Immunomodulation with Toll-like receptor 7 ligands in infectious and allergic diseases

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements of the degree of Doctor of Philosophy

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August 2005

Your file Votre référence
ISBN: 978-0-494-25213-0
Our file Notre référence
ISBN: 978-0-494-25213-0

To Jill,

For everything ...

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Abstract

Modulation of the immune response to treat various infectious and allergic diseases as well as certain forms of cancer has been employed since William B. Coley injected a mixture of bacteria into patients with large inoperable tumors over 100 years ago. The imidazoquinoline compounds are nucleoside analogs with strong type I interferon inducing activity. Recently, these compounds have been found to mediate their effects through a subset of the Toll-like receptor (TLR) family. A topical cream containing imiquimod, a member of the imidazoquinoline family, is currently employed in humans for the treatment of genital warts caused by the human papilloma virus, against actinic keratosis as well as for topical treatment of primary superficial basal cell carcinoma. The goal of the present study was to determine the potential of imidazoquinolines in the treatment of other infectious and allergic diseases as well as their mechanism of action. Furthermore, the role of possible modulators of response to these chemicals has been studied. We show that imidazoquinoline treatment can increase mycobactericidal activity in mice carrying a resistant (wild type) allele of SLC11A1 (formerly natural resistance associated macrophage protein 1 (NRAMP1)). NRAMP1 affected responsiveness to imidazoquinolines in macrophages by modulating P38 MAPK activation as well as atypical PKC activity both of which are required for optimal immunodulatory activity by these TLR ligands. Imidazoquinoline treatment also prevented development of atopic allergic asthma in mice sensitized and challenged with ovalbumin by preventing inflammatory cytokine production as well as eosinophil infiltration in the lungs. Interestingly, this drug when used as a therapeutic agent against allergic asthma was equally efficient in mice carrying the wild type (resistant) or the

susceptible allele of the *Nramp1* gene. This suggests that NRAMP1 modulates response to imidazoquinoline treatment in models dependent on macrophage subsets which require NRAMP1 function to become activated to an appropriate degree. Taken together, the presented study broadens the possible applicability of imidazoquinolines to the treatment of mycobacterial infections as well as to allergic asthma treatment and defines an important role for NRAMP1 in regulating responses to this family of pharmaceutical compounds.

Résumé

Depuis que le Dr. William B. Coley injecta un mélange de bactéries à des patients ayant de larges tumeurs inopérables au début des années 1890, l'approche immunothérapeutique pour le traitement des maladies infectieuses et allergiques a gagné de plus en plus en popularité. Les imidazoquinolines sont des analogues de nucléotide avec une capacité d'induction de haut niveau d'interféron de type I. Récemment, l'effet immunomodulateur de ces molécules a été attribué à leur activation de certains membres des récepteurs de la famille Toll. Une crème topique est actuellement utilisée chez l'humain pour le traitement des verrues génitales causées par le virus du papillome humain, les carcinomes de cellules basales et contre la kératose actinique. Le but de la présente étude est de déterminer le potentiel des imidazoquinolines dans le traitement des maladies infectieuses et allergiques et leurs mécanismes d'action. De plus, nos études ont tenté de déterminer les modulateurs de réponse à ces produits pharmaceutiques. Nos données démontrent que le traitement avec les imidazoquinolines induit une activité mycobactéricide seulement dans les souris exprimant l'allèle sauvage du gène *Slc11a1* (aussi connu sous le nom de natural resistance associated macrophage protein 1 (*Nramp1*)). La modulation de la réponse des macrophages aux imidazoquinolines par NRAMP1 est liée à une réduction de l'activité des PKC atypiques et une réduction de l'activation de P38 MAPK. Le traitement avec les imidazoquinolines prévient aussi le développement de l'asthme allergique dans un modèle murin qui se traduit par le recrutement des cellules inflammatoires dans les poumons ainsi que par une réduction des cytokines inflammatoires. Les souris exprimant l'allèle sauvage ou

mutant de NRAMP1 étaient protégées au même niveau contre le développement de l'asthme allergique par les imidazoquinolines. Cette dernière observation suggère que l'activité de NRAMP1 est requise pour une réponse optimale aux imidazoquinolines seulement dans des modèles dépendants sur des classes de macrophages requérant ce gène pour atteindre un niveau d'activation adéquat. La présente étude devrait pouvoir élargir les possibilités d'applications cliniques des imidazoquinolines contre les infections aux mycobactéries ainsi que contre l'asthme allergique et définit un rôle important pour NRAMP1 dans la modulation de la réponse à ces molécules.

Preface and Acknowledgements

As described in the *Thesis Preparation Guidelines*, the author will present a manuscript-based thesis. Jacques Moisan is the principal author on all of the manuscripts presented in the thesis. The work presented in Chapter 2 has been published in *Antimicrobial Agent and Chemotherapy* (AAC, 2001, 45(11) pp. 3059-3064). All of the work was performed by the author. The work in Chapter 3 is currently prepared for submission. Most of the data presented in this chapter has been performed by the thesis author. The TLR4 and TLR7 mRNA expression analysis was performed by Thusanth Thuraisingam. He has also generated the protein extracts for P38 and ERK MAPK activation analysis as well as contributed to the P38 MAPK inhibition studies. The data presented in chapter 4 are ready to be submitted for publication. The thesis author has played a major role in the generation of the data presented in Chapter 4. Pierre Camateros prepared lung homogenates for cytokine analysis as well as helped during animal challenge with ovalbumin and sample collection at the end of experiments. Hossein Khoosari contributed to lung parameter measurement using the Flexivent apparatus.

I would like to specially thank Thusanth Thuraisingam and Pierre Camateros for their invaluable help on projects they are now continuing very successfully. I would like to acknowledge Wojciech Wojciechowski who first taught me everything when I started in the lab and influenced the way I do research to this day. I would also like to acknowledge Patricia Martin, Dominique Marion and Marie-Linda Boghdady for their excellent technical help throughout most of the studies. I would like to thank all lab members, past and present which, through discussions and suggestions, have contributed

to the progression of the various studies. Finally, but most importantly, I would like to thank my supervisor, Danuta Radzioch, for being always there to discuss experiments, review manuscripts or just take a cup of coffee and chat. She has taught me to persevere in the face of adversity and to face the challenges that arise on a daily basis in the world of scientific research.

General Introduction

Modulation of the immune response to treat various diseases is an approach that has been employed since William B. Coley, at the turn of the century, injected filtrates from *Streptococcus pyogenes* and *Bacillus prodigiosus* cultures into tumors. The concept of manipulating the immune response to achieve resolution of various autoimmune and infectious diseases has been the focus of intense research for the past 40 years. Advances in immunology and microbiology have led to the identification of microbial products such as lipopolysaccharide and bacterial DNA that induce an inflammatory response when administered in the host. These molecules have the potential of altering an already established immune response that may be detrimental to an individual or to generate a protective response that may not be present initially.

The discovery of the role of the Toll protein in resistance to fungi and gram-positive infection in *Drosophila* rejuvenated the field of innate immunology. Positional cloning of the *Lps* locus in lipopolysaccharide hyporesponsive C3H/HeJ mice demonstrated the crucial role of Toll-like receptors in mammalian recognition and sensing of a wide class of pathogens. The discovery of TLR4 as the receptor for LPS provided a plausible mechanism of action for Coley's toxin. A lot of focus in the past 10 years has been the characterization of the various ligands for the 13 members of the mammalian TLR family which have yielded new insights into the mechanism of pathogen sensing and transduction of these signals to the adaptive arm of the immune system.

The imidazoquinoline family of compounds are nucleoside analogs with potent immunomodulatory activities through cytokine induction as well as direct cell activation. They are currently employed in humans for the treatment of HPV-induced warts, superficial basal cell carcinoma and actinic keratosis. Like for Coley's toxin, the mechanism of action for this class of compounds remained unclear until the discovery that they initiate signal transduction through TLR7/8. Previous studies had pointed to the potential of imidazoquinoline in the treatment of cutaneous leishmaniasis caused by *Leishmania major*, an obligate intracellular pathogen. Our hypothesis was that these compounds could prove beneficial in the treatment of other intracellular pathogens as well as other diseases such as allergic asthma that could benefit from the spectrum of cytokines induced by the imidazoquinolines. The first goal of the present study was to determine the potential of imidazoquinolines in the treatment of another intracellular pathogen, namely *Mycobacterium bovis* Bacille Calmette Guérin (BCG), as well as any factor which could modulate the response to these drugs. Secondly, our studies focused on the intracellular molecular events triggered through TLR7 by imidazoquinolines. Lastly, because of the spectrum of cytokine induced which was skewed toward a T_H1 response, we wanted to test the ability of imidazoquinolines to prevent the development of atopic allergic asthma, a disease predominated by T_H2 cytokines.

In chapter 2, we demonstrate that imidazoquinoline treatment can reduce splenic bacterial loads in mice infected with *M. bovis* BCG. Interestingly, mice lacking the *Nramp1* gene were found to be refractive to imidazoquinoline treatment. Macrophage cytokine responses to these drugs were also found to be dependent on the presence of a functional *Nramp1* allele. Only escalating the imidazoquinoline dose by a factor of 250

resulted in a small, but significant, decrease in splenic *M. bovis* BCG levels. In light of their ability to signal through TLR7/8, the role of NRAMP1 in signal transduction by imidazoquinolines was further characterised in chapter 3. As described in Annexe 1, imidazoquinoline signalling requires ligand uptake as well as endosomal maturation prior to signal transduction. Lack of NRAMP1 function did not affect TLR7 ligand uptake or intracellular localisation in murine macrophage cell lines. P38 MAPK activation, but not ERK1/2, was found to be reduced following imidazoquinoline stimulation. The effect was specific to TLR7 as P38 MAPK activation by LPS was normal in wild type and *Nramp1* knockout macrophages. Levels of activated PKCζ were also found to be reduced in resting *Nramp1* KO macrophages. The confirmation of the importance of these pathways in cytokine induction by imidazoquinoline was obtained from inhibitor studies. Taken together, we have characterised the importance of NRAMP1 in macrophage cytokine responses to TLR7/8 ligands which leads to a failure of mycobacterial infection treatment in Nramp1 KO mice.

Asthma is one of the leading causes of childhood hospitalisation in North America. Current treatments aim at inhibiting the T_H2 dominated chronic inflammatory response present in the lungs of affected individuals. Imidazoquinolines have been shown to induce a T_H1 skewed cytokine response characterised by IL-12 and IFNγ production. As many cell types involved in asthma pathogenesis such as mast cells and eosinophils express TLR7/8, we hypothesised that imidazoquinoline treatment could prevent development of allergic asthma in a murine model. Surprisingly, imidazoquinoline treatment led to a complete inhibition of the inflammatory reaction induced following

ovalbumin sensitization and challenge. Inhibition of both T_H1 and T_H2 lung cytokine production led to the prevention of inflammatory cells, especially eosinophils, infiltration in the lungs of challenged animals. Increases in lung resistance and elastance as well as serum IgE levels normally induced by OVA sensitization and challenge were also prevented by imidazoquinoline treatments. This suggests that imidazoquinolines, through TLR7 activation, induce regulatory mechanisms which prevent most of the asthmatic phenotype in a murine model of atopic allergic asthma. Interestingly, NRAMP1 was not required for treatment efficacy as mice lacking NRAMP1 function responded equally well to treatment. Taken together, the data presented suggest a clear potential for imidazoquinoline in the treatment of asthma and the mechanism of action is independent of NRAMP1 protein suggesting macrophages might not be the primary effector cells.

We believe that the data presented in this thesis broadens the clinical applicability of the imidazoquinoline family of compounds as well as further our knowledge of Toll-like receptor-mediated signal transduction. Future work should focus on pre-clinical studies to test the exact mechanism of action of imidazoquinolines in both infectious and allergic diseases. Furthermore, the contribution of NRAMP1 responses in human macrophages should also yield important information characterising the response of an individual to TLR7/8 ligands.

Chapter 1

Literature Review

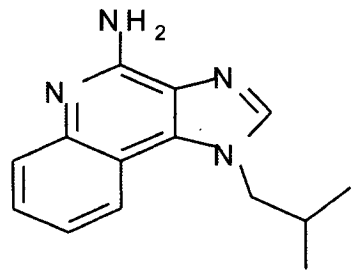
Abstract

Compounds that belong to the imidazoquinoline family are known as potent immunomodulators by their ability to induce a plethora of cytokines that contribute to the activation and differentiation of immune cells. Administration of imidazoquinolines has been shown to be an efficient treatment for many viral, bacterial and parasitic infections. A topical form of imiquimod, a member of the imidazoquinoline family, commercially known as ALDARA™, is currently administered to humans to treat genital warts caused by the human papilloma virus (HPV). ALDARA™ has also been recently approved by the FDA for the treatment of actinic keratosis as well as for topical treatment of primary superficial basal cell carcinoma. Until recently, the mechanism of action of these compounds was still uncertain, but recent research has pointed to the imidazoquinolines as ligands for the Toll-like receptor 7 (TLR7), a member of the Toll receptor family. The Toll-like receptor family members recognize conserved patterns on pathogens and therefore represent an important component of the innate immune response which has profound effects on the adaptive arm of the immune system. This review will focus on the potential of imidazoquinolines in modulating the immune response for treatment of infectious and allergic diseases, in the light of their ability to signal through TLR7. This review will also touch on some of the genetic components that have been described as modulators of the response to these compounds.

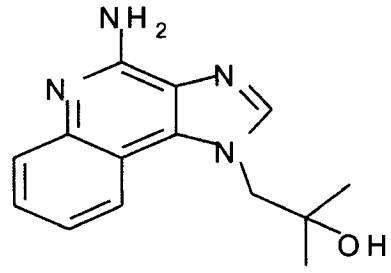
1. Imidazoquinoline Family

The concept of manipulating the immune response to control viral and bacterial infection as well as autoimmune disease has been thought of and tried by many investigators, with various levels of success. Injection of high concentrations of one or more cytokines was shown to lead to severe toxicity which was not always associated with resolution of the condition initially targeted by the treatment. The focus has thus shifted towards identifying natural or synthetic compounds which could modulate the immune response to induce the desired patterns of cytokine production and cellular activation without the toxicity associated with other methods.

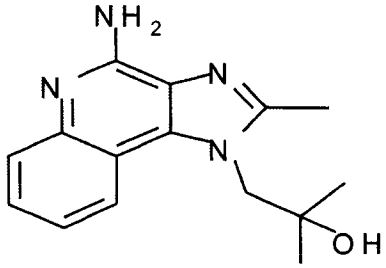
A screen of chemicals with nucleoside analog structures in the 1980s revealed imiquimod (R837, S26308), an imidazoquinoline, which could induce a whole spectrum of immunomodulatory activity without the toxicity associated with other immunomodulatory agents such as double stranded RNA (Poly I:C) [1]. Subsequently, many different derivatives of this initial compound were synthesized and shown to exhibit a spectrum of potencies (Figure 1).



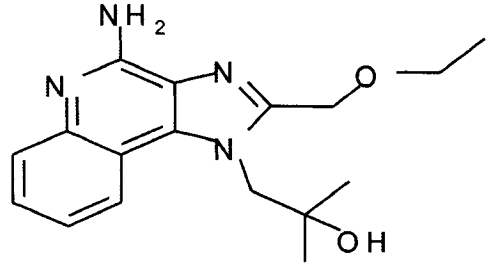
Imiquimod (R-837, S26308)



R-842



S27609



Resiquimod (R-848, S28463)

Figure 1: Structure of Various Imidazoquinoline Family Members

Imiquimod and its hydroxylated derivative R842 were shown to induce a similar cytokine pattern and share similar potency in inducing an immune response [2]. Another member of this family, S27609, was obtained by adding a methyl group at position 2 which leads to a 5- to 10-fold increase in its potency [3]. S28463 (R848, resiquimod), which has an alkoxyalkyl group substitution at position 2, further increases its potency by 20 to 100 times compared to imiquimod [4]. Currently, only imiquimod has been approved for use in humans, although resiquimod is currently being tested in clinical trials.

2. Immunomodulatory Effects

2.1 Cytokine Induction

The imidazoquinoline family of compounds was discovered by their ability to induce high levels of type I interferon (IFN) [5]. This was reproduced in many species including the mouse [6], monkeys [7] as well as in humans [2]. These increases in circulating levels of IFN could be achieved by administering the drug either orally, topically or systemically. Although very high concentrations were achieved in the sera, there was a very low level of toxicity observed. For example, in mice, a single oral dose of 100 mg/kg of imiquimod could induce 5780 U/ml of IFNα in the sera of animals after 2 hours without detectable toxicity [8]. The LD_{50} for a single oral dose in this model was about 500 mg/kg.

Further studies were undertaken to better characterize the cytokines induced by the imidazoquinolines. It could be appreciated that these compounds induced a wide spectrum of cytokines. In mice, imiquimod was shown to increase tumor necrosis factor (TNF) and interleukin (IL)-1α serum levels [6]. Splenic and bone marrow cultures exposed to imiquimod or S28463 were shown to produce IL-6, TNF, IFNα and IL-12 [6;9]. S28463 could induce a similar pattern of cytokines, but exhibited a higher potency as well as an enhanced induction of IFNγ of which imiquimod was a poor inducer [4;9]. S28463 also inhibited IL-5 production in splenic cultures and this effect required the presence of both macrophages and CD4+ lymphocytes [9]. The induction of IL-12 and IFNγ as well as inhibition of IL-5 clearly demonstrates the ability of imidazoquinolines

to skew the immune response toward a T_H1 phenotype. S28463 was also shown to stimulate the production of nitric oxide (NO) in bone marrow macrophages, enhancing their leishmanicidal activity [10]. Gene expression array analysis of bone marrow macrophages stimulated with S28463 demonstrated the induction of genes mostly involved in the inflammatory response such as inducible NO synthase (iNOS), CD40, macrophage inflammatory protein (MIP)-1α, MIP-1β, MIP-2α, IL-1β as well as plasminogen activator inhibitor 2 (PAI-2) [11]. Topical application of imiquimod in hairless rats led to a significant production of IFNα and TNF at the site of application [12]. These cytokines were found at a much reduced concentration at sites far removed from the area receiving the drug and were undetectable in the sera of the animals Thus, applied topically, these compounds locally induce the same spectrum of cytokines.

Oral administration of imidazoquinolines to cynomolgus monkeys led to increased serum levels of IFN, TNF, IL-1 receptor antagonist and IL-6 [7]. Furthermore, cultures of peripheral blood mononuclear cells (PBMC) stimulated with either imiquimod, S27609 or S28463 showed increased secretion of IFN, TNF, IL-1β, IL-6 and IL-8. This increased secretion was correlated with an increase in the mRNA levels for IFNα and MxA [7]. Topical application of S28463 in these monkeys led to increased IFNα, myxovirus resistance 1 (MxA) and IL-6 mRNA levels. Human responsiveness to the imidazoquinoline family of compounds was studied by analyzing local cytokine production in the skin of patients as well as by isolated PBMC. Topical application led to an increase in the mRNA for IFNα, IFNγ and 2'-5' oligoadenylate synthetase (2'-5'-AS) [13]. Isolated human PBMC stimulated with imiquimod, S27609 or S28463

secreted enhanced levels of IFNα, TNF, IL-1β, IL-1RA, IL-8, IL-10, IL-12, granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage CSF and macrophage inflammatory protein 1α (MIP-1α) [3;4;9]. From these studies, it becomes apparent that the different derivatives of imiquimod differ not only in their potency but also in the spectrum of cytokines they induce. Imiquimod is a poor inducer of IFNγ, TNF and IL-1α, all of which are readily induced by S28463 and S27609 [3;9]. As will be discussed below, these differences in cytokine induction may be due to differential receptor utilisation between imiquimod and resiquimod. The spectrum of cytokine induced by a specific imidazoquinoline may lead to a different spectrum of efficacy against particular pathogens.

Studies were undertaken to determine which cell type was responsible for the increase in cytokine secretion. Initial reports using cell type specific depletion of human PBMC suggested that the major target for imidazoquinoline-induced cytokine production was the monocyte/macrophage [14]. Further analysis found that the major cell type responsible for type I IFN production from PBMC was the plasmacytoid dendritic cell (pDC) [15] while other proinflammatory cytokines such as TNF and IL-12 were mainly produced by monocytes as well as various subset of dendritic cells [14;16]. Human epidermal carcinoma cell line COLO-16 as well as primary human keratinocytes have been shown to secrete IL-1α, IL-8 and TNF following stimulation with imiquimod or S28463 [17;18]. A more recent analysis revealed no TLR7 or TLR8 mRNA expression in primary human keratinocytes which correlated with inability of these cells to respond to resiquimod [19] which suggests a small amount of contaminating Langerhan's cells in

the previous study. All the data confirm that imidazoquinolines are potent inducers of cytokines that may play a crucial role in their immunomodulatory effects. It can also be observed that these compounds have a very general mechanism of action which is conserved across species from chickens [20] to humans.

2.2 Cellular Effects of Imidazoquinolines

The ability of imidazoquinolines to induce cytokine secretion in various cell types is clearly important to their immunomodulatory activity and may have a very profound effect on the outcome of the immune response of an individual. Besides the effect of these cytokines on immune cells, many studies have brought to light the ability of these compounds to directly influence the activation and differentiation status of immune cells. These observations add another layer to the immune modifying ability of imidazoquinolines.

As stated previously, the major cells responsible for cytokine production are antigen presenting cells (APC), but they are definitely not the only cells which have the capacity to respond to these compounds. Studies using B-cells have shown that imidazoquinolines can induce the proliferation of B-cells from T-cell depleted splenic cultures [21]. Imiquimod stimulated proliferation of preactivated B-cells while S28463 could affect both resting and preactivated B-cells. The proliferation of B-cells was also associated with the expression of class II major histocompatibility factor (MHC) and the costimulatory molecules B7.2 [21]. S28463 could induce the secretion of IgM from B-cells, but not imiquimod. The induction of naïve B cell proliferation and immunoglobulin production by imidazoquinolines appear to be at least in part dependent

on type I IFN produced by pDC which sensitizes the B cell to respond to these compounds by upregulating TLR7 mRNA expression [22]. Other cell types have been shown to be affected by imidazoquinolines. Indeed, it has been observed that both imiquimod and resiquimod can lead to splenomegaly in animals receiving one or two weeks of treatment [23;24]. Cellular splenic content revealed an increased amount of DC, natural killer (NK) cells, granulocytes and macrophages following imidazoquinoline administration [24]. Furthermore, S28463 could stimulate *in vitro* isotype switching towards either a T_H1 or T_H2 pattern, depending on whether the cells were given IFNγ or IL-4 [21]. Resiquimod also has the potential to shift an allergen-specific T_H2 $CD4^+$ T cell to a T_H0 or T_H1 cytokine producing cell [25]. When mice were injected with S28463 and OVA, the antibody response was skewed toward a T_H1 phenotype characterized by increased production of IgG2a and a reduction in IgE levels [21]. Confirming this observation, human PBMC from normal and atopic donor produced reduced levels of IgE following CD40 and IL-4 stimulation *in vitro* [26]. Thus, imidazoquinolines have the inherent ability to induce either a T_H1 or T_H2 immune response, depending on the cytokine environment, although, *in vivo*, they tend to favour the skewing of the response toward a T_H1 phenotype.

Macrophages and dendritic cells are an important part of the innate immune system and they play an essential role in presenting antigens to naïve T-cells after antigen capture and migration to the draining lymph node from the site of immunological insult. Topical application of imiquimod has been shown to stimulate migration of Langerhans cells (LC) from the skin to the draining lymph node in a murine model [27]. This migration

was not associated with an increase in expression of costimulatory molecules. Although no phenotypic difference was observed in Langerhans cells isolated from human skin treated with imidazoquinolines, these cells exhibited an increased capacity to stimulate the proliferation of T lymphocytes in a mixed lymphocyte reaction [28]. Once more, a skew in the response toward a T_H1 phenotype was observed, which led to a decreased response to IL-4 in lymphocytes exposed to LC treated with S28463 while responding normally to IFNγ treatment. Monocyte-derived dendritic cells (MO-DC) are obtained by treating monocytes from PBMC with IL-4 and GM-CSF [29]. MO-DC stimulated with S28463 showed an increased surface expression of CD80, CD83, CD86 and CD40 [30]. As was observed with Langerhans cells, MO-DC treated with imidazoquinoline could stimulate stronger proliferative response from T lymphocytes. Treatment with imidazoquinolines also induces the migration of other APC into important immune organs to trigger activation of B and T lymphocytes. Topical imiquimod administration increased splenic and dermal accumulation of pDC-like cells expressing CD4, Gr-1, B220, MHC class II and low levels of CD11c [24]. Once in the spleen, pDC formed clusters in the marginal zone as well as around the outer T cell area while $CD11c^+$ conventional dendritic cell (cDC) migrated within the T cell area following S28463 treatment [31]. Intravenous injection of S27609 led to IL-12 cytokine production by $CD11c^+CD11b^+CD8^-$ dendritic cells (DC) which was also associated with B7-1, B7-2 and MHC class II upregulation in both $CD8^-$ and $CD8^+$ $CD11c^+CD11b^+$ DC [16]. Although initial reports had shown that $CD8^+CD11c^+CD11b^+$ DC did not express TLR7 mRNA and failed to respond to imidazoquinolines [32], further analysis using quantitative realitime PCR (QPCR) revealed TLR7 mRNA expression in both $CD8^-$ and

$CD8^+$ $CD11c^+CD11b^+$ DC [16]. Furthermore, $CD11c^+CD11b^+CD8^-$ DC also migrated to the T cell area in the spleens of animals receiving imidazoquinolines.

Taken together, these observations clearly demonstrate the ability of imidazoquinolines to enhance migration, proliferation and differentiation of DC and B-cells, leading to an increased innate and adaptive immune response both in terms of cytokine induction and cell maturation as demonstrated by upregulation of surface markers and costimulatory molecules.

3. Tumor Treatment Using Imidazoquinolines

The spectrum of cytokines as well as activation of the adaptive arm of the immune system make imidazoquinolines good candidates for the treatment of various tumors by breaking tolerance. Most studies have focused on superficial premalignant and malignant epithelial tumors. Initial animal studies demonstrated that imiquimod treatment was tumoricidal against MC-26 colon carcinoma, RIF-1 sarcoma as well as Lewis lung carcinoma cells, but was ineffective against P388 leukemia cells [8]. Human trials for treatment of actinic keratoses, premalignant lesions with potential to progress into squamous cell carcinoma, with imiquimod revealed complete clearance in 84% of patients treated for 12 weeks [33]. Gene expression analysis revealed increased levels for IFNα, IL-6, IL-10RI and TLR7 mRNA in imiquimod-treated lesions [34]. The anti-apoptotic genes hurpin and HS-1-associated protein X-1 (HAX-1) were downregulated by imiquimod treatment. Numerous clinical trials have been performed to determine the efficacy of imiquimod treatment against superficial basal cell carcinoma [35-38].

Depending on length and frequency of treatment, imiquimod application resulted in complete clearance in 69 and 100% with once daily application leading to complete clearance in 88% of patients [39]. Based on the human clinical trials data, the FDA has approved the use of ALDARA™ cream in the treatment of superficial basal cell carcinoma. Regression of superficial basal cell carcinoma was associated with infiltration of $CD4^+$ T cells, dendritic cells and macrophages [40]. Interestingly, imiquimod treatment upregulated expression of the opioid growth factor receptor (OGFR) on tumor cells as well as cells infiltrating the tumor [41]. Expression of OGFR was associated with a reduction in tumor recurrence rate. Imidazoquinoline treatment has also been associated with inhibition of tumor-induced angiogenesis as well as *in vivo* tumoricidal activity against vascular tumors associated with an increased expression of tissue inhibitor of matrix metalloproteinase (TIMP)-1 and concurrent decrease in matrix metalloproteinase (MMP)-9 activity [42;43]. Furthermore, imidazoquinolines may have direct antitumoricidal activity on malignant melanoma. Imiquimod, but not resiquimod, resulted in apoptosis of tumor cells after 48 hours of incubation [44]. This observation was confirmed in human trials as imiquimod treatment of basal cell carcinoma resulted in a decrease in Bcl-2 expression as well as an increase in the apoptotic index [45]. Taken together, the data point toward a role for the immunomodulatory activity as well as direct anti-tumoricidal activity of imidazoquinolines in the treatment of various types of topical tumors.

4. Infectious Diseases and Imidazoquinolines

The ability of imidazoquinolines to induce a wide spectrum of cytokines as well as to directly modulate the activation and differentiation of many types of immune and non-

immune cells make them good candidates for the design of an effective treatment against various infectious diseases. Skewing of the immune response toward a T_H1 phenotype suggests that these compounds may be best suited for treatment of viral and bacterial infections as well as allergic diseases which could benefit by this kind of response.

4.1 Imidazoquinoline in the Treatment of Viral Diseases

ALDARA™, an imiquimod cream, is currently approved for the treatment of HPV-induced genital warts, actinic keratosis and primary superficial basal cell carcinoma. The efficacy of this cream in humans in treating HPV-induced warts has been determined through six clinical trials [46]. Briefly, results from five clinical trials demonstrated that treatment with imiquimod led to a complete clearance of genital warts in 51% of human immunodeficiency virus (HIV) negative patients. Warts were completely cured and did not recur after 10 to 16 weeks after the end of treatment. Interestingly, the cream seemed more effective in women than in men. 5% imiquimod cream resulted in at least a 50% reduction in wart area in 72% of patients compared with 20% in the placebo group [46]. The side effects associated with usage of ALDARA™ included itching, erythema, burning and erosion at the site of application. Overall, ALDARA™ appears to be an effective treatment for HPV-induced warts, resulting in only mild side effects.

Herpes viruses are enveloped icosahedral, double-stranded DNA viruses that replicate within the nucleus of the infected cell. They have the ability to cause lifelong infection within their host through establishment of latency interspersed with reactivation events. The alphaherpesviruses are neurotropic and rapidly replicating viruses. Herpes simplex

viruses 1 and 2 (HSV-1 and HSV-2) are the most notable members of this group and are the leading cause of facial and genital lesions, respectively. It has been estimated by seroprevalence that more than 45 million people in the United States are infected with HSV-2 [47]. An effective treatment for HSV should be able to control both the acute and the latent phases of the infection. Imiquimod and S28463 have been extensively studied in an intravaginal HSV-1 or HSV-2 infection model in the guinea pigs. Topical administration of imidazoquinoline between 72 hours prior to infection and up to 24 hours after infection was effective in reducing the number of lesions and of virus-positive lumbar spinal cord homogenates [48]. Treatment with imiquimod 12 hours after infection led to a decrease in the numbers of infected neurons and a reduction in the recurrence rate after the end of treatment [49]. Imidazoquinolines appear to have no direct antiviral effect [48;50]. If treatment was delayed until the appearance of lesions, no effect of imidazoquinolines on infection could be observed. However, combination therapy using both imiquimod and acyclovir was shown to have a protective effect against disease progression, even when treatment was initiated after lesion development [51]. Thus, imidazoquinoline either alone or in combination with other drugs can reduce acute phase HSV infection.

When treatment was initiated only once latency was established, imidazoquinoline treatment for 21 days decreased the recurrence of HSV by 67% over an 8 week period, whereas 5 days of treatment was shown to be ineffective [52]. Imidazoquinoline treatment did not affect the number of neurons infected. There was also an increased production of IL-2 by PBMC exposed to HSV antigen in the group receiving treatment for 21-day. S28463 treatment of latent HSV for a 3-week period led to a decrease in

recurrence rate by more than 80% [53]. Interestingly, daily, every other day or weekly treatment with the same dose of S28463 led to a similar reduction in recurrence rate. This last observation and the fact that imidazoquinolines prevent recurrence for many weeks after treatment is terminated [52] suggests that these compounds not only induce cytokine production, but also generate a long-lasting change in the immune status of the host. Human studies have been less clear about potential benefits of imidazoquinolines for the treatment of herpes virus infection. Imiquimod has shown beneficial effect in two case studies [54;55]. There have also been reports of patients experiencing worsening of their condition [56]. In a phase II clinical trial, S28463 (resiquimod) had generated promising results for the treatment of HSV in humans leading to a reduction in the recurrence rates [57]. Phase III clinical trials were undertaken under a 3M-Lilly joint venture which was stopped in Febuary 2003 stating lack of efficacy of the chosen dose of resiquimod [1].

Imidazoquinolines have also been tested against other viral infections, although few studies have been performed. A slight improvement in cytomegalovirus (CMV) in the guinea pig was observed when treated with another derivative of imiquimod, S26308 [50]. The treatment resulted in the reduction of the pathological consequences of infection that manifest themselves as an absence of splenic inclusions in the animals treated with imidazoquinoline.

4.2 Imidazoquinoline in the Treatment of Bacterial and Parasitic Infections

The broad pattern of immunomodulatory activity associated with imidazoquinolines makes them good candidates for treatment of diseases which could benefit from the T_H1

cytokine spectrum associated with these compounds. Leishmaniasis is associated with infection by various species of *Leishmania* protozoa. Cutaneous leishmaniasis can be caused by many species of *Leishmania*, including *Leishmania major*, while visceral leishmaniasis is associated with *Leishmania donovani*. The type of immune response generated by the host plays a determining role in the outcome of disease. In experimental models, animals that could generate a stronger T_H1 immune response were more efficient at containing the protozoa while a T_H2 response was associated with increased protozoa growth and more severe pathological changes [58]. Bone marrow macrophages isolated from the *Leishmania* susceptible strain of mice (BALB/c) exhibited enhanced leishmanicidal activity when treated with S28463 [10]. As previously stated, this enhanced killing of protozoa was associated with an increase in nitric oxide (NO) production by macrophages. Topical administration of imiquimod to BALB/c mice infected in the footpad with *Leishmania major* prevented surface lesions and caused a decrease in footpad thickness compared to the placebo control [10]. In humans, imiquimod was able to completely cure 6 of 12 patients with meglumine antimoniate-resistant lesions [59]. Patients who responded to imiquimod treatment also displayed a re-epithelization of the lesion, which suggests that the imidazoquinolines induce factors important for tissue regeneration. This effect could possibly be explained by the induction of plasminogen activator inhibitor by imidazoquinolines [11]. Recently, a randomized double-blind clinical trial of imiquimod and meglumine antimoniate for the treatment of cutaneous leishmaniasis demonstrated an almost complete healing of lesions by one to three months in patients receiving both treatments which took up to a year when only antimoniate was administered [60]. Further studies on the potential mechanism of tissue regeneration induced by imidazoquinolines as well as on the effects

of imidazoquinoline treatment on a wider range of *Leishmania* infection-induced lesions, including some which do not contain parasites that are resistant to antimony treatment, are necessary to fully understand the mechanism and potential benefits of imidazoquinoline treatment.

Leishmania protozoa infect and replicate exclusively inside macrophages. Since the immunomodulatory activity of imidazoquinolines is mediated at least in part by macrophages and that these compounds were shown to be effective at stimulating killing of *Leishmania*, infections with various bacteria that replicate within macrophages would also be good candidates for imidazoquinoline treatment. *Mycobacteria spp.* are intracellular bacteria that replicate within macrophages. The most well-known member of this family is *Mycobacterium tuberculosis*, the causative agent of tuberculosis which infects nearly a third of the world population and is one of the most pressing health issues worldwide. *Mycobacterium bovis* BCG is the vaccine strain still in use in Europe and Asia for the prevention of tuberculosis. Mice infected with *M. bovis* BCG and treated intraperitoneally with S28463 for 2 weeks showed a reduction in the bacterial load of their spleens [23]. As will be discussed further below, the genetic background of the animal is crucial for the treatment to be effective.

All of the above presented studies point toward the effectiveness of imidazoquinolines in enhancing clearance as well as in altering the course of various viral, bacterial and parasitic infections. Some of the observations made using animal models could be applied in humans, although, as is the case with HSV infection, the choice of imidazoquinoline derivative as well as the dosing may prove to be crucial in determining

the outcome of treatment. Resolution of disease requires a T_H1 immune response of the host in response to these pathogens. Since imidazoquinolines consistently induce T_H1 skewing of response *in vivo*, it is thus not surprising that these compounds improve host response against these pathogens. It is clear that the immunomodulatory function of imidazoquinolines allow for the induction of an immune response which is tailored to better eliminate the invading organisms.

5. Adjuvant Activities of Imidazoquinolines

Over the past decade, much effort has been put forward to develop new vaccine adjuvants that can induce a more defined immune response to specific antigens while minimizing side effects in the patients receiving vaccines. Some promising compounds include nonmethylated CpG containing oligodeoxynucleotides (ODN), as well as monophosphoryl Lipid A (MPL) from *Salmonella minnesota*, which has a lipid A moiety homologous to the lipid A of other lipopolysaccharide (LPS) molecules. The ability to activate proliferation of B-cells as well as to induce immunoglobulin secretion [21] made imidazoquinolines potentially useful adjuvants. Injection of the Type 2 glycoprotein from HSV-2 in complete Freund's adjuvant with or without imiquimod was tested in a guinea pig HSV infection model. Imiquimod-HSV-2 vaccination increased the level of protection afforded by the already active vaccine, resulting in a 3-log reduction of viral shedding by day 1 after infection. It also resulted in the absence of recurrent disease when compared to the group receiving only the vaccine [61]. In another study, only imiquimod was used as the adjuvant for the HSV vaccine, and this vaccine was administered to animals with already established latent infections. This study demonstrated an 80% reduction in recurrence rate compared to the placebo control

[62]. The response to HSV when imidazoquinolines are given as adjuvants was characterized by increased levels of circulating T_H1 antibody (IgG2a) and a reduction in IgE levels [63]. Other promising uses of imidazoquinoline include particle-mediated DNA vaccines, transcutaneous peptide immunization as well as enhancement of immunogenicity of certain peptide-adjuvant combinations. Injection of imiquimod or resiquimod at the site of plasmid DNA delivery using a gene gun resulted in a two-fold increased in co-stimulatory molecule expression in the draining lymph node as well as increased $CD4^+$ T lymphocyte proliferation [64]. Imiquimod and S27609 have also been found to enhance DNA vaccine efficacy against mammary tumors in a murine model [65]. Topical peptide application in the presence of imiquimod led to increases in CTL responses against the selected antigen [66]. The advantage of this method is that systemic administration of the adjuvant is not required which should minimize side effects. Of particular interest, it was described that using S28463 in conjunction with the HIV-1 Gag protein, very low adjuvanticity was observed until the imidazoquinoline was physically conjugated to the Gag protein where it could induce very strong T_H1 and $CD8^+$ T cell responses [67]. Finally, addition of imiquimod to a melanoma peptide with FMS-like tyrosine kinase 3 (Flt3) ligand as an adjuvant resulted in an increased number of patients with $CD8^+$ T cells specific for the antigen compared to those receiving only Flt3 ligand as adjuvant [68]. These studies suggest a very good potential for imidazoquinolines as adjuvants if the side effects associated with systemic administration of these drugs can be kept within acceptable limits [69].

