Implication of Intracellular Signalling Pathways

in Allergic Asthma Pathogenesis

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A thesis submitted to the Graduate and Postdoctoral Studies Office to obtain the degree of

Doctor of Philosophy

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Montréal

June 2008

Your file Votre référence
ISBN: 978-0-494-66684-5
Our file Notre référence
ISBN: 978-0-494-66684-5

To everyone who supported me during this work (you recognize yourselves),
and to the little one I now support

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List of abbreviations

AHR: airway hyperresponsiveness

AM: Alveolar Macrophage

AMP: adenosine monophosphate

APC: antigen presenting cell

ATP: adenosine triphosphate

BAL: bronchoalveolar lavage

bpV(phen): bisperoxovanadium phenantroline

bpV(pic): bisperoxovanadium picolinic acid

CBP: cAMP-response element-binding protein

CD: cluster differentiation (identification of cell markers)

Csk: c-terminal src kinase

DAG: diacyl glycerol

EAR: early asthmatic response

ECP: eosinophil cationic protein

EPO: eosinophil peroxidase

ERK: extracellular signal-regulated kinase

FAK: focal adhesion kinase

FcεR1: crystallisable fraction epsilon receptor 1 or high affinity IgE receptor

FcεR2: crystallisable fraction epsilon receptor 2 or low affinity IgE receptor

FeNO: fraction of exhaled NO

FEV1: forced expiratory volume in 1 second

Fyn: protein-tyrosine kinase fyn

Gads: Grb2-related adaptor downstream of Shc

GAS: gamma-interferon activation sequence

GATA-3: GATA binding protein 3

G-CSF: granulocyte colony stimulating factor

GM-CSF: granulocyte macrophage colony stimulating factor

Grb2: growth factor receptor-bound protein 2

GRE: glucocorticoid receptor elements

GRs: glucocorticoid receptors

ICS: inhaled corticosteroid

IFN-γ: interferon-gamma

IFN-γR: interferon-gamma receptor

Ig: immunoglobulin

IL: interleukin

IM: interstitial macrophage

i.n.: intra-nasal

iNOS: inducible nitric oxide synthase – NOS2

i.p.: intra-peritoneal

IP3: inositol 1,4,5- triphosphate

ITAM: immunoreceptor tyrosine-based activation motif

ITIM: immunoreceptor tyrosine-based inhibition motif

i.v.: intra-venous

LABA: long-acting β_2-agonists

LAR: late asthmatic response

LAT: linker for activation of T cells

Lck: leukocyte-specific protein tyrosine kinase

LIF: Leukemia inhibitory factor

LPS: lipopolysaccharide

LT: leukotriene

LTRAs: leukotriene receptor antagonists

MAPK: mitogen-activated protein kinase

MBP: major basic protein

M-CSF: macrophage colony stimulating factor

MEK: MAPK-ERK kinase

MHC: major histocompatibility complex

MIP: macrophage inflammatory protein

MMP: matrix metalloprotease

MRP: myeloid-related protein

Mθs: macrophage

NFAT: nuclear factor of activated T cells

NF-κB: nuclear factor kappa B

NOS: NO synthases

NO: nitric oxide

OVA: ovalbumin

PAF: platelet-activating factor

PAG: phosphoprotein associated with glycosphingolipid-enriched microdomains

PBMC: peripheral blood mononuclear cell

PC20: provocative challenge causing 20% decrease in FEV1

Penh: Pause enhancement

PG: prostaglandine

PIP2: phosphatidylinositol 4,5-bisphosphate

PKC: protein kinase C

PLC: phospholipase C

PTEN: phosphatase and tensin homologue

PTP: protein tyrosine phosphatase

PTP-1B: protein tyrosine phosphatase 1B

PTP-PEST: PTP proline-, glutamine-, serine-, threonine-rich sequence

Pyk2: proline-rich tyrosine kinase 2

Raf: protein kinase raf 1

RANTES: regulated on activation normal T cell expressed and secreted

Ras: resistance to audiogenic seizure

ROS: reactive oxygen species

SABA: short acting β_2-agonists

SFK; Src family kinase

SH2: src homology 2 domain

Shc: src homologuous and collagen

SHP: src homology 2 domain-containing phosphatase

STAT: signal transducer and activator of transcription

TC-PTP: T cell protein tyrosine phosphatase

TCR: T cell receptor

TGF-β: tumor growth factor beta

Th: T helper cell

TLR: toll-like receptor

TNF: tumor necrosis factor

Tregs: regulatory T lymphocytes

ZAP-70: Zeta-chain-associated protein kinase 70

Abstract

The regulation of systemic immune responses is dependent on individual cell responses that will concur to induce a coherent response against a stimulus. In turn, cell response is dependent on the processing of intracellular signals generated at the cell membrane and transmitted through successive protein modifications to the nucleus in order to activate gene transcription. This is referred to as intracellular signalling. Tight control of these mechanisms is required to generate an appropriate cell response to environmental stimulations and globally to establish an appropriate immune response. Among protein modifications used to transmit a signal to the nucleus, protein tyrosine phosphorylation represents a pivotal method used by immune cells to rapidly induce signalling. While protein tyrosine kinases (PTKs) phosphorylate proteins, protein tyrosine phosphatases (PTPs) regulate the signalling by removing the phosphate group. The goal of this study was to better characterize intracellular signalling events involved in allergic asthma, a chronic inflammatory disease involving a Th2 immune response. In a first time, we investigated the role of PTPs in the development of asthma. We show that inhibition of global PTP activity in mice, during either the allergen sensitization or the allergen challenge phase, reduces asthma development and is linked to an increased Th1 response in the spleen and lung. Secondly, we revealed that TC-PTP inhibition reduces asthma development, while PTP-1B inhibition exacerbates inflammatory cells recruitment to the lung. Inhibition of either SHP-1 or PTP-PEST activity did not significantly modulate asthma development in our model. In a third set of experiments, we got interested in the signalling pathways triggered by the pro-inflammatory molecules myeloid-related proteins (MRPs) 8 and 14. MRPs are small cytosolic proteins recently described to have

extracellular functions. MRP8 expression is resistant to corticosteroid treatment, and potentially promotes inflammation in corticosteroid-treated patients. We identified that MRPs induce signal through the action of TLR-4 and trigger the activation of MEK/ERK and JNK pathways that lead to NF-κB translocation. Collectively, our data provide a new characterization of signalling pathways engaged in allergic asthma. This should be helpful in the elaboration of new therapeutic approaches targeting precise pathways to inhibit mechanisms of inflammation.

Résumé

La régulation de la réponse immunitaire dépend de la réponse de chaque cellule qui collabore à l'élaboration d'une réponse systémique contre un stimulus. En retour, l'établissement de la réponse cellulaire dépend de l'intégration de signaux intracellulaires générés à la membrane et transmis au noyau par l'activation successive de protéines impliquées dans la signalisation. Ces processus sont appelés signal intracellulaire. Un contrôle strict de ces mécanismes est nécessaire pour la génération d'une réponse cellulaire adéquate aux stimuli environnementaux et globalement, pour la réponse immune. Parmi les modifications protéiques utilisées dans ces mécanismes, la phosphorylation de résidus tyrosils est une méthode de choix dans les cellules immunitaires pour l'induction rapide d'un signal. Alors que les protéines tyrosine kinases (PTKs) ajoutent un groupement phosphate aux protéines, les protéines tyrosine phosphatases (PTPs) régulent le signal en les enlevant. Le but de cette étude est de mieux caractériser certains événements signalétiques impliqués dans l'asthme allergique, une maladie inflammatoire chronique à prédominance Th2. En premier lieu, nous avons étudié le rôle des PTPs dans le développement de l'asthme. Nous avons démontré que leur inhibition soit durant la sensibilisation allergénique, soit durant l'exposition allergénique, réduit le développement de l'asthme et correspond à une augmentation de la réponse Th1 dans la rate et le poumon. Ensuite, nous avons observé que l'inhibition de TC-PTP réduit le développement de l'asthme alors que l'inhibition de PTP-1B augmente le recrutement cellulaire pulmonaire. L'inhibition de SHP-1 ou de PTP-PEST n'a pas significativement modulé le développement de l'asthme dans notre modèle. En troisième lieu, nous avons étudié les voies signalétiques induites par les *myeloid-related proteins*

(MRPs) 8 et 14. Des fonctions extracellulaires ont récemment été décrites pour ces petites protéines cytosoliques. Les niveaux de MRP8 résistent au traitement avec les corticostéroïdes et promeuvent probablement l'inflammation chez les patients traités aux corticostéroïdes. Nous rapportons que les MRPs activent les phagocytes via l'activation de TLR4 et des voies MEK/ERK, JNK et la translocation de NF-κB. Collectivement, nos résultats caractérisent de nouvelles voies signalétiques impliquées dans l'asthme, ce qui pourra être utile afin d'établir de nouvelles approches thérapeutiques spécifiques.

Preface and Acknowledgement

As prescribed by the actual *Thesis Preparation Guidelines*, available through the Graduate and Postdoctoral Studies Office of McGill University, the author presents a manuscript-based thesis. Philippe Pouliot is the principal author on all the manuscripts included in this thesis and is recognized to have performed the majority of the work presented in these manuscripts.

The manuscript presented in Chapter 2 is prepared for submission. The author of the thesis acknowledges the assistance of Pierre Camateros, from Dr Danuta Radzioch's laboratory (McGill University) for the whole-body plethysmograph measurements of the pause enhancement on animal subjects.

The manuscript presented in Chapter 3 is prepared for submission. The thesis author acknowledges the collaboration of: Dr Michel L. Tremblay's laboratory (McGill University) in providing the mutant mouse involved in this study; Dr Sébastien Bergeron from Dr André Marette's laboratotory (Laval University) for training the author on the preparation of adenoviruses; Pierre Camateros, from Dr Danuta Radzioch's laboratory (McGill University) for the whole-body plethysmograph measurements of the pause enhancement on animal subjects.

Chapter 4 presents a manuscript that is now re-submitted to the *Journal of Immunology* after revision (resubmitted on May 8, 2008). Acknowledgements for assistance must be made to: Marie-Astrid Raquil from Dr Philippe Tessier's laboratory for the preparation of

recombinant MRP proteins used in the described experiments; Isabelle Plante from Dr Martin Olivier's lab for generating preliminary results of this work (many replicates of figures 1, 2 and 3).

I would like to thank the laboratory assistants (Marianne Godbout and Caroline Martel) with who I worked for their occasional help in experimentations, but also for their constant and careful management of the lab activities and equipment. I would like to thank Dr Olivier's research team for the many discussions, and extensive helpful interactions we shared. Particularly, I thank Dr Marina Tiemi Shio for clever suggestions on experimental approaches; Maria Adelaida Gomez, Issa Abu Dayyeh and Irazu Contreras for many discussions throughout the years that helped my progress. Out of my laboratory, I have to thank Pierre Camateros for the physiology of the mice, but also for all the discussions we shared on these long days. Marceline Côté from Dr Liu lab was of an invaluable help for planning the production of my adenoviruses as well as a great scientist with who to discuss experiments.

Finally, but not the least, I would also like to thank my supervisor, Dr Martin Olivier for his help in my work; for insightful discussions on the design of our experimentations; for expanding my understanding of the world of academic research by multiple discussions; for insights on career choices; for his time taken out of normal (or what could be called "decent") work hours; for making himself available in critical periods and, well, for being the supervisors he is.

General Introduction

The immune system refers to a complex array of cells that interact together in order to protect the integrity of the host organism. These individual cells can all sense their environment by various methods and communicate together in order to coordinate their response in a coherent whole. However, as individual cells, they are also discrete entities, just as their host is a discrete individual of a group of other individuals. Therefore, the signals of their extracellular milieu, originating from the environment or from other cells, do not have a direct access to the cell nucleus where the response to the stimulation will be initiated. Therefore, evolution favoured the apparition of complex mechanisms that allow the cell nucleus to be "informed" of what happens at the cell periphery in order to create a reaction. It also appears that these mechanisms are actually "creating" a lot of the cell response by integrating the various simultaneous stimulations of the cell and activate specific transcription factors in function of the triggered cell receptor. The nucleus also has a role to play in the decision, as not all transcription factors-binding sequences are available at a defined time or in a defined cell. Here, let's consider a unique cell receptor located at the cell membrane that is bound by its ligand, freshly added to the cell media. Upon ligation, the signal must be transmitted to the nucleus in order for the cells to mount up a response to this environmental trigger. This implies that modifications of the protein pool of the cytoplasm must occur, as no cell reaction is observed in the resting state, before cell stimulation. Obviously, to modify the protein pool, no new protein is involved as the signal has not reached the nucleus and no gene transcription can occur at this moment. Other mechanisms have to be found. The study of what happens between the cell receptor to the gene transcription is a long-standing field.

A partial answer to how proteins are modified came with the discovery that some enzymes required the presence of a co-factor. Nevertheless, here again, how was this co-factor brought to the enzyme? Although incomplete at the time, a new discovery by Krebs and Fischer in 1956 was about to change the world of intracellular signalling. Working on phosphorylase β and phosphorylase α, they discovered that phosphorylase α was actually phosphorylase β to which a phosphate group was added (radiolabelled ATP has been added to the preparation and the bound phosphate group was detected by autoradiography). Phosphorylase α exhibits potent phosphorylase activity while phosphorylase β is dramatically less efficient. This was the first demonstration that phosphorylation could be involved in the modification of protein enzymatic activity.

Further studies on the subject showed a variety of ways on how phosphorylation of proteins can modify protein functions. For example, the ligation of a receptor can induce oligomerization of these receptors, bringing their cytoplasmic domains into proximity. This proximity can allow receptors to *trans*-activate each other by phosphorylation, if their cytoplasmic domains have kinase activity (which is the classic activation pattern of receptor tyrosine kinases). It can also induce a conformational change that allows another cytosolic protein to phosphorylate their cytoplasmic tail and hence create a docking site for another cytosolic kinase that can now phosphorylate protein targets in the surroundings. These are but only a few examples that reflect the possibilities provided by protein phosphorylation.

Proteins are formed of amino acids, but the side chains of the various amino acids have radically different properties, which suggest that phosphorylation cannot happen on any residue. First identified to happen on serine and threonine, phosphorylation was later described to happen on tyrosine by Eckhart, Hutchisson and Hunted in 1979. Tyrosine phosphorylation then began to be recognized as a prominent method of signal transduction in immune cells. It is an obligatory event of TCR signalling and is the first detectable biochemical reaction to occur upon TCR stimulation. The phosphorylation of tyrosine residues is performed by protein tyrosine kinases (PTKs) while protein tyrosine phosphatases (PTPs) remove the phosphate group. Interestingly, although PTKs are numerous in resting T cells, the level of phosphotyrosil residues is very low, around 0,01% of total tyrosil residues. This shows the importance of a housekeeping PTP activity in the regulation of protein tyrosine phosphorylation. This also highlights the importance of tyrosine phosphorylation in quick changes of the resting state of T cells as these proteins are readily available for new phosphorylation. In comparison, the level of phosphoserine residues reaches 95% and phosphothreonine 5% in resting state, suggesting that their role is important in the resting state but has less potential of modulation for quickly induced events.

The various protein interactions that are initiated by phosphorylation are often described as a signalling cascade: a first protein phosphorylates a second, which phosphorylates a third and so on. The use of such protein interactions is useful in amplifying the signal that emerges from the ligation of possibly just one receptor. Such succeeding steps also open possibilities for the integration of signals from many different receptors that converge or diverge from these pathways in order to induce or prevent gene expression. Interesting

examples exist where the ligation of a receptor bearing an immunoreceptor tyrosine-based inhibitory motif (ITIM) prevents the signal transduction of another receptor that usually has activator functions. These mechanisms underscore very well the possibilities for fine-regulation of intracellular pathways that can affect the outcome of the cell response. We must keep in mind that depending on the cell response, the whole immune response can be modulated.

It is in this context that we were interested to investigate the implication of signalling pathway elements in the regulation of the immune responses leading to allergic asthma. It was previously reported that many kinases had a role to play in asthma. Although their role in first events of phosphorylation was intuitively suggesting that their inhibition would prevent the development of a cell response, and hence a proper asthma development, their role in the phosphorylation of ITIMs and inhibitory phosphotyrosil residues on proteins create a more complex picture. Still, inhibition of the majority of the kinases reported, resulted in reduction of asthma development. On the other hand, it is interesting to observe that at the exception of PTEN and SHP-1, the role of protein tyrosine phosphatases (PTPs) has been largely overlooked. Our laboratory previously reported that inhibition of PTPs by peroxovanadium compounds favoured a Th1 profile of gene expression in the spleen. As a Th2 response is usually recognized to be involved in asthma, we hypothesized that inhibition of PTPs could be beneficial in asthma by antagonizing the Th2 response and reducing asthma development.

In chapter 2, we demonstrate that inhibition of PTPs by an inhibitor, which specifically inhibits all PTPs, can prevent the development of asthma. To achieve this, we inhibited

PTPs during either allergen sensitization or during allergen challenge. We then observed that: IgEs were reduced (if the inhibition is performed during allergen sensitization only); cells found in the bronchoalveolar lavage fluid (BALf) were decreased; lung eosinophilia was reduced; tissue inflammation was normalized and development of airway hyperresponsiveness was prevented. We observed that treatment with PTP inhibitors favoured spleen expression of Th1 cytokines (IFN-γ and IL-12) while it decreased Th2 cytokines expression (IL-4, IL-10). We also noted an increase in IFN-γ presence in the BALf, which strongly suggests that the Th1/Th2 balance of the lung had been modified by PTP inhibitors treatment.

In chapter 3, we pursued the investigation on PTPs modulation of asthma development by characterizing the effects of the reduction of individual PTP activities. We uncovered that the regular activity of PTP-1B is beneficial to reduce inflammatory cells recruitment, as we noted that PTP-1B-deficient mice had exacerbated inflammatory cells recruitment to the lungs. Reduction of PTP-PEST and SHP-1 activity did not affect asthma development in our model. On the other hand, reduction of TC-PTP activity markedly reduced asthma development as shown by a reduction in serum IgEs and reduced lung inflammation. Collectively, chapter 2 and 3 show the importance of PTPs for the development of immune response, and suggests that PTPs could be new therapeutic targets for asthma.

Therapeutic approaches are actually used or investigated for asthma and most of them interfere with extracellular events or with gene expression. For example, corticosteroids interfere with the expression of inflammatory genes under the control of certain

transcription factors like NF-κB. In this context, it is sometimes observed that the expression of proteins with pro-inflammatory properties is resistant to corticosteroid treatment. Namely, MRP8 (myeloid-related protein 8) or S100A8, an intracellular calcium-binding protein, has been observed to be maintained at high levels in subjects under corticosteroids therapy. MRP8 exerts strong chemotactic and pro-inflammatory functions on neutrophils, probably recruiting them to the site of inflammation. As neutrophil apoptosis is delayed by corticosteroids, they have more time to produce pro-inflammatory mediators, and most interestingly, neutrophils can secrete MRP8, which can actually represent 40% of neutrophils granules. This provides a positive feedback loop that avoids control by corticosteroids. As it is currently unknown how MPRs activate cell response, we hypothesized that a better characterisation of signal pathways triggered by MRPs would be beneficial to our understanding of their resistance to corticosteroid treatment and could eventually provide new therapeutic targets to prevent their detrimental pro-inflammatory effects.

Chapter 4 presents our results on the investigations of signalling pathways triggered by MRPs. We observed for the first time that MRPs induces nitric oxide (NO) production and we report that this induction is mediated through TLR-4. The signalling pathways triggered passes by MEK/ERK as well as JNK, which results in activation of NF-kB. We also observed a very potent synergistic effect between MRP stimulation and IFN-γ stimulation resulting in NO production and expression of TNF-α. This is the first report that depicts, from receptor to transcription factor, the signalling pathways used by MRPs to induce NO production in macrophages. This data will allow us to better understand the

effect of MRPs in regard of their corticosteroid-resistance, but also better understand the role of MRPs in other contexts, such as the induction of bactericidal functions of macrophages.

We believe that the work presented in this thesis challenges the vision of PTPs as non-specific dephosphorylating enzymes and contributes to confer them their rightful status of critical regulator of cell response and consequently, immune response. This work also opens possibilities for therapeutic approaches that would aim at modulating intracellular signalling in order to modify cell responses. Our results open many possibilities for future studies, beginning with the investigation of cell reactions that are critically affected by PTP inhibition. The identification of TC-PTP as a needed PTP for asthma development also invites to the elucidation of mechanisms that are under its control and that can affect the immune response so critically. We are also quite confident that our characterization of MRPs-induced pathways and the discovery of their induction of macrophages microbicidal functions will allow a better understanding of MRPs extracellular roles.

Chapter 1: Literature review

1. Abstract

Allergic asthma is a chronic inflammatory disease characterized by a Th2 type of inflammation. This disease is also characterized by the recruitment of inflammatory cells to the lung, such as lymphocytes, eosinophils and, in severe forms of the disease, neutrophils. These cells, and many others, play a role in the pathogenesis of the disease, and their interactions are needed for asthma development. However, in order to interact, cells have to integrate adequately the various signals of their environment. This integration of signals is mediated by various intracellular signalling pathways that sense environmental stimuli and transmit it to the nucleus for the generation of an adequate cell response. These signalling events are dependent on many protein interactions and heavily rely on protein phosphorylation, particularly protein tyrosine phosphorylation to affect proteins functions and transmit a signal to the nucleus. This review of literature will discuss important aspects of inflammation, allergic asthma, pharmacologic control of the symptoms and disease exacerbation. Then we will address the role of signalling pathways in the immune response leading to asthma and we will review how signalling pathways have been modulated to change the disease outcome.

2. Inflammation

In the development of many diseases or upon injuries, inflammation is regarded as a source of pain and is often counteracted by the use of anti-inflammatory drugs. For the public, it is viewed as a symptom to treat. This is obvious from the definition given in the Oxford English Dictionary: "Inflammation: a morbid process affecting some organ or part of the body, characterized by excessive heat, swelling, pain, and redness; also, a particular instance or occurrence of this". Even among immunologists, this vision of inflammation is well present. The classic immunology textbook "Immunobiology" by Janeway and colleagues describes inflammation as: "traditionally defined by the four Latin words *calor*, *dolor*, *rubor*, and *tumor*, meaning heat, pain, redness, and swelling, all of which reflect the effects of cytokines and other inflammatory mediators on the local blood vessels" (1). As it can be observed, the definitions are quite similar even if one is coming from a lay source and the other one from a scientific (immunologic) source. Interestingly though, the immunology textbook added the notion of inflammatory mediators and cytokines, showing that inflammation is not to be understood as a mere symptom, but as the result of a complex reaction of the organism to a trauma or an insult to its integrity. This shows that, even for specialists of immunology, inflammation is a phenomenon encompassing many concepts and cannot be expressed in a very precise form. Interestingly, these two definitions refer to inflammation as a manifestation of the most visible symptoms. Nevertheless, inflammation seen by fundamental immunologists would be better described by the actual events involved in inflammation and not the apparent result. In the present work, we got interested to the inflammation generated by the activation of the immune system. This inflammation can result from a breach in the

integrity of the body, or from an immune reaction against an agent that passes the physical barriers of the organism or yet from an acquired sensitivity to agents that are recognized by the immune system. Such immune responses can take many forms and are usually adapted to the insult suffered by the body (2). In the case of a scratch in the skin, the response will indeed involve the classically described heat, pain, redness, and swelling. The necrosis of the epithelium will activate an immune reaction leading to the dilatation of the blood vessels (redness), increased effusion of liquids in the tissue (swelling), increased blood flow (heat and redness) as well as an increased stimulation of pain sensors by the mediators released by the immune and local cells (pain) (2). In the case of internal organs, inflammation exists, but will not show these classical features, revealing the limitations of this definition.

There exist many different inflammatory contexts, depending on the trigger. For example, inflammation can ensue a bacterial pneumonia (3), an autoimmune reaction like type-1 diabetes (4) or an allergic reaction (5). In each case, the ensuing inflammation is expressed differently as the trigger is not the same. In this work, we have been interested by the mechanisms involved in the inflammation observed after an allergic sensitization, often referred to as allergic inflammation.

Allergic inflammation is also commonly referred to as a Th2 inflammation. This is due to the fact that allergies are immune reactions involving a predominant response from the Th2 arm of the immune system (6). Many types of allergic inflammations exist: asthma, hay fever, eczema, urticaria, food allergies and anaphylaxis (2). All these disorders have in common to be IgE-mediated, which is also referred as Type 1 hypersensitivity (1;7).

The Th2 aspect of this immune response is visible by the fact that IgEs are produced by B cells upon the stimulation of Th2 polarized T cells. T cells of the Th2 profile can induce B cells to adopt the production of IgEs, while other types of T cells would have favoured other types of immunoglobulins (2). In all of the mentioned allergies, the disorder is triggered by the recognition of an allergen by the IgEs bound to a receptor on mast cells. This ligation cross-links the receptor, which triggers mast cell degranulation, initiating the inflammatory reaction (2).

Although some properties seem to be common in allergens (8), the definition of an allergen remains a functional one: "an allergen is an antigen that induces an allergic reaction in sensitized individuals but not in others" (2). As an allergen needs to induce the cross-linking of the IgE receptor on the mast cell, it implies that they have to contain more than one epitope per allergen molecule (referred to as a multivalent antigen), or at least to aggregate together in order to exhibit many epitopes (2). In atopic dermatitis, or eczema and in acute urticaria, the allergen induces IgE-dependant cutaneous inflammation (9), although it seems that not all forms of urticaria have an allergic nature (10). Food allergy and anaphylaxis are also triggered through IgE recognition of an allergen, but the inflammation now occurs inside the body, not on the skin (2). It is noteworthy that while asthma and hay fever both depend on inhalable allergens, hay fever is an acute form of inflammation, while asthma is usually chronic with acute exacerbations (2;11).

As asthma is a chronic disease, there is a long-term immunological reaction happening in the lung, which leads to tissue disruption and destruction. This damage to the lung tissue

is then followed by tissue repair and potentially tissue remodelling, which alters the physiology of the lung and induces changes in the respiratory capacity and reactivity (12). This complex situation of chronicity with acute exacerbations and tissue destruction/remodelling renders this disease difficult to characterize and obviously creates difficulties in adopting effective therapeutic approaches. In consequence, asthma is one of the most studied allergic inflammatory disorders, but still has no cure.

3. Asthma

The last Canadian Asthma Consensus Guidelines describes asthma as following:

> *Asthma is characterized by paroxysmal or persistent symptoms such as dyspnea, chest tightness, wheezing, sputum production and cough associated with variable airflow limitations and a variable degree of airway hyper-responsiveness to endogenous or exogenous stimuli. Inflammation and its resultant effects on airway structure are considered the main mechanisms leading to the development and maintenance of asthma (13).*

While this definition is adequate for medical purposes, it does not discuss the implication of immunological events in the disease, although it acknowledges the preponderant role of inflammation. From an immunological standpoint, asthma is a chronic inflammatory disorder presenting an increase prevalence of Th2 cytokines and Th2 T cells (14). This does not exclude a role for Th1 cells and Th1 cytokines, but highlights that the Th2 response is predominant. Interestingly, Th1 immune response in established asthma

induces exacerbation of the disease (15). This demonstrates the primary role of inflammation regardless of the polarization of the immune response in the established disease. Asthma can be divided in two groups if we consider the allergic origin of the disease: there is extrinsic or atopic asthma, that is an allergic response with an increase in IgE serum titres and intrinsic or non-atopic asthma, that does not exhibit an increase in IgEs and presumably does not result from allergy (16). An allergic origin can usually be found in 80% of asthma patients, which makes extrinsic asthma the most prevalent form of the disease. This type of asthma will be referred as allergic asthma in this text. Both allergic and non-allergic asthma shows very similar symptoms and characteristics if IgE titre is not considered. Again, this highlights the importance of inflammatory processes in asthma development, regardless of the origin of inflammation (16).

3.1. Hallmarks of allergic asthma

The phase of early bronchoconstriction after allergen exposition is probably the best known aspect of the disease, but many others exist that are observed even when there is no acute exacerbation of the disease. Here are reviewed some of these characteristics.

3.1.a. IgEs

In a first place, we can consider the IgE levels. The presence of IgEs in the serum of a subject reflects its previous sensitization to one or more allergens. An increase in IgE levels in an individual, without an apparent reason, does not necessarily imply an allergy, but is referred to as atopy (7). Atopy is defined by this increase in IgEs, but is not necessarily linked to a disease condition, although atopics have a higher prevalence of

allergy and asthma (17). On the other hand, as allergic asthma is dependent on a sensitization to an allergen, asthmatic (allergic) subjects are therefore also atopics as they exhibit high IgE titre. IgE levels are usually high in allergic asthma and allergen-specific IgEs can be used to identify the allergen that causes the allergic reaction, often helping the subject to reduce its asthma exacerbations and improve its quality of life. On the other hand, allergen avoidance does not guarantee an improvement in chronic asthma as the inflammation often appear to also be self-perpetuating and therefore persist even in absence of allergen re-exposition (18).

The increase in serum IgE concentrations depends on the antibody isotype switching in antibody-secreting B cells (19). While IFN-γ favours the isotype switching towards IgG_{2a} (mouse) and TGF-β towards IgA, IL-4 is a potent inducer of isotype switching towards IgE (and IgG_1 in mouse or IgG_4 in human) (20;21). The produced IgE will then bind the IgE high affinity receptors (FcεR1) located on many cells such as basophils and mast cells (2). These cells are now said to be "sensitized" to the allergen recognized by the IgE. Before the binding of allergen-specific IgEs, these cells were unable to "sense" the presence of the allergen. The term sensitized is important in the context of allergies, as it is used to described the status of immunization toward the allergen: a subject is said to be sensitized to the allergen, the same way that we would have said another subject is immunized to an antigen (a vaccine for example, involving protective IgG production). Interestingly, the predisposition of atopic subjects to have an elevated IgE titre seems to be influenced by genetic components (22;22-24). For example, variations in HLA class 3 have been linked to atopy (25). Variations in chemokine receptors have been described

(26), as well as variations in leukotriene receptors (27). Other genetic associations have been made with some components of IL-4 and IL-13 signalling, such as STAT-6 (28), IL-13Rα1 (29) and other components (30). While all of these variations have been noted, they often need to be regrouped in order to have a significant effect on the outcome of atopy or asthma (30), demonstrating that atopy and asthma are multigenic diseases.

After allergen sensitization, subsequent allergen challenges can increase IgE levels (31), which can worsen the reaction observed at the next allergen encounter. Using the primordial role of IgEs in the development of lung allergic disease, drugs were actually developed to counteract the biological events mediated through IgEs, by using monoclonal anti-IgEs (32). In fundamental research, serum IgE levels are a common and practical tool to assess the allergic sensitization of the subject or the degree of successive exposure in a chronic model of allergen exposition (33-36). It is often useful to differentiate between total serum IgEs and Ag-specific IgE in order to ascertain that what is observed is an effect specific to the Ag used for immunization and not an atopic reaction (where total IgE would be increased, but not allergen-specific IgEs). The two levels also allow us to evaluate the proportion of allergen-specific IgEs in the total IgE pool.

3.1.b. Immune cells involved in asthma

Inflammation in the asthmatic lung is responsible for the recruitment of inflammatory cells. In animal models, cells of the lung are available through a bronchoalveolar lavage (BAL) with physiologic solution. Analysis of the retrieved BAL fluid (BALf) shows that the normal lung lumen will contain a proportion of more than 95% alveolar macrophages

(AMs), less than 5% lymphocytes and less than 1% eosinophils and neutrophils (36). These proportions change in the asthmatic lung where AMs can range 50-80%, lymphocytes 5-25%, eosinophils 5-25% while neutrophil presence should remain negligible (35). However, other cells are important in the lung, even if they are not recruited freshly but are rather residents of the lung tissue. Of them, mast cells (37) and dendritic (38) cells are very important cells.

Lymphocytes

Lymphocytes are present in the normal lung in small proportions, usually around 5% in the BALB/c mouse model (39). Upon sensitization and further allergen challenge, lymphocyte presence is accentuated (39). The Th1/Th2 polarization of T cells in asthma is skewed toward the Th2 profile (40) and studies now suggest that specific chemokine receptors are expressed on Th2 clones (41), facilitating their preferential recruitment to the allergic lung. The importance of lymphocytes in asthma development was clearly established by Dr Martin's team at McGill University as well as by other groups (42-44). The adoptive transfer of lymphocytes from sensitized rat to a naive rat conferred cardinal asthma features of asthma to the naïve rat upon allergen challenge (42-44). The CD4+ T cells were taken from an allergic rat and transferred to a naive one before allergen challenge was performed. This transfer allowed asthmatic features such as lung eosinophilia and increase in lung resistance to be observed in the naive rat (45). Although these experiments did not reveal which antigen presenting cell (APC) is needed to induce Th2 lymphocytes in the first place, it clearly showed that CD4+ T cells were sufficient for establishing the critical airway features of the disease.

With the increasing interest in regulatory T cells (Tregs), the role of lymphocytes in asthma is now being reviewed to include the possibility that the regulatory functions of this subset of T cells is modulating asthma development (46). As their role is expected to result in reduction of immune reactions, they might be important for reducing the inflammation observed in allergies. In a recent report, it was observed in dermatophagoides-sensitive asthmatics, that the percentage of Tregs in the peripheral blood mononuclear cells (PBMC) pool was increased, but that their suppressive capacity was actually reduced (47), suggesting a decreased regulation of the immune response in asthma. This reduced suppressive effect was normalized after allergen immunotherapy (47), showing in a first time that immunotherapy can induce Treg activity, and second, that this Treg activity can reduce asthma. In a similar model, the resolution of acute reaction to an allergen of *Dermatophagoides pteronyssinus*, Der p1, was linked to the activity of Tregs as their depletion exacerbated the allergen challenge and prevented the timely resolution of the allergen challenge (48).

The implication of Tregs in the resolution of allergic inflammation and the implication of Th2 T cells in the pathology of asthma does not reveal which APC is actually inducing the allergen-specific t cells.

Dendritic cells

The APC needed for induction of response to the allergen remained controversial for a while. But strong indications that DCs were important for antigen presentation in the lung were provided by the work of Dr Holt's team which reported already in 2001 that human DCs were recruited to the *lamina propria* in biopsy sections of humans challenged with

the allergen Der p1 (49). They also showed that DCs form a dense network below the basal membrane (50), locating DCs in the right environment to pick up allergens. Others have shown that DCs can open the epithelial tight junctions to expand their dendrites toward the airway lumen across the epithelium while preserving the integrity of the tight junction by expressing occludin, claudin-1 and zonula occludens-1 which are tight junction proteins (51). This enables DCs to sample the lumen of the airways and therefore encounter inhaled allergens. Upon encounter of the allergen, the immature DC, that has a high phagocytic activity, will engulf the allergen (52) and its subsequent migration depends on its maturation.

In the context of allergies, the trigger for maturation is not yet clear and we can only speculate that microenvironment events release a "danger signals" in this context. Interestingly, Eisenbarth and colleagues reported that intra-nasal administration of ovalbumin (OVA) to mice resulted in different immunological responses depending on the level of lipopolysaccharide (LPS) that was co-administered during intra-nasal instillation of the ovalbumin. If the OVA used contained no LPS, allergy to OVA was unable to develop. If low doses of LPS were found in the OVA (0,1 μg), allergies developed and the immune response was of a Th2 nature (increase of IL-5 and IL-13, presence of eosinophils). Mice in which high dose of LPS was used (100 μg) showed no apparent allergy and had signs of a Th1 immune response (IFN-γ and neutrophils) (53). This study elegantly shows the importance of small amounts of maturation-inducing substances for establishment of immune responses that depend on DC to induce them. It also brings molecular evidence to the hygiene hypothesis initially proposed by Strachan

(54) and then expanded (55), which draws a link between hygienic societies (low LPS levels) and the increase in allergies. So although the precise reason why some individuals develop allergies while others do not is complex and still eludes clear understanding, the role of DCs and their antigen presentation capacity are accepted to be instrumental to allergen sensitization (52). Upon encounter with the allergen, the DC will phagocytose it and after proper stimulation it will mature and migrate to the lymph node, in the T cell rich area, where it will be able to induce proliferation of allergen-specific T cells, mounting an immune response that results in allergen sensitization (52;56). The polarisation of the T cell clone toward Th1 or Th2 is then dependant on the microenvironment created by the DC and is also influenced by the formation of the immunological synapse (57). It has been reported that the molecular configuration of the immunological synapse between the DC and the T cell is important in the polarisation of the T cell. Co-polarisation of the IFN-γR with the TCR/MHC complex would favour Th1 expansion while IL-4R and TCR/MHC complex co-polarisation would favour Th2 expansion (57). It also appears that distinct subsets of DCs may induce different polarisation in T cells.

Monocyte-derived DCs were shown to induce Th1 T cells while plasmacytoid-derived DCs were observed to induce preferentially Th2 T cells (58). A similar observation was made regarding CD8+ and CD8- DCs where CD8+ directed the immune response toward Th1 while Th2 was favoured by CD8- DCs (59). This capacity of CD8+ or CD8- DCs was found to be dependent on the expression of cytokines from these two subsets, IL-12 and IFN-γ being crucial for CD8+ DCs and IL-10 for CD8- DCs (60). Apparently, more

plasmacytoid DCs circulate in human peripheral blood of asthmatics (in ratio with myeloid DCs) as compared with normal subjects (61). But as appealing as it might seem, upon influenza virus infection, plasmacytoid DCs were observed to induce a Th1 response, which strongly suggests that although DC subsets have a predisposition in their response, this is dependant on stimulation and microenvironment (62). Although the mechanisms of induction of a Th2 clonal expansion by DCs remains to be investigated more thoroughly, it has reached consensus that DCs are necessary for allergic sensitization. Interestingly, it was observed that even in the allergen challenge post sensitization, DCs in the lung could have an important role to play in inducing local immune reactions (38;52). Even though DCs exhibit such a potent effect on the induction of immune responses in the lung, other APCs do exist in the lung and their activity is also modulated in asthma.

Macrophages

Macrophages (Mθs) are present across the whole body and patrol most organs. Their phenotype vary depending on the microenvironment in which they reside (7). Historically, they even got different names depending in which organ they were found. For example, conjunctive tissues have histiocytes, the liver hosts Kupffer cells, mesangial phagocytes are found in the kidney, microglial cells in the brain and the osteoclasts are macrophage-like cells of the bone (2;7). In the lungs, Mθs found in the lumen of the airways are called Alveolar Macrophages (AMs) while Mθs found deeper in the lung parenchyma are called interstitial macrophages (IM). IMs have a reduced phagocytic capacity when compared to AMs, they are smaller and also adhere less than AMs (63;64). Some of their surface markers are different and consequent to their reduced phagocytic

capacity, IMs express less lysosome (63). Only AMs are accessible by bronchoalveolar lavage (BAL) and most studies investigate this population. They can represent more than 85% of BAL-extracted cells in rodents (35) and around 90% in humans (65;66).

Although AMs have a high phagocytic activity, they have not been shown to migrate to the lymph nodes upon stimulation and phagocytosis. AMs do not express sufficient CD80/CD86 proteins at their surface to induce the co-signal to T cells (67). This antigen presentation capacity is therefore probably insufficient to allow allergen sensitization, an observation coherent with the data involving DCs in this process (52). Interestingly, it has been previously observed that MΘs tend to favour a Th1 response upon allergen stimulation, while induction of a Th2 response relied mostly on DCs (68). Supporting this concept, the depletion of AMs before allergen challenge allowed a stronger increase in IgE levels than if AMs were present (69).

In previous studies, we showed that AMs from normal subjects prevent development of AHR (70), which shows that theses cells nonetheless have an important immunomodulatory role. We previously reported that AMs from asthmatic subjects were observed to adopt an alternative activation state in the allergic lung due to the Th2 environment (36). This alternative activation is not an irreversible process and extracting AMs from this environment reveals to be sufficient to release AMs from this alternative activation and confer them back their capacity to modulate AHR (36). To add to complexity of AMs role in the lung homeostasis, we also reported that human AMs produce IL-4 (71), the prototypic Th2 cytokine, suggesting that they could be the initial

source of Th2 skewing in the lung, or at least that once influenced by the Th2 environment of the allergic lung, they could actively support this type of response.

