

Figure 4.7

OVA-induced cytokine levels in BAL fluid are decreased in animals receiving SIP-1-treated $CD4^+$ T cells.

BAL fluid levels of (A) IL-4, and *(B)* IFNγ were quantified. Mean values from three independent experiments are shown (± SEM). * $p < 0.001$.

A)


IL-4
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10
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pg/ml
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TREATMENT:
CHALLENGE:
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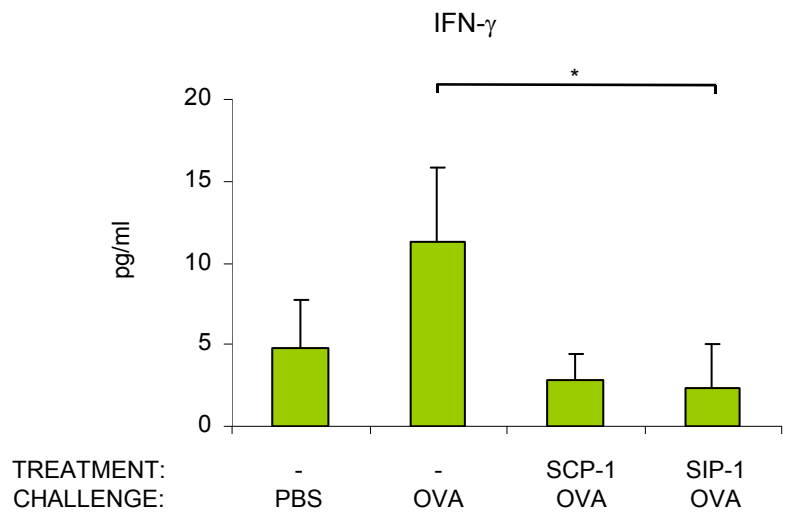
OVA

B)


IFN-γ
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SIP-1
OVA

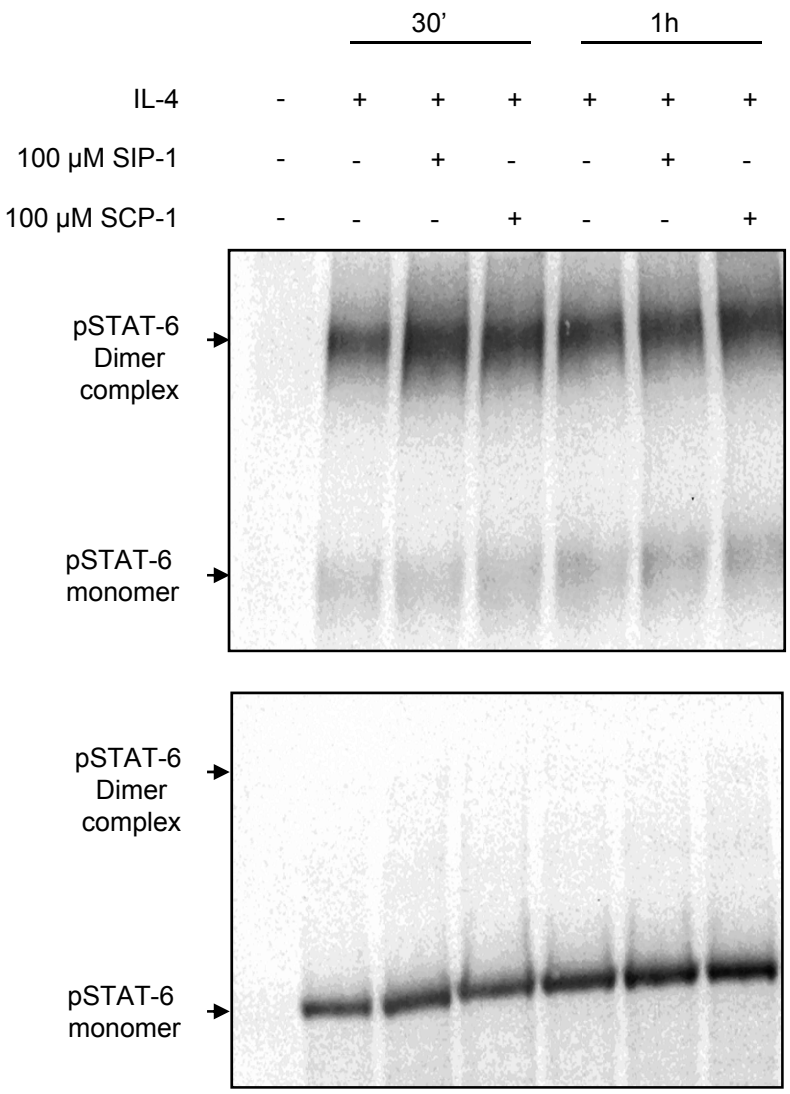
SIP-1 fails to inhibit the formation of a STAT-6 dimer complex in IL-4 stimulated splenocytes

We next sought to determine the mechanism by which SIP-1 inhibits STAT-6 activity and STAT-6-dependent IL-4 production. We harvested splenocytes from BN rats and pretreated them with SIP-1 or SCP-1 (100μM) for 4 hours. Pretreated cells were then stimulated with IL-4, lysed in a buffer containing the crosslinker DSP, and tyrosine phosphorylated STAT-6 detected by western immunoblotting. Stimulation with IL-4 lead to an increase in phospho-STAT-6 dimers as shown by the presence of a protein complex at approximately 200kDa (**Figure 4.8**, upper panel) with few phosphoSTAT-6 monomers remaining. Formation of this phosphoSTAT-6 complex was not inhibited by either the SIP-1 or SCP-1. When DSP was reduced by incubating lysates under reducing conditions, the phosphoSTAT-6 dimers were disrupted leaving monomers that migrated at approximately 100kDa (**Figure 4.8**, lower panel). These data also demonstrate that the SIP-1 did not inhibit IL-4-induced STAT-6 tyrosine phosphorylation in these cells.

Figure 4.8

SIP-1 does not inhibit formation of a STAT-6 dimer complex in IL-4 stimulated splenocytes.

Splenocytes were treated with 100μM of SIP-1 and SCP-1 for 4 hours. Cells were then stimulated with IL-4 for 30mins or 1 hour after which cells were lysed in buffer containing 2.5mM of the crosslinker DSP. Equal quantities of protein were resolved by SDS-PAGE and then transferred onto PVDF membranes in order to assess the position of tyrosine phosphorylated STAT-6 by immunoblot analysis. In the upper panel, lysates were resolved under nonreducing conditions in order to visualize cross-linked STAT-6 complexes. In the lower panel, cell lysates were resolved under reducing conditions, in the presence of β-mercaptoethanol, to reduce DSP in order to assess levels of phospho-STAT-6 monomers.


30'
1h
IL-4
- + + + + + +
100 μM SIP-1
- - + - - + -
100 μM SCP-1
- - - + - - +
pSTAT-6
Dimer
complex
pSTAT-6
monomer
pSTAT-6
Dimer
complex
pSTAT-6
monomer

Discussion

Cell permeable peptides, with the ability to disrupt specific protein:protein interactions, have been used to characterize the role of several proteins in different *in vitro* responses. These peptides have also proven useful in defining molecular mechanisms that contribute to disease pathogenesis *in vivo* in animal models, thus providing evidence that CPPs may also have therapeutic potential in humans. In this study, we designed an inhibitory chimeric cell permeable peptide consisting of the nona-arginine PTD conjugated to a peptide sequence predicted to bind to and inhibit the STAT-6 transcription factor. This STAT-6 inhibitory peptide, SIP-1, efficiently transduced primary $CD4^+$ T cells *in vitro* and inhibited STAT-6-dependent IL-4 production both *in vitro* and *in vivo.* In addition, the SIP-1 peptide inhibited the ability of adoptively transferred T cells to induce airway inflammation *in vivo* demonstrating it's efficacy as an inhibitor of T cell specific STAT-6 dependent airway inflammatory responses. These studies further validate the use of cell permeable peptides for investigating the role of signaling molecules *in vivo*.