6. Mechanism of Action of Imidazoquinolines

6.1 Toll-Like Receptor Family

The primary role of the immune system is to recognize and destroy invading pathogens that could compromise the survival of the organism. To achieve this goal, two arms have evolved with very specific functions that work together to efficiently sense and kill pathogens as well as discriminate "self" from "non self". The adaptive arm of the immune system is designed to recognize specific antigens presented at the surface of antigen presenting cells (APC) through somatically recombined receptors, which, depending on the presence of co-stimulatory molecules (CD40, CD80, CD86) may lead to either anergy or cellular activation. The innate component of the immune response is mainly composed of cells of myeloid origin which can be further subdivided into mononuclear (monocytes, macrophages, dendritic cells) and polymorphonuclear cells (neutrophils, eosinophils, basophils). These cells express germline encoded receptors that recognize various conserved molecules found at the surface of phylogenically distant organism [70]. How recognition of invading pathogens by innate immune cells leads to activation of the adaptive immune response has recently received a lot of attention through the discovery of the Toll-like receptor (TLR) family. Initially discovered in *Drosophila*, the Toll protein was found to control resistance to fungal and gram-positive bacteria as well as play an important role in dorsoventral polarity [71]. To date, 13 members have been described in mammals (TLR1 to 13) [72]. TLR1 to TLR9 are shared between mouse and human. TLR10 appears to be functional only in humans

[73], while TLR11 to TLR13 appear to be specific to the mouse [72]. These receptors are characterized by the presence of 19-25 leucine rich repeats (LRR) domain with a consensus sequence of XLXXLXLXX in their extracellular domain as well as a Toll/IL-1R (TIR) domain in their cytoplasmic region. During the past few years, significant progress has been made to characterize the various ligands for these receptors (Table 1). Based on sequence homology and the type of ligands (protein, lipid or nucleic acid) recognized, the Toll receptor family can be subdived into five families: TLR2 (TLR1, TLR2, TLR6, TLR10), TLR3, TLR4, TLR5 (TLR5, TLR11), TLR9 (TLR7, TLR8, TLR9).

6.1.1 TLR4 and Gram-Negative Bacteria Sensing

Initially described as a heat stable derivative of microbes, lipopolysaccharide (LPS) is the major cell wall component of gram-negative bacteria as well as a crucial factor in the induction of septic shock. The search for the receptor of LPS was resolved when it was found by positional cloning that C3H/HeJ mice, which were known to be very resistant to the toxic effects of LPS, had a mutation in the *Tlr4* gene resulting in a substitution of a proline with a histidine at position 712 [74;75]. These findings were confirmed in TLR4 knockout (KO) mice which were unresponsive to LPS treatment [76]. Two single nucleotide polymorphisms have been described for the human *Tlr4* gene (Asp299Gly and Thr399Ile) which leads to reduced responsiveness of immune cells to LPS treatment [77]; further lending support to TLR4 as the receptor for LPS both in mice and in humans. Recently, many host proteins such as heat shock protein (HSP) 60 and soluble hyaluronan have been described as signaling through either TLR4 or TLR2. Unfortunately, the presence of bacterial LPS as well as lipoprotein in the preparations of

the recombinant protein suggests that the role of host protein in TLR activation needs to be revisited to eliminate the possible contribution of bacterial contaminants [78].

Table 1: Toll-Like Receptor Family and Ligands [Adapted from [79;80] and Updated [73;81]]

Receptor	Ligand	Origin
TLR1 (with TLR2)	Triacyl lipopeptides	Bacteria and Mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoprotein/lipopeptides	Various Organisms
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria*
	Lipopolysaccharide	*Leptospira interrogans*
	Lipopolysaccharide	*Porphyromonas gingivalis*
	Zymosan	Fungi
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	F protein	Respiratory syncytial virus
	Env protein	Mouse mammary tumor virus
TLR5	Flagellin	Bacteria
TLR6 (with TLR2)	Diacyl lipopeptides	*Mycoplasma*
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline	Synthetic compound
	Loxoribine	Synthetic compound
	Bropirimine	Synthetic compound
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline (Resiquimod)	Synthetic compound
	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria, Herpes simplex virus 1 and 2
TLR10 (potentially with TLR1 and TLR2)	Unknown	Unknown
TLR11	Profilin-like protein	*Toxoplasma gondii*
	Unknown	Uropathogenic bacteria
TLR12	Unknown	Unknown
TLR13	Unknown	Unknown

6.1.2 TLR2 Family: Recognition Through Cooperation

The TLR2 family is characterized by a broad spectrum of ligand recognition through interaction between the various members. Recognition of lipoteichoic acid as well as diacylated lipopeptides is recognized through a TLR2/TLR6 heteromeric complex [82;83]. Interestingly, CD36 has been shown to participate in the recognition of diacylated bacterial lipoproteins by interacting with the TLR2/TLR6 complex [84]. Although CD36 and TLR6 were not essential for the response, they greatly enhanced cytokine production induced by the lipoprotein. TLR2 complexed with TLR1 has been implicated in the response to triacylated bacterial lipoproteins [85]. The TLR2/TLR6 and TLR2/TLR1 complexes do not discriminate between di- and triacylated lipoproteins, but rather recognize the general structure of both the lipid and peptide chain [86]. TLR10 has been found not only to homodimerize, but also associate with TLR1 and TLR2 [73]. The *Tlr10* gene appears to be functional only in humans because of a retroviral insertion present at this locus in the mouse genome [73]. Although no ligands have been identified for TLR10, genetic variation in the *Tlr10* gene was shown to be associated with susceptibility to asthma in two asthmatic populations [87]. TLR2 has also been implicated in recognition of peptidoglycan, a major cell wall component of gram-positive bacteria [83]. A recent study has found that highly purified peptidoglycan from eight strains of bacteria did not activate TLR2, TLR2/TLR6 or TLR2/TLR1 [88]. The study concludes that commercial preparations of peptidoglycan probably contain lipoproteins that signal through TLR2. Peptidoglycan can still be recognized by the nucleotide-binding domain (NOD) 1 and NOD2 proteins located in the cytoplasm [89]. Finally, TLR2 in association with TLR6 plays an important role in fungal recognition through sensing of zymosan, an important cell wall component [82].

6.1.3 TLR3 Family and Virus Sensing

Double-stranded RNA (dsRNA) is a common product of viral replication and infection. TLR3 knockout mice were shown to be unresponsive to poly I:C, a synthetic dsRNA molecule, stimulation. Interestingly, contrary to TLR2 and TLR4 which are mainly localized at the plasma membrane, TLR3 has an intracellular localization in accordance with its role in detection of viral infection [90;91]. As will be discussed below, TLR3 is also unique among other TLRs because it does not employ the adaptor molecule MYD88, depending instead on the adaptor TRIF for signal transduction [92;93].

6.1.4 TLR5 Family and Protein Recognition

The TLR5 family is unique in its ability to recognize protein components of pathogens. TLR5 has been shown to recognize bacterial flagellin [94]. Only the monomeric form of flagellin can activate TLR5 while filamentous flagellin is inactive. The recognition site for TLR5 recognition is buried within the core of the flagella which is not accessible in the polymerized form explaining the inability of filamentous molecules to activate immune cells [95]. It has also been observed that TLR5 can form complexes with TLR4 which appears to be required for nitric oxide (NO) induction by flagellin in macrophages [96]. TLR11 has initially been shown to play a role in the resistance of mice to uropathogenic bacteria infection [97]. It was observed that a stop codon exists in the human *tlr11* gene which suggests that it is nonfunctional. Recently, a profilin-like protein from *Toxoplasma gondii* was found to induce IL-12 secretion from dendritic cells in a TLR11-dependent manner. To date, TLR5 and TLR11 are the only known TLR which specialize in protein recognition.

6.1.5 TLR9 Family and Nucleic Acid Sensing

Bacterial extracts have been known to induce strong inflammatory reactions when injected to mice or humans and displayed tumoricidal activity against certain types of tumors. The immunostimulatory activity of *M. bovis* BCG extracts was found to reside with the DNA component [98]. Further studies characterized that CpG-containing oligodeoxynucleotide (ODN) could mimic the stimulatory activity of bacterial DNA [99]. These CpG motifs are found at a much lower frequency in eukaryotes and are methylated in mammals which render them immunologically inert. Later, it was found that TLR9 KO mice failed to respond to CpG ODN [100]. Sequence analysis revealed three classes of immunostimulatory sequences which induced various spectra of immunological effects [99]. CpG-A ODN induce mainly type I IFN production and induce NK cell activation. These ODN are characterized by a phosphodiester backbone which is capped at the 3' and 5' end by phosphorothioated bonds to prevent nuclease digestion. On the other hand, CpG-B ODN have an entirely phosphorothioated backbone and have been found to affect strongly B cell proliferation and activation. Finally, a third class, CpG-C ODN, combines features of the other two and induces broader immunological activity [101]. Recently, it was found that the phosphorothiate backbone itself is recognized by TLR9 which may explain the different activities of ODN [102]. Furthermore, initial species-specific sequences which were characterized between mice and humans appear only to depend on the presence of the phosphorothioate backbone; both species responding to the same optimal sequence when the ODN backbone is changed to phosphodiester.

Although the imidazoquinoline family of compounds have been studied for many years and are currently being administered for the treatment of HPV-induced warts in humans, no precise mechanism of action had been defined until it was observed that TLR7 KO mice were completely unresponsive to stimulation with this class of compounds [103]. Furthermore, a role for TLR8 in imidazoquinoline response in humans was also described shortly after [104]. It is still unclear whether murine TLR8 is non-functional or if it is involved in recognizing different ligands from its human homolog. Interestingly, different members of the imidazoquinoline differ in their TLR receptor usage in humans. Imiquimod signals strictly through TLR7, while it was found that resiquimod can activate through both TLR7 and TLR8 [104]. The differential receptor usage may explain the difference in potency and biological activities induced by imiquimod and resiquimod. Furthermore, other nucleoside analogs such as loxoribine were found to signal through the TLR7 pathway [105]. Recently, ssRNA of viral origin were described as ligands for TLR7 and TLR8 [106;107]. Certain short inhibitory RNA (siRNA) sequences have also been found to activate plasmacytoid dendritic cells through TLR7 which is of particular importance in gene silencing experiments [108].

A common feature of all of the members of the TLR9 family is their intracellular localization [90]. Accordingly, ligand uptake and endosomal maturation are required before signal transduction can occur (see annex and [105;109]). Following fluorescent-labeled CpG ODN treatment, both TLR9 and MYD88 were found to co-localize to lysozome-associated membrane protein (LAMP)1 positive vesicles [110]. TLR9 has been found to localize in the endoplasmic reticulum in resting cells and to translocate to lysosomes containing CpG DNA as early as five minutes after exposure [111].

Treatment with TLR7 ligands recruited the MYD88 adapter molecule to LAMP1 positive late endosomes [105]. Although to this date, no direct measurements have been performed to directly assess the localization of both TLR7 ligand and receptor due to the lack of specific antibodies. It is thought that through their endosomal localization, the TLR9 family of receptors sense the presence of nucleic acid which is not normally found in these compartments but may arise through bacterial and viral infections.

6.2 Tissue and Cellular Distribution of TLR

Much effort has been put into defining the TLR receptor expression profile both in mice and in humans. The expression profiles are mostly based on mRNA expression analysis which should be taken with caution as they do not always correlate with protein levels. Some studies are now starting to validate functional TLR expression in various cell types by treating them with receptor-specific ligands. Differences in TLR expression between mouse and humans have emerged and they will be discussed where appropriate [112].

Murine TLR2 (mTLR2) has been found to be highly expressed in the spleen, lung and thymus [113]. Lower levels of mTLR2 expression were found in brain and skeletal muscle. Very low levels were found in peripheral blood leukocytes (PBL). Using various murine cell lines, mTLR2 expression was found in dendritic cells, macrophages, mast cells and neutrophils and to a lower extent in B and T cells [114]. Contrary to mTLR2, human TLR2 (hTLR2), was highly expressed in PBL, spleen, lung and was barely detectable in the thymus [115]. Expression of hTLR2 was highest in monocytes and polymorphonuclear cells (PMN) with weak expression in DC and was barely

detectable in T and B cells [116]. TLR3 expression in human tissues has been detected in spleens, lung, pancreas, placenta and testis. Human TLR3 is almost exclusively expressed in dendritic cells and is not detected in any other immune cell types [116;117]. Fibroblasts have been shown to express TLR3 as well as TLR9 on their surface [118]. Murine TLR3 is expressed at high levels in both macrophages and DC [112;114]. Murine fetal skin-derived mast cells have also been reported to produce cytokine following poly I:C stimulation [119]. Mouse and human TLR4 shows a very similar pattern of expression being mainly expressed in cells of myeloid origin such as macrophages, DC and granulocytes [112]. Human TLR4 is expressed at high levels in PBL and spleen and to a lesser extent in the lungs [115]. Mouse TLR4 is mainly expressed in the heart, brain, spleen and lung with detectable expression in liver and skeletal muscle [75].

Human TLR tissue expression, carried out using quantitative PCR analysis, revealed that TLR7 is mainly expressed in the spleen, lung, placenta and in PBL [115]. TLR8 was found mainly in PBL, lung, spleen and placenta. Further analysis revealed that TLR7 expression was found in plasmacytoid dendritic cells (pDC), B cells and also at very low levels in monocytes, while TLR8 was expressed almost exclusively in monocytes [120]. Human eosinophils have been found to express TLR7 while neutrophils express both TLR7 and TLR8 [121]. Murine TLR7 expression revealed high levels mainly in the heart and spleen [103]. Analysis of TLR expression in murine cell lines revealed that TLR7 expression was found mainly in dendritic cells and macrophages [114]. Murine $CD45RB^{low}$ $CD25^{+}$ regulatory T cells (Treg) and connective tissue type mast cells also express TLR7 and respond to imidazoquinoline treatment [119;122]. Although

keratinocytes have been shown previously to respond to imidazoquinolines [17;18], no TLR7 mRNA was detected suggesting contamination with other cell types [19]. The downside to all these studies is that they are limited to mRNA expression, it is thus imperative to develop antibodies against TLR7 and TLR8 to further characterize their role in imidazoquinoline signalling.

Human TLR9 shows a very restricted pattern of expression which can be detected in the spleen, PBL and ovaries [115]. TLR9 expression in immune cells is restricted to pDC and B lymphocytes [120]. Contrary to its human counterpart, murine TLR9 is highly expressed only in the spleen [100]. TLR9 expression can be readily detected in macrophages, both myeloid DC and pDC as well as in B cells [114;123]. The more restricted pattern of TLR9 expression in humans could limit the clinical applicability of CpG ODN treatments and suggest that murine studies employing TLR9 ligands should take this into consideration.

6.3 Signal Transduction Through TLR: Specificity Through Adaptors

The intracellular events leading to cell activation and cytokine production following TLR activation have been the focus of intense research in the past years. Initially described as sharing all of their signaling components, it has now become clear that various members of the TLR family activate specific signal transduction events which explain their differential activity both *in vivo* and *in vitro*. One major aspect of signal transduction specificity between various TLR is their usage of different adaptor proteins which recruit and activate specific downstream kinases and transcription factors leading to the induction of specific genes and ultimately effector functions (Figure 2).

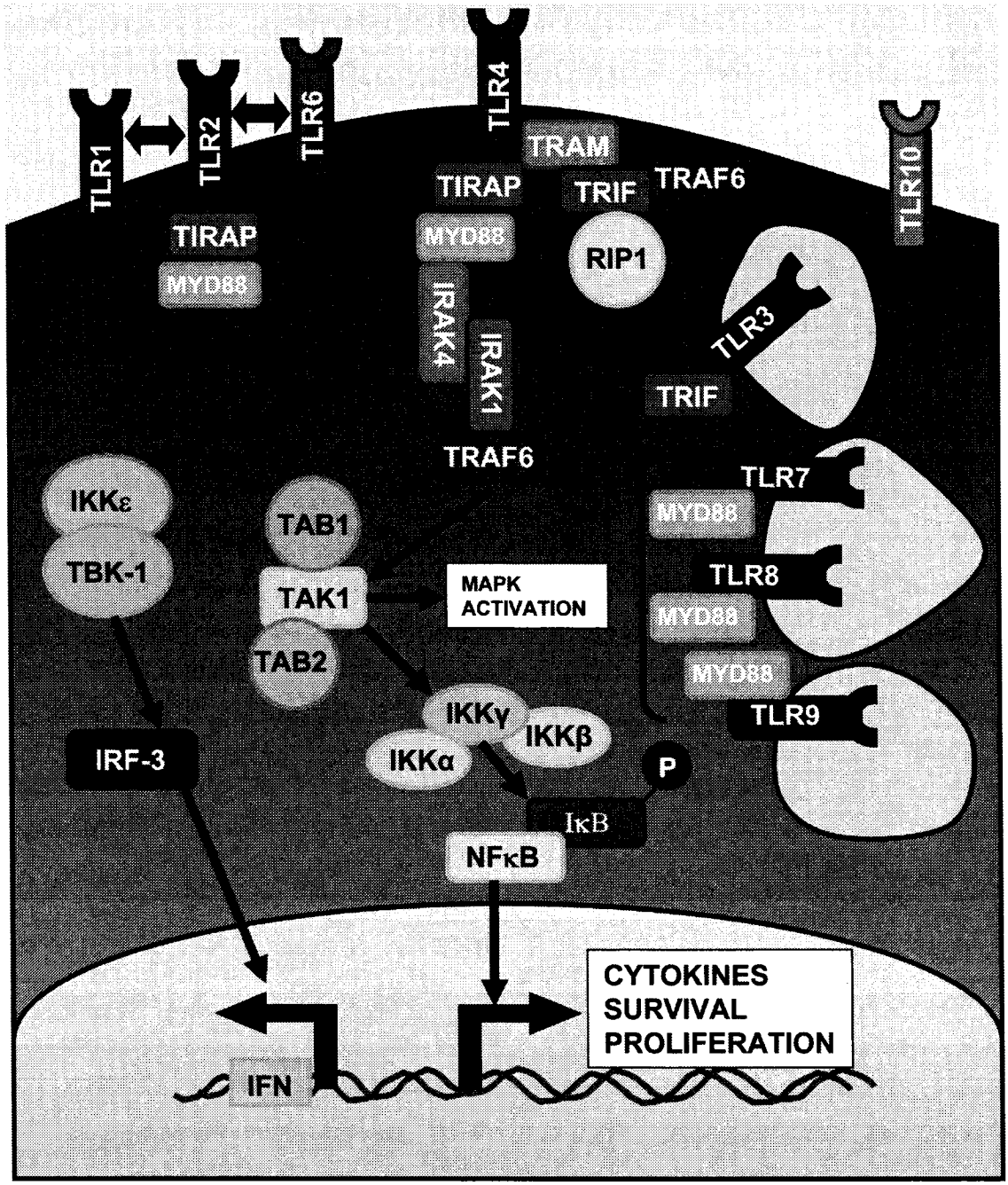


Figure 2: Signal transduction events triggered by various Toll-like receptors

MYD88 is an adaptor protein shared by all TLR except for TLR3. It is recruited, upon ligand interaction, to the receptor via its Toll/IL-1R (TIR) domain. Through its N-terminal death domain (DD), it can interact with IL-1R-associated kinase (IRAK)4

which then activates IRAK1. This leads to the recruitment of TNF receptor-associated factor (TRAF)6 and the dissociation of the complex from the receptor. The TRAF6-IRAK1 complex then interacts with the TAK1-TAB1-TAB2/TAB3 complex [79]. Activation of the TAK1 kinase occurs through polyubiquination mediated by the ubiquitin ligase activity of TRAF6 [124]. TAK1 activation leads to the phosphorylation of IKKβ resulting in the phosphorylation and degradation of IκB releasing NFκB which translocate to the nucleus to induce transcription of immune genes [125]. TAK1 also has the ability to phosphorylate and activate MKK6 leading to P38 and JNK MAPK activation [124].

Even though MYD88 KO mice were found to be resistant to LPS-induced shock and failed to produce proinflammatory cytokines following LPS challenge, IRF3 phosphorylation, NFκB as well as delayed MAPK activation could still be observed in these animals [126]. Five TIR containing proteins (MYD88, TIRAP (MAL), TRIF (TICAM-1), TRAM and SARM) are found in the mouse and human genomes [72]. Knockout animals as well as mutagenesis studies were undertaken to assess the possible contribution to TLR signal transduction by these various TIR containing proteins. Initially considered to be responsible for MYD88-independent responses, TIRAP KO mice displayed the same response pattern to TLR2 and TLR4 agonists as the MYD88 KO animals suggesting that it lays within the MYD88-dependent pathway [127]. The discovery of TRIF as the adaptor protein responsible for the MYD88-independent TLR4 responses was discovered by both gene deletion as well as large scale mutagenesis screens [92;93]. TRIF deficient animals are relatively resistant to endotoxic shock

induction by LPS and failed to induce type I IFN following LPS stimulation [92]. Interestingly, TLR3 was found to depend on TRIF, but not MYD88 or TIRAP, to initiate signal transduction following dsRNA stimulation [92;93]. Recently, another adaptor molecule termed TRAM was found to be specifically involved in the TLR4-dependent MYD88-independent pathway [128]. Thus through receptor expression profiles, cellular localization and differential adaptor usage, different TLR can initiate specific cellular responses.

6.4 Downstream Effectors of Imidazoquinoline Signalling

The downstream targets activated by imidazoquinolines have been studied well before the discovery that these compounds were TLR7/8 ligands. It was described that imiquimod stimulated binding of NFκB to their target sequences [103;129]. Later, phosphorylation of the inhibitor of NFκB (IκB) subunit was observed following imidazoquinoline stimulation [10]. Further studies identified AP-1 as another transcription factor activated by imidazoquinoline treatment [10]. Initial observations had indicated that STAT-1 was required for induction of cytokine production [130], although it was later shown that imidazoquinoline treatment did not lead to any phosphorylation of STAT-1 [10]. This discrepancy could be explained by the observation that STAT-1 is required for basal expression of many genes and that this activity does not require prior phosphorylation of the protein [131-134]. Thus, STAT-1 knockout mice might be lacking expression of many important signaling components preventing them from properly responding to the stimuli. However, it would be inappropriate to conclude from these results that STAT-1 activation represents one of

the steps activated during the signal transduction mechanism initiated by imidazoquinolines. Besides transcription factors, imidazoquinolines have also been found to activate numerous kinases (Figure 2). In accordance with signalling through a TLR, the IL-1 receptor-associated kinase (IRAK) kinase activity was shown to be induced following S28463 treatment [103]. Recently, activation of IRF-5 and IRF-7 was demonstrated following activation of TLR7 and TLR8 leading to type I IFN production [135]. Activation of IRF5/7 required the activity of IRAK1 and TRAF6. TLR7/8 activation of interferon response factor (IRF5) does not appear to be shared by other TLR members. A role for protein kinase C has also been suggested, although the study employed a very general inhibitor [129]. Since there are twelve isoforms of PKC which differ in tissue distribution, substrates and cofactor requirements, the analysis of the precise isoform(s) involved in imidazoquinoline signalling is required. Imidazoquinoline treatment has also been shown to activate numerous members of the mitogen-activated protein kinase (MAPK), which are stress response kinases activated by a number of growth factors as well as cytokine receptors [136]. Imidazoquinolines were found to stimulate phosphorylation of c-jun N-terminal kinase (JNK), as well as p38 MAPK, in both splenic B cells and in a B cell line [137]. In BMM, imidazoquinoline treatment led to the activation of JNK [10;103]. Activation of ERK MAPK also occurs following imidazoquinoline stimulation of murine macrophages (see Annex 1 and [105]). In summary, most of the downstream targets activated by imidazoquinolines are in agreement with signaling initiated by binding of this ligand to TLR, although much research is still warranted to better characterize the signal transduction cascade activated by this family of compounds.

7. Genetic Factors Modulating Responses to Imidazoquinolines

In mice, susceptibility to infection with *Leishmania donovani*, *Mycobacterium bovis* BCG and *Salmonella enterica* serovar Typhimurium was shown to be under the control of an autosomal dominant gene located in the B*cg/Ity/Lsh* locus on chromosome 1 [138-140]. Positional cloning led to the characterization of the natural resistance-associated macrophage protein 1 (NRAMP1, renamed to solute carrier family 11 member 1 (SLC11A1)) which encodes a macrophage and neutrophil-restricted transmembrane protein that shares homology with other eukaryotic transporter proteins of the non-ATP-binding cassette type [141-143]. In mice, two alleles of the *Nramp1* gene have been described. The wild-type allele (*Nramp1*^{r}), conferring resistance to infection, has a glycine residue at position 169, while the other allele (*Nramp1*^{s}) has an aspartic acid residue at the same position, leading to susceptibility to intracellular pathogens [144]. This aspartic acid substitution was found to be in absolute association with the *M. bovis* BCG susceptibility phenotype in all tested mice strains. The confirmation of *Nramp1* as the gene responsible for resistance to *M. bovis BCG*, *Salmonella enterica* serovar Typhimurium and *Leishmania donovani* was obtained by knockout and transgenic studies [145;146]. NRAMP1 is localized to LAMP1-positive late endosomal and lysosomal compartments [147]. Interestingly, it was observed that mice carrying the susceptible allele had a very similar infection kinetic compared to mice in which *Nramp1* had been deleted [146]. This suggested that the mutation of the glycine residue at position 169 for aspartic acid led to a functionally null allele. Recently, detailed analysis has revealed that the susceptible allele of *Nramp1* leads to the production of a protein which is retained in the endoplasmic reticulum preventing lysosomal localization and interaction with pathogen-containing vacuoles [148].

The mechanism of action of NRAMP1 in disease resistance has not yet been fully elucidated. Phagosomes from *Nramp1*$^{+/+}$ macrophages infected with *M. bovis* BCG displayed a lower pH which was thought to hinder bacterial growth [149]. *Salmonella*-containing vesicles (SCV) did not show this alteration in pH, but rather the presence of NRAMP1 induced phagosome maturation and lysosome fusion which was not observed in macrophages lacking functional NRAMP1 [150]. The discovery of NRAMP2 (SLC11A2) as a divalent cation transporter required for iron uptake from endosomes suggested a possible mechanism for NRAMP1 function [151;152]. Based on sequence homology to NRAMP2, NRAMP1 was proposed to function as a divalent cation transporter which was confirmed subsequently by many studies [153-156]. A lot of debate exists as to which direction NRAMP1 moves these divalent cations, namely Mn^{2+} and Fe^{2+}. The first model proposes that NRAMP1 transport divalent cations, especially Fe^{2+}, into phagosomes containing bacteria and drive the production of toxic radicals through the Fenton reaction [155;156]. Another model proposes that NRAMP1 transport divalent cations such as Fe^{2+} and Mn^{2+} from the lumen of the phagosome into the cytosol. This would deprive the bacteria of essential co-factors required for replication as well as survival within the toxic environment of the phagosome [154]. In support of this model, it was observed that iron chelators mimic the effect of NRAMP1 on the maturation of vesicles containing *Salmonella enterica* serovar Typhimurium [157]. Interestingly, it appears that iron chelators also mimic the effect of NRAMP1 on the bacteria residing within the phagosomal compartment resulting in the upregulation of virulence factors [158]. A third model suggests that NRAMP1 functions as an antiporter which transports divalent cations in either direction against a proton gradient [153]. All

models tend to agree on the requirement for a proton gradient for efficient divalent cation transport by NRAMP1. It should be kept in mind that no studies have directly assessed the requirement of NRAMP1 divalent cation activity in intracellular pathogen resistance and most studies infer this requirement. Analysis of an NRAMP1 protein lacking divalent cation transport capacity, but retaining proper intracellular localization, should be able to shed some light on the exact contribution of Mn^{2+} or Fe^{2+} transport by NRAMP1 in controlling intracellular pathogen infection.

NRAMP1 has been shown to play a role in many different aspects of the immune response, mainly by modulating responses of macrophages. Many early studies have used congenic mice strain to study the biology of NRAMP1 on macrophage function. It is unclear whether the effects observed in these studies were the result of NRAMP1 function or of other genes present within the 13 to 40 cM interval different between the two strains of mice. We will focus on studies which have used knockout animals or transfection to study NRAMP1 function. Presentation of antigen through MHC class II is impaired in *Nramp1*^{s} and *Nramp1*$^{-/-}$ macrophages [159;160]. Furthermore, induction of MHC class II by IFNγ is also impaired in macrophages lacking a functional NRAMP1 protein. Dramatically reduced responsiveness to IFNγ was linked to a reduction in STAT1 phosphorylation [159]. Nitric oxide production following IFNγ stimulation by bone marrow-derived macrophage cell lines was also impaired in *Nramp1*$^{-/-}$ and *Nramp1*^{s} cells (Chapter 2 and [161]). Ribozyme-mediated knock-down of Nramp1 in macrophages led to a reduction in NO, TNF and MHC class II induction following IFNγ

treatment [162]. Thus, NRAMP1 expression modulates macrophage responses to IFNγ in murine macrophages.

The initial studies in mice suggested that *Nramp1* may play a crucial role in innate resistance to intracellular pathogen resistance in humans. The lack of a functionally null allele of *Nramp1* in humans as well as the endemicity of certain pathogens such as *M. tuberculosis* has not revealed the same striking phenotype observed in mice. *Nramp1* could thus be a major player in protection against intracellular pathogens in immunologically naïve populations. As an example, a striking effect for *Nramp1* in *M. tuberculosis* resistance was found in an aboriginal population not previously exposed to this pathogen [163]. Analysis of the *Nramp1* gene in humans has revealed many polymorphisms across the gene, but only one was found in the coding sequence [164]. A Z-DNA forming promoter polymorphism which can act as an enhancer has been linked to both autoimmune and infectious disease. To date seven alleles which differ in sequence and length of a GT repeat have been characterized [165]. Allele 3 leads to higher transcriptional activity of the *Nramp1* promoter and, based on luciferase reporter assay, is believed to lead to higher protein expression and enhanced inflammatory responses [166]. As such, allele 3 has been found to be associated with protection from infectious diseases such as pulmonary tuberculosis, leprosy as well as visceral leishmaniasis [165]. On the other end of the spectrum, allele 2 was shown to be a very weak promoter and as such has been linked to susceptibility to autoimmune diseases such as rheumatoid arthritis as well as type I diabetes [165]. This association is not perfect and may vary depending on the population studied. Other alleles of *Nramp1* have

also been associated with disease, although their functional activities have not been assessed.

The data presented in chapter 2 demonstrates that when *Nramp1*^{r} and *Nramp1*$^{-/-}$ mice were infected with *M. bovis* BCG and treated with imidazoquinoline (S28463), only the mice carrying the resistant allele of *Nramp1* displayed a decrease in splenic bacterial load. Increasing the dose by 25-fold did not lead to a reduction in the bacterial load of *Nramp1*$^{-/-}$ mice. As expected with a dominant phenotype, the F1 cross between these two strains of mice exhibited normal responsiveness to imidazoquinoline treatment (Chapter 2). This study suggests that NRAMP1 is important for the response to imidazoquinolines and that in the light of these compounds using the TLR7 pathway, the discovery of the factors contributing to the response will prove invaluable in dissecting the mechanism of action as well as in predicting how an individual will respond to treatment. One useful approach would be the use of recombinant congenic strains of mice, which could allow for the mapping of chromosomal loci important for the response to imidazoquinolines as well as other TLR ligands [167].

The ability of imidazoquinolines to effectively treat various conditions is intimately related to the ability of an individual to respond efficiently to these compounds. Already, it has been observed that the result of treatment varied between different individuals. Analysis of complete and incomplete responders in humans has shown a correlation between the expression levels of IFNγ signalling components and efficacy of treatment [168]. Complete responders were observed to have increased levels of signal transducer

and activator of transcription 1 (STAT1) and interferon response factor 1 (IRF-1), while incomplete responders had higher levels of STAT3, IRF2 and protein inhibitor of activated STAT1 (PIAS-1) [168].

8. Unresolved Issues and Future Perspective

In conclusion, although much has been learned in the last few years on the mechanism of action of imidazoquinolines, many questions still remain to be answered. The exact mechanism how imidazoquinolines induce signal transduction from TLR7 or 8 should be studied. It would be especially interesting to learn if these compounds directly bind to these receptors, and also if there is cooperation between multiple TLRs for effective signal transduction to occur as is the case for TLR2 [169]. The recent discovery of ssRNA as a natural ligand for TLR7 and TLR8 warrants more study into the contribution of these receptors in viral defense. The potential of these compounds to treat more virulent mycobacterial infections such as *M. bovis* or *M. tuberculosis* and other parasitic infections could lead to potential new treatments that do not directly target the pathogen, but rather enhance the ability of the host to defend itself against the infectious agent. Finally, the differences and similarities between the various TLR signalling pathways could help in better understanding the role of each of these receptors in innate and acquired immunity. The imidazoquinoline family of compounds has shown their potential in the treatment of infectious diseases and has unexpectedly demonstrated the role of TLRs in modulating the immune status of the host.

Chapter 2

Clearance of Infection with *M bovis* BCG in Mice is Enhanced by the Treatment with S28463 (R-848) and its Efficiency Depends on the Expression of a Wild Type (Resistant Allele) of the *Nramp1* (*Slc11a1*) Gene

In this chapter, imidazoquinoline treatment is applied to a macrophage-restricted intracellular pathogen, namely *M. bovis* BCG. As resistance to *M. bovis* BCG in mice is under the control of the *Nramp1* gene (also known as *Slc11a1* or *Bcg/Ity/Lsh*), mice carrying the wild type (wt) allele as well as mice deficient in *Nramp1* have been employed in the study. Imidazoquinoline treatment led to a reduction in splenic bacterial load only in mice carrying a functional *Nramp1* allele after two weeks of treatment. Bone-marrow-derived macrophage cell lines generated from both strains of mice revealed increased nitric oxide production in *Nramp1* wt macrophages. Mice lacking *Nramp1* are not completely unresponsive to imidazoquinoline treatment as increasing the dose by a factor of 250 did induce a significant reduction in splenic bacterial load. The reduction in *M. bovis* BCG numbers observed in *Nramp1* KO was still lower than what was observed in wild type mice at a significantly lower dose of imidazoquinoline. High imidazoquinoline dosage led to splenomegaly which could allow *Nramp1* KO mice to bypass reduced macrophage-dependent responses to imidazoquinolines by recruitment of other immune cell types to the spleen.

Abstract

The mouse *Slc11a1* (formerly *Nramp1/bcg/ity/lsh*) host resistance gene is known to control the activation of host macrophages for killing of intracellular parasites like *Leishmania donovani* as well as intracellular bacteria including *M. bovis* BCG and *Salmonella enterica* serovar Typhimurium. It has been shown that imidazoquinoline compounds, including S28463, are able to improve the clearance of a number of intracellular pathogens such as HSV-2, HPV and *Leishmania*. The goal of this study was to determine whether S28463 is effective against infection with another intracellular pathogen, in this case *M. bovis BCG*, and to determine the molecular basis underlying this effect. To achieve this, B10.A(*Nramp1*^{r}) and B10.A(*Nramp1*$^{-/-}$) mice were infected with *M. bovis* BCG and treated with S28463. The bacterial content in the spleen from these mice was assayed by a colony-forming assay. In addition, *in vitro* experiments were performed using bone marrow derived macrophage cell lines from these mice. These cells were treated with S28463 and/or IFNγ and nitric oxide production was measured. Our study was able to show that S28463 acts in synergy with IFNγ to increase the production of NO *in vitro*. We were also able to demonstrate that mice carrying the resistant (wild type) allele of the *Nramp1* gene and that were infected with *M. bovis* BCG responded to treatment with S28463 resulting in a decreased bacterial load after two weeks of treatment. Mice that do not express the *Nramp1* gene responded only to a very large dose of S28463 and the response was not as efficient as that observed in mice carrying a wild type *Nramp1* allele. Our data provide evidence for the potential of S28463 as an immunomodulator that may be helpful in designing efficient strategies to improve host defence against mycobacterial infection.

Introduction

S28463 (R-848), an analog of imiquimod, is a member of the imidazoquinoline family that has been described as immune response modifiers. Numerous members of this family of compounds exhibit potent antiviral [48;49], tumoricidal [8;35;170] as well as adjuvant activities [61;63]. An imiquimod cream, ALDARA™, is currently being administered in humans for the treatment of external genital warts caused by the human papilloma virus (HPV) [171-173] .