AMs can also participate in allergic inflammation by their activation through the low affinity IgE receptor (FcεR2) (72). This receptor is usually weakly expressed on these cells, but AMs from asthmatic subjects have an increased expression (73). Finally, it is worth mentioning that free radicals (reactive oxygen species (ROS) and nitric oxide (NO)) production by AMs is increased in asthma (74), potentially collaborating to the increased exhaled NO observed in asthma (75).

Mast cells

Mast cells are generated from CD34+ progenitors (76) and are found in their mature form in mucosal and conjunctive tissues (reviewed in (37)). They have long been recognized to play an important role in the early asthmatic response (77). It is through IgEs that mast cells can react to allergen presence in the environment. Upon encounter of the allergen, mast cells will recognize it by their bound IgEs. This binding will cause the aggregation of the FcεR1 receptors if the allergen is multivalent (pre-requisite for FcεR1 activation) and the aggregation will cause the degranulation of the mast cells. Mast cell degranulation is the initial event of bronchoconstriction that occurs in asthma (2;7). When they degranulate upon recognition of the allergen, mast cells release histamine, which is one of the major bronchoconstrictor molecules that induce the early asthmatic response. Rapidly after degranulation, mast cells synthesize *de novo* lipid mediators as prostaglandine D_2 (PGD_2) and leukotriene C_4 (LTC_4), as well as platelet-activating factor (PAF) (reviewed in (37)). These mediators strongly favours inflammation and can support the

bronchoconstriction as well (37;77). Later on (after hours), mast cells will also secrete cytokines, supporting the Th2 inflammation, namely IL-4 (78) and IL-13 (79). The subsequent recruitment of immune cells as lymphocytes and eosinophils is a second critical step in asthma, which is initiated by mast cells pro-inflammatory functions.

Eosinophils

Eosinophils are the classical cellular hallmark of asthma (reviewed in (19)). They are not usually observed in the healthy lung, but are found in the asthmatic lung in the vast majority of cases (80). Upon the original recruitment of other inflammatory cells such as lymphocytes, a strong Th2 environment settles and among the important cytokines produced by the Th2 lymphocytes and the concurrent Th2 inflammation, IL-5 is noticeable for recruiting and supporting eosinophil survival, which are usually short-lived (81). Lung eosinophilia is a classical hallmark of asthma and eosinophils can be found both in lung tissue and in the bronchoalveolar lavage fluid (BALf) (82). Eosinophils have an important baggage of proteases and can produce lipid mediators as well as cytokines (reviewed in (77;83)). Release of these mediators in the lung environment upon eosinophil degranulation or through their secretion can strongly enhance the actual inflammation. For example eosinophils degranulate proteases that have an important effect on the lung. The eosinophil cationic protein (ECP), eosinophil peroxidase (EPO) and the major basic protein (MBP) are important proteases released by the eosinophils that damage surrounding tissues, potentially altering the epithelial barrier (84). MBP is known to participate to lung inflammation by damaging the epithelial barrier and it can act directly on the airway smooth muscle to cause their contraction (85). Lipid mediators are also released by eosinophils, such as PAF, LTC_4 and PGE_2. The PAF (86) and LTC_4

(87) are bronchoconstrictors and participate in the generation of a second phase of bronchoconstriction, known as the delayed phase or late asthmatic response (LAR). Although eosinophils are generally recognized as a hallmark of asthma (19), it has been observed that AHR can be generated in their absence, casting doubts on their role in AHR development (88). In absence of eosinophils, neutrophils are often observed to be increased (89). Although the reason of this different recruitment of neutrophils instead of eosinophils might reveal an external cause to the observed asthma, the proteases of the neutrophils are also deleterious for the host and can probably compensate the absence of eosinophil's proteases.

Neutrophils

It was previously unusual to discuss the involvement of neutrophils in asthma, but it is now fairly clear that some subsets of asthma exhibit an important neutrophilia (89). Neutrophils are not usually observed in the lung of mild asthmatics, but are sometimes observed in the lung of severe asthma patients (90;91). Most strikingly, they are observed in the lung of patients who died of an acute and fatal exacerbation of asthma (status asthmaticus) (92). Neutrophils can produce a plethora of mediators that can be very deleterious in the context of the lung. For example, the proteases released by neutrophils can have adverse effects on the epithelium (93): elastase, collagenase and matrix metalloprotease 9 (MMP-9). Of these, elastase exhibit very severe effects: it can break down collagen type 3, forcing the remodelling of this collagen layer; it can proteolyse surfactant proteins, that have important roles both for the surface tension of the mucus and as signalling molecules; and it can finally also activate various matrix metalloproteases (93). Neutrophils can also produce lipid mediators like LTA_4 and LTB_4,

PAF and thromboxane A_2. Interestingly, PAF is a bronchoconstrictor agent (77), conferring to neutrophils the capacity to affect important physiological processes like smooth muscle cells contraction. They are also a source of pro-inflammatory cytokines (IL-1β, IL-6, IL-8 and TNF-α). Notably, they also produce ROS and NO (93). All of it confers to neutrophils an important potential to damage the lung tissue. It was previously thought that their short survival in the tissue could prevent them from inducing severe damage in the lungs, but more recently, it was reported that glucocorticosteroids enhance neutrophil survival by inhibiting their apoptosis (94). This is further interesting, as neutrophilia is increasingly observed in subjects treated with glucocorticosteroids (90;91), probably due to their longer survival upon treatment. Interestingly, not only neutrophils are resistant to glucocorticosteroid treatment, but the levels of S100A8 proteins are also resistant to glucocorticosteroid treatment (95). As this protein (aka myeloid-related protein 8 –MRP8) is produced by neutrophils, where it can constitute up to 40% a of the cell granule content long with the related MRP14 (96), it is interesting to speculate that there is a link between the increased survival of neutrophils to glucocorticosteroids treatment and the resistance of S100A8 to treatment to the same drug.

A role for MRPs in neutrophil presence in the asthmatic lung

MRPs are known under various names: S100A8 is MRP8 or calgranulin A while S100A9 is MRP14 or calgranulin B and their heterodimer is called MRP8/14, S100A8/A9 or calprotectin (97). They were previously identified as intracellular cytoplasmic proteins with a calcium-binding capacity, of a size of 10,8 kDa for S100A8 and 13,2 kDa for S100A9 (98). Their intracellular role is not yet clear, but it is speculated that they could inhibit casein kinase 1 and 2, therefore inhibiting the phosphorylation of many proteins

important for metabolic activity of the cell. This hypothesis is very appealing as it could involve MRPs in the events leading to a short lifespan in neutrophils (98). It is further interesting if we consider that MRPs can form 40% of neutrophil proteins while it is less than 1% in monocytes and that these two cell types have very different longevity (98). More recently, S100A8 was reported to inhibit telomerase activity, revealing another intracellular role (99). Of outstanding interest is the report that S100A9 is implicated in the reorganisation of microtubules in Mθs and granulocytes (100). This last data is interesting because it would suggest that MRPs act both at the intracellular level and at the extracellular level to modulate cell chemotactism. As a matter of fact, one of the main extracellular roles discovered to date for MRPs is the induction of chemotactism in neutrophils (101). Although it is MRPs antibacterial capacity (102) that was first noted, their chemotactic capacity is now drawing the attention (103;104). MRP8 has been observed to activate neutrophil chemotaxis (105) while both MRP8 and MRP14 are known to have important roles in the induction of inflammation (106-109). It was also shown that inhibition of MRP8 and MRP14 by antibody injection prevented neutrophil migration in response to LPS injection in an air pouch model (110), clearly establishing their role in neutrophil recruitment.

3.1.c. Bronchoconstriction

Bronchoconstriction is probably the most remarkable hallmark of asthma. It is the feature that anyone can notice in an acute exacerbation of asthma, commonly referred to as an "asthma crisis". Although this episode is the one mostly known, asthma actually consist of two phases of bronchoconstriction, this first one, part of the early asthmatic response (EAR) and a second one three to eight hours later, called the late asthmatic response

(LAR) (111). This is summarized in Figure 1. The bronchoconstriction is due to the narrowing of the airways by the contraction of the smooth muscle cells that are in the airways. This contraction reduces the diameter of the airway lumen and therefore limits the flow of air (111). While causing laborious breathing and wheezing this increase in airways contraction can also be clinically observed by a diminution of the forced expiratory volume in 1 second (FEV_1), as the contraction of the airways does not allow the same flow of air to exit the lung (111). The first bronchoconstriction is induced seconds to minutes after the inhalation of the allergen when it cross-links the IgEs that are bound to the FcεR1 receptor of mast cells that degranulate and release bronchoconstrictor agents such as histamine (37;111). It is a transient bronchoconstriction that usually resolves by itself, but that can also be resolved by the use of medication.

The second bronchoconstriction is happening usually three to eight hours later and is not as sudden, but it can reduce the FEV_1 by as much as 60% (111). This second phase is generally thought to be due to the inflammation induced by mast cells degranulation, such as the recruitment of new inflammatory cells that release mediators that have bronchoconstrictive potential (leukotrienes, prostaglandines...) (37;77;111).

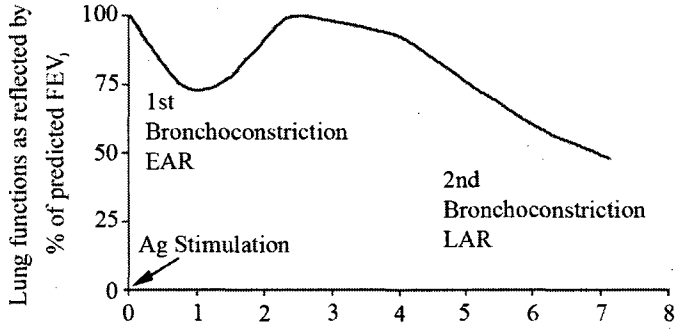

Figure 1: *Events of bronchoconstriction in asthma. Adapted from Nadel and Busse 1998 (111).*

3.1.d. Airway hyperresponsiveness

Airway hyperresponsiveness (AHR) is the predisposition of a lung to react in an exaggerated manner to a bronchoconstriction stimulus (reviewed in (112)). It has been suggested that the observed AHR is divided in two components, a variable component and a persistent component (112). According to this theory, the variable component is dependent on the exacerbation of lung inflammatory status while the persistent component is dependent on lung remodelling that increases sensitivity to direct smooth muscle stimulation of contraction. Allegedly, both components can be evaluated by different stimulations: a direct stimulation with a bronchoconstrictor agent (methacholine or acetylcholine) will reveal more of the persistent component while stimulation with an indirect stimulus as AMP will involve the inflammation and reveal more of the variable component (112). It was reported already in 1976 that bacterial or viral infections can increase the hypersensitivity of airways to bronchoconstriction (113), which would depend on the variable component of AHR. The persistent AHR on the other hand could depend predominantly on the lung restructuration, that happens in allergic asthma due to the chronicity of the disease, which is called lung remodelling (114). Consequently, the method that would preferentially measure this persistent AHR component would be a direct stimulation of the smooth muscle cells (SMCs) that cause the contraction with agents such as methacholine or acetylcholine (112). For practical purpose, as airway remodelling is largely due to damage induced by previous exacerbations of the disease and consequently of exacerbations of inflammation, AHR in its whole can be considered to reflect the status of past and present lung inflammation (112). If the measurement of airway responsiveness is made with a direct stimuli (methacholine, acetylcholine) during

active inflammation, it will express the global AHR of the lungs as during inflammation SMCs are more sensitive to contraction due to inflammatory stimulus (115). This approach is experimentally the most followed, as AHR measurements are taken shortly after allergen challenge, when inflammation is still exacerbated, but measured by the administration of increasing methacholine doses.

AHR is defined in humans by the dose of methacholine needed to diminish the forced expiratory volume in one second (FEV_1) by 20%, which is called the provocative challenge causing 20% decrease in FEV_1, or PC_{20}. Asthmatics are usually identified by a PC_{20} value of 8 mg/ml of methacholine or less while normal subjects should have a value higher than 8 mg/ml, normally even more than 16 mg/ml (116). This PC_{20} value usually correlates with asthma severity (116). This logically implies that asthmatics are hyperreactors, but we should keep in mind that hyperreactors are not necessarily asthmatics. In animal models, AHR is usually measured by the dose of methacholine needed to double lung resistance (35) or by the increase in the Pause enhancement (Penh) value (117). In Balb/c mice, these 2 measures correlate well and Penh can be used as representative of the lung resistance. Lung resistance is usually measured by the evaluation of the volume of air displaced out of the lungs in a defined time, while the Penh value is derived from the change in the flow of exhaled air. As lung resistance is affecting the flow of air that can be exhaled, both measurements do correlate if other variables are not affecting the system. Hamelmann et al. showed the reliable use of Penh in the Balb/c model (118), but in other animal models, the correlation has not necessarily be found to be reliable.

3.1.e. Nitric oxide production

Being an inflammatory disorder, asthma is characterized by an increase in many inflammatory mediators (reviewed in (119)). The presence of NO in exhaled air is now increasingly used to assess the status of inflammation as its presence is increased in asthmatics and usually inversely correlates with disease control (119). NO is synthesized by NO synthases (NOS) through the oxidation of L-arginine to L-citrulline (reviewed in (120)). The synthesis of NO made by NOS1 (neuronalNOS-nNOS) and NOS3 (endothelialNOS-eNOS) is calcium-dependant, requiring calmodulin, while NOS2 (inducibleNOS-iNOS) is not dependent on calcium increase (120;121). NOS 1 and 3 produce lower concentrations of NO (pM), while NOS2 catalyses the formation of greater concentrations (μM) of NO (121).

NO had controversial beginnings, as it was found to exhibit very similar biological activities to endothelium-derived relaxation factor (122), which history finally identified to be the same molecule (123-125). It is therefore interesting to note that now recognized as a potent anti-microbial compound produced in inflammatory situations (126), NO was first known for its physiological relaxation role (123). Therefore, it is important to remember that out of immunological field, the vasodilatator and bronchodilatator capacity of NO is of important physiological consideration (120;124;127). These beneficial effects of NO on vasodilatation and bronchodilatation are usually observed with low concentrations of NO, presumably through the synthesis from NOS1 or NOS3, while high concentrations of NO usually through NOS2 can lead to its pro-inflammatory activities and to nitrosylation of proteins (126).

In the context of asthma, increased NO in exhaled breath is a well conserved characteristic feature in asthmatics (75). Allergen challenge can even further increase NO in exhaled breath (128). Exhaled NO is believed to be a very reliable marker for the status of lung inflammation (129;130) and it is now reliably used to assess asthma control in patients (129;130) in order to get a quantifiable and non-biased evaluation. The measurement of the fraction of exhaled NO (Fe_{NO}) inversely correlates lung capacity (FEV_1) but correlates with AHR and eosinophilia (131-133). The source of this high amount of NO in the lung is not yet clear, but it is known that inflammatory cells such as Mθs have an increased expression of NOS2 in the asthmatic lung (121) and that lung epithelial cells also exhibit a stronger expression of NOS2 (134;135). High concentrations of NO increases the likelihood of NO to react with superoxide anions to form peroxynitrite, which can cause the nytrosilation of various proteins and therefore induce tissue damage (136;136;137). It also seems that NO exhibit some anti-inflammatory properties in the immune system that could be beneficial in the context of asthma. Among others, NO can downregulate Mθ production of pro-inflammatory cytokines (121;138;139).

Now, the final effect of NO in the asthmatic lung is still unravelled: although it can be beneficial for its vasodilatation and bronchodilatation or for a down-regulation of some inflammatory capacities, the peroxynitrates formed upon reaction with superoxide ions can damage the tissue. The conclusion that can be drawn about NO is that it is dependent on subjacent inflammation and that usually, reduction of inflammation by corticosteroid can reduce NO in exhaled air (90).

3.2. Th2 inflammation

As previously stated, asthma is a chronic inflammatory disease involving a Th2 polarized immune response (19). Although the role of Th2 inflammation is decisive for the support of chronic inflammation, it is unsure how the immune response is initially polarized toward this response. Some investigations in animal models even reported establishment of asthma-like phenotype by viral infection, in absence of allergen, suggesting a non-Th2 initiation of the disease (140). In this model, it appeared that viral infection and lung repair mechanisms were sufficient to induce long-term AHR, as observed in asthma. It remains true that Th2 immune response is more important in asthma and that such a response is involved in the maintenance of the disease (141), but the initiation of the disease possibly does not rely on an event that would be Th2 polarizing. The nature of the disease has probably more to do with inflammation and inflammation-induced damage, than with the original immune polarization. Indeed, immune stimulations, even of Th1 profile, can exacerbate asthma in allergic subjects. This is true in animal models where exposition to bacterial stimulation with *Mycobacterium vaccae* or Bacille Calmette-Guérin leads to exacerbation of symptoms (15;142). This is also observed in humans where viral infections exacerbate asthma symptoms (143). It then appears that the beneficial effect of bacterial exposition is in young age, or at least before establishment of allergy in animal models (55).

It is important to keep in mind that the polarisation of Th1/Th2 response was originally observed in murine T cells by Mosmann and Coffman in 1986 (144). They noticed this polarization by a differential pattern of cytokine secretion by different clones of T cells.

Since then, this concept has been applied largely to the immune system, but it is useful to keep in mind that while other cells can contribute to the polarization or can affect it. Only T cells have been reliably classified in this dichotomic scheme, a feature greatly dependent on the activity of different transcription factors: T-bet for Th1 clones and GATA-3 for Th2 clones (145). When discussing the Th1/Th2 status of an immune response, it is valid to discuss of the output of the response: the produced mediators. But that does not imply that only one type of T cells is playing a role in the observed effect: the two subsets could be involved in the response, but one subtype will predominate.

When they were first described in mouse (144;146), then in humans (147), Th1 and Th2 T cells were observed to produce different cytokine patterns. Th1 cells produced IL-2, IFN-γ, GM-CSF and IL-3 while Th2 cells produced IL-3 and B cell stimulation factor 1 (BSF1) (144). BSF1 is now known to be IL-4 (148) and it is now recognized that type 1 response is characterized by IL-2, IFN-γ and lymphotoxin while type 2 is characterized by IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 production (149). In humans, the patterns are similar, but IL-2, IL-6, IL-10 and IL-13 are also produced by other cell types (149), which confer other cell types the possibility to modulate Th1/Th2 balance. Here we will review quickly the various roles of these prototypical Th1/Th2 cytokines (summarized in Table 1).

3.2.a. Th1 cytokines

IFN-γ

IFN-γ is the classical Th1 type cytokine. It shows antagonistic effects on the development of Th2 clones as it inhibits their proliferation (150), but it can't inhibit Th1 proliferation as these clones do not have the IFN-γR-β subunit. The absence of the receptor renders them insensitive to IFN-γ-mediated inhibition of proliferation (151). IFN-γ is also antagonising IL-4 production, which confers a potent inhibitory effect on Th2 response to this cytokine (152). IFN-γ is mostly produced by NK cells, lymphocytes and to a lesser extent by cells of monocytic lineage such as Mθs (152). IFN-γ is primordial in Mθ activation: it is a strong inducer of Mθ microbicidal functions and upon IFN-γ stimulation, Mθs increase their antigen processing as well as their antigen presentation (153). Although they are very sensitive to IFN-γ, Mθs produce minute amounts of this cytokine, reducing the possibility of autocrine activation. On the other hand, IFN-γ induces production of IL-12 in Mθs, which stimulates production of IFN-γ from T cells, creating a feedback loop (152). It has been shown experimentally that an increased expression of IFN-γ in a murine model decreases the severity of asthma (154).

IL-12

IL-12 is the most representative Th1 cytokine that is not produced by T cells, but by Mθs, monocytes, DCs, neutrophils and B cells (2). It is an active dimer of the two monomeric sub-units p35 and p40 (152). Active IL-12 is therefore often referred to as IL-12 p70. The regulation of IL-12 production is made at the level of the expression of the p40 subunit

which can be induced while the p35 subunit is not inducible (152). The role of IL-12 in the support of a Th1 response passes through the induction of IFN-γ from T cells, but also through an inhibition of IgE synthesis that is usually induced by IL-4 (155;156).

3.2.b. Th2 cytokines

IL-4

In pathologic situations, the Th2 cytokines produced by the lymphocytes are instrumental to the installation of an effective lung Th2 environment and to the maintenance of this Th2 inflammation. IL-4 is the prototypic Th2 cytokine as it is a differentiation factor for naïve CD4+ T cells (157). Interestingly, IL-4 is also a factor for growth of other Th2 inflammation cells as mast cells and B cells and enhances their *in vitro* survival (158;159). Furthermore, IL-4 has the potential to inhibit the expansion of Th1 T cells, highlighting its potent effect in promoting Th2 polarization of the immune response (152). Its secretion by Th2 cells also induces IgE class switching in B cells (160), an effect that was long thought to be exclusive to IL-4, but that has been demonstrated to also be induced by other pathways (161). IL-4 expression is increased in PBMCs of asthmatics, which correlates well with an increase in Th2 inflammation (162). IL-4 is sharing many common effects with IL-13, another prototypic Th2 cytokine, which is now better understood as their respective receptors use a common subunit. Indeed, the IL-4Rα is used in both receptors, IL-4R (IL-4Rα and γ_c) and IL-13R (IL-4Rα and IL-13Rα1) (163).

IL-5

Th2 T cells also produce IL-5, which induces eosinophils recruitment to the lung and increases their survival (152;164). By increasing eosinophils survival, lymphocytes support the inflammation caused by these cells and contribute to eosinophils role in both late phase reaction and AHR (77). Eosinophils have important beneficial effector functions in a Th2 response against helminths (165), but in asthma, their activation has been found to be partly responsible for the lung inflammation (77).

IL-10

IL-10 is increasingly known for its anti-inflammatory functions, but it is also important for the establishment and maintenance of the Th2 response (152). One of the ways that IL-10 promotes Th2 response is by inhibiting preferentially the production of Th1 cytokines by Th1 clones over the production of Th2 cytokines by Th2 clones, which is not significantly modulated (166). Interestingly, the same group observed later that IL-10 was able to inhibit the production of proinflammatory mediators from Mθs (IL-1α and -β, IL-6, IL-8, IL-12, GM-CSF, G-CSF, M-CSF, TNFα, MIP-1α and -1β, MIP-2, RANTES and LIF) (167-169), which clearly illustrates the potent anti-inflammatory role of IL-10. Its capacity to down-regulate pro-inflammatory functions of cells that would support a Th1 response also indirectly supports the Th2 response, for example, by orienting Mθs toward an alternative activation pattern (170). This cytokine has also been described to favour Th2 development by its requirement in the induction of Th2 clones by CD8- DCs (60). But the roles of IL-10 are complex and its presence has been observed to limit the survival of granulocytes, including eosinphil populations, which is not typically a Th2

function (152). Lymphocytes apart, IL-10 is mainly produced by AMs in the lung (81). Finally, although IL-10 roles are complex, it is generally accepted that it exhibits an important support to Th2 immune responses, in mice, although it is not a consensus in humans (171).

IL-13

IL-13 is also a very characteristic Th2 cytokine and shares many effects with IL-4, due to their common use of the IL-4Rα (163). Although IL-13 does not directly act on Th1 clones to inhibit the Th1 response as IL-4 does (152), it can still counter-act the Th1 response as it can prevent the production of many pro-inflammatory cytokines (IL-1α, IL-1β, TNF-α, and IL-8), but also the production of IL-12 (163). IL-13 is able to induce class switching to IgEs and can inhibit Th1 cytokines production (172). The promotion of many physiological roles are also attributed to IL-13: AHR (12); airway inflammation (12); airway remodelling (12) and mucus hypersecretion (173). Upon injection of an anti-IL-13 monoclonal antibody, these features of asthma are abolished (12).

In the establishment of the chronic Th2 inflammation observed in asthma, the interactions between many mediators, such as those presented, creates the decisive chronic Th2 inflammation. The maintenance of a Th2 response is also a characteristic feature of the disease, which suggests a deregulation in the immune system as normally the immune response should slowly disappear and re-equilibrate.

Table 1: *Overview of selected Th1 and Th2 cytokines.*

Cytokine	Support Th1/Th2	Mechanism
IFN-γ	Th1	Inhibition of Th2 clones expansion (150). Activates Mθ inflammatory functions (153).
IL-12	Th1	Induction of IFN-γ production by Th1 clones (155). Inhibition of IgE production (156).
IL-4	Th2	Induction of isotype switching to IgE (160). Inhibition of Th1 clones proliferation (152).
IL-5	Th2	Activates eosinophils recruited to the lungs and increases eosinophil survival (152).
IL-10	Th2	Preferential inhibition of Th1 cytokines (166).
IL-13	Th2	Promotion of airway hyperresponsiveness, airway inflammation and airway remodelling (12).

4. Pharmacological control of asthma

It is useful for the understanding of the disease to discuss briefly the pharmacologic approaches used to control asthma. In the treatment for asthma, one must keep in mind that presently, there is no cure: there is no intervention that heals the disease, but control of symptoms can be achieved. Rather, the current treatments aim at controlling the exacerbations (asthma crisis) that a person has, and to reduce the inflammation of the lung.

In a first time, for intermittent asthma, guidelines recommend the use of short acting β-agonists, or SABA (174;175). This medication is used by inhalation and can be used *pro re nata* (PRN or as needed) (174;175).

In a second step, for subjects where asthma is not intermittent but persistent, the recommendations advise the use of therapy for prevention of symptoms on a daily basis. In a first attempt of treatment, the administration of low-dose inhaled corticosteroid (ICS) is advised. If unsuccessful alone, this ICS treatment can be given along with leukotriene receptor antagonists (LTRAs), Cromolyn or Theophylline (174;175). Depending on the response of the subject, it is then advised to increase the dose of ICS and to use long-acting β-agonists (LABA). In such cases, Zileuton can be added to treatment. For subjects where the symptoms are difficult to control, it is possible to administer high-dose ICS, plus LABA and oral corticosteroid (therefore systematic delivery of these corticosteroids). If the patient has allergies, it is also possible to use Omalizumab, a humanized anti-IgE, monoclonal antibody (174;175). Figure 2 shows the general scheme of treatment. The effects of the different classes of drugs will now be discussed briefly.

4.1. Short acting β-agonists (SABA) and long-acting β -agonists (LABA)

In situations where asthma is exacerbated and there is bronchoconstriction, it is important to relieve quickly this bronchoconstriction to allow normal airflow. This is where the β_2-agonists come into play, in the form of inhaled drugs. β_2-agonists are able to relax the contraction of smooth muscle cells through the stimulation of the β_2-adrenoceptors (176). β_2-adrenoceptors are G-coupled proteins that can quickly signal in the cell and induce the relaxation of the smooth muscle cell, therefore reducing the airway contraction (176). Their use is usually prescribed as needed for SABA, or they are administered regularly in case of moderate to severe asthma in the form of LABA, to relieve the more predominant contraction sometimes leading to chest tightness (174;175). Their administration is made by inhalation to insure a deliver to the lungs.

4.2. Inhaled corticosteroids (ICS)

ICS can be administered in various doses, depending on the need of the subject, but the low dose is prescribed if symptoms to control are not severe (174;175). The corticosteroids are anti-inflammatory drugs that are administered daily to maintain the level of inflammation low, in order to prevent exacerbation and worsening of the disease (175). The mode of action involves the activation of the glucocorticoid receptors (GRs) (177). Activation of the receptors results in their translocation to the nucleus and their binding to glucocorticoid receptor elements (GRE), a binding that activates the transcription of anti-inflammatory genes or repress the transcription of pro-inflammatory genes. GRs can also bind co-activators, such as CBP (cAMP-response element-binding protein), preventing their interaction with pro-inflammatory transcription factors such as NF-κB (177). For specific lung activity, they can be delivered by inhalation. They now form the fundamental treatment of asthma and the dose given will vary depending on the severity of the disease (174;175;177).

4.3. Cromolyn

Cromolyn is used in asthma since 1967 shortly after it was first synthesized (178). Early experiments showed its potency to inhibit asthma in subjects with severe asthmatic disease (179). It was discovered to be a mast cell membrane stabilizer (179-181). Therefore Cromolyn prevents mast cell degranulation, which consequently prevents the cascade of events leading to asthma exacerbation upon encounter of the allergen. As Cromolyn shows similar efficacy to corticosteroids, but mostly act on the acute phase of allergen encounter, corticosteroid treatment is considered in the first line of medication.

Corticosteroid can then be complemented with cromolyn if needed in individuals that do not control their asthma exacerbations with corticosteroids alone (174;175;182).

4.4. Leukotriene receptor agonists (LTRAs) and Zileuton

Leukotrienes are lipid mediators that are quickly generated after mast cell activation (37). They have important roles in inflammation in general, but LTC_4, LTD4 and LTE_4 are of special interest in asthma as they also have potent bronchoconstriction effect (LTC_4 and LTD_4 being a thousand fold more potent than histamine for similar molarities (183)). Moreover, their presence in asthma seems to be insensitive to corticosteroid treatment (reviewed in (184)). Viewing the important effects of LTs in the asthmatic response, designer drugs have been prepared to inhibit the activity of their receptors (185). The inhibition of LTRs results in bronchodilatation, reduction of inflammation and reduction of AHR (185). Like Cromolyn, these drugs are used to complement the effects of ICS (174;175) and essentially aim at reducing the inflammatory response engaged at allergen exposition.

Zileuton acts on the same mechanisms, but at the level of inhibition of LT synthesis, by inhibiting the activity of the 5-lipoxygenase (184). The 5-lipoxygenase is responsible for converting arachidonic acid, allowing its further transformation to various LTs (184). The rationale of its use in asthma is very similar to the one of LTRs (174;175).

4.5. Theophylline

Theophylline is used in asthma treatment for more than seventy years (186). This product shows bronchodilatator properties as well as anti-inflammatory properties (186). One of

the drawbacks of this medication resides in the observed side-effects: nausea and headaches happen at recommended doses (186). Also, their anti-inflammatory capacity is not as important as the one of ICS and their bronchodilatation effect is not as potent as β_2-agonists (186). Their incorporation in a regime of ICS treatment has been reported to be equivalent to doubling the ICS dose, which confers this drug a sound use as a complementary treatment in cases where ICS at low doses is not sufficient (174;175;187).

4.6. Omalizumab

Omalizumab is a humanized monoclonal $IgG_1\kappa$ antibody that can bind free IgEs, but not FcεR-bound IgEs, therefore does not induce receptor activation (188). Its use can down-regulate FcεR1 expression on mast cells, basophils and dendritic cells (189), reducing the exacerbations linked to the allergen exposition. The prescribed use of Omalizumab is in subjects where allergies are known to contribute to the disease (189), if the disease severity is moderate to severe (174;175). It is now recognized as a potent complementary drug and showed to permit a diminution in the dose of corticosteroids (189).

This review of the drugs used to treat asthma highlights that current treatment aims at releasing the symptoms, but that not much can be done to stop the disease itself. For allergic asthmatics, there is increasing interest in the use of immunotherapy to modulate the allergen reactivity and reduce the allergic component (190).

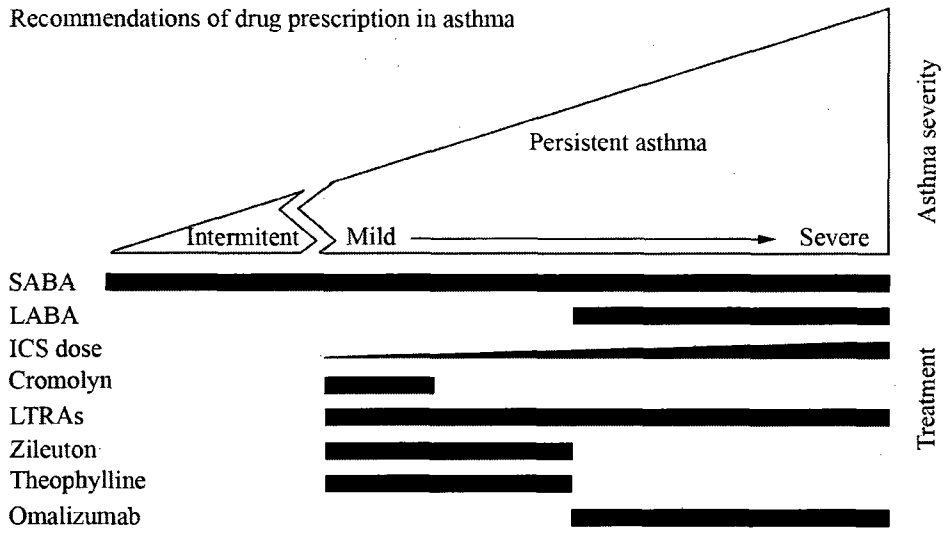

Figure 2: *Summary of therapies used for the treatment of asthma. Adapted from Becker and colleagues; Schatz and colleagues (174;175).*

5. Exacerbations of the disease

Whether asthma is medically controlled or not, it is possible to experience asthma exacerbation: an event in which an asthmatic subject gets bronchoconstriction following a trigger event. This is usually resolved in medicated patients by the use of bronchodilatators (β_2-agonists), and can also vanish with time. In the case of severe asthma exacerbations, or status asthmaticus, this exacerbation is not controlled by the usual medication (191). This is actually the definition of status asthmaticus: *unresponsiveness of the exacerbation to repeated courses of β_2-agonist therapy or epinephrine* (191). As can be imagined, such exacerbations do require hospitalization and close surveillance of patients. In about 50% of the cases, there is a concomitant infection in the patient's lungs, that probably served as a trigger for the exacerbation (191). It is interesting to note that when the trigger of the exacerbation is found to be a respiratory

tract infection, an increase in neutrophils can be found in the sputum (hypertonic solution-induced spitting of high trachea content) of patients (192). But it is also noticed that neutrophils are present in situations where an infectious agent was not found, suggesting that neutrophils are a hallmark of such acute exacerbations (92;191).

Many studies divided these severe asthma exacerbation in slow-onset and sudden onset. While slow-onset is hypothesized to be initiated by inadequate medication, inadequate compliance to the medication or to psychological factors, the sudden-onset is hypothesized to depend on exposition to various allergen insults or to many unidentified triggers (193). Other studies showed that the predominance of neutrophils in the BALf (193;194) and bronchiole parenchyma (193;195) of patients appears to be in the sudden-onset groups, while the slow-onset group have an increase in their lung eosinophilia, but no significant modulation of their neutrophilia (196;197). This situation suggests that neutrophilia is due to an increased inflammatory status in the lung (not necessarily allergen-mediated). What is the causative agent of such an inflammatory exacerbation remains largely unknown (193).

Severe acute exacerbations are life-threatening for the patient and often the inflammation does not respond well to medication (191;193). It was found that corticosteroid treatment of severe acute exacerbation patients reduced their eosinophilia (196;198), but was unable to reduce their neutrophilia and the presence of neutrophil-related proteins such as the neutrophil elastase (196;198). Very interestingly, it was previously described that neutrophil survival is increased by corticosteroid treatment (90;94), providing a possible explanation on the increased presence of neutrophils in the lung during these

exacerbations even though corticosteroid treatment is performed. Although the presence of neutrophils is increased in severe asthma and in some subtypes of acute exacerbations, the role of this neutrophilia is not clear, but is most probably sustaining inflammation (199).

6. Control of immune reactions by intracellular signalling.

As the development of any immune response relies on the appropriate interaction of immune cells in order to obtain a systemic reaction that will be adapted to the triggering event, the cellular response to environmental stimuli is of primordial importance. The possibility to modify cell response by interfering with intracellular signalling events attracted researchers in the perspective to affect the global immune response and prevent the development of asthma.

In this context, we previously discussed the importance of various cell types and their mediators in orchestrating the reaction to allergens that will lead to the Th2 inflammation observed in asthma. In turn, the interactions of these cells are highly dependent on cell-based sensing and analysis of various environmental signals transmitted in the organism. For example, a Mθ would sense an intruding bacteria (Gram negative) through the ligation of its TLR4 receptor (200). This ligation will in turn trigger the activation of an intracellular signalling cascade resulting in the expression of pro-inflammatory genes. Cells are constantly stimulated in this manner by a plethora of environmental stimuli, and the way cells integrate all of the signals decides of their response to these extracellular stimulations. It is also important to consider the regulation of these signalling pathways,

because receptor-induced signal must stop in absence of trigger. In order to achieve this, a variety of mechanisms exist, from internalisation of the receptor for recycling (or degradation), to dephosphorylation of activated proteins (201). In this next section, we will review the signalling pathways involved in the mechanisms mostly targeted by the experimental attempts to modulate asthma development.

6.1. Induction of immune responses requires induction and control of the intracellular signal.

In the unfolding of events leading to asthma, many processes can be affected by alteration of the intracellular signalling pathways. In this section, we review some of the most important events involved in the establishment of allergic inflammation in order to highlight how the immune reaction can be manipulated by modifying the signalling pathways at these critical steps.

6.1.a. Regulation of signal in APCs

As we discussed previously, the induction of an allergic response to an allergen requires this allergen to be taken up by an APC, which needs to be activated in order to stimulate an immune response (1;2). Usually performed by DCs, the antigen uptake activates the DC only if it has intrinsic ability to bind activating receptors such as the TLRs (200). It is why allergen sensitization with OVA in animal models is commonly performed with a co-administration of the alum adjuvant, to enhance the response to OVA (202). Therefore, in animal models, we make use of alum to modify the usual response to OVA and favour the Th2 response in order to get a sensitization against this antigen. The use of OVA given intranasally does not induce asthma even after repeated challenges, unless the

administered OVA contains a minimum level of endotoxins (202). A similar picture arises with intra-tracheal administration of OVA where the immune response will be oriented toward Th1 or Th2 depending on the dose of endotoxins present in the OVA preparation (53). Eisenbarth and colleagues observed that low levels of endotoxins were pushing the immune reaction toward the Th2 profile, while high levels of endotoxins were favouring the Th1 profile (53). This shows the necessity of a signal from activating receptors, like TLRs. This signal can be modulated by a modification of intracellular signals, for example by inhibiting it to completely abolish immunization towards OVA, or to increase TLR signal to skew the reaction towards Th1.

6.1.b. Intracellular signal of the T cell

Another relevant example is the integration of signals from T cells. It is known that the signal from the TCR is not sufficient to drive activation of T cells (1;2;7). The signal of co-stimulatory molecules must also be sensed by the T cell, otherwise naïve T cells are driven to anergy, a state of non-responsiveness to subsequent stimulations (1;2;7). Of particular relevance in asthma, Kuchroo and colleagues have reported that the co-signal generated by APCs will influence the polarization of the T cell toward either Th1 or Th2 profile depending on which B7 molecule is engaged (203). They originally reported that B7.1 (CD80) co-stimulation favoured the development of Th1 T cells while B7.2 (CD86) co-stimulation favoured the development of Th2 clones (203), suggesting that the signals received by the naïve Th cell are different and lead to a different cell fate. Since these original findings, other groups examined the question and did not always reproduce the observations (204). Schweitzer and colleagues reported that the situation was more complex: while CD80 and CD86 have no noticeable effects in optimal situation (high

antigen dose with optimal quantity of APCs), the role of CD80 and CD86 ligation can be noticed at sub-optimal conditions. In such context, both molecules do play a role in the production of both subsets of Th cells (204). They noticed though that CD86 had a prominent effect on both responses while CD80 presence had less impact on the intensity of IL-4 or IFN-γ production in their experiments (204). These findings were further investigated and it was found that B7 co-stimulation with both CD80 and CD86 was important for the development of T regs populations (204). Therefore, experiments with cells deficient for the expression of one or both molecules were affected by the lack of Tregs, which could help explain the discrepancies between some experiments. Modulating the intensity and nature of the co-signal seems to be affecting the outcome of the polarization of Th cells. Upon a precise characterization of this mechanism, it is realistic to think that modulating the underlying signalling pathways could affect the outcome of this immunological event.

Once T cells are polarized, their actual response to a stimulus can still vary if cells are not of the same type. One very interesting finding was brought in 2002 by Hannier and colleagues (205). In a first set of experiments, they showed that upon TCR ligation, Th1 clones were expressing more CD25 than Th2 clones, a marker of effector T cell activation. But more interestingly for our context, they observed that Th1 and Th2 cell lines were not exhibiting the same activation of intracellular signal upon TCR engagement. Upon TCR stimulation (α-CD3), Th1 cell lines exhibited stronger phosphotyrosine content than Th2 cell lines, suggesting a different intensity of signal. They repeated this experiment with Th clones and observed the same phenomenon. They

also used both TCR and co-stimulation (α-CD3 and α-CD28) which resulted in similar observations (205). This clearly suggested that Th1/Th2 polarization results in a different reorganization of intracellular signalling events and that this difference can be exploited to modulate the reaction of T cells.

