The role of Rip2 kinase and bacterial infections in shaping innate and adaptive immune responses

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Presented by
CHIARA NEMBRINI
Dipl. Natw. ETH
born August 7, 1980
citizen of Camorino (TI)

Accepted on the recommendation of
Prof. Dr. Manfred Kopf, examiner
Dr. Benjamin J. Marsland, co-examiner
Dr. Federica Sallustio, co-examiner

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1. Summary

Innate and adaptive immune responses tightly regulate each other to ensure a high degree of protection against pathogens or appropriate responsiveness to allergens. Several effector proteins play a crucial role in the initiation of both innate and adaptive immune responses. One such protein is the kinase Rip2, which was initially described to induce inflammation upon Toll-like and Nod-like receptor stimulation, as well as to mediate signaling downstream of the T cell receptor. In the first part of this thesis, we sought to study the role of Rip2 in regulating T cell activation, proliferation and cytokine production in response to a variety of stimuli. Moreover, we investigated the importance of Rip2 kinase activity in mediating signaling downstream of Nod1 and Nod2. Our results show that Rip2 kinase is dispensable for the generation of adaptive T cell responses both in vitro following polyclonal or specific antigen stimulation, as well as in vivo upon LCMV or *Listeria monocytogenes* infections. We could further demonstrate that the kinase activity of Rip2 is critical for the stability of the protein. Bone marrow-derived dendritic