The imidazoquinolines have no inherent antiviral activity but seem to stimulate the innate and acquired arm of the immune response [13]. They were first described as potent inducers of type I interferon in the sera of mouse [6], chicken [20] and humans [2] that had been orally treated with these compounds. The imidazoquinolines have also been shown to induce the secretion of a whole spectrum of cytokines such as IL-1, IL-6, IL-8 and TNF-α in a number of animal models such as the mouse, the guinea pig and the monkey [2;3;6;12;18]. Imidazoquinolines can also induce cytokine secretion as well as maturation of dendritic cells as shown by increased T cell proliferation in the presence of imiquimod treated dendritic cells [28;30]. B cell maturation and antibody production are also enhanced by these compounds [21]. Furthermore, Langerhans cell migration to the draining lymph node is increased in imiquimod treated mice [27].

Recently, imidazoquinolines have been shown to mediate their effects through members of the Toll-like receptor family (TLR7 and TLR8) [103;104]. A number of transcription factors and protein tyrosine kinases have been shown to be important for some but not

all of the effects of these compounds. Some of these crucial factors include STAT1 [130], NFκB and AP-1 [10] which are transcription factors controlling the expression of a number of immune response genes. Protein kinase C (PKC) [129], Jun kinase (JNK) and the mitogen activated protein kinase p38 [21] have all been shown to play a role in the response to the imidazoquinolines.

The macrophage has been characterized as one of the major proinflammatory cytokine source following imidazoquinoline treatment [14] and the cytokines produced in response to these compounds such as IL-12 are able to skew the immune response toward a T_H1 phenotype [9]. This type of response is crucial against intracellular pathogens such as viruses and intracellular parasites. In accordance with these findings, S28463 has been shown to effectively activate macrophage killing of *Leishmania* both *in vivo* and *in vitro* through inducing the synthesis of nitric oxide (NO) [10]. More recently, through a gene array approach, it was revealed that S28463 induced the expression of numerous genes which are associated with macrophage activation and the inflammatory response [11]. Based on these observations, we hypothesized that S28463 may also be effective against *M. bovis* BCG.

The susceptibility to infection with *Leishmania donovani*, *M. bovis* BCG and *Salmonella enterica* serovar Typhimurium was shown to be regulated by an autosomal dominant gene located on chromosome 1 in the mice [138-140;174]. The natural resistance associated macrophage protein 1 (NRAMP1) encodes a macrophage restricted transmembrane protein that shares homology with other eukaryotic transporter proteins

of the non ATP binding cassette type [141;142]. In mice, two alleles of the *Nramp1* gene have been described. The wild type allele (*Nramp1*^{r}) conferring resistance to infection has a glycine residue at position 169 while the other allele (*Nramp1*^{s}) has an aspartic acid residue at the same position leading to susceptibility to intracellular pathogens [144]. This aspartic acid substitution was found to be in absolute association with the *M. bovis* BCG susceptibility phenotype in all tested mice strains.

Mice lacking the *Nramp1* gene (*Nramp1*$^{-/-}$) exhibit numerous defects at the immunological level and have been previously reported to be as susceptible to *M. bovis* BCG infection as *Nramp1*^{s} mice [146]. Availability of *Nramp1*$^{-/-}$ mice has provided a genetically homogenous model to study the consequence of *Nramp1* gene deletion on susceptibility to infection with *M. bovis* BCG, *Salmonella enterica* serovar Typhimurium and *Leishmania donovani*. In fact, for all these pathogens, the kinetics of infection in *Nramp1*$^{-/-}$ mice was the same as that observed previously in *Nramp1*^{s} mice [146]. In *Nramp1*^{s} macrophages, induction of MHC class II expression by IFNγ is much less pronounced then what is observed in *Nramp1*^{r} macrophages [159;160;175]. *Nramp1*^{s} macrophages treated with IFNγ are less efficient in phosphorylating STAT1 [159] and consequently exhibit lower levels of a number of cytokines such as iNOS, IL-1β and TNF-α compared to *Nramp1*^{r} macrophages [161;162;164;176;177].

Studies of the yeast homologs of *Nramp1*, *smf1* and *smf2*, suggest that the NRAMP1 protein might act to transport divalent cations such as Fe^{2+}, Mn^{2+} or Zn^{2+} [178]. Since NRAMP1 is localised in the late endosomal/lysosomal vesicle [179], it could modulate

the intravesicular environment and control the proliferation of some intracellular pathogens.

Since imidazoquinolines (S28463 and imiquimod) were shown to be effective against *Leishmania spp.* [10] and because resistance to this pathogen is controlled at the *Nramp1* locus [180], we hypothesized that imidazoquinolines may affect the *Nramp1*-dependent macrophage activation during the course of infection with *M. bovis* BCG or other intracellular pathogens such as *Leishmania donovani.*

In this study, we demonstrate that the expression of the NRAMP1 protein is required for the optimal induction of nitric oxide production in macrophages treated with S28463. Our results also demonstrate that the *Nramp1* gene plays an important role in the regulation of the efficiency of imidazoquinoline-induced macrophage activation in mice as seen by the inability of *Nramp1*$^{-/-}$ mice to reduce *M. bovis* BCG proliferation in response to imidazoquinoline treatment.

Materials and Methods

Bacteria and Reagent. *Mycobacterium bovis* BCG substrain Montreal was cultivated using constant rotation at 37°C for 2 weeks in Middlebrook 7H9 broth supplemented with 10% Middlebrook OADC Enrichment (Becton Dickinson, Cockeysville, Maryland) and containing 0.05% Tween-80. After culture reached an OD_{600} of 0.6-1.0, cells were collected, briefly sonicated to disrupt bacterial clumps and filtered through 5 μm syringe filter to eliminate remaining clumps. After estimation of bacterial concentration the culture was aliquoted and frozen in 15% glycerol solution. S28463 is propriety of 3M Pharmaceuticals and was kindly provided by Dr. R. Miller (3M Pharmaceuticals). IFNγ was purchased from Invitrogen (Burlington, Ontario).

Mice. B10.A(*Nramp1*^{r}) mice expressing the wild type allele of the *Nramp1* gene have been previously described [181]. B10.A(*Nramp1*$^{-/-}$) mice generated from 129/J mice which had the *Nramp1* gene disrupted [146] and backcrossed for 16 generations to the B10.A(*Nramp1*^{r}) genetic background were bred according to animal care committee protocol in the Montreal General Hospital Research Institute Animal Facility under specific pathogen free (SPF) conditions.

Cells. Macrophage cell lines were derived from the bone marrow of B10.A(*Nramp1*^{r}) mice denoted B10A.*Nramp1*^{r} cell lines and of B10.A(*Nramp1*$^{-/-}$) mice denoted B10A.*Nramp1*$^{-/-}$ cell line. Cell lines were cultured in Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% of heat inactivated fetal bovine serum

(from Hyclone, Logan, Utah) and 1% penicillin/streptomycin antibiotic mixture (Invitrogen). Subconfluent cell cultures were used for all the experiments.

Quantification of nitrite production by macrophages. Two to four hours prior to stimulation, B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ cell line were plated at a concentration of 1 million cells/ml. The cells were subsequently treated with IFNγ (10 U/ml) and/or S28463 (25 ng/ml) for 24 hours. The estimation of NO_2^- in supernatants of stimulated and nonstimulated macrophages was performed by colorimetric spectrophotometry at 543 nm using the Griess reagent. Background values for the media were subtracted from those obtained for all experimental samples. Results are expressed as nitrite production in the supernatant (μM) per microgram of protein in the cell lysate. Protein concentrations were determined using the Bio-Rad protein assay.

Infection of mice and determination of spleen CFUs. B10.A(*Nramp1*^{r}), B10.A(*Nramp1*$^{-/-}$) and F1 B10.A(*Nramp1*^{r}) x B10.A(*Nramp1*$^{-/-}$) mice were infected intravenously with *M. bovis* BCG at the indicated dose in the results section. They were subsequently injected intraperitoneally every 2 days with varying doses of S28463 for 2 weeks (see results section). The mice were then sacrificed by CO_2 inhalation. The spleens were removed and homogenised in 4 ml of 0.25% saponin solution using a polytron. Subsequently, various dilutions of the homogenized samples were plated on Dubos solid agar. Plates were incubated at 37°C for two weeks and the number of CFUs were counted to assess bacterial burden.

Statistical Analysis. A Mann and Whitney non parametric test was performed using the SigmaStat software (SPSS, Chicago, Illinois) to calculate statistical significance. Potential differences among various treatments are considered significantly different from the others when the p values calculated are lower than 0.05.

Results

S28463 induces NO production alone in B10A.*Nramp1*^{r} and in B10A.*Nramp1*$^{-/-}$ macrophage cell line and acts synergistically with IFNγ. It has previously been shown that imidazoquinolines could induce the secretion of numerous cytokines by various cell types including macrophages [2;6;12;182] and it has been observed that *Nramp1*^{s} macrophages do not respond efficiently to IFNγ treatment resulting in low levels of NO upon stimulation [161]. To determine whether the presence of the *Nramp1* gene was essential for activation of macrophages by S28463 and also to study the possible interplay between imidazoquinolines and IFNγ, B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ cells were treated for 24 hours with S28463 (25 ng/ml) with or without IFNγ (10 U/ml)(Figure 1). S28463 alone or in combination with IFNγ induced a significant increase in NO production ($p<0.001$ in all cases) in B10A.*Nramp1*^{r} (0.5 μM/μg protein and 1.5 μM/μg protein, respectively) compared to B10A.*Nramp1*$^{-/-}$ (0.23 μM/μg protein and 0.84 μM/μg protein, respectively). *Nramp1*$^{-/-}$ macrophages produced significantly less NO in response to S28463 and IFNγ ($p<0.001$ in both cases). Synergy between S28463 and IFNγ could still be observed in B10A.*Nramp1*$^{-/-}$ albeit to a lower extent then that seen in B10A.*Nramp1*^{r} macrophages ($p<0.001$).

S28463 reduces *M. bovis* BCG load in B10.A(*Nramp1*^{r}) and in F1 B10.A(*Nramp1*^{r})xB10.A(*Nramp1*$^{-/-}$) mice. S28463 treatment was shown to be effective against various intracellular pathogens [4;5;10;52;172;182;183]. To determine whether it could be effective against *M. bovis* BCG infection, B10.A(*Nramp1*^{r}) and F1

B10.A(*Nramp1*^{r}) x B10.A(*Nramp1*$^{-/-}$) mice were infected intravenously with $5x10^5$ CFUs of *M. bovis* BCG and injected intraperitoneally every two days with S28463 (2 μg/mouse) for a period of 2 weeks before being sacrificed. The spleens were homogenized, plated, incubated at 37°C and the CFUs were determined 2 weeks later. Treatment with S28463 significantly reduced the amount of CFUs present in the spleen of B10.A(*Nramp1*^{r}) from 13,432 to 4,959 CFUs per spleen ($p<0.001$, n=13 for PBS injected and n=12 for S28463 treatment) (Figure 2A). Since the B10.A(*Nramp1*^{r}) mice are naturally resistant to *M. bovis* BCG infection, an infectious dose five times higher than that used with the B10.A(*Nramp1*$^{-/-}$) mice was administered, although similar effects were observed in these mice at an infectious dose of 10^5 CFUs of *M. bovis* BCG (data not shown). F1 B10.A(*Nramp1*^{r})xB10.A(*Nramp1*$^{-/-}$) mice also showed a significant reduction in the bacterial load from 10,400 to 5,600 CFUs ($p<0.001$, n=7 for both groups) (Figure 2B). These findings show that S28463 is effective against *M. bovis* BCG infection and that the presence of one functional allele of the *Nramp1* gene is sufficient for complete responsiveness to this compound.

S28463 fails to reduce bacterial load in B10.A(*Nramp1*$^{-/-}$) mice.

Nramp1^{s} and *Nramp1*$^{-/-}$ mice have previously been shown to display numerous differences in their immunological response when compared to wild type mice. Some of these differences include the production of some crucial cytokines such as IFNγ [184]. To determine the role of *Nramp1* in the responsiveness to imidazoquinolines *in vivo*, B10.A(*Nramp1*$^{-/-}$) mice were infected intravenously with 10^5 *M. bovis* BCG and injected

intraperitoneally every two days with different doses of S28463 (from 2 μg to 50 μg). After 2 weeks of treatment the animals were sacrificed, the spleens were collected, homogenized and plated. The CFUs were subsequently counted after 2 weeks. No significant reduction in bacterial load could be observed with the dose of S28463 shown to be effective in B10.A(*Nramp1*^{r}) mice (2 μg) or doses 4, 8 or 25 times higher ($p>0.05$ for all the doses tested) (figure 3). These results and those presented in figure 2 demonstrate that the *Nramp1* gene plays an important role in the regulation of the imidazoquinoline-induced protection against mycobacterial infection.

High dose of imidazoquinolines leads to a small reduction of the bacterial load in B10.A(*Nramp1*$^{-/-}$) mice infected with *M. bovis* BCG. Even though B10.A(*Nramp1*$^{-/-}$) mice failed to respond to a dose of S28463 effective in B10.A(*Nramp1*^{r}) mice (2 μg), it is still possible that dramatically higher doses of imidazoquinolines could rescue these mice from their lack of responsiveness. To test this hypothesis, B10.A(*Nramp1*$^{-/-}$) mice were infected with 10^5 *M. bovis* BCG and treated every two days with 500 μg of S28463, which is 250 times higher than the effective dose used in mice carrying a wild type allele of *Nramp1*. After two weeks, spleens were collected to measure bacterial load and the splenic ratio was calculated. Treatment with a high dose of S28463 led to a small, but significant decrease in the amount of bacteria found in the spleen from 232,500 to 175,250 CFUs ($p=0.034$, $n=11$ for PBS treatment and $n=12$ for S28463 treatment) (Figure 4, panel A), albeit to a much lower level than what is observed in B10.A(*Nramp1*^{r}). At this dose (500 μg), S28463 induced a twofold increase in the splenic ratio of both B10.A(*Nramp1*$^{-/-}$) ($p<0.001$) (Figure 4, panel B). Taken together,

these results demonstrate that at much higher doses, B10.A(*Nramp1*$^{-/-}$) mice are able to respond to S28463 although less efficiently than B10.A(*Nramp1*^{r}) mice.

Discussion

The study presented here demonstrates that S28463 alone can induce NO production in B10A.*Nramp1*^{r} macrophages and that a synergistic effect is observed when S28463 is combined with IFNγ to increase NO secretion. Although it had previously been demonstrated that S28463 alone could induce NO production in bone marrow derived macrophages (BMM) [10], the effect of S28463 in conjunction with IFNγ has never been reported.

The response to S28463 in *Nramp1*$^{-/-}$ macrophage cell line was also analyzed. It was observed that lower levels of NO were produced in response to IFNγ, which is in agreement with what has previously been observed in our laboratory [161]. Treatment with S28463 induced production of NO, but to a lesser extent than that observed in B10A.*Nramp1*^{r} macrophages. Treatment with IFNγ and S28463 induced a significant production of NO from *Nramp1*$^{-/-}$ macrophages indicating that S28463 may be bypassing the IFNγ signalling pathway and activating downstream effector molecules. These components may include the NFκB transcription factor as well as AP-1 and c-fos, all of which were shown to be activated by S28463 [10;21].

The effect of S28463 on *M. bovis* BCG infection was also tested in this study. We demonstrate that S28463 is able to reduce splenic *M. bovis* BCG loads in B10.A(*Nramp1*^{r}) and F1 B10.A(*Nramp1*^{r}) x B10.A(*Nramp1*$^{-/-}$) mice 14 days post-infection. This effect is probably due to the immunomodulatory activity of S28463. A possible hypothesis to explain our observations could be that by inducing

proinflammatory cytokines and favoring a T_H1 response, S28463 increases the amount of IFNγ present and from our results, this would lead to an increase in NO production by macrophages. Since NO was found to be directly toxic to *M. bovis* BCG, an increase in NO production would result in a more efficient destruction of the bacteria.

We showed that S28463 was able to reduce the bacterial load in B10.A(*Nramp1*^{r}) mice, but not in B10.A(*Nramp1*$^{-/-}$) mice at a dose of 2 μg per injection. A number of factors may explain this discrepancy. First, it was previously showed that *Nramp1*^{s} mice produce less IFNγ early in infection compared to *Nramp1*^{r} mice [184], which could have an effect on the NO production by the macrophages. Secondly, S28463 was shown to induce IL-12 production [9], which triggers CD4 T cells to produce IFNγ. Susceptible mice may have a defect in the production of IL-12 that would decrease the amount of IFNγ present. Differences in *M. bovis* BCG infection kinetics between resistant and susceptible mice could potentially account for varying imidazoquinoline treatment potency. Since B10.A(*Nramp1*$^{-/-}$) mice exhibit hundred fold more CFUs in their spleen than in B10.A(*Nramp1*^{r}), the bacterial burden, which is controlled by the *Nramp1* gene, differs between these two mouse strains and could interfere with the immunomodulatory function of S28463.

Of note is the observation that treatment of B10.A(*Nramp1*$^{-/-}$) mice with a dose 250 times higher than that used for the B10.A(*Nramp1*^{r}) mice leads only to a small, but significant, decrease in bacterial load at the end of infection with *M. bovis* BCG. This clearly demonstrates that the lack of the *Nramp1* gene in these mice leads to a defect that

cannot easily be overcome by using a higher dose of imidazoquinolines. Since *Nramp1* is expressed almost exclusively in macrophages [142] and since these cells are also important effectors in the response to imidazoquinolines [14], any defect in *Nramp1* gene expression or function will lead to a severe impairment in the *in vivo* response to these compounds. It was also observed after exposure to a high dose of S28463 that the splenic ratio increased two-fold in B10.A(*Nramp1*$^{-/-}$) mice. A previous analysis had found that splenomegaly following imidazoquinoline treatment was linked to an increase in dendritic, NK, granulocytes and macrophages [24]. Although this increase in inflammatory cells recruitment to the spleen would be expected to increase the clearance of *M. bovis* BCG, it did not have a major effect on the course of *M. bovis* BCG infection in B10.A(*Nramp1*$^{-/-}$) mice.

Studies have already shown that the responsiveness to imidazoquinolines varies from patients to patients and that the interferon response might be involved [168]. Our model allows to study these differences in an animal model as well as allowing us to search for the critical pathway used by the imidazoquinolines. The data presented in this study suggest an important role for the *Nramp1* gene in controlling the responsiveness to imidazoquinolines. This may be of crucial importance in determining the outcome of treatment for patients receiving imidazoquinolines as treatment for warts caused by HPV or for cancer treatment.

Figure 1: Effect of S28463 on NO production. B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages were plated on 24 well plates ($1x10^6$ cells/well) and let to adhere for 2-4 hours. The cells were then treated for 24 hours with S28463 (25 ng/ml) with or without IFNγ (10 U/ml). Each stimulus was done in quadruplicate. The amount of NO_2^- produced per amount of total protein was determined by utilizing the Griess reagent. Data are presented as the mean+SD of two independent experiments (n=8 for each groups). There was a significant difference in NO production between B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages following S28463 treatment alone ($p<0.001$) or following treatment with IFNγ and S28463 ($p<0.001$).

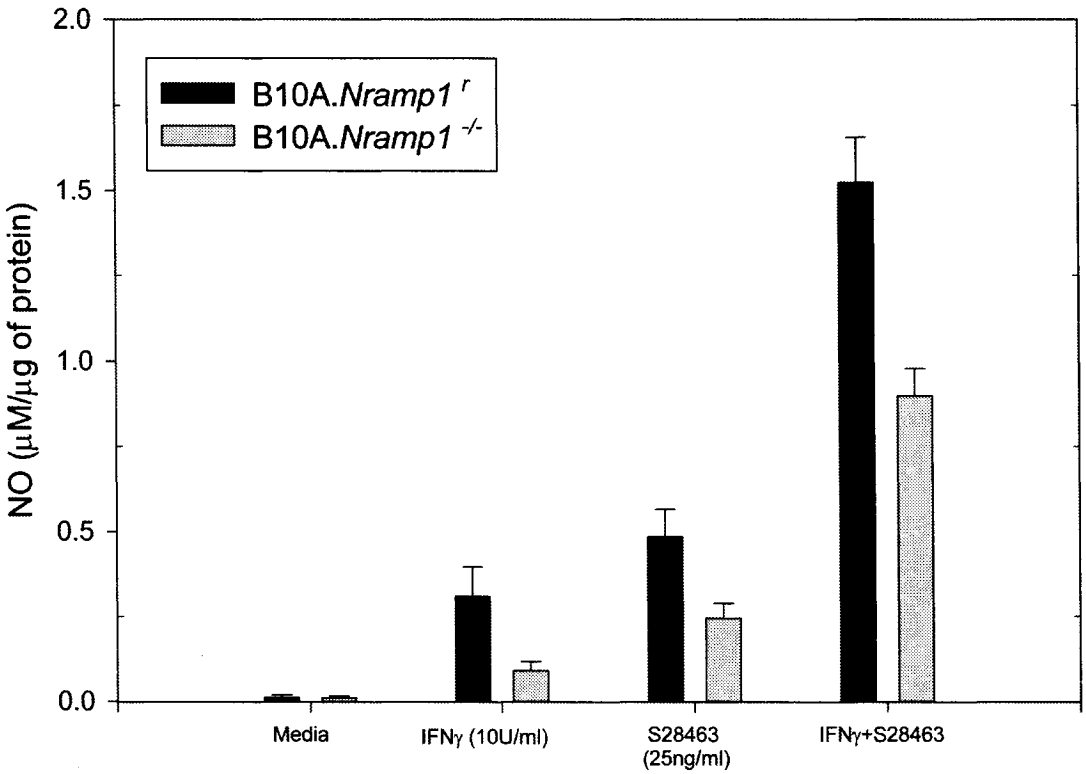
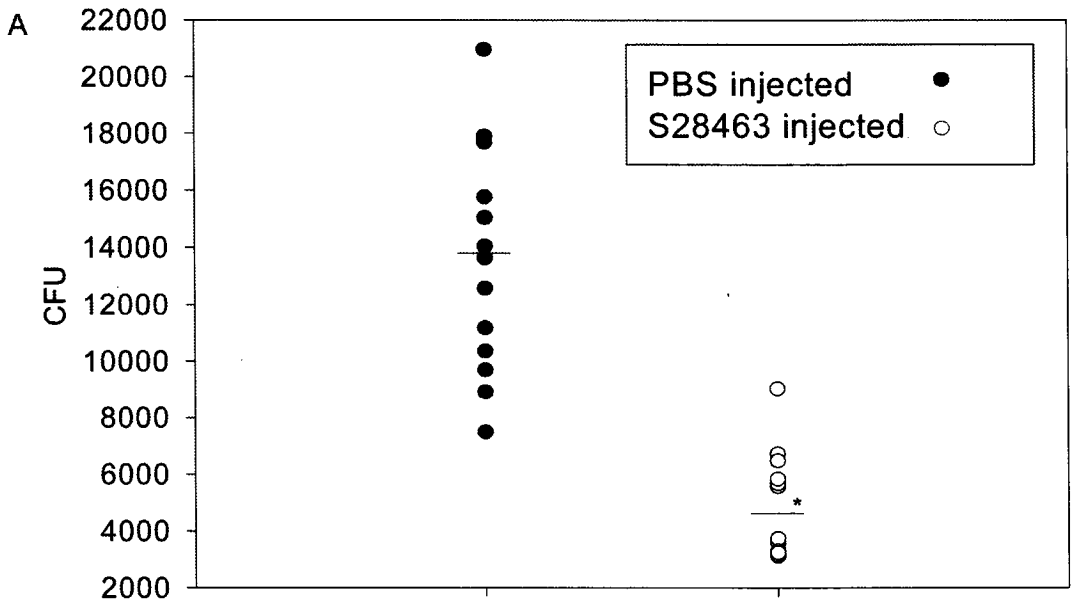

2.0
1.5
1.0
0.5
0.0
NO (μM/μg of protein)
B10A.Nramp1 r
B10A.Nramp1 -/-
Media
IFNγ (10U/ml)
S28463
(25ng/ml)
IFNγ+S28463

Figure 2: Splenic bacterial load of B10.A(*Nramp1*^{r}) and F1 B10.A(*Nramp1*^{r})xB10.A(*Nramp1*$^{-/-}$) mice treated with S28463. B10.A(*Nramp1*^{r}) (Panel A) or F1 B10.A(*Nramp1*^{r})xB10.A(*Nramp1*$^{-/-}$) mice (Panel B) were infected intravenously with $5x10^5$ *M. bovis* BCG and injected intraperitoneally every 2 days with S28463 (2 µg/mouse). Once the mice were sacrificed, the spleen was removed, homogenized, plated at various dilutions and incubated for 2 weeks. The colonies were then counted which enabled the determination of the total amount of bacteria. Data are presented as the median from three and two independent experiments for panel A (n=13 for PBS and n=12 for S28463 group) and panel B (n=7 for both groups) respectively. Differences in CFUs between PBS treated and S28463 treated mice were significant ($p<0.001$) in panel A and panel B.

A


22000
20000
18000
16000
14000
12000
10000
8000
6000
4000
2000
CFU
PBS injected
S28463 injected
*

B

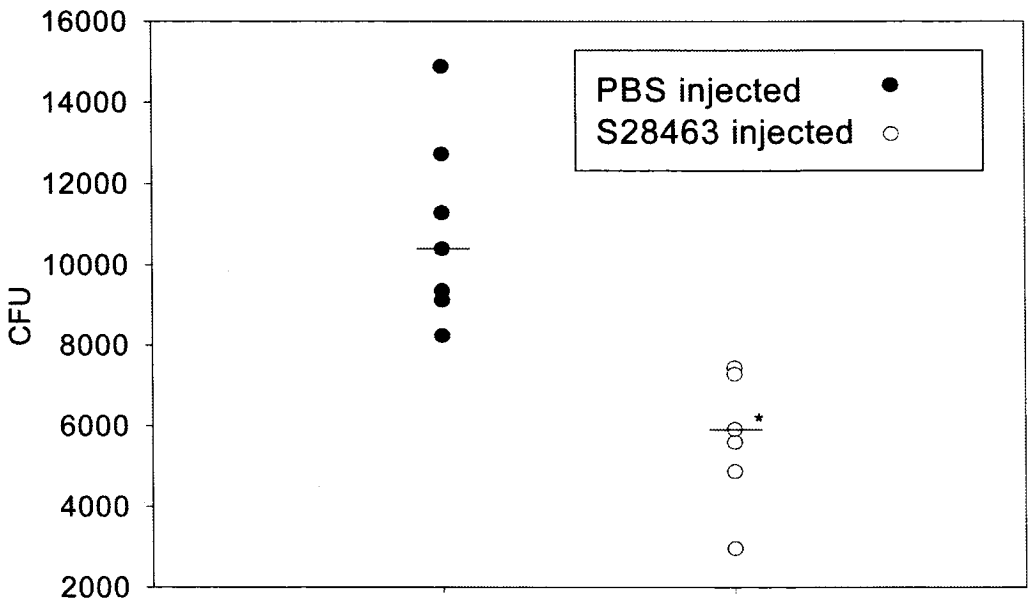

16000
14000
12000
10000
8000
6000
4000
2000
CFU
PBS injected
S28463 injected
*

Figure 3: Bacterial load of B10.A(*Nramp1*$^{-/-}$) mice treated with various doses of S28463. B10.A(*Nramp1*$^{-/-}$) mice were infected intravenously with $1x10^5$ *M. bovis* BCG and injected intraperitoneally every 2 days with 2μg (panel A), 8 μg (panel B), 16 μg (panel C) or 50 μg (panel D) of S28463. The mice were sacrificed and the spleen removed. The spleens were homogenized and various dilutions were plated and left to grow for 2 weeks. The colonies were then counted and the total amount of bacteria determined. Results from four different experiments are shown. No significant decrease in CFU could be detected for any of the doses tested ($p>0.05$ in all cases).

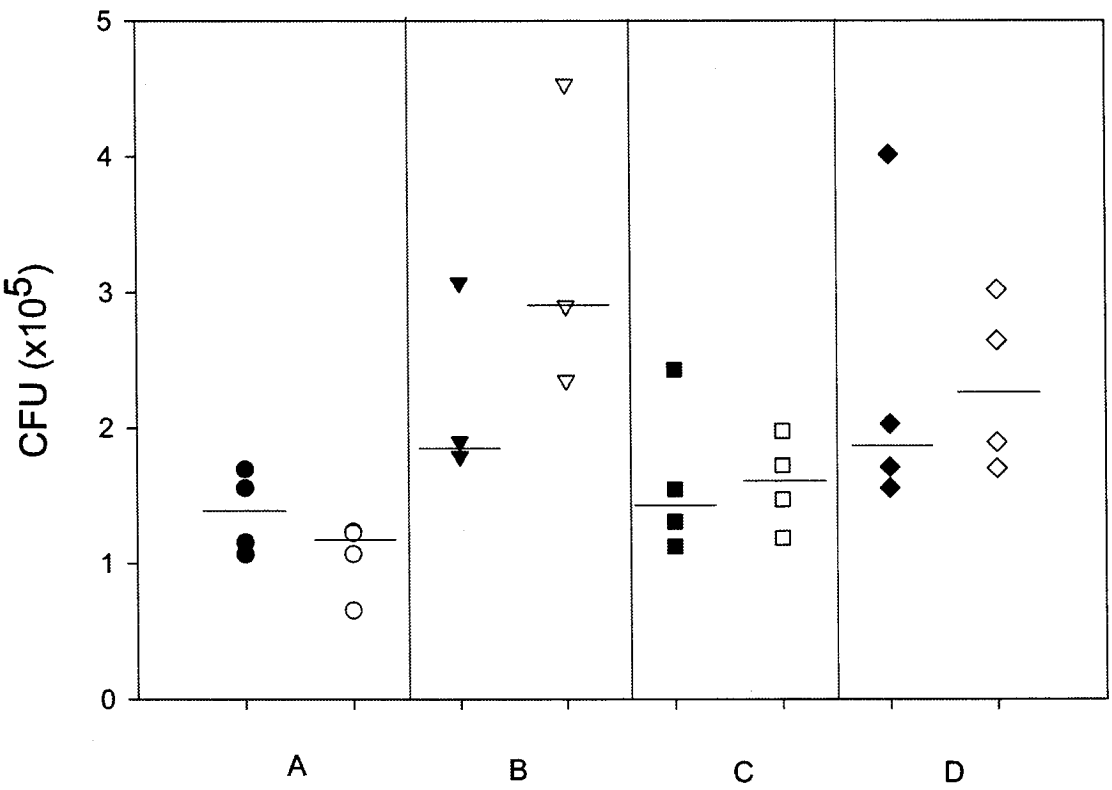
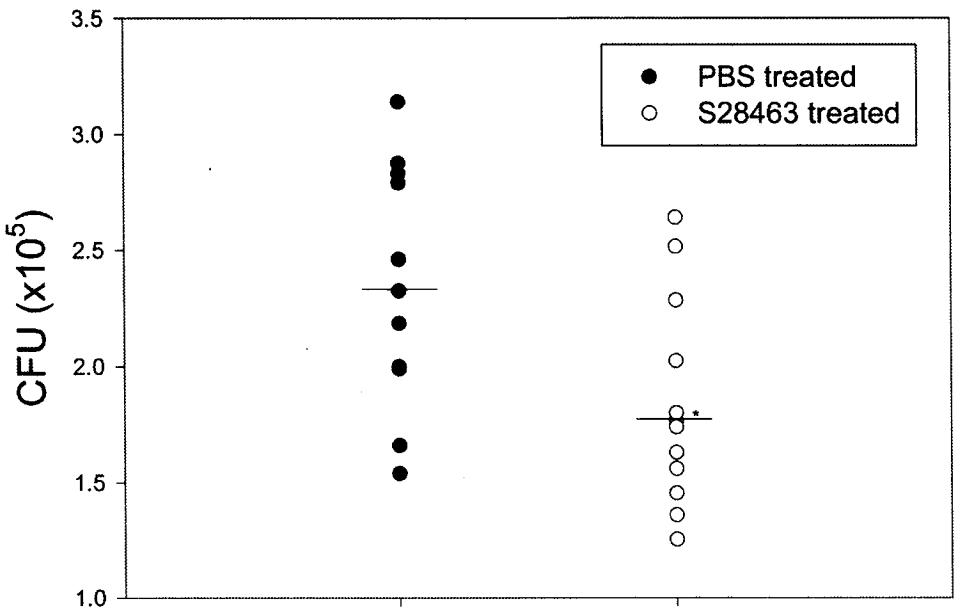

5
4
3
2
1
0
CFU (x10^5)
A
B
C
D

Figure 4: Effect of high doses of S28463 on bacterial load in B10.A(*Nramp1*$^{-/-}$) mice.

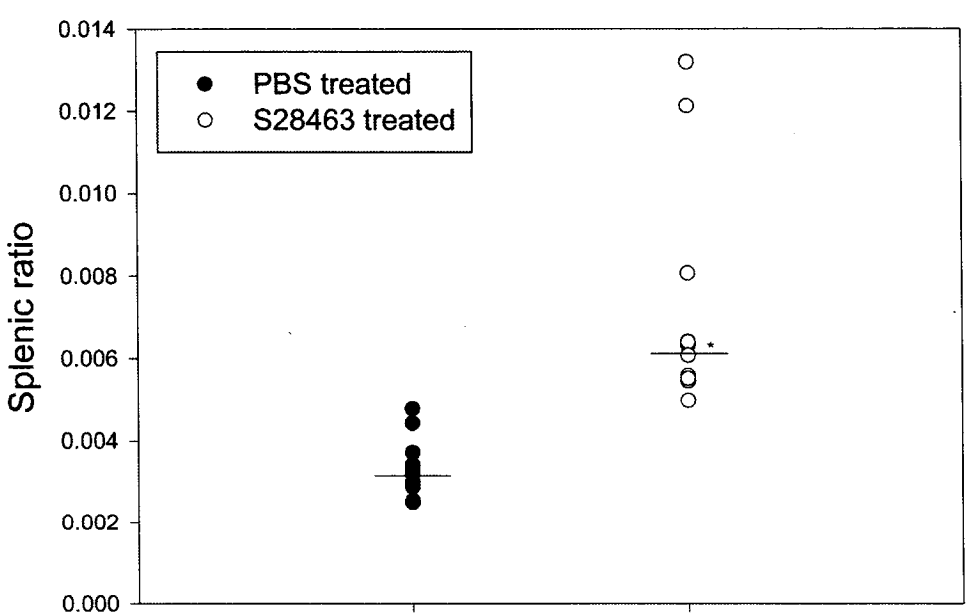
Panel A. B10.A(*Nramp1*$^{-/-}$) mice were infected intravenously with $1x10^5$ *M. bovis* BCG and injected intraperitoneally every 2 days with S28463 (500µg/mouse). Once the mice were sacrificed, the spleen was removed, homogenized, plated at various dilutions and incubated for 2 weeks. The colonies were then counted which enabled the determination of the total amount of bacteria. Data are presented as the median from three independent experiments (n=11 for PBS and n=12 for S28463 group). S28463 treatment led to a significant reduction in CFUs ($p=0.034$).

Panel B. Splenic ratios were calculated from the PBS and S28463 treated mice by dividing the weight of the spleen by the total body weight of the mouse. Data are presented as the median from three independent experiments. Increasing the dose to 500 µg of S28463 led to a significant increase in the splenic ratio of B10.A(*Nramp1*$^{-/-}$) ($p<0.001$).

A


3.5
3.0
2.5
2.0
1.5
1.0
CFU (x10^5)
PBS treated
S28463 treated

B


0.014
0.012
0.010
0.008
0.006
0.004
0.002
0.000
Splenic ratio
PBS treated
S28463 treated

Chapter 3

Role of SLC11A1 (Formerly NRAMP1) in Regulation of Signal Transduction Induced by Toll-Like Receptor 7 Ligands

The data presented in the previous chapter pointed to a role for NRAMP1 in modulating the response of macrophages to imidazoquinoline stimulation. The discovery that imidazoquinolines transduce signals through the TLR7/8 receptor suggests that NRAMP1 modulates responses to TLR7 ligands. The response of macrophages to the recently discovered TLR7/8 ligands, viral ssRNA, was analyzed in *Nramp1* wt and KO macrophage cell line. We demonstrate that just like with imidazoquinolines, *Nramp1* modulates also macrophage responses to ssRNA. Using fluorescently labeled ssRNA, we were able to address intracellular uptake and localization in *Nramp1* wt and KO cells. We demonstrate that Nramp1 does not affect TLR7 ligand uptake and intracellular localization. On the other hand, P38 MAPK, but not ERK1/2, activation was significantly reduced in Nramp1 KO macrophages. Furthermore, levels of activated atypical PKC in resting macrophages were found to be significantly higher in cells carrying a functional *Nramp1* allele. Further experiments were performed to confirm the biological significance of these two pathways in TLR7 responses. Taken together, we characterize the role of NRAMP1 in modulating P38 MAPK and atypical PKC activity which leads to reduced cytokine production specifically to TLR7 ligands. The data suggests a mechanism for the failure of imidazoquinoline treatment against *M. bovis* BCG infection in *Nramp1* KO mice presented in the previous chapter.