The regulation of the T cell response is also critically regulated by the activation of its TCR. The TCR of Th cells consist of the αβ heterodimer receptor plus three CD3 proteins (2ε, 1δ) and two ζ homodimers (206). The ligation of the TCR (concomitant to CD4 or CD8) induces the activity of Lck and Fyn that phosphorylate ITAMs in the TCR (206). The phosphorylated immuno-tyrosine based activation motifs (ITAMs) of the ζ chain of the TCR allow the recruitment of ZAP-70, which phosphorylates LAT (linker for activation of T cells). LAT is now able to bind PLCγ1, Grb2 and the Grb2-related adaptor downstream of Shc (Gads). This association then allows PLCγ1 to produce DAG and IP_3 from PIP_2. DAG activates PKC which will lead to NF-κB activation while IP_3 leads to the release of Ca^{2+} from intracellular stores, which activates calcineurin, responsible for NFAT activation and translocation (2;206). Grb2 recruitment by LAT will lead to the ERK MAPK activation through activation of the Ras/Raf pathway (2;206). Gads recruitment on the other hand will lead to activation of RhoA, involved in the reorganization of actin structures (2;206) (summarized in Figure 3). Of interest, the early events of this TCR signalling are strongly dependent on tyrosine phosphorylation, as Lck, Fyn and ZAP-70 are PTKs and have obligatory roles in this system (206).

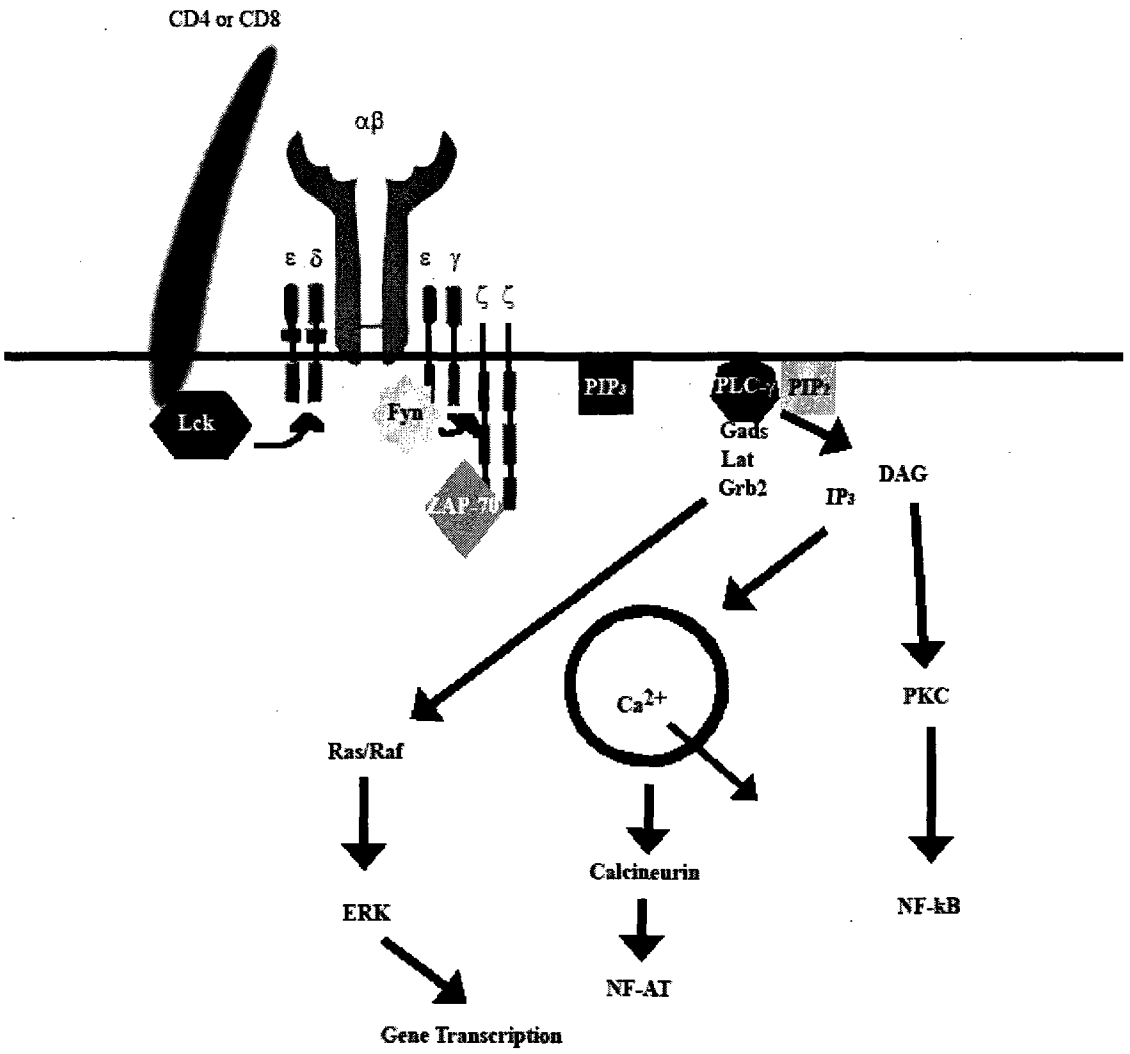

Figure 3: *TCR signalling. Adapted from Mak and colleagues; Mustelin and colleagues (2;206).*

6.1.c. Intracellular signalling of mast cells

Obviously, as asthma is a disease involving an adaptive immune response, the interactions between APCs and T cells are of importance for the induction of the immune

response and the control of these interactions by adequate intracellular signalling is primordial. But we must also consider the signalling pathways triggered in other immune cells. The mast cell is another important mediator of asthma and its reaction to immune stimulations is strongly mediated by its intracellular signal. Mast cells have high affinity IgE receptors (FcεR1) that bind IgEs, substantially increasing the half-life of these circulating IgEs (2). It is classically composed of three different subunits: $\alpha_1\beta_1\gamma_2$ (2). Upon encounter of a multivalent allergen, these IgEs will be aggregated and intracellular signalling will be induced, which results in mast cell degranulation (2). This process is critical for asthma exacerbation, but also highly depends on intracellular signal. The signalling pathways engaged in the signal transmission from the FcεR1 receptor to the nucleus in order to generate a response also provide an interesting possibility for modulation.

Initially, the FcεR1 is crosslinked by an allergen recognized by the bound IgEs. This will induce phosphorylation of the tyrosil residues of ITAMs found on the β and γ subunits of the FcεR1 by the cytoplasmic, receptor-associated, tyrosine kinase Lyn (207). These phosphorylated ITAMs create SH2 docking sites that can now be bound by the SH2 domains of Syk (on the γ subunit), another PTK (208). The association of Syk SH2 domains to the ITAMS induces a change of conformation in the kinase and Syk auto-phosphorylates its activation loop, gaining complete enzymatic activity (209). Syk in turn activates many other proteins, as LAT, PLC–γ1, PLC-γ2, SLP-76 and Vav (207). Concomitantly, receptor aggregation induces the activity of Fyn PTK, which phosphorylates Gab2, an adaptor protein that has no catalytic activity, but serves to

recruit PI3K to the membrane-proximal area. PI3K is responsible for the production of PIP_3, which diffuses in the membrane and recruits other proteins such as Btk and PLC-γ to the membrane region through their PH domain (207). PLC-γ is then involved in the formation of IP_3 and DAG from PIP_2 at the membrane level. IP3 induces the release of Ca^{2+} from internal stores, while DAG activates PKC, involved in NF-κB activation (2). The activated LAT, SLP-76 and Btk are responsible for a sustained Ca^{2+} influx needed for exocytosis of granules. Syk will also activate the pathway of Vav, Grb2 and Sos, which are responsible for G-protein Ras activation. Vav promotes activation of the G-protein Rac. Ras and Rac pathways will lead to downstream activation of cytokines production and production of arachidonic acid. Ras signals through the activation of the MAPK ERK, while Rac passes through the JNK MAPK (207) (summarized in Figure 4).

We saw through this simplified picture of the FcεR1 how the initial FcεR1 ligation can result in the degranulation of pre-formed histamine, formation of lipid mediators from arachidonic acid and later production of cytokines by signal amplification. It is important to note that the early events of this signal depend on the action of protein tyrosine kinases (PTKs), such as Lyn and Syk. All of these biochemical processes happen in the cell and their fine-tuning is primordial for appropriate cell response. At the beginning of this reaction, investigators reported a role for CD45, a protein tyrosine phosphatase (PTP) (210). It appears that Lyn harbours a tyrosine residue that inhibits its activity when phosphorylated and that CD45 is necessary to keep this site non-phosphorylated in order to maintain Lyn activity (210;211), adding a level of regulation to the described pathways.

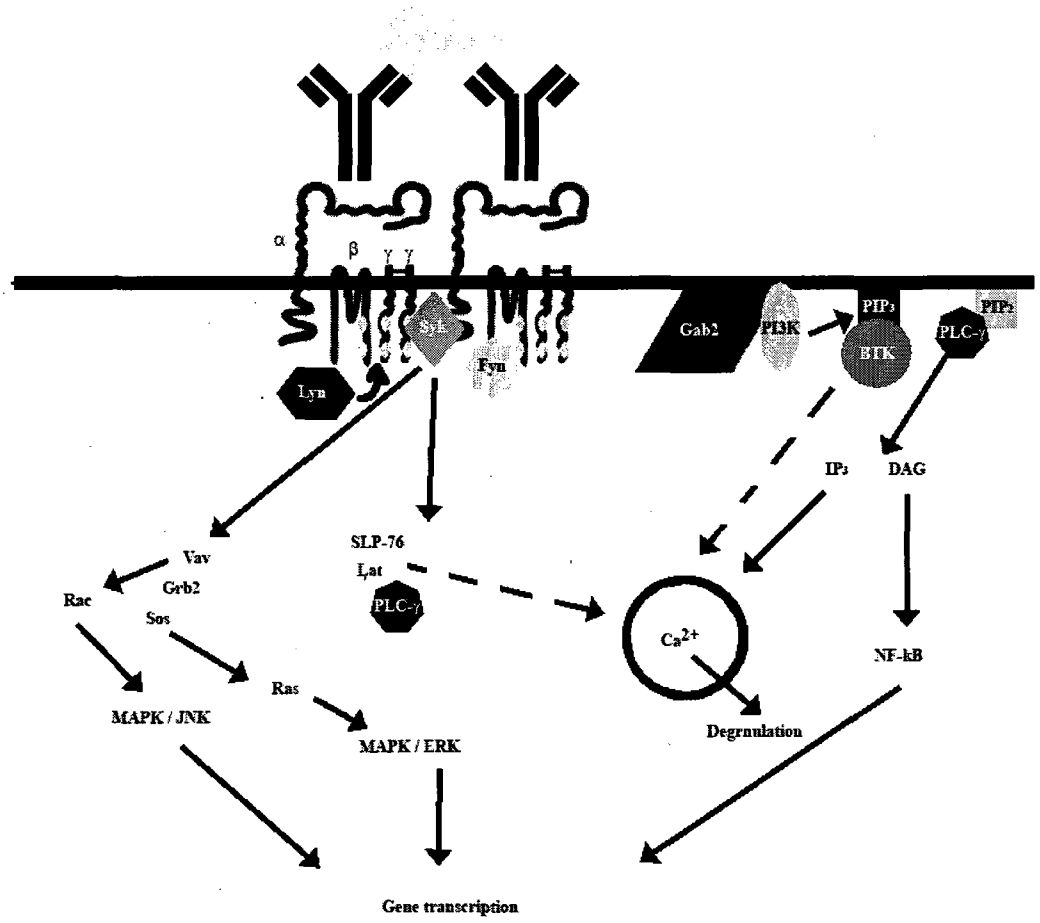

Figure 4: *FcεR1 signalling. Adapted from Mak and colleagues; Siraganian and colleagues (2;207).*

It should now appear obvious, through the examples given, that intracellular signalling involving tyrosine phosphorylation is of prime importance in the proper development of an immune response, whether this immune response is leading to protection or is detrimental to the host. The next section will address the role of tyrosine phosphorylation in the control of immune reactions.

6.2. Implication of tyrosine phosphorylation in the regulation of immune responses.

Protein phosphorylation is a mechanism commonly used to modulate protein activity (201). This phenomenon was first observed with the enzyme phosphorylase, which exists in two interconvertible forms, *a*, which is active and *b* which is inactive. At the time, co-factors were discovered and the regulation of many enzyme activities was linked to the presence or absence of the co-factor. In their seminal experiment, Krebs and Fischer added radiolabelled ATP to their phosphorylase preparation and observed the conversion from inactive *b* to active *a* form (212). Although the precise mechanism of phosphorylation was not clear at the moment, this is the first published demonstration that protein phosphorylation regulates protein activity.

The process of phosphorylation is now known to depend on protein kinase activity. Protein kinases add a phosphate group, taken from ATP, to specific sites on proteins. This phosphorylation can occur on three possible sites in proteins: on serine, threonine or tyrosine residues (201). These residues are of a neutral pH and have a polarity: due to this polarity, their positioning in proteins is usually toward the outside, allowing their interaction with kinases and phosphatases (201). The addition of a phosphate group adds a negative charge to the residue, which can modify protein conformation, possibly leading to a change of protein properties (201). This can confer protein activity, like in the discussed example of phosphorylase (213), but it can also modify the protein in order to create a site for protein interaction.

A clear example of such case is found with the phosphorylation of ITAMs. ITAM are motifs found in the intra-cytoplasmic area of cell transmembrane proteins, usually immunoglobulin-like receptors. These motifs have the consensus sequence: YxxI/L$x_{(6-12)}$YxxI/L (214). The two tyrosines of the conserved sequence are critical and can be phosphorylated. Their phosphorylation modifies locally the protein and this phosphorylated ITAM domain can now be recognized by other protein domains such as the src-homology 2 (SH2) domain, present on kinases like ZAP-70, Syk and Btk (2). These events of phosphorylation are often critical to the early steps of signalling and are tightly regulated by events of dephosphorylation as we will see later (215).

The events of phosphorylation are mediated by kinases, and phosphatases are responsible for the dephosphorylation of the proteins (201). Many cell processes are controlled by balance of these events, and the residue targeted for phosphorylation is not always the same, as mentioned previously. The phosphorylation events in the immune system make an extensive use of the tyrosil residue as targets for phosphorylation (215). The TCR signalling requires tyrosine phosphorylation to induce signalling, through the actions of ZAP-70, Lck and Fyn (216;217).

Interestingly, although it is crucial in the regulation of many other cellular and molecular processes, tyrosine phosphorylation was discovered only after the serine or threonine phosphorylation (218). In 1979, Eckhart and colleagues reported that the polyoma T antigen (middle T antigen) of the polyoma virus was becoming phosphorylated on a tyrosine residue, which, until then, has never been observed (218). Nowadays, the number

of known kinases has increased tremendously, reaching 518 kinases in the human genome, with 90 PTKs (219).

Table 2: *Classification of kinases. Adapted from Manning et al. 2002 (219).*

Group	Families of interest	Sub-families of interest	Number of members
AGC	PKA, PKB, PKC		63
CAMK	MAPKAK (MAPK activated kinase)		74
CK1			12
CMGC	MAPK	Erk, Jnk, p38	61
STE			47
TK (Tyrosine Kinase)	Jak, Src, Tec		90
TKL (Tyrosine Kinase-like)	IRAK		43
RGC			5
Atypical			40
Other			83

In 2002, Manning and colleagues published in Science a reference paper on the classification of kinases. They established their classification based on the sequence of their catalytic domains, then global sequence homology followed by domain structure (excluding pre-considered catalytic domain), biological functions and finally linked with classifications in other organisms (219). Their classification resulted in the formation of 10 groups further divided in families and subfamilies. The ten groups are: AGC, which contain the PKA, PKB and PKC families; CAMK, which contains the MAPKAPK

family; CK1; CMGM, containing the MAPK family which is further divided in ERK, JNK and p38 subfamilies; STE; Tyrosine Kinases, which contains the JAK, Src and Tec families; Tyrosine Kinase-like, which contains the IRAK family; RGC; Atypical Kinases and finally the Other kinases group (219) (see summary in Table 2).

6.2.a. Tyrosine phosphorylation: protein tyrosine kinases

Tyrosine phosphorylation is a pivotal element of immune responses, and this phosphorylation is logically performed by tyrosine kinases, members of the TK family of kinases. One of the best-characterized pathways is the JAK/STAT pathway, for example downstream of the IFN-γ receptor. In this case, the ligation of IFN-γ to its receptor (IFN-γR) will induce the activation of JAK1 and JAK2, which phosphorylate STAT1 on tyrosine residues. Phosphorylated STAT1 dimerize and translocate to the nucleus where it can bind to the gamma-interferon activation sequence (GAS) and induce gene expression (220). Although this is one of the canonical pathways identified to date, its signalling is not so simple, and other pathways interfere with this otherwise straightforward system. As a matter of fact, we and others have observed that STAT1 also needs to be phosphorylated on Serine 727 to gain full activation and this phosphorylation is achieved by ERK2, itself activated by Pyk2 after stimulation of the IFN-γR (221-223). Inhibition of this serine phosphorylation reduces STAT1 induction of transcription by up to 80%, showing an important, but not obligatory role for this serine phosphorylation in STAT1 biological activity (224). This example shows how pathways are intertwined and how PTKs can influence or can be influenced by other kinases (here MAPKs, which are serine-threonine kinases).

It was established for a long time that TCR activation results in increase of tyrosine phosphorylation before an increase in intracellular Ca^{2+} can be noticed (225;226). This can obviously be due to an increase of PTK activity, but recently, a better characterization of PTPs suggest that PTP activity reduction could also account for a significant part of this phenomenon (206;227;228). Indeed, protein phosphorylation is tightly regulated in space and time and PTP activity can be altered in order to favour phosphorylation or to decrease it (229). As discussed before, CD45 is important in FcεR1 signalling to keep Lyn in an active state (210). Apparently, CD45 plays a similar role in the TCR signalling but with the PTK Fyn and Lck. Fyn is kept in an active conformation by CD45 which dephosphorylates an inhibitory site (230). On the other side, Lck is kept inactive by a phosphotyrosine residue on the protein. This phosphotyrosine residue allows Lck SH2 domain to bind itself and therefore prevents ligation of Lck SH2 domain to the proper ITAM of the TCR complex. The role of CD45 in steady-state is therefore to remove this inhibitory phosphotyrosine (231). But upon triggering of TCR signal, CD45 is excluded of the area of TCR, which is most probably to prevent dephosphorylation of other necessary phosphorylation events. Consequently, segregation will also prevent that CD45 keep Lck active (232). It is therefore obvious that PTPs have an important role to play in the TCR signalling, and in other events.

6.2.b. Tyrosine phosphorylation and protein tyrosine phosphatases

PTPs are a large group of different proteins which share one essential domain: the PTP domain (215). With the exception of Eyes PTPs, that were shown to have a PTP domain relying on an aspartic acid-based PTP domain (233-235), all PTPs have an enzymatic activity that relies on a conserved cysteine residue (215). In 2004, Alonso and colleagues,

from Dr Mustelin's laboratory (215), reported a classification of PTPs, based on the sequence similarity of the PTP domain. One hundred seven human PTPs were identified in this classification. Of these, 11 show no PTP activity, 2 are mRNA phosphatases, 13 have their catalytic activity on inositol phospholipids, leaving 81 proteins that actively show a PTP activity (215). This amount of proteins is very similar to the number of PTKs previously reported (90 PTKs (219)), suggesting that both types of enzymes have similar substrate specificity. PTPs are divided in four classes and each class of PTPs contains PTPs that are expected to have evolved from a common ancestor. PTPs of Class1 have a cysteine-based PTP domain and are further subdivided in classical PTPs or VH1-like PTPs. Classical PTPs have a strict specificity for substrate as they only dephosphorylate tyrosine residues, while the VH1-like group show a double specificity as PTPs of this class can also dephosphorylate serine/threonine residues, mRNAs or phosphoinositols. The second class is a one PTP class, made for the low molecular weight PTP (LMPTP), which is also cysteine-based and exhibit a phosphotyrosine-exclusive PTP activity. Class three contains the CDC25 PTPs that are also cysteine-based, but show a phosphotyrosine/threonine activity. The final class four contains the 4 Eyes PTPs that have an aspartic acid-based PTP domain and show a specificity to phosphotyrosine and phosphoserine (233-235). This classification is summarized in Table 3. The other features of PTPs are quite heterogenous and presumably reflect the need of particular domains for their cellular activity.

In fact, many protein domains can be found on PTPs in addition to the PTP domain itself. In class 1 PTPs, the class can be sub-divided in receptor PTPs (RPTPs) and non-receptor PTPs (NRPTPs) or cytosolic PTPs. Obviously, the RPTPs contain minimally a PTP

domain and a transmembrane domain. For example, CD45 harbours three fibronectin-like domains. The terminal (membrane-distal) region of CD45 is highly glycosylated and is involved in the recognition of galectin-1 (236). The NRPTPs show a similar heterogeneity in the other protein domain that they contain. The prototypic PTP-1B does not have other particular domains, but contains some sequences useful for its activity. For instance, a hydrophobic stretch allows it to be retained in the endoplasmic reticulum while a nuclear localisation signal allows it to reach the nucleus (237). The PTPs SHP-1 and SHP-2 contain an important domain for their function: the SH2 domain. These PTPs actually contain two SH2 domains one C-terminal and an N-terminal. In the inactive conformation of the protein, the N-terminal SH2 domain sits in the catalytic domain of the protein, preventing its activity, but both SH2 domains have their phosphotyrosine binding section oriented outside, allowing them to find and bind phosphotyrosines, in activated ITIMs for example. Upon binding, this induces a change of conformation and the catalytic domain becomes available (238). In summary, although all PTPs share a catalytic site with a conserved high homology, the other domains or protein sequences are of prime importance for the regulation of their activity (215).

Although the human genome contains 107 PTPs, not all of them are expressed at the same time in a specified cell. In the T cells for example, it was found that around 45 PTPs are actually expressed, with about the same number in B cells (229). Their role in lymphocytes is better known than in many other cell types, and PTP involvement in the activation of T cells expresses the complexity of their regulation of signalling events and therefore of immune responses. In T cell activation, SHP-1 is reported to inhibit TCR signalling by binding to ITIM-containing receptors and dephosphorylating ZAP-70 as

well as Syk (239). On the other hand, SHP-2, a very similar PTP, has a positive regulatory role on TCR signalling. It has been reported to increase Ras and Src family kinase (SFK) activation through the dephosphorylation of PAG (phosphoprotein associated with glycosphingolipid-enriched microdomains) (240). By dephosphorylating PAG, SHP-2 prevents the recruitment of Csk that would phosphorylate SFK on an inhibitory tyrosine, therefore promoting SFK activity that is necessary for PLCγ1 activation and subsequent induction of Ras and Erk activation (240).

Table 3: *Classification of PTPs. Adapted from Alonso and al. (215)*

Class	Division	Sub-division	Substrate specificity	Cys/Asp-based PTP domain
Class 1	Classical	RPTPs	pTyrosine	Cysteine
		NRPTPs	pTyrosine	Cysteine
	Dual specifities/ VH1-like	MKPs	pTyrosine, pThreonine	Cysteine
		Atypical Dual specifities	pTyrosine, pThreonine, mRNA	Cysteine
		Slingshots	pSerine	Cysteine
		PRLs	pTyrosine	Cysteine
		CDC14s	pSerine, pThreonine	Cysteine
		PTENs	Phosphoinositides	Cysteine
		Myotubularins	PI3P	Cysteine
Class 2	LMPTP		pTyrosine	Cysteine
Class 3	CDC25		pTyrosine, pThreonine	Cysteine
Class 4	Eya		pTyrosine, pSerine	Aspartic acid

Inhibitors of protein tyrosine phosphatases

The fact that the PTP domain is a highly conserved feature of these proteins is an advantage that can be used for their inhibition. It is long known that vanadate can inhibit PTP activity (241), and sodium orthovanadate is regularly added to protein lysates in order to prevent dephosphorylation of phosphotyrosines in proteins lysates (221;241;242). This inhibition was shown to be competitive, probably due to a similar structure to the target phosphate group in the catalytic pocket and it was reversible by the addition of EDTA, which chelate vanadate (243). Based on the observation that hydrogen peroxide and vanadate had a stronger inhibitory activity on PTPs, Dr Posner's team established that this was due to formation of aqueous peroxovanadium complexes that had more potent activity than hydrogen peroxide or vanadate alone (244). Then, they produced various peroxovanadium compounds with variations in the ancillary ligands. The resulting compounds showed that the inhibitory activity was modulated by the group that was placed in the ancillary ligand position (see Fig. 5) (244). Among the produced compounds, bisperoxovanadium phenantroline (bpV(phen)) and bisperoxovanadium picolinic acid (bpV(pic)) showed a very potent inhibitory capacity on the dephosphorylation of the insulin receptor (244). The inhibitory effect of these compounds was at least 100 fold more important than the effect of vanadate alone (244). Of special interest, the effect of these compounds is not competitive and it showed to be permanent (243). This permanent inhibition of PTP catalytic activity was traced back to the oxidation of the critical cysteine residue in the PTP catalytic pocket, therefore preventing the activity of the enzyme (243). Our laboratory previously demonstrated that peroxovanadium can be used for long term administration in animals without apparition

of undesired effects (245;246). We showed it in a first set of experiments where the inhibitor was administered safely to mice for six weeks (42 days) (245). We later used the same bpV(phen) compound for 38 days without undesired side-effects (246). These long-term experiments demonstrate the therapeutic potential of peroxovanadium compounds.

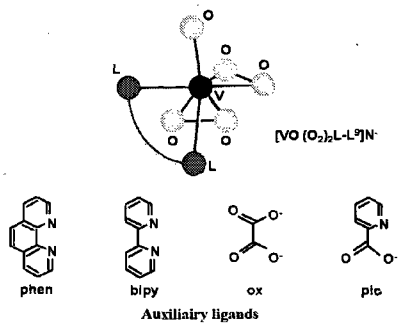

Figure 5: *Structure of peroxovanadium compounds.*

6.3. Inhibition of signalling pathways in asthma development.

As we discussed, signalling pathways involving phosphorylation and furthermore tyrosine phosphorylation are involved in almost all cellular events and their control is of critical importance for the proper functioning of cellular responses. In allergic inflammation, many attempts have been made to modify signalling events involved in the process of immune responses leading to asthma development. Most of these attempts have been made to inhibit the role of kinases involved in the various processes leading to asthma (247). Here we will have a quick overview of what has been attempted previously in the manipulation of intracellular pathways in asthma.

6.3.a. General Tyrosine kinases inhibition

Genistein is a broad-range inhibitor of PTKs and will inhibit PTKs at large with a varied efficacy. Its use in an animal model would reveal the functions that are PTK-dependant. Duan and colleagues (248) used this inhibitor in an experimental model of asthma in order to investigate the involvement of PTKs in disease development. Interestingly, they noticed a decrease in lung inflammation as reflected by a decrease in total inflammatory cells recruitment, including a reduction in eosinophils. They also observed a reduction in allergen-induced bronchoconstriction and AHR, two characteristics that are dependent on the inflammation induced by the allergen challenge.

6.3.b. Tyrosine kinases inhibition

Specific inhibitors for PTKs have been synthesized and some of them have been tried in animal models of asthma. Adachi and colleagues were interested to inhibit Lyn activity downstream of the IL-5R, in order to inhibit the signal of this receptor in eosinophils. They used a Lyn blocking peptide, which was synthesized upon the Lyn-binding site of the IL-5R. Injection of this blocking peptide inhibited the binding of Lyn to the IL-5R. It reduced eosinophil survival and prevented eosinophilia, presumably through the usual effect of Lyn in IL-5R signalling (249). This shows an interesting very specific inhibition of Lyn function, resulting in prevention of a particular trait of asthma.

As opposed to what is usually observed with Lyn peptide inhibitor, a deficiency in Lyn cause more severe asthmatic disorder (250). Complete deficiency of Lyn in mice

sensitized to Ova and further challenged with it intensifies lung eosinophilia, mast cell degranulation and bronchospasm while it also increases IgE levels in the serum. Interestingly, DCs have been found to exhibit an immature phenotype in these mice and they preferentially polarize T cells to the Th2 profile (250). This striking difference between Lyn inhibitory peptide and the deficient mice probably reflect the difference between an acute inhibition using the inhibitor specific for both Lyn and the IL-5R and the effect of life-long inhibition of all Lyn activities in the deficient mice. These results are also in striking contrast with the inhibition of many other kinases, but nicely demonstrate the limited knowledge that we have on the fine tuning of immune reactions by the balance of phosphorylation. It also suggests that phosphatases could also be implicated in events where they were not classically expected to interact.

Using an inhibitor of Syk PTK, it was shown *in vitro* that mast cell degranulation can be inhibited (251). It also appears that other mast cells functions (production of IL-13, TNF, IL-2 and IL-6) were inhibited by the use of this inhibitor (251). When administered *in vivo*, the Syk inhibitor was able to limit lung eosinophilia, as well as goblet cell hyperplasia and development of AHR, clearly suggesting an early inhibition of asthma symptoms, consistent with its action on mast cells *in vitro* (251).

The JAK family of PTKs is well known to mediate the signal downstream of many cytokines receptors. Kumano and colleagues (252) reported that JAK2 inhibition with Ag490 prevents eosinophilia in the lung by altering IL-5R signalling. They found that IL-5 production was normal, but that eosinophils were unresponsive to this cytokine (252). Working on a different family member, others have shown that inhibition of JAK3 also

resulted in a decreased eosinophilia, and a decrease in AHR post allergen challenge. This inhibition was proposed to be dependent on the inhibition of mast cell leukotriene production, for which they showed a dependence on JAK3 activity (253).

6.3.c. MAPK inhibition

MAPKs are a class of kinases encompassing the JNK, ERK and p38 families and they are common converging effector signalling molecules for many pathways. The initiation of pathway leading to the involvement of MAPKs in the signalling is often dependent on upstream PTKs activation (206;217;225). The inhibition of their function could be expected to reduce some characteristics of asthma.

By using inhibitors of p38 or ERK, investigators showed the implication of these two MAPKs in the recruitment of inflammatory cells to the lung as their inhibition prevented lung eosinophilia (254-256). Using the SB202190 inhibitor of p38, it was shown that eosinophil recruitment to the lung was reduced upon exposition to the allergen (254). Using a different inhibitor of p38 (SB239063), other investigators also observed a reduction of lung eosinophilia (256), confirming this function of p38. This inhibition was traced back to the integration of the signal induced by eotaxin stimulation, usually inducing the chemotactism (254;256). It was also noted that p38 inhibition was increasing eosinophil apoptosis to normal values (eosinophil apoptosis is usually reduced in the asthmatic lung) (256). Some investigators noted that ERK1/2 inhibition by PD98059 also resulted in the inhibition of eotaxin-mediated lung eosinophilia (254;255). Interestingly, inhibition of ERK with yet another inhibitor (U0126) was able to reduce total inflammatory cell recruitment to the lung, including the reduction in eosinophilia

observed by others. This team also noted a reduction in allergen-specific IgE and IgG_1 titres in the serum, as well as a reduced production of IL-4, IL-5, IL-13 and eotaxin, and a reduction in AHR (257).

Inhibition of ERK by U0126 was reported to be important in the events following FcεR1 engagement in the mast cell. Chue and colleagues observed that ERK1/2 inhibition, but not p38 inhibition, was able to reduce the bronchoconstriction that followed allergen challenge and the subsequent production of leukotrienes, while histamine release was only slightly reduced (258).

Investigating the implication of JNK in their model, Eynott and colleagues reported that JNK inhibition with SP600125 is able to inhibit inflammatory cell recruitment to the lung, including the recruitment of eosinophils, but that single administration of the inhibitor was not able to reduce other characteristics of the disease such as AHR or expression of inflammatory cytokines (259). Another team reported that inhibition of JNK by SP600125 reduced BAL and lung tissue eosinophilia as well as lymphocyte recruitment to the BAL. Their results also show that it prevents goblet cells hyperplasia and reduced AHR (260).

These various experiments with inhibitors of kinases showed interesting results, and other similar experiments provided other encouraging results (261;262). The effect of kinases and particularly PTKs in asthma is a field that has been studied quite a bit now and it

helped to unravel the functioning of many receptors. On the other hand, the study of the involvement of PTPs in asthma lags behind the study of kinases.

6.3.d. Inhibition of PTPs

The inhibition of PTPs in allergic asthma is still a largely unexplored field of study. However, one interesting study involves the description of PTEN role in asthma. PI3K and PTEN are antagonistic kinases and phosphatases. While PI3K phosphorylates phosphatidyl inositol, PTEN dephosphorylates it. By using inhibitors of PI3K (wortmannin or LY-294002) in an asthma model, Kwak and colleagues observed a reduction of lung inflammation, eosinophil recruitment and AHR (263). This is coherent with the important role of PI3K in creating phosphoinositides that are essential to the recruitment of many proteins to the membrane in order to activate numerous pathways. This result revealed the role of PI3K, but did not show how PI3K is involved in asthma. In another set of experiments, Kwak and colleagues showed that after allergen challenge, PTEN protein presence dropped, while apparent PI3K activity increased (263). This was a significant observation, which established the mechanism behind the apparent increase in PI3K activity. Subsequently, they performed experiments where they inhibited PI3K or where they increased PTEN activity through an adenoviral-driven expression. Both inhibition of PI3K and expression of PTEN resulted in very similar inhibition of asthma development (263). This last set of data clearly demonstrates that the balance of tyrosine phosphorylation is dependent on the interaction of both kinases and phosphatases activity.

In regard to another PTP, the investigation of asthma development in an animal having minimal SHP-1 activity resulted in exacerbated asthma development (264). Kamata and

colleagues used the *motheaten* mouse model where SHP-1 activity is absent due to a mutation in the gene. As *motheaten* homozygous (*Ptpn6*$^{me/me}$) mice are short lived, the investigators used heterozygous mice (*Ptpn6*$^{me/+}$) to perform their experiments. The *Ptpn6*$^{me/+}$ mice have about a third of SHP-1 activity. In this context, they observed an increase in the development of asthma phenotype. Although interesting, this model has limitations as it is well known that *Ptpn6*$^{me/me}$ mice die after exhibiting serious inflammatory disease: autoimmunity, patchy dermatitis, progressive arthritis. It is usually a hemorrhagic pneumonitis that causes the death in these animals, by three weeks for *Ptpn6*$^{me/me}$ or nine weeks for *motheaten viable* homozygous (*Ptpn6*$^{mev/mev}$) (265). *Motheaten* mice have no SHP-1 protein, while *motheaten viable* mice have a catalytically inactive SHP-1 (266). Although *Ptpn6*$^{me/+}$ mice have a longer longevity than their homozygous counterparts, they also exhibit some inflammatory disorders later in their life (264). This suggests that although their study reveals a general role for SHP-1, it may hide more subtle roles for this protein.

The studies performed on the role of PTPs in asthma are very limited and this field would deserve more investigations. As studies now unravel more about the fine-tuning and the contextual interactions of PTKs and PTPs, there is an urgent need to characterize better the roles of PTPs.

Chapter 2: Protein Tyrosine Phosphatases Regulate Asthma Development in a Murine Asthma Model

Tyrosine phosphorylation is a predominant mechanism of regulation used in signalling pathways. Protein phosphorylation is mediated by PTKs, while PTPs dephosphorylate their targets. To date, the role of various PTKs in the development of asthma has been studied and many PTKs were shown to be necessary for development of allergic asthma. Interestingly, the role of PTPs is still largely unknown. In regard to the little information available on the role of PTPs in the regulation of allergic asthma, we have been interested to investigate their implication in this disease. In order to reveal their role, we used the specific PTPs inhibitor, bpV(phen). We were interested to understand the role of PTPs in both the allergen sensitization and the allergen challenge phases. To achieve this, we inhibited PTP activity with bpV(phen) during both phases separately and observed how the development of lung disease was affected. This permitted us to depict the roles of PTPs in allergic asthma development. This chapter presents how we performed this experimentation and the results obtained.

This chapter is submitted to Journal of Immunology. In this study, Dr Martin Olivier designed the project, supervised the work of the thesis author and analysed the data with P. Pouliot. Pierre Camateros provided technical assistance in the measurement of the pause enhancement on animal subjects by whole-body plethysmography, this work was necessary for figures 3 and 8. Dr Radzioch allowed us to use her laboratory equipment and personnel (P. Camateros) for measuring the Penh. The thesis author designed the project and experiments along with Dr Olivier, performed the experiments described here and analysed the results. An approximative ratio of work could be established as following: Pierre Camateros, 4%; Dr Radzioch, 1%; Dr Olivier, 10%; Philippe Pouliot, 85%.

Protein Tyrosine Phosphatases Regulate Asthma Development in a Murine Asthma Model

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Running title: PTPs regulate asthma development

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Keywords: Protein tyrosine phosphatases, signal transduction, asthma.

Abstract

Allergic asthma is a chronic inflammatory disease characterized by a Th2 inflammation. Although the cellular interactions are now well studied, the intracellular signalling involved in asthma development is still a developing field. Protein tyrosine kinases (PTKs) are actually the main focus of research and their inhibition shows improvement of asthma features. Interestingly, very little attention was given to protein tyrosine phosphatases (PTPs) -the counter-parts of PTKs- in the development of asthma. Previous studies from our laboratory showed that pharmacological inhibition of PTPs induced a transient Th1 response in the spleen. Therefore, we hypothesized that modulation of PTPs could influence asthma development. In order to assess PTP functions, we used the PTP inhibitor peroxovanadium bpV(phen) in an asthma murine model during either allergen sensitization or at allergen challenge. Inhibition of PTPs during allergen sensitization resulted in the reduction of several key events of allergic asthma such as serum IgE levels, lung tissue inflammation, eosinophilia and airway hyperresponsiveness. Of utmost interest, PTP inhibition at allergen challenge resulted in a very similar improvement of asthma features, taken apart an absence of effect on serum IgE titres. Of further importance, we observed that bpV(phen) treatment modulated cytokine expression in the spleen and favoured Th1 cytokines while inhibiting Th2. Collectively, in this study we showed for the first time that intact activity of PTPs is a prerequisite for a complete induction of asthma in a mouse model, clearly suggesting that PTPs have a pivotal regulatory role to play in the development of this disease, possibly opening new therapeutic avenues.

Introduction

Allergic asthma is a chronic inflammatory disease characterized by a sustained Th2 type of immune response (52). Its development relies first on the sensitization to an allergen, and subsequently on the IgE-mediated response to the allergen (77). The mast cell degranulation following allergen recognition by membrane-bound IgEs leads to bronchoconstriction and recruitment of inflammatory cells. Recruited eosinophils and Th2 cells further promote the allergic inflammation and favour tissue damage, which can lead to lung remodelling (77). Although the various cell interactions leading to asthma development are now better understood, very few is known regarding the intracellular events regulating cell responses involved in asthma.

The importance of intracellular signalling in cell regulation is decisive and its alteration can result in severe dysfunction of the immune response. Of the events involved in this signal transmission from the membrane to the nucleus, it appears that tyrosine phosphorylation is a critical step that is often obligatory for many cell receptors related to immunity (216;217;225;226). Among cell receptors, tyrosine phosphorylation in the TCR complex is the first detectable event happening after receptor ligation (217;225). Therefore, the regulation of tyrosine phosphorylation is tightly regulated by a balance between the protein tyrosine kinases (PTKs) that add a phosphate group and the protein tyrosine phosphatases (PTPs) that remove it (267;268). In addition to their role in TCR signal, tyrosine phosphorylation has also been shown to be a critical event in signals from IL-4, IL-5, IL-13 and IFN-γ receptors (220) as well as for the FcεR1 (207), which all have a potent role in the modulation of the immune response. PTKs were characterized some

ten years before PTPs (269-271) and as can be expected, their role in asthma development is better understood (272). The inhibition of many PTKs was evaluated in asthma and many inhibitors successfully reduced asthma symptoms in animal models (272).