Many methods currently used to manipulate protein expression or function rely on the introduction of nucleic acids into target cells. However, some cells, such as primary lymphocytes, are difficult to transfect and thus the modulation of protein expression or function in these cells is more challenging. Protein transduction technology has provided an alternative means to introduce into these and other difficult-to-transfect cells, molecular cargo such as proteins or peptides with the ability to modify the function of intracellular target proteins. Although

PTDs may use several mechanisms to gain entry into cells, endocytotic processes seem to be involved in the uptake of most PTDs (1, 8, 22). Arginine-based peptides, such as that used in this study, have been shown to bind to heparan sulfate on the cell surface after which they are internalized by endocytosis. Within the endocytic vesicle, heparan sulfate is degraded by heparanases allowing the free nona-arginine peptides to move from the vesicles into the cytoplasm (8). Under some conditions they may also enter cells via an endocytosis-independent pathway(s), possibly translocating directly through the plasma membrane. Our uptake studies using FITC-conjugated SIP-1 showed rapid and efficient intracellular transport into primary $CD4^+$ T cells. Peptide uptake was both time- and concentration-dependent with maximum transduction occurring using 10-100μM peptide and within one hour. Interestingly, although there was successful cell transduction of $CD4^+$ cells, the CD4 negative cell population, containing antigen presenting cells, had consistently higher mean fluorescence intensity, most likely due to the increased endocytic activity in these cells.

STAT-6 regulates expression of Th2 cytokines and chemokines that promote allergic airway inflammation and is thus a promising target for allergy treatment (23, 24). STAT-6 is expressed by $CD4^+$ T cells that orchestrate allergic airway inflammation as well as by structural cells in the lung, including airway epithelial cells, fibroblasts and smooth muscle cells (15, 25). In experimental asthma, recruitment of inflammatory cells to the airways is mediated in part by STAT-6-dependent production of chemokines from airway structural cells (26). Moreover, IL-13-induced activation of STAT-6 in airway epithelial cells alone is

sufficient to induce airway hyperresponsiveness and mucus production (27). In this study we targeted STAT-6 activity specifically in the $CD4^+$ T cells that induce and maintain allergy (28, 29). Our data demonstrate that OVA-induced IL-4 production from cultured $CD4^+$ splenocyte T cells was reduced by the SIP-1 peptide. These data are consistent with that showing that STAT-6 deficient T cells are unable to produce IL-4 in response to antigen stimulation (30) and also cannot differentiate into Th2 cells secreting IL-4 and IL-5 (31). While STAT-6 activity in structural cells of the airways promotes airway inflammation (27), our data demonstrate that T cell specific STAT-6 activity is also required for the recruitment of both lymphocytes and eosinophils to the airways following antigen challenge. Our data also suggest that blocking T cell specific inflammatory mediators in the lung could have substantial therapeutic effect on the inflammatory responses during asthma (32, 33).

We are interested in the mechanism by which the SIP-1 peptide inhibits STAT-6 activity and attenuates IL-4 production in antigen-stimulated splenocytes. Current understanding of the life cycle of STAT-6 predicts that IL-4 stimulation via the IL-4 receptor leads to phosphorylation of STAT-6 with subsequent dimerization and nuclear localization prior to DNA binding (34). Therefore, SIP-1 could inhibit antigen-induced STAT-6 activity at several different stages including phosphorylation, dimerization, nuclear localization, DNA binding or protein degradation. In our studies examining STAT-6 dimerization we did not detect decreased levels of the phospho-STAT-6 dimer complex following IL-4 stimulation of SIP-1 treated cells. Similarly, total levels

of phospho-STAT-6 were unaffected by the presence of SIP-1. The ability of SIP-1 to inhibit phospho-STAT-6 nuclear localization, DNA binding, and protein stability is currently under investigation.

Our data using adoptively transferred T cells pre-treated with SCP-1 suggest that SCP-1 may have an effect on the ability of these cells to induce airway inflammation (Figure 4.5C). Our data from the bronchoalveolar lavage fluid from OVA challenged recipients also showed that SCP-1 could inhibit OVA-induced interferon gamma production. We do not know the mechanism by which SCP-1 is able to do this and, although we hypothesize that SCP-1 is unable to bind to phosphorylated STAT-6, we can not discount the possibility that its ability to modify airway inflammatory responses may be due to SCP-1 binding to, and possibly inhibiting, other as-yet-unidentified proteins

In addition to increased numbers of $CD4^+$ T cells, T cell-derived Th2 cytokine levels are also increased in the airways of asthmatics (18, 35, 36). Abundant evidence from animal models has established the importance of Th2 cytokines in asthma pathogenesis. In adoptive transfer models, the ability of antigen-specific T cells to induce allergic asthma is dependent upon the cytokines they produce (37). For example, adoptive transfer of T cells that express Th1 cytokines fails to induce experimental asthma and may actually counteract the effects of Th2 dependent inflammation, whereas T cells expressing Th2 cytokines induce allergic inflammation following antigen challenge (18). In the BN rat, data from Molet et al demonstrate that antisense inhibition of IL-4 expression in $CD4^+$ T cells prior to adoptive transfer impairs the ability of these cells to induce

airway inflammation and the late response to antigen challenge (38). Our data now show that targeting the STAT-6 transcription factor, which regulates Th2 cytokine production, has a similar effect and leads to reduced levels of Th2 cytokines in the airway and decreased inflammatory responses to antigen.

In conclusion, we have developed a cell permeable peptide, SIP-1, targeting the STAT-6 transcription factor. Our data demonstrate that the SIP-1 peptide inhibited STAT-6-dependent cytokine production *in vitro* as well as $CD4^+$ T cell-dependent inflammatory responses *in vivo*. These data validate the use of cell permeable peptides as a tool to investigate the role of signaling molecules T cell-dependent airway inflammatory responses in experimental asthma.

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Chapter 5:
Conclusion

The objective of this thesis was to expand upon current knowledge regarding the mechanism by which adoptively transferred T cells induce allergic airway responses. To do this, we tested the hypothesis that modulating the transcription factors that regulate Th2 cytokine production would alter the ability of antigen-specific T cells to induce airway inflammation *in vivo*. We utilized different methodologies to modulate the expression levels or activity of two transcription factors, GATA-3 and STAT-6, respectively. To modulate expression of GATA-3 in antigen-specific T cells we developed methodology using centrifugal concentration to generate high titer retroviral preparations for the efficient transduction of primary $CD4^+$ T cells (Chapter 2). We utilized retrovirally-transduced antigen-specific T cells in the Brown Norway rat model of experimental asthma and showed that antigen-specific T cells overexpressing GATA-3 successfully trafficked to the lung where they induced airway eosinophilic inflammation (Chapter 3). The induction of this inflammatory response was dependent upon antigen challenge and was enhanced in animals receiving T cells overexpressing GATA-3. Finally, we utilized a cell penetrating peptide to modulate the activity of STAT-6 in primary rat T cells destined for adoptive transfer (Chapter 4). The chimeric cell penetrating peptide (SIP-1) consists of a poly-arginine protein transduction domain (PTD) coupled to a peptide sequence predicted to bind to phosphorylated STAT-6. Our *in vitro* studies showed that the SIP-1 peptide inhibited antigen-induced IL-4 production from primary rat splenocytes. Furthermore, *ex vivo* treatment with the SIP-1 peptide inhibited the ability of adoptively-transferred $CD4^+$ T cells to induce

inflammatory responses following antigen challenge. Taken together, these data demonstrate that we have successfully modified T cell specific cytokine output by modulating expression or activity of the Th2 cell specific transcription factors, GATA-3 and STAT-6. In turn, this has modified the ability of these cells to induce airway allergic inflammatory response upon adoptive transfer and antigen challenge.