Abstract

Modulation of immune responses using Toll-like receptor ligands is fast becoming one of the main new approaches for treatment of infectious and allergic diseases. Characterizing the role of genetic factors in modulating responses to these ligands will be crucial in determining the efficacy of a particular treatment. Our previous findings have shown that treatment of *M. bovis* BCG infection with a synthetic TLR7 ligand resulted in a reduction of the splenic bacterial load only in mice carrying a wild type allele of *Nramp1*. To further understand how NRAMP1 modulates responses to TLR7 ligands, we have analysed various important TLR7 signal transduction events in macrophage cell lines derived from B10.A(*Nramp1*^{r}) and B10.A(*Nramp1*$^{-/-}$) mice. The *Nramp1* genotype did not affect TLR7 receptor expression, ligand uptake or intracellular processing. Following TLR7 ligand stimulation, P38 MAPK activation was significantly reduced in B10A.*Nramp1*$^{-/-}$ macrophages compared to B10A.*Nramp1*^{r} cells. The biological significance in TLR7 response was confirmed by inhibiting P38 MAPK in B10A.*Nramp1*$^{-/-}$ macrophages as well as studying response of MAPKAP2 (MK2) knockout macrophages to imidazoquinolines. Interestingly, levels of PKCζ activation were also found to be lower in B10A.*Nramp1*$^{-/-}$ macrophages and inhibition of this kinase in B10A.*Nramp1*^{r} cells led to a reduction in nitric oxide production. Taken together, the data demonstrate a role for NRAMP1 in modulating P38 MAPK and PKCζ activity, which leads to reduced cytokine induction by TLR7 ligands and can account for the lack of treatment efficacy by TLR7 ligand against *M. bovis* BCG infection in B10.A(*Nramp1*$^{-/-}$) mice.

Introduction

The imidazoquinoline family of compounds has been shown to exhibit immunomodulatory effects both *in vivo* and *in vitro*. Numerous derivatives exist which vary in structure and potency. S27609 has a chemical structure comparable to other imidazoquinolines and induces a similar cytokine pattern being ten times more potent than imiquimod and ten times less potent than R-848 (S28463) [7]. The imidazoquinolines are potent inducers of a whole spectrum of cytokines such as interleukin (IL)-1, IL-6, IL-8 and tumor necrosis factor (TNF) in a number of animal species such as the mouse, the guinea pig and the monkey [1]. Administration of these compounds tends to skew the immune response towards a T_H1 phenotype [9]. Imidazoquinolines have been shown to have positive effects in numerous infectious disease models including herpes simplex virus (HSV), *Leishmania spp.* as well as *Mycobacterium bovis* BCG [10;23;48;59]. An imiquimod cream, ALDARA, is currently being administered to humans for the treatment of external genital warts caused by the human papilloma virus (HPV) [171-173]. Interestingly, Toll-like receptor (TLR) 7 knockout (KO) mice were shown to be unresponsive to imidazoquinolines and failed to induce cytokine production in the presence of these compounds [103].

The Toll-like receptor family has been shown to play a crucial role in both the innate and adaptive immune responses [185;186]. These receptors are considered to be sensors of the extracellular environment, which trigger an immune response to conserved motifs on the surface of invading pathogens, thus their classification as pattern recognition receptors (PRR). To date, thirteen members have been characterised, although certain members are not functional in some species [187]. Most of the ligands defined for these

receptors are pathogen derived products. TLR4 is important for the response to LPS from gram-negative bacteria [76]. TLR2 transduces signals for lipoprotein and cell wall products from gram positive bacteria as well as lipoarabinomannan (LAM) from mycobacteria [188;189]. TLR9 has been found to be responsible for the response to unmethylated CpG oligodeoxynucleotides in both mice and humans [100;190], while TLR3 was described as playing a major role in double-stranded RNA recognition [191]. More recently, TLR7 and 8 have been implicated in recognizing single-stranded RNA of viral origin [106;107].

The susceptibility of mice to infection with *Leishmania donovani*, *M. bovis* BCG and *Salmonella enterica* serovar Typhimurium is under the control of an autosomal dominant gene located on chromosome 1 [138-140]. The natural resistance associated macrophage protein 1 (NRAMP1) encodes a macrophage restricted transmembrane protein that shares homology with other eukaryotic transporter proteins of the non ATP binding cassette type [141;142]. In mice, two alleles of the *Nramp1* gene have been described. The wild type allele (*Nramp1*^{r}) confers resistance to infection and has a glycine residue at position 169 while the susceptibility conferring allele (*Nramp1*^{s}) has an aspartic acid residue at this position [144]. Mice lacking the *Nramp1* gene (*Nramp1*$^{-/-}$) displayed the exact same pattern of susceptibility to intracellular pathogen as *Nramp1*^{s} mice [146]. The availability of *Nramp1*$^{-/-}$ mice has provided a genetically homogenous model for the study of the consequences of *Nramp1* gene deletion on susceptibility to infection with *M. bovis* BCG, *Salmonella enterica* serovar Typhimurium and *Leishmania donovani*. Studies have shown that NRAMP1 acts as a divalent cation transporter for Fe^{2+} and Mn^{2+} [192]. Although the exact mechanism through which

NRAMP1 regulates innate resistance to certain intracellular pathogens has still not been completely elucidated, it has been proposed that, through its transporter activity and because of its late phagosomal localisation [179], it could modulate the intravesicular environment and control the proliferation of some intracellular pathogens.

Our previous findings showed that imidazoquinoline treatment of *M. bovis* BCG infection results in a reduction of the splenic bacterial load only in mice carrying a wild type allele of *Nramp1*. *Nramp1*$^{-/-}$ mice were refractory to treatment with imidazoquinolines and only responded when the dose was increased 250 fold [23]. In the present study, the mechanism through which NRAMP1 modulates TLR7 signal transduction was investigated at various levels. Both the uptake and intracellular localization of the TLR7 ligand were unaffected by the *Nramp1* genotype. TLR7 receptor expression was identical in both B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. The activation of P38 mitogen activated protein kinase (MAPK), but not ERK1/2, was significantly reduced following treatment with a TLR7 ligand in B10A.*Nramp1*$^{-/-}$ macrophages. The importance of P38 MAPK pathway activation in TLR7-induced cytokine production was confirmed by inhibitor studies as well as studying the responses of macrophages from MK2 KO mice. Furthermore, levels of activated PKCζ were reduced in B10A.*Nramp1*$^{-/-}$ cells. Inhibition of protein kinase C zeta (PKCζ) activation in B10A.*Nramp1*^{r} macrophages confirmed the importance of this pathway in TLR7 mediated nitric oxide induction. Overall, the data point toward a role for NRAMP1 in modulating PKCζ activity as well as P38 MAPK activation following TLR7 ligand stimulation which leads to reduced cytokine production by B10A.*Nramp1*$^{-}$

$^{-/-}$ cells. The present study provides a mechanism for the observed lack of efficacy of imidazoquinoline treatment against *M. bovis* BCG infection in B10.A(*Nramp1*$^{-/-}$) mice.

Materials and Methods

Reagents. S27609 and S28463 were graciously provided by Dr. R. Miller and are the property of 3M Pharmaceutical. Phosphorothioate stabilised single-stranded RNA (5'-GCCCGUCUGUUGUGUGACUC-3') as well as a Cy-3-labelled version were synthesised by Curevac (Tübingen, Germany). PhosphoPlus p44/p42 MAPK (Thr202/Tyr204), PhosphoPlus p38 MAPK (Thr180/Tyr182) and Phospho-PKC zeta antibody kits were purchased from Cell Signaling Technology (Beverly, MA). Total PKC zeta antibody was acquired from Santa Cruz (Santa Cruz, CA). FITC-LAMP1, Rab5 and FITC anti-mouse IgG were purchased from BD Biosciences (Missisauga, Ontario, Canada). The PKC inhibitor bisindolylmaleimide I, P38 MAPK inhibitor III and the PKA inhibitor PKI 14-22 were purchased from Calbiochem (Hornby, Ontario, Canada). The myristoylated PKC zeta pseudosubstrate peptide (Myr-Ser-Ile-Tyr-Arg-Arg-Gly-Ala-Arg-Arg-Trp-Arg-Lys-Leu-OH) was obtained from Medicorp (Montreal, Quebec, Canada).

Cells. Macrophage cell lines were derived from the bone marrow of B10.A(*Nramp1*^{r}) mice (refered to as B10A.*Nramp1*^{r}) and B10.A(Nramp1$^{-/-}$) mice (refered to as B10A.*Nramp1*$^{-/-}$). The latter were generated from 129/J mice which had the *Nramp1* gene disrupted [146] and backcrossed for 16 generations to the B10.A(*Nramp1*^{r}) genetic background. Bone marrow-derived macrophage cell lines from MAPKAP2 (MK2) knockout mice as well as littermate controls were also generated in a similar manner. Cell lines were cultured in Dulbecco's Modified Eagle Medium (Invitrogen, Burlington,

Ontario, Canada) supplemented with 10% heat inactivated fetal bovine serum (from Hyclone, Logan, Utah) and 1% penicillin/streptomycin antibiotic mixture (Invitrogen). Subconfluent cell cultures were used for all the experiments.

Single-Stranded RNA Stimulation. Twenty four hours prior to stimulation, B10A.*Nramp1*^{r} and B10A.Nramp1$^{-/-}$ cells were plated at a concentration of 0.2 million cells/ml in 96 well plates (VWR, Montreal, Quebec, Canada). The cells were stimulated with a mixture of single-stranded RNA and DOTAP transfection reagent (Roche, Laval, Quebec, Canada) as previously described [106]. Briefly, a diluted ssRNA solution (40 μg/ml) was mixed with equal volume of DOTAP solution (100 μg/ml) and incubated at room temperature for 15 minutes. The mixture was further diluted with an equal volume of complete media. The cells were subsequently treated with IFNγ (10 U/ml) (R&D, Minneapolis, MN) and/or the RNA-DOTAP mixture for 24 hours. Supernatants were collected and quantitation of nitric oxide and TNF was performed as previously described [23].

RNA Analysis. Total RNA from untreated macrophages was purified using Trizol reagent (Invitrogen). Following DNase digestion of 1 μg of total RNA (DNA-free, Ambion, Austin, TX), reverse transcription was performed using random primers (Stratascript First Strand cDNA synthesis kit, Stratagene, LaJola, CA). TLR4 and TLR7 mRNA levels were determined by real-time PCR analysis performed using the Brilliant SYBR Green QPCR kit according to the manufacturer's protocol (Stratagene) on a Stratagene MX-4000 apparatus. The levels of TLR mRNA were adjusted for differences

in GAPDH expression and normalised to levels of TLRs mRNA in untreated B10A.*Nramp1*^{r} macrophages. Primer Sequences:

TLR4-F (5'-AACTTCAGTGGCTGGATTTA-3'),

TLR4-R (5'-GTTTGAGAGGTGGTGTAAGC-3'),

TLR7-F (5'-TCTAGAGTCTTTGGGTTTCG-3'),

TLR7-R (5'-TTCTGTCAAATGCTTGTCTG-3').

TLR7 Ligand Delivery. Twenty four hours prior to stimulation, B10A.*Nramp1*^{r} and B10A.Nramp1$^{-/-}$ cells were plated at a concentration of 0.75 million cells/ml in 48 well plates (VWR). The cells were subsequently stimulated for 2.5 hours with a Cy-3-ssRNA-DOTAP mixture prepared as described above. Following treatment, the media was removed, the cells were washed, resuspended in PBS and incubated on ice for 10 minutes. The cells were then detached by gentle pipetting, spun down and resuspended in FACS buffer (PBS containing 0.1% BSA and 0.1% NaN_3) supplemented with 50 mg/ml dextran sulfate to remove surface bound oligonucleotides. After a 15 minute incubation period on ice, the cells were spun down and washed twice in FACS buffer. Analysis of intracellular fluorescence levels was performed using flow cytometry on a BD FACScan (BD Biosciences) and the relative intensity was calculated by multiplying the percentage of positive cells by the mean fluorescent intensity.

Immunofluorescent Microscopy. Twenty four hours prior to stimulation, B10A.*Nramp1*^{r} and B10A.Nramp1$^{-/-}$ cells were plated at a concentration of 0.1 million cells/ml in 12 well plates (VWR) containing glass coverslip (Fisher, Nepean, Ontario, Canada). The cells were stimulated with ssRNA (10μg/ml of which 1:100 is Cy-3

labelled)-DOTAP solution for 45 minutes. Cell monolayers were then washed three times with cold PBS, fixed with 2% paraformaldehyde (5 min, 4°C), followed by acetone (5 min, -20°C). Fixed cells were incubated with PBS/3% BSA containing Fc block antibody (BD Biosciences) for 20 min. LAMP1 localisation was directly detected by a fluoresceinated antibody. Mouse anti-Rab5 IgG was used as an early endosomal marker. IgG binding was revealed with a fluoresceinated goat anti-mouse IgG (BD Biosciences). Visualisation was performed on Nikon eclipse E600 fluorescent microscope using MetaMorph 4.6r9 software (Universal Imaging Corporation, Downingtown, PA).

Atypical PKC and P38 MAPK Inhibition. Two hours prior to stimulation, B10A.*Nramp1*^{r} and B10A.Nramp1$^{-/-}$ cells were plated at a concentration of 1 million cells/ml in 24 well plates (VWR). The cells were then stimulated with S28463, a synthethic TLR7 ligand, in the presence of either PKC or PKA inhibitors. The levels of nitric oxide present after 18 hours were quantitated using the Greiss method. For P38 MAPK inhibition studies, the inhibitor was added one hour prior to stimulation with the various TLR ligands for 18 hours. Supernatants were collected and quantitation of nitric oxide and TNF was performed as previously described [23].

Western Blotting. $2x10^6$ macrophages were plated in complete media on 6-well plates (VWR). After 2 hours, the cells were washed and fresh media added. Following stimulation, the media was removed; the cells were washed with PBS and lysed in SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol. The cells were scraped off the

plate and the samples were boiled for 5 minutes. The lysates were subsequently sheered using a 26G needle. Samples were loaded onto 4-12% Bis-Tris NuPAGE gels (Invitrogen). Following electrophoresis, semi-dry transfer onto Immobilon-P membranes (Fisher) was performed. The membrane was subsequently blocked in 5% milk and probed with antibodies according to the manufacturer's protocol. Blots were subsequently scanned and densitometric analysis (ImageQUANT, GE Healthcare, Piscataway, NJ) was performed to quantitate the level of MAPK activation.

Statistical Analysis. A Mann-Whitney non parametric test was performed using SigmaStat software (SPSS, Chicago, Illinois) to calculate statistical significance. Differences among treatments are considered significant if $p \leq 0.05$.

Results

Cytokine Induction by ssRNA in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages.

Our previous observations showed that macrophages from *Nramp1*^{r} mice produced more nitric oxide (NO) following imidazoquinoline stimulation than mice in which the *Nramp1* gene had been deleted [23]. This deletion also led to the failure of imidazoquinoline treatment for *M. bovis* BCG infection in *Nramp1*$^{-/-}$ mice. Recently, single-stranded RNA of viral origin has been shown to induce cytokine production through the TLR7/8 pathway. Macrophage responsiveness to this new TLR7/8 ligand was analysed using bone marrow derived-macrophage cell lines from B10.A(*Nramp1*^{r}) and B10.A(*Nramp1*$^{-/-}$) mice. Following 24 hours of stimulation with ssRNA packaged in DOTAP, B10A.*Nramp1*^{r} macrophages responded dramatically better to this TLR7 ligand than their B10A.*Nramp1*$^{-/-}$ counterparts, as measured by nitric oxide (NO) secretion (Figure 1 panel A, 9.4±0.9 μM and 0.96±0.23 μM respectively, $p<0.05$) as well as TNF production (Figure 1 panel B, 31.6±4.7 ng/ml and 4.8±0.9 ng/ml respectively, $p<0.05$). Interestingly, co-stimulation with IFNγ rescued the NO response in B10A.*Nramp1*$^{-/-}$ macrophages, but reduced TNF secretion was not rescued. Single-stranded RNA having a phosphodiester backbone and 2-O-methyl modifications had no immunomodulatory activity (data not shown). Transfection of the ssRNA with the DOTAP reagent was also required for cytokine induction as the ssRNA alone did not induce any detectable levels of these cytokines (data not shown). These data demonstrate that *Nramp1* gene expression is critically required for response to both synthetic TLR7 ligands (S28463) and ssRNA that is considered to be a natural ligand for TLR7.

TLR4 and TLR7 mRNA expression in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. To confirm that the difference in cytokine production following imidazoquinoline or ssRNA treatment was not due to a difference in TLR7 expression, the levels of TLR4, as a reference, and TLR7 were analysed in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages by quantitative PCR. The efficiency of the primer sets for TLR4 and 7 were found to be between 85 to 95% (data not shown). TLR expression values were adjusted to the levels of GAPDH and normalised to the levels observed in B10A.*Nramp1*^{r} cells. Both TLR4 and TLR7 mRNA were found to be expressed at similar levels in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages (Figure 2, p=0.690 for TLR4 and p=0.151 for TLR7 receptor expression). Overall, the observed difference in cytokine production following TLR7 ligand treatment in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages cannot be attributed to difference in levels of TLR7 mRNA expression.

Delivery of TLR7 ligand in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. It has become evident over the last few years that the TLR7 receptor is found within the cell and that ligand uptake and further endosomal processing is required for signal transduction (Annexe 1 and [105;193]). Since our results demonstrated the importance of NRAMP1 for response to TLR7 ligands and because NRAMP1 was shown to be localised in late phagosomal compartments [179], we have assessed its role in TLR7 ligand uptake and further intracellular processing by both flow cytometry and immunofluorescent microscopy. B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages were

stimulated with a Cy-3 labelled ssRNA molecule packaged in DOTAP for 2.5 hours and fluorescence was measured by flow cytometry to assess the amount of ssRNA present in the cells. Similar levels of fluorescence was detected in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ cells demonstrating that a similar amount of ssRNA was delivered (Figure 3, 350.7±40.4 and 461±15.8 respectively, $p>0.05$). Packaging of the ssRNA with DOTAP resulted in a 7- to 10-fold increase in fluorescence compared to the ssRNA alone (Figure 3). These data clearly show that the delivery of ssRNA which activates the TLR7 receptor is not affected by the *Nramp1* genotype.

Intracellular localization of ssRNA in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. Previous studies have shown that upon TLR7 ligand stimulation, the adaptor protein MYD88 is recruited to LAMP1 positive endosomal structures [105]. NRAMP1 has also been shown to co-localise with this late endosomal marker [179]. The effect of NRAMP1 on TLR7 ligand localisation was thus studied in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophage cell lines. Cy-3 labelled ssRNA displayed a punctuated pattern of staining within the cells (Figure 4 panel A and B, first column). Staining of the TLR7 ligand displayed extensive colocalisation with the late phagosomal marker LAMP1 in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages, with no apparent difference in fluorescence staining pattern (Figure 4 panel A, merged image). No colocalisation between the early endosomal marker Rab5 and the TLR7 ligand was observed in either B10A.*Nramp1*^{r} or B10A.*Nramp1*$^{-/-}$ macrophage cells (Figure 4 panel B, merge image). Taken together, these observations demonstrate that NRAMP1 does not affect the intracellular localisation of TLR7 ligands in murine macrophages.

MAPK Activation Following TLR7 Treatment in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. Activation of the MAPK pathway following TLR ligand treatment is a shared event among all members of this family of receptors and has been found to play a crucial role in cytokine induction [194-196]. The levels of activated P38 and ERK1/2 MAPK after TLR7 ligand treatment was analysed using phospho-specific antibodies recognising activation-associated phosphorylated forms of P38 or ERK1/2 MAPK (Figure 5 panel A and B, respectively). Treatment with a synthetic TLR7 ligand (S28463) led to the activation of P38 MAPK in B10A.*Nramp1*^{r} macrophages from 30 minutes and remaining through to 60 minutes post-treatment (Figure 5 panel A). Levels of activated P38 MAPK in B10A.*Nramp1*$^{-/-}$ macrophages remained significantly lower at both 30 and 60 minutes following TLR7 ligand treatment, as compared to B10A.*Nramp1*^{r} cells. The activation of ERK1/2 MAPK by the TLR7 ligand was not affected by the *Nramp1* genotype and revealed similar levels and kinetics in both B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages (Figure 5 panel B). Reduced P38 MAPK activation in Nramp1$^{-/-}$ macrophages appeared to be specific for TLR7 signal transduction as activation of P38 MAPK by LPS occurred at similar levels between B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. Thus, NRAMP1 is required in macrophages for efficient P38 MAPK activation in response to TLR7 ligand treatment.

Role of the P38 MAPK Axis in TLR-Mediated Cytokine Induction. To confirm the biological importance of reduced P38 MAPK activation in B10A.*Nramp1*$^{-/-}$

macrophages following TLR7 activation, chemical inhibition studies were undertaken in B10A.*Nramp1*^{r} cells. Cells were pre-treated with the P38 MAPK inhibitor III for one hour prior to stimulation with TLR4, TLR7 and TLR9 ligands for 18 hours. Blockade of P38 MAPK activation led to an almost complete inhibition in both NO and TNF secretion by B10A.*Nramp1*^{r} cells (Figure 6, Panel A and B). All tested TLR were susceptible to P38 MAPK inhibition suggesting a conserved mechanism of cytokine induction. One of the downstream targets of P38 MAPK is MAPKAP2 (MK2) [197]. Further confirmation of the importance of the P38 MAPK axis in TLR-induced cytokine production was obtained by stimulating peritoneal macrophages from MK2 KO mice with TLR4, TLR7 and TLR9 ligands. TNF induction by all three TLR ligands was significantly reduced in MK2 KO macrophages compared to their littermate controls (Figure 7). Taken together, the data demonstrate the importance of P38 MAPK as well as MK2 activation in TLR7-induced cytokine production.

Atypical PKC Activity in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages. The PKC family of kinases is comprised of twelve isoforms which are subdivided depending on their co-factor requirements [198]. The conventional PKCs (α, βI, βII, γ) are DAG/phorbol ester and Ca^{2+}-dependent while the novel PKCs (δ, ε, θ, η, μ) are only dependent on the presence of DAG/phorbol ester for enzymatic activity. Finally, the activity of the atypical PKCs (ζ, λ) is independent of DAG/phorbol ester and Ca^{2+}. Previous work from our laboratory has demonstrated that macrophages from *Nramp1*^{s} mice display reduced PKC activity compared to their *Nramp1*^{r} counterparts, although no difference at the protein level was found [199]. Atypical PKC activity has previously

been shown to play a very important role in cytokine induction by TLR2 and TLR4 [200;201]. The level of PKCζ activity in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages was quantitated using a phospho-specific antibody recognising phosphorylation at Thr410 of PKCζ, which is known to be correlated with enzymatic activity [202]. Unstimulated B10A.*Nramp1*^{r} macrophages (Figure 8, lane 1 and 3) consistently displayed increased phosphorylation of PKCζ compared to B10A.*Nramp1*$^{-/-}$ cells (Figure 8, lane 2 and 4). Overall, the current findings point to a role for NRAMP1 in modulating the activation status of atypical PKC in murine macrophages.

Inhibition of Atypical PKC Inhibits Cytokine Production in B10A.*Nramp1*^{r} macrophages. To confirm the biological significance of reduced PKCζ activity in B10A.*Nramp1*$^{-/-}$ macrophages, the effect of PKCζ inhibition on cytokine production induced by a TLR7 ligand (S28463) was determined in B10A.*Nramp1*^{r} cells. The contribution of different PKC isoforms to NO secretion was determined by using various concentrations of the bisdolylmaleimide I (BIM) PKC inhibitor which at low concentrations (200 nM) inhibits the activity of both conventional and novel isoforms of PKC, but not atypical isoforms [203]. A dose of 6 μM of BIM results in inhibition of all isoforms of PKC. Imidazoquinoline induction of NO secretion by B10A.*Nramp1*^{r} macrophages was unaffected by a dose of 200 nM of BIM (Figure 9 panel A). Interestingly, increasing the dose of BIM to 6 μM resulted in a significant reduction in the amount of NO secreted following 18 hours of stimulation with a synthetic TLR7 ligand (Figure 9 panel A, from 27.9±0.5 μM to 10.3±0.6 μM with inhibitor, $p<0.05$). To confirm that the observed inhibitory effect was due to inhibition of atypical PKC

activity, production of nitric oxide by macrophages treated with a TLR7 ligand was analysed in the presence of a specific PKCζ pseudosubstrate inhibitory peptide. Treatment with S28463 in the presence of the PKCζ inhibitory peptide resulted in a significant reduction in NO secretion by B10A.*Nramp1*^{r} macrophages (Figure 9 panel B, from 27.9±0.5 μM to 16.5±0.8 μM with inhibitor, $p<0.05$). As a control, a PKA inhibitory peptide (PKI 14-22) was found to have no effect on NO induction by the TLR7 ligand (Figure 9 panel B). Taken together, these data show that reduced PKCζ activity correlates with a reduction in nitric oxide production in macrophages stimulated with a TLR7 ligand and suggest a possible molecular mechanism dependent on NRAMP1 which is essential for response to TLR7 ligands to occur.

Discussion

We previously demonstrated that mice carrying the resistant allele of the *Nramp1* gene, but not *Nramp1* knockout mice, infected with *M. bovis* BCG responded to TLR7 ligand (S28463) resulting in a significant decrease in splenic mycobacterial load after two weeks of treatment [23]. The exact molecular events controlled by NRAMP1 that would explain this difference have not yet been elucidated. Therefore, the goal of the present study was to characterize the involvement of NRAMP1 in TLR7 signal transduction. Many different aspects of TLR7 signalling were studied to determine the level at which NRAMP1 modulates the response.

The rate of TLR7 ligand uptake as well as intracellular localization was found to be very similar in *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages. TLR7 receptor expression was also found not to be affected by the *Nramp1* genotype. We found that, following treatment of macrophages with a TLR7 ligand, P38 MAPK, but not ERK1/2, was differentially activated in macrophages derived from *Nramp1* expressing mice compared to *Nramp1* knockout. Furthermore, levels of active form of PKCζ were much higher in resting B10A.*Nramp1*^{r} cells compared to B10A.*Nramp1*$^{-/-}$ macrophages. Inhibition of PKCζ activity in B10A.*Nramp1*^{r} cells led to reduced ability of macrophages to produce nitric oxide mimicking what is found in B10A.*Nramp1*$^{-/-}$ macrophages. Altogether, the data demonstrate a crucial role for P38 MAPK activation and atypical PKC activity in TLR7 signal transduction that are dependent on the presence of NRAMP1 in macrophages.

Analysis of the imidazoquinoline response in *Nramp1*^{r} or *Nramp1*$^{-/-}$ macrophages had previously revealed reduced nitric oxide induction in *Nramp1*$^{-/-}$ macrophages compared to *Nramp1*^{r} [23]. The recent observation that ssRNA of viral origin can stimulate through TLR7 [106;107] prompted the characterization of the role of *Nramp1* in response to these ligands. Interestingly, similarly to what we observed for a synthetic TLR7 ligand (S28463), *Nramp1*$^{-/-}$ macrophages were almost completely unresponsive to ssRNA stimulation. In fact, the difference in macrophage response to ssRNA was even more striking than what we previously observed for synthetic TLR7 ligand treatment [23]. Differential responsiveness of *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages to TLR7 ligands cannot be explained by differential TLR7 expression since no statistically significant difference in TLR7 mRNA expression was found. Analysis of TLR7 protein levels is still warranted to confirm the mRNA expression analysis. Co-stimulation with IFNγ could restore induction of NO, but not TNF after TLR7 stimulation. This dichotomy might suggest that two different signal transduction pathways are affected by NRAMP1, where only one is activated by IFNγ treatment.

Previous analysis of TLR7 signal transduction has revealed a requirement for ligand uptake and further processing within the cell prior to the activation of intracellular signalling cascades [105;193]. Localisation of NRAMP1 to LAMP1-positive phagosomal structures [179] where MYD88 is also recruited following TLR7 ligand stimulation [105], suggested that intracellular processing could be affected by the *Nramp1* genotype. Flow cytometry and fluorescent microscopy have revealed that the uptake and localisation of the TLR7 ligand to LAMP1 positive vacuoles was virtually

identical in *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages. These data strongly suggest that very similar events take place prior to receptor ligation in *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages, although this analysis cannot unequivocally exclude an influence of NRAMP1 in affecting the intravesicular environment through its divalent cation transporter activity.

Previous analyses found a greater level of activated P38 MAPK in untreated *Nramp1*^{r} macrophages [204]. Our data also support this observation. Furthermore, we demonstrate that TLR7-induced P38 MAPK activation is also compromised in *Nramp1*$^{-/-}$ cells. Modulation of P38 MAPK by NRAMP1 is specific to TLR7 agonists, as LPS treatment resulted in similar activation in both *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages. We had previously observed a difference in ERK1/2 activity between macrophages expressing the resistant allele (B10R macrophage cell line) and macrophages expressing the susceptible allele (B10S macrophage cell line) of the *Nramp1* gene [204]. In the present analysis, we did not observe any difference in ERK1/2 activation in either resting or in TLR7 ligand treated macrophages. Therefore the previously described difference most likely resulted from the differences in expression of genes other than *Nramp1* that are known to be different between B10R and B10S macrophages (approximately 13 cM on chromosome 1). In this study, we used 129Sv.*Nramp1*$^{-/-}$ mice that were backcrossed to B10.A(*Nramp1*^{r}) mice for 16 generations resulting in a very homogenous genetic background. Activation of P38 MAPK was shown to be crucial for the TLR-induced immunomodulatory activity for various TLR family members [194-196]. We demonstrate both by direct inhibition of P38 MAPK as well as in MK2 KO

macrophages that responses to TLR7 ligands are severely impaired without activation of the P38 MAPK axis. Therefore, our findings suggest that lower levels of P38 MAPK activity following TLR7 ligand stimulation can account, at least in part, for the difference in responsiveness between *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages.

Our data further describe that, in *Nramp1*$^{-/-}$ macrophages, the baseline level of atypical PKC activity is 2- to 3- fold lower than in *Nramp1*^{r} cells. Previous studies from our laboratory found reduced PKC enzymatic activity in macrophages from susceptible mice compared to their resistant counterpart [199]. The protein levels for these kinases were found to be comparable between the two macrophage cell lines. In this study, although the total amount of PKCζ was similar between *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages, the level of phosphorylation at position Thr410 found on the activated PKCζ kinase were 2 to 3 fold higher in *Nramp1*^{r} cells. The biological relevance of this observation was confirmed by inhibiting PKCζ activity with two different inhibitors in *Nramp1*^{r} macrophages leading to a reduction in nitric oxide production following TLR7 ligand treatment. This defect in atypical PKC activation does not reflect a general inhibition of kinase activation in *Nramp1*$^{-/-}$ macrophages as ERK1/2 MAPK activation following TLR7 stimulation was found to be virtually identical between *Nramp1*^{r} and *Nramp1*$^{-/-}$ macrophages. PKCζ is activated following endotoxin treatment of macrophages and is a crucial activator of downstream signal transduction pathways [205]. Atypical PKCs also regulate TLR4 recruitment to lipid rafts following LPS stimulation in human macrophages [200]. Therefore, our data reveal an important role for atypical PKC in controlling responsiveness of macrophages to TLR7 ligands.

To summarise, NRAMP1 modulation of TLR7 signal transduction occurs at the level of P38 MAPK activation and atypical PKC activity, which consequently may be responsible for the differences at the level of nitric oxide and TNF production that are both essential in clearance of mycobacterial infection. As TLR7 ligands are currently being used clinically in humans, an understanding of factors which control the response to these compounds, as well as their mechanism, will aid in the development of these treatments as well as improve our understanding of the pharmacogenetics of TLR7 ligands.

Figure 1: Cytokine Induction by ssRNA in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages Murine macrophage cell lines derived from *Nramp1* wild type (B10A.*Nramp1*^{r}) or *Nramp1* knockout (B10A.*Nramp1*$^{-/-}$) mice were treated with phosphorothioated ssRNA packaged in DOTAP transfection reagent. Twenty-four hours after stimulation, supernatants were analysed for nitric oxide (Panel A) and TNF secretion (Panel B). Macrophages lacking the *Nramp1* gene produced significantly less NO and TNF in response to the TLR7 ligand. Data are represented as mean ± SEM of two independent experiments (n=6 for all groups) (* = $p<0.05$ between the B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ group).

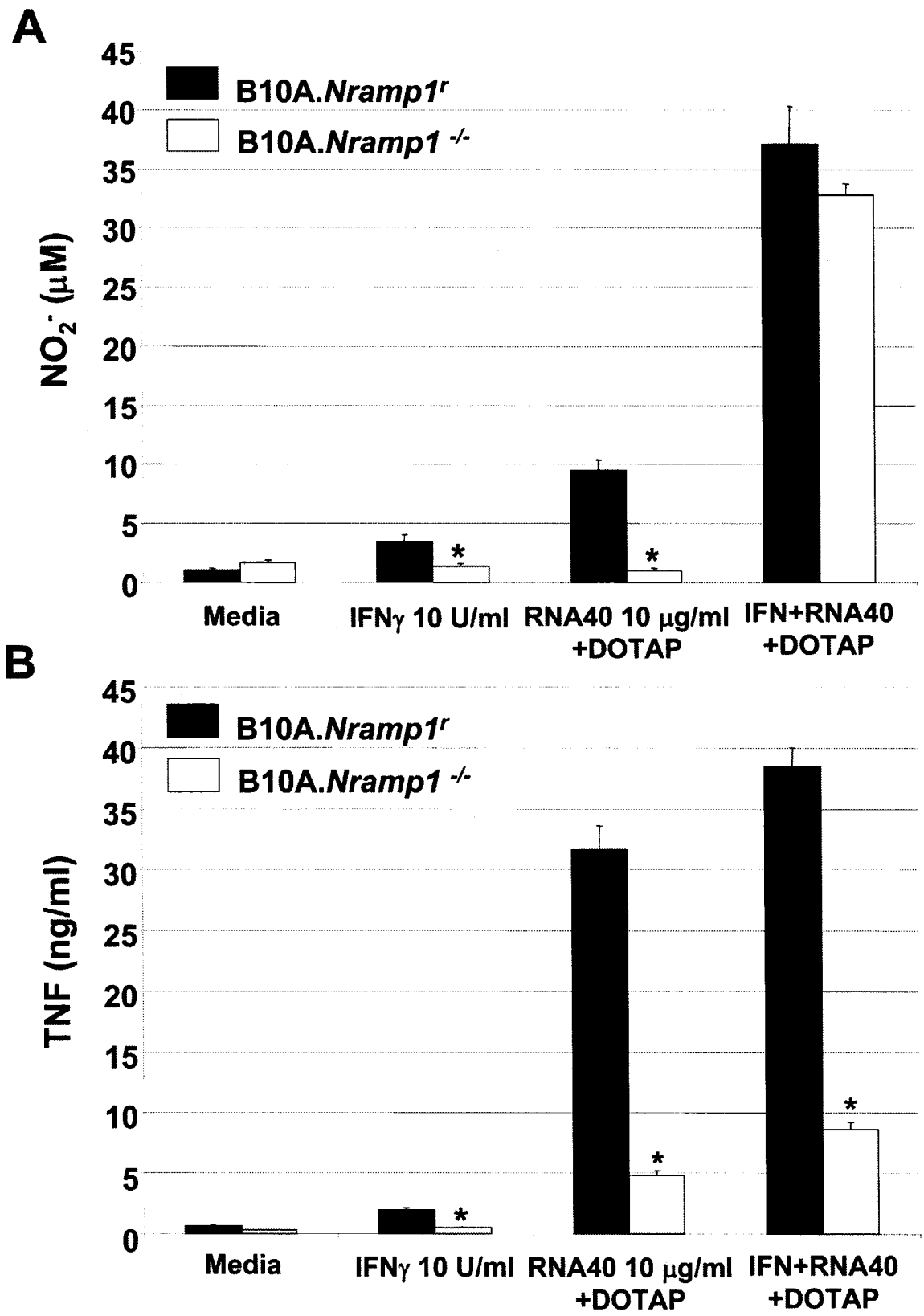

A
B10A.Nramp1r
B10A.Nramp1 -/-
NO2- (μM)
45
40
35
30
25
20
15
10
5
0
Media
IFNγ 10 U/ml
RNA40 10 μg/ml +DOTAP
IFN+RNA40 +DOTAP
B
B10A.Nramp1r
B10A.Nramp1 -/-
TNF (ng/ml)
45
40
35
30
25
20
15
10
5
0
Media
IFNγ 10 U/ml
RNA40 10 μg/ml +DOTAP
IFN+RNA40 +DOTAP

Figure 2: TLR7 mRNA expression in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages Total RNA from untreated macrophages was purified using Trizol reagent. Real-time PCR analysis was performed using a Statagene MX-4000 apparatus. Levels of TLR4 and TLR7 mRNA were adjusted for differences in GAPDH expression and normalised to levels in untreated B10A.*Nramp1*^{r} macrophages. Levels of TLR4 and TLR7 were found to be similar between B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages ($p>0.05$). Data are represented as mean ± SEM of five independent samples.