On the other hand, very few is known about the role of PTPs in asthma. Upon allergen challenge, PTEN (phosphatase and tensin homologue) was shown to be down-regulated in a murine model of asthma, favouring development of the disease (263). Its over-expression using an adenoviral vector diminished the asthmatic phenotype. SHP-1 has also been shown to play a role in asthma development. If SHP-1 activity is reduced (in *Ptpn6*$^{me/+}$mice), investigators have noted an exacerbated asthma development (264). We previously reported (273;274) that PTP inhibitors of the peroxovanadium class alone or in combination with various stimuli (*e.g.* IFN-γ) can significantly augment signalling and transcriptional events, resulting in the promotion of specific cellular functions. Experiments in mouse model with *i.p.* injection of the peroxovanadium bpV(phen) triggered a preferential expression of Th1 type cytokines over Th2 in the spleen (274). Therefore, we hypothesized that PTP inhibition could favour a Th1 immune response and affect asthma development.

In order to better understand the role of PTPs in asthma pathogenesis, we used a murine model of asthma where PTPs were inhibited during either the sensitization phase (from allergen injection to allergen challenge) or during the challenge phase where the lung disease is developing. Our data indicates that PTP inhibition during allergen sensitization leads to a significant reduction of several asthma-related features including serum IgE

titre (total and allergen-specific), inflammatory cells recruitment to the lungs, lungs eosinophilia and substantially prevented the development of AHR. These results are accompanied with an increase in the level of IFN-γ in the bronchoalveolar lavage fluid (BALf). Inhibition of PTPs during allergen challenge phase resulted in similar observations with the exception of IgE levels that were not modulated at this stage. IFN-γ levels in the BALf were also increased upon treatment with bpV(phen). Collectively, our data point to a role for PTPs in regulating the Th1/Th2 response in a mouse model of allergic asthma.

Material and methods

Chemicals and reagents

Ovalbumin (OVA) grade V and aluminium hydroxide gel were purchased from Sigma-Aldrich (Sigma-Aldrich, Oakville, ON, Canada). Bis-peroxovanadium bpV(phen) was synthesized as described previously (244). PCR primers were ordered from IDT (IDT, Coralville, IA, USA).

Animal sensitization protocol and allergen challenge add references

Balb/c mice were purchased 6-8 wks of age from Charles River Canada (Saint-Constant, Qc, Canada), and housed in McGill University animal facility in accordance with the Canadian Council on Animal Care guidelines. Mice were injected i.p. on day 0 and 7 with 40 µg of OVA and 2,6 mg of aluminium hydroxide in a total of 200 µl injection saline. Allergen challenges were performed by nebulization of 5% OVA in saline for 5 minutes. Inhibition with bis-peroxovanadium bpV(phen) was performed daily by s.c. injection of 2,5 µmole/ 30g body weight with a total of 250 µl in saline. Inhibition was performed from day -1 to day 19 (included) for inhibition during sensitization or from day 20 to day 23 (included) for inhibition at allergen challenge.

Animal airway hyperresponsiveness measurement

Forty eight hours after the last allergen challenge, mice were put in a whole-body plethysmograph chamber (Buxco Research Systems, Willington, NC, USA). Penh was measured after each nebulization of increasing methacholine doses and this measure was

used to evaluate AHR. Animals were sacrificed after analysis for evaluation of other parameters.

Serum IgEs measurement

Serum was obtained from mice and was used to measure IgEs. Total IgEs were measured using the ELISA technique with BD Pharmingen antibodies following recommended procedure (capture antibody clone R35-72 and detection antibody clone R35-118) (BD Biosciences, Missisauga, ON, Canada). Specific IgE titre was measured using the same capture antibody, but the detection antibody was replaced by 10 µg/ml of biotinylated OVA. OVA grade 5 was obtained from Sigma-Aldrich and conjugated to biotin using the biotin conjugation kit from Sigma-Aldrich (Sigma-Aldrich). A ratio of 4 biotin/OVA was achieved as calculated by the extinction coefficient of avidin-HABA .

Bronchoalveolar lavage procedure

Lungs were lavaged with 1ml saline. Bronchoalveolar lavage fluids (BALfs) were spun and the cell pellet was resuspended in 100 µl PBS, counted and applied on microscope glass-slide using a Cytospin apparatus. The slide was stained using the Diff-Quik stain and blind differential count was performed on these slides. After BAL procedure, the lung was inflated with paraformaldehyde at a pressure of 25cm H_2O and let in paraformaldehyde for 48h to achieve tissue fixation. After fixation, the lung was processed in paraffin, cut into 5 µm sections and mounted on slides before staining with H&E using standard procedure. Inflammation was evaluated on a scale of 0-4 for perialveolar/peribronchial or perivascular recruitment.

Cytokine mRNA expression analysis

At sacrifice, organs were retrieved and flash-frozen. RNA was extracted using a tissue homogenizer and the TRIzol reagent (Invitrogen Canada, Burlington, ON, Canada). Reverse transcriptase was performed using oligodT as previously described (71). Quantitative relative PCR was performed using Invitrogen Platinum® qPCR SuperMixes and 0,4 µM primer in 25 µl. qPCR program goes as follow: 50°C 2 min; 95°C 3 min; (95°C 20 sec, 60°C 30 sec, 72°C 20 sec, 80°C-reading step- 20 sec) for 40 cycles followed by a melting curve. All primers annealing temperature was 60°C. Oligo sequences go as following: GAPDH: 5'-CGG ATT TGG CCG TAT TGG GCG CCT-3' and 3'- ACA TAC TCA GCA CCG GCC TCA CCC-5'; IL-4: 5'- AACATGGGAAAACTCCATGC-5' and 3'- TTGCATGATGCTCTTTAGGC-5'; IL-10: 5'- GGT TGC CAA GCC TTA TCG GA-3' and 3'- ACC TGC TCC ACT GCC TTG CT-5'; IL-12: 5'- GGA AGC ACG GCA GCA GAA TA-3' and 3'-AAC TTG AGG GAG AAG TAG GAA TGG-5'; IFN-γ: 5'- GCGTCATTGAATCACACCTG-3' and 3'- TGAGCTCATTGAATGCTTGG-5'.

Protein phosphatase activity measurement by pNPP hydrolysis

Analysis of phosphatase activity was performed with a variation of our previously reported method (275), tissues were collected and flash-frozen in liquid nitrogen. Tissue samples were then homogenized in PTP lysis buffer (50 mM Tris, pH 7, 0.1 mM EDTA, 0.1 mM EGTA, 0.1% 2-mercaptoethanol, 25 µg/ml aprotinin, and 25 µg/ml leupeptin) using a PRO 200 tissue homogenizer (Pro Scientific inc., Oxford, CT, USA). 1% Igepal was added after homogenization. Samples where then kept on ice for 45 min with agitation each 10 min. Then lysates were centrifuged and supernatant was used. Protein

concentration was determined by the Bradford method following manufacturer's protocol (Biorad Laboratories Canada, Mississauga, ON, Canada). Twenty μg of protein was used to evaluate phosphatase activity. Incubation was performed in phosphatase reaction buffer (50 mM Hepes, pH 7.5, 0.1% 2-mercaptoethanol, 10 mM pNPP) at 37°C. OD was acquired each 15 min at 405 nm by an automated Powerwave 340 absorbance reader (Biotek Instruments inc, Winooski, VT, USA).

IFN-γ and IL-4 measurement in the BALf

After sacrifice, supernatant from BALf were frozen at -80°C until used for cytokine measurement by ELISA. IFN-γ ELISA (BD Pharmingen, Mississauga, ON, Canada) and IL-4 ELISA (eBioscience, San Diego, CA, USA) were performed as specified by the manufacturer of the kit with detection limit of 10 pg/ml and 4 pg/ml respectively.

Statistical analysis

Statistically significant differences were identified using the ANOVA module of StatView from the SAS institute (version 5, SAS Institute, NC). *P* values of ≤ 0.05 were considered statistically significant. All data are presented as mean ± SEM.

Results

Inhibition of PTP activity during allergen sensitization.

In a first set of experiments, PTP activity was inhibited by daily injection (s.c.) of bpV(phen) as we previously performed knowing that this inhibition is transient over a 24-hour period ((274) and data not shown). BpV(phen) was administered to mice daily from day -1 to abolish PTP activity prior to sensitization, and treatment was terminated on day 19 of the sensitization protocol in order to ascertain that PTPs inhibition was not exceeding during allergen challenge (day 21-23).

Ovalbumin (OVA) sensitization was first evaluated by measurement of serum IgE level. As shown in Figure 1, in absence of bpV(phen) treatment, OVA sensitization induces an increase in total and specific IgE levels when compared to saline-sensitized animals, as expected in this model. Animals treated with bpV(phen) and sensitized to saline showed no difference with saline-treated/saline-sensitized controls. But interestingly, animals sensitized to OVA and treated with bpV(phen) had a lower level of both total and OVA-specific IgEs, with an effect slightly more important in OVA-specific IgEs. Thus, PTP inhibition prevented the usual production of IgEs observed in this asthma model.

Next, we evaluated inflammatory cells recruitment to the lung by analysing the cells found in the BAL fluid (BALf) (1 ml). As reported in Fig. 2A, OVA sensitization prior to OVA challenge increased the total number of cells found in the BALf and more specifically induced the recruitment of lymphocytes and eosinophils, as expected in this model. Treatment with bpV(phen) during sensitization did not affect cellular recruitment

if animals were not sensitized to OVA (Saline control), but reduced total cell infiltration in the BALf of OVA sensitized animals. More importantly, eosinophils in the BALf were reduced to about a third of what is observed in the OVA control while lymphocytes were reduced by half, showing a considerable effect of PTP inhibition on asthma development. We further our observations concerning the reduced inflammatory cell recruitment by the evaluation of the inflammation on H&E stained lung microscopic histological slides. This allowed us to observe that although perivascular and peribronchial infiltrations were important in the OVA control, they were dramatically reduced upon PTP inhibition during sensitization (Fig. 2B), suggesting a role for PTP in full development of allergic asthma disease.

Then, we investigated the effect of PTP inhibition on airway hyperresponsiveness (AHR) development. BpV(phen) treatment in unsensitized animals did not affect AHR (Fig. 3), but PTP inhibition in animals sensitized to OVA resulted in a strong reduction of AHR, confirming the potency of bpV(phen) in affecting the development of this aspect of the disease.

In order to assess the physiological effect of bpV(phen), we investigated the production of cytokines in the BALf, namely IFN-γ and IL-4 to verify the status of the Th1/Th2 balance. As shown in Fig. 4, IFN-γ levels in BALf remained low in Saline-challenged animals as well as in OVA-challenged animals in absence of bpV(phen) injection. Of utmost interest, bpV(phen) treatment during sensitization significantly increased IFN-γ levels in BALf after allergen challenge. IL-4 levels on the other hand showed significant

modulation (Fig. 4B). This set of results clearly illustrates the fact that bpV(phen) can alter the balance of the Th1/Th2 response in mice if administered during allergen sensitization.

Effective inhibition of PTP activity in the lungs and Th1 polarization in the spleen.

Previous work from our laboratory revealed that bpV(phen) s.c. injection was reducing PTP activity in the spleen as reflected by transient hyperphosphorylation of tyrosyl substrate and the modulation of Th1 type cytokine gene in the spleen(274), a potential important issue during OVA sensitization. However, the capacity to inhibit PTP activity in the lungs has not been established in our previous study. In Figure 5A, we show that bpV(phen) injection substantially diminishes the total lung phosphatase activity by around 50% over a 24h period, in a manner similar to the inhibition observed in the spleen (40%). This confirmed that bpV(phen) was able to reach the lung and inhibit PTP activity, securing that the treatment will efficiently inhibit PTPs in both organs during the allergen challenge phase.

We wanted to extend our previous findings regarding the induction of Th1 cytokine expression in the spleen (274) after bpV(phen) treatment. Here we evaluated by qRT-PCR the expression of IFN-γ, IL-12, IL-4 and IL-10 in the spleen after peroxovanadium treatment. As seen in Fig. 5B, IL-12 expression is increased after PTP inhibitor injection and this increased is sustained for about 24h. The same observation is made with IFN-γ, which suggests a support to a Th1 response as we reported previously (274). Very interestingly, IL-4 expression in the spleen is markedly reduced by PTP inhibitor treatment, as well as IL-10, an effect that further increase the Th1 polarisation favoured

by the inhibitor. As the balance between these mediators is crucial for a proper allergic inflammation to be established, these early results reinforced our hypothesis and provide a consistent mechanism for the reduction of the asthmatic phenotype in these mice.

Inhibition of PTP activity during allergen challenge.

If, as we hypothesized, PTP inhibition favoured a Th1-type response, it is consequent that inhibition during allergic sensitization would alter asthma development as it may reduce the extent of the Th2 response that is allowed to develop. What remained to be verified is if this inhibition of PTP activity was able to affect local lung allergic disease development induced by allergen challenge in previously sensitized mice. We inhibited PTP activity by daily s.c. injection of bpV(phen) beginning one day before the first allergen challenge up to the last day (days 20-23), with the PTP inhibitor administered 6h before the allergen challenge. Mice were sacrificed 48h after the last allergen challenge.

Our first parameter of analysis in this second set of experiments was to verify serum IgE levels. As shown in Fig 6, all groups showed production of IgEs as all of them were sensitized to OVA. Notably, the two OVA-challenged groups show a trend of increased IgE production (total IgEs), an observation that does not reach statistical significance. Consequently, bpV(phen) treatment did not significantly affect IgE levels, neither total IgEs, nor OVA-specific IgEs that were induced by the previous i.p. sensitization.

We then evaluated the cells found in the BALf. As observed in Fig. 7A, OVA challenge in OVA-sensitized control animals results in an important recruitment of total inflammatory cells, with a typical increase in lymphocyte and eosinophil populations.

Treatment of these animals with our PTP inhibitor resulted in a marked diminution of total inflammatory cells found in the BALf with a dramatic decrease in lymphocytic population and an almost complete disappearance of eosinophils. Then we verified on histological slides if lung tissue inflammation was affected by PTP inhibition. As seen in Figure 7B, the prominent perivascular and peribronchiolar infiltration observed in OVA-sensitized/OVA-challenged animals was completely abolished in their bpv(phen)-treated counterparts, revealing the effect of inhibiting PTPs in asthma development.

To complete the evaluation of the effect of bpV(phen) on allergic asthma development, we investigated the AHR expressed by these animals. Fig. 8 shows that OVA-sensitized/OVA-challenged animals exhibit strong AHR, while OVA-sensitized/Sal-Challenged (with or without bpV(phen)) exhibited no AHR. PTP inhibition in OVA-sensitized/OVA-challenged animals resulted in a normalisation of AHR in these animals that no longer showed significant AHR.

Here also, we investigated the production of cytokines in the BALf, (IFN-γ and IL-4) to verify the status of the Th1/Th2 balance. Fig. 9 shows that in this context, bpV(phen) treatment was sufficient in both Saline- and OVA-challenged animals to induce IFN-γ production, which present a more prominent Th1 response. IL-4 protein in the lung remained under the detection limit of our assay (data not shown).

Discussion

Asthma is known to be a chronic inflammatory disease sustained by a Th2 response (77). Previous observations of our laboratory reported that inhibition of PTPs, by the use of the inhibitor bpV(phen), favoured the expression of Th1 cytokines in the spleen (274). This suggested that PTP inhibition could antagonize the development of asthma by preventing the establishment of the required Th2 response. Therefore, in the actual study, we report that PTP inhibition prevented the development of an asthmatic status in a mouse model. In a first set of experiment, we showed that inhibition during allergen sensitization could reduce serum IgE concentration, diminish BALf inflammatory cells recruitment and eosinophilia, reduce lung tissue inflammation and prevent development of AHR. IgEs are mainly produced during allergen sensitization, therefore PTP inhibition during this period might have hampered the process of IgE production. But it is interesting to note that the other features of the disease (recruitment of inflammatory cells, tissue inflammation and AHR) develop after allergen sensitization, as consequence of the allergen challenge, and PTP inhibition was not performed during this phase. This illustrates that bpV(phen) inhibitor injected during allergen sensitization profoundly altered the immune response induced by sensitization in a way that cannot be reversed at allergen challenge. As we previously showed that the Th1/Th2 balance is affected by bpV(phen) treatment (274), we speculated that a more pronounced Th1 reaction was created during sensitization, resulting in a weaker asthma phenotype at later allergen challenge. In support of this, we observed the expression of critical cytokines in the spleen upon treatment with bpV(phen) and found that Th1 cytokines (IFN-γ and IL-12) expression was increased while Th2 cytokines (IL-4 and IL-10) were dramatically reduced, giving support to our hypothesis.

In a second set of experiments, we showed that treatment with bpV(phen) can reduce BALf inflammatory cells recruitment and eosinophilia, reduce lung tissue inflammation and prevent development of AHR but did not significantly reduce serum IgE titre. It was not surprising to find no significant effect on IgE as they are mainly produced during the sensitization phase and these animals had PTP inhibition only during allergen challenge. It is known that upon subsequent allergen exposition, like with allergen seasons, IgE levels will rise (276). In our model, OVA challenge shows a non-significant increase in IgE levels, and it is interesting to note that animals treated with bpV(phen) do not show a similar trend.

In this context, as the allergen is nebulized to the lung, the events occurring to the lungs are of prime importance. Therefore, we verified that the phosphatase activity was indeed inhibited in the lung upon s.c. injection of bpV(phen). We observed a substantial decrease of the phosphatase activity over 24h, allowing us to conclude that PTPs in the lungs were inhibited by our treatment. It is interesting to note that the levels of IFN-γ are also increased in the BALf in this context, indicating that inhibition of PTPs at allergen challenge also affected the Th1/Th2 balance. This piece of data clearly demonstrates the potency of PTPs in the fine regulation of immune responses as the immune sensitization was performed identically in all mice with alum, priming T cells in a Th2 phenotype (277). This indicates that bpV(phen) can somehow prevent the development of a Th2-oriented immune response in the lung. It was previously reported that Th1 and Th2 clones do not exhibit the same pattern of tyrosine phosphorylation in the early events of the TCR (205): Th1 clones had stronger phosphorylation profile than Th2 clones. Therefore, it is tempting to suggest that the use of PTP inhibitors in such a situation can increase the

basal phosphorylation level of all clones, hence preventing Th2 clones from being activated with their classical faint phosphorylation level. Although requiring more investigation, such a mechanism could explain a prevalence of a Th1 response, without implying a quantitative modulation of the populations.

Interestingly, other studies on the role of PTPs in asthma have reported that inhibition of SHP-1 or PTEN favoured asthma development (263;264). The inhibition of SHP-1 in *motheaten* heterozygous (*Ptpn6*$^{me/+}$) mouse resulted in a marked increased of allergic inflammation reflected by increased eosinophilia and AHR (264). In a mouse model of asthma, it was reported that PTEN protein expression was down-regulated in the lung upon allergen challenge. If PTEN levels were restored or if PI3K inhibitors were used, asthma development was reduced, showing that PTEN is necessary to counter-act PI3K and prevent asthma development (263). As our model with a global inhibition of PTPs show a protective effect, we expect that such global inhibition prevents early events involved in asthma development, so that inhibition of SHP-1 and PTEN activity does not cause exacerbation of the disease.

Our results seem to contrast with previous results where inhibition of PTKs reduced asthma development (248;252;253;257;259;278). However, we do not consider it as surprising, but as highlighting the refined role of PTPs in the regulation of signalling pathways. Our data clearly shows the necessity of a functional protein tyrosine phosphatome in the establishment of allergic asthma. Interestingly, inhibition of PTPs either during sensitization or during lung allergen challenge resulted in reduction of the pathology, most probably resulting from different mechanisms. In the inhibition during

antigen sensitization, we observed an increase in IFN-γ presence in the BALf, which might be due to an increase in Th1 clonal expansion, but this mechanism is less probable for the inhibition during allergen challenge.

PTP inhibition during allergen sensitization diminished IgE levels, but this feature was not observed in mice in which inhibition was performed solely during the allergen challenge, showing that lower IgE levels could be involved in the reduction of the pathology in the first case, but not in the second, therefore suggesting that more than just IgE levels are affected by the treatment.

Of interest, we observed that FceR1-mediated degranulation in RBL-2H3 (a cancer basophil-like cell line used for the investigation of FcεR1-induced degranulation) was not affected by bpV(phen) treatment at any dose studied (data not shown), suggesting that reduction of mast cell degranulation is probably not involved in the reduction of pathology. Using MF2.2D9 MHC-II-restricted T-T hybridoma and DC2.4 dendritic cell lines (279), we also investigated the effect of bpV(phen) on antigen presentation by dendritic cells to T cells. We found that treatment of cells with subcytotoxic doses of bpV(phen) were not affecting cell proliferation (data not shown), further suggesting that the observed reduction of pathology in our model is not due to a quantitative reduction of allergen-induced cellular proliferation. To expand this observation, we also used the transfer of CFSE-labelled OT-II T cells into C57/B6 mouse prior to OVA sensitization. This enabled us to observe if bpV(phen) treatment was affecting any of the processes involved in allergen uptake to allergen presentation in the lymph node. Our results (not

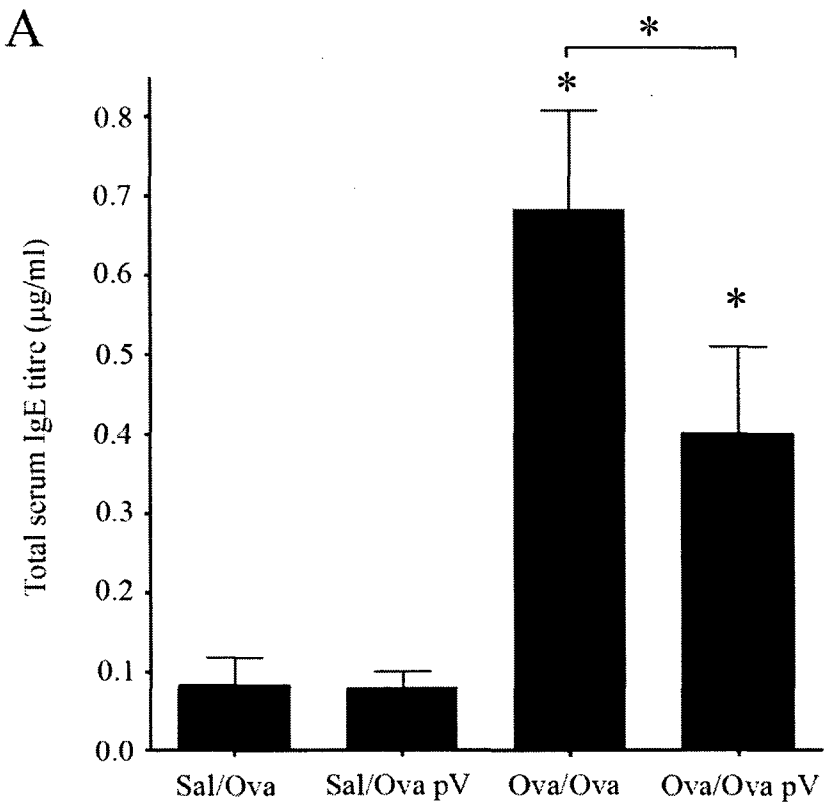
shown) show no difference in the proliferation after 3 days between bpV(phen)-treated and non-treated mice, restricting furthermore the effect to a modification of the Th1/Th2 balance.

We also performed preliminary experiments where bpV(phen) was administered i.n. during allergen challenge in mice previously sensitized to OVA. We observed that lung inflammation observed in tissue sections was reduced in the group treated with bpV(phen), suggesting that restricted local PTP inhibition could also exhibit beneficial effects (data not shown). We are actually investigating the optimal administration regimen in this context to undertake further experiments using this approach.

Our study is the first to reveal the important role of PTPs in the development of allergic asthma in a mouse model. This is an important observation as it suggests that PTPs might be used as therapeutic targets. The fact that bpV(phen) can prevent lung allergic disease in pre-sensitized individual is even more interesting as it suggests that such therapies can be envisaged using PTP inhibition. On a more fundamental aspect, it highlights the importance of PTPs in regulating the type of immune responses towards a precise antigen. Finally, further investigations using mice deficient for various PTPs should permit to firmly identify the PTPs involved in this effect, and therefore allow the development of potentially important therapeutic approaches to treat asthma.

Figure 1: *Serum IgEs in mice with PTP inhibition during allergen sensitization.*

Blood was collected at sacrifice and serum obtained. A. Total IgE and B. Ovalbumin-specific IgE levels were measured by a sandwich ELISA. Data shown represents the average of 13-14 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).


A
Total serum IgE titre (µg/ml)
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
*
*
*
Sal/Ova
Sal/Ova pV
Ova/Ova
Ova/Ova pV

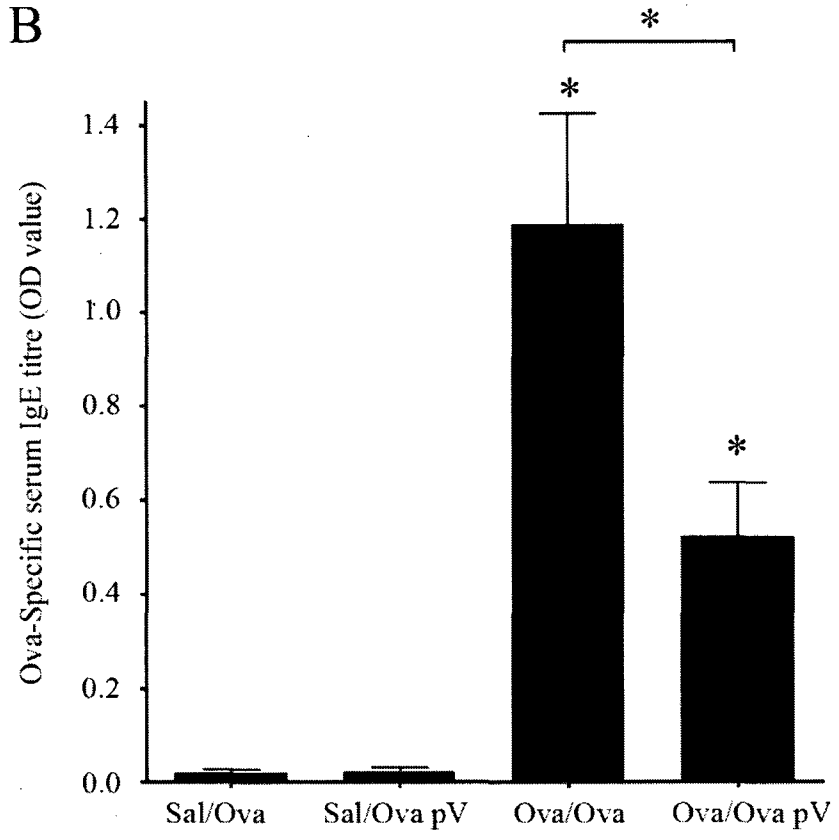

B
Ova-Specific serum IgE titre (OD value)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
*
*
*
Sal/Ova
Sal/Ova pV
Ova/Ova
Ova/Ova pV

Figure 2: *Lung inflammation of mice with PTP inhibition during allergen sensitization.*

Inflammatory cells recruitment to the lung was evaluated. A. Recruitment of inflammatory cells to the bronchoalveolar lavage fluid compartment was evaluated by differential count and total cell count. Data shown represents the average of 13-14 animals/group. B. Local tissue inflammation was evaluated on histology cuts. Observations were made at 400x on 5 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

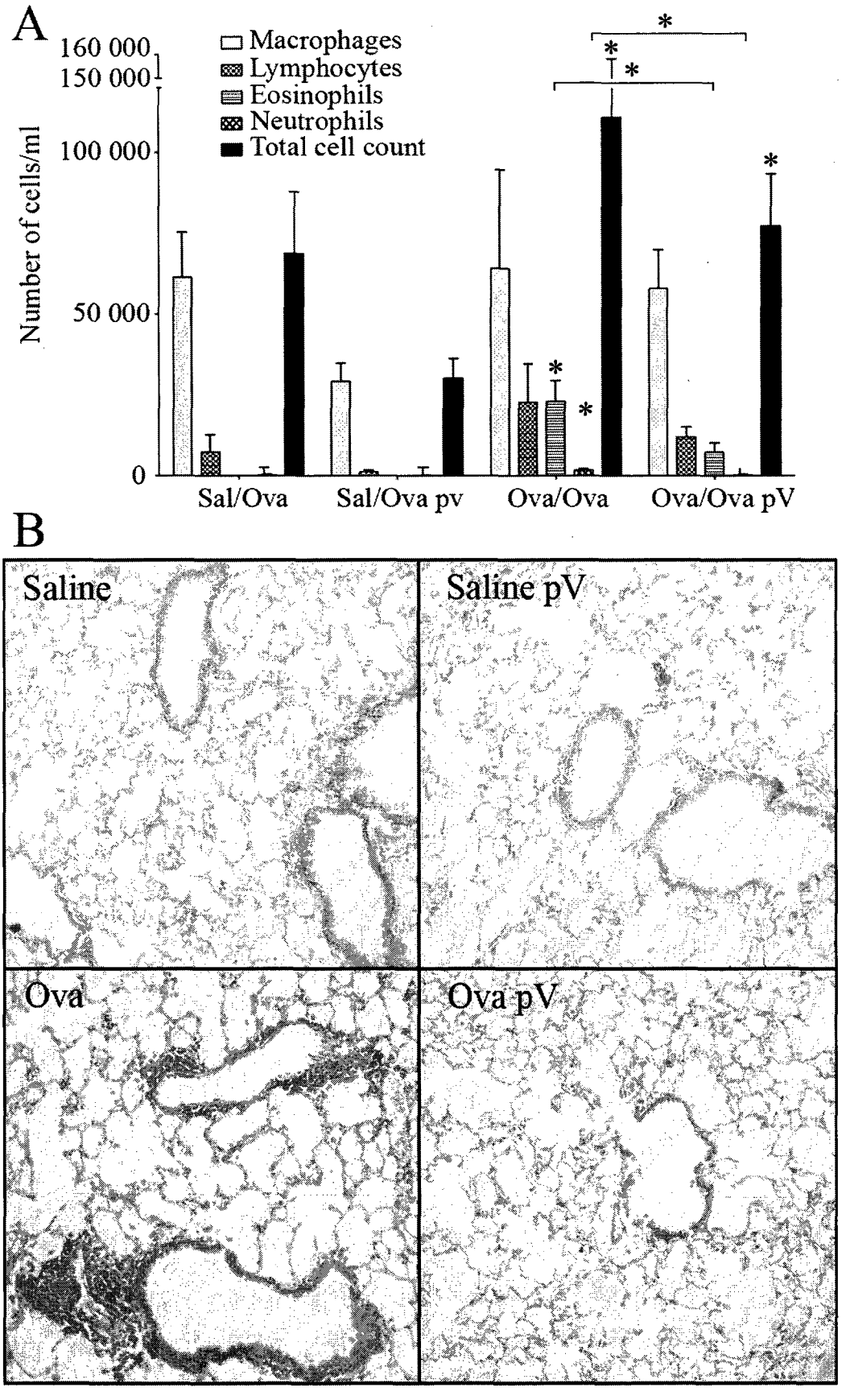

A
160 000
150 000
100 000
50 000
0
Number of cells/ml
Macrophages
Lymphocytes
Eosinophils
Neutrophils
Total cell count
*
*
*
*
*
*
*
Sal/Ova
Sal/Ova pv
Ova/Ova
Ova/Ova pV
B
Saline
Saline pV
Ova
Ova pV

Figure 3: *Airway hyperresponsiveness of mice treated with PTP inhibitor during allergen sensitization.*

Mice were evaluated for airway hyperresponsiveness to methacholine in a whole-body plethysmograph. After recording the values of baseline breathing and the values of PBS only, increasing doses of methacholine were nebulized to the chamber and respiratory pattern was recorded. Penh value was then used as a measure of airway hyperresponsiveness. Data shown represents the average of 9-14 mice/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

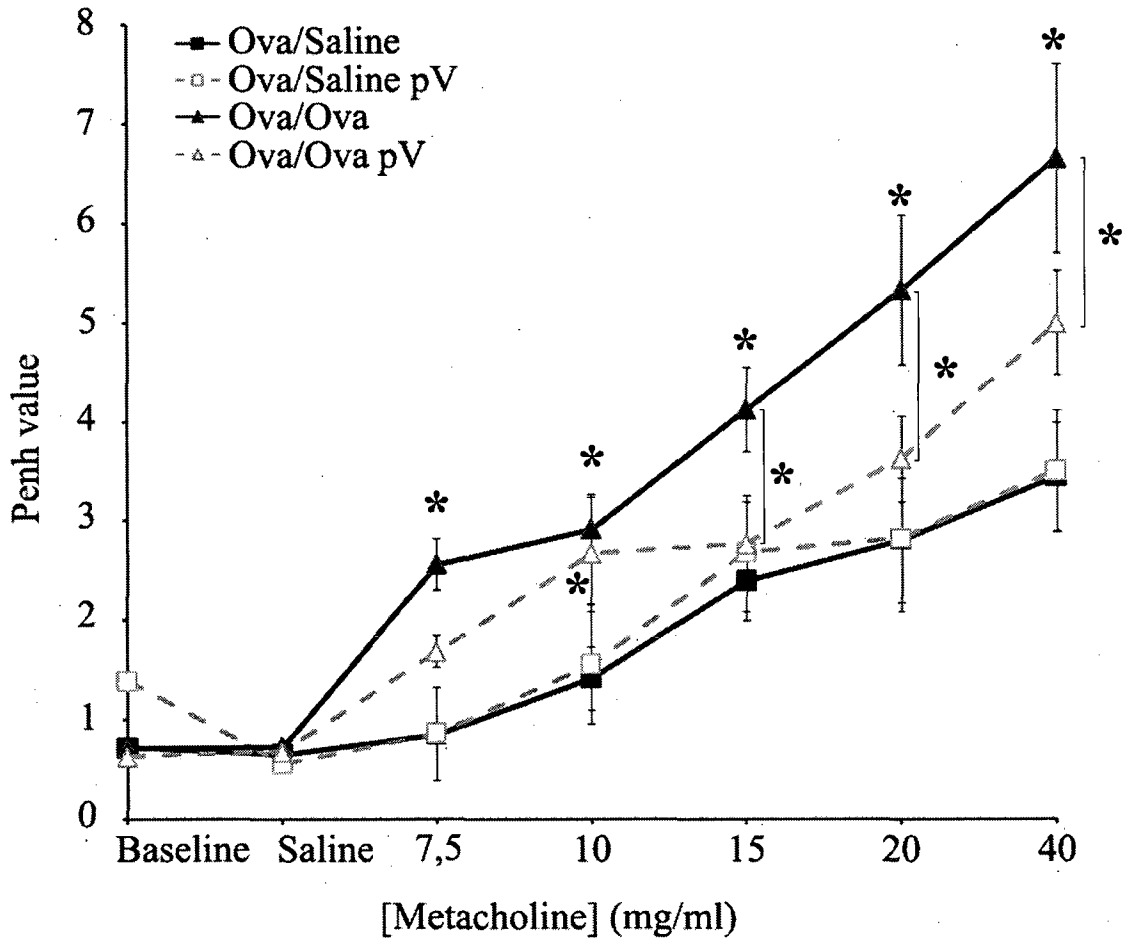
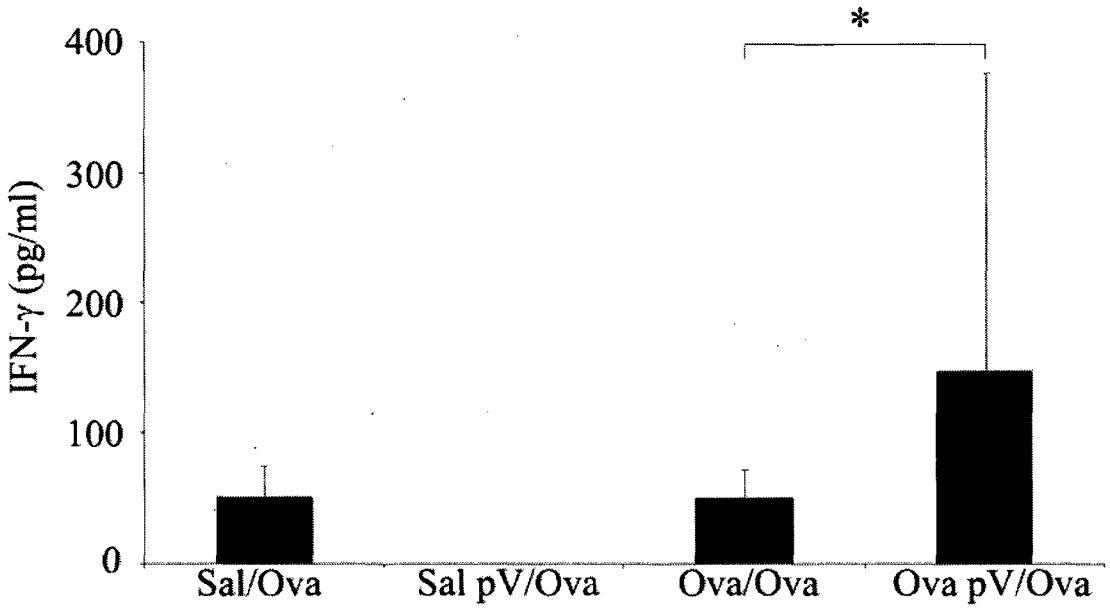

Ova/Saline
Ova/Saline pV
Ova/Ova
Ova/Ova pV
Penh value
0
1
2
3
4
5
6
7
8
Baseline
Saline
7,5
10
15
20
40
[Metacholine] (mg/ml)
*

Figure 4: *Cytokines present in the BALf of mice treated with bpV(phen) during allergen sensitization.*

BALf supernatants were evaluated for the presence of cytokines by ELISA. IFN-γ levels (A) and IL-4 levels (B) were evaluated. Data represents the average of 6-9 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).