Enhanced transduction of T lymphocytes with recombinant retroviruses concentrated by centrifugal filtration

Retroviruses are one of the most widely used vehicles for gene transfer and their proven success in gene therapy clinical trials suggests that retroviral vectors will continue to be utilized for gene delivery (1). One of the factors that limit their use, however, is the challenge that arises from the low titer retroviruses that are obtained from current retroviral packaging systems. In particular, ecotropic and amphotropic retroviruses are limited in their ability to transduce primary T cells. In our studies, retroviral titers from supernatants harvested directly from Phoenix ecotropic packaging cell lines ranged between 1.1-1.5 x 10^6 CFU/ml. Retroviral transduction with these preparations led to transduction of a relatively low percentage of antigen-stimulated splenocytes (4.8-8.8%) (Chapter 1). To obtain enough cells for *in vivo* experiments we increased transduction efficiencies by using centrifugal filtration to concentrate low titer retroviral preparations. Using this process, we were consistently able to increase retroviral

titers ten fold (8.0-11.5 x 10^6 CFU/ml). Importantly, this increase in viral titer led to a concomitant increase in splenocyte transduction efficiency (25.7-34.7%).

In addition to increasing the concentration of retroviral particle preparations, centrifugal filtration also provides an additional advantage since it removes a number of low molecular weight and potentially toxic impurities such as the protein contaminants from cell culture media including serum (2), as well as DNA contaminants from the packaging cell line and plasmid retrovectors. Thus, centrifugal filtration provides added quality control for the production of retroviral preparations since it produces consistently high concentrations of retroviral particle preparations with fewer impurities.

We found that retroviral particle damage following the concentration process was relatively low and, compared to their nonconcentrated counterparts, the concentrated retroviral preparations: (i) retained the ability to transduce cells; (ii) transduced splenocyte cell populations with greater efficiency; iii) successfully modified expression of the proviral genes they encoded (EGFP and GATA-3); and (iv) induced higher levels of transgene expression in target cells. Thus, concentrating retroviral preparations in this way increased the efficiency with which retroviral transduction coupled with T cell stimulation generated gene-modified antigen-specific T cells.

The role of GATA-3 in the ability of antigen specific T cells to induce airway inflammation

When we utilized gene-modified antigen-specific T cells in adoptive transfer experiments, we found that following intraperitoneal (i.p.) injection, the *in vitro* stimulated retrovirally-transduced T cells trafficked to the lung where they induced allergic airway inflammation in OVA challenged naïve recipients. It is important to note that although the retrovirally transduced T cells induced allergic inflammation, it was significantly lower than the airway inflammation induced by T cells harvested from non-transduced sensitized donors (See Chapter 4). It is probable that retroviral transduction of splenocyte cultures induced production of cytokines such as IL-12 and interferon- α (3), which subsequently had a negative effect on the Th2 inflammatory response induced by the antigen specific T cells following adoptive transfer and challenge. Regardless of this, it is clear from our data that the retrovirally-transduced T cells induced an increased inflammatory response that was OVA dependent, eosinophilic in nature, and greatly enhanced by T cells overexpressing GATA-3.

The ability of these antigen-specific T cells to home to the lung is consist with results from Wise and colleagues (4, 5) who also generated similar antigen-specific populations that consisted of both central memory and effector $CD4^+$ T cells. Nevertheless, we cannot discount that, rather than trafficking to the lung, these adoptively transferred T cells entered the lung due to passive accumulation as a result of the increased vasodilation that follows antigen challenge. Since we detected larger numbers of the transduced cells in the antigen challenged animals

however, we believe that retention of the transferred T cells in the lung is dependent upon antigen challenge, consistent with data suggesting that survival of these cells *in vivo* is contingent upon antigen reactivation by resident lung DCs (6, 7). Although we also have no direct evidence for this reactivation, we believe that following re-encounter with antigen in the lung, the adoptively transferred T cells become re-activated and produce T cell cytokines. Furthermore, we believe that the lung is the primary site of antigen re-encounter since we did not detect $EGFP^+$ cells in the draining lymph nodes or spleens of antigen challenged animals. Data from others also suggest that adoptively-transferred antigen-specific T cells remain in the lung and produce cytokines and yet are unable to return to the draining lymph nodes or the circulating pool of lymphocytes (4). Conclusive evidence that the adoptively transferred cells in our system remained in the lung and did not travel to other lymphoid organs will require time-course experiments following the migratory pathway of these cells following transfer.

The exact mechanism by which adoptively-transferred antigen-specific T cells induce inflammation is still not fully understood and several factors, including the expression of adhesion molecules and chemokine receptors, may influence the ability of these T cells to induce allergic inflammation. The ability of T cells overexpressing GATA-3 to enhance eosinophilia is likely dependent upon all three of the Th2 cytokines whose expression is induced by GATA-3, IL-4, IL-5 and IL-13. Within the lung, re-activated antigen-specific T cells are predicted to produce IL-4 and IL-13, which would then induce expression of eosinophil chemoattractants such as eotaxin-1, eotaxin-2, and eotaxin-3 on lung

epithelial, endothelial, and other structural cells (3, 8, 9). In addition, these T cells are predicted to produce abundant IL-5, which would promote eosinophil recruitment, activation and survival. Thus, T cells overexpressing GATA-3 are predicted to influence the lung microenvironment by producing greater levels of Th2 cytokines and thereby augmenting the recruitment of eosinophils and other inflammatory cells.

In addition to increasing eosinophil recruitment to the lung, our data in Chapter 3 demonstrate that T cells overexpressing GATA-3 increased the influx of $CD4^+$ T cells to the lung. This enhanced influx of $CD4^+$ T cells could again be due to the cytokine dependent expression of T cell chemoattractants by epithelial cells or endothelial cells. T cells express several chemokine receptors including CCR3, CCR4, and CCR8 and migrate to their ligands: eotaxin, monocyte-derived chemokine (MDC) and thymus- and activation-regulated chemokine (TARC), and I-309 (10). Ligands for CCR3, CCR4, and CCR8 have been implicated in allergic inflammation where they work in concert to increase airway inflammation (10-13). Deletion of single chemokine receptors does not completely abolish the recruitment of inflammatory cells to the lung (11, 13, 14). We did not examine if T cells overexpressing GATA-3 had modified chemokine receptor expression or if these cells regulated chemokine expression in the lung following antigen challenge. Further studies could address these questions by examining chemokine receptor expression on adoptively transferred T cells and the endogenous T cells recruited to the lung as well as by assessing chemokine expression in the lungs following antigen challenge.

It is also possible that the adoptively-transferred, antigen-specific T cells induced airway inflammation by enhancing dendritic cell maturation in the lung. The presence of IL-4 and IL-13 within the airway influences the rate at which DCs mature (15) and inhibition of IL-13 using soluble IL-13Rα2 inhibits DC maturation within the lungs of antigen challenged animals (16). Migration of DCs from the lung to the lymph nodes is also dependent on the inflammatory conditions in the lung since only mature DCs expressing high levels of MHC II can migrate to the draining lymph nodes (15, 17). The presence of antigen-specific T cells overexpressing GATA-3 and Th2 cytokines could lead to rapid maturation of DCs within the lung with consequently higher numbers of DCs migrating to the draining lymph nodes to activate T cells. This hypothesis is supported by our data from *in vitro* DLN cultures. DLN cells from animals receiving T cells transduced with the GATA-3 retrovirus, when cultured with OVA, showed enhanced production of IL-13 as well as decreased production of IFN-γ, suggesting that a greater number of endogenous T cells with a Th2 phenotype responded to *in vitro* antigen re-stimulation. Interestingly, the production of IL-13 has been shown to enhance the capacity of DCs to suppress T cell secretion of IFN-γ (15). This could explain the lower levels of IFN-γ that were detected in cultures from animals receiving transduced T cells overexpressing GATA-3. Overall, this suggests that GATA-3 overexpressing T cells may influence both the infiltration of $CD4^+$ T cells to the lung and also the development of antigen responsive Th2 cells within the DLNs.