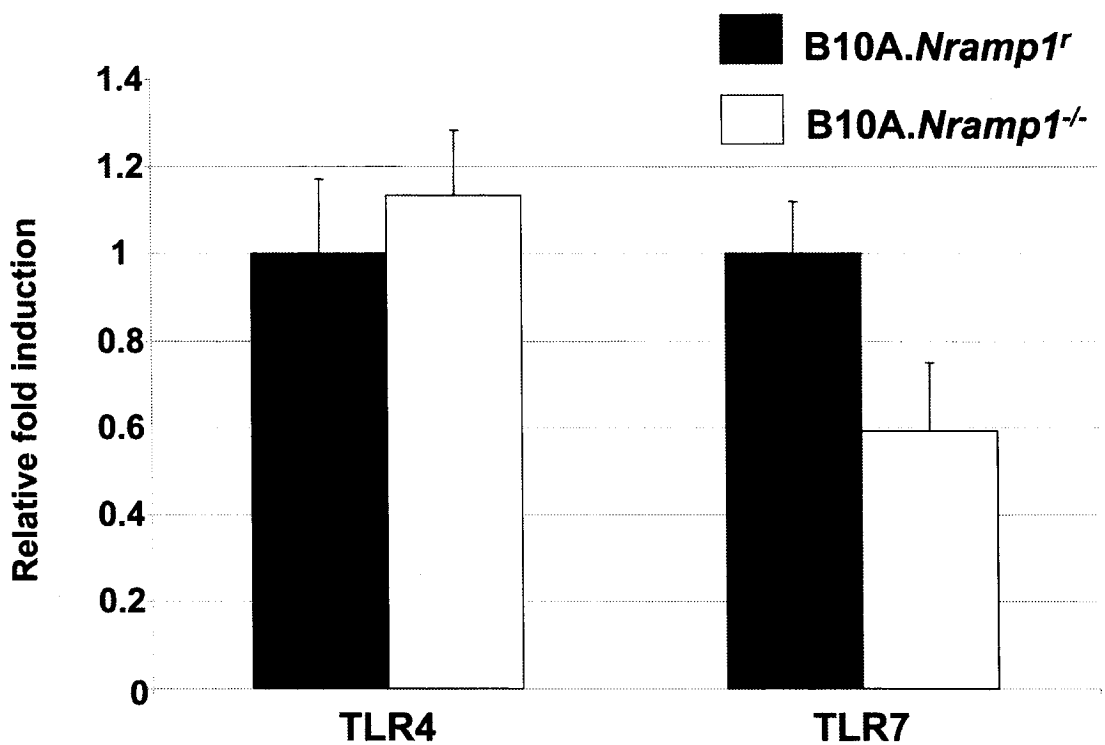

B10A.Nramp1r
B10A.Nramp1-/-
Relative fold induction
1.4
1.2
1
0.8
0.6
0.4
0.2
0
TLR4
TLR7

Figure 3: Delivery of ssRNA to B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages

B10A.*Nramp1*^{r} or B10A.*Nramp1*$^{-/-}$ macrophage cell lines were treated with a Cy3-labelled phosphorothioated ssRNA packaged in DOTAP transfection reagent for 2.5 hours. Samples were prepared as described in the *Materials and Methods* section and analysed by flow cytometry. The relative intensity was calculated by multiplying the mean fluorescent intensity by the percentage of positive cells. No difference in TLR7 ligand delivery was observed between B10A.*Nramp1*^{r} or B10A.*Nramp1*$^{-/-}$ macrophages. Data for treatment with Cy3-RNA40 represent two individual samples. Data with ssRNA/DOTAP treatment are represented as mean ± SEM of three independent experiments (n=9 for both B10A.*Nramp1*^{r} or B10A.*Nramp1*$^{-/-}$ macrophages).

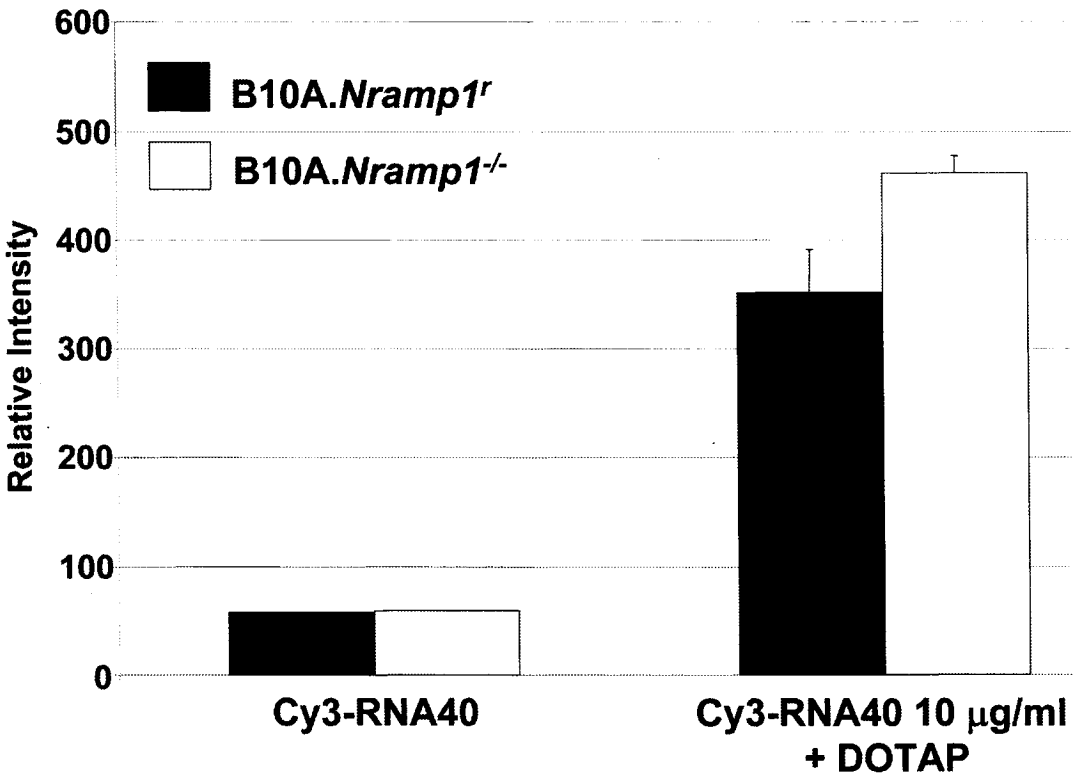

600
500
400
300
200
100
0
Relative Intensity
B10A.Nramp1r
B10A.Nramp1-/-
Cy3-RNA40
Cy3-RNA40 10 µg/ml
+ DOTAP

Figure 4: Intracellular localization of ssRNA in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages B10A.*Nramp1*^{r} or B10A.*Nramp1*$^{-/-}$ macrophage cell lines were treated with a Cy3-labelled phosphorothioated ssRNA packaged in DOTAP transfection reagent for 45 minutes. The cells were then fixed with 2% paraformaldehyde and permeabilized with acetone. Following incubation with an FcR blocking antibody, the cells were incubated with either a FITC-labelled LAMP1 (Panel A) or a Rab5 antibody followed by a FITC-labelled secondary antibody (Panel B). Data shown are representative pictures of two independent experiments done in duplicate (n=4 for each group).

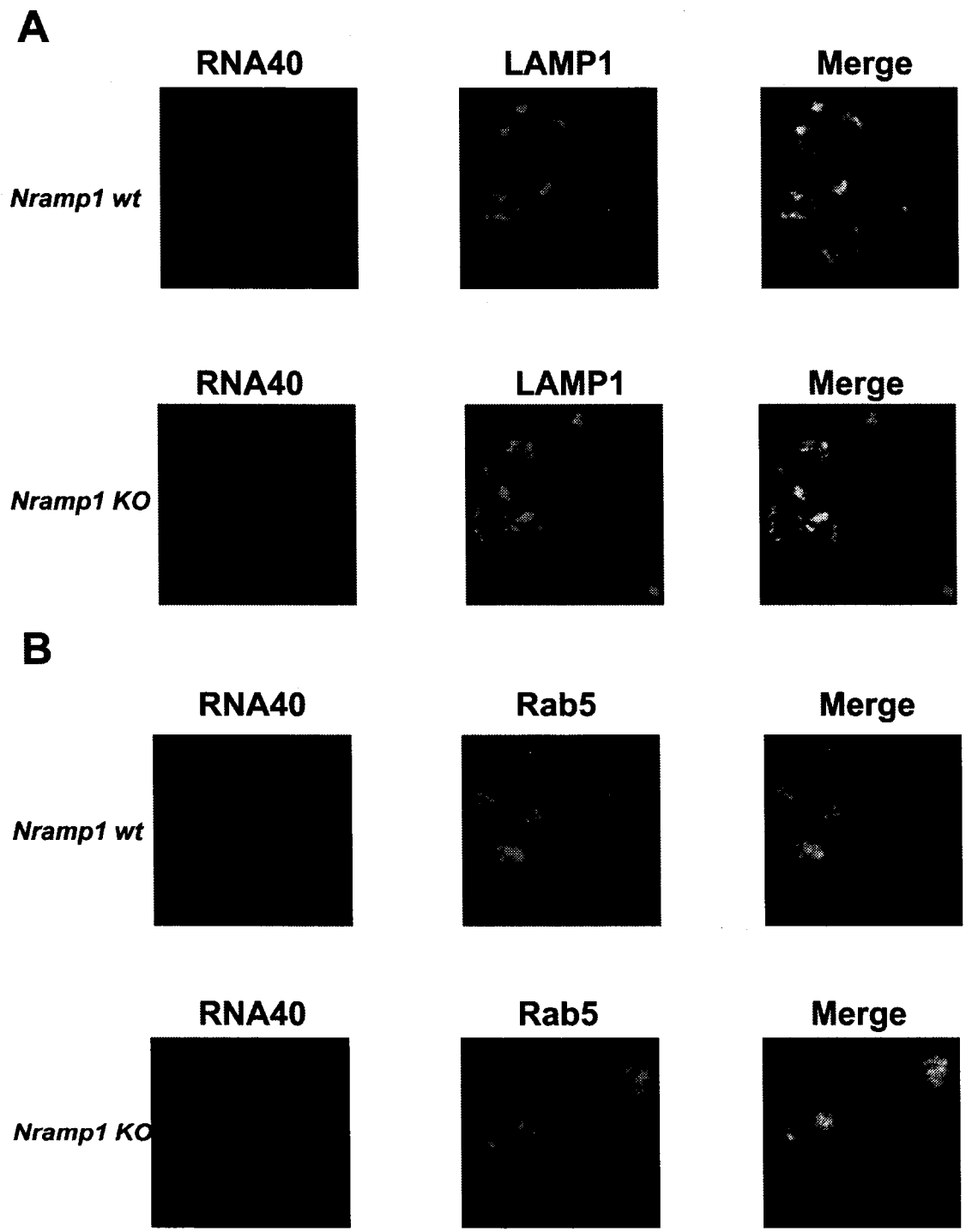

A
RNA40
LAMP1
Merge
Nramp1 wt
RNA40
LAMP1
Merge
Nramp1 KO
B
RNA40
Rab5
Merge
Nramp1 wt
RNA40
Rab5
Merge
Nramp1 KO

Figure 5: MAPK Activation Following TLR7 Treatment in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages B10A.*Nramp1*^{r} or B10A.*Nramp1*$^{-/-}$ macrophages were treated with S28463 for 30 or 60 minutes. Levels of activated P38 (Panel A) and ERK1/2 (Panel B) MAPK were determined by Western blotting using phospho-specific antibodies that recognise activating phosphorylation events. The membranes were subsequently stripped and probed with an antibody recognising both the phosphorylated and unphosphorylated form of the kinase. Blots were scanned and densitometry analysis was performed to quantitate the level of MAPK activation. Untreated B10A.*Nramp1*^{r} macrophages (lane 1-2) had higher P38 activation levels than B10A.*Nramp1*$^{-/-}$ group (lane 3-4). Treatment with S28463 activated P38 and ERK1/2 MAPK at both 30 (lane 5-6) and 60 minutes (lane 9-10) in B10A.*Nramp1*^{r} cells. B10A.*Nramp1*$^{-/-}$ macrophages displayed reduced activation of P38 MAPK, but not ERK1/2, after 30 (lane 7-8) and 60 minutes (lane 11-12) following TLR7 ligand treatment. Data illustrated in the graph combined the results from testing four samples of each group collected from two independent experiments. Data are represented as mean ± SEM of four samples (* = represents $p<0.05$ between the B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ group). The picture below each of the graph illustrates results of a representative experiment.

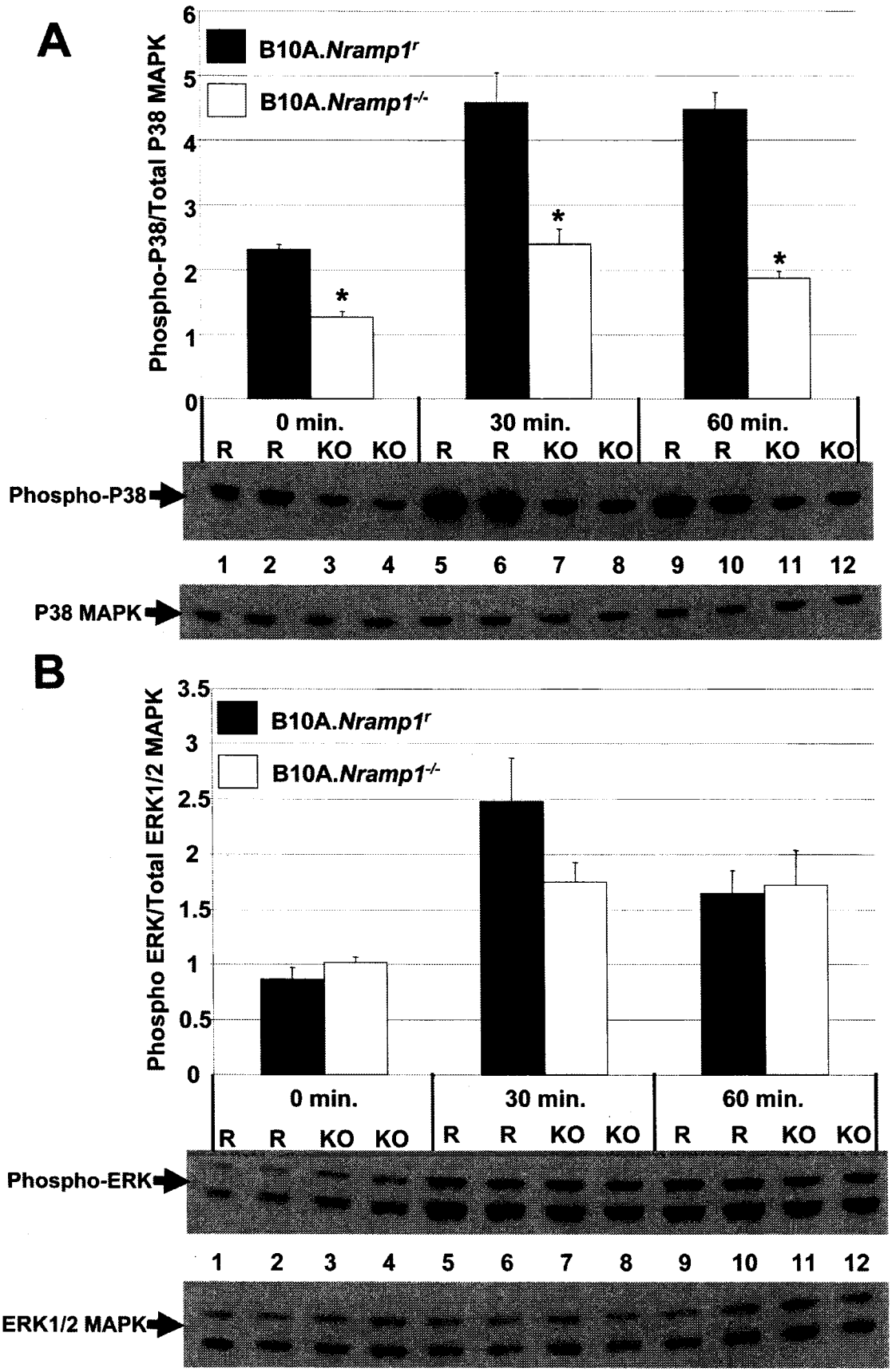

A
B10A.Nramp1r
B10A.Nramp1-/-
Phospho-P38/Total P38 MAPK
0
1
2
3
4
5
6
*
*
*
0 min.
30 min.
60 min.
R R KO KO R R KO KO R R KO KO
Phospho-P38
1 2 3 4 5 6 7 8 9 10 11 12
P38 MAPK
B
B10A.Nramp1r
B10A.Nramp1-/-
Phospho ERK/Total ERK1/2 MAPK
0
0.5
1
1.5
2
2.5
3
3.5
0 min.
30 min.
60 min.
R R KO KO R R KO KO R R KO KO
Phospho-ERK
1 2 3 4 5 6 7 8 9 10 11 12
ERK1/2 MAPK

Figure 6: Role of P38 MAPK in TLR-Induced Cytokine Induction Bone-marrow derived macrophage cell lines were generated from B10.A(*Nramp1*^{r}) mice. Cells were treated with 10 μM of P38 MAPK inhibitor III for one hour prior to stimulation with LPS, CpG ODN or S28463 for 18 hours. Supernatants were collected and NO (Panel A) and TNF (Panel B) levels were quantitated. Inhibition of P38 MAPK led to a significant reduction in both NO and TNF secretion from macrophages treated with TLR4, TLR7 or TLR9 ligands. Data are represented as mean ± SD from four experiments (n=12 for all the groups) (* = represents $p<0.05$ between the control and the P38 MAPK inhibitor III group).

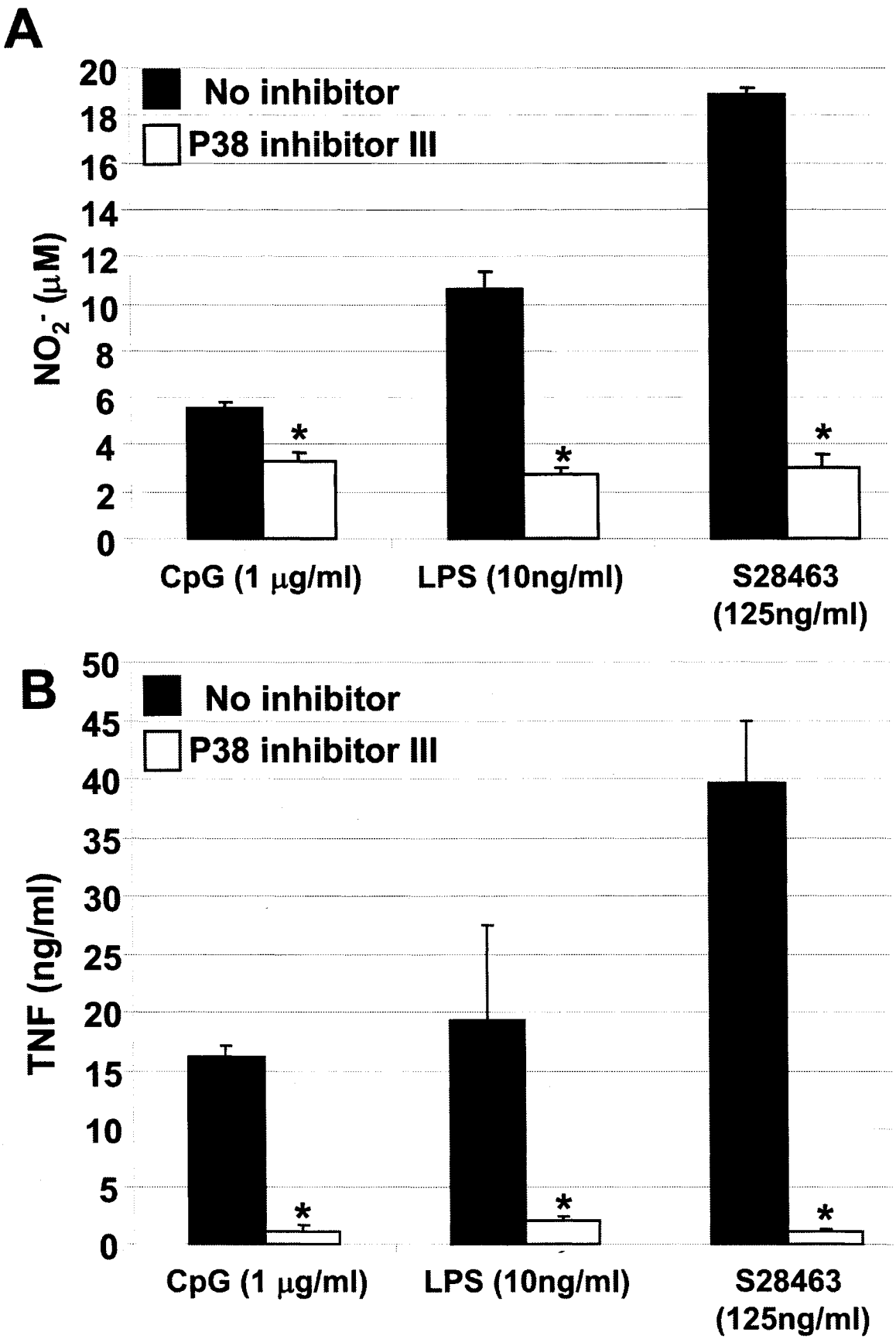

A
No inhibitor
P38 inhibitor III
NO_2^- (μM)
20
18
16
14
12
10
8
6
4
2
0
*
*
*
CpG (1 μg/ml)
LPS (10ng/ml)
S28463 (125ng/ml)
B
No inhibitor
P38 inhibitor III
TNF (ng/ml)
50
45
40
35
30
25
20
15
10
5
0
*
*
*
CpG (1 μg/ml)
LPS (10ng/ml)
S28463 (125ng/ml)

Figure 7: TNF Induction by TLR Ligands in MAPKAP2 Knockout Macrophages

Thioglycollate-elicited peritoneal macrophages were stimulated for 18 hours with a TLR4, TLR7 or TLR9 ligand. TNF levels were measured by ELISA. MK2 KO macrophages produced significantly less TNF following stimulation with all three TLR ligands. Data are represented as mean ± SD from four experiments (n=12 for all the groups) (* = represents $p<0.05$ between the MK2 wild type and MK2 KO macrophages).

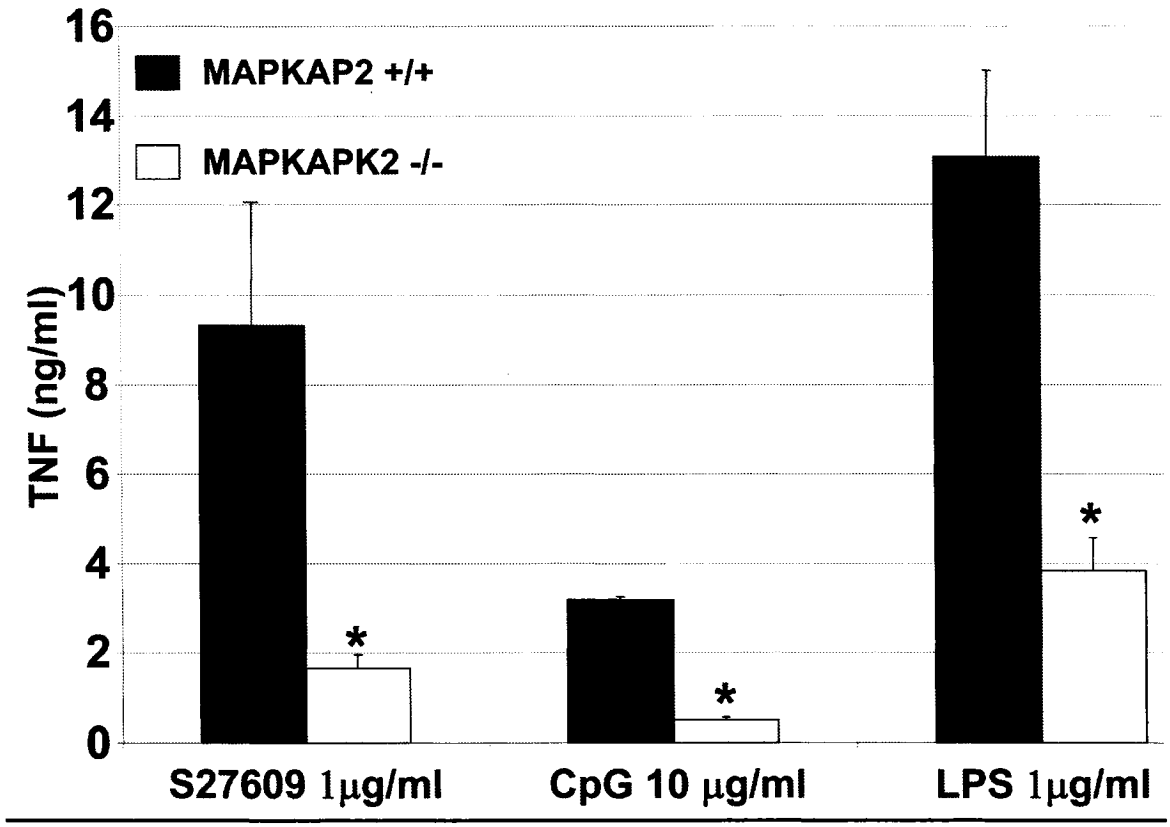

16
14
12
10
8
6
4
2
0
TNF (ng/ml)
MAPKAP2 +/+
MAPKAPK2 -/-
*
*
*
S27609 1μg/ml
CpG 10 μg/ml
LPS 1μg/ml

Figure 8: Atypical PKC activity in B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages Atypical PKC activity was measured in unstimulated B10A.*Nramp1*^{r} and B10A.*Nramp1*$^{-/-}$ macrophages using a phosphospecific activation epitope on PKCζ. Western blot analysis revealed increased phospho-PKCζ levels in B10A.*Nramp1*^{r} (lane 1 and 3) compared to B10A.*Nramp1*$^{-/-}$ macrophages (lane 2 and 4).

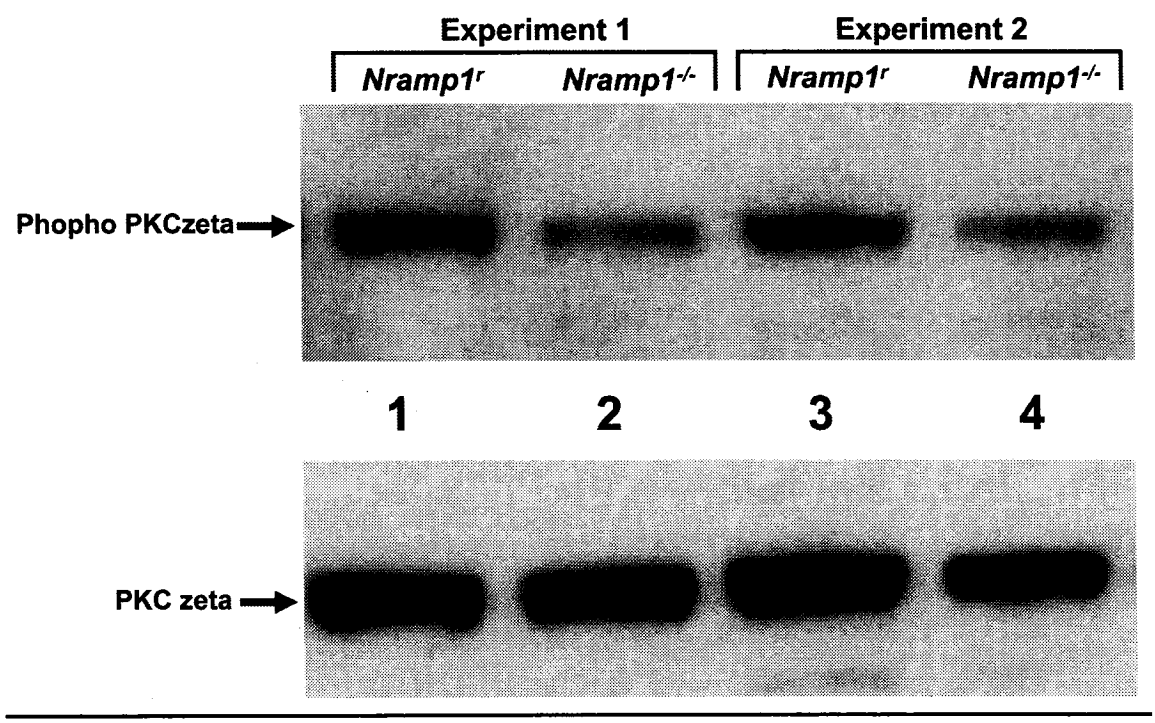
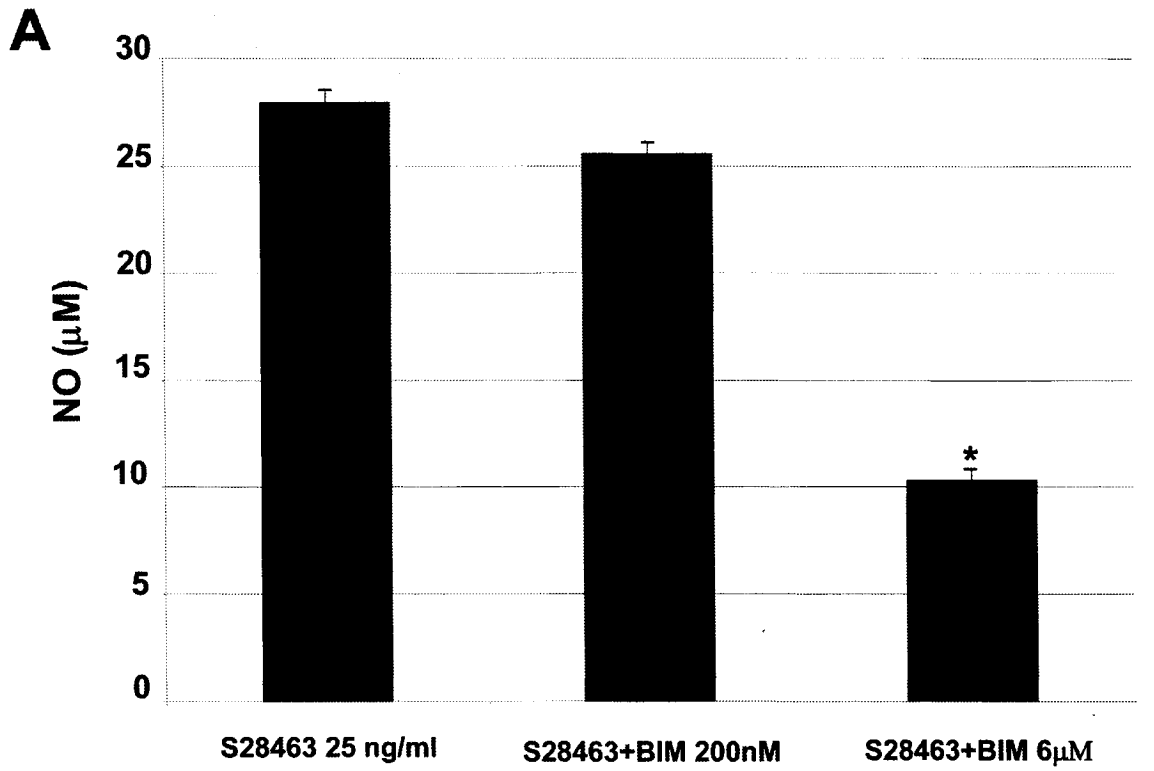
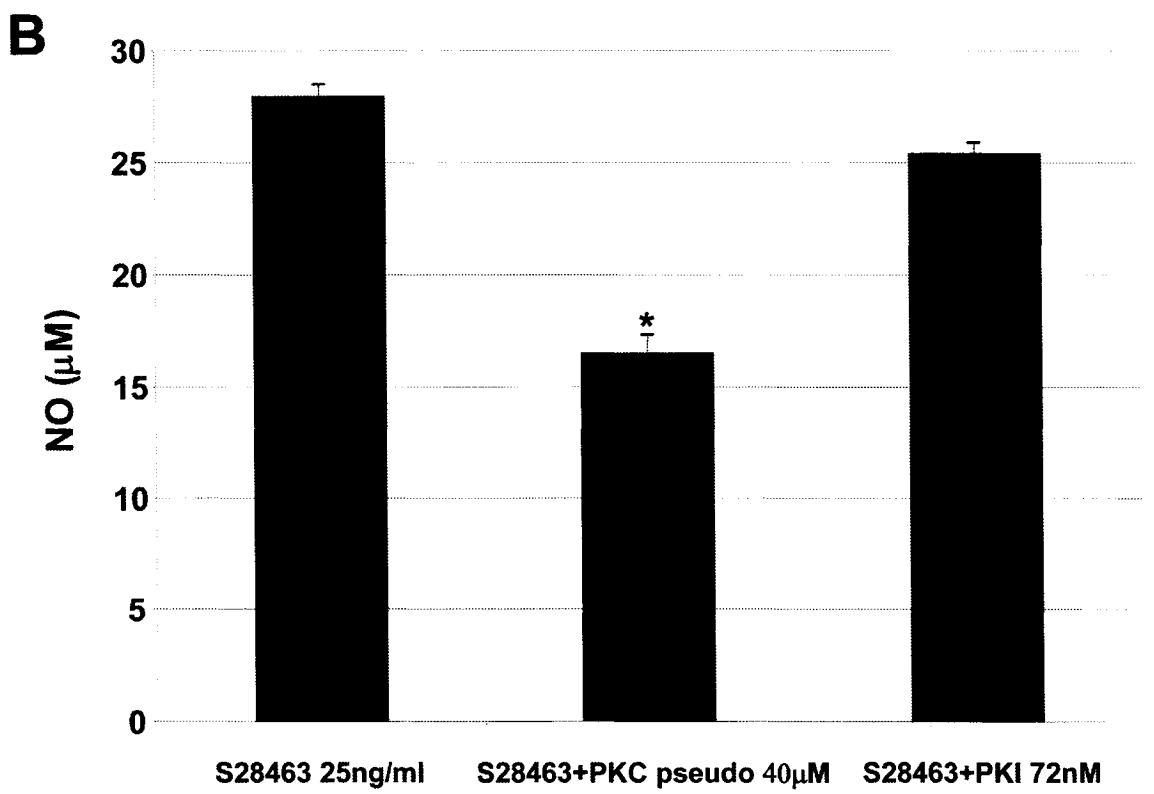

Experiment 1
Experiment 2
Nramp1r
Nramp1-/-
Nramp1r
Nramp1-/-
Phopho PKCzeta
1
2
3
4
PKC zeta

Figure 9: Inhibition of atypical PKC in B10A.*Nramp1*^{r} macrophages following TLR7 ligand treatment NO induction by TLR7 ligand in the presence of PKC inhibitors was analysed in the B10A.*Nramp1*^{r} macrophage cell line. Chemical inhibition revealed that high dose (6 μM) inhibition of all PKC isoform with bisindolylmaleimide I (BIM) inhibitor significantly reduced NO induction by TLR7 ligand (Panel A). A low dose of BIM (200 nM), which inhibits conventional and novel PKC, had no effect on NO induction (Panel A). Specific inhibition of atypical PKC with a myristoylated pseudosubstrate also significantly reduced NO induction by imidazoquinolines (Panel B). Inhibition of PKA activity with PKI 14-22 peptide had no effect on NO induction (Panel B). Data are represented as mean ± SEM of three independent experiments (n=8 for all the groups) (* = represents $p<0.05$ between the S28463 group and S28463 stimulated macrophages in the presence of inhibitor).


A
30
25
20
15
10
5
0
NO (μM)
*
S28463 25 ng/ml
S28463+BIM 200nM
S28463+BIM 6μM


B
30
25
20
15
10
5
0
NO (μM)
*
S28463 25ng/ml
S28463+PKC pseudo 40μM
S28463+PKI 72nM

Chapter 4

Toll-Like Receptor 7 Ligand Treatment Prevents Allergen-Induced Airway Hyperresponsiveness and Eosinophilia in Allergic Asthma

Previous studies have assessed the potential of TLR4 and TLR9 ligands in the treatment of allergic asthma. Originally believed to act by inducing a T_H1 cytokine profile to counteract the T_H2 dominated response that characterizes asthmatics, regulatory mechanism such as IL-10 and TGFβ have been shown to be crucial mediators of allergic asthma resolution by TLR ligands. Since imidazoquinolines induce a very similar cytokine profile to TLR9 ligands, we hypothesized that they could prove good candidates to treat allergic asthma. The amount of toxicology studies as well as the fact that the human lung expresses very high level of TLR7 suggests a greater clinical applicability of imidazoquinolines in the treatment of allergic asthma compare to either TLR4 or TLR9 ligands. We demonstrate that imidazoquinoline treatment completely prevented the development of allergic asthma in both A/J and C57BL/6 mice. Both lung resistance and elastance were reduced in mice receiving imidazoquinoline treatment after antigenic challenge. Furthermore, both T_H1 and T_H2 cytokines were reduced following ovalbumin challenge in animals receiving imidazoquinoline treatment. Contrary to what we observed with treatment of *M. bovis* BCG infection, *Nramp1* function was not required for protection as C57BL/6 mice, which carry a susceptible allele of this gene, were equally protected by imidazoquinoline administration against development of atopic allergic asthma. The macrophage-restricted expression of *Nramp1* suggests that protection to allergic asthma by imidazoquinoline occurs through a macrophage-independent mechanism.

Abstract

Asthma is one of the leading causes of childhood hospitalisation and its incidence is on the rise throughout the world. Currently, the standard treatment for asthma is the use of corticosteroids to try to suppress the inflammatory reaction taking place in the bronchial tree. Using a murine model of atopic allergic asthma employing a methacholine hyperresponsive (A/J) as well as a hyporesponsive (C57BL/6) strain of mice sensitised and challenged with ovalbumin, we show that treatment with a synthetic Toll-like receptor 7 ligand (S28463, a member of the imidazoquinoline family) leads to a reversal of the asthmatic phenotype. Treatment with S28463 resulted in a reduction of airway resistance and elastance following ovalbumin sensitisation and challenge. This was accompanied by a dramatic reduction in infiltration of leukocytes, especially eosinophils, into the lungs of both C57BL/6 and A/J mice following OVA challenge. Treatment with S28463 also abolished both the elevation in serum IgE level as well as the induction of IL-4, IL-5 and IL-13 by OVA challenge. The protective effects of S28463 were also observed in MK2 knockout, but not MYD88 knockout mice. We did not observe a switch in cytokine profile from T_H2 to T_H1, as both IL-12p70 and IFN-gamma levels were reduced following S28463 treatment. These results clearly demonstrate the anti-inflammatory effect of imidazoquinolines in an allergic asthma model as well as the clinical potential of TLR7 ligands in the treatment of allergic diseases.