*
400
300
200
100
0
IFN-γ (pg/ml)
Sal/Ova
Sal pV/Ova
Ova/Ova
Ova pV/Ova

B

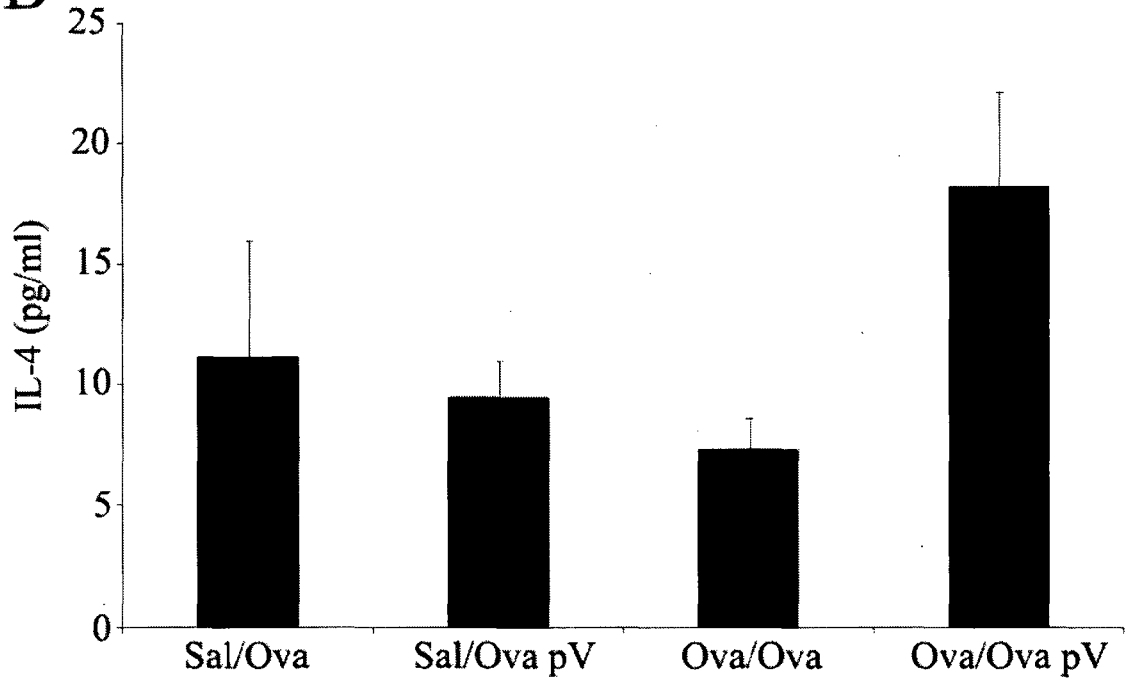

25
20
15
10
5
0
IL-4 (pg/ml)
Sal/Ova
Sal/Ova pV
Ova/Ova
Ova/Ova pV

Figure 5: *Modulation of phosphatase activity and cytokine expression by bpV(phen) injection.*

A. Phosphatase activity was investigated in spleen and lung by cleavage of the pNPP substrate in a time-dependant manner. Data represents the average of 3 animals/time-point. B. Modulation of cytokine mRNA expression in time after bpV(phen) treatment was evaluated in the spleen. IFN-γ and IL-12 were assessed for the Th1 cytokines while IL-4 and IL-10 were used as Th2 cytokines. Data represents the average of 6 animals/time-point. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

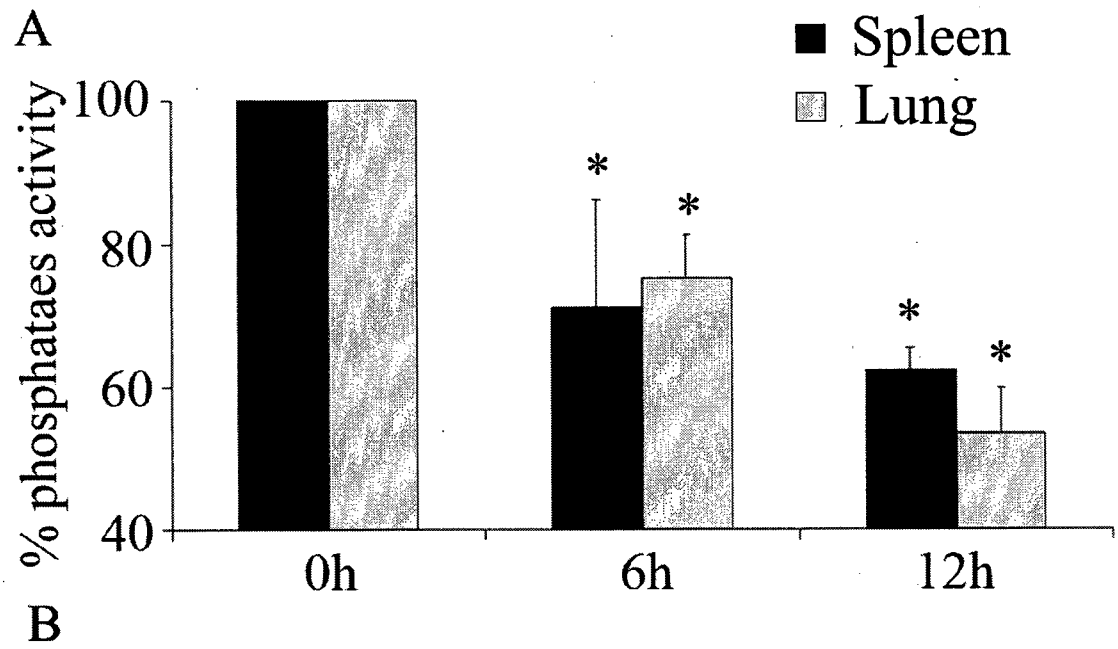
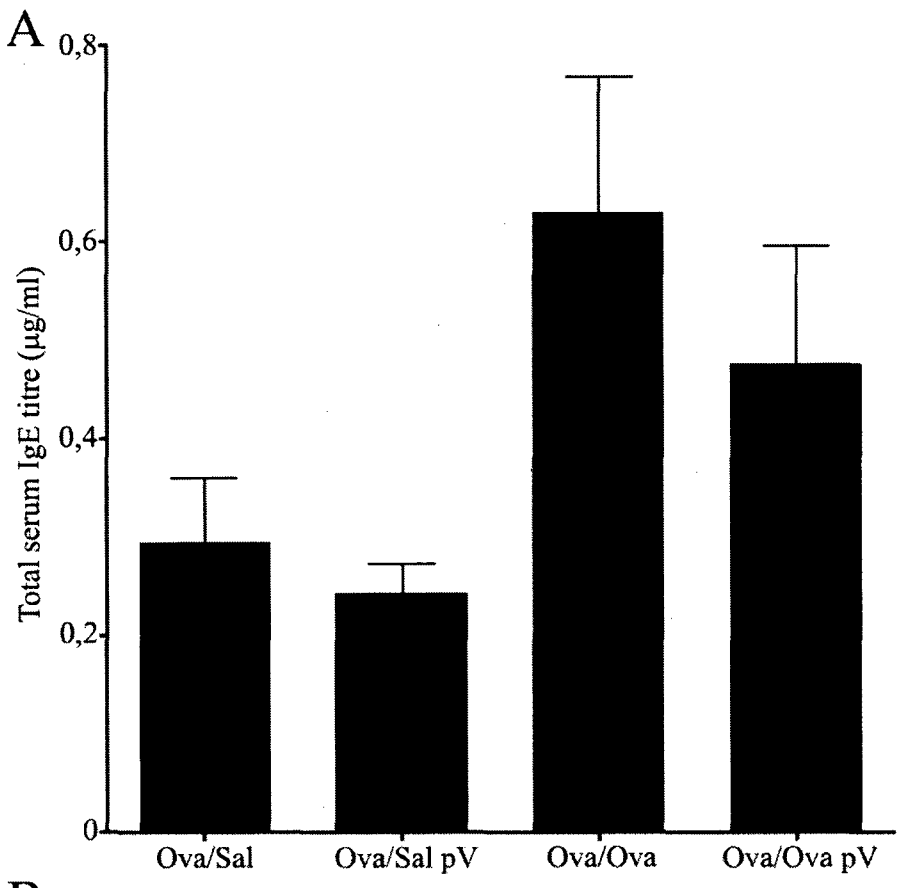

A
Spleen
Lung
% phosphataes activity
100
80
60
40
0h
6h
12h
B
IL-12
IFN-γ
IL-4
IL-10
mRNA expression (A.U.)
2,5
2,0
1,5
1,0
0,5
0,0
0h
3h
6h
12h
24h

Figure 6: *Serum IgEs in mice with PTP inhibition at allergen challenge.*

Blood was collected at sacrifice and serum obtained. A. Total IgE and B. Ovalbumin-specific IgE levels were measured by a sandwich ELISA. Data shown represents the average of 15-17 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).


A
0,8
0,6
0,4
0,2
0
Total serum IgE titre (µg/ml)
Ova/Sal
Ova/Sal pV
Ova/Ova
Ova/Ova pV

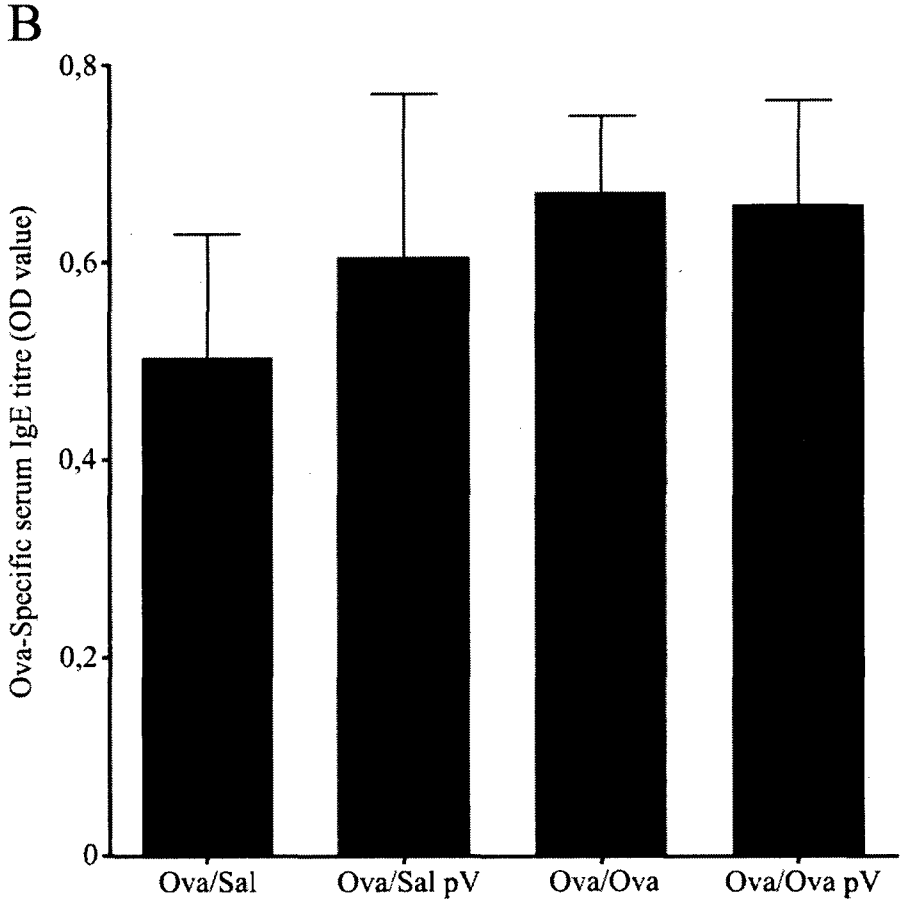

B
0,8
0,6
0,4
0,2
0
Ova-Specific serum IgE titre (OD value)
Ova/Sal
Ova/Sal pV
Ova/Ova
Ova/Ova pV

Figure 7: *Lung inflammation of mice with PTP inhibition at allergen challenge.*

Inflammatory cells recruitment to the lung was evaluated. A. Recruitment of inflammatory cells to the bronchoalveolar lavage fluid compartment was evaluated by differential count and total cell count. Data shown represents the average of 6-7 animals/group. B. Local tissue inflammation was evaluated on histology cuts. Observations were made at 400x on 5 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

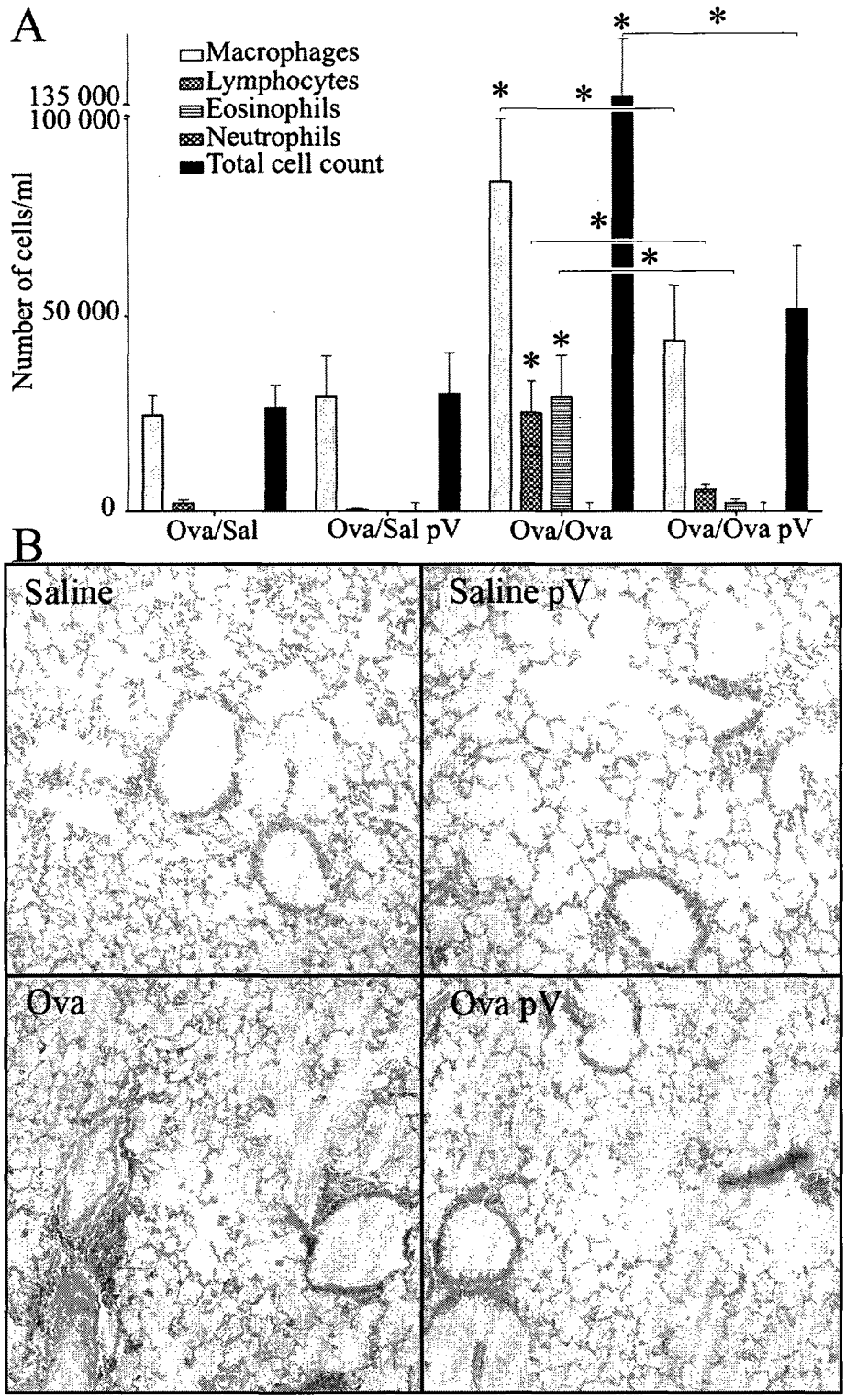

A
Macrophages
Lymphocytes
Eosinophils
Neutrophils
Total cell count
135 000
100 000
50 000
0
Number of cells/ml
Ova/Sal
Ova/Sal pV
Ova/Ova
Ova/Ova pV
B
Saline
Saline pV
Ova
Ova pV

Figure 8: *Airway hyperresponsiveness of mice treated with PTP inhibitor at allergen challenge.*

Mice were evaluated for airway hyperresponsiveness to methacholine in a whole-body plethysmograph. After recording the values of baseline breathing and the values of PBS only, increasing doses of methacholine were nebulized to the chamber and respiratory pattern was recorded. Penh value was then used as a measure of airway hyperresponsiveness. Data shown represents the average of 10-11 mice/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

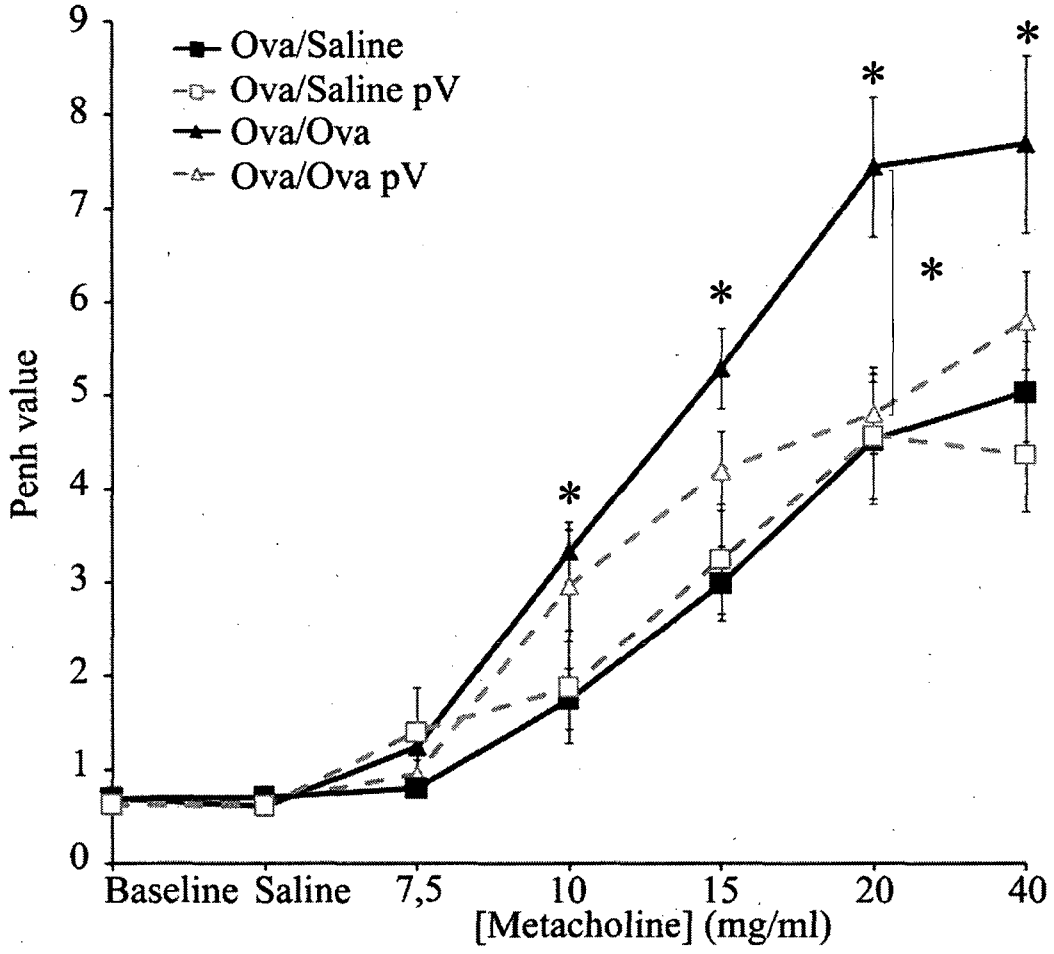
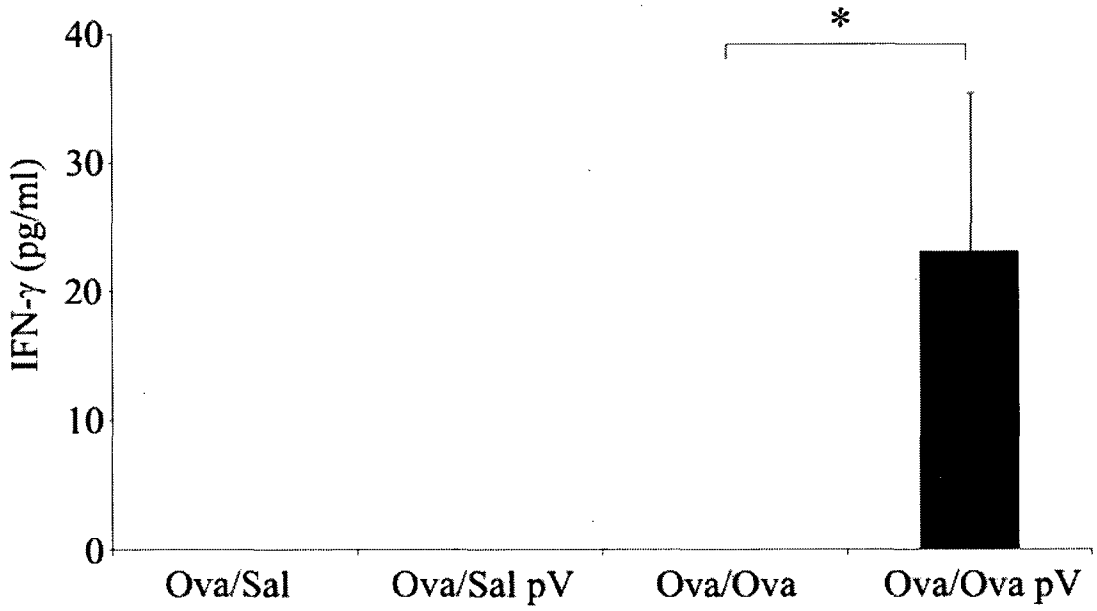

Ova/Saline
Ova/Saline pV
Ova/Ova
Ova/Ova pV
Penh value
0
1
2
3
4
5
6
7
8
9
Baseline
Saline
7,5
10
15
20
40
[Metacholine] (mg/ml)
*
*
*
*
*

Figure 9: *Cytokines present in the BALf of mice treated with bpV(phen) at allergen challenge.*

BALf supernatants were evaluated for the presence of cytokines by ELISA. IFN-γ levels were evaluated. Data represents the average of 5-9 animals/group. IL-4 levels remained below detection limit. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).


*
40
30
20
10
0
IFN-γ (pg/ml)
Ova/Sal
Ova/Sal pV
Ova/Ova
Ova/Ova pV

Chapter 3: The role of protein tyrosine phosphatases in the regulation of allergic asthma: implication of TC-PTP and PTP-1B in the modulation of disease development

In this chapter, we pursued the observations made in chapter 2. Here, we investigated the roles of individual PTPs that had previously revealed their importance in the regulation of immune responses: PTP-1B, PTP-PEST, TC-PTP and SHP-1. As the three first ones were available as mutant mice, we used these models. A mutant mouse for SHP-1 is known and was previously used for this type of investigation where they noted that SHP-1 inhibition resulted in an exacerbation of asthma. SHP-1 *motheaten* homozygous mutants are known to die of inflammatory disorders, therefore the inflammatory status could have altered this study. We decided to inhibit SHP-1 only during allergen challenge, in order to limit the possible inflammatory events linked to SHP-1 absence. Here we report the results of our investigations on these PTPs and their implication in the development of allergic asthma.

The work presented in this chapter is ready to be submitted for publication. In this study, Dr Martin Olivier designed the project, supervised the work of the thesis author and analysed the data with P. Pouliot. Sébastien Bergeron trained Philippe Pouliot on the use of adenoviruses. Dr Marette provided Dr Olivier's laboratory with original stocks of adenoviruses and allowed S. Bergeron to train the thesis author. Dr Tremblay provided us with the PTP mutant mice. The thesis author designed the project and experiments along with Dr Olivier, performed the experiments described here and analysed the results. An approximative ratio of work could be established as following: Sébastien Bergeron, 2%; Dr Marette, 1%; Dr Tremblay, 2%; Dr Olivier, 10%; Philippe Pouliot, 85%.

The Role of Protein Tyrosine Phosphatases in the Regulation of Allergic Asthma: Implication of TC-PTP and PTP-1B in the Modulation of Disease Development

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Running title: TC-PTP and PTP-1B modulate asthma development.

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Keywords: Protein tyrosine phosphatases, signal transduction, asthma.

Abstract

Protein tyrosine phosphorylation is an important and often obligatory event in the signal transduction of many receptors involved in immune responses, such as TCR, cytokine receptors and FcεR1. This protein tyrosine phosphorylation is performed by protein tyrosine kinases (PTKs) while protein tyrosine phosphatases (PTPs) dephosphorylate the target protein. The implication of PTKs in allergic asthma is well documented, but the role of PTPs is largely unknown. It has been previously reported that PTEN protein expression was diminished upon allergen challenge and that deficiency in SHP-1 resulted in exacerbation of asthma severity in mouse models. We recently reported that global PTP inhibition during either the allergen sensitization or the allergen challenge phase reduced the development of asthma, correlating with an increase of Th1 response in both the lung and spleens. Here we have been interested to elucidate the importance of key individual PTPs in the regulation of allergic immune response. We observed that PTP-1B deficiency resulted in an increased lung infiltration of inflammatory cells, while PTP-PEST deficient mice exhibited similar phenotype to WT mice. We also report for the first time that reduced expression of TC-PTP dramatically reduces IgE production and reduces recruitment of inflammatory cells to the lung, conferring an important role for TC-PTP in development of allergic asthma. SHP-1 inhibition during allergen challenge did not affect outcome of the disease. Collectively, our results underscore the importance of PTPs in the development of allergic asthma and open a promising avenue for the development of new therapies.

Introduction

Tyrosine phosphorylation is a critical event in the signalling cascades triggered by many receptors in immune cells, such as TCR (217;225), cytokine receptors (280) and FcεR1 (207). The cell reaction to the ligation of these receptors is in turn important for the orchestration of an immune response. Protein tyrosine phosphorylation is mediated through the protein tyrosine kinases (PTKs), while the removal of the phosphate group from phosphotyrosil residues is performed by protein tyrosine phosphatases (PTPs) (229). These two groups of proteins have antagonistic functions, and are both used to fine-tune the cell response to the many signals received *in vivo*, therefore influencing the outcome of immune responses (229).

In the context of allergic asthma, tyrosine phosphorylation has been found to be a crucial signalling event for the development of the disease (261). The role of PTKs has been addressed and many PTKs (e.g. JAK2, Lyn, Fyn) have been shown to play an obligatory role in the development of asthma. Genistein, a general inhibitor of PTKs has been demonstrated to prevent inflammatory cells recruitment to the lungs, to reduce allergen-mediated bronchoconstriction and to prevent the development of airway hyperresponsiveness (AHR) (248). Inhibition of particular PTKs also showed potent effects in the reduction of asthma features: among many inhibition experiments, both a Lyn inhibitor peptides (249) and a JAK2 inhibitor prevented eosinophilia (252); a Syk inhibitor prevented mast cell degranulation and cytokine production *in vitro* while inhibiting both AHR and eosinophilia *in vivo* (251). Studies on the roles of kinases in

asthma have been abundantly investigated (247), but studies on the roles of PTPs lag behind.

Of the 105 PTPs found in the mouse genome (215), very few have been shown to have a role in allergic disease development. The role of phosphatase and tensin homologue (PTEN) in asthma development has been addressed previously and it revealed that PTEN protein expression was reduced in asthmatic lung upon allergen challenge, allowing PI3K to induce signalling more efficiently (263). Artificial overexpression of PTEN in this model prevented asthma development. Others reported that a reduction of SHP-1 activity in heterozygous *motheaten* (*Ptpn6*$^{me/+}$) mice exacerbated asthma development, conferring a role for SHP-1 in the down-regulation of events leading to asthma development (264). In a different context, our laboratory previously noted that inhibition of PTPs in mice resulted in a preferential increase of Th1 cytokines in the spleen (274). This led us to investigate the role of PTP inhibition in an asthma model. Using bpV(phen), an inhibitor of PTPs, we were able to show that global PTP activity is necessary during both the allergen sensitization and the allergen challenge phases. In these two situations, inhibition of PTP activity reduced inflammatory cells recruitment to the lung, prevented development of AHR and reduced IgE production in mice treated during allergen sensitization (P. Pouliot P. Camateros, D. Radzioch and M. Olivier, submitted). Here, we propose to investigate the role of individual PTPs in allergic asthma development.

Our attention was brought to four specific PTPs in this study, given their known importance in the modulation of immune responses. The small PTPs TC-PTP, PTP-1B (281-283) and PTP-PEST (284) are known to modulate the activity of JAK family

proteins and to have a potent regulatory capacity on large scale inflammatory events (285). This might confer them the possibility to regulate complex immune responses like asthma. We were also interested to investigate the implication of SHP-1, a cytosolic PTP also known to target JAK2 (242) and MAPK family kinases (286). SHP-1 is recognized as an important modulator of immune responses due to its implication in a broad range of immunological situations (239;242).

Here, we report that mice deficient for PTP-1B exhibit an increased recruitment of inflammatory cells to the lung, suggesting that this PTP may play a role in control of inflammatory cell migration. On the other hand, mice that are heterozygous TC-PTP mutant exhibit a weaker recruitment of inflammatory cells to the lungs, exhibit less inflammation in lung tissue and produce a lower titre of allergen-specific IgEs, fitting better with the protective effect we previously described using PTP inhibitors. Of importance, heterozygous expression of PTP-PEST did not alter asthma development and inhibition of SHP-1 activity by an adenovirus-delivered shRNA before allergen challenge showed no increase in severity at the contrary of a previous report (264).

Material and methods

Chemicals and reagents

Ovalbumin (OVA) grade V and aluminium hydroxide gel were purchased from Sigma-Aldrich (Sigma-Aldrich, Oakville, ON, Canada).

Animals and sensitization protocol add references

Balb/c mice aged 6-8 wks were purchased from Charles River Canada (Saint-Constant, Qc, Canada), and housed in McGill University animal facility in accordance with the Canadian Council on Animal Care guidelines. Mice with homozygous deletion of PTP-1B (287), heterozygous deletion of TC-PTP (288) and PTP-PEST (289) were generated as reported previously, backcrossed in the Balb/c background 4 times from their 129sv ancestry. Mice were injected i.p. on day 0 and 7 with 40 μg OVA and 2,6 mg aluminium hydroxide in a total of 200 μl injection saline. Allergen challenges were performed by nebulisation of 5% OVA in saline for 5 minutes on days 21, 22 and 23 before sacrifice on day 25.

Adenovirus preparation and treatment.

Adenoviruses encoding SHP-1-specific shRNA and GFP protein were prepared using the AdEasy system, as we reported (290). On day 18 of the sensitization protocol, $6x10^{10}$ viral particles/100 μl were injected to the tail vain animals to establish an inhibition of SHP-1 mRNA expression during allergen challenge.

Animal airway hyperresponsiveness measurement

Forty-eight hours after the last allergen challenge, mice were put in a whole-body plethysmograph chamber (Buxco Research Systems, Willington, NC, USA). Penh was measured after each nebulisation of increasing methacholine doses and this measure was used to evaluate AHR. Animals were sacrificed after Penh measurements for evaluation of other parameters.

Serum IgEs measurement

Serum was obtained from mice and was used to measure IgEs. Total IgEs were measured using the ELISA technique with BD Pharmingen antibodies following recommended procedure (capture antibody clone R35-72 and detection antibody clone R35-118) (BD Biosciences, Missisauga, ON, Canada). Specific IgE titre was measured using the same capture antibody, but the detection antibody was replaced by 10 µg/ml of biotinylated OVA. OVA grade 5 was obtained from Sigma-Aldrich and conjugated to biotin using the biotin conjugation kit from Sigma-Aldrich (Sigma-Aldrich). A ratio of 4 biotin/OVA was achieved as calculated by the extinction coefficient of avidin-HABA.

Bronchoalveolar lavage procedure add reference

Lungs were lavaged with 1ml saline. BALfs were spun and the cell pellet was resuspended in 100 µl PBS, counted and applied on microscope glass-slide using a Cytospin apparatus. The slide was then stained using the Diff-Quik stain and blind differential count was performed on these slides. After BAL procedure, the lung was inflated with paraformaldehyde at a pressure of 25cm H_2O and let in paraformaldehyde for 48h to achieve tissue fixation. After fixation, the lung was processed in paraffin, cut

into 5 μm sections and mounted on slides before staining with H&E using standard procedure. Inflammation was evaluated on a scale of 0-4 for perialveolar/peribronchial or perivascular recruitment.

Western Blotting

Western blotting was performed as previously described (291). Briefly, organs were extracted, flash-frozen in liquid nitrogen and kept at -80°C until further use. Then tissues were homogenized with a PRO 200 tissue homogenizer (Pro Scientific inc., Oxford, CT, USA) in ice-cold lysis buffer without NP-40: 20mM Tris-HCl (pH 8.0), 0.14 M NaCl, 10% glycerol (v/v), 1mM PMSF, 1mM sodium orthovanadate (Na_3VO_4), 1μM NaF and protease inhibitors (40μg/ml aprotinin and 20μg/ml leupeptin). 1% NP-40 (v/v) was added to the lysate after homogenization. The lysates (30μg/lane) were separated by SDS-PAGE and proteins transferred to polyvinylidene difluoride membranes (PVDF, Millipore). Membranes were blocked in Tris-buffered saline/0.1% Tween containing 5% milk for 1 hour at room temperature. Then membranes were washed and incubated with monoclonal antibody for 1 hour α-SHP1, (Cell Signalling, New England Biolabs, Pickering, ON, Canada). After washing, membranes were incubated with anti-rabbit horseradish peroxidase (HRP)-conjugated antibody (GE healthcare, Baie d'Urfé, QC, Canada) for 1 hour and proteins visualized with the use of the ECL Plus Western Blotting reagent (GE Healthcare). Membranes were then stripped using the Restore Western Blot Stripping Buffer (Pierce, Nepean, ON, Canada) and blotting was done again using α-actin antibody (Sigma-Aldrich).

Statistical analysis

Statistically significant differences were identified using the ANOVA module of StatView from the SAS institute (version 5, SAS Institute, NC). *P* values of ≤ 0.05 were considered statistically significant. All data are presented as mean ± SEM.

Results

Development of asthma in PTP-1B K/O mice.

As an indicator of sensitization to the allergen, the production of IgEs by PTP-1B K/O mice was investigated in comparison to their WT counterparts. Figure 1A shows the production of total serum IgEs. WT animals had a significant increase in total IgE production upon ovalbumin (OVA) sensitization. Serum IgEs were also increased in PTP-1B K/O animals sensitized to OVA when compared to the saline-sensitized mice. Interestingly, the IgE level in PTP-1B K/O mice was increased in OVA-sensitized animals when compared to the WT counterparts, an increase that was not significant in saline-sensitized animals. We then investigated the OVA-specific serum IgEs found in these animals (Fig. 1B). Both OVA-sensitized groups showed a significant increase in OVA-specific IgEs when compared to their saline-sensitized controls, but no significant difference was noted between the WT animals and the PTP-1B K/O ones.

The establishment of lung disease was also investigated by the analysis of cells recruited to the bronchoalveolar lavage fluid (BALf) compartment after allergen challenge. Figure 2A shows that PTP-1B K/O animals have a dramatic and significant increase in inflammatory cells recruitment to the lungs. Notably, lymphocyte and eosinophil presence is 5 fold more important in K/O animals when compared to their WT counterparts. In Figure 2B, we evaluated the lung tissue inflammation by microscopic analysis of tissue sections. The perialveolar/peribronchial and perialveolar inflammation was noted on a scale from 0 to 4. Only OVA-sensitized animals exhibited inflammation and although PTP-1B K/O animals tended to exhibit more infiltration from inflammatory cells, this was

not significantly different from the WT control. It was impossible to perform analysis of lung reactivity on these mice due to the contaminating background of the parental strain used to generate the K/O mice. As AHR is linked to a multitude of genes (292), this contaminating background of genes probably interfered with AHR development, as no reliable observations were obtained with neither non-invasive nor invasive AHR evaluation techniques.

Development of asthma in PTP-PEST$^{+/-}$ mice.

As the PTP-PEST complete K/O is embryonic lethal (289), we could only investigate PTP-PEST involvement in asthma by the use of PTP-PEST$^{+/-}$ mice. As described above, the first hallmark of asthma that we assessed after sensitization and allergen challenge is the IgE serum titre. In Figure 3A, we can see that total IgEs are similarly increased in OVA-sensitized animals from both PTP-PEST$^{+/-}$ and WT background. As shown in Figure 3B, OVA-specific titre is not significantly modulated by the reduced PTP-PEST activity. Therefore, IgE production upon allergen sensitization does not appear to be affected in these PTP-PEST$^{+/-}$ mice.

Then, we evaluated lung inflammatory cells recruitment. The populations of cells retrieved from the BALf compartment in OVA-sensitized animals reflected the allergic status as eosinophils could be found in these BALf. But there was no significant difference between WT and PTP-PEST$^{+/-}$ mice, suggesting that reduction of PTP-PEST activity does not hamper the recruitment of inflammatory cells to the lungs (Fig.4A). Figure 4B shows that local lung tissue inflammation is not significantly affected by a

reduced PTP-PEST activity as inflammation indexes are similar in both OVA-sensitized groups of mice.

Development of asthma in TC-PTP$^{+/-}$ mice.

IgE serum titres were evaluated in TC-PTP$^{+/-}$ as it is shown in Figure 5. Upon OVA-sensitization and challenge, the WT mice increased their total IgE titre, but interestingly, TC-PTP$^{+/-}$ mice were unable to mount up an equivalent IgE response. Indeed, as seen in Figure 5A, TC-PTP$^{+/-}$ OVA-sensitized group produced IgEs in concentrations that do not differ from their saline-sensitized controls. The observation is further interesting when paralleled by OVA-specific IgE titres where OVA-sensitized mice do produce some OVA-specific IgEs, but this concentration is significantly reduced when compared to their WT counterparts (Fig. 5B). This clearly shows that TC-PTP activity is necessary for IgE production, and consequently, optimal sensitization.

We then investigated the lung inflammation in TC-PTP$^{+/-}$ mice, beginning with BALf cells. As reported in Figure 6A, OVA-sensitized WT animals showed a classic recruitment of inflammatory cells to the BALf compartment with an increase in lymphocytes, eosinophils and even a light neutrophilia. On the other hand, TC-PTP$^{+/-}$ mice were unable to recruit similar numbers of inflammatory cells to the lung and are therefore different from their WT counterparts. When we assessed tissue inflammation (Fig. 6B), we observed that although lung inflammation was present in TC-PTP$^{+/-}$ mice, it was significantly lower than in the WT animals, showing the implication of TC-PTP in the recruitment of inflammatory cells to the lung.

Inhibition of SHP-1 in the development of asthma.

Studies on *Ptpn6*$^{me/+}$ mice where SHP-1 activity is about a third of WT activity have been reported to exacerbate asthma severity (264). As *motheaten* (*Ptpn6*$^{me/me}$) mice are known to die of inflammatory disorders, we hypothesized that acute inhibition of SHP-1 activity for a short period, during allergen challenge, could present a different outcome than its inhibition on the whole lifetime of the mouse. For that purpose, we used adenoviruses that induce expression of a shRNA directed to SHP-1 mRNA in order to inhibit SHP-1 activity. We previously reported that three days post tail vein injection, the adenoviruses were able to alter SHP-1 activity in the liver (290). To assess the efficiency of this approach, we wanted to confirm that the inhibition of SHP-1 activity was also occurring in lymphoid organs such as the spleen. Figure 7A shows that inhibition of SHP-1 protein expression is reduced by day 3 and that this SHP-1 protein reduction is further augmented and maintained until day 7. Therefore, injection of adenoviruses was scheduled on day 18 of the sensitization protocol so that from the first challenge (day 21, 3 days post-injection), to the sacrifice (day 25, 7 days post-injection) SHP-1 activity would be reduced.

We investigated IgE production in these animals to assess the allergen sensitization. We observed no difference between the groups in regard to total serum IgE titres (Fig. 7B). Injection with shRNA-encoding adenoviruses or with their control GFP-encoding adenoviruses did not affect OVA-specific IgE titres neither, as expected by the similar sensitization for all animals Fig. 7C).

Next, we assessed the lung inflammation, firstly by the investigation of the recruitment of inflammatory cells to the BALf compartment. Injection of adenoviruses did not modulate the basal level of cells found in the BALf (Fig. 8A) in mice challenged with saline. While OVA challenge induced an increase in inflammatory cells recruitment, typically lymphocytes and eosinophils, the inhibition of SHP-1 activity did not affect cell recruitment to the lung.

In a second approach to investigate lung inflammation, we evaluated the lung tissue inflammation on tissue sections. As can be seen on Figure 8B, OVA challenge induced a marked inflammation in mice. This inflammation was reduced in adenoviruses-injected mice in a similar level between GFP-encoding and shRNA-encoding adenoviruses. Therefore, this reduction in lung inflammation is probably not specific to SHP-1 activity.

In a third approach to the evaluation of lung inflammation, we evaluated the AHR in these mice. OVA challenge induced an increase in airway reactivity expressed by an increase of Penh value at low doses of methacholine (Fig. 8C). This AHR increase was paralleled in mice that received both types of adenoviruses, showing that inhibition of SHP-1 activity did not affect AHR in these mice.

Discussion

The necessity of PTK activity in asthma development has been previously investigated to some extent (248;249;252;253;261), but the implication of PTP activity still remains obscure, for having been reported only in a few studies (263;264). Having previously established that abolition of PTP activity during either allergen sensitization, or allergen challenge, resulted in a decreased allergic asthma phenotype in our mouse model (P. Pouliot P. Camateros, D. Radzioch and M. Olivier, submitted), we were interested to depict the roles of individual PTPs in allergic asthma development.