Nevertheless, considering the time period during which these experiments were performed (the animals were sacrificed 3 days after adoptive transfer and antigen challenge), it seems unlikely that all of the host T cells infiltrating the lung were antigen specific. Little information is available on the activation of naive T cells *in vivo* since antigen specific cells are difficult to detect due to their low frequency. Studies in which antigen loaded DCs are adoptively transferred into naïve animals in a single intratracheal injection demonstrate that the mature DCs migrate to the mediastinal lymph nodes where they are detected as early as 24 h after injection. Strikingly, at this time point, antigen-responsive T cells have already undergone as many as six cell divisions (18). Hence, although the host T cells recruited to the lung in our study may not be fully differentiated T cells, they may be cells that proliferated in response to antigen, that were then recruited to the lung in response to DC activation within the lymph nodes.

The enhancement of allergic airway inflammation by T cells overexpressing GATA-3 suggests that cytokine production by small numbers of antigen specific T cells may strongly influence the level of airway inflammation induced by environmental antigens. The mechanism by which exposure to allergens leads to allergic airways disease has not been fully elucidated. It is almost certain that, among the repertoire of T cells present in the lung, those which are allergen-specific, are the primary regulators of the immune response to that allergen. Antigen specific T cells present in the asthmatic lung express a range of cytokines including IL-4, IL-10 and IFN-γ. Compared to normal human subjects, the dominant repertoire of allergen specific T cells in asthmatics produce

IL-4 (19). Not surprisingly, levels of GATA-3 are increased in asthmatic patients compared to controls with higher levels of cytoplasmic and nuclear levels of GATA-3 and a greater number of GATA-3 DNA binding complexes (20). Results from our work using antigen specific T cells overexpressing GATA-3 suggest that the magnitude of the inflammatory response to allergens is dependent upon Th2 cells. Data from other laboratories suggest that antigen-specific Th2 cells in the lymph node enhance the development of T cells with specificity to neo-allergens (21, 22), thus leading to the activation of a larger repertoire of T cells. This process, known as phenotype spread, explains why patients with atopy often develop immune responses to neo-allergens with time. Our data suggest that the enhancement of the allergic response initiated by relatively small numbers of antigen specific Th2 cells leads to the recruitment of antigen non-specific T cells to the site of inflammation. Following exposure to additional antigens, the transduced T cells could influence the lung microenvironment and enhance the activation of T cells to neo-allergens. Future experiments in which alternate antigens are utilized to challenge recipients of GATA-3 transduced T cells would help determine if the endogenous T cells recruited to the lung in our study can participate in phenotype spread. Alternatively, culture of GATA-3 overexpressing T cells with T cells from naïve animals in the presence of both OVA and alternate antigens could also demonstrate their ability to enhance proliferative responses to new antigens.

In addition to enhancing allergic inflammation, the presence of a large repertoire of T cells in the asthmatic lung may also influence the development of

another significant feature in asthma pathology, airway remodeling. We have demonstrated that adoptively-transferred, antigen-specific T cells can induce airway smooth muscle (ASM) remodeling, possibly by direct contact with smooth muscle cells in vivo (23, 24). Antigen stimulated T cells also regulate the expression of adhesion molecules (25), T cell co-stimulatory molecules, as well as smooth muscle responsiveness to bronchoconstrictors (26). In addition, activated T cells induce increases in smooth muscle expression of IL-5 and IL-1B, which then act in an autocrine fashion to alter SMC responsiveness to contractile agonists (27). Conversely, SMC may also influence T cell survival (24) as well as T cell activation within the lung. SMC express both MHC Class II and the co-stimulatory molecules required for antigen presentation to T cells (28). Altogether, these data suggest that T cells influence ASM proliferation and responsiveness and that these processes are dependent on the direct interplay between T cells and ASM cells. The endogenous T cells recruited by $CD4^+$ T cells overexpressing GATA-3, may enhance AHR and airway remodeling by driving increases in airway smooth muscle mass. Indeed, studies using transgenic animals where overexpression of GATA-3 is restricted to T cells, show that T cells overexpressing GATA-3 can enhance AHR, subepithelial fibrosis, ASM hyperplasia, goblet cell hyperplasia, and mucus hypersecretion (29, 30). However, these studies did not examine the antigen specificity of the T cells recruited to the lung following antigen exposure.

The role of STAT-6 in the ability of antigen specific T cells to induce airway inflammation

We also chose to modulate the activity of another transcription factor that regulates Th2 cytokine expression, STAT-6. STAT-6 is activated following binding of either IL-4 or IL-13 to their cognate receptors. STAT-6 is also required for IL-4 production as well as differentiation of antigen-stimulated splenocytes. Our data demonstrate that an inhibitory chimeric peptide targeting STAT-6 effectively inhibited IL-4 production from rat splenocytes cultured with antigen. Uptake studies using the FITC-conjugated peptide demonstrated efficient cellular internalization. Microscopic observation of the fluorescent peptide showed diffuse cytosolic and nuclear distribution without the punctuate staining observed in some cases (31). A possible explanation for this observation may be due to the fact that we used relatively high concentrations of the poly-arginine peptide in our studies. Increasing the peptide concentration enhances peptide localization to the cytosol and leads to a more diffuse staining (32). Peptide treatment was also performed at 37^0C. Physiological temperatures may contribute to peptide distribution since higher temperatures lead to peptide degradation thereby reducing the fraction that is able to migrate to the nucleus and the nucleolus (32).

Regardless of the exact intracellular localization, the STAT-6 inhibitory peptide-1 (SIP-1) peptide transduced cells and inhibited antigen-induced, STAT-6-dependent, IL-4 production. Though the exact mechanism is poorly understood, there are several stages within the life cycle of STAT-6 where inhibition by SIP-1 may occur. In IL-4/IL-13 stimulated cells, monomers of STAT-6 are constantly

cycling by binding to SH2 domains, dimerizing, and translocating to the nucleus, where they bind DNA. Once STAT-6 is dephosphorylated in the nucleus, it is exported to the cytoplasm where it joins the pool of STAT-6 monomers. The SIP-1 peptide may inhibit STAT-6 activity by interfering at one or more of these stages. Inhibition may take place in the cytoplasm or in the nucleus since the peptide likely crosses the nuclear membrane. Our data thus far suggest that SIP-1 does not inhibit IL-4/IL-13-induced Tyr phosphorylation or dimerization of STAT-6. It may inhibit STAT-6 activity by preventing DNA binding. To clarify this, experiments testing the ability of the SIP-1 peptide to inhibit IL-4/IL-13-induced STAT-6 DNA binding in electrophoretic mobility shift or chromatin immunoprecipitation assays could be done.

The ability of SIP-1 to inhibit STAT-6 is also supported by our *in vivo* studies, where we noted a decrease in the level of inflammation, both lymphocytic and eosinophilic, in the animals that received SIP-1 treated $CD4^+$ cells. The ability of adoptively transferred T cells to induce allergic inflammation is dependent on Th2 cytokines such as IL-4 and IL-13, it is likely that the inhibition of STAT-6 by SIP-1 inhibits cytokine dependent recruitment of inflammatory cells to the lung following antigen challenge. It is also possible that the decrease in inflammatory cells in the airway is due to a decrease in chemokine production by the adoptively transferred T cells themselves since they may also be a potential source of chemokines such as TARC/CCL17(33). Since we did not track OVA specific T cells *in vivo*, there is also a possibility that SIP-1 treated cells simply fail to migrate to the lung. To determine if the trafficking of adoptively transferred

T cells to the lung is inhibited by the SIP-1 peptide, cells could be identified after labeling with a fluorescent dye such as CFSE.