Introduction

1. Epidemiology and Pathogenesis of Asthma

Asthma is a common disease, which frequently appears in childhood, affects millions of people worldwide, and is a leading cause of hospitalization of children in westernized countries. While asthma remains more common in industrialized and urbanized nations, its incidence has increased around the world over the last 20 years [206-209]. Asthma is characterized by episodes of intermittent reversible airway obstruction, excessive airway mucous production, airway smooth muscle hyperplasia and chronic inflammation of the bronchi involving infiltration of the submucosa by neutrophils, monocytes and eosinophils [210]. There is convincing evidence that the development and progression of asthma depends on the expression of several genes interacting with multiple environmental factors. Airway hyperresponsiveness (AHR) and atopy represent two important genetically-regulated phenotypes characteristic of asthma.

1.1. Airway Hyperresponsiveness (AHR)

Airway responsiveness is the ability of the airways to respond to bronchoconstricting stimuli by reducing their diameter. AHR is the term used to describe overly forceful airway responses to direct and indirect bronchoconstricting stimuli. Direct stimuli induce a response by ligating airway smooth muscle cell receptors [211] and include histamine, cholinergic agonists such as acetylcholine and methacholine, prostaglandin D_2 [212] and F_2 [213], as well as leukotrienes C_4 and D_4 [214]. Indirect stimuli elicit a response by stimulating the release of bronchoconstrictors from airway cells [211] and include

exercise [215], cold air [216], distilled water, adenosine monophosphate [217], pollutants and allergens [218].

AHR in response to direct stimuli has long been known to be a risk factor for the development of asthma [219]. In fact, it has been demonstrated that AHR in response to histamine, in infants as young as 4 weeks old, is significantly associated with the development of asthma by age six [220]. It is well documented that children with AHR are more likely to develop asthma than children with low airway responsiveness [221]. Furthermore, there are several lines of evidence which indicate that AHR is a heritable trait in both man and mouse. For over a decade, AHR has been known to precede the development of asthma [222] and for many years it has been known that nonasthmatic parents of asthmatic children have higher levels of AHR than nonasthmatic adults with no familial history of asthma [223;224]. More recently, several studies demonstrated genetic linkage of specific loci and AHR in response to histamine and methacholine [225;226]. Taken together, these findings demonstrate that AHR is genetically determined and often precedes the development of asthma. In mice, clear evidence of the heritability of AHR comes from several groups who have compared the AHR of various inbred strains of mice [227;228].

However, it is important to keep in mind that, in humans and in murine models of asthma, AHR is also affected by environmental factors. For example, in virtually all mouse models of allergic asthma, an increase in airway reactivity is observed after the animal has been antigen-sensitized and challenged [229], and allergen challenge in

humans similarly leads to an increase in AHR [230]. It has been hypothesized that the increase in AHR seen in asthmatics results from structural changes of the large and small airways, and that these structural changes are a result of acute-on-chronic inflammation caused by repeated exposure to allergens or agents which induce inflammation [211].

1.2. Atopy

Atopy is a predisposition of individuals to produce an immunoglobulin E (IgE) antibody-mediated response to common, innocuous antigens. Like AHR, there is strong evidence that atopy is in large part genetically determined in both human subjects and murine models of allergic disease [231-234]. Atopy, and its markers, are associated with a heightened risk of developing asthma in man [235;236].

A defining characteristic of atopy is the shift in the T helper 1 (T_H1) cell/T helper 2 (T_H2) cell balance in favour of T_H2 cells and mediators favouring IgE production. $CD4^+$ T cells can be divided into several separate subpopulations, including T_H1, T_H2 and T_H3 cells, based on their cytokine production profiles [237]. The different cytokine production profile of the CD4+ T cell subpopulations allows each to exhibit distinct functional properties. Furthermore, the cytokines produced by the T_H1 and T_H2 subpopulations promote the growth and function of that T-cell subpopulation while antagonizing the growth and function of the other [238]. Thus, T_H1 cytokines promote

T_H1 cell growth and function while inhibiting T_H2 cell growth and function, and vice versa.

T_H1 cells are known to produce interferon-γ (IFN-γ) and interleukin (IL)-2, and they are principally involved in fighting intracellular pathogens such as viruses and certain bacteria. Defence against intracellular pathogens is achieved by providing help to B cells which leads to isotype switching favouring opsonizing antibodies, and by activating macrophages to kill bacteria, such as *Mycobacterium tuberculosis*, efficiently. T_H1 mediated immune responses commonly involve a strong inflammatory reaction that may lead to tissue damage and destruction [239].

T_H2 cells characteristically produce IL-4, IL-5, IL-6, IL-10 and IL-13 and they are principally involved in defence against helminths. T_H2 cells also provide B cell help, but they induce isotype switching to a different set of isotypes, including IgE. In addition, several T_H2 cytokines have strong anti-inflammatory properties, including IL-13 and, especially, IL-10. Finally, IL-5 is a potent growth and differentiation factor for eosinophils [239].

T_H3 cells are characterized by their production of transforming growth factor-β (TGF- β) and appear to have a regulatory role in autoimmune disease [240]. Other non-T_H3 $CD4^+$ regulatory T-cells, including $CD25^+$ and $CD25^-$ cells, exist. Some of these cells exert their regulatory effects through the production of IL-10 and TGF-β while others act

through a contact dependent mechanism. All these cells are believed to be capable of regulating autoimmune and allergic diseases, however, the potential role in the modulation of the pathogenesis and severity of asthma of $CD4^+$ regulatory T-cells has not yet been fully elucidated [241-243].

1.3. Immunopathogenesis of Asthma

1.3.1. Atopy and AHR

Several studies using human clinical samples [244-246] and experimental animal models [247-249], together, strongly implicate high levels of T_H2 cells and their soluble products in the pathology of asthma. The first mechanism, by which T_H2 cells are thought to contribute to the pathology of allergic asthma, and in particular AHR, involves mast cell activation. In this model, T_H2 cells produce IL-4 and provide B-cell help leading to the production of allergen-specific IgE antibodies, which subsequently bind to FcεR expressed on the surface of mast cells. In the presence of allergen, or anti-IgE antibodies, FcεR-mediated mast cell degranulation is able to induce AHR in an IL-5 and eosinophil independent manner [250]. It had been postulated that the mast cells, which secrete IL-4 following FcεR activation, were the initial source of IL-4 [251]. However, the results from reconstitution experiments using IL-4 deficient mice, in which only transferred T cells could produce IL-4, indicate that IL-4 producing T cells are sufficient to restore the ability to develop AHR [252].

The second mechanism through which T_H2 cells are thought to contribute to AHR is by the recruitment of eosinophils to the airways, mediated principally by IL-5. The release of mediators, including major basic protein, from the eosinophils then leads to AHR [253]. The central importance of T cells in eosinophil recruitment to the airways, as well as to the development of AHR after methacholine challenge, has been demonstrated by several experiments which showed that both eosinophil recruitment and AHR were abrogated in mice whose T cells were depleted after monoclonal anti-CD4 antibody treatment [254].

1.3.2. Hygiene Hypothesis

As mentioned above, T_H1 cytokines have the ability to downregulate T_H2 responses. Given that T_H2 cells and their soluble mediators are known to be necessary for the development of allergic asthma pathology, it has been suggested that a relatively lower number of T_H1 cells, or decreased T_H1 cell activation, and a relatively enhanced number of T_H2 cells, or augmented T_H2 cell function, could at least partly be responsible for the development of AHR and atopy.

Asthma is not the only atopic disease in which T_H2 cells have been implicated. The fact that many allergic disorders are mediated by T_H2 cell products and that the incidence of these diseases has been increasing in westernized countries has led to the proposal that this increase is due to an imbalance between the numbers of T_H1 and T_H2 cells caused by environmental factors.

The hygiene hypothesis states that a lack of childhood exposure to infectious microorganisms will increase susceptibility to allergic diseases [255]. Since many bacteria and viruses elicit T_H1 responses, it was originally thought that insufficient stimulation of T_H1 immunity by these organisms led to insufficient T_H1-mediated downregulation of T_H2 immune responses which in turn led to allergic disease [256]. There are at least two observations which contradict this mechanistic explanation of the hygiene hypothesis. The first is that the incidence of T_H1 mediated autoimmune diseases such as multiple sclerosis (MS) [257;258], type I diabetes [259] and inflammatory bowel disease (IBD) [260] have increased concurrently with an increase in T_H2-mediated allergic diseases in westernized countries. The second is that the introduction of antigen-specific T_H1 cells into an antigen sensitized and challenged mouse not only failed to reduce AHR but led to an increase in airway inflammation [261].

An alternative mechanistic explanation for the hygiene hypothesis has been suggested to address the contradictions of the original formulation. The mechanism proposed is one in which insufficient downregulation of T helper cell-mediated immune responses by (as yet unidentified) regulatory T cells leads to allergic or autoimmune disease by allowing the response to escalate out of control, and that colonization or infection by certain microorganisms can induce the development, or promote the function, of regulatory T cells [262-265]. In other words, the imbalance would occur between T_H1 or T_H2 cells and regulatory T cells; T_H1 cells and regulatory T cells in the case of autoimmunity, and T_H2 cells and regulatory T cells in the case of allergic diseases.

2. Role of Toll-Like Receptors (TLR) Signalling in the Pathogenesis of Asthma

2.1. TLR Ligands

During the past decade, the TLR family has been shown to play a crucial role in both innate and adaptive immune responses [185;186]. They have the ability to modulate the immune response through the induction of cellular activation, as well as cytokine secretion. These receptors are considered to be sensors of the extracellular environment which trigger an immune response to conserved motifs on the surface of invading pathogens, hence their classification as pattern recognition receptors (PRR). Toll-like receptors are therefore some of the first response modulators to interact with environmental immunological stimuli. The TLR family of receptors is broadly expressed in many tissues and cell types, although the exact combination expressed by any given cell can vary greatly. TLR expression in the lungs as well as in immune cells known to traffic to this organ will be discussed here because of its relevance to asthma.

To date, thirteen TLR family members have been characterized in mammals (TLR1-TLR13) [72]. Mice express TLR1 to TLR9 as well as TLR11 to TLR13. Humans express TLR1 to TLR10. Most of the ligands defined for these receptors are pathogen-derived products. TLR4 is crucial for the response to lipopolysaccharides (LPS) from Gram-negative bacteria [76], and TLR2 transduces signals from lipoprotein as well as lipoteichoic acid and lipoarabinomannan (LAM) from mycobacteria [83;188]. TLR5, complexed to TLR4, recognizes flagellin derived from Gram-negative bacteria [96]. TLR9 has been found to be involved in the response to CpG oligodeoxynucleotides (ODN) [100]. Three categories of CpG ODN have been defined based on their

backbone (phosphodiester or phosphorothioate), their sequences and the cell types mediating the response to these oligonucleotides [101]. Interestingly, TLR7 knockout (KO) mice were unresponsive to a class of pharmaceutical compounds belonging to the imidazoquinoline family and failed to induce cytokines in the presence of these compounds [103]. In humans, TLR8 might also play a role in fine-tuning the response to these compounds [104]. Recently, TLR7 and TLR8 have been shown to mediate the response to viral RNA sequences [106;107;266].

2.2. TLR and the Pathogenesis of Asthma

Most studies analyzing the expression of TLRs are based on mRNA expression analyses and only a few have corroborated mRNA expression with protein analysis. Human lungs have been shown to express most TLRs with varying levels of expression [115]. TLR1-5, as well as TLR7 and TLR8, have been shown to be expressed at relatively high levels. Primary human airway epithelial cells express TLR2-6 as well as TLR9 [267;268]. Tracheal smooth muscles have been shown to express TLR2 and TLR4, with the former mediating responses to lipoteichoic acid [269]. Alveolar macrophages have been described as being able to respond to TLR2, TLR4 and TLR9 ligands, although the other receptors were not tested for [270]. An mRNA expression study indicated that the expression of various combinations of TLR1-7 and 9 was possible in murine macrophages [114]. Mouse neutrophils have been shown to respond to LPS through TLR4 [271] and expression studies using human neutrophils indicate that they express mRNA transcripts for TLR1,2 and TLR4-10 [272].

Of particular interest in relation to asthma, human mast cells have been shown to respond to TLR2 and TLR4 ligands [273] and murine mast cells were shown to express mRNA transcripts for TLR1, 2, 4 and 6 [114]. It has also been demonstrated that human primary eosinophils respond to S28463 (R-848), a member of the imidazoquinoline family and a TLR7 ligand [121]. Dendritic cells, which are involved in allergen presentation, express a wide range of TLRs (1-7 and 9) [114;123] and can respond to several of their ligands, including CpG ODN (TLR9 ligand) [120] and S28463 (R-848) and imiquimod (TLR7 ligands) [15].

B-cells are very important in the pathogenesis of asthma as a result of their ability to produce IgE antibodies. It has been demonstrated that both human and mouse B-cells respond to CpG ODN through TLR9 [120], and murine B-cells have been shown to respond to LPS [274]. Finally, there is evidence that several T-cell subsets express TLRs and are capable of responding to TLR ligands [114]. Human T-cells that are known to respond to TLR ligands include $CD8^+$ T-cells, which can respond to imiquimod through TLR7 [275], and $CD4^+CD25^+$ regulatory T-cells, which can respond to LPS through TLR4 [122]. Taken together, these observations suggest that the lung constitutes an environment where TLR ligands can modulate the responses of various cell types and potentially alter the asthmatic phenotype.

2.3. TLR Signalling and Immunotherapy of Asthma

Dendritic cells express several TLRs (see above), are at the forefront of the innate immune system, and play a pivotal role in activating and directing both the innate and adaptive immune responses. In the periphery, dendritic cells are highly endocytic and

efficiently take up antigen. Upon activation, most commonly by a pathogen component signalling through a TLR, these cells migrate to a local lymph node and present antigen (in the context of MHC molecules) and co-stimulatory molecules to naïve T-cells [276]. A dendritic cell's ability to direct the activation of T_H cells to a particular subtype depends on the types of co-stimulatory molecules expressed and the cytokines secreted. These in turn depend on the type of dendritic cell and the microbial environment in which the dendritic cell was activated. Specifically, myeloid dendritic cells secrete the T_H1 cytokine IL-12 in response to TLR7 or TLR9 ligation while plasmacytoid dendritic cells secrete IFNα [276]. With this in mind, several TLR ligands have been used in immunomodulatory treatment of murine models of asthma over the past few years in an effort to direct the immune response away from the allergic T_H2 arm of the adaptive immune system. It is important to note that, as discussed above, many immune and non-immune cells also express TLRs. While dendritic cells are well characterized with respect to their expression and response to TLR ligands, they are by no means the only cell type that could be affected by TLR ligand treatment.

Several groups have used murine models of asthma, principally in the BALB/c and C57BL/6 strains, to demonstrate the protective effect of TLR ligands on the development of the asthma phenotype. There are almost as many murine models of asthma used as there are groups using them, but most involve sensitization of the animal with one to three weekly injections of ovalbumin (OVA) adsorbed in aluminium hydroxide. The sensitization(s) are followed by one or more challenge of the airways, either by aerosol or intranasal delivery, with a solution of OVA in normal saline. Allergic asthma in mice can also be induced with house dust mite, cockroach and

Aspergillus fumigatus-derived antigens [277-279]. These protocols of sensitization and challenge have led to the development of an asthma-like phenotype which includes allergen-induced AHR, inflammation of the lungs and airways, the production of T_H2 cytokines and an increase in serum IgE levels.

2.3.1. Toll-Like Receptor 2

Like TLR4, the role of TLR2 signalling in the development and pathogenesis of asthma remains controversial. Using a mouse model, some studies have shown that the activation of TLR2 aggravates asthma by inducing the T_H2 cytokines IL-13 and IL-5, inhibiting the T_H1 cytokines IL-12 and IL-18 and increasing AHR [280]. On the contrary, other studies have shown that, *in vitro*, TLR2 ligands induce the production of the T_H1 cytokine IFN-γ but not the T_H2 cytokines IL-4 and IL-5. In addition, the use of TLR2 ligands inhibited IgE production in an allergic model *in vivo* [281].

2.3.2. Toll-Like Receptor 3

Evidence of the effect of TLR3 signalling in asthma is beginning to accumulate. *In vitro* studies have shown that TLR3 ligands can enhance murine tracheal smooth muscle responses to bradykinin, suggesting that TLR3 signalling could increase AHR [282]. The induction of RANTES expression by airway epithelial cell lines, in response to TLR3 ligands, has also been demonstrated *in vitro* [283]. Unlike TLR7 and TLR9, TLR3 signals, based on these observations, appear to aggravate the asthma phenotype. Several respiratory viruses, such as Rhinoviruses which have dsRNA genomes that can interact with TLR3, are known to exacerbate asthma in human adults [284]. This would

suggest that blockade of TLR3 activation is a viable therapeutic approach to the treatment of virus-induced exacerbations of asthma.

2.3.3. Toll-Like Receptor 4

Epidemiological evidence, though often contradictory [285-287], suggests a role for LPS in the pathology of asthma. It has been shown that airway macrophages become activated *in vitro* in a TLR4 dependent manner by the low levels of bacterial products that contaminate air pollution particles [288]. Taken together, this suggests that environmental endotoxin is capable of exerting physiologically relevant effects on the airways; however, the circumstances under which, as well as the level of, exposure required to protect against asthma have yet to be established [289]. Like the epidemiological evidence, the experimental evidence for the role of LPS in asthma is contradictory. Studies have found that LPS administered with antigen during the sensitization or challenge phase, in a murine model of asthma, prevented the development of AHR and inflammation of the lungs [290;291]. On the other hand, TLR4-defective mice, which cannot respond to LPS, also develop very little AHR and airway inflammation [292]. These dichotomous results cannot simply be explained by the existence of two independent signalling pathways. While MYD88-dependent TLR4 signalling has recently been demonstrated to be required for the development of a T_H2 mediated allergic response in a mouse model of asthma, in which sensitization is carried out by intra-nasal delivery of OVA containing LPS [293], other models clearly demonstrate that a lack of MYD88 leads to an increase in T_H2 cytokine production [294]. These disparate results might be reconciled by the observation that, in some murine asthma models, low levels of LPS in the antigen preparation are required to

induce the T_H2 response characteristic of asthma while high levels of LPS inhibit it [295]. While TLR4 signal modulation may turn out to have therapeutic potential, it is unclear, at the present time, how TLR4 signalling could be used to alleviate asthma.

2.3.4. Toll-Like Receptor 9

Treatment of sensitized animals with CpG ODN, a TLR9 ligand, either systemically by injection or by tracheal instillation before, or concurrently with, allergen challenge, leads to a decrease in airway inflammation that can be observed by histological analysis or by quantifying the number of inflammatory cells in broncho-alveolar lavage fluid (BALF) [296;297]. Production of the T_H2 cytokines IL-4 and IL-5 was abrogated, following allergen challenge, while levels of the T_H1 cytokines IFN-γ and IL-12 were increased and total serum IgE levels decreased [296;297]. Finally, the increase in AHR induced by allergen challenge was also greatly reduced in CpG ODN treated animals [297]. Follow-up studies indicate that a single CpG ODN administration can protect sensitized animals from allergen challenge for 6 – 8 weeks following treatment [298;299]. Evidence from some studies suggest that dendritic cells may be involved in promoting a T_H1 response and inhibiting the T_H2 response after TLR9 ligand treatment [300]. Others have shown increased levels of transforming growth factor β (TGF-β) in the BALF of treated animals [301], which indicates that regulatory T-cells may be involved. Recently, Hayashi *et al.* have demonstrated that, in a BALB/c model of acute allergic asthma, TLR9 ligation induces indoleamine 2,3-dioxygenase (IDO) activity in the lung and in $CD11c^+$ splenocytes. This enzyme is involved in tryptophan catabolism and has been shown to induce regulatory T-cells [302]. In this model, the TLR9 induced

increase in IDO activity mediated the suppression of allergic inflammation and AHR [303].

Also recently, Agrawal *et al.* have generated immunomodulatory oligonucleotides based on the active structures of classical CpG ODN, which appear to have more potent but similar biological activity, suggesting that compounds exhibiting an improved therapeutic potential could be generated [304]. A potential caveat to the successful treatment of asthma by TLR9 ligands in humans is that human lungs appear to express relatively little TLR9 compared to other Toll-like receptors, such as TLR2 and TLR7 [115]. As a whole, there is strong evidence which suggests that the modulation of TLR signalling can result in a reduction of asthma development and pathology. Both natural and artificial ligands for these receptors have successfully been used to treat allergic asthma in mice and are therefore prime candidates for continued development into therapeutics for human patients.

In the present study, using mice sensitised and challenged with ovalbumin, we demonstrate that imidazoquinoline treatment prevents increased lung resistance and elastance in mice that exhibit low (C57BL/6) as well as high (A/J) naive lung AHR. TLR7 ligand administration also led to a decrease in serum IgE levels. TLR7 activation prevented lung cellular infiltration; completely abrogating lung eosinophil recruitment. Furthermore, complete inhibition of T_H2 as well as T_H1 cytokines following imidazoquinoline treatment in OVA-challenged animals suggest that TLR7 triggering in the context of allergic asthma plays an anti-inflammatory role.

Material and Methods

Mice. Eight to ten-week-old male A/J and C57BL/6 mice were purchased from Harlan (Indianapolis, IN). C57BL/6 MAPKAP-2 knockout mice previously generated by Kotlyarov *et al.* [305] were bred at the Research Institute of the McGill University Health Center. MYD88$^{-/-}$ mice were generated in C57BL/6 x 129/SvJ [306] and subsequently backcrossed six times to the C57BL/6 background at the Weill Medical College of Cornell University. All animals were specific pathogen free and were maintained according to the standards of the Animal Care Committee of the McGill University Health Center.

Challenge Protocol and Respiratory System Physiology. Mice were sensitized once a week for three consecutive weeks by intraperitoneal injections of 100 μg ovalbumin adsorbed to 1.5 mg of aluminium hydroxide (Imject Alum, Pierce, Rockford, IL) in a total volume of 0.2 mL of sterile PBS. Seven days following the final sensitization mice were challenged on three consecutive days by aerosol exposure to either a 1% ovalbumin solution (OVA-OVA and OVA-S28 group) or PBS alone (OVA-PBS group) for 30 minutes. One group of mice was injected intraperitoneally with 100 μg of S28463 (generously provided by Dr. R. Miller, 3M Pharmaceuticals, St. Paul, MN) on 3 consecutive days starting one day prior to the first ovalbumin challenge (OVA-S28 group). Measurements of respiratory system resistance (Rrs) and elastance (Ers) were measured using a Flexivent small animal ventilator (SAV) (Scireq, Montreal, PQ, Canada). Briefly, 48 hours after final challenge, animals were anaesthetized,

tracheotomized and connected to the ventilator. Mechanical ventilation was carried out and peak resistance and elastance were measured after intra-jugular administration of increasing doses of methacholine (0-320 μg/kg).

Histology. Immediately following sacrifice of the mouse, the lungs were removed, inflated with 10% buffered formalin, dehydrated, mounted in paraffin and sectioned. Deparaffinized and hydrated sections were stained with hematoxylin and eosin (H&E) and Congo red (CR) stains using standard procedure. For quantitation, 300 infiltrating cells in the lungs were counted by two blinded observers and the amount of Congo red positive cells were quantitated.

Bronchial Alveolar Lavage Fluid Analysis. After the final antigen challenge, mice were sacrificed and the BAL analysis was performed. Following insertion of a catheter into the trachea, the lungs of the animal were lavaged using 1.2 mL of Hank's Balanced Salt Solution (Invitrogen, New York) and the samples were kept on ice for further processing. The lavage fluid was centrifuged at 250×g for 10 minutes and the cell pellet was resuspended in RPMI media (Invitrogen, New York). Cells were then loaded onto a cytospin machine and spun onto slides at 350 rpm. The slides were subsequently stained with a Diff-Quik stain set (Dade Behring, Newark) and a differential cell count was performed to determine the cellular makeup of the inflammatory cells.

Cytokine and IgE Measurements. Immediately following sacrifice of the mouse, the lungs of the animals were removed and placed in PBS containing protease inhibitors

(Complete inhibitor, Roche Diagnostics, Laval, PQ, Canada). Shortly thereafter, the lungs were homogenized using a polytron and the homogenates were stored at –80°C. Cytokine production was measured 3 and 6 hours after the last OVA challenge. IL-4, IL-5, IL-6, CCL5 (RANTES) and IL-10 levels were quantitated using Lincoplex murine cytokine analysis (Linco Research Inc., St. Charles, MO). IL-12p70, IL-13 and IFNγ production was measured using Beadlyte cytokine kits (Upstate, Charlottesville, VA). Serum IgE levels were measured 48 hours following the last challenge using the BD OptEIA ELISA kit (BD Biosciences, Missisauga, ON, Canada) according to the manufacturer's protocol.

RNA Analysis. Total lung RNA was purified using Trizol reagent (Invitrogen). Following DNase digestion of 4 μg of total RNA (DNA-free, Ambion, Austin, TX), reverse transcription was performed using random primers (Stratascript First Strand cDNA synthesis kit, Stratagene, LaJola, CA). CCL5, CCL11, CCL17 and CCL24 mRNA levels were determined by real-time PCR analysis performed using Brilliant SYBR Green QPCR kit according to manufacturer's protocol (Stratagene) on Statagene MX-4000 apparatus using the following cycling conditions: denaturation at 95°C for 30 seconds, annealing at 56°C for 60 seconds and extension at 72°C for 30 seconds. Primer sequences employed in this study are presented in Table 1. Levels of chemokine mRNA were adjusted for differences in GAPDH expression and normalised to the levels in the A/J OVA-PBS group.

Table 1: Primer sequence for real time quantitative PCR

Gene name	Sense 5' to 3'	Antisense 5' to 3'	Product size (bp)
CCL5	TCGTGCCCACGTCAAGGAGTATTT	TCTTCTCTGGGTTGGCACACACTT	107
CCL11	TATTCCTGCTGCTCACGGTCACTT	TCTCTTTGCCCAACCTGGTCTTGA	192
CCL17	AGGGATGCCATCGTGTTTCTGACT	ATGTTGAAACCATGGACAGCAGCC	162
CCL24	TTGACGCTTTACCAGGCTGCTTTG	ATGCTTGCAGCTCACTCAGGTTCT	128
GAPDH	ATGTGTCCGTCGTGGATCTGA	TTGAAGTCGCAGGAGACAACCT	145

Statistical Analysis. A Mann and Whitney non parametric test was performed using the SigmaStat software (SPSS, Chicago, Illinois) to calculate statistical significance for all analyses except gene expression where an unpaired *t* test using Welch correction was applied (Prism 4, GraphPad Softwares, San Diego, CA) . Differences among treatments are considered significant if $p \leq 0.05$.

Results

Imidazoquinoline Treatment Prevents Ovalbumin-Induced Increases in Airway Hyperresponsiveness. Previous studies have indicated the potential of TLR signalling in modulating allergen-induced airway hyperresponsiveness. TLR4 and 9 ligands have been shown to down regulate allergic immune responses in murine models of asthma [290;297;307;308]. In this study, the potential of TLR7 ligands to regulate allergic diseases was investigated in A/J and C57BL/6 mice. All animals were sensitised once a week for 3 consecutive weeks. Mice were then separated into three groups receiving either a challenge with PBS, with 1% OVA or treatment with S28463 followed by challenge with OVA. Treatment with OVA induced an increase in lung responsiveness that could be prevented by TLR7 ligand treatment (Figure 1 panel A). There was a statistically significant increase in lung resistance values in mice receiving OVA challenge versus PBS at 320 μg/kg of methacholine (30.4±4.2 and 12.5±3.1 $cmH_2O/ml/s$, respectively, $p<0.05$). Interestingly, the animals receiving S28463 treatment prior to OVA challenge had significantly lower lung resistance than the OVA challenged group at the same methacholine dose (30.4±4.2 for OVA-OVA and 17.3±3.6 $cmH_2O/ml/s$ for the OVA-S28 group, $p<0.05$). This reduction in lung resistance by S28463 could also be observed at the 160 μg/kg methacholine dose (12.1±3.2 for the OVA-OVA group and 4.6±0.7 $cmH_2O/ml/s$ for the OVA-S28 group, $p<0.05$). Similar effects were observed in C57BL/6 mice treated with TLR7 ligand and challenged with OVA (data not shown). Concurrently with modulation of airway resistance, OVA challenge also increased lung elastance which could also be prevented by S28463

treatment (Figure 1 panel B). These data suggest that TLR7 signalling is able to prevent airway hyperresponsiveness following allergen challenge in a murine model of asthma.

TLR7 Signalling Abrogates Increased Serum IgE Levels Following OVA Challenge. One of the hallmarks of asthma and other atopic diseases is the presence of increased levels of IgE [309]. A/J mice sensitised and challenged with OVA displayed increased levels of serum IgE compared to naïve animals (Figure 2). Challenge with ovalbumin resulted in an increase in the levels of IgE compared to the group receiving PBS challenge (8959±1837 ng/ml versus 3161±710 ng/ml, respectively). Imidazoquinoline treatment resulted in a dramatic reduction in IgE levels compared to the OVA challenged group (1266±282 ng/ml and 8959±1837 ng/ml, respectively), bringing the levels into the range of the PBS-challenged group. Interestingly, both the A/J and C57BL/6 strains display a similar pattern of response, except that OVA challenge induced 4 to 5 times more IgE in A/J as compared to C57BL/6 mice. This data demonstrates that TLR7 signalling can reduce the increase in IgE levels induced by OVA challenge even in a genetically predisposed strain of mice such as A/J.

Treatment with TLR7 Ligand Prevents Induction of both T_H2 and T_H1 Cytokines Following OVA Sensitisation and Challenge. Cytokine profiling in asthmatic patients has pointed to a role for T_H2 cytokines such as IL-4, 5 and 13 in the pathology of this disease [310]. Studies with TLR ligands have shown a tendency to skew the asthmatic response from a T_H2 to a T_H1 immune response resulting in prevention of the asthmatic phenotype in animal models. We assessed the ability of TLR7 ligands to modulate cytokine production in animals sensitized and challenged with OVA. IL-4, 5, 6 and 13

were all strongly induced 6 hours following OVA challenge in both A/J and C57BL/6 mice (Figure 3 panel A-D). PBS challenge did not lead to any increase in these cytokines. Treatment with S28463 completely prevented induction of these cytokines following OVA challenge. TLR7 activation did not lead to induction of T_H1 cytokines as both IFNγ and IL-12p70 levels were reduced by S28463 treatment (Figure 3 panel E-F). Levels of IL-10 were only slightly elevated following OVA challenge and returned to PBS levels in animals treated with imidazoquinolines (Figure 3 panel G). Imidazoquinoline treatment also led to a significant induction of CCL5 (RANTES) protein levels in the lungs of challenged animals (Figure 3 panel H) suggesting that the mode of action of these compounds is not through induction of a general immunosuppressed state. Furthermore, mRNA expression analysis in lungs from A/J and C57BL/6 mice revealed an upregulation by TLR7 ligand treatment of CCL5 (RANTES) mRNA (Figure 4 panel A) as well as chemokines normally associated with a T_H1 response (CCL4, data not shown). Moreover, we found that imidazoquinoline treatment led to inhibition in the mRNA levels of chemokines normally involved in eosinophil recruitment and T_H2 cell infiltration (CCL11, CCL17 and CCL24) following OVA challenge (Figure 4 panel B, C and D, respectively). Taken together, our data demonstrate that TLR7 ligand treatment results in an almost complete prevention of the inflammatory reaction following OVA sensitization and challenge; characterized by an inhibition of induction of several T_H2 and T_H1 cytokines in the lungs.

TLR7 Ligand Treatment Prevents Airway Inflammation. Proinflammatory cytokines such as IL-5 are known to be important in the recruitment of inflammatory cells to the

lung [311]. The inhibition of cytokine induction by OVA challenge in imidazoquinoline treated animals suggested that this might lead to a change in the cellular influx to the lungs of antigen challenged animals. OVA challenge induced a significant increase in the amount of cells in the BAL. Total BAL cell numbers went from 28,295±18,942 in the PBS group to 115,682±35,825 for OVA challenged A/J mice and from 13,766±1,995 to 283,807±138,121 in C57BL/6 (Figure 5 panel A). Treatment with imidazoquinolines completely prevented cellular infiltration in the BAL of both strains of mice. Analysis of cellular composition revealed that OVA challenge induced a significant increase in eosinophil composition of the BAL representing 31±7% in A/J (Figure 5 panel B) and 30±8% in C57BL/6 mice (Figure 5 panel C). Treatment with a TLR7 ligand completely prevented eosinophil recruitment in both strains of mice. OVA challenge also increased the relative amounts of lymphocytes in the BAL of both strains of mice which was not prevented by imidazoquinoline treatment. We did not observe any significant modulation of neutrophil and basophil levels by any of the treatments (data not shown). These results demonstrate the ability of imidazoquinolines to prevent influx of inflammatory cells, mainly eosinophils, to the lumen of airways in both A/J and C57BL/6 mice.

Imidazoquinoline Treatment Prevents Lung Inflammatory Cell Recruitment in OVA-Challenged Mice. Paraffin embedded lungs from animals sensitised and challenged with OVA displayed increased cell infiltration in both strains of mice as shown by H&E staining (Figure 6). The recruited cells tended to accumulate around the blood vessels close to airways (Figure 6 panel B and E). Imidazoquinoline treatment almost completely abrogated the recruitment of cells to the lungs around both

blood vessels and the surrounding airways (Figure 6 panel C and F). Challenge with PBS alone did not induce any significant cellular recruitment to the lungs (Figure 6 panel A and D). Flow cytometry analysis of digested lungs revealed an increased influx of CD45-positive cells following OVA challenge that could be prevented by imidazoquinoline treatment (data not shown), further confirming these observations. In addition, MYD88$^{-/-}$ mice displayed significant cellular infiltration to the lungs following OVA challenge which was not prevented with imidazoquinoline treatment. These findings further confirm the involvement of Toll-like receptors in the mechanism of action of imidazoquinolines in the treatment of asthma (Figure 6 panels H and I, respectively). Interestingly, although very different in their lung AHR, A/J and C57BL/6 mice exhibit very similar cellular infiltration following OVA challenge.

Eosinophil Infiltration is Abrogated Following TLR7 Ligand Treatment. Eosinophilic infiltration has been implicated in many of the pathological features of asthma. They release mediators such as leukotriene C_4, major basic protein as well as many cytokines which contribute to the asthmatic phenotype [311]. Histological analysis of lungs following OVA challenge revealed a significant increase in eosinophilic infiltration (Figure 7). Eosinophils represented 42.2±5.7% of infiltrating cells in the lungs of A/J mice and 39.8±4.7% in C57BL/6. Treatment with S28463 completely prevented eosinophil infiltration into OVA-challenged lungs in both strains of mice. MYD88$^{-/-}$ mice sensitized and challenged with OVA displayed a similar increase in lung eosinophilia which could not be prevented by imidazoquinoline treatment (Figure 7). Interestingly, MAPKAP-2 (MK2) knockout mice responded normally to S28463 treatment as shown by a reduction in airway eosinophilia (Figure 7) as well as AHR

(data not shown). PBS challenge did not induce any detectable levels of eosinophils in any of the strains tested. These data demonstrate that Toll-like receptor 7 ligand treatment prevents eosinophil recruitment to the lungs leading to attenuated AHR levels in both A/J and C57BL/6 mice which is dependent on MYD88, but independent of MK2.

Discussion

The results presented in this study demonstrate that TLR7 ligand treatment in the context of atopic allergic asthma leads to a reversal of the acute inflammatory state. The inhibition of both T_H2 (IL-4, 5, 6 and 13) and T_H1 (IL12p70 and IFNγ) cytokines prevents infiltration of inflammatory cells, especially eosinophils, to the lungs of animals sensitized and challenged with OVA. The inhibition of early inflammatory events associated with OVA challenge led to a reduction in serum IgE levels as well as a reduced lung response to methacholine challenge in both A/J and C57BL/6 mice. Furthermore, the protective effect of imidazoquinolines was found to be dependent on the presence of MYD88, but not on the MK2 pathway.

In both A/J and C57BL/6 strains, we did not observe any induction of T_H1 cytokines following imidazoquinoline treatment. A previous study looking at resiquimod treatment of allergic asthma in BALB/c mice has shown a decrease in Penh induction following OVA challenge associated with a shift in cytokine profile from T_H2 to T_H1 [312]. In the previous study, responsiveness to methacholine was measured using the whole-body plethysmography method which does not directly measure any lung parameter, but rather measures differences in respiratory pattern. This method of measurement has been found to behave in a strain specific manner and to correlate poorly with changes in lung properties [313;314]. To illustrate the drawbacks of relying on the Penh value to approximate lung parameters, we have observed that, although lung resistance and elastance are increased following OVA challenge in A/J mice, Penh values for these

animals were actually decreased following challenge (data not shown). This observation in A/J mice has also been reported by another laboratory [228]. Furthermore, the possible discrepancy in cytokine profile can be attributed to the fact that cytokine production was measured from *in vitro* cultured lung cells in the previous study rather than direct lung measurement as presented here. It is of great clinical importance whether TLR7 ligand treatment induces a shift in cytokine production or a complete block of the inflammatory reaction. We have used two different mouse strains, directly measured lung parameters, as well as measured cytokines *in vivo* which we believe gives a better approximation of the physiological effect of TLR7 ligand in the lungs.