PTP-1B is now established as a potent regulator of insulin signalling (293), but in regard to its role in the regulation of the immune system, information is still limited. It has been previously reported that PTP-1B is able to bind p130cas (294) and negatively modulate integrin signalling upon fibronectin binding in experiments where it was overexpressed (295). Subsequently, experiments performed with fibroblasts deficient for PTP-1B did not support this role of PTP-1B and even revealed no effect of PTP-1B absence or simply a delay in cell response to fibronectin binding (296). Therefore, a definitive role for PTP-1B in cell adhesion still needs to be established. The effect of PTP-1B on cell adhesion is an interesting avenue in our observed phenotype. Indeed, as we noticed an increased recruitment of inflammatory cells to the BALf compartment, an increase in adhesion and migration of the infiltrating cells could be coherent with our model. On the other hand, PTP-1B has also been reported to be a modulator of JAK2 (282;283), and Tyk2 (283) in fibroblasts upon IFN-α, IFN-γ or insulin stimulation. We also know that in absence of PTP-1B, Mθ sensitivity to LPS is increased, resulting in a more pronounced production of

NO *in vitro* or IL-12 and IFN-γ *in vivo* (297). These findings are also interesting in our context as JAK signalling is utilized downstream of many cytokine receptors and inhibition of PTP-1B activity might result in stronger or longer signal emanating from these receptors, potentially increasing the cell response to the stimulation. This might correlate very well with our observed increased infiltration of cells in the BALf compartment. It is also appealing to speculate that increased activation of Mθs by low levels of contaminant endotoxins in the OVA could influence the immune reaction toward a Th1 response, as it was previously shown by others (53).

PTP-PEST K/O mice exhibit embryonic lethality, highlighting the importance of this PTP in embryogenesis (289). PTP-PEST was very recently shown to regulate T cell response upon TCR activation (298). Expressed in resting T cells, PTP-PEST protein is down-regulated upon initial activation, probably to allow the processing of a stronger signal: if PTP-PEST protein level is reintroduced in Jurkat cells (that do not express PTP-PEST at basal level), TCR signal is reduced (298). In addition, if PTP-PEST is reintroduced in activated cells, their secondary response is dampened. Apparently, PTP-PEST can also regulate TCR signal through an inhibition of Ras signalling and by dephosphorylation of src homologuous and collagen (Shc), protein tyrosine kinase 2 (Pyk2), focal adhesion kinase (FAK) and $p130^{cas}$ (299). FAK and p30cas were also reported to be important for the effect of PTP-PEST on the regulation of integrin signal and focal adhesion (300). In our context, PTP-PEST did not appear to modulate asthma development although allergen sensitization is expected to strongly rely on TCR signalling. One plausible reason is that

heterozygous expression of PTP-PEST is sufficient for the accomplishment of PTP-PEST function in the regulation of TCR signal.

TC-PTP-/- mice can reach birth, but rapidly exhibit serious health problems and die at a young age, usually around 25 days (288). Among the defects noted is a gradual amplification of inflammatory disorders (285). These disorders result in an increase infiltration of tissues by mononuclear cells and a marked increase in inflammatory mediators such as IFN-γ, TNF-α and NO (285). In these complete K/O mice, B cell population is also reduced in the spleen and their presence keeps reducing with age (288;301). Interestingly, both T cells and B cells response to concavalin A is reduced in TC-PTP$^{-/-}$ mice (288;301).

In our model, TC-PTP$^{+/-}$ mice exhibited an important reduction in asthma development, as noted by a reduction in IgEs, BALf inflammatory cells recruitment and lung tissue inflammation. Our results seem to indicate an obligatory role for TC-PTP in the establishment of allergic disease, possibly during both allergen sensitization and allergen challenge. The effect of TC-PTP$^{+/-}$ mice on IgE production and lung inflammation was not complete, but the mice are heterozygous, therefore still exhibit some TC-PTP activity. Our results with TC-PTP mice are coherent with the reported response of B and T cells to concavalin A (288;301), as such a reduced response would prevent the usual development of an allergic sensitization. Our observations regarding IgE levels tend to support this hypothesis. It would have been interesting to evaluate the effect of a complete absence of TC-PTP in this context, in order to see if asthma development is more severely hampered,

but our present data already clearly provides a demonstration of the role of TC-PTP in the development of allergic asthma.

It was impossible to perform analysis of AHR in these mutant mice strains. AHR is a phenomenon highly dependent on genetic factors and the response of mouse lab strains to methacholine challenge varies greatly (292). Our mice were backcrossed (more than 4 times) in the Balb/c background, but apparently, some genetic contamination from the other strain used for generation of the mice prevented a reliable expression of AHR in these mice. Whole-body plethysmograph and invasive lung resistance methods proved to be unsuccessful in monitoring a reliable pattern of AHR (data not shown).

SHP-1 is long known for its regulation of immune responses of various contexts (266;302), and it has been recognized as an important regulator of the TCR activation threshold (302-304). It has also been found to be the active protein that mediates TCR antagonism in the context of altered peptide ligands (305). We took part in the elucidation of SHP-1 role in the regulation of inflammatory events by describing its implication in the dephosphorylation of JAK2 (242) and ERK (286) and its implication in the modulation of inflammatory and microbicidal response of Mθs (306). In the context of asthma, Kamata and colleagues reported previously that reduction of SHP-1 activity exacerbated the severity of asthma (264). They used mouse heterozygous for the *motheaten* allele ($Ptpn6^{me/+}$) mice where they report that SHP-1 activity is a third of the activity in WT mouse. Using this model, they observed that eosinophilia was increased as well as mucus production and airway hyperresponsiveness (264). The major problem with this study is the fact that the *motheathen* mutation, if homozygous ($Ptpn6^{me/me}$), results in severe

chronic inflammation and auto-immune disease (266). According to the investigators, lymphocyte development appeared normal in *Ptpn6*$^{me/+}$ mice. However, it is practically impossible to rule out the possibility that chronic inflammation is already programmed in these mice and that induction of allergic asthma is actually a trigger for this inflammation, a situation that makes impossible the distinction between origins of inflammatory events.

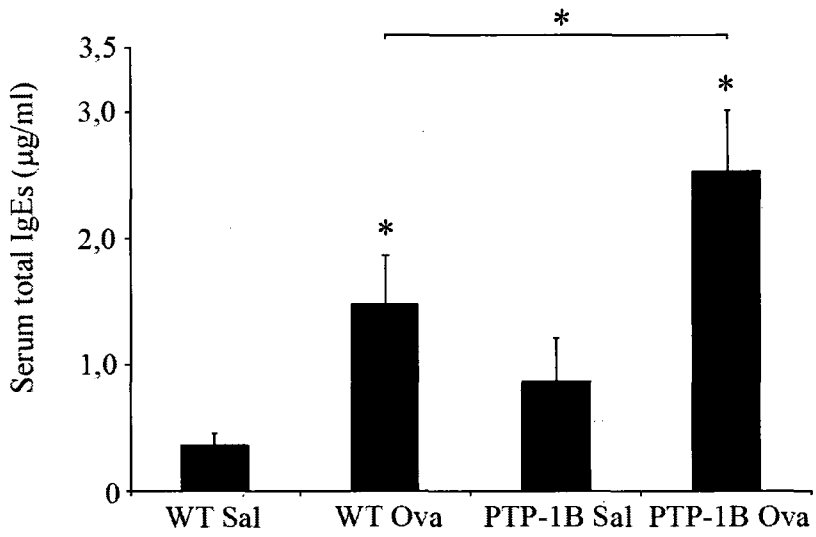
We therefore proposed to verify the effect of acute SHP-1 inhibition at allergen challenge. To achieve this, we used adenoviruses encoding shRNA directed at SHP-1 and their control adenovirus encoding GFP, as we previously reported (290). In order to confirm the effective inhibition of SHP-1 activity, we verified the protein presence in the spleen and noticed its disappearance between day 3 and day 7 post-adenovirus injection. We also verified SHP-1 expression in the lung, but basal level of SHP-1 in this organ was not detected by western blotting (data not shown). Our data indicates that inhibition of SHP-1 during allergen challenge did not result in an exacerbation of asthmatic features, in fact inhibition of SHP-1 during this phase did not appear to modulate the outcome of the disease. Lung tissue inflammation is significantly different in adenovirus-injected OVA-sensitized mice, but is similar between GFP- and shRNA-encoding adenoviruses, suggesting that this observation is an artefact of the technique. One limitation of our approach is that freshly recruited cells might not be inhibited for SHP-1 activity as adenoviruses were administered three days before the first challenge and that they are in finite number as they are non-replicative. Therefore, our results suggest that inhibition of SHP-1 in endogenous tissues cell populations does not appear to modulate asthma development, but it would be hazardous to conclude about freshly recruited inflammatory cells.

Collectively, this study provides data supporting that PTPs are important for the unfolding of immune events leading to allergic asthma. While in our context, PTP-PEST and SHP-1 inhibitions were not observed to modulate asthma development, TC-PTP activity appeared to play a role in the development of allergic asthma, whereas in absence of PTP-1B activity, recruitment of inflammatory cells to the BALf compartment is exacerbated. These data reveal important PTP-specific roles in asthma development and suggest that therapeutic approaches aimed at inhibiting specific PTPs could be envisaged in the treatment of allergic disorders as asthma.

Figure 1*: Serum IgEs of PTP-1B$^{-/-}$ mice.*

Blood was collected at sacrifice and serum obtained. A. Total IgE and B. Ovalbumin-specific IgE levels were measured by a sandwich ELISA. Data shown represents the average of 15-16 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

A


Serum total IgEs (µg/ml)
3,5
3,0
2,0
1,0
0
*
*
*
WT Sal
WT Ova
PTP-1B Sal
PTP-1B Ova

B

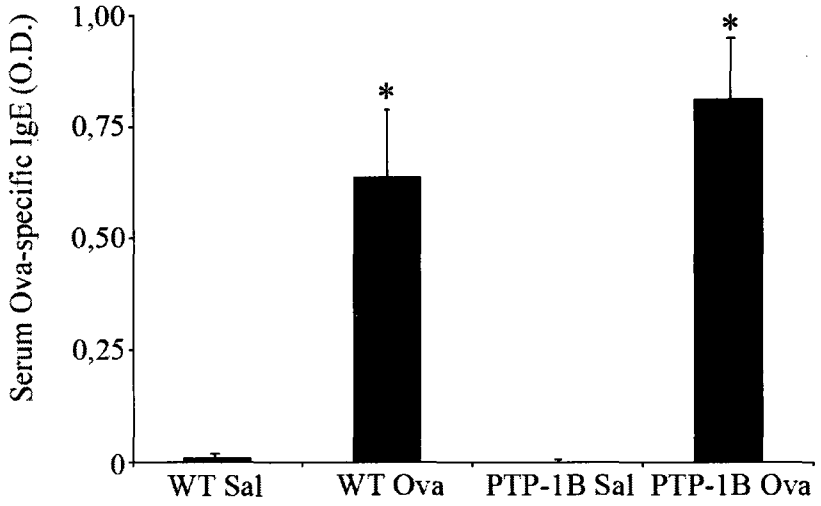

Serum Ova-specific IgE (O.D.)
1,00
0,75
0,50
0,25
0
*
*
WT Sal
WT Ova
PTP-1B Sal
PTP-1B Ova

Figure 2: *Lung inflammation of PTP-1B$^{-/-}$ mice.*

Inflammatory cells recruitment to the lung was evaluated. A. Recruitment of inflammatory cells to the bronchoalveolar lavage fluid compartment was evaluated by differential count and total cell count. Data shown represents the average of 7-9 animals/group. B. Local tissue inflammation was evaluated on histology cuts. Observations were made at 400x on 7-9 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

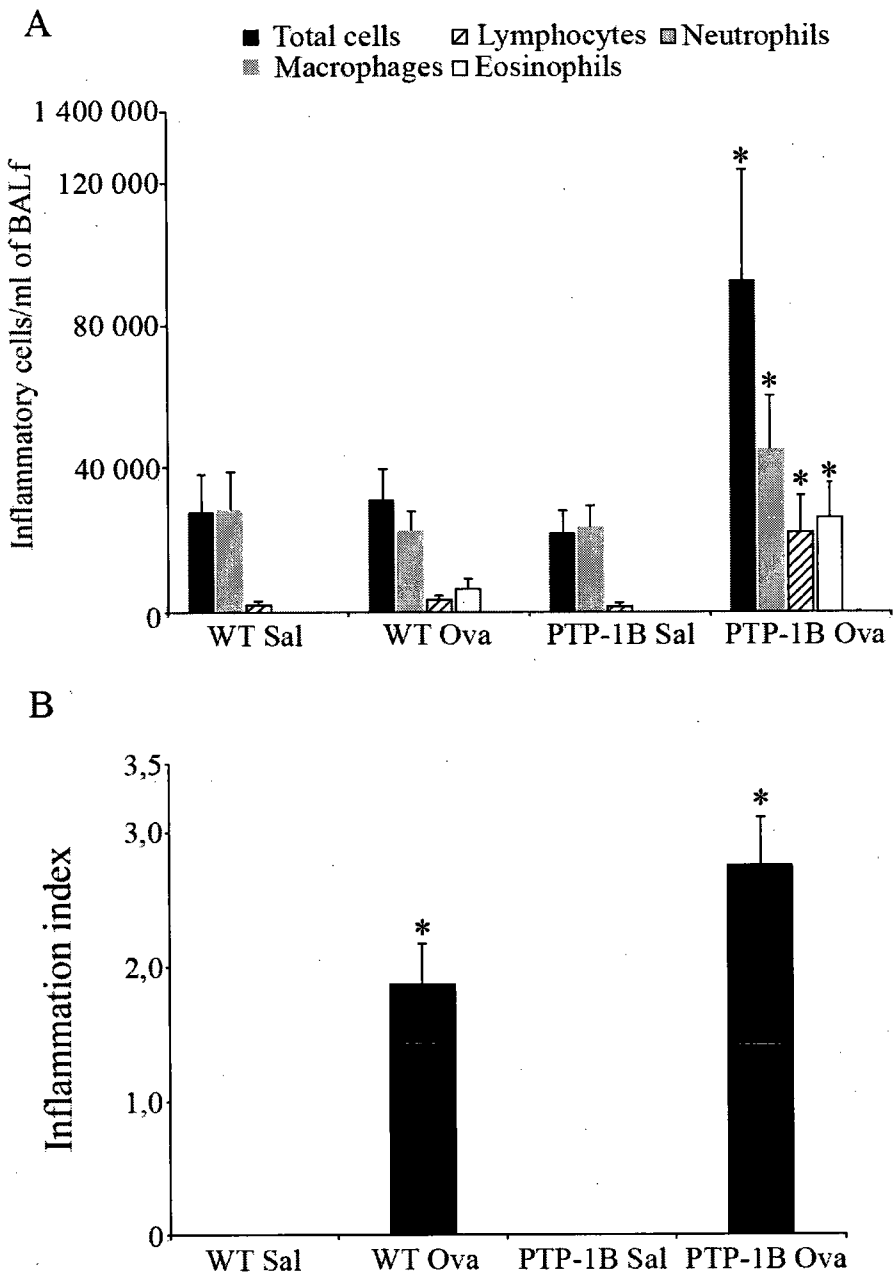

A
Total cells
Lymphocytes
Neutrophils
Macrophages
Eosinophils
1 400 000
120 000
80 000
40 000
0
Inflammatory cells/ml of BALf
WT Sal
WT Ova
PTP-1B Sal
PTP-1B Ova
B
3,5
3,0
2,0
1,0
0
Inflammation index
WT Sal
WT Ova
PTP-1B Sal
PTP-1B Ova

Figure 3: *Serum IgEs of PTP-PEST$^{+/-}$ mice.*

Blood was collected at sacrifice and serum obtained. A. Total IgE and B. Ovalbumin-specific IgE levels were measured by a sandwich ELISA. Data shown represents the average of 8-11 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

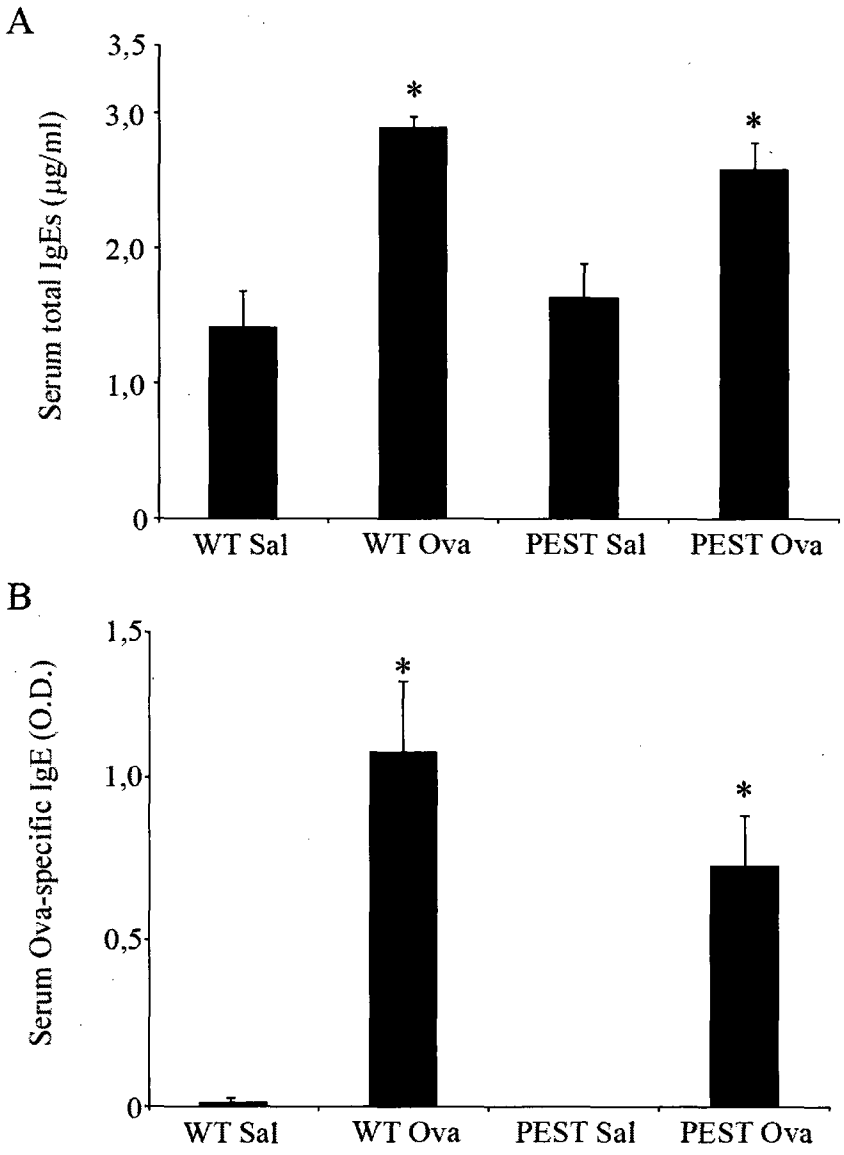

A
Serum total IgEs (µg/ml)
3,5
3,0
2,0
1,0
0
*
*
WT Sal
WT Ova
PEST Sal
PEST Ova
B
Serum Ova-specific IgE (O.D.)
1,5
1,0
0,5
0
*
*
WT Sal
WT Ova
PEST Sal
PEST Ova

Figure 4: *Lung inflammation of PTP-PEST$^{+/-}$ mice.*

Inflammatory cells recruitment to the lung was evaluated. A. Recruitment of inflammatory cells to the bronchoalveolar lavage fluid compartment was evaluated by differential count and total cell count. Data shown represents the average of 6-10 animals/group. B. Local tissue inflammation was evaluated on histology cuts. Observations were made at 400x on 4 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

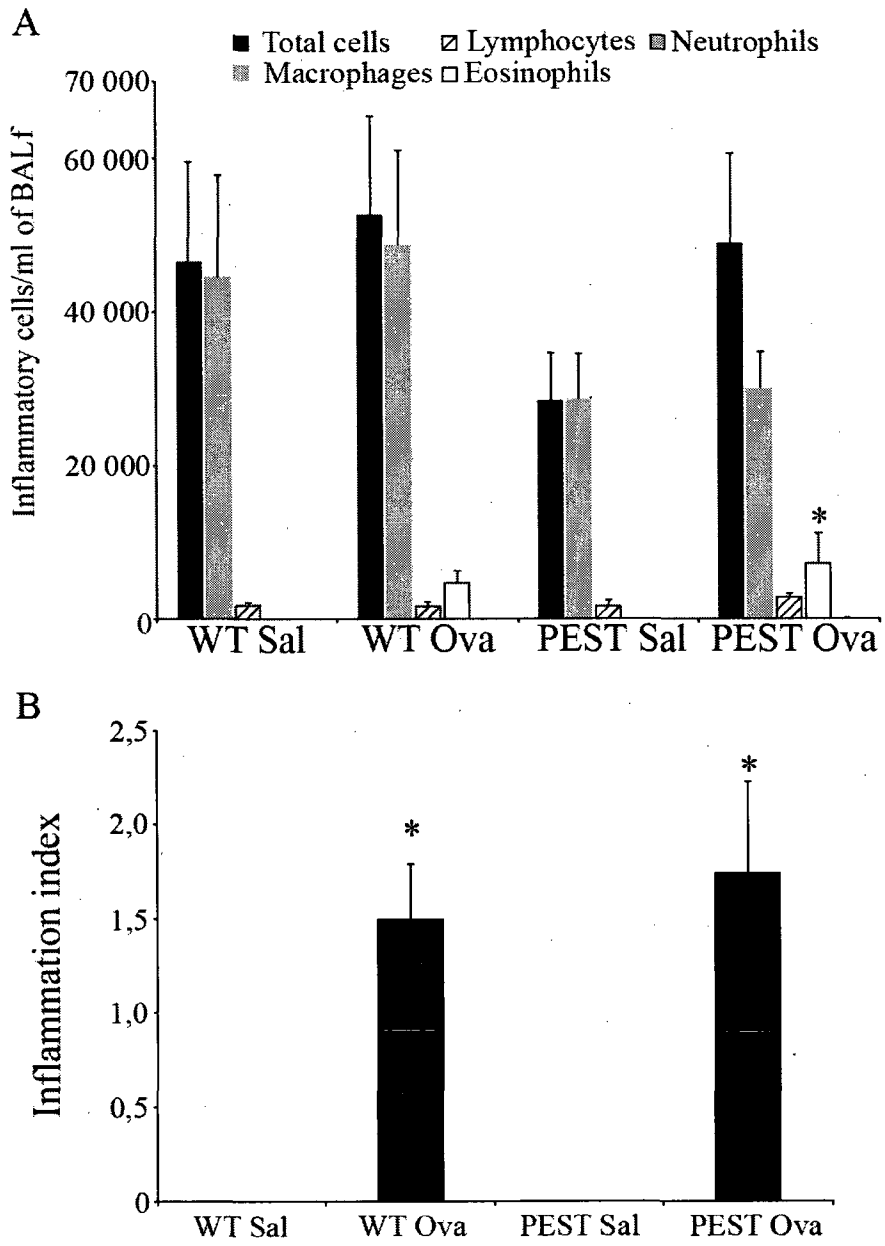

A
Total cells
Lymphocytes
Neutrophils
Macrophages
Eosinophils
Inflammatory cells/ml of BALf
70 000
60 000
40 000
20 000
0
*
WT Sal
WT Ova
PEST Sal
PEST Ova
B
Inflammation index
2,5
2,0
1,5
1,0
0,5
0
*
*
WT Sal
WT Ova
PEST Sal
PEST Ova

Figure 5: *Serum IgEs of TC-PTP$^{+/-}$ mice.*

Blood was collected at sacrifice and serum obtained. A. Total IgE and B. Ovalbumin-specific IgE levels were measured by a sandwich ELISA. Data shown represents the average of 8-12 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

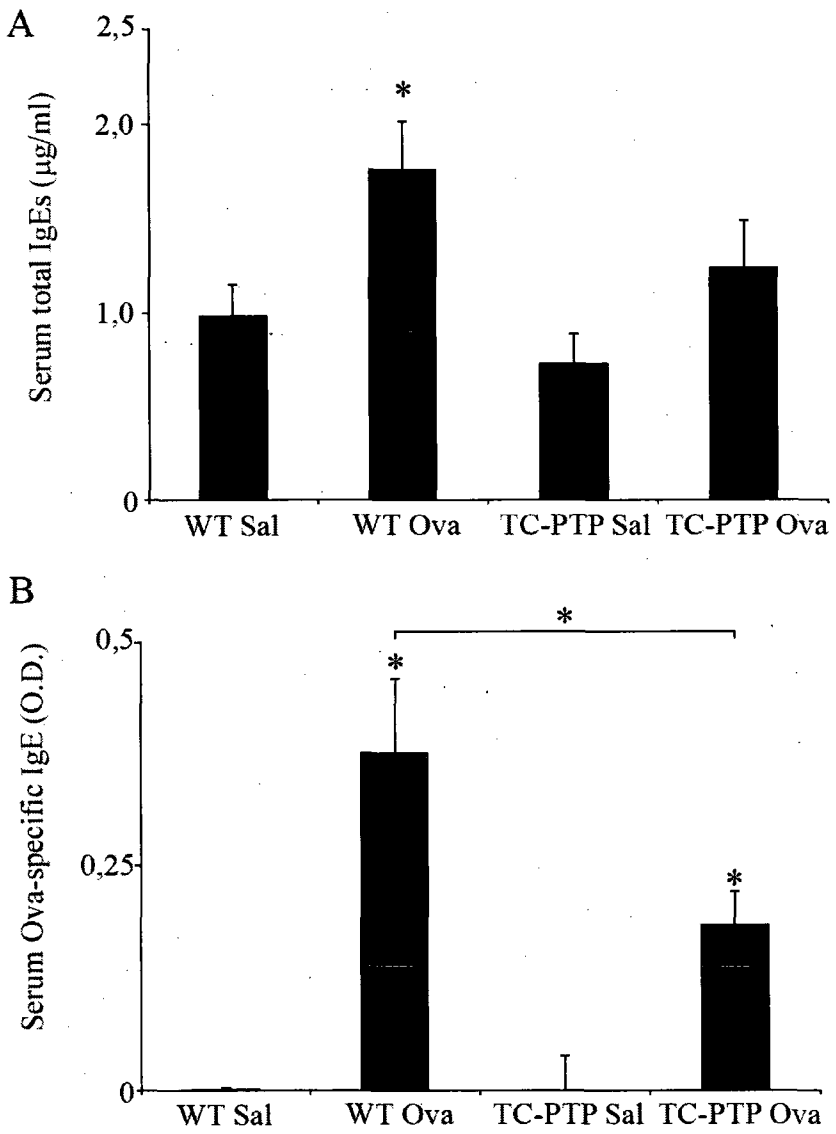
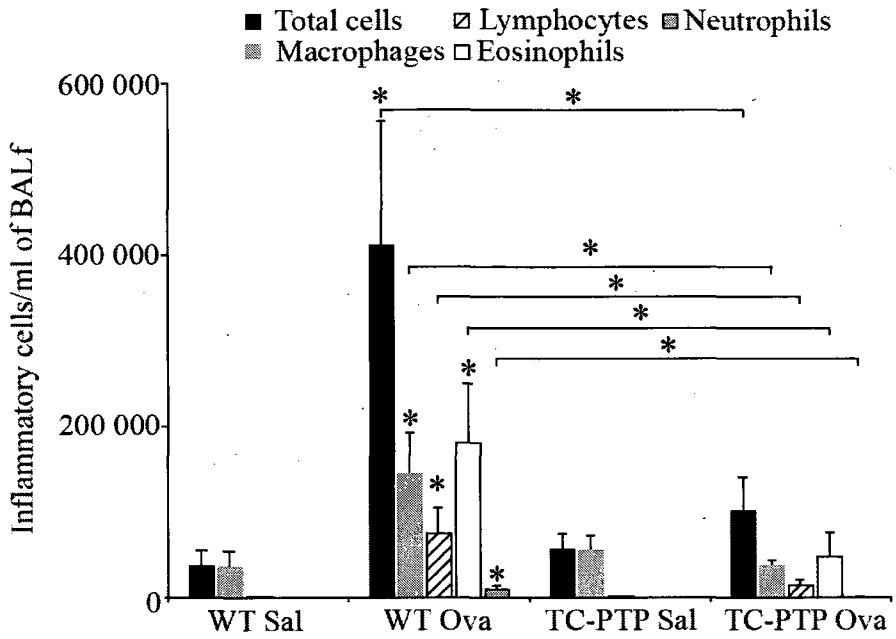

A
Serum total IgEs (µg/ml)
2,5
2,0
1,0
0
*
WT Sal
WT Ova
TC-PTP Sal
TC-PTP Ova
B
Serum Ova-specific IgE (O.D.)
0,5
0,25
0
*
*
*
WT Sal
WT Ova
TC-PTP Sal
TC-PTP Ova

Figure 6: *Lung inflammation of TC-PTP$^{+/-}$ mice.*

Inflammatory cells recruitment to the lung was evaluated. A. Recruitment of inflammatory cells to the bronchoalveolar lavage fluid compartment was evaluated by differential count and total cell count. Data shown represents the average of 5-10 animals/group. B. Local tissue inflammation was evaluated on histology cuts. Observations were made at 400x on 8-10 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).


Total cells
Lymphocytes
Neutrophils
Macrophages
Eosinophils
Inflammatory cells/ml of BALf
600 000
400 000
200 000
0
WT Sal
WT Ova
TC-PTP Sal
TC-PTP Ova

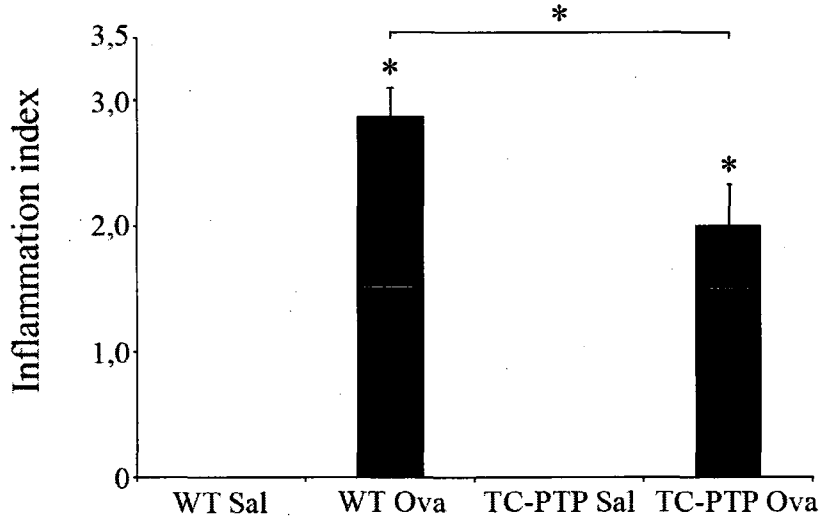

Inflammation index
3,5
3,0
2,0
1,0
0
WT Sal
WT Ova
TC-PTP Sal
TC-PTP Ova

Figure 7: *Inhibition of SHP-1 expression by adenoviruses encoding SHP-1-specific shRNA and related Serum IgEs titres.*

A. SHP-1 protein presence in the spleen was evaluated by Western blot on days 0, 3, 5 and 7 post-injection of adenoviruses. Actin is shown as loading control. Data shown represents 4 separate experiments. B. Blood was collected at sacrifice and serum obtained. Total IgE and C. Ovalbumin-specific IgE levels were measured by a sandwich ELISA. Data shown represents the average of 4 animals/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).

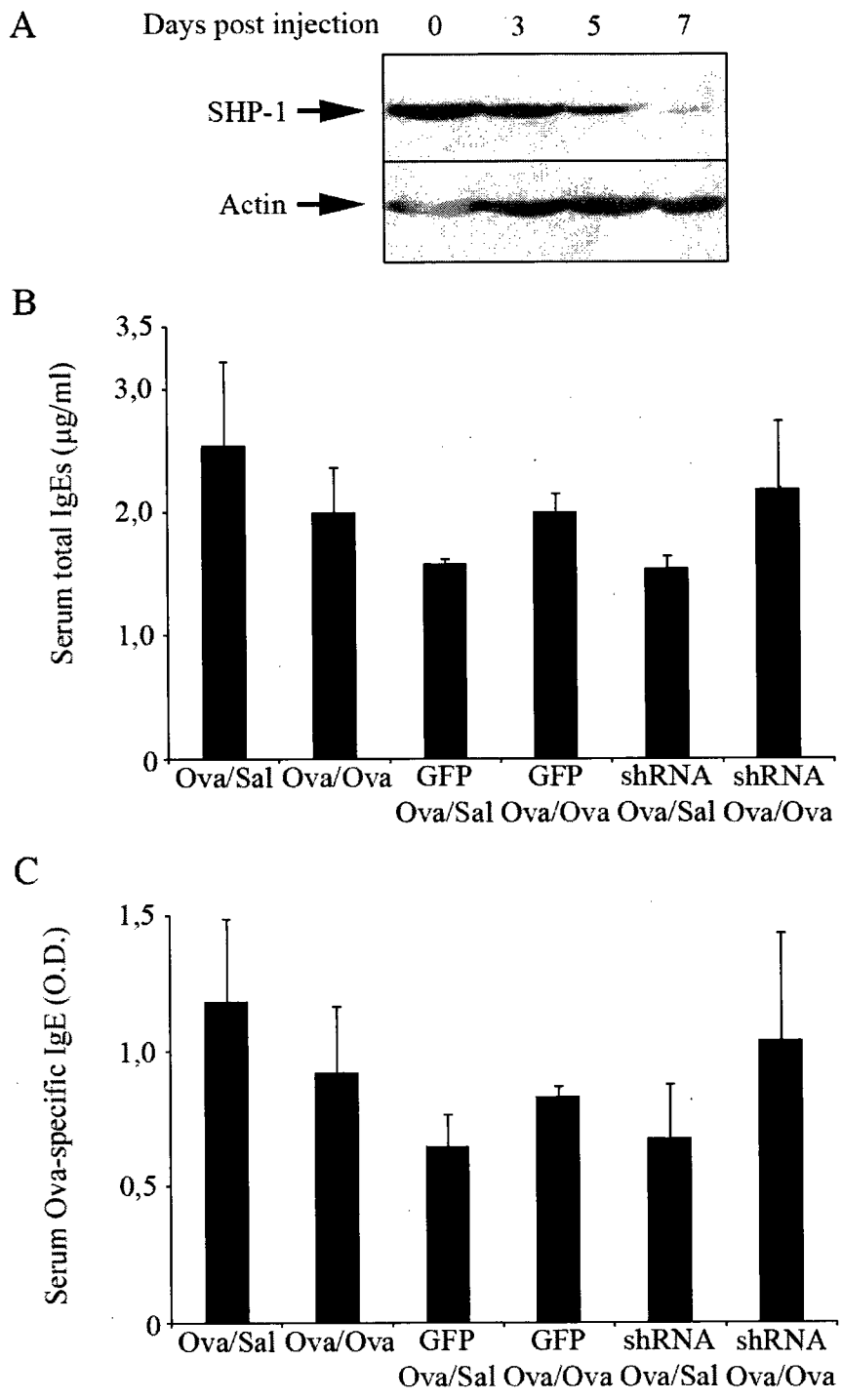
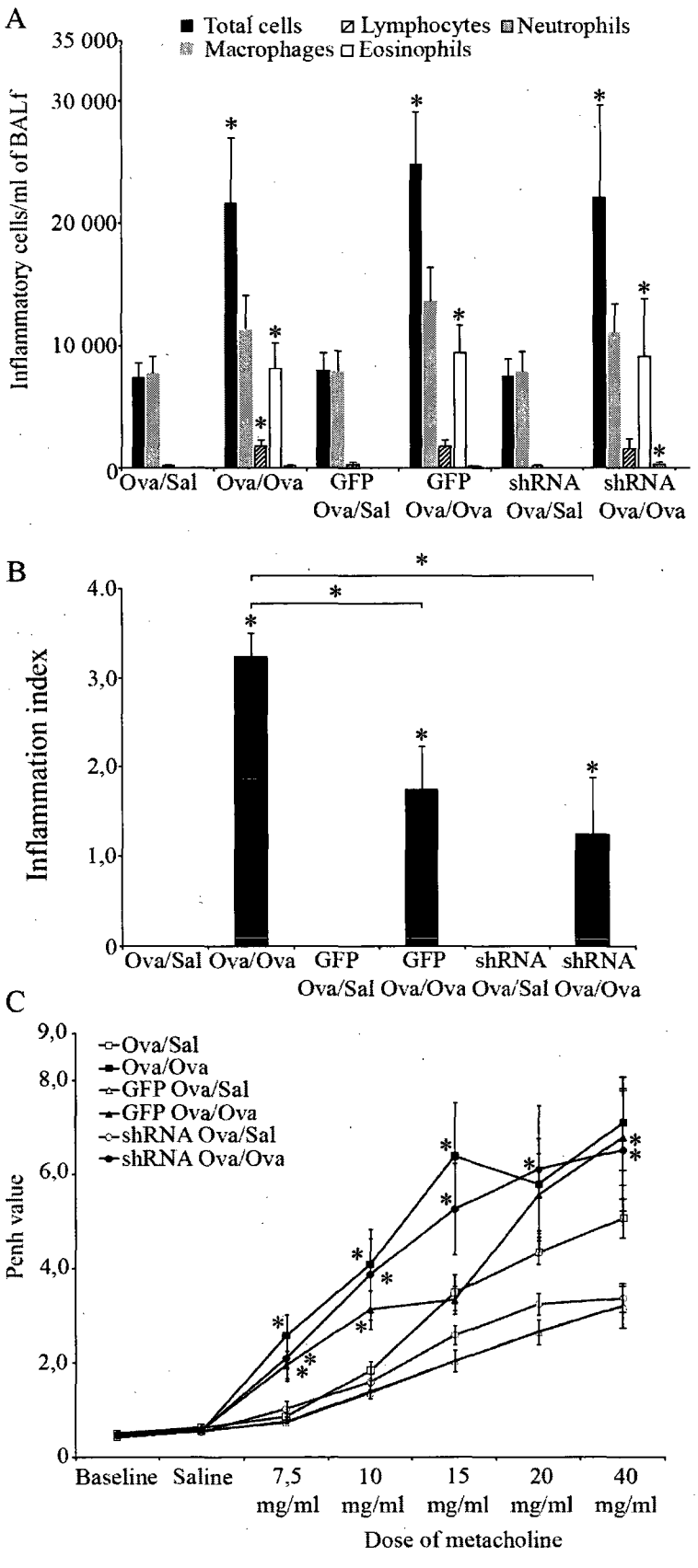

A
Days post injection 0 3 5 7
SHP-1
Actin
B
Serum total IgEs (µg/ml)
3,5
3,0
2,0
1,0
0
Ova/Sal Ova/Ova GFP GFP shRNA shRNA
Ova/Sal Ova/Ova Ova/Sal Ova/Ova
C
Serum Ova-specific IgE (O.D.)
1,5
1,0
0,5
0
Ova/Sal Ova/Ova GFP GFP shRNA shRNA
Ova/Sal Ova/Ova Ova/Sal Ova/Ova

Figure 8: *Lung inflammation of mice injected with adenoviruses encoding SHP-1-specific shRNA.*

Inflammatory cells recruitment to the lung was evaluated. A. Recruitment of inflammatory cells to the bronchoalveolar lavage fluid compartment was evaluated by differential count and total cell count. Data shown represents the average of 7-8 animals/group. B. Local tissue inflammation was evaluated on histology cuts. Observations were made at 400x on 4 animals/group. C. Mice were evaluated for airway hyperresponsiveness to methacholine in a whole-body plethysmograph. After recording the values of baseline and PBS only, increasing doses of methacholine were nebulised to the chamber. Penh value is used as a measure of airway hyperresponsiveness. Data shown represents the average of 8 mice/group. * = significant difference with appropriate control or identified sample ($p \leq 0,05$).