The ability of SIP-1 to inhibit the adoptively transferred T cells from inducing allergic inflammation suggests that cell penetrating peptides may have therapeutic benefit in allergic disease. In fact, intranasal instillation of cell permeable peptides and full-length proteins targeting a variety of different signaling molecules has now been shown to prevent several indices of allergic disease (34-37). Additional work from our laboratory has also demonstrated that intranasal delivery of a related STAT-6 inhibitory peptide (37) inhibits antigen dependent airway inflammation, and AHR in murine models of allergic airways disease (and data not shown). Our data demonstrating that SIP-1 effectively inhibits IL-4 production from cultured splenocytes suggests that cell penetrating peptides could be useful in dissecting STAT-6 dependent signalling pathways both *in vivo* and *in vitro*. Thus, our work with cell penetrating peptides suggests that they may not only have potential therapeutic benefit in allergic disease but may also help dissect the signalling pathways that regulate allergic airway responses induced by antigen exposure.

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Statement of Originality

In Chapter 2 we demonstrated that concentration of retroviral preparations using centrifugal filters can lead to a ten fold increase in the titers harvested from packaging cell lines. More importantly this increase in viral titers leads to a concomitant ten fold increase in the transduction of antigen stimulated splenocyte cultures. This chapter contributes to the literature by establishing detailed methodology for the use of centrifugal filters in concentrating retroviral particle preparations and has been published in the *Journal of Immunological Methods*.

I utilized the methodology in Chapter 2 to generate for the first time of antigen specific T cells that overexpress GATA-3. Adoptive transfer of these transduced antigen specific T cells leads to enhanced airway inflammation consisting of an increase in $CD4^+$ T cells and eosinophils. I also demonstrate that these $CD4^+EGFP^+$ T cells traffic to the lung and can be detected by cytofluorescence. Thus the majority of the $CD4^+$ T cells that infiltrate the lung following antigen challenge are endogenous T cells. I also demonstrate that T cells overexpressing GATA-3 influence the maturation/proliferation of DCs in the lung and lead to increased numbers ($CD4^-$ and $CD4^+$) in the DLNs of challenged animals that are not activated since they lack OX-40 expression. At the time of thesis submission this manuscript was accepted for publication in the *Clinical and Experimental Allergy.*

Finally, I utilized an inhibitory STAT-6 peptide (SIP-1) to investigate the ability of STAT-6 to modulate adoptively transferred T cells in experimental allergic asthma. We demonstrate that the inhibitory peptide is able to prevent antigen dependent inflammation induced by adoptively transferred T cells. This

also demonstrates that inhibitory peptides may be used investigate the signaling pathways induced during *in vivo* reactivation of adoptively transferred T cells. Chapter 3 is currently being prepared for submission.

Contribution of Authors

The following original manuscripts are in press or have been published, and are part of the thesis work presented by Margaret Kinyanjui:

1. M.W. Kinyanjui, Meiyo Tamaoka and E.D. Fixman. CD4+ T cell specific overexpression of GATA-3 enhances antigen-dependent recruitment of inflammatory cells in experimental asthma. Clinical Experimental Medicine, In Press.

MWK participated in designing and performing experiments as well as writing and editing the manuscript. MT assisted with the intubation of animals during adoptive transfer experiments. EDF oversaw the experimental research and preparation of the manuscript.

2. M.W. Kinyanjui*, D. Ramos-Barbón*, A. Villeneuve, and E.D. Fixman. Enhanced transduction of antigen-specific T lymphocytes with recombinant retroviruses concentrated by centrifugal filtration. Journal of Immunological Methods 314:80-89.
* Co-authorship

DRB proposed the use of Centricon filters for retroviral supernatant concentration and was responsible for initial experiments (Figure 1-3). MWK was responsible for performing the research experiments to obtain the data for Figures 2-4 in NIH3T3s and Figure 5 in splenocytes. MWK and DRB both participated in the preparation of the manuscript. AV generated the retroviral packaging cell line. EDF oversaw the experimental research and preparation of the manuscript.

Research Compliance Certificates

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

Protocol #: 4344
Investigator #: 923
Approval End Date: Aug. 31, 2003
Facility Committee: MCL

Title: THE ROLE OF T CELL ACTIVATION IN THE DEVELOPMENT OF AIRWAY REMODELING IN ASTHMA
(must match the title of the funding source application)

☐ New Application ☒ Renewal of Protocol # 4344 ☐ Pilot Category *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Elizabeth Fixman and James Martin **Phone #:** 398-3864 EXT 00140
Department: Medicine/Meakins-Christie Laboratories **Fax#:** 398-7483
Address: 3626 St. Urbain, Montreal Quebec H2X 2P2 **Email:** elizabeth.fixman@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Elizabeth Fixman **Work #:** 398-3864 ext 00140 **Emergency #:** 481-6583
Name: David Ramos-Barbon **Work #:** 398-3864 ext 00128 **Emergency #:** 522-7258

3. Funding Source:

External ☒
Source (s): CIHR/ASTRA-ZENECA
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 07/00-09/02

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

For Office Use Only:

ACTION	✓	DATE
CCC	✓	July 5 02
DE		
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Elizabeth D Fixman **Date:** May 21, 02

Approved by:

Chair, Facility Animal Care Committee:		**Date:** 2002/06/25
University Veterinarian:		**Date:** June 14/02
Chair, Ethics Subcommittee (as per UACC policy):		**Date:**
Approved Animal Use	**Beginning:** Sept. 1, 2002	**Ending:** Aug 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

JUL 08 2002

ACTION	514-398
CC	✓ Dec 10 '02
DE	
APPROVED	

McGill University
AMENDMENT to Animal Use Protocol
University Animal Care Committee www.mcgill.ca/ogs/animal

Principal Investigator: Elizabeth Fixman

Protocol #: 4344 exp Dec '03

Protocol Title: THE ROLE OF T CELL ACTIVATION IN THE DEVELOPMENT OF AIRWAY REMODELING IN ASTHMA

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Fax: 398-7483

Email: elizabeth.fixman@mcgill.ca

Level: C

Funding: CIHR

1. ADDITIONAL ANIMALS REQUESTED *(Justify additional animal numbers in Box 5 below)*

Species	Strain	Supplier / Source	# Animals Purchased	# By Breeding	Age	Sex	Weight	# Needed at One Time	#/Cage	Total Per Year

2. ADDITIONAL PERSONNEL *(Indicate any training required (e.g. workshops, lectures, etc.)*

Name	Classification	Training	Signature
Annie Villeneuve	Research Assistant	McGill rat workshop	Annie Villeneuve

3. CHANGE IN FUNDING SOURCE AND/OR TITLE *(use of grant must appear on the cover page of the animal protocol)*

4. OTHER *(including housing, procedure, anaesthetic/analgesic, problems anticipated) If additional procedures, specify how many and which animals are to be used*

In the original protocol we indicated that gene-modified T cells would be introduced into Brown Norway (BN) rats by IP injection. We now wish to introduce the T cells by IV injection into the tail vein. Rats will be anaesthetized as per SOP2 (included). Approximately 70 BN rats will undergo this procedure. VOLUME = 0.3 to 0.5 ml

5. WHY ARE THESE AMENDMENTS NECESSARY? *If requesting additional animals, justify the numbers*

We wish only to modify the method by which T cells are introduced into recipient rats

Approval Signatures

		Date
Principal Investigator/ Course Director	Elizabeth D Fixman	Dec 4, 02
Chair, Facility Animal Care Committee		2002 / 12 / 4
UACC Veterinarian		Dec 9, 2002
Chairperson, Ethics Subcommittee (D level or Teaching Protocols Only)		Date

Form revised January 2002

Note: the above modifications are valid until the expiration date of the main protocol.