Both TLR4 and TLR9 activation have been associated with positive outcome in allergic asthma models [290;307]. Initial observations had suggested that the mechanism of action involved a shift in cytokine production, although further analysis revealed that T_H1 cytokines could not account for the observed beneficial effect associated with both LPS and CpG treatment of allergic asthma. This suggests that T_H2 cytokine inhibition and induction of regulatory mechanism account for the observed amelioration [290;307]. IL-10 has been shown to be crucial in mediating CpG efficacy in preventing allergic asthma [315]. We saw very little modulation in the levels of this cytokine suggesting an alternative mechanism for TLR7 ligand action in allergic asthma treatment. The fact that imidazoquinolines are currently employed in humans for the treatment of human papilloma virus (HPV)-induced warts and the observation that human lungs have ten fold higher mRNA levels for TLR7 than TLR9 suggests a greater clinical applicability of imidazoquinolines for asthma treatment [115].

Although A/J and C57BL/6 mice displayed comparable inflammatory responses following OVA sensitization and challenge, serum IgE levels were found to be differentially induced in these animals. OVA sensitization induced five times more serum IgE in A/J mice compared to C57BL/6. OVA challenge maintained the difference in serum IgE between the two strains. Levels of serum IgE are also associated with differences in airway resistance and elastance between A/J and C57BL/6 following OVA challenge. Imidazoquinoline treatment prevented increases in IgE levels as well as improved the lung physiology in both strains of mice. Using S28463 as an adjuvant in conjunction with Alum, it had been reported that TLR7 ligands tended to favor IgG2a production and led to reduced IgE production in a murine model [63]. Human PBMC stimulated *in vitro* with CD40 and IL-4 also produced significantly less IgE in the presence of TLR7 ligands [26]. We demonstrate that administration of TLR7 ligands, even after sensitization, leads to a dramatic reduction in serum IgE levels induced following OVA challenge.

OVA challenge induced a dramatic influx of cells both in the BAL and in the interstitial lung environment. The main recruited cell types were eosinophils and macrophages. OVA challenge also led to a significant increase in lung responsiveness to methacholine in A/J mice following OVA challenge. Both effects were greatly diminished by imidazoquinoline treatment. This was also associated with a reduction in CD45 positive lung cells in both A/J and C57BL/6 animals (data not shown). The complete lack of eosinophils as well as IL-5 and IL-13 can account for the observed reduction in lung AHR levels [311;316]. Because of their role in airway remodeling and lung inflammation [317], inhibition of eosinophilia through lack of cytokine (IL-5) and

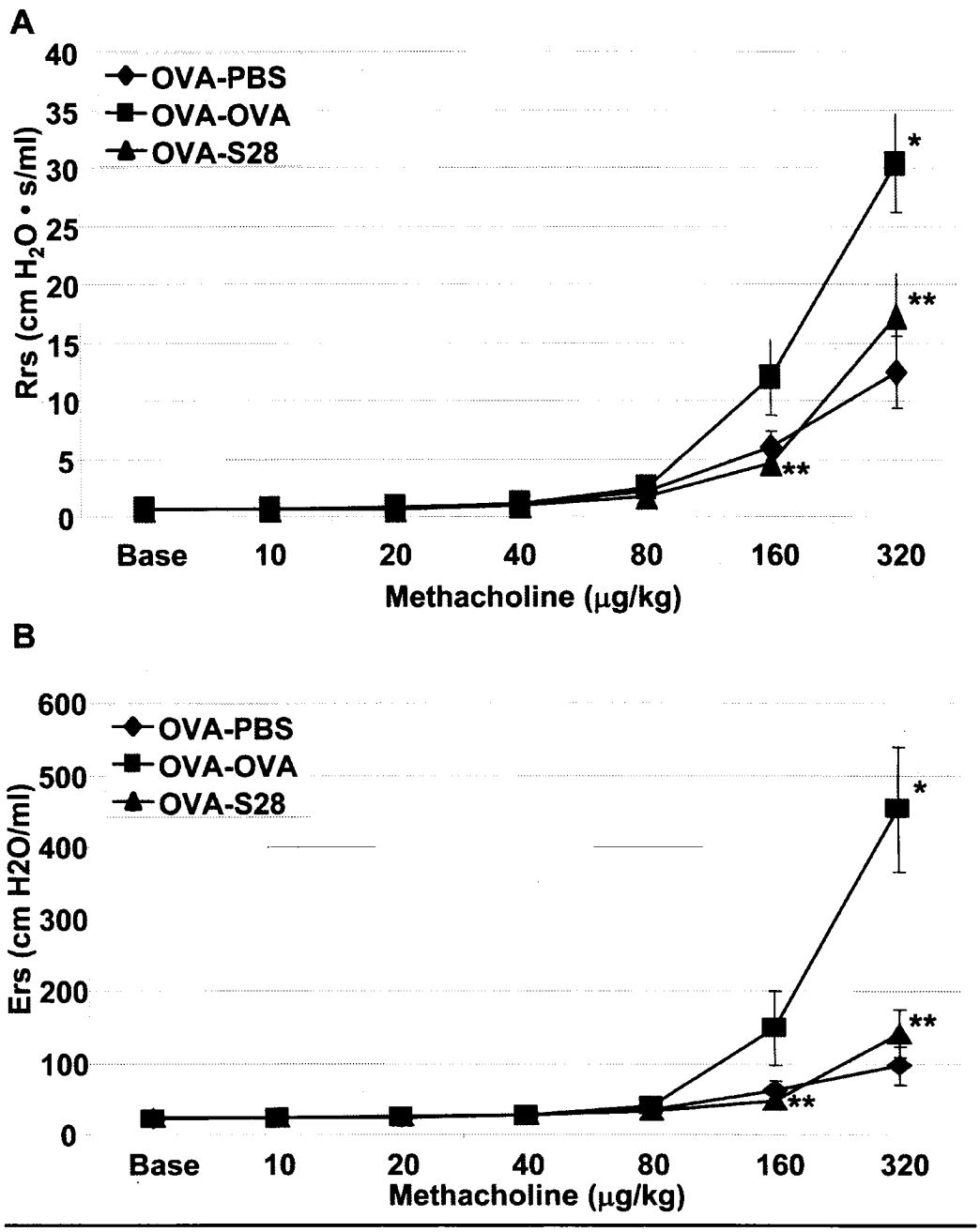
chemokine (CCL11, CCL17 and CCL24) induction following TLR7 ligand treatment provides a plausible mechanism for the protective effect of imidazoquinolines in the treatment of allergic asthma [318].

The mechanism of action of imidazoquinoline treatment in allergic asthma was also addressed using mice lacking important signal transduction components which have been previously shown to play a major role in immunomodulatory effects induced by various TLR. MYD88 is a crucial adapter molecule linking the TLR with other signal transduction components. TLR7 has been previously shown to be dependent on the presence of MYD88 for signal transduction to be initiated following imidazoquinoline stimulation [103]. We demonstrate that in the context of atopic allergic asthma, the protective effect of imidazoquinoline on lung cellular infiltration and eosinophilia is dependent on the presence of a functional MYD88. The P38 MAPK substrate, MAPKAP-2 (MK2), has previously been shown to be important in cytokine induction by TLR [305]. Using MK2 KO mice, we demonstrate that this kinase was not required for the protective effect of imidazoquinoline in allergic asthma as measured by both lung eosinophilia and AHR. A recent study has demonstrated a role for P38 MAPK in the induction of allergic asthma [319]. MK2 KO mice displayed normal increases in lung resistance following OVA challenge (data not shown). Furthermore, MK2 KO mice exhibited increased lung eosinophilia following OVA challenge. This suggests that P38 MAPK, but not MK2 is important for allergic asthma induction in mice. This is the first study describing the role of various signal transduction molecule in the protective effect of Toll-like receptor 7 ligands in the treatment of allergic asthma.

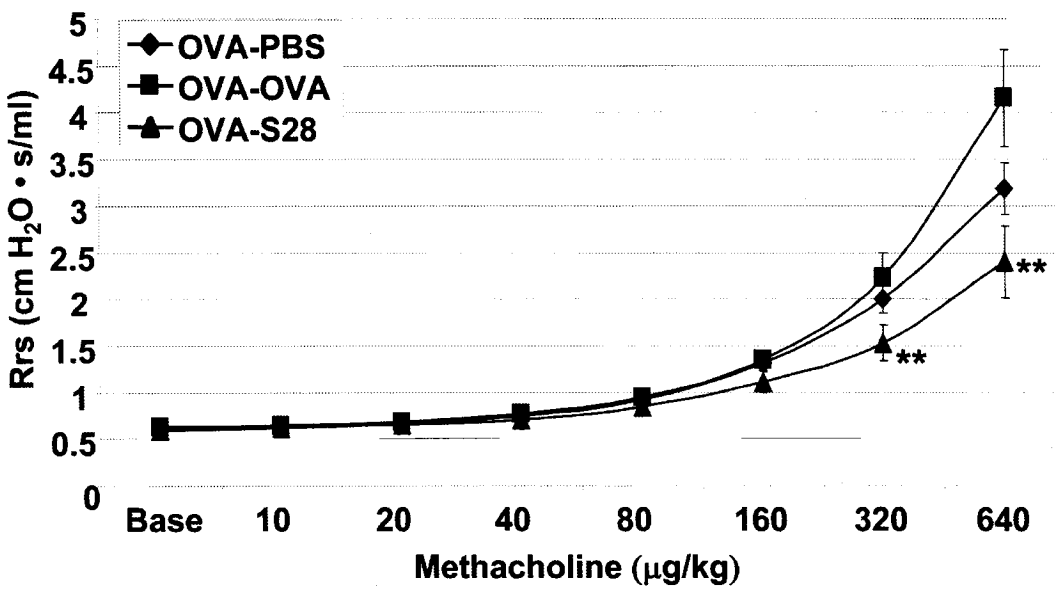
Previous studies in our laboratory have pointed to a role of NRAMP1 (Natural Resistance-Associated Macrophage Protein 1 or SLC11A1) in modulating responsiveness to TLR7 ligands in mycobacterial infections [23]. Mice carrying the wild type (or resistant) allele of *Nramp1* displayed increased responsiveness to imidazoquinoline treatment than mice carrying the susceptible allele of this gene. NRAMP1 expression in mice is restricted to macrophages. In the present study, both A/J and C57BL/6 mice responded equally to S28463 treatment even though they carry different *Nramp1* alleles. This might suggest that the response of macrophages to TLR7 ligands in the lungs might not be critical for its protective effect against allergic asthma, although the contribution of NRAMP1 could be complemented by other genes that are different between the two strains of mice.

Taken together, we demonstrate that the ability of TLR7 to reverse acute atopic allergic asthma through inhibition of cytokine production as well as cellular infiltration leading to a reduction in airway hyperresponsiveness is dependent on MYD88, but does not require functional MK2. The data presented in this study warrant further clinical investigation of the potential for TLR7 ligands in the treatment of allergic asthma.

Figure 1: Treatment with S28463 Abrogates Allergen Induced AHR in Response to Methacholine. A/J mice were sensitized with OVA and challenged by aerosol exposure to PBS (OVA-PBS) or OVA (OVA-OVA), as described in the *Materials and Methods* section. One group of mice received 100 μg of S28463 intraperitoneally one day prior to each OVA challenge (OVA-S28). Respiratory system resistance (Rrs) (Panel A for A/J and Panel C for C57BL/6 mice) and elastance (Ers) (Panel B for A/J and Panel D for C57BL/6 mice) in response to increasing doses of methacholine (MCh) was measured 48 hours after the last PBS or OVA aerosol challenge. Results are presented as mean ± SEM from three independent experiments (n=10) (* represents $p<0.05$ between OVA-PBS and OVA-OVA group. ** represents $p<0.05$ between OVA-OVA and OVA-S28 group).


A
OVA-PBS
OVA-OVA
OVA-S28
Rrs (cm H2O • s/ml)
40
35
30
25
20
15
10
5
0
*
**
**
Base
10
20
40
80
160
320
Methacholine (μg/kg)
B
OVA-PBS
OVA-OVA
OVA-S28
Ers (cm H2O/ml)
600
500
400
300
200
100
0
*
**
**
Base
10
20
40
80
160
320
Methacholine (μg/kg)

C


OVA-PBS
OVA-OVA
OVA-S28
Rrs (cm H2O • s/ml)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
**
**
Base
10
20
40
80
160
320
640
Methacholine (μg/kg)

D

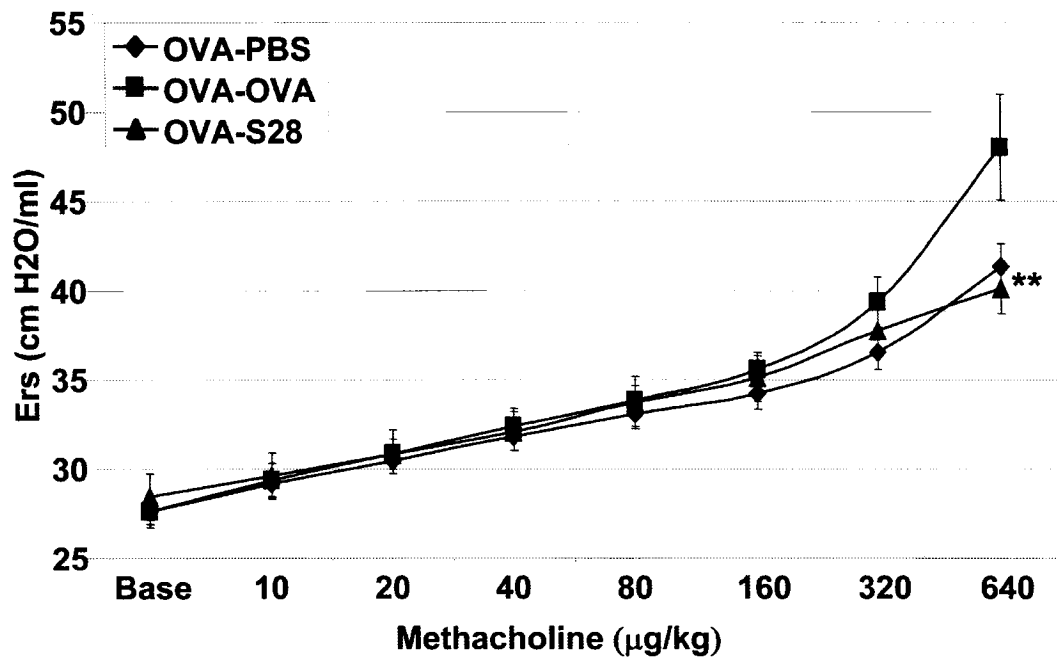

OVA-PBS
OVA-OVA
OVA-S28
Ers (cm H2O/ml)
55
50
45
40
35
30
25
**
Base
10
20
40
80
160
320
640
Methacholine (μg/kg)

Figure 2: IgE Levels in OVA Challenged A/J and C57BL/6 mice. Mice were sensitized with OVA and challenged with PBS (OVA-PBS) or OVA (OVA-OVA) and serum was collected as described in the *Materials and Methods* section. One group of mice received 100μg of S28463 intraperitoneally one day prior to each OVA challenge (OVA-S28). Total serum IgE levels were determined by ELISA 48 hours after PBS or OVA challenge. OVA challenge in A/J mice led to a significant increase in IgE levels (n=13) compared to PBS challenge (n=11). S28463 treatment led to a dramatic reduction in serum IgE levels (n=12). A similar pattern was observed in C57BL/6 mice for OVA-PBS (n=24), OVA-OVA (n=26) and OVA-S28 (n=25) group. Results are presented as mean ± SEM from 5 independent experiments (* represents $p<0.05$ between OVA-PBS and OVA-OVA group. ** represents $p<0.05$ between OVA-OVA and OVA-S28 group).

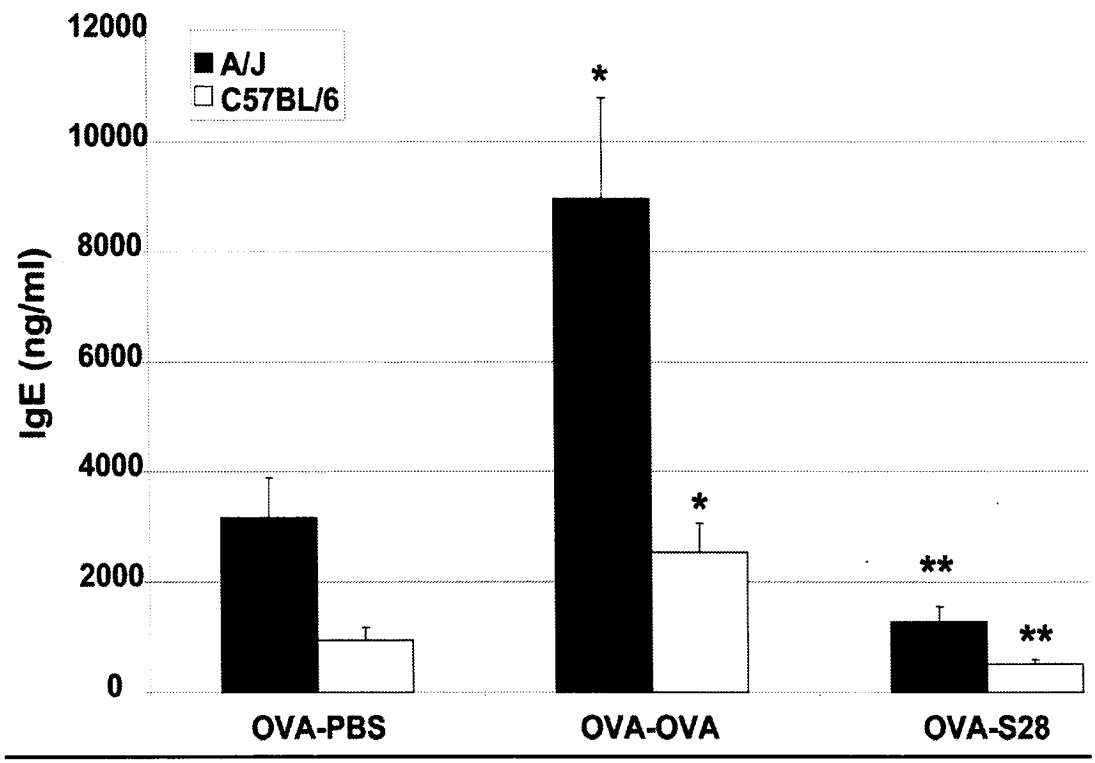

12000
10000
8000
6000
4000
2000
0
IgE (ng/ml)
A/J
C57BL/6
*
*
**
**
OVA-PBS
OVA-OVA
OVA-S28

Figure 3: Cytokine Levels Following OVA Challenge. Following sensitisation and challenge with OVA or PBS, whole lung tissue homogenates from A/J (solid bars) and C57BL/6 (open bars) were collected as described in the *Materials and Methods* section. Lung IL-4 (Panel A), IL-5 (Panel B), IL-6 (Panel C), IL-10 (Panel G) and RANTES (Panel H) levels were determined by Lincoplex murine cytokine array 6 hours after PBS or OVA challenge. IL-13 (Panel D), IL-12p70 (Panel E) and IFNγ (Panel F) levels were quantitated using the Beadlyte cytokine kits. Results are represented as mean ± SEM from four animals (* represents $p<0.05$ between OVA-PBS (PBS) and OVA-OVA (OVA) group. ** represents $p<0.05$ between OVA-OVA and OVA-S28 group).

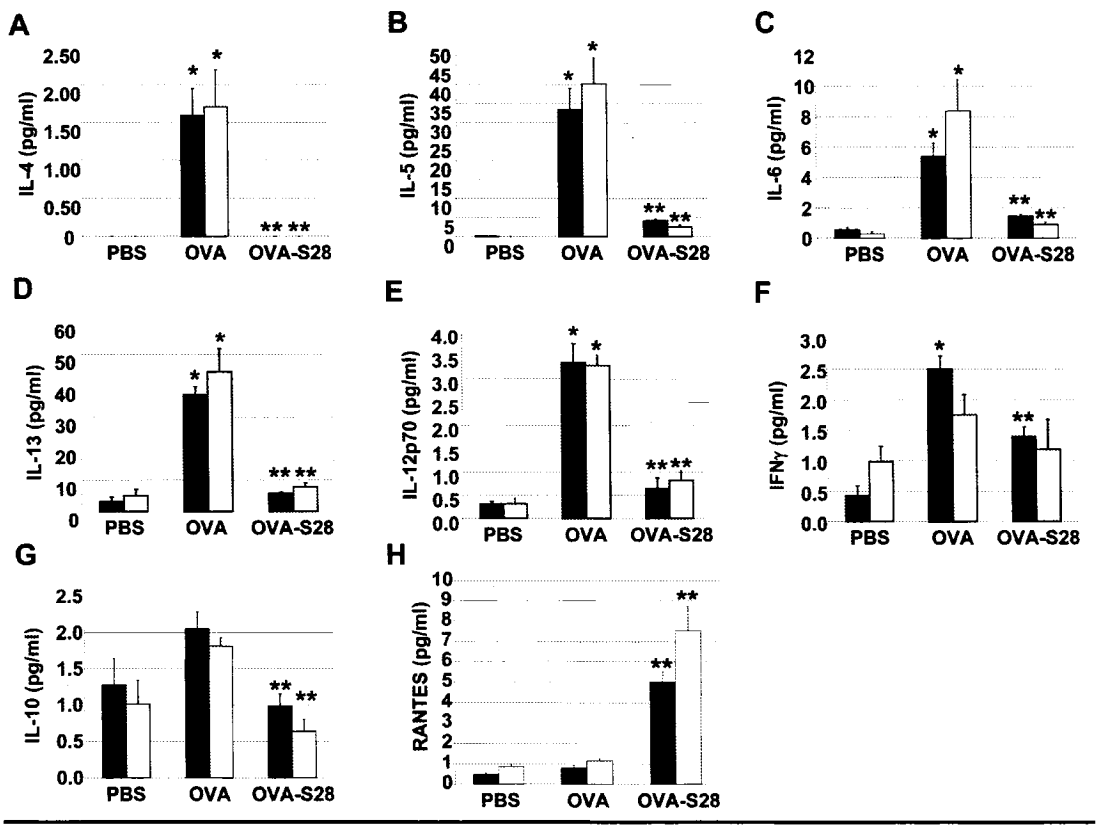

A
IL-4 (pg/ml)
2.50
2.00
1.50
1.00
0.50
0
PBS
OVA
OVA-S28
B
IL-5 (pg/ml)
50
45
40
35
30
25
20
15
10
5
0
C
IL-6 (pg/ml)
12
10
8
6
4
2
0
D
IL-13 (pg/ml)
60
50
40
30
20
10
0
E
IL-12p70 (pg/ml)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
F
IFNγ (pg/ml)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
G
IL-10 (pg/ml)
2.5
2.0
1.5
1.0
0.5
0.0
H
RANTES (pg/ml)
10
9
8
7
6
5
4
3
2
1
0

Figure 4: Chemokine mRNA Expression Following OVA Challenge. Total lung RNA from A/J (solid bars) and C57BL/6 (open bars) mice was purified using Trizol reagent. Real-time PCR analysis was performed using the Statagene MX-4000 apparatus. Levels of CCL5 (Panel A), CCL11 (Panel B), CCL17 (Panel C) and CCL24 (Panel D) mRNA were adjusted for differences in GAPDH expression and normalised to the levels of the A/J OVA-PBS group. Results are represented as mean ± SEM from three animals for each group (* represents $p<0.05$ between OVA-PBS and OVA-OVA group. ** represents $p<0.05$ between OVA-OVA and OVA-S28 group).

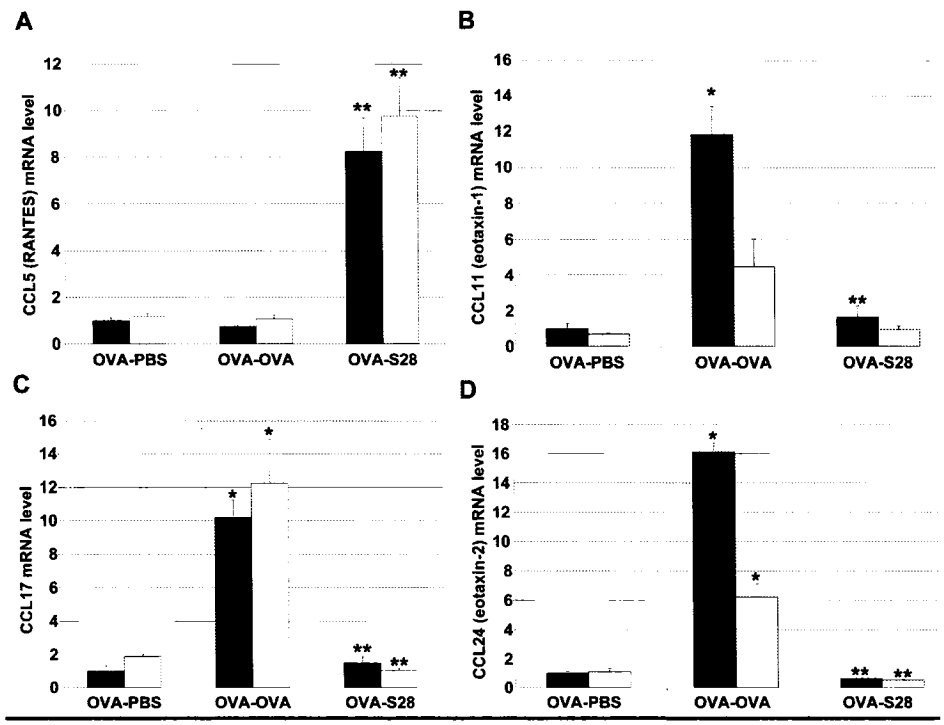

A
CCL5 (RANTES) mRNA level
12
10
8
6
4
2
0
OVA-PBS
OVA-OVA
OVA-S28
B
CCL11 (eotaxin-1) mRNA level
16
14
12
10
8
6
4
2
0
OVA-PBS
OVA-OVA
OVA-S28
C
CCL17 mRNA level
16
14
12
10
8
6
4
2
0
OVA-PBS
OVA-OVA
OVA-S28
D
CCL24 (eotaxin-2) mRNA level
18
16
14
12
10
8
6
4
2
0
OVA-PBS
OVA-OVA
OVA-S28

Figure 5: BAL Analysis. Analysis of inflammatory cells found in bronchioalveolar lavages following OVA challenge. Total number of cells was increased in both A/J and C57BL/6 following OVA challenge (Panel A). Treatment with imidazoquinoline significantly inhibited influx of inflammatory cells to the lungs. Analysis of cell types in BAL from A/J (Panel B) and C57BL/6 (Panel C) revealed influx of eosinophil following OVA challenge which was prevented by TLR7 ligand treatment. Results for total BAL cell count are represented as mean ± SEM from three independent experiments (n=8 for all groups) (* represents $p<0.05$ between OVA-PBS and OVA-OVA group. ** represents $p<0.05$ between OVA-OVA and OVA-S28 group).

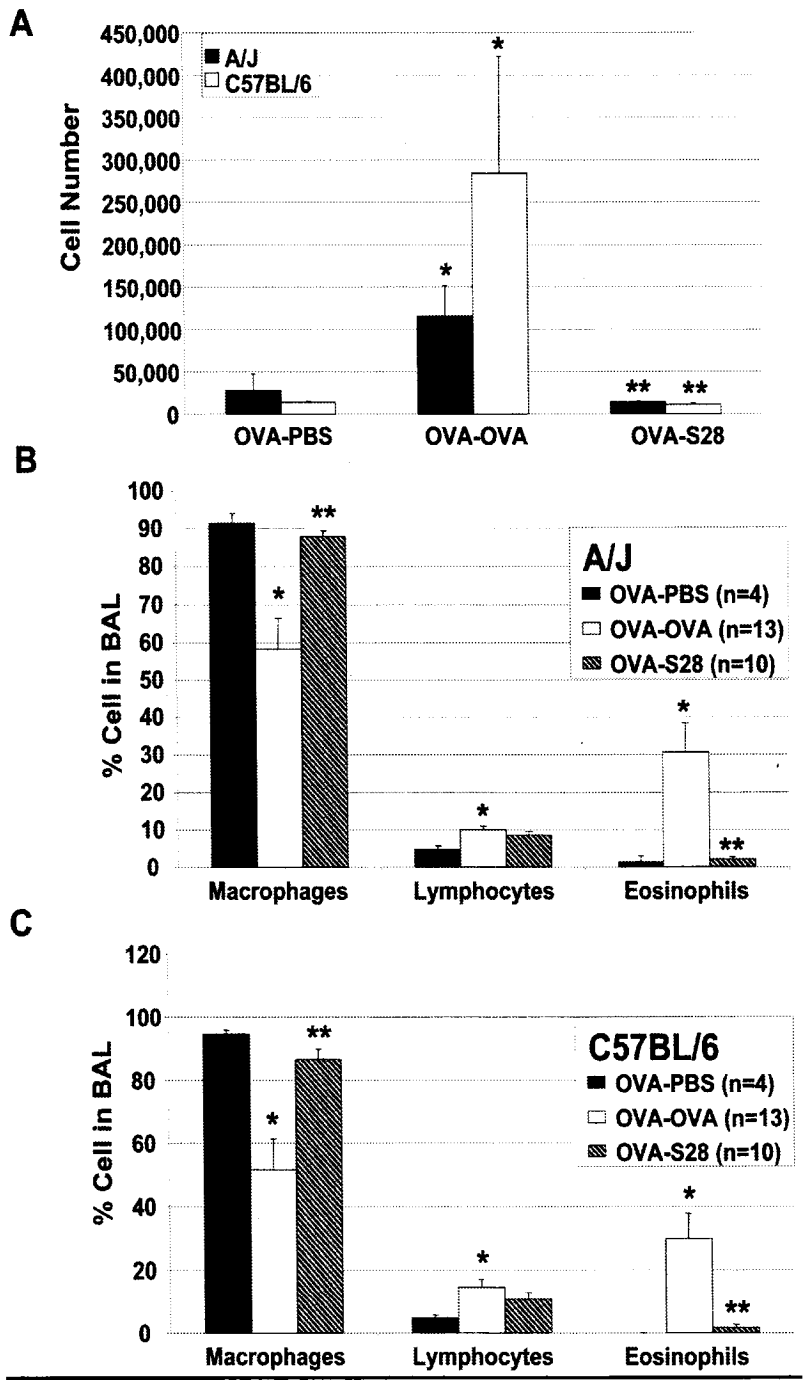

A
Cell Number
450,000
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
A/J
C57BL/6
OVA-PBS
OVA-OVA
OVA-S28
B
% Cell in BAL
100
90
80
70
60
50
40
30
20
10
0
A/J
OVA-PBS (n=4)
OVA-OVA (n=13)
OVA-S28 (n=10)
Macrophages
Lymphocytes
Eosinophils
C
% Cell in BAL
120
100
80
60
40
20
0
C57BL/6
OVA-PBS (n=4)
OVA-OVA (n=13)
OVA-S28 (n=10)
Macrophages
Lymphocytes
Eosinophils

Figure 6: Histological Analysis of Lungs from OVA Challenged mice. Pictures illustrate representative hematoxylin- and eosin- (H&E)-stained lung sections prepared from mice sensitized and challenged as described in the *Materials and Methods* section. The three top panels are from A/J mice, the middle panels represent lungs from C57BL/6 and the lower panels represent lungs from MYD88 knockout mice. The panels are representative lung sections of H&E stains (200X magnification) from OVA-PBS (Panel A, D and G), OVA-OVA (Panel B, E and H) and OVA-S28 (Panel C, F and I) mice 48 hours after challenge. OVA challenged A/J and C57BL/6 mice displayed a markedly increased cellular infiltration around blood vessels (indicated by black arrows) and airways when compared to both OVA-PBS and S28463 treated lungs. Lungs from $MYD88^{-/-}$ mice displayed a marked cellular infiltration following OVA challenge that could not be reversed by S28463 treatment.

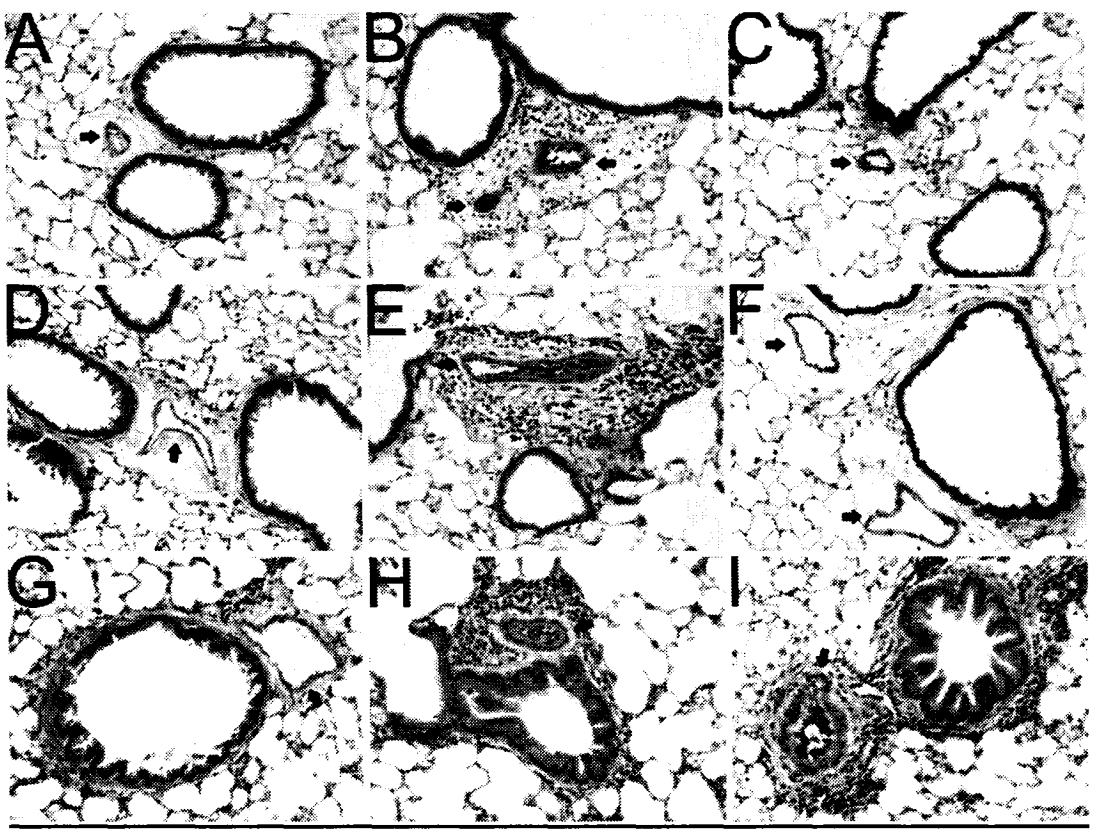
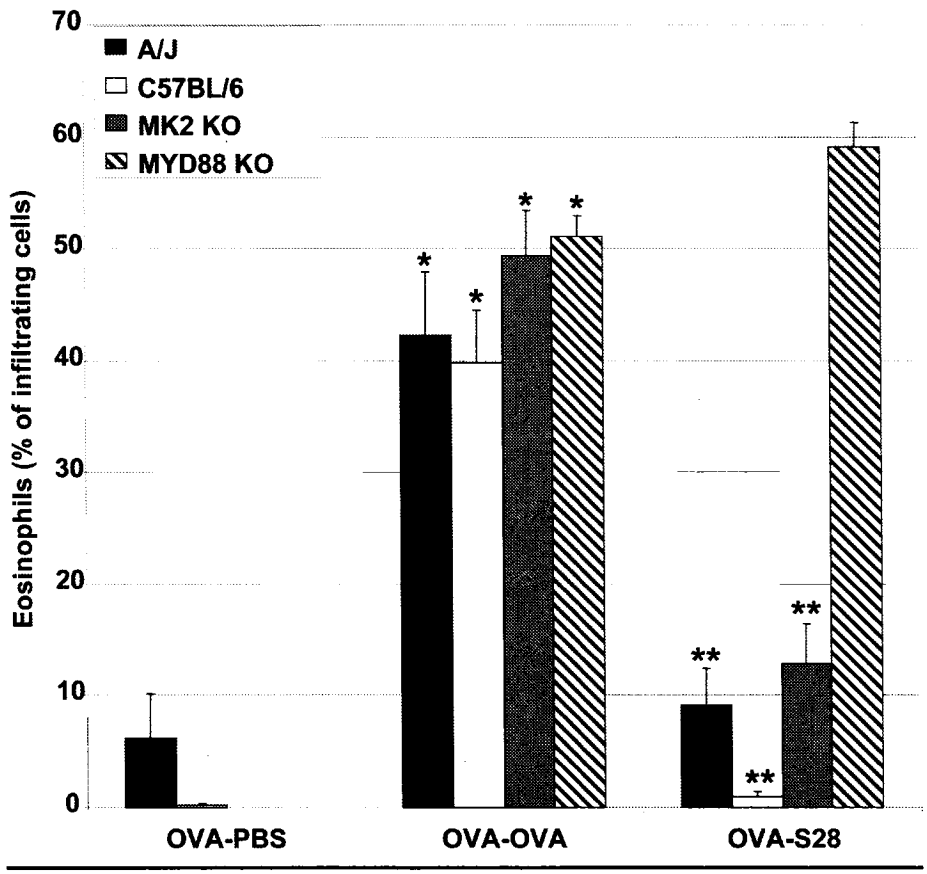

A
B
C
D
E
F
G
H
I

Figure 7: Lung Eosinophilia Following OVA Challenge is Inhibited by Imidazoquinoline Treatment. Imidazoquinoline treatment resulted in a dramatic reduction in eosinophil level following OVA challenge (Figure 7). Quantitation of Congo red positive cells revealed a similar amount of eosinophil infiltration in both A/J (n=8 for PBS, n=9 for OVA and n=7 for OVA-S28) and C57BL/6 (n=9 for PBS, n=8 for OVA and n=9 for OVA-S28) mice following OVA challenge that was prevented by S28463 treatment. $MYD88^{-/-}$ mice also displayed similar eosinophil infiltration following OVA challenge that could not be prevented by imidazoquinoline challenge (n=3 for all groups). MAPKAP-2 KO mice responded normally to S28463 treatment resulting in reduction of airway eosinophilia following OVA challenge (n=3 for PBS, n=3 for OVA and n=8 for OVA-S28). Data for A/J and C57BL/6 are represented as mean ± SEM from three experiments (n=8 for all groups) (* represents $p<0.05$ between OVA-PBS and OVA-OVA group. ** represents $p<0.05$ between OVA-OVA and OVA-S28 group).