A
Total cells
Lymphocytes
Neutrophils
Macrophages
Eosinophils
Inflammatory cells/ml of BALf
35 000
30 000
20 000
10 000
0
Ova/Sal
Ova/Ova
GFP Ova/Sal
GFP Ova/Ova
shRNA Ova/Sal
shRNA Ova/Ova
B
Inflammation index
4,0
3,0
2,0
1,0
0
C
Ova/Sal
Ova/Ova
GFP Ova/Sal
GFP Ova/Ova
shRNA Ova/Sal
shRNA Ova/Ova
Penh value
9,0
8,0
6,0
4,0
2,0
0
Baseline
Saline
7,5 mg/ml
10 mg/ml
15 mg/ml
20 mg/ml
40 mg/ml
Dose of metacholine

Chapter 4: Myeloid-Related Proteins Rapidly Modulate Mθ Nitric Oxide Production During Innate Immune Response

In this chapter, we wanted to investigate the signalling pathways triggered by the MRPs, proteins that could have a role in severe asthma exacerbations. In acute asthma exacerbations, it has been reported that levels of MRP8 are resistant to corticosteroid treatment, and lung neutrophilia also. Knowing both the potent chemotactic and activatory capacities of MRPs on neutrophils, and the abundant production of MRPs by neutrophils, it is probable that the presence of MRP8 is elevated due to their release by neutrophils and that lung neutrophilia is sustained by the secretion of MRP. As neutrophil apoptosis is reduced by corticosteroids, their presence in the lung is prolonged and the detrimental effects of neutrophils are possibly increased. Although this remains to be clearly demonstrated, this hypothesis is appealing. Preliminary work in the laboratory also suggested that MRPs were able to increase NO production by Mθs. This observation is also very intriguing as NO is known to be increased in asthma. Furthermore, the elevated levels of NO observed in asthma are most probably to be attributed to NOS2 activity as this enzyme produces high concentrations of NO. Such NOS2 activity in asthma was reported in the epithelium, but also in inflammatory cells. Taken together, this suggests that MRPs could have a role in increasing the production of NO in the lung. Therefore, we have been interested to investigate the signalling pathways triggered by MRPs, in order to eventually reduce the production of NO in asthma and prevent the feedback loop produced by MRPs and neutrophils in severe asthma exacerbations. The receptor for MRPs is still debated, but a thorough demonstration of the implication of TLR4 was recently published. In the present manuscript, we describe the pathways employed by MRPs to signal from the membrane to the nucleus.

As this work also has implications in the modulation of inflammatory and microbicidal functions of Mθs, the manuscript was written with this perspective.

This chapter is now published in the *Journal of Immunology* (2008 Sep 1;181(5):3595-601). In this study, Dr Martin Olivier designed the project, supervised the work of the thesis author and analysed the data with P. Pouliot. Isabelle Plante performed numerous experiments that represent many replicates of figures 1, 2 and 3. Marie-Astrid Raquil prepared the recombinant MRP proteins used in the described experiments. Dr Tessier provided our laboratory with the recombinant MRP proteins. The thesis author designed experiments along with Dr Olivier, performed the experiments described here and analysed the results. An approximative ratio of work could be established as following: Isabelle Plante, 18%; Marie-Astrid Raquil, 1%; Dr Tessier, 1%; Dr Olivier, 10%; Philippe Pouliot, 70%.

Myeloid-Related Proteins Rapidly Modulate Macrophage Nitric Oxide Production During Innate Immune Response

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Running title: MRPs induce macrophage nitric oxide production

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Keywords: Nitric oxide, cell activation, inflammation, signal transduction.

Abstract

S100A8 and S100A9 are intracellular calcium-binding proteins produced by myeloid cells that promote neutrophil/monocyte recruitment at inflamed tissues by enhancing attachment to endothelial cells. Although intracellular functions of these proteins (*a.k.a.* myeloid-related protein 8 and 14; MRP8 and MRP14) are not completely understood, they exhibit prominent extracellular cytokine-like functions and are considered reliable markers of inflammation in diverse diseases. As S100A8 and S100A9 have been reported to be rapidly released in response to components derived from infectious agents, we hypothesized that they play an important role in the modulation of key microbicidal phagocyte functions. Here we report for the first time that MRPs are powerful inducers of nitric oxide (NO) production by murine macrophages (Mθs). This increase in NO production was linked to an increased iNOS expression both at gene and protein level. This induction was concomitant with an important phosphorylation of SAPK/JNK, but also of MEK and ERK kinases. Upon stimulation with MRPs, NF-κB was rapidly translocated to the nucleus (30 min). When Mθs were treated concomitantly with IFN-γ, another activator of Mθ functions, we observed a strong synergy in NO production, synergy that resulted from the engagement of exclusive signalling pathways: SAPK/JNK, ERK and NF-κB were involved in signalling of MRPs while IFN-γ uses the JAK/STAT pathway. This suggests that the synergy results from interactions of transcription factors in the promoter region. Finally, we observed this effect to be dependent on TLR4. Collectively, our study unravels the importance of MRPs as potent new inducers of Mθ NO production.

Introduction

S100 proteins form a family of small intracellular proteins (10-12 kDa) of approximatively 20 members. These proteins are formed of two Ca^{2+}-binding domains which are separated by a hinge region (97). While their action have been mainly reported to occur intracellularly, more attention is now given to their important extracellular roles (307). For instance, S100A8 and S100A9 (*a.k.a.* myeloid-related proteins-MRP-8 and -14) which are found as homo- or heterodimers (308), exhibit antimicrobial properties (102). The heterodimeric form is usually translocated to the membrane in response to intracellular Ca^{2+} mobilization prior to their release through a golgi-independent secretion system involving cytoskeleton-membrane interactions (309).

S100A8 and S100A9 also exhibit important functions in inflammation (106-109;310), where S100A8 is known to induce polymorphonuclear (PMN) chemotactism (105). In particular, we have previously reported that S100A8 and S100A9 are important neutrophil recruitment mediators in response to LPS as treatment with α-S100A8 and α-S100A9 considerably reduced neutrophil recruitment, and as MRPs induced the release of neutrophils from bone marrow to the blood stream (110). Although MRPs are mostly known to be produced by neutrophils where they can represent as much as 30 to 40% of cytosolic proteins (96), they are also produced by cells of the monocytic lineage (311). It is still unclear which cell receptor is relevant for S100A8/A9 extracellular roles. CD36 was found to bind the heterodimer and facilitate uptake of lipids (312), while it was also noticed that S100A8/A9 could bind heparan sulfate glycosaminoglycans (313) or carboxylated glycans (314) on endothelial cells. As RAGE (receptor for advanced

glycation end product) is the receptor of S100A12 (315) , it is often speculated that it might act as the receptor for S100A8/A9. This topic is still controversial and recently, S100A8 and S100A8/A9 have been shown to be endogenous ligands of TLR4 (316), suggesting an interesting mechanism for their extracellular role.

Knowing MRPs potential to activate PMNs during inflammatory processes and knowing that MRPs secretion can rapidly be induced by pathogen by-products (*e.g.* LPS and *Plasmodium* hemozoin) (317), we were interested to determine whether MRPs can also be important activator of macrophage (Mθ) functions, which is a key player of innate immune response. Due to its importance as an antimicrobial agent, nitric oxide (NO) was of critical interest.

In this paper, we report for the first time that MRP homodimers (S100A8 and S100A9) or their heterodimer can induce NO production from Mθ in an iNOS expression-dependent manner. Of further interest, IFN-γ and S100A8/A9 showed a synergistic effect on NO generation. In an attempt to delineate the signalling pathways underlying MRP-induced NO by Mθ, we observed an important phosphorylation of SAPK/JNK kinases as well as a more modest, but conserved phosphorylation of MEK1/2 and ERK1/2, concurrent with NF-κB nuclear translocation. Of importance, IFN-γ and MRPs engage distinct pathways, suggesting that the synergy results from interactions between transcription factors in the promoter region of the iNOS gene.

At the view of this finding, it is clear that MRPs play a crucial role in cytokine-mediated phagocytes activation during innate immune response, and that further investigation concerning its potential as modulator of antimicrobial functions should be considered.

Material and methods

Reagents

Murine recombinant S100A8 and S100A9 were produced as described previously (318) and found to contain less than 1 pg endotoxin/μg protein as we previously reported (319). Recombinant murine IFNγ was purchased from Fitzgerald (Concord, MA, USA). The α-iNOS and α-pJAK2 (Tyr 1007/1008) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnologies, Santa-Cruz, CA, USA), while α-pSTAT-1α Tyr701, α-pSTAT-1α Ser 727, α-pSAPK/JNK (Thr 83/Tyr185), α-pMEK1/2 (Ser217/221) and α-pERK1/2 (Thr202/Tyr204) and their α-protein antibodies were purchased from Cell Signaling (Cell Signaling, Danvers, MA, USA). Oligonucleotides containing STAT-1, NF-κB, CREB and AP-1 consensus binding sequences came from Santa Cruz Biotechnology. Inhibitors of MAPKs, SP600125 (25 μM), PD98059 (100 μM), Apigenin (50 μM) and SB203580 (1 μM) were bought from Biomol (Plymouth Meeting, PA, USA). All material for cell culture was bought from Gibco (Gibco-Invitrogen, Burlington, ON, Canada).

Cell culture and nitric oxide assay

B10R murine bone-marrow derived Mθ (BMDM) cell line was grown as described previously (320) and kept in DMEM culture media supplemented with 10% FBS, penicillin (100U/ml), streptomycin (100μg/ml) and 2 mM L-glutamine. B10Rs were seeded in 24 wells plate at $1x10^5$ cells/well overnight. The next day, MRPs were added to the wells at specified concentration for 24h. B10R TRL4 K/O Mθs obtained as we

previously reported (321) were also used in similar conditions. BMDMs were obtained from Balb/c mice using standard protocol (302). In situations where IFN-γ was used, it was added concomitantly to MRPs at 100 U/ml. At 24h of incubation, plates were centrifuged and supernatant collected for nitrite measurement by Griess reaction as we reported previously (322). When MAPKs inhibitors were used, they were added 1h before the MRP treatment for 24h at optimal subcytotoxic concentrations. The maximal subcytotoxic concentration was assessed by the XTT reduction assay. An inhibitor concentration was considered cytotoxic if viability was less than 90% of untreated control after 24h.

Western Blotting

Western blotting was performed as previously described (291). Briefly, cells were plated overnight at $5x10^5$/well in a 6 well plate and stimulated the next day with MRPs and/or IFN-γ, as described in legend figures. After the defined time, cells were lysed in cold buffer containing 20mM Tris-HCl (pH 8.0), 0.14 M NaCl, 10% glycerol (v/v), 1% NP-40 (v/v), 1mM PMSF, 1mM sodium orthovanadate (Na_3VO_4), 1μM NaF and protease inhibitors (40μg/ml aprotinin and 20μg/ml leupeptin). The lysates (30μg/lane) were separated by SDS-PAGE and proteins transferred to polyvinylidene difluoride membranes (PVDF, Millipore). Membranes were blocked in Tris-buffered saline/0.1% Tween containing 5% milk (or 1,5% fetal bovine albumin for α-SAPK/JNK antibody), for 1 hour at room temperature. Then membranes were washed and incubated with monoclonal antibody for 1 hour (phospho-JAK2, α-phospho-ERK1/2, α-phospho-MEK1/2, α-phospho-STAT-1α Ser and α-phospho-STAT-1α Tyr) or overnight at 4°C

for α-phospho-SAPK/JNK. After washing, membranes were incubated with anti-rabbit or anti-mouse horseradish peroxidase (HRP)-conjugated antibody (GE healthcare, Baie d'Urfé, QC, Canada) for 1 hour and proteins visualized with the use of the ECL Plus Western Blotting reagent (GE Healthcare). Membranes were then stripped using the Restore Western Blot Stripping Buffer (Pierce, Nepean, ON, Canada) and blotting was done again using α-proteins antibodies (same manufacturer as α-phosphoproteins).

Electrophoretic Mobility Shift Assay

EMSA was performed as we previously described (221). Briefly, $1x10^6$ cells were plated in a 25 cm^2 flask and let to adhere overnight before treatments. Cells were then treated with IFN-γ and/or MRPs. Cells were washed with ice-cold PBS then scraped in 1 ml PBS. After centrifugation, cells were resuspended in 400µl of ice-cold buffer A (10mM Hepes, pH 7.9, 10mM KCl, 0.1mM EDTA, 0.1mM EGTA, 1mM dithiothreitol (DTT) and 0.5mM PMSF), and incubated on ice for 15 min. Twenty-five µl of 10% IGEPAL (Sigma) were then added and tubes vortexed for 10 sec, and centrifuged at maximum speed for 30 sec. Nuclear fractions were resuspended in 50µl of ice-cold buffer C (20mM Hepes, pH 7.9, 400mM NaCl, 1mM EDTA, 1mM EGTA, 1mM DTT and 1mM PMSF) and incubated at 4°C on a shaking platform for 15 min. After centrifugation at 12000x*g* for 5 min at 4 °C, the supernatants were stored at -70°C until further use. Six µg of these nuclear protein extracts were mixed with a γ^{32}P-labelled oligonucleotide containing a consensus binding sequences for STAT-1α, NF-κB, CREB or AP-1. Complexes were then resolved by electrophoresis on a 4% non-denaturing polyacrylamide gel. The gels were dried and visualised by autoradiography. The consensus sequences for NF-κB,

STAT-1, CREB and AP-1 were 5'-AGT-TGA-GGG-GAC-TTT-CCC-AGG-C-3', 5'-AAG-TAC-TTT-CAG-TTT-CAT-ATT-ACT-CTA-3', 5'-AGA GAT TGC CTG ACG TCA GAG AGC TAG-3' and 5'-CGC-TTG-ATG-ACT-CAG-CCG-GAA-3', respectively.

Northern blotting

Northern blotting was performed as described previously (245). Cells were plated at $1x10^6$ Mθs/25cm^2 flask and stimulated for 8h (unless specified otherwise) before being washed in ice-cold PBS. RNA was then extracted using TRIzol (Invitrogen) following manufacturer's protocol). Twenty μg of RNA were loaded onto 1% agarose gels, equal loading and RNA integrity were confirmed by ethidium bromide staining. RNA was then transferred onto Hybond-N filter paper and hybridized with random primer-labeled cDNA probes. Equal loading of RNA was also confirmed by hybridization with glyceraldehyde-3-phosphate dehydrogenase cDNA probe. All washes were performed under stringent conditions. The mRNA hybridizing with the cDNA probe was visualized by autoradiography. Probes have been kindly provided by Dr. Danuta Radzioch from the Montreal General Hospital Research Center (McGill University, Montréal, Québec, Canada).

Statistical analysis

Statistically significant differences were identified using the ANOVA module of StatView from the SAS institute (version 5, SAS Institute, NC). *P* values of <0.05 were considered statistically significant. All data are presented as mean ± SEM.

Results

Induction of nitric oxide production by MRPs.

Initially, we have been interested to test whether MRPs, (S100A8, S100A9 and the heterodimer S100A8/A9) had intrinsic capacity to induce NO generation. As reported in Figure 1A, all MRPs utilized were shown to be strong inducer of iNOS gene expression to a level even slightly more elevated than our positive control, IFN-γ (100 units/ml). This increase in iNOS gene expression was followed by an increase in protein expression (Fig 1B). Then NO production was investigated (Fig. 1C) and MRPs used alone were observed to induce NO production in Mθs in amounts comparable with IFN-γ-treated Mθs. This observation paralleled iNOS expression which shows the implication of iNOS in the observed increase of NO in MRP-stimulated Mθs. Taken together, this initial set of data showed for the first time that MRP proteins are important inducer of iNOS and consequently NO.

The concentration of MRPs used initially (10 μg/ml) was in the physiological range as measured previously in our air-pouch model upon stimulation with pro-inflammatory compound (317), but we decided to perform a dose-response experiment in order to fully determine MRPs NO-induction potency on Mθs. As can be seen in Fig. 2A, iNOS gene expression was increased by all MRP compounds at the level of IFN-γ with doses as low as 1 μg/ml of MRPs and increased substantially until our maximal assessed dose of 50 μg/ml. NO production (Fig. 2B) matched closely the increase in iNOS gene expression and was undetectable at low doses (0,01 to 0,1 μg/ml), but started to be detected at 1 μg/ml to reach a plateau at the highest physiological doses used (10 to 50 μg/ml). This

trend is conserved for all MRPs used: S100A8, S100A9 and their heterodimer. Data reported in this section clearly reveals the potency of MRPs as iNOS/NO inducer in comparison to IFN-γ (100 U/ml), a known iNOS-inducer in Mθs (322). Noticeably, even at low doses, MRPs were inducing comparable iNOS expression as IFN-γ-stimulated cells. As the heterodimer S100A8/A9 was representative of the effects induced by all MRPs preparation, this preparation was used throughout the next experimentations.

Thereafter we were interested to determine the time-response effect of MRPs on Mθs in order to appreciate the rapidity of their modulation over a 24h period. Additionally we wanted to compare MRPs NO-production potency to IFN-γ, a known NO inducer to better appreciate their potency. As revealed in Figure 3A, 100 U/ml IFN-γ stimulation increased iNOS mRNA by 8h post-stimulation, while stimulation with 10μg/ml of S100A8/A9 alone induced iNOS expression as soon as by 4h. Most interestingly, stimulation with both compounds resulted in a very rapid (as soon as by 2h) and dramatic increase in iNOS mRNA production that was sustained for 24h. The mRNA induction was not restricted to iNOS as we also observed an induction of TNF-α mRNA upon treatment with IFN-γ or MRPs. Interestingly, stimulation with both compounds resulted in a stronger and more sustained presence of TNF-α mRNA, correlating observations with iNOS. Protein production was then confirmed by immunoblotting (Fig. 3B). Stimulation with IFN-γ induced iNOS production beginning around 8h, while MRPs induced iNOS protein as soon as by 4h, but this production decreased after an optimum around 8h, correlating well with mRNA expression. On the other hand, when both IFN-γ and MRPs were used, iNOS protein was observed early at 4h at a level higher than any

compound alone, reached an all-condition maximum at 8h before reducing for 24h, as opposed to the sustained mRNA presence, suggesting that some post-transcriptional regulation mechanisms limit the iNOS production from mRNA. Final NO production was measured (Fig. 3C) and observed at 24h for IFN-γ stimulation and earlier (8h) for MRPs stimulation, reflecting observations at gene and protein level. When both compounds were used, NO production began at 8h at a level higher than any stimulation alone and was also increased at 24h, suggesting some synergy between the compounds.

IFN-γand MRPs synergistic effect on Mθ-derived NO production.

At the view of a possible synergy between IFN-γ and MRPs, we have been interested to further investigate this important phenomenon. Using a fixed dose of IFN-γ (100 U/ml), which provides an adequate NO production, and increasing concentrations of S100A8/A9 (0,01 μg/ml to 10 μg/ml), we have evaluated whether MRPs and IFN-γ can synergistically induce NO. To illustrate the synergy, cells were stimulated with IFN-γ and MRPs alone or in combination. NO production values after stimulations with compounds alone were summed to provide the expected additive value then the observed NO production upon combined stimulation was expressed as a percentage of this observed additive value. Fig. 4 shows a clear synergistic NO production by cells stimulated with both compounds simultaneously. All doses used result in a clear increase in NO production (more than 100% of observed additive value), but low doses of MRPs (0,01 and 0,1 μg/ml) did not reach statistical significance. This illustrates that IFN-γ and MRPs can have a synergistic effect on Mθ NO production that is more than additive.

Deciphering the signalling pathways involved in MRPs-induced NO generation.

As this is the first report that MRPs are powerful inducer of NO, we aimed at determining the pathways engaged in Mθs after MRP stimulation in Mθs, a task even more necessary as pathways involved in MRP signalling are not clearly identified. Furthermore, given the synergy observed with IFN-γ, it was also of paramount importance to determine whether the signalling pathways used by these two S100 proteins were similar to the known signalling events triggered upon stimulation of Mθs by IFN-γ. As both IFN-γ and MRPs were increasing NO production, we first monitored whether the IFN-γ-induced JAK2/STAT-1 pathways was also induced by MRPs. As shown in Figure 5, IFN-γ was concurring to its known phosphorylation of Mθs STAT-1α on both Tyr 701 and Ser 727 residues, sign of STAT-1α activation. This was also accompanied by the phosphorylation of JAK2 (Tyr 1007/1008). Interestingly, MRPs alone did not induce any phosphorylation on these proteins. A combination of IFN-γ and MRPs did not further increase the phosphorylation observed with IFN-γ alone. Therefore this set of data strongly suggests that JAK2/STAT-1α pathway is not involved in MRPs-induced signalling concurring to NO generation, even in the context of a synergistic effect.

In parallel, we also investigated the various MAPKs pathways. First we were interested to decipher the MEK/ERK pathway. We observed that MEK1/2 phosphorylation (Ser 217/221) was more induced by MRPs in comparison to IFN-γ. This MRPs-induced phosphorylation was observed already at 15 min post-stimulation and was sustained up to 1h to a comparable level in samples stimulated with MRPs alone or in combination with IFN-γ (Fig. 5). On the other hand, activation by IFN-γ *per se* was only noticeable by 1hr

post-stimulation. Thereafter, downstream phosphorylation of ERK1/2 (Thr 202/Tyr 204) was also monitored. As observed for MEK1/2, a similar MRPs-induced ERK1/2 phosphorylation was seen from 15 min to 1h after stimulation (Fig. 5). Here again, IFN-γ was observed to induce ERK1/2 phosphorylation later in time and to a lesser extent than MRP alone or in combination with IFN-γ. We also investigated the involvement of SAPK/JNK kinase pathway in this experimental context. Of great interest, we discovered that MRPs can induce SAPK/JNK (Thr183/Tyr185) phosphorylation in a similar manner as observed for MEK/ERK kinases from 15 min to 1h with an optimal peak at about 30 min post-stimulation (Fig. 5). Of utmost importance, IFN-γ was shown not to cause SAPK/JNK phosphorylation. Therefore, at the difference of IFN-γ, the MRPs seem to selectively exploit the SAPK/JNK pathways to induce NO generation. Differential use of JAK2/STAT-1 pathway by IFN-γ and SAPK/JNK pathway by MRPs, may concur to amplify the signal leading to NO generation in a synergistic manner. As both molecules use different signalling pathways, these data suggest that the synergistic effect observed does not involve a greater induction of a pathway engaged by both molecules, but might rather depend on engagement of transcription factors.

Pharmalogical inhibition of proteins involved in MRP signalling.

As our results point to a role for SAPK/JNK, MEK1/2 and ERK1/2 in the transduction of signal (Fig. 5), we wanted to confirm the implication of MAPKs in NO production upon MRP stimulation. To achieve this, we chose to inhibit these kinases using specific inhibitors used at optimal sub-cytotoxic concentrations (data not shown). Figure 6 shows that inhibition of SAPK/JNK (SP600125) and ERK1/2 (Apigenin) resulted in a complete

abrogation of NO production by MRPs-stimulated cells, in accordance with the observed increase in their phosphorylation. Interestingly, MEK1/2 inhibition (PD98059) did reduce NO production, but not to the extent of ERK1/2 inhibition, possibly suggesting a mechanism of ERK1/2 activation independent of MEK1/2, a possibility also supported by protein phosphorylation patterns where ERK1/2 phosphorylation is more prominent than MEK1/2 phosphorylation. As suggested by preliminary immunoblottings (data not shown), p38 MAPK was not involved in MRP-mediated NO generation, as reflected by the conserved NO production upon p38 inhibition (SB203580).

Transcription factors involved in MRPs-induced NO generation.

In order to complete the signalling picture of MRPs in Mθs, we investigated the down-stream activation of transcription factors usually involved in IFN-γ-induced iNOS expression (STAT-1, NF-κB, CREB and AP-1). As a prominent inflammatory transcription factor, NF-κB involvement was monitored. As expected, IFN-γ-induced NF-κB translocation was detected at late time-points (2-4h) (Fig. 7), but strikingly, in response to MRPs stimulation, we observed a stronger and quicker induction of Mθs NF-κB activity that was sustained for 30 min to 1h, and decreased to a lesser level by 4h post-stimulation. A very similar level of induction was observed for IFN-γ/MRPs-stimulated cells, identifying MRPs as a potent inducer of NF-κB. Thereafter, we have been interested to confirm both the induction of STAT-1α translocation by IFN-γ stimulation and the incapacity of MRPs to do so, as suggested by the incapacity of MRPs to induce JAK2/STAT-1 phosphorylation. As shown in Figure 7, STAT-1α translocation is, as expected, observable as soon as 1h upon IFN-γ stimulation and sustained up to 4h. STAT-

1α activation was confined to IFN-γ and IFN-γ/MRPs treated cells as MRPs *per se* were not inducing its activation, therefore confirming that JAK2/STAT-1 pathway is exclusively used by IFN-γ in this context. Other transcription factors involved in the regulation of iNOS were investigated (AP-1 and CREB), but MRPs were not significantly modulating these factors (data not shown).

Nitric oxide production in bone marrow-derived Mθs and implication of TLR4 in MRPs-induced nitric oxide production.

Knowing the pathways triggered by MRPs, we were interested to investigate the effect of MRPs on NO production in bone marrow-derived Mθs (BMDMs). As shown in Figure 8A, stimulation of BMDMs with MRPs induced NO production. IFN-γ on the other hand, did not *per se* induce NO production, reflective of an IFN-γ-priming, as BMDMs could still be activated by LPS. Very interestingly, stimulation with both IFN-γ and MRPs induced a striking synergistic NO production, confirming our observations in B10R Mθs. It is also noteworthy that MRPs and LPS co-stimulation does not further increase the NO production induced by LPS alone. As TLR4 was reported to be the receptor for MRPs (316), this observation might suggest that NO production can not be increased anymore, due to the fact that both compounds signal through TLR4 pathway. In order to verify this possibility, we used a Mθ cell line with a TLR4 gene deletion (in the B10R background) and observed NO production after MRP stimulation. As can be seen in figure 8B, LPS stimulation induced NO production only in WT B10Rs. MRPS-induced NO production was observed in WT B10R Mθs, but was not observed in TLR4 K/O Mθs, revealing a necessary role for TLR4 in sensing extracellular presence of MRPs. Costimulation of

both cell lines with MRPs and LPS was not observed to further stimulate NO production, as observed in Fig. 8A.

Discussion

The S100 protein family is a wide group of intracellular proteins involved in many cellular events such as contraction, motility, secretion, and cell differentiation (reviewed in (97;323)). Among this family, S100A8, S100A9 and S100A12 are reported to be important in the immunological context (324). For instance, these proteins were reported to be chemotactic for neutrophils and to be produced by these cells (101;109;319;325), creating a positive feedback loop. More recently, we reported that S100A8, S100A9 and their heterodimer were strongly induced by hemozoin injection (a malaria-related metabolic waste) in the context of an air pouch model (317). Among the cells recruited in this system were neutrophils, as well as monocytic cells (317). Therefore, in the present study, we have been interested to test whether S100A8 and S100A9 may have immunostimulatory effects on cells of monocytic lineage as Mθs.

As NO plays a key role in Mθs microbicidal functions during innate immune response, we investigated this mediator to assess whether MRPs can be an important player in Mθs activation. Of utmost interest, the present study reports the first observation that MRPs are effectively important inducers of NO, an observation even more interesting given the fact that they were used at physiologically relevant doses (10 μg/ml) as we previously measured *in vivo* (317). For instance, we observed that all MRPs used in our study were similarly inducing NO production in correlation with equivalent increase in iNOS gene expression. This finding is of paramount importance as epithelial cells are known to secrete MRPs and therefore it suggests that this could be sufficient to rapidly induce microbicidal functions of local Mθs in response to infectious agents.

The activation induced by MRPs alone showed to be even faster than IFN-γ-driven Mθ activation (Fig.3), suggesting an important role *in vivo* for MRPs. MRPs might have a preponderant role as they can activate freshly recruited cells very quickly in addition to their chemotactic capacity for inflammatory cells. Moreover, we observed that if both IFN-γ and MRPs (S100A8/A9) are present, Mθs produce even more NO, and as with MRPs alone, begin more quickly. Upon recruitment to the site of ongoing inflammation, a mediator cocktail containing IFN-γ and MRPs might be very potent at activating recruited Mθs. Most interestingly, the interaction of the two products induced a remarkably rapid and strong induction of iNOS gene, beginning at 2h and quickly resulting in more mRNA expression than with each compounds alone. Importantly, the fact that maximal iNOS mRNA expression in response to IFN-γ/MRPs cocktail does not result in proportional translation of iNOS protein indicates that post-transcriptional events regulate the production of iNOS protein and probably represents a negative feedback loop that prevents excessive production of NO, an event that could potentially drive NO-induced apoptosis in producing cells. This negative feedback mechanism would deserve further investigation in a near future.

The enhanced production of NO proved to be synergistic at doses of MRPs between 1 and 10 μg/ml where the produced NO is well over the expected additive value. This clearly suggests an important *in vivo* role as these two mediators could easily be inducing strong pro-inflammatory capacities of freshly recruited Mθs and it clearly states that MRPs have more functional extracellular capacities that the mere chemotactic and adhesion inducer effects generally recognized. Although the synergy was consistently observed through our

experiments, a variation in its intensity was noted. We attribute this variation in MRP stimulatory activity to variations in biological activity between MRP preparations. We are actually working on a method to standardize this biological activity.

As we established that NO is induced upon stimulation with MRPs, we attempted to identify the signalling pathways involved in response to S100A8/A9. The receptor for S100A8/A9 is still elusive although it is known that the heterodimer can bind heparan sulfate by the S100A9 subunit (313) and carboxylated N-glycans on endothelial cells (314). Some speculated that the RAGE might be the receptor for S100A8/A9 as it is the receptor for S100A12 (315), but other studies were unable to demonstrate its implication in the response induced by S100A8/A9 (326). A recent report also indicates that S100A8/A9 is an endogenous ligand of TLR4 (316). Here we focused on identifying the pathways engaged by the recognition of the MRPs complex and to see if they were entangled with the ones engaged by IFN-γ stimulation which could help us to understand their synergy. IFN-γ and MRPs induced the activation of different and exclusive pathways. As generally recognized, IFN-γ stimulation resulted in JAK2/STAT-1α pathway activation. On the other hand, S100A8/A9 engaged MEK1/2 & ERK1/2 pathway at an early time-point (15 min) and this pathway's phosphorylation is almost back to normal by 2h. This is in agreement with the recently suggested induction of the ERK/MEK pathway by MRPs in bone marrow cells (19). It is interesting to note that MEK phosphorylation is not as prominent as ERK phosphorylation and that pharmacologic inhibition of MEK does not results in complete abrogation of NO production whereas inhibition of ERK does. Although MEK/ERK is often viewed as

linear, recent results show that some proteins, as $p56^{lck}$ and PKCε can bypass MEK to activate ERK (327). Such a mechanism could explain why ERK inhibition is more potent than MEK inhibition in our model.

(316). Very interestingly, SAPK/JNK pathway was activated rapidly with an optimal phosphorylation around 30 min but was more prominently phosphorylated than the MEK/ERK pathway. The fact that IFN-γ and MRPs engage exclusive pathways could suggest that the co-stimulation will result in an additive effect. But as we observed a synergy, it strongly suggests that both pathways collaborate to activate iNOS gene and that this collaboration results in an enhanced capacity to produce iNOS mRNA as observed in Fig. 3A which shows a tremendous increase in iNOS mRNA. This raised the question of which transcription factors are engaged by these signalling pathways.

As iNOS contains promoter binding sequences for STAT-1α, NF-κB, CREB (cAMP-responsive element binding protein) and AP-1 (activating protein-1), we drew our attention on these transcription factors (328). CREB and AP-1 were not significantly modulated by MRPs (data not shown) and therefore did not retain our attention any further. STAT-1α showed to be modulated (Fig 6) only by IFN-γ and to follow induction of JAK2 and STAT-1α phosphorylation (Fig5). NF-κB (p65/p50 form, consensus sequence) was observed to be modulated only by the heterodimer S100A8/A9. Its induction was very rapid as it showed strong DNA-binding capacity after 30 min. Here again we show that the ways to activate iNOS expression are distinct. Although other factors might intervene, we can speculate that synergy between the 2 pathways is the

result of the interaction of these two transcription factors that might increase the availability of the binding site for one another. As a matter of fact, their binding sites can be found within 800bp in the iNOS promoter (328). Recently, we and others have reported that NF-κB was activated upon stimulation with S100A8/A9 and have shown that this activation was not mediated through RAGE, a proposed receptor for S100A8/A9 (326;328;329). Therefore it appears that NF-κB is consistently involved in cell responses to MRPs. Whereas STAT-1α, CREB and AP-1 are not modulated upon MRPs stimulation, suggesting a very specific role of NF-κB in this signalling pathway. Taken together, our work seem to support the recent finding that MRPs are endogenous ligands of TLR4 (316) as NF-κB, SAPK/JNK and MEK/ERK pathways are activated, which correlates with the effect of TLR4 ligation (330).

After establishing a coherent signalling pathway, we confirmed in BMDMs that MRPs induce NO production. Interestingly, we observed that the synergistic effect between IFN-γ and MRPs is even more striking in this context as IFN-γ only primed these BMDMs, without inducing NO production *per se*. The resulting NO production upon costimulation was therefore even more potent. As NO production upon LPS and MRPs costimulation was not increased when compared to LPS alone, it raised the possibility that MRPs and LPS are signalling through the same receptor. Therefore, we used TLR4 K/O B10R Mθs to verify NO production upon MRPs stimulation. As Vogl and colleagues reported (316), our results also support the role for TLR4 in the recognition of MRPs as TLR4 K/O Mθs were unresponsive to MRPs.

Collectively, our study represents the first demonstration that MRPs can induce Mθs microbicidal functions through TLR4 as potently as IFN-γ, and that in combination, these cytokines further synergize NO production by Mθs suggesting that this activation process may play a crucial role in innate immune response concurring to control infectious agents.

Figure 1: *Production of nitric oxide by B10R Mθs following stimulation with MRPs.* A. iNOS gene expression by Mθs upon stimulation with IFN-γ (100 U/ml) or MRPs (10 μg/ml). Cells were stimulated for 8h with indicated stimuli and iNOS mRNA expression was assessed by northern blotting, using GAPDH as housekeeping gene. B. iNOS protein expression in Mθs stimulated with IFN-γ or MRPs was assessed after 8h of treatment. C. Mθs were stimulated with IFN-γ, S100A8, S100A9 or S100A8/A9 for 24h and nitrite were measured in the supernatants as reflective of nitric oxide. Results shown are representative of at least 3 independent experiments performed in triplicate. * = statistically significant from nil ($p \leq 0,05$).

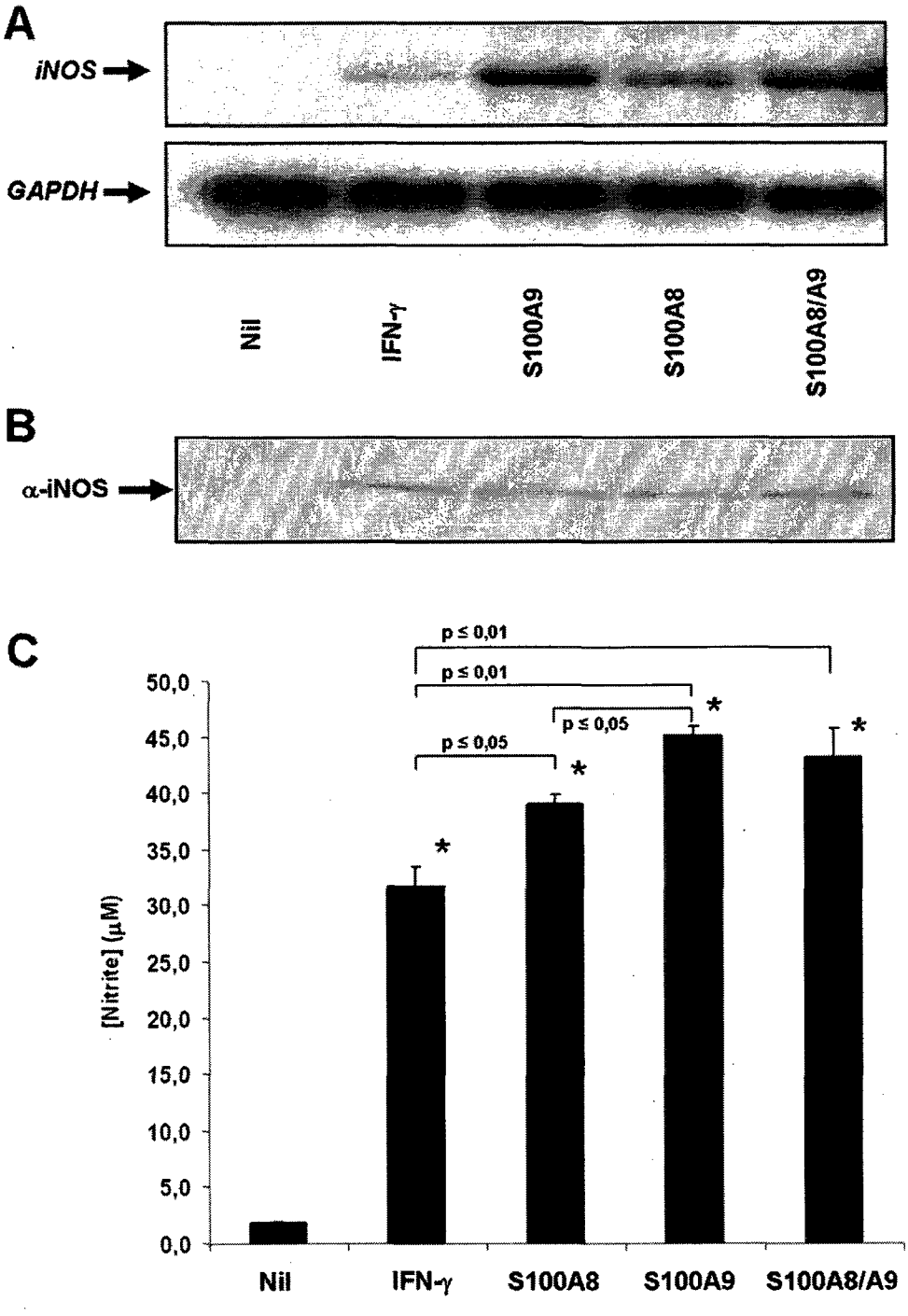

A
iNOS
GAPDH
Nil
IFN-γ
S100A9
S100A8
S100A8/A9
B
α-iNOS
C
p ≤ 0,01
p ≤ 0,01
p ≤ 0,05
p ≤ 0,05
[Nitrite] (μM)
50,0
45,0
40,0
35,0
30,0
25,0
20,0
15,0
10,0
5,0
0,0
Nil
IFN-γ
S100A8
S100A9
S100A8/A9

Figure 2: *Production of NO after MRP stimulation is dose-dependant.*

iNOS protein levels were evaluated by immunoblotting after stimulation with doses of MRPs (0,01 to 50 μg/ml) for 24h. B. NO production was measured after the same the same treatment. Results shown are the average of 2 independent experiments performed in triplicate. * = statistically significant from nil ($p \leq 0,05$).