DEC 06 2002

McGILL UNIVERSITY
UNIVERSITY ANIMAL CARE COMMITTEE

Standard Operating Procedure #UACC-2

January 2002 form version

GENERAL ANAESTHESIA IN ADULT RODENTS (RATS AND MICE)

1. INTRODUCTION

Standard Operating Procedures (SOPs) provide a detailed description of commonly used procedures. SOPs offer investigators an alternative to writing detailed procedures on their protocol forms. Any deviation from the approved procedures must be clearly described and justified in the Animal Use Protocol form. Approval of the protocol indicates approval of the deviation from the SOP for that project only. A signed SOP form (cover sheet only) must be attached to the AUP form. The relevant SOP number must be referred to in Section 9 of the Animal Use Protocol form.

2. INFORMATION REQUIRED

2.1 **Species/strain(s)**: BROWN NORWAY RAT *(can use column # of section 6c in main protocol)*

2.2 **Anaesthesia chosen:**

☒ Ketamine/acepromazine/xylazine Dose:50/5/1mg/kg Route: ☐ IM ☒ IP

☐ Ketamine/xylazine Dose: Route: ☐ IM ☐ IP

☐ Pentobarbital (Somnotol ®) Dose: Route: IP

☐ Tribromoethanol (Avertin ®) Dose: Route: IP

☐ Hypnorm ® (Fentanyl-fluanisone and Diazepam) Dose: Route: IP

☐ Other, specify: Dose: Route:

2.3 **There are changes to this SOP indicated in the AUP form:** ☐ YES ☒ NO

2.4 **Signature:** Elizabeth Fixman **Date:** Dec 5, 02

PLEASE ATTACH ONLY THIS SIGNED COVER SHEET TO THE BACK OF EACH RELEVANT AUP FOR ANIMAL CARE COMMITTEE APPROVAL.

Guidelines for completing the form are available at www.mcgill.ca/rgo/animal

McGill University
Animal Use Protocol – Research

Protocol #: 4344
Investigator #: 923
Approval End Date: Aug. 31, 2003
Facility Committee: MCL

Title: THE ROLE OF T CELL ACTIVATION IN THE DEVELOPMENT OF AIRWAY REMODELING IN ASTHMA
(must match the title of the funding source application)

☐ **New Application** ☒ **Renewal of Protocol #** 4344 ☐ **Pilot** **Category** *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Elizabeth Fixman and James Martin **Phone #:** 398-3864 EXT 00140
Department: Medicine/Meakins-Christie Laboratories **Fax#:** 398-7483
Address: 3626 St. Urbain, Montreal Quebec H2X 2P2 **Email:** elizabeth.fixman@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Elizabeth Fixman **Work #:** 398-3864 ext 00140 **Emergency #:** 481-6583
Name: David Ramos-Barbon **Work #:** 398-3864 ext 00128 **Emergency #:** 522-7258

3. Funding Source:

External ☒
Source (s): CIHR/ASTRA-ZENECA
Peer Reviewed: ☒ YES ☐ NO**
Status : ☒ Awarded ☐ Pending
Funding period: 07/00-09/02

Internal ☐
Source (s):
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period:

For Office Use Only:

ACTION	✓	DATE
CC	✓	July 5 02
DE		
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Forms are available at www.mcgill.ca/rgo/animal

Proposed Start Date of Animal Use (d/m/y): or ongoing ☒

Expected Date of Completion of Animal Use (d/m/y): or ongoing ☒

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Elizabeth D Fixman **Date:** May 21, 02

Approved by:

Chair, Facility Animal Care Committee:		**Date:** 2002/06/25
University Veterinarian:		**Date:** June 14/02
Chair, Ethics Subcommittee (as per UACC policy):		**Date:**
Approved Animal Use	**Beginning:** Sept. 1, 2002	**Ending:** Aug 31, 2003

☐ This protocol has been approved with the modifications noted in Section 13.

JUL 08 2002

McGill University Animal Care Committee

RENEWAL of Animal Use Protocol

For: Research ☒ Teaching ☐ project

For Office Use Only:
Protocol #: 4344
Approval end date: Aug 31, 2007
Facility Committee: MCL
Renewal#: 1st (2nd)

Principal Investigator: Elizabeth D. Fixman
Protocol # 4344
Protocol Title: The gene modified T cell: a tool to study airway responses and smooth muscle remodeling in asthma
Phone: 514 498 3864 ext 00140
Unit, Dept. & Address: Meakins-Christie Labs/Medicine
Fax: 514 398-7483
Email: elizabeth.fixman@mcgill.ca **Level:** C
Funding source: CIHR 10747 17511
Start of Funding: October 2003 **End of Funding:** September 31, 2006
Emergency contact #1 + work AND home phone #s: Margaret Kinyanjui, work # 398-3864 ext 00128; home # 514 287 9262
Emergency contact #2 + work AND home phone #s: Hector Valderrama-Carvajal, work #398-3864 ext 00128; home # 514 284 5431

1. Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to www.animalcare.mcgill.ca for details. Each person listed in this section must sign. *(Space will expand as needed)*

Name	Classification	Animal Related Training Information	Occupational Health Program *	Signature *"Has read the original full protocol"*
Margaret Kinyanjui	doctoral student	rat workship McGill	none	MKinyanjui
Hector Valderrama	doctoral student	rat workshop Mcgill	none	
Jichuan Shan	research associate	rat workshop McGill	none	JShan
Elizabeth Fixman	principal investigator	none (no contact with animals)	none	EFixman

All have taken on-line theory course.

* Indicate for each person, if participating in the local OHP Program, see http://www.mcgill.ca/rgo/animal/occupational/ for details.

Approved by:

2. Approval Signatures

Principal Investigator/ Course Director	Elizabeth D Fixman	Date: May 8, 06
Chair, Facility Animal Care Committee		Date: July 2 2006
UACC Veterinarian		Date: 2nd 1/06
Chairperson, Ethics Subcommittee *(D level or Teaching Protocols Only)*	—	Date: —
Approved Animal Use Period	Start: Sept 1, 2006	End: Aug 31, 2007

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Elizabeth D. Fixman TELEPHONE: 398-3864 ext 00140

   ADDRESS: Meakins-Christie Labs 3626 St. Urbain FAX NUMBER: 398-7483

   E-MAIL: elizabeth.fixman@mcgill.ca

   DEPARTMENT: Medicine

   PROJECT TITLE: The gene-modified T cell: a tool to study airway responses and smooth muscle remodeling in asthma

2. FUNDING SOURCE: (CIHR) NSERC NIH FCAR FRSQ

   INTERNAL OTHER

   Grant No.: MOP-64362 Beginning date October 1, 2003 End date September 30, 2006

3. Indicate if this is

   Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.

   Approval End Date _____

   (New funding source:) project previously reviewed and approved under an application to another agency.

   Agency Richard and Edith Strauss Canada Foundation Approval End Date September 30, 2006

   (This project is still ongoing. The biohazard procedures for the two projectes are the same.)**

   New project: project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: Elizabeth D Fixman (SIGNATURE) date: 22 Sept 03 (day month year)

DR. DALIUS J. [illegible]

Chairperson, Biohazards Committee: _____ (SIGNATURE) date: 06-09-03 (day month year)

Approved period: beginning 06-11-03 (day month year) ending 30-09-06 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Elizabeth Fixman	Medicine	X				
David Ramos-Barbon	Experimental Medicine				X	
Margaret Kinyanjui	Experimental Medicine				X	
Annie Villeneuve	Medicine		X			

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Elizabeth Fixman Phone No: work: 398-3864 ext 00140 home: 481-6583

Name: David Ramos-Barbon Phone No: work: 398-3864 ext 00128 home: 522-7258

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

Phoenix Eco Cells (ATCC #SD3444). This cell line is derived from human embryonic kidney (HEK) 293 cells, an immortalized cell line containing transforming regions of adenoviral 5 DNA. Like the parental HEK 293 cells, they are designated as Biosafety risk group 2. The Phoenix Eco cells are used to generate recombinant, non-replicating retroviruses. They are a standard retroviral packaging cell line, containing integrated copies of retroviral sequences encoding gag-pol and, separately, env. Biosafety risk group 2. The ecotropic retroviruses they produce transduce rodent cells only. Biosafety risk group 2.

ii. the procedures involving biohazards

The Phoenix Eco cells are used to generate recombinant, non-replicating ecotropic retroviruses. Phoenix-Eco cells are transfected with a plasmid containing the retroviral genome. Ecotropic retroviruses are then packaged and produced by the cells. Ecotropic retroviruses are used to transduce primary rat spleen cells harvested from Brown Norway rats in order to modify the profile of proteins/cytokines expressed by T cells from the spleen. The spleen cells are cultured for approximately one week to 10 days before being injected into recipient Brown Norway rats. The recipient rats are challenged with aerosolized ovalbumin and subsequently sacrificed. For some experiments rats will be sacrificed the 48 hours after receiving T cells. For others recipient rats will be sacrificed after 15 days. Once the recombinant retrovirus infects the target T cell, no progeny virus is made.

iii. the protocol for decontaminating spills

Any spill of cells, solutions that have come into contact with cells, or virus-containing cell supernatents is cleaned with bleach and 70% ethanol.