70
60
50
40
30
20
10
0
A/J
C57BL/6
MK2 KO
MYD88 KO
Eosinophils (% of infiltrating cells)
*
*
*
*
**
**
**
OVA-PBS
OVA-OVA
OVA-S28

Chapter 5

Summary and General Discussion

The major findings presented in this thesis can be summarized as follows:

- Treatment of mice infected with *M. bovis* BCG with an immune modulator of the imidazoquinoline family of compounds results in a reduction of splenic mycobacterial counts after two weeks. Efficacy of treatment required the presence of a functional *Nramp1* allele as shown by the observation that *Nramp1* knockout mice respond only to a dose 250 times what is found to be effective in mice carrying a wild type allele of the *Nramp1* gene.

- Lack of imidazoquinoline treatment efficacy in *Nramp1* KO mice can be traced back to macrophage responsiveness to these TLR7 ligands. Macrophage cell lines derived from *Nramp1* KO mice displayed reduced production of nitric oxide and TNF following stimulation with imidazoquinolines or ssRNA of viral origin.

- Analysis of TLR7 signal transduction in *Nramp1* wild type and KO macrophages revealed a crucial role for P38 MAPK as well as atypical PKC for cytokine induction by imidazoquinoline. Interestingly, NRAMP1 function was required for full P38 MAPK activation following TLR7 ligand stimulation. Furthermore, levels of activated PKCζ in unstimulated *Nramp1* KO macrophages were significantly lower compared to their wild type counterparts. The modulation of P38 MAPK as well as PKCζ activity can account for the lack of treatment efficacy against *M. bovis* BCG infection in *Nramp1* KO mice.

- Immunomodulatory activities of imidazoquinolines were also successful in preventing the development of atopic allergic asthma using two strains of mice with different baseline lung responsiveness to methacholine challenge. Imidazoquinoline treatment prevented increased lung resistance and elastance observed following antigenic challenge. Increased serum IgE as well as both T_H1 and T_H2 cytokine production were completely abolished in animals receiving TLR7 ligand treatment. Recruitment of inflammatory cells, especially eosinophils, to the lungs was also completely prevented by imidazoquinoline treatment. Surprisingly, contrary to what we observed in the *M. bovis* BCG model, NRAMP1 function was not required for the protective effect of imidazoquinolines in mice against allergic asthma development.

Over the last few decades, many immunotherapeutic approaches have been developed to treat various infectious, autoimmune and allergic diseases. Immunotherapy for the treatment of cancer has also been the focus of intense research. Passive immunotherapy involves the transfer of immune cells, such as dendritic cells, or antibodies to ameliorate the disease status of a patient. This approach does not lead to any long lasting immunological memory. Active immunotherapy involves the development of a long lasting immune response through vaccination or broad immune mediators such as bacterial products or cytokine administration. The data presented in this thesis using imidazoquinoline administration falls in the active immunotherapy category. Imidazoquinoline have been shown to induce a long lasting effect in various disease models. Many patients receiving imiquimod treatment against HPV-induced warts

showed a markedly lower frequency of wart reappearance after the end of treatment [46]. Furthermore, relapse rates in HSV-2 infected animals were significantly decreased, even though the treatment had been discontinued [53]. Clearly, these observations demonstrate the ability of TLR7 ligands to induce a long lasting immunological state that is maintained even after the removal of the drug. Activation of other TLR, such as TLR4 and TLR9, has also shown long lasting immune effects in humans and animals. The pattern of receptor expression in certain organs or cell type as well as the toxicology of the ligands used will determine which TLR should be targeted for a particular disease. A good example would be the use of TLR agonist as a therapeutic approach against allergic asthma. Although both TLR7 and TLR9 ligands have been found to be beneficial in the treatment of allergic asthma in murine models (Chapter 4 and [301;307]), the high levels of TLR7 expression in the human lungs [320] and the lack of TLR9 expression on myeloid dendritic cells [120] suggests a greater potential for TLR7 ligands in the treatment of human allergic asthma. One of the major hurdles for the application of broad immunomodulators in the treatment of human diseases is the relatively high toxicity that may be encountered due to the induction of inflammatory mediators. Septic shock has long been known to be the result of systemic infection with mainly gram positive or negative bacteria which leads to the production of many inflammatory cytokines such as TNF resulting in massive organ failure and possibly death [321]. For a broad immunomodulator to be a candidate for human treatment, toxicity associated with immune system activation must be kept as minimal as possible. A monophosphoryl Lipid A (MPL) from *Salmonella Minnesota* has been found to retain immunomodulatory activity without the toxicity associated with LPS and is currently under consideration as a vaccine adjuvant [322]. To date, imidazoquinolines have been

approved only for the topical treatment of HPV-induced warts, superficial basal cell carcinoma and actinic keratosis. The data presented in this thesis has mainly employed a systemic administration of imidazoquinoline to affect disease state in either the spleen or the lungs (refer to chapter 2 and 4). Initial phase I clinical trials with imiquimod employed systemic oral administration of the drug. Of the three trials, two had weekly or bi-weekly schedules and another gave the drug on a daily basis with drug doses ranging from 25 to 500 mg [69;323;324]. Daily administration of the drug does not appear to be a feasible approach as it leads to severe side effects characterised by flu-like symptoms and lymphopenia. Low dose administration of imidazoquinolines either weekly or twice weekly appear to be a more promising approach for systemic treatment with imidazoquinolines. Another approach would be to deliver the drug locally. In the allergic asthma model presented in chapter 4, our preliminary data suggests that direct delivery of the drug to the lungs of the animals is as effective as systemic administration (data not shown). Using a nebulizer, such as the ones currently employed in human asthma treatment, levels of drug reaching the blood stream could be kept low while achieving high concentration in the target organ. We believe that oral or systemic drug administration is feasible in the models presented in this thesis and should warrant more research on the potential of imidazoquinoline in the treatment of allergic asthma and mycobacterial diseases in humans.

The data presented point to a role for NRAMP1 function in modulating macrophage responses to TLR7 ligands. Although the exact mechanism of action of NRAMP1 is still unknown even in terms of innate resistance to intracellular pathogen resistance, it is possible to propose some hypotheses on the interaction between the NRAMP1 and

TLR7 pathways based on previous findings as well as data from our laboratory. We have shown in chapter 3 that ssRNA, a TLR7 ligand, co-localises with the late endosomal marker LAMP1. A previous study has shown that MYD88 is recruited to LAMP1 positive vacuoles following imidazoquinoline stimulation [105]. NRAMP1 has also been detected in LAMP1 positive vacuoles [179]. Thus, both the TLR7 and the NRAMP1 pathway co-localise at the level of the late phagosomal compartment. To date, the most solid biological functions attributed to NRAMP1 is its ability to pump divalent cations as well as modulate phagosomal maturation of intracellular pathogen-containing vesicles [150;325]. We have shown in chapter 3 that NRAMP1 function was not required for proper intracellular targeting of TLR7 ligands in macrophages. A possibility that remains is that through divalent cation pumping, NRAMP1 modulates the redox potential of the macrophages, enhancing specific signal transduction pathways. It is well established that iron can catalyse the production of reactive oxygen intermediates (ROI) which then have the ability to modulate signal transduction [326]. One of the major effects of ROI on signal transduction is the inactivation of phosphatases by oxidation of a critical cysteine residue [327]. Previous observations have found that macrophages expressing a wild type allele of the *Nramp1* gene exhibited increased PKC activity compared to macrophages expressing a susceptible allele [199]. Furthermore, this increased PKC activity was dependent on reactive oxygen intermediate generation [328]. We have found that one of the PKC isoforms modulated by NRAMP1 is a member of the atypical PKC family (Chapter 3). One possible mechanism for modulation of TLR7 signalling by NRAMP1 could involve generation of higher ROI levels following TLR7 ligand treatment which would then deactivate a series of phosphatases leading to increased PKCζ activity as well as P38 MAPK activation. A potential drawback to this

model is the fact that we observe normal ERK1/2 MAPK activation following TLR7 treatment of *Nramp1* wt and KO macrophages. The exact reason why only one of two known redox sensitive MAPK pathways would be affected is still unclear. Studies looking at the levels of ROI as well as specific phosphatase activity should help clarify the veracity of this model. The use of iron chelators should also determine the exact contribution of iron levels to NRAMP1-mediated modulation of TLR7 signal transduction. Another possibility is that lack of NRAMP1 on the phagosomal membrane prevents the recruitment of signal transduction components to the vesicle leading to reduced activity of PKCζ and P38 MAPK. Further immunofluorescent microscopy analysis should be able to shed more light into this hypothesis. In the context of *M. bovis* BCG infection, it is possible that the block in phagosomal maturation and acidification which is observed in infected susceptible macrophages leads to the sequestration of the TLR7 ligands from their receptor located in LAMP1 positive late endosomal compartments (Chapter 3 and [149]). Macrophages expressing the wild type allele of *Nramp1* do not display this halt in phagosomal maturation and acidification of mycobacteria-containing vesicle which could lead to enhanced responsiveness to imidazoquinoline responsiveness *in vivo*.

The presented studies have defined a new role for NRAMP1 in modulating responsiveness to TLR7 ligands in murine models. The next important question will be to determine the exact contribution of NRAMP1 in human responsiveness to TLR7 ligands. The lack of a functionally null allele of *Nramp1* in humans suggests that the levels of NRAMP1 protein expression will be the determining factor in controlling

responses to imidazoquinolines. Indeed, promoter polymorphism affecting levels of *Nramp1* transcription should be studied for their association with imidazoquinoline responsiveness. The generally broader expression of NRAMP1 in PBMC in humans compared to mouse suggests that other cell types than macrophages may respond differently to TLR7 ligands [329].

Taken together, the data presented broaden the clinical potential of imidazoquinolines for the treatment of both mycobacterial infections and allergic asthma. We have also characterized the crucial role of NRAMP1 in modulating signal transduction to TLR7 ligands in macrophages which may affect treatment efficacy in macrophage-dependent models. Analysis of the role of human NRAMP1 in regulating responses to TLR7 ligands would be the next logical step to confirm the clinical importance of the presented findings. Further pre-clinical studies as well as toxicological analysis in humans will show the real potential of imidazoquinoline treatment against both infectious and allergic diseases. The presented data confirms the potential of broad immunomodulating agents in modifying existing immune responses to ameliorate various diseases. Defining pathways modulating responses to these compounds may provide, in the near future, another tool in the clinician's arsenal to improve the quality of life for patients with infectious, allergic and autoimmune diseases.

Contribution to Original Knowledge

The work presented in this thesis has broadened the clinical applicability of TLR7 ligands to the treatment of mycobacterial and allergic diseases. Furthermore, a role for NRAMP1 in modulating responses to imidazoquinolines will further our understanding of a patient's response to this class of compounds. The major original findings presented in this thesis are:

- The author was the first to describe that treatment with imidazoquinolines resulted in increased mycobactericidal activity in mice infected with *M. bovis* BCG.

- The author was the first to describe a role for NRAMP1 in modulating responses to imidazoquinolines and ssRNA, both TLR7 ligands. NRAMP1 function was required for optimal atypical PKC activity and P38 MAPK activation both of which are required for TLR7-induced cytokine production.

- The author contributed to demonstrating the potential of imidazoquinolines in preventing allergic asthma in mice. Our study focused mainly on *in vivo* lung and immunological parameters. The author was able to show that both lung resistance and elastance were reduced in mice treated with imidazoquinoline prior to antigenic challenge. Furthermore, the data demonstrated inhibition of both T_H1 and T_H2 cytokines which led to reduced inflammatory cell recruitment to the lungs.

Annex 1

Early Signalling Events Triggered by Toll-Like Receptor 7 Ligands in Murine Macrophages

Abstract

The imidazoquinoline family of compounds are immunomodulatory agents capable of inducing a wide spectrum of cytokines and activation of immune cells. Recently, signal transduction by these compounds was shown to be dependent on MYD88 and Toll-like receptor 7 (TLR7). To further study the mechanism of TLR7 signal transduction in macrophages, the requirement for cellular uptake and endosomal maturation prior to signal transduction was analysed. We demonstrate that a non-stimulating GpC-containing oligonucleotide can block cytokine induction by imidazoquinolines. Furthermore, inhibition of endosomal maturation by bafilomycin A1 leads to a block in cytokine production. We also report that the activation of ERK and P38 MAPK can be inhibited by bafilomycin A1. These findings demonstrate that TLR7 signaling requires uptake of the ligand followed by endosomal maturation of the vesicle before initiation of signal transduction.

Introduction

The discovery of the Toll family of receptors demonstrated their importance in the immune response against invading pathogens. These receptors are conserved across many species including *Drosophila*, mice and humans [330]. To date, twelve members have been characterised in mice (TLR1-9, 11-13) [331] and ten have been described in humans (TLR1-10) [330]. TLR4 was shown to be the main receptor involved in signal transduction initiated by lipopolysaccharides found at the surface of gram negative bacteria [76]. Similarly, TLR2 was described as being essential in the recognition of gram positive bacteria through lipoprotein and lipoteichoic acid recognition [331] as well as mycobacterial lipoarabinomannan [332]. TLR3 was found to play a major role in double-stranded RNA recognition [191] while TLR9 senses CpG-containing DNA motif occurring at a high frequency in bacterial DNA [100]. Recently, TLR7 has been shown to be important in mediating signal transduction by pharmaceutical compounds of the imidazoquinoline family [103]. Interestingly, single stranded RNA of viral origin appears to be the natural ligand for TLR7 [106;107;266].

The imidazoquinolines are a family of small compounds which have been shown to exhibit immunomodulatory effects both *in vivo* and *in vitro*. Numerous derivatives exist which vary in structure and potency. S27609 has a chemical structure comparable to other imidazoquinolines and induces a similar cytokine pattern being ten times more potent than imiquimod and ten times less potent than R-848 (S28463) [7]. Imidazoquinolines induce a wide spectrum of cytokines such as IFNα, TNFα, IL-1α, IL-1β, IL-6 and IL-12 [333]. Administration of these compounds tends to skew the

immune response towards a T_H1 phenotype [9]. Imidazoquinolines have been shown to have positive effects against infectious diseases including herpes simplex virus (HSV), *Leishmania spp.* as well as M. *bovis* BCG [10;23;48;59]. Imiquimod is currently used in the treatment of human papilloma virus (HPV)-induced warts.

Study of TLR4 and TLR9 signaling has suggested very different intracellular localisation of the receptors [110]. TLR4 signalling occurs mainly at the surface of the cell where the signal transduction events are initiated. Conversely, TLR9 appears to be located intracellularly within the endosomal/phagosomal compartment which leads to a requirement for the ligands to be taken within the cell for signal transduction to occur. Supporting this hypothesis is the observation that blockade of endosomal maturation by inhibiting endosomal acidification prevents activation of downstream signalling events by CpG DNA [109]. Furthermore non-stimulating oligonucleotides can compete for endosomal access and inhibit uptake of CpG DNA. We hypothesised that TLR7 might also be located intracellularly and that initiation of signal transduction would require prior endosomal maturation. Our data establish the importance of endosomal maturation in TLR7 signaling and sheds some light on the mechanism of initiation of signal transduction by the imidazoquinoline compounds.

Material and Methods

Reagents. S27609 was graciously provided by Dr. R. Miller and is the propriety of 3M Pharmaceutical. Lipopolysaccharide from *E. coli* serotype 0111:B4 and bafilomycin A1 were purchased from Sigma (Oakville, Ontario, Canada). Phosphorothioate stabilised CpG-containing oligonucleotide (5'-TCCATGACGTTCCTGACGTT-3') as well as GpC oligonucleotide (5'-TCCAATGAGCTTCCTGAGTCT-3') were synthesised and HPLC purified by Biosource International (Nivelle, Belgium). PhosphoPlus p44/p42 MAPK (Thr202/Tyr204), PhosphoPlus p38 MAPK (Thr180/Tyr182) and PhosphoPlus IκB-alpha (Ser32) Antibody Kit were purchased from Cell Signaling Technology (Beverly, MA).

Cells. Macrophage cell lines were derived from the bone marrow of C57BL/10A.*Nramp1*^{r} mice as previously described [334]. Cell lines were cultured as previously described [23] and plated at a concentration of $0.5x10^{6}$ cells/ml for stimulation. Peritoneal exudates cells were obtained by injecting 1ml of 4% Brewer Thioglycollate (VWR, West Chester, PA) solution intraperitoneally to C57BL/10A.*Nramp1*^{r} mice. Four days later, the peritoneal cavity was flushed using HBSS (Invitrogen) supplemented with 1% penicillin/streptomycin. Cells were spun down and red blood cells were lysed by adding 10-15ml of ACK lysing buffer (Cambrex, Belgium) and incubating for 3-5 minutes on ice. Cells were washed and plated onto 24 well plates at a concentration of $1x10^{6}$ cells/ml in RPMI1640 (Invitrogen) supplemented with 6% FBS and 1% penicillin/streptomycin. Cells were left to adhere

for 2 hours, washed and fresh media was added. All inhibitors were added 30 minutes prior to stimulation.

Cytokine Production Measurement. Supernatants from cells stimulated for the indicated amount of time were collected and spun at 13,000 rpm for 5 minutes. A fraction of the supernatant was used to measure NO_2^- concentration using the Griess method. TNFα production was measured using a sandwich ELISA described elsewhere [335].

Western Blotting. $6x10^6$ peritoneal exudates cells were plated in 4ml of RPMI1640 media supplemented with 6% FBS and 1% penicillin/streptomycin on 6-well plates (VWR). After 2 hours, the cells were washed and fresh media added. Following stimulation, the media was removed, the cells were washed with PBS and lysed in SDS-PAGE sample buffer containing 2.5% 2-mercaptoethanol. The plate was scraped and the samples were boiled for 5 minutes. The lysates were subsequently sheared using a 26G needle. Samples were loaded onto 10% Bis-Tris NuPAGE gels (Invitrogen). Following electrophoresis, semi-dry transfer was performed onto Immobilon-P membranes (Fisher, Nepean, Ontario, Canada). The membrane was subsequently blocked in 5% milk and probed with antibodies according to manufacturer's protocol.

Statistical Analysis. A Kruskal-Wallis one way analysis of variance on ranks was performed using the SigmaStat software (SPSS, Chicago, IL) to calculate statistical

significance. Potential differences among various treatments are considered significantly different from the others when the p values calculated are lower than 0.05.

Results

Non-Stimulating GpC-Containing Oligonucleotides Inhibit TNFα and NO Secretion Induced by Imidazoquinoline in a Macrophage Cell Line. Analysis of CpG-induced cellular activation demonstrated that uptake of the oligonucleotide could be competed by adding a non-CpG containing oligonucleotide [109]. To test whether TLR7 signalling might require uptake of the imidazoquinoline compound for cytokine induction, bone-marrow-derived macrophage cell lines were pre-treated with a non-stimulating GpC-containing oligonucleotide prior to imidazoquinoline treatment. Pre-treatment with the oligonucleotide reduced, in a dose-dependent fashion, NO and TNFα production by macrophages following S27609 stimulation. The IC50 for the GpC oligonucleotide was approximatively 2.5μg/ml and 1 μg/ml for NO and TNFα respectively (Figure 1 panel A). At a concentration of 5 μg/ml of the GpC DNA, the induction of cytokines by imidazoquinoline was almost completely abolished. As expected, CpG DNA cytokine induction was also inhibited by the non-stimulating oligonucleotide. NO and TNFα secretion were also completely inhibited by a concentration of 5 μg/ml of the non-stimulating oligonucleotide with an IC50 of 1 μg/ml for both cytokines (Figure 1 panel B). To confirm that the non-stimulating oligonucleotide mechanism of inhibition occurs by actually preventing uptake of the imidazoquinoline into the cell, signalling through TLR4, a surface expressed receptor, was also analysed. Addition of the GpC DNA did not have any significant inhibitory effect on NO and TNFα secretion induced by LPS (Figure 1 panel C). These data

suggest that TLR7 signalling requires uptake of its ligand for cytokine induction to occur and that this uptake can be competed by a non-stimulating oligonucleotide.

Inhibition of Endosomal Maturation Prevents Cytokine Induction by S27609. Previous observations have suggested that TLR3, 7 and 9 might be located intracellularly [336]. In agreement with this hypothesis, CpG DNA was shown to require endosomal maturation for cytokine induction [109]. Since GpC DNA could inhibit cytokine production by TLR7 and TLR9 ligands, the requirement of TLR7 for endosomal maturation in macrophages was further studied. Endosomal acidification is a crucial step in the maturation of endosomes/phagosomes and blockade of this process has been linked with a block in maturation of the vesicles. Two known inhibitors of endosomal acidification are chloroquine and bafilomycin A1. Chloroquine acts as a base to neutralize the intravesicular pH [337], while bafilomycin A1 specifically inhibits the vesicular hydrogen ion transporter [338]. Pre-treatment of macrophage cell line with bafilomycin A1 resulted in a complete inhibition of TNFα secretion after 6 hours of treatment with S27609 (Figure 2 panel A). This observation was also reproduced in primary peritoneal macrophages. Longer time points were not performed because of the toxicity associated with long-term bafilomycin treatment. In accordance with previous reports, CpG cytokine induction was completely abolished by bafilomycin A1 treatment (Figure 2 panel B). Chloroquine pre-treatment also led to inhibition of TNFα secretion by CpG DNA (Figure 2 panel B). S27609 was resistant to this same dose of chloroquine (Figure 2 panel A). Higher concentration of chloroquine could inhibit TNFα secretion (data not shown), but this might be due to non-lysosomotropic effects that have been

reported for chloroquine at higher doses [339;340]. These observations suggest that TLR7 and TLR9 could reside in different vesicular compartments which show differential sensitivity to general pH neutralisation while being sensitive to inhibition of the vacuolar ATPase. LPS induction of TNFα was not inhibited by these two inhibitors. Bafilomycin A1 actually led to a small but significant increase in TNFα secretion while chloroquine treatment had no effect on LPS induction (Figure 2 panel C). These observations confirm the specificity of the inhibitory effect on TLR7 and TLR9 receptors.

Endosomal Maturation Inhibition Prevents MAPK Activation by a TLR7 Ligand. One of the earliest events following Toll-like receptor engagement involves the phosphorylation of MAPK as well as activation of the NFκB transcription factor [79]. Since these factors have also been shown to become activated following imidazoquinoline stimulation, we investigated whether blockade of endosomal maturation could block these events. S27609 stimulation as well as CpG stimulation of primary peritoneal macrophages led to phosphorylation of ERK (Figure 3 panel A, lane 2 and 5 respectively) and P38 MAPK (Figure 3 panel B, lane 2 and 5 respectively). Basal levels of phosphorylation in unstimulated cells were very low for both of these kinases (Figure 3 panel A lane 1 and Figure 3 panel B lane 1). Bafilomycin A1 treatment led to a complete inhibition of ERK and P38 MAPK phosphorylation by S27609 (Figure 3 panel A lane 4 and Figure 3 panel B lane 4, respectively). Conversely, pre-treatment of peritoneal macrophages with chloroquine did not prevent MAPK activation by imidazoquinolines, even though cytokine induction could be inhibited by this higher

dose. These findings suggest that the inhibition observed at higher doses of chloroquine occurs through an effect unrelated to the lysosomotropic effect of this inhibitor. As expected, CpG-induced activation of ERK and P38 MAPK were inhibited by bafilomycin A1 and chloroquine treatment (Fig3a lane 6 and 7; Fig.3b lane 6 and 7, respectively). Conversely, LPS activation of these MAPK was unaffected by inhibition of endosomal maturation (Figure 3 panel A lane 9 and 10; Figure 3 panel B lane 9 and 10). Taken together, these observations suggest that endosomal maturation is required prior to initiation of signal transduction from TLR7 and TLR9, which is not the case for the surface expressed TLR4.

Discussion

The data presented in this report demonstrate that cytokine induction by imidazoquinolines, known TLR7 ligands, can be blocked by adding a non-stimulating oligonucleotide. Furthermore, inhibition of endosomal acidification by bafilomycin A1 also resulted in a complete inhibition of cytokine production by imidazoquinolines. MAPK activation was also blocked by bafilomycin A1. These observations demonstrate that TLR7 signaling is preceded by non-specific uptake of its ligand followed by endosomal maturation prior to signal transduction.

The fact that a GpC-containing oligonucleotide can inhibit cytokine secretion following S27609 as well as CpG suggest that uptake of these two compounds could occur through a similar mechanism. The GpC oligonucleotide has no stimulatory activity on its own (data not shown). It has been observed by others that the non-stimulatory oligonucleotide could prevent uptake of a CpG DNA and hence inhibit its stimulatory activity [109]. The uptake mechanism does not seem to be restricted to a particular molecule as it seems responsible for internalisation of CpG DNA, GpC DNA as well as imidazoquinolines. As expected, TLR4 was unaffected by the GpC DNA owing to its plasma membrane localisation.

Endosomal maturation has previously been recognised as a crucial step preceding signal transduction initiated by CpG DNA [109]. Localisation of TLR9 to intracellular vesicles suggests that this step is required for the ligand to come in contact with the receptor [110]. Our data demonstrate that TLR7 also requires endosomal maturation prior to signal transduction in macrophages. The observation that imidazoquinoline appears

resistant to the lysosomotropic effects of chloroquine suggest that TLR9 and TLR7 might be within different intracellular compartments. Inhibition of cytokine production induced by imidazoquinolines by increasing concentration of chloroquine is not correlated with reduction in MAPK activation (Figure 3) which suggests that this inhibition occurs through another mechanism that does not require blockade of endosomal maturation. These findings are consistent with previously reported effects of chloroquine on MAPK activation and TNFα induction by LPS in various human and murine cells [339;340]. A recent study has demonstrated that chloroquine could partially inhibit NFκB activation by R-848 [341]. These seemingly contradictory data might be due to several factors. Firstly, a human B cell line as well as transfected HEK cells were employed compared with murine macrophages in the present study. Furthermore, the use of a relatively high dose of chloroquine might account for the apparent inhibition which could be independent of the effect of chloroquine on endosomal maturation. Conversely, specific inhibition of the vacuolar ATPase involved in endosomal acidification and maturation by bafilomycin A1 completely blocked cytokine production induced by imidazoquinolines and CpG DNA. However, no inhibitory effect by chloroquine and bafilomycin A1 was observed for LPS induced cytokine production (Figure 2 panel C) demonstrating the very different mechanism of signal transduction between TLR4 and TLR7/9.

The complete inhibition of cytokine induction following endosomal maturation blockade suggested that it was affecting a very early step in the signal transduction pathway. This hypothesis was confirmed when MAPK activation as well as NFκB activation were

analysed following imidazoquinoline stimulation. Interestingly, endosomal maturation inhibition led to a complete block in both P38 and ERK MAPK phosphorylation. These results clearly point out that inhibition of endosome maturation leads to a block in the activation of the earliest events associated with signal transduction normally initiated by the imidazoquinolines.

Taken together, our data show that TLR7 requires endosomal processing for signal transduction to occur. This observation suggests some common feature between TLR7 and TLR9 in the initiation of signaling while showing some difference in the susceptibility of their compartment to pH neutralisation. Our data shed some light into the mechanism of signal transduction by TLR7 and imidazoquinolines which may provide hints as to the nature of its natural ligand.

Figure 1: Effect of GpC DNA on NO and TNFα production induced by Toll ligands in macrophages. B10A.*Nramp1*^{r} macrophage cell line were stimulated for 18 hours with 250 ng/ml S27609 (Panel A), 1 μg/ml CpG DNA (Panel B) or 50 ng/ml LPS (Panel C) with or without various concentrations of non-stimulating GpC DNA. NO production was analysed using the Greiss method and TNFα secretion was measured by ELISA. Results are presented as mean+SD from one experiment which was representative of three independent experiments (n=7 for all groups).

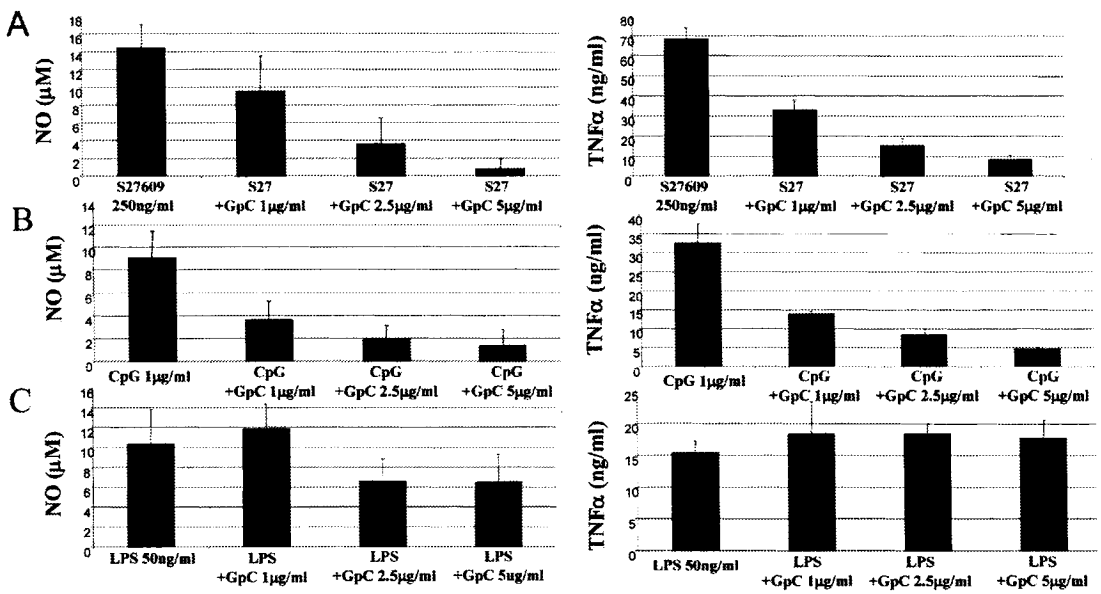

A
NO (μM)
S27609 250ng/ml
S27 +GpC 1μg/ml
S27 +GpC 2.5μg/ml
S27 +GpC 5μg/ml
TNFα (ng/ml)
B
CpG 1μg/ml
CpG +GpC 1μg/ml
CpG +GpC 2.5μg/ml
CpG +GpC 5μg/ml
C
LPS 50ng/ml
LPS +GpC 1μg/ml
LPS +GpC 2.5μg/ml
LPS +GpC 5μg/ml

Figure 2: Effect of blockade of endosomal maturation on TNFα induction by Toll ligands. B10A.*Nramp1*^{r} macrophage cell line were stimulated for 6 hours with 100 ng/ml S27609 (Panel A), 1μg/ml CpG DNA (Panel B) or 50 ng/ml LPS (Panel C). Chloroquine (Chl) (1μg/ml) and bafilomycin A1 (Baf) (100 nM) were added 30 minutes prior to stimulation. TNFα secretion was measured using a sandwich ELISA. Results are presented as mean+SD from one experiment which was representative of three independent experiments (n=9 for all the groups).

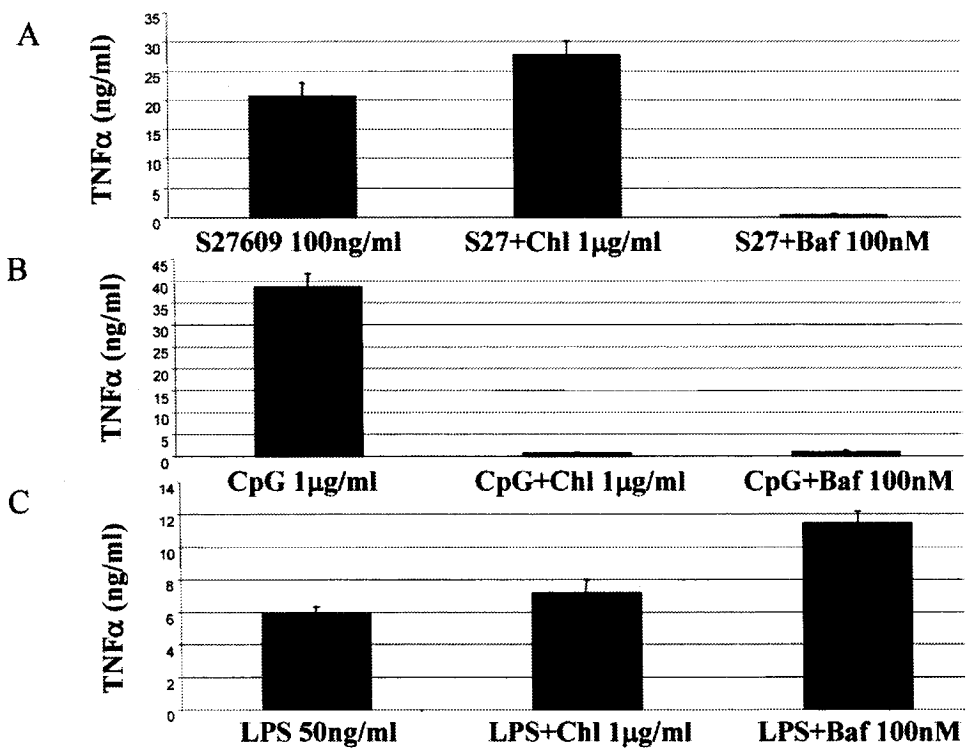
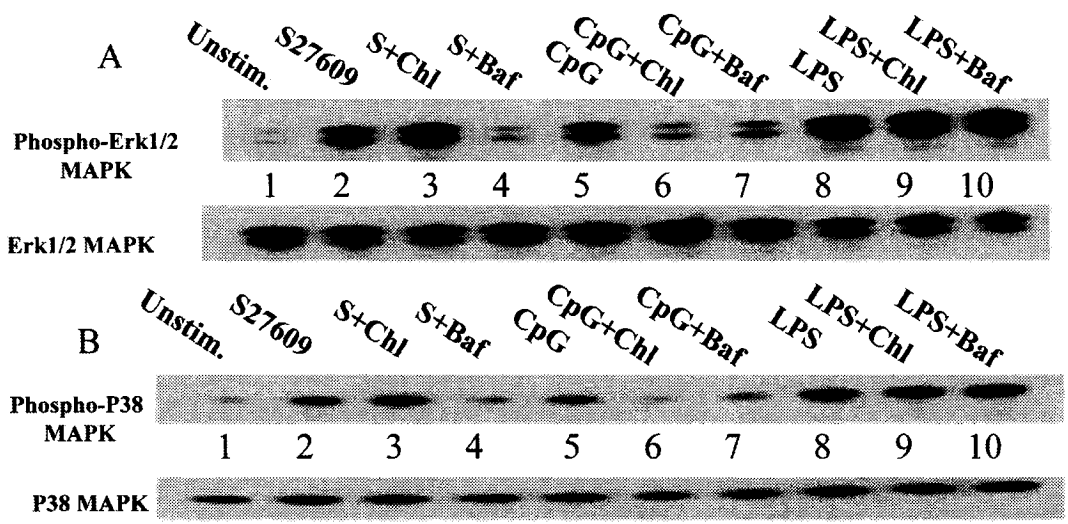

A
TNFα (ng/ml)
35
30
25
20
15
10
5
0
S27609 100ng/ml
S27+Chl 1μg/ml
S27+Baf 100nM
B
TNFα (ng/ml)
45
40
35
30
25
20
15
10
5
0
CpG 1μg/ml
CpG+Chl 1μg/ml
CpG+Baf 100nM
C
TNFα (ng/ml)
14
12
10
8
6
4
2
0
LPS 50ng/ml
LPS+Chl 1μg/ml
LPS+Baf 100nM

Figure 3 Effect of endosomal maturation on MAPK activation by Toll ligands.

Primary peritoneal macrophages were stimulated for 45 minutes with S27609 (1 μg/ml), CpG DNA (10 μg/ml) and LPS (1 μg/ml). Bafilomycin A1 (Baf) (100 nM) and chloroquine (Chl) (5 μg/ml) were added 30 minutes prior to stimulation. Whole cell lysates (10 μg/lane) were analysed by Western blot using antibodies specific for the phosphorylated form of ERK and P38. The membrane were subsequently stripped and blotted with antibodies that recognise these proteins independently of their phosphorylation status. Data presented is representative of three independent experiments.


A
Unstim.
S27609
S+Chl
S+Baf
CpG
CpG+Chl
CpG+Baf
LPS
LPS+Chl
LPS+Baf
Phospho-Erk1/2 MAPK
1 2 3 4 5 6 7 8 9 10
Erk1/2 MAPK
B
Unstim.
S27609
S+Chl
S+Baf
CpG
CpG+Chl
CpG+Baf
LPS
LPS+Chl
LPS+Baf
Phospho-P38 MAPK
1 2 3 4 5 6 7 8 9 10
P38 MAPK

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Appendix 1:

Ethics Certificates