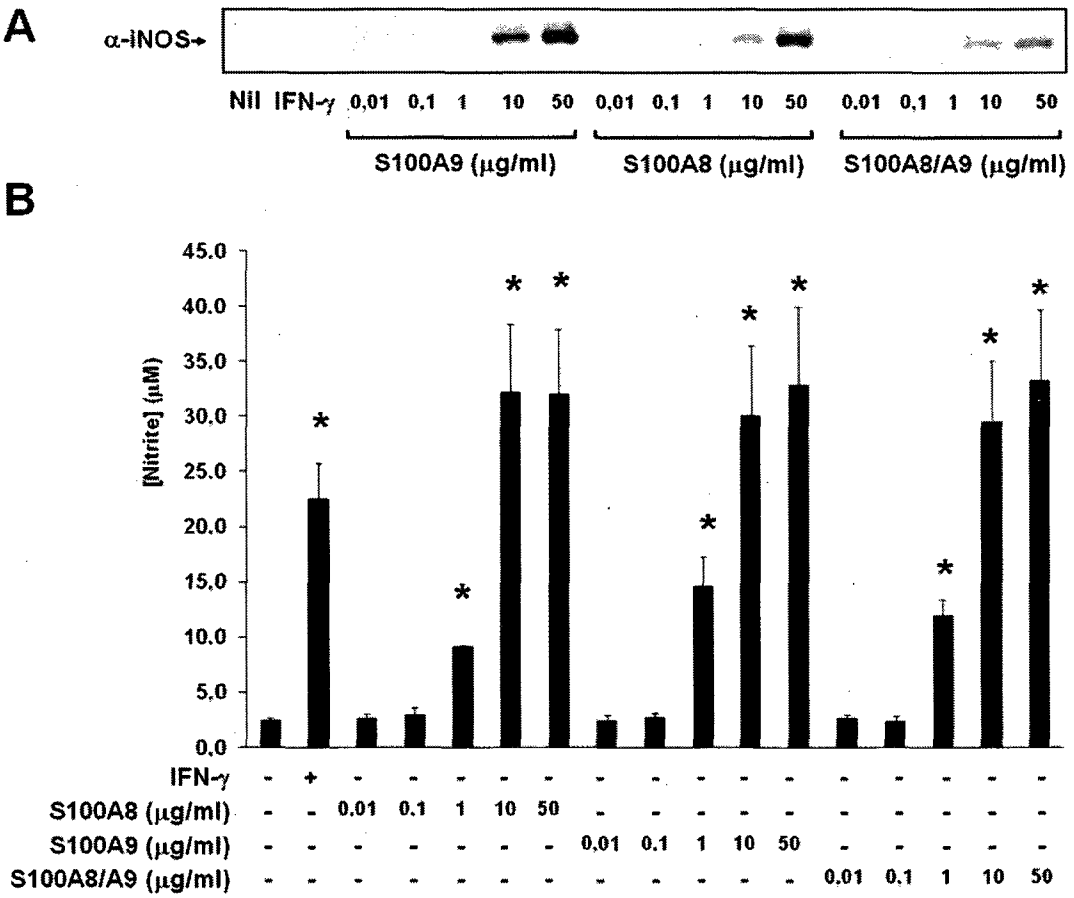

A
α-iNOS→
Nil IFN-γ 0,01 0,1 1 10 50 0,01 0,1 1 10 50 0,01 0,1 1 10 50
S100A9 (μg/ml)
S100A8 (μg/ml)
S100A8/A9 (μg/ml)
B
[Nitrite] (μM)
45.0
40.0
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
IFN-γ - + - - - - - - - - - - - - - - - -
S100A8 (μg/ml) - - 0.01 0.1 1 10 50 - - - - - - - - - - -
S100A9 (μg/ml) - - - - - - - 0.01 0.1 1 10 50 - - - - -
S100A8/A9 (μg/ml) - - - - - - - - - - - - 0.01 0.1 1 10 50

Figure 3: *NO production after various durations of stimulation with S100A8/A9.*

A. Macrophages were stimulated with IFN-γ (100 U/ml) or S100A8/A9 or both for 2, 4, 8 or 24h after what iNOS gene expression was monitored by Northern blot. TNF-α was also investigated for the same time-points. GAPDH served as a housekeeping gene. B. iNOS protein production was evaluated by immunoblotting after the same time points. C. NO production was monitored after the same time of stimulation by Griess reaction. Results shown are representative of at least 3 independent experiments performed in triplicate. * = statistically significant from nil ($p \leq 0,05$).

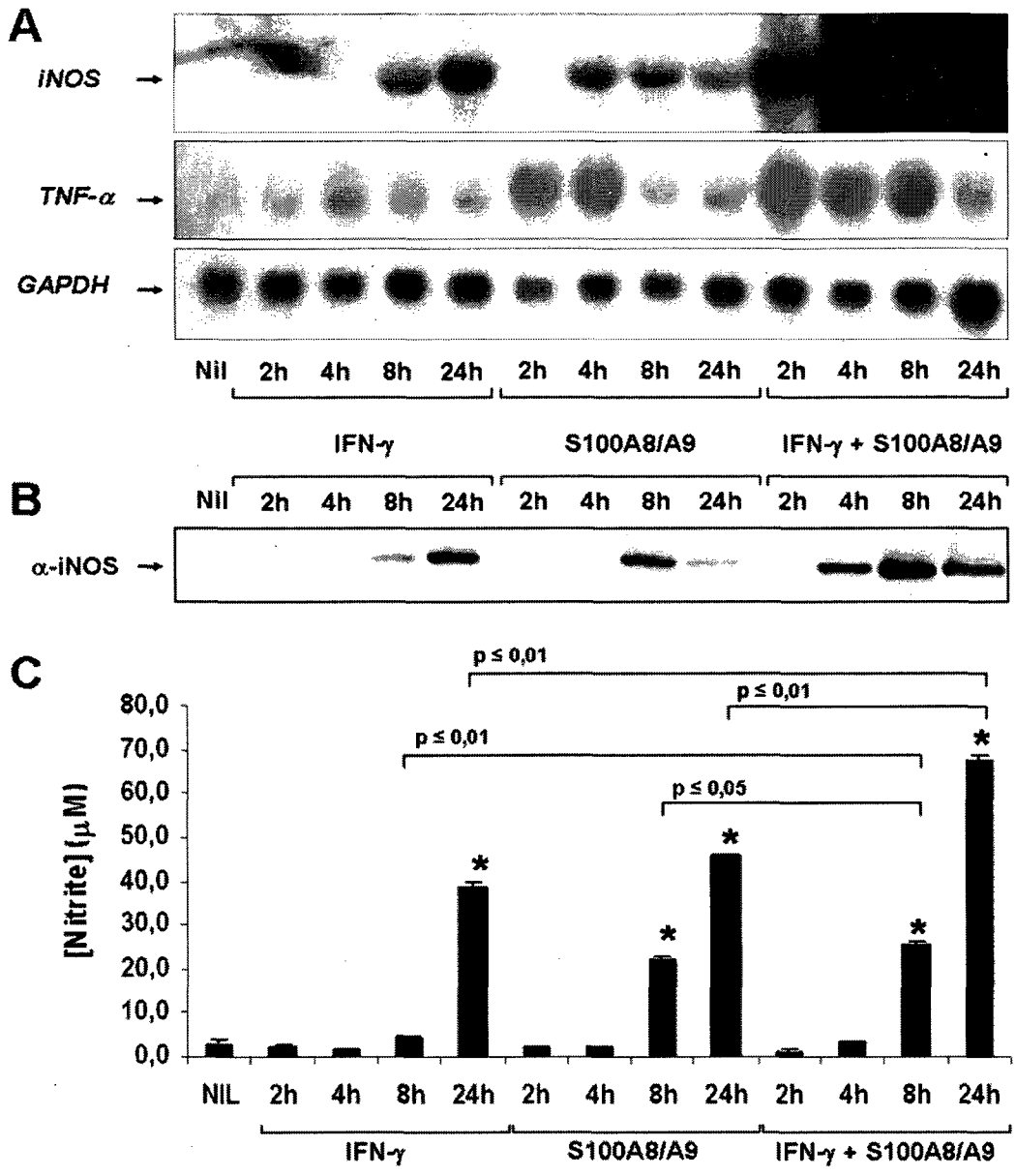

A
iNOS
TNF-α
GAPDH
Nil 2h 4h 8h 24h 2h 4h 8h 24h 2h 4h 8h 24h
IFN-γ
S100A8/A9
IFN-γ + S100A8/A9
B
Nil 2h 4h 8h 24h 2h 4h 8h 24h 2h 4h 8h 24h
α-iNOS
C
p ≤ 0,01
p ≤ 0,01
p ≤ 0,01
p ≤ 0,05
[Nitrite] (μM)
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
NIL 2h 4h 8h 24h 2h 4h 8h 24h 2h 4h 8h 24h
IFN-γ
S100A8/A9
IFN-γ + S100A8/A9

Figure 4: *Synergistic nitric oxide production by macrophages upon stimulation with IFN-γand various concentrations of MRPs.*

Cells were stimulated for 24h with or without 100 U/ml IFN-γ and various concentrations of S100A8/A9. Results are shown as percentage value of nitric oxide production over the additive value of observed nitric oxide production of B10Rs stimulated with IFN-γ 100 μg/ml alone and B10Rs stimulated with MRPs alone at precised concentration. Percentage is issued from this calculation: 100 x [NO] of (IFN-γ (100 U/ml) + MRP (X μg/ml) stimulation) / ([NO] of IFN-γ (100 U/ml) stimulation + [NO] of MRP (X μg/ml) stimulation). Mean of 3 independent experiments performed in triplicate. * = statistically significant ($p \leq 0,05$).

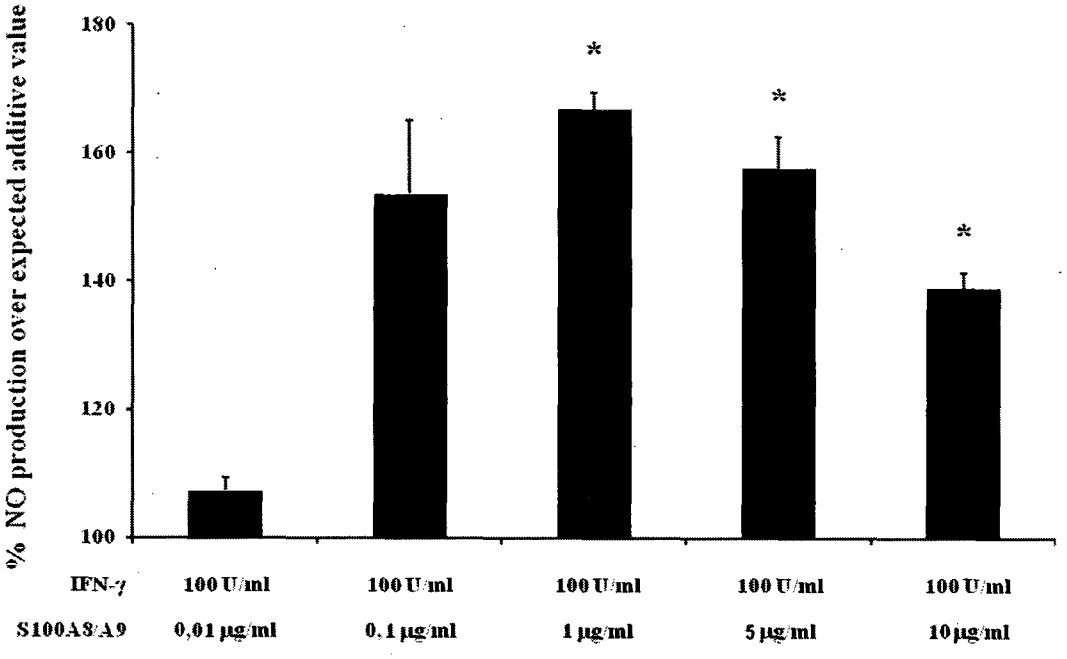

% NO production over expected additive value
180
160
140
120
100
*
*
*
IFN-γ
S100A8/A9
100 U/ml
0,01 µg/ml
100 U/ml
0,1 µg/ml
100 U/ml
1 µg/ml
100 U/ml
5 µg/ml
100 U/ml
10 µg/ml

Figure 5: *MRPs-induced signalling.*

Macrophages were treated with IFN-γ (100 U/ml) and/or MRPs (10 μg/ml) during 15min, 30 min, 1h or 2h. Protein lysate was then investigated for phosphorylation of STAT-1α, JAK2, MEK1/2, ERK1/2 and SAPK/JNK by immunoblotting. Blots shown are representative of at least 3 separate experiments.

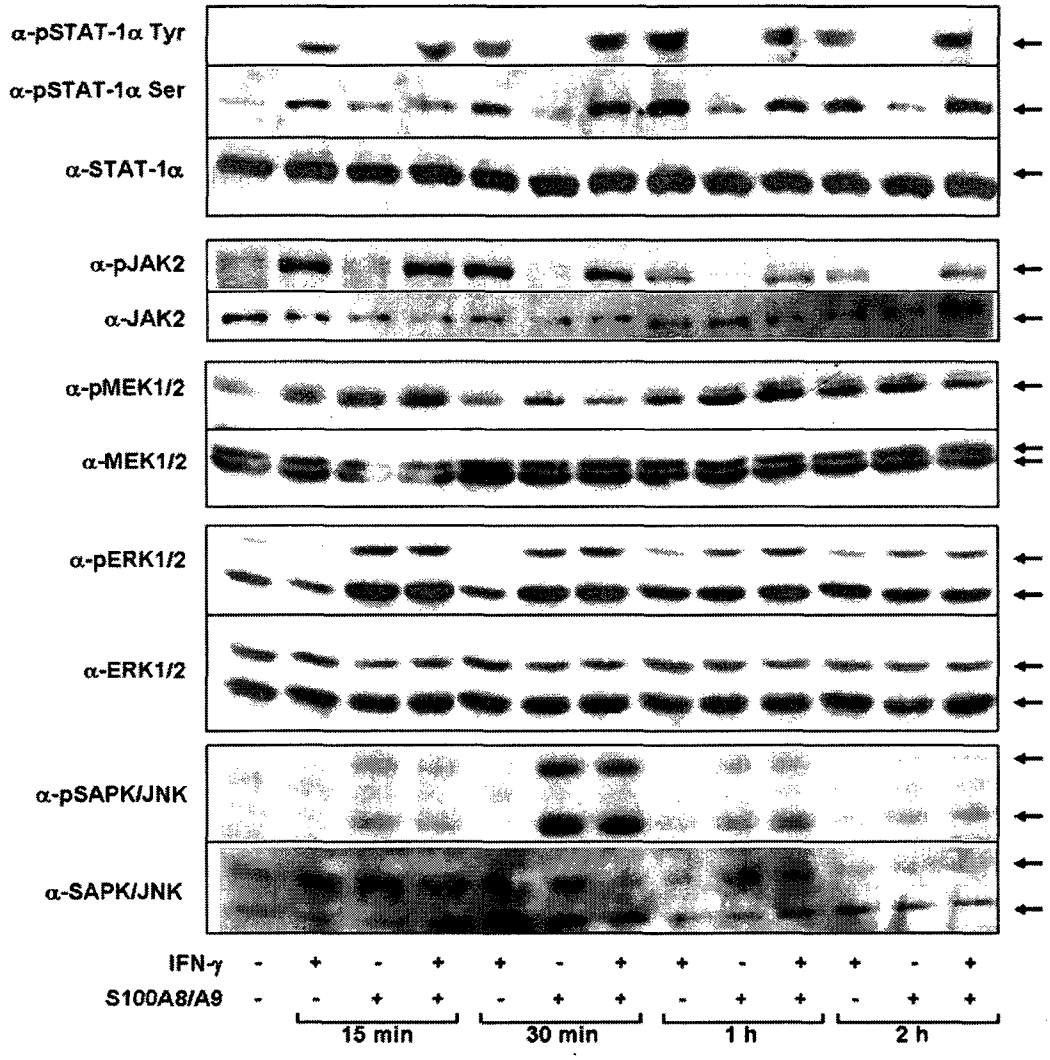

α-pSTAT-1α Tyr
α-pSTAT-1α Ser
α-STAT-1α
α-pJAK2
α-JAK2
α-pMEK1/2
α-MEK1/2
α-pERK1/2
α-ERK1/2
α-pSAPK/JNK
α-SAPK/JNK
IFN-γ - + - + + - + + - + + - +
S100A8/A9 - - + + - + + - + + - + +
15 min
30 min
1 h
2 h

Figure 6: *Pharmacologic inhibition of MAPKs.*

B10R macrophages were treated with various inhibitors 1h before addition of S100A8/A9 at 10 μg/ml for 24h after which NO production was measured. SP600125 (25 μM), PD98059 (100 μM), Apigenin (50 μM) and SB203580 (1 μM) inhibit SAPK/JNK, MEK1/2, ERK1/2 and p38 respectively. Inhibitors were all used at maximal subcytotoxic doses for a total of 25 hrs. Average of 4 individual experiments done in triplicate. * = statistically different ($p \leq 0,05$) from control.

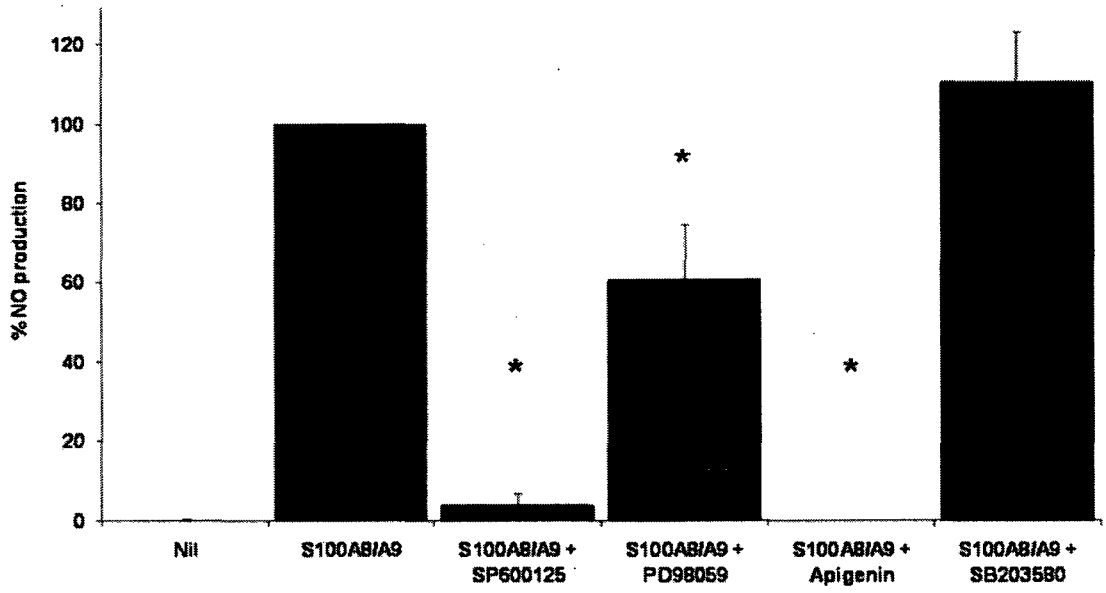

% NO production
120
100
80
60
40
20
0
*
*
*
Nil
S100A8/A9
S100A8/A9 + SP600125
S100A8/A9 + PD98059
S100A8/A9 + Apigenin
S100A8/A9 + SB203580

Figure 7: *Nuclear factors translocation and binding activity.*

Macrophages were treated with IFN-γ (100 U/ml) and/or MRPs (10 μg/ml) during 30 min, 1h, 2h or 4h. Nuclear proteins were then submitted to electrophoretic mobility shift assay with STAT-1α or with NF-κB (p65/p50) consensus oligonucleotide. Results shown are representative of at least 3 independently performed experiments.

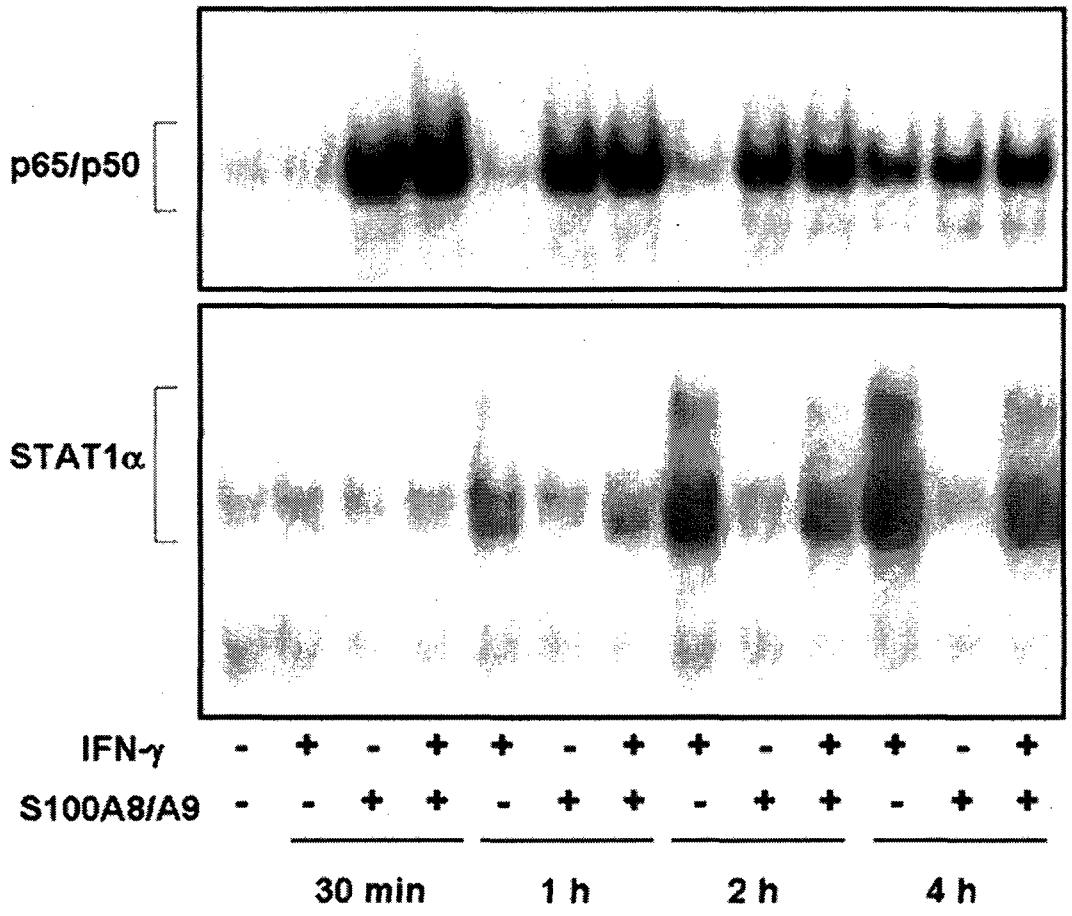
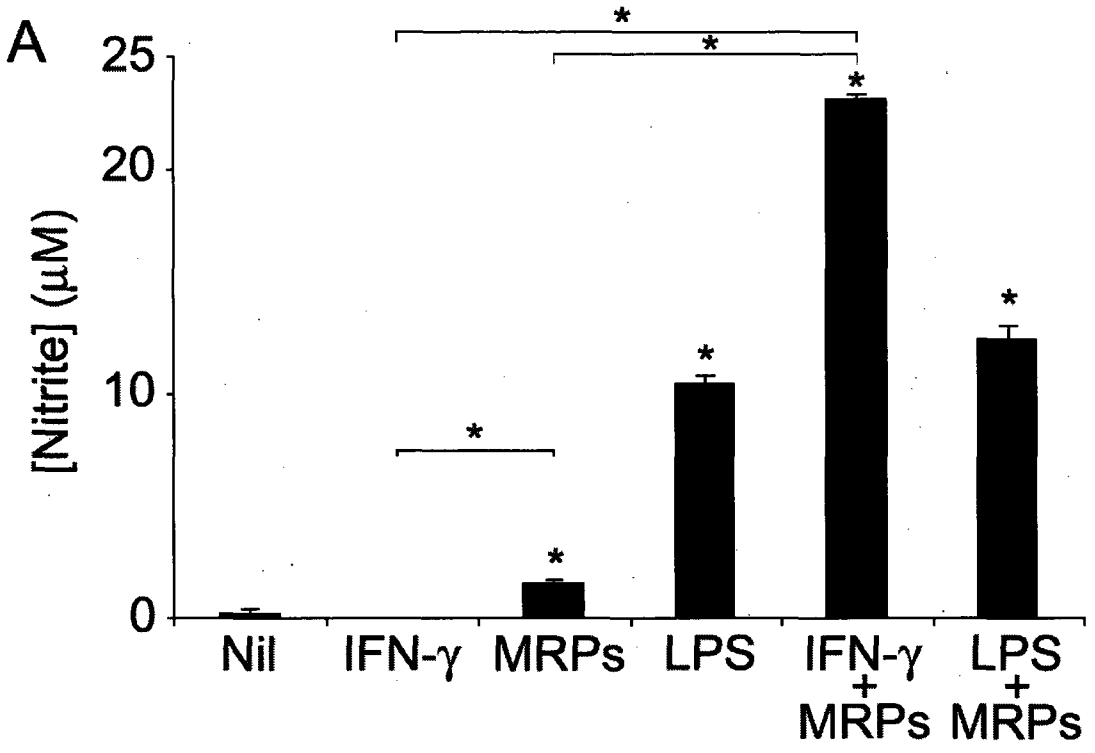
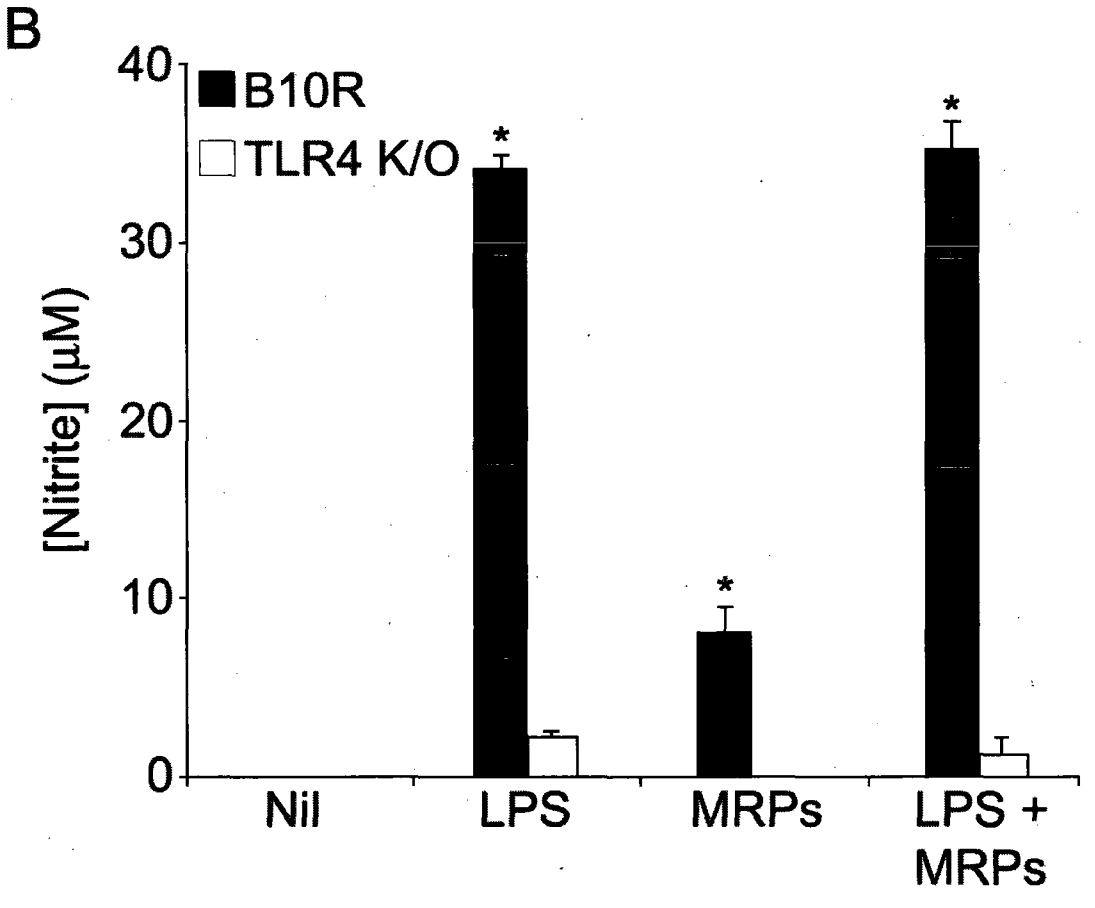

p65/p50
STAT1α
IFN-γ - + - + + - + + - + + - +
S100A8/A9 - - + + - + + - + + - + +
30 min
1 h
2 h
4 h

Figure 8: *Nitric oxide production in bone marrow-derived macrophages and the implication of TLR4 in myeloid-related proteins-mediated NO production in macrophages.*

A. Bone marrow-derived macrophages or B. B10Rs and TLR4 K/O B10Rs macrophages were stimulated with IFN-γ (100 U/ml), MRPs (25 μg/ml) and LPS (100 ng/ml) or combinations of these compounds. Nitric oxide was measured 24h later. Results shown are representative of at least 3 independently performed experiments. * = statistically different ($p \leq 0,05$) from control.


A
25
20
10
0
[Nitrite] (μM)
*
Nil
IFN-γ
MRPs
LPS
IFN-γ + MRPs
LPS + MRPs


B
40
30
20
10
0
[Nitrite] (μM)
B10R
TLR4 K/O
*
Nil
LPS
MRPs
LPS + MRPs

Chapter 5: Summary and General Discussion

The major findings of the work presented in this thesis can be summarized as following:

- This is the first report in a mouse model showing that inhibition of the global PTP activity during OVA sensitization can prevent subsequent development of allergic asthma in mice challenged with OVA: serum IgEs are reduced; lung eosinophilia is greatly reduced; lung tissue inflammation is normalized and AHR development is not significant in mice treated with the PTP inhibitor bpV(phen).
- This is the first report in a mouse model showing that inhibition of the global PTP activity during OVA challenge, in mice pre-sensitized to OVA, can also prevent the development of allergic asthma in a murine model: lung eosinophilia is largely reduced; lung tissue inflammation is normalized and AHR development is not significant in mice treated with the PTP inhibitor bpV(phen), but IgE titres are not significantly affected by the treatment. This represents an excellent therapeutic avenue.
- PTP inhibition can modulate the cytokine expression in the spleen in order to favour the expression of Th1 cytokines (IFN-γ and IL-12) while decreasing the expression of Th2 cytokines (IL-4 and IL-10).
- PTP inhibition by bpV(phen) modulates the immune responses and promotes the production of the Th1 cytokine in the lung of allergic, bpV-treated animals, as demonstrated by the increase of IFN-γ in the BALf fluids of these animals.
- In WT mice, PTP-1B limits the accumulation of inflammatory cells to the BALf compartment of the lung, as revealed by the fact that its deletion increases such cell recruitment.

- TC-PTP activity is necessary for the development of allergic asthma: with reduced TC-PTP activity, less IgEs are produced and less inflammatory cells are recruited to the lung.
- MRPs are potent and rapid inducers of NO production in Mθs. Their effect on NO production is even more rapid than IFN-γ, a renowned NO-inducer.
- MRPs and IFN-γ can synergize to increase NO production in Mθs, but use different and exclusive signalling pathways.
- MRPs induce NO production through TLR-4.
- MRPs signal through the activation of the MEK/ERK and JNK pathways, resulting in NF-κB translocation to the nucleus and activation of its DNA-binding activity.

In a first study (Chapter 2), we used bpV(phen) to inhibit PTPs in order to unravel their role in allergic asthma. In this study, we were able to demonstrate the potency of bpV(phen) to favour expression of Th1 cytokines in the spleen, while simultaneously repressing expression of Th2 cytokines. These observations complemented our previous results showing that bpV(phen) was able to induce IL-12 production in the spleen (274). When PTP activity was inhibited during allergen sensitization, we observed an important reduction in all asthma features investigated: IgEs, inflammatory cells recruitment, lung tissue inflammation, and AHR. As inhibition was performed during sensitization, it might suggest that some early events of asthma development were inhibited and it is why we observe a reduction in all asthma characteristics investigated. As we observed that terminal lung environment was oriented toward a Th1 response (increased IFN-γ

concentration in the BALf), and that Th1 cytokines expression is favoured in the spleen upon bpV(phen) treatment, we hypothesize that the promotion of a Th1 response is the driving force that prevents the development of the Th2 response needed for development of asthma. This hypothesis fits well with the marked decrease in IgE production that we observed, IgE isotype switching being dependent on Th2 response.

Then we have been interested to see if the inhibition of PTP activity in sensitized mice, during the allergen challenge, could also prevent the development of asthma. We first confirmed that s.c. injection of bpV(phen) could result in PTP inhibition in the lung, just as we previously knew that it does in the spleen (274). We show that this inhibition also resulted in the reduction of all investigated asthma features, at the exception of IgEs, which were not expected to be modulated. This set of data is of utmost importance, as it shows that PTP inhibition can prevent the development of asthma in pre-sensitized individuals. Interesting therapeutic avenues that make use of this potential during acute allergen expositions can be envisioned. For example, it could be interesting to inhibit PTPs during pollen season in individuals that are allergic, but that have not developed allergic asthma yet, in order to prevent the development of lung disease. Regarding such possibilities, further studies investigating the precise pathways to inhibit in order to gain the best effect are definitively needed, but global PTP inhibition might represent a serious possibility. In fact, we previously demonstrated an innocuous long term use of peroxovanadium in animals without apparition of undesired effects for 19 days (Chapter 2), 38 days (246) and 42 days (245). Although further experiments are required to fully confirm the innocuousness of this approach, PTP inhibition might represent a tangible therapeutic possibility.

These results also open the possibility that local lung delivery of the drug could mediate a beneficial effect. We decided to attempt preliminary studies using an intra-nasal (i.n.) instillation of the drug to the mice during allergen challenge. As a therapeutic approach, this would have the benefit of delivering the drug where the allergen is initially triggering immune reactions. In these preliminary experiments, we observed that i.n. administration of bpV(phen) was effectively able to reduce lung tissue inflammation (data not shown). Our laboratory is currently pursuing experiments with a wide array of bpV(phen) doses and a varied regimen of administration to verify if this technique could also prevent development of asthma, which would render bpV(phen) even more interesting for therapy.

The many investigations made since the discovery of PTPs have highlighted their implication in the fine regulation of many processes (331). Given that PTPs are in similar numbers (107 in humans) (215) in the genome than their kinase counterparts (around 90 in humans) (219), their specificity should be expected to be similar to that of PTKs. In fact, many experiments of PTP overexpression were not observed to interfere with TCR signal, showing PTP specificity (331). Our data also clearly shows the implication of PTPs in the fine regulation of immune response and their specific roles. The fact that global inhibition of PTPs prevents asthma development highlights that the activity of at least some PTPs are necessary for the reaction to occur. The precise role could be restricted to one PTP acting upstream in the signalling pathways, for example CD45 regulating the phosphorylation of Lck and of Fyn in TCR signal (230;231). But the explanation could also involve more PTPs at many steps of signalling events. One such

option is that a decreased PTP activity allows PTKs to increase the general level of tyrosine phosphorylation in the cell. Interestingly, Th1 and Th2 cells do not exhibit the same extent of phosphorylation upon activation of their TCR (205). Th1 clones undergo a more extensive phosphorylation than Th2 clones. This suggests that inhibition of PTP activity could induce a Th1-like signalling in Th2 clones, probably not converting them to a Th1 phenotype, but potentially preventing their activation toward the classical Th2 clonal activation. This hypothesis suits well with our results, as upon bpV(phen) treatment, expression of Th1 cytokines in the spleen is lightly increased (Chapter 2, (274)), which might reflect a more potent activation of Th1 clones by a slightly more intense phosphorylation than in absence of bpV(phen). Of utmost interest in this context, Th2 cytokines were dramatically reduced, which might reflect a reduced expression of Th2 type cytokines due to a more extensive phosphorylation in Th2 clones in presence of bpV(phen).

Obviously, depicting the mechanisms involved in the reduction of asthma upon PTP inhibition is a fastidious and complicated task, as 107 PTPs exist and the possibilities of affected cell reactions are numerous. Therefore we decided to begin investigations with inhibition of individual PTPs that already showed strong potential in the regulation of immune responses. Our experiments have permitted to reveal that reduction of TC-PTP activity in mutant mouse was coherent with our global inhibition of PTPs, therefore reduction of TC-PTP activity correlates with reduced asthma development. Interestingly, total inhibition of PTP-1B showed an increase in inflammatory cells, something that is at the opposite of what we saw by global PTP inhibition. This could suggest many things, but probably reflects the fact that PTP-1B action in this context is happening downstream

of another event that is normally happening, but that is not initiated in bpV(phen)-treated mice. This also suggests quite strongly that PTP-1B could be involved in the mechanisms leading to cell recruitment, such as chemokine production or chemokine receptor signalling. Together with our results of mice treated with bpV(phen), the results with PTP deficient mice highlights the importance of PTPs in the regulation of immune responses.

After studying the importance of signalling events in the sensitization and allergen challenge, we got interested to study other events of the disease where a better characterization of signalling pathways could be beneficial. It has been noted in severe asthma exacerbations that MRP8 levels are resistant to corticosteroid treatment. MRP8 production is made by myeloid cells, such as neutrophils and monocytes/Mθs. It is possible that neutrophils, which produce high amounts of MRPs and are also resistant to corticosteroid treatment, produce a feedback loop with MRPs that perpetuate each other's presence in the lung. Preliminary results from our laboratory also provided evidence that MRPs can induce production of NO in Mθs. Given these possibilities for a role of MRPs in asthma pathogenesis, we were interested to depict the signalling pathways triggered by MRPs. This characterization could potentially be used to inhibit the recruitment of neutrophils to the lung and could also possibly be used to reduce NO production in the lung.

Here, we report that we uncovered the signalling pathways triggered by MRP stimulation on Mθs. This work was never performed from a membrane-to-nucleus approach as we performed it. Our approach allowed us to unravel the implication of TLR-4, MEK/ERK and JNK pathways and NF-κB in MRPs-induced NO production by Mθs. Other groups

previously reported the engagement of some signalling molecules upon stimulation with MRPs, so that NF-κB and p38 were reported to be involved in MRPs-induced signalling pathways in monocytes (329). While we also observed the activation of NF-κB, our results rule out the involvement of p38 in Mθs. Interestingly, they report that p38 inhibition in their monocytes was not preventing NF-κB activation, suggesting that both signalling elements are engaged in different pathways. This further suggests that cell maturation and/or activation status might change the pathways involved in the cell response and that while pathways leading to NF-κB are preserved between their monocytes and our Mθs, p38 is not involved in our cells. In human prostate cancer cells, investigators reported an activation of p38 and ERK1/2 upon MRPs stimulation as well as the involvement of NF-κB (326). They also showed that RAGE was co-localizing with MRPs upon stimulation. Our results are therefore diverging. In a first step, our results tend to identify TLR-4 as the receptor for MRPs, as was previously reported (316), and not RAGE, that was also previously reported not to be implicated in signalling events of monocytes (329). As the induction of NO in our model was completely abolished in TLR-4K/O Mθs, it shows that TLR-4 is obligatory for MRPs-induced NO production. As investigated cells differ, it is probable that this can be a reason why our results also differ.

Our results are interesting in the perspective that MRPs level, precisely MRP8, has been reported to be resistant to corticosteroid treatment in severe asthma. As these molecules have a potent chemotactic effect on neutrophils, they could represent a mechanism of persistence of neutrophil populations in subjects exhibiting severe asthma exacerbations that do not respond to corticosteroid. While this is already a remarkable observation and that these pathways could be exploited to reduce this corticosteroid-resistant neutrophilia,

our observation that MRPs induce NO production is of interest, both in asthma and in other immunological reactions. First, in asthma, NO levels are known to be increased (75;128). As it is increased after allergen challenge (128), which also increases inflammation, it is expected that this increased in NO is due to inflammation and probably NOS2. Such high levels of NO exceed the beneficial dose for tissue relaxation and induce peroxynitrite formation, contributing to lung damage (136). This could represent a harmful effect of MRPs in asthma. On the other hand, the induction of microbicidal functions in Mθs can be very attractive in infection models where NO is important for killing the intracellular invader (274). Therefore our findings can be applied to larger perspectives and this promise to be an interesting finding for the field of anti-microbial response.

Taken together, the results presented here contribute to further our knowledge on the signalling mechanisms regulating cells involved in immune responses. It supports an important role for PTPs in modulating the immune response, a role that is both required for allergen sensitization, and most interestingly, for establishing asthma upon allergen challenge. It also identifies MRPs as a new powerful modulator of host immune cells such as Mθs and the induction of NO, an important mediator involved in asthma. A better understanding of these signalling mechanisms might allow us to develop therapies altering specific signalling pathways in order to prevent asthma development or reduce the severity of acute asthma exacerbations. For example, inhibition of PTPs in sensitized individuals during periods of possible allergen exposition (e.g. pollen season) could prevent development of lung disease, as we showed that this inhibition prevents asthma development. On the other hand, inhibition of signalling pathways triggered by MRPs in

acute asthma exacerbations could complement corticosteroid treatment in resistant individuals in order to dampen lung inflammation.

Claims to Originality

The work presented here increases the scientific knowledge of signalling events and how they affect cell response and eventually immune responses. Although made in the context of allergic asthma, extrapolation of these results to other immunological situations is more than likely to be made. To our knowledge, the findings have not been reported previously, or were partially reported in a different context. To our knowledge, this thesis reports for the first time:

- The modulation of asthma development upon sensitization by inhibition of PTP activity.
- The prevention of asthma development in pre-sensitized animals upon allergen challenge.
- The involvement of TC-PTP in the regulation of asthma development.
- The implication of PTP-1B in the control of immune cell recruitment to the lung.
- The induction of NO production by Mθs upon MRPs stimulation.
- The synergistic interaction of MRPs with IFN-γ in the generation of NO in Mθs.
- A membrane-to-nucleus characterization of the signalling pathways triggered by MRPs in Mθs.

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Appendix 1: Ethics Certificates