McGill University

University Biohazards Committee

APPLICATION TO USE BIOHAZARDOUS MATERIALS*

No project should be commenced without prior approval of an application to use biohazardous materials. Submit this application to the Chair, Biohazards Committee, one month before starting new projects or expiry of a previously approved application.

1. PRINCIPAL INVESTIGATOR: Elizabeth Fixman TELEPHONE: 398-3864

   ADDRESS: Meakins-Christie Labs 3626 St. Urbain FAX NUMBER: 398-7483

   E-MAIL efixman@meakins.lan.mcgill.ca

   DEPARTMENT: Medicine

   PROJECT TITLE: Role of T Cell Cytokines in Airway Remodelling

2. FUNDING SOURCE: (MRC) NSERC NIH FCAR FRSQ

   INTERNAL OTHER (specify) _____

   Grant No.: MOP 31511 Beginning date October 1, 1999 End date Sept 30, 2002

3. Indicate if this is

   Renewal use application: procedures have been previously approved and no alterations have been made to the protocol.
   Approval End Date _____

   New funding source: project previously reviewed and approved under an application to another agency.

   Agency _____ Approval End Date _____

   (New project): project not previously reviewed or procedures and/or microorganism altered from previously approved application.

CERTIFICATION STATEMENT: The Biohazards Committee approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the "Laboratory Biosafety Guidelines" prepared by Health Canada and the MRC, and in the "McGill Laboratory Biosafety Manual".

Containment Level (circle 1): 1 (2) 3 4

Principal Investigator or course director: Elizabeth B Fixman (SIGNATURE) date: 08 12 99 (day month year)

Chairperson, Biohazards Committee: _____ (SIGNATURE) date: 13-12-99 (day month year)

Approved period: _____ beginning 13-12-99 (day month year) ending 30-09-2002 (day month year)

* as defined in the "McGill Laboratory Biosafety manual"

4. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Check appropriate classification				Fellow
		Investigator	Technician & Research Assistant	Student		
				Undergraduate	Graduate	
Elizabeth Fixman	Medicine	X				
David Ramos-Barbon	Experimental Medicine				X	
Rame Taha	Medicine					X

5. EMERGENCY: Person(s) designated to handle emergencies

Name: Elizabeth Fixman Phone No: work: 398-3864 ext 09392 home: 481-6583

Name: David Ramos Barbon Phone No: work: 398-3864 ext 3007 home: 844-0881

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues) & designated biosafety risk group

non replicating recombinant ecotropic retrovirus

Biosafety risk group 2

ii) the procedures involving biohazards T cells will be harvested from the lymph nodes of ovalbumin sensitized Brown Norway Rats. They will be purified and then infected with recombinant ecotropic retroviruses engineered to over- or underexpress cytokines implicated in airway hyperresponsiveness and structural remodeling in asthma. T cells will be cultured for up to 3 weeks before they are injected into recipient animals. These animals will subsequently be challenged up to three times with aerosolized ovalbumin over the course of 9 days. On the 10th day the animals will be sacrificed.

The retrovirus is made following transient transfection of HEK293T cells with one plasmid containing the retroviral genome and a second plasmid encoding the packaging proteins. Once recombinant virus infects the recipient T cell, no progeny virus is made.

iii) the protocol for decontaminating spills any spill of virus-containing cell supernatents will be cleaned with bleach and 70% ethanol.

Guidelines for completing the form are available at www.mcgill.ca/research/compliance/animal/forms

McGill University
Animal Use Protocol – Research

For Office Use Only:
Protocol #: 5364
Approval End Date: Feb 28, 2008
Facility Committee: ACL

Title: Cell permeable peptides targeting STAT-6 and inhibition of allergic airways disease
(must match the title of the funding source application)

☒ **New Application** ☐ **Renewal of Protocol #** _____ ☐ **Pilot** **Category** *(see section 11)*: C

1. Investigator Data:

Principal Investigator: Elizabeth Fixman **Phone #:** 398-3864 ext 00140
Unit/Department: Medicine/Meakins-Christie Laboratories **Fax#:** 398-7483
Address: 3626 St. Urbain **Email:** elizabeth.fixman@mcgill.ca

2. Emergency Contacts: Two people must be designated to handle emergencies.

Name: Jichuan Shan **Work #:** 398-3864 09395 **Emergency #:** (514) 982-6994
Name: Elizabeth Fixman **Work #:** 398-3864 00140 **Emergency #:** (514) 481-6583

3. Funding Source:

External ☒ 17387
Source (s): CIHR
Peer Reviewed for the project proposed in this Animal Use Protocol: ☒YES ☐NO**
Status : ☒ Awarded ☐ Pending
Funding period: April 2007-March 2010

Internal ☐
Source (s): _____
Peer Reviewed: ☐ YES ☐ NO**
Status: ☐ Awarded ☐ Pending
Funding period: _____

For Office Use Only:

ACTION	✓	DATE
CCs		
DB	✓	Mar 13/07
APPROVED		

** All projects that have not been peer reviewed for scientific merit by the funding source require 2 Peer Review Forms to be completed e.g. Projects funded from industrial sources. Peer Review Form available at www.mcgill.ca/research/compliance/animal/forms

Proposed Start Date of Animal Use (d/m/y): April 1, 2007 **or ongoing** ☐
Expected Date of Completion of Animal Use (d/m/y): March 31, 2010 **or ongoing** ☐

Investigator's Statement: The information in this application is exact and complete. I assure that all care and use of animals in this proposal will be in accordance with the guidelines and policies of the Canadian Council on Animal Care and those of McGill University. I shall request the Animal Care Committee's approval prior to any deviations from this protocol as approved. I understand that this approval is valid for one year and must be approved on an annual basis.

Principal Investigator's signature: Elizabeth Fixman **Date:** Feb 2, 2007

Approved by:

Chair, Facility Animal Care Committee:		**Date:** Mar 6, 07
University Veterinarian:		**Date:** 13/03/07
Chair, Ethics Subcommittee (as per UACC policy):	—	**Date:** —
Approved Animal Use	**Beginning:** MARCH 1, 2007	**Ending:** Feb 28, 2008

☐ This protocol has been approved with the modifications noted in Section 13.

☐ **Renewal requires submission of full Animal Use Protocol form**

May 2006

ML Rec 13/03/07

13 MAR. 2007

4. Research Personnel and Qualifications

List the names of the Principal Investigator and of all individuals who will be in contact with animals in this study and their employment classification (investigator, technician, research assistant, undergraduate/ graduate student, fellow). Indicate if Principal Investigator is not handling animals. If an undergraduate student is involved, the role of the student and the supervision received must be described. Training is mandatory for all personnel listed here. Refer to *www.animalcare.mcgill.ca* for details. Each person listed in this section must sign to indicate that s/he has read this protocol. ***(Space will expand as needed)***

Name	Classification	Animal Related Training Information		Occupational Health Program *	Signature
		UACC on-line Theory course	Workshops + others		
Jichuan Shan	research associate	yes	mice, rats	no	
Margaret Kinyanjui	graduate student	yes	rats	no	MKinyanjui
Emily Nakada	graduate student	yes	rats	no	
Elizabeth Fixman	PI	yes	none (no animal handling)	no	ElizabethFixman

*** Indicate for each person, if participating in the local Occupational Health Program, see *www.mcgill.ca/research/compliance/animal/occupational* for details.**

5. Summary (in language that will be understood by members of the general public)

5 a) AIMS AND BENEFITS: Describe, in a short paragraph, the overall aim of the study and its potential benefit to human/animal health or to the advancement of scientific knowledge.

Asthma and allergies affect up to 30% of the general population with significant morbidity. Abundant evidence suggests that these allergic airway diseases are initiated and exacerbated by inflammatory cells called Th2 cells. STAT-6 is a transcription factor that regulates development of Th2 cells as well as airway inflammation and airway hyperresponsiveness in animal models of asthma. In this proposal we are testing a novel approach to inhibit STAT-6 in order to better understand how STAT-6 contributes to asthma pathogenesis. We are using a STAT-6 inhibitory peptide that has the ability to enter cells and bind to and inhibit STAT-6. Our data demonstrate that intranasal delivery of this STAT-6 inhibitory peptide inhibits inflammation and airway hyperresponsiveness in a murine model of rhinitis and asthma. We will now test if this peptide has the ability to inhibit experimental asthma when it is administered after inflammation is already established. Structural changes to the airways, termed airway remodeling, are thought to occur as a consequence of airway inflammation induced by Th2 cells. We will now use the STAT-6 inhibitory peptide to define the role of STAT-6 in airway remodeling. We believe these studies will provide evidence that inhibition of STAT-6 using this approach can be used to ameliorate bothacute and chronic aspects of asthma pathogenesis and thus may offer an important new therapeutic approachfor the treatment of allergic airways disease in humans.

5 b) SPECIFIC OBJECTIVES OF THE STUDY: Summarize in point form the primary objectives of this study.

Specific Aims
1) To investigate the mechanism by which the STAT-6-IP inhibits STAT-6 activity in vitro
2) To investigate the mechanism by which STAT-6-IP inhibits STAT-6 activity as well as airway inflammation and AHR in vivo
3) To investigate the ability of the STAT-6-IP to inhibit airway remodeling in the BN rat

5 c) Indicate if and how the current goals differ from those in last year's application.

This is a new application.

5 d) List the section / subsection numbers where significant changes have been made

not applicable

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: FIXMAN, Elizabeth PHONE: 398-3864 ext 00140
DEPARTMENT: Medicine FAX: 398-7483
ADDRESS: Meakins-Christie Labs, 3626 St. Urbain E-MAIL: elizabeth.fixman@mcgill.ca

PROJECT TITLE(S):Cell permeable peptides targeting STAT-6 and inhibition of allergic airways disease

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Jichuan Shan Phone No: work: 398-3864 ext 09395 home: (514) 982-6994
Name: Elizabeth Fixman Phone No: work: 398-3864 ext 00140 home: (514) 481-6583

3. FUNDING SOURCE OR AGENCY: list all sources when information in Sections 5-12 is identical:

Source	CIHR	Grant No.	MOP-82766	Start date	April 1, 07	End date	Mar 31, 10
Source		Grant No.		Start date		End date	
Source		Grant No.		Start date		End date	

4. Indicate if this is

☒ Renewal: procedures previously approved without alterations.
Approval End Date: February 28, 2007
☐ New funding source: project previously reviewed and approved under an application to another agency.
Agency: Approval End Date:
☐ New project: project not previously reviewed.
☐ Approved project: change in biohazardous materials or procedures.
☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the Public Health Agency of Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: Elizabeth Fixman SIGNATURE date: 28 (day) 02 (month) 07 (year)

Approved by Environmental Health & Safety: C. St. Louis SIGNATURE date: 09 (day) 03 (month) 07 (year)

Expiry: 31 (day) 03 (month) 10 (year)

5. RESEARCH PERSONNEL: (attach additional sheets if preferred)

Name	Department	Job Title/Classification	Trained in the safe use of biological safety cabinets within the last 3 years? If yes, indicate training date.
Jichuan Shan	Medicine	Research Associate	no
Margaret Kinyanjui	Medicine	Doctoral Student	no
Emily Nakada	Medicine	MSc Student	no

6. Briefly describe:

i) the biohazardous material involved (e.g. bacteria, viruses, human tissues, toxins of biological origin) & designated biosafety risk group

We are testing the ability of peptides containing protein transduction domains to inhibit IL-4/IL-13 dependent responses in vitro and in vivo using muring and rat models of allergic rhinitis and asthma. The protein transduction domains allow the uptake of peptides into cells. The protein we are targeting is the STAT-6 transcription factor, which regulates allergic responses induced by IL-4/IL-13. Proteins or peptides containing protein transduction domains are considered to be Class 2 Biohazards. They are not infectious.

ii) the procedures involving biohazards

For the in vivo experiments, peptides are administered intranasally to mice or rats. For the in vitro experiments, petpides are incubated with cultured murine splenocytes or immortalized epithelial cell lines.

iii) the protocol for decontaminating spills

We routinely use 70% ethanol to clean areas where peptides have been used.

McGill University

APPLICATION TO USE BIOHAZARDOUS MATERIALS

Projects involving potentially biohazardous materials should not be commenced without approval from Environmental Health & Safety. Submit applications before 1) starting new projects, 2) renewing existing projects, or 3) changing the nature of the biohazardous materials within existing projects.

1. PRINCIPAL INVESTIGATOR: FIXMAN, Elizabeth PHONE: 398-3864 ext 00140
DEPARTMENT: Medicine FAX: 398-7483
ADDRESS: Meakins-Christie Labs, 3626 St. Urbain E-MAIL: elizabeth.fixman@mcgill.ca

PROJECT TITLE(S):Cell permeable peptides targeting STAT-6 and inhibition of allergic airways disease

2. EMERGENCY: Person(s) designated to handle emergencies

Name: Jichuan Shan Phone No: work: 398-3864 ext 09395 home: (514) 982-6994
Name: Elizabeth Fixman Phone No: work: 398-3864 ext 00140 home: (514) 481-6583

3. FUNDING SOURCE OR AGENCY: list all sources when information in Sections 5-12 is identical:

Source	CIHR	Grant No.	MOP-82766	Start date	April 1, 07	End date	Mar 31, 10
Source		Grant No.		Start date		End date	
Source		Grant No.		Start date		End date	

4. Indicate if this is

☒ Renewal: procedures previously approved without alterations.
Approval End Date: February 28, 2007
☐ New funding source: project previously reviewed and approved under an application to another agency.
Agency: Approval End Date:
☐ New project: project not previously reviewed.
☐ Approved project: change in biohazardous materials or procedures.
☐ Work/project involving biohazardous materials in teaching/diagnostics.

CERTIFICATION STATEMENT: Environmental Health & Safety approves the experimental procedures proposed and certifies with the applicant that the experiment will be in accordance with the principles outlined in the Public Health Agency of Canada's "Laboratory Biosafety Guidelines" and in the "McGill Laboratory Biosafety Manual".

Containment Level (select one): ☐ 1 ☒ 2 ☐ 2 with additional precautions ☐ 3

Principal Investigator or course director: Elizabeth Fixman (SIGNATURE) date: 28 (day) 02 (month) 07 (year)

Approved by Environmental Health & Safety: C. St. Louis (SIGNATURE) date: 09 (day) 03 (month) 07 (year)

Expiry: 31 (day) 03 (month) 10 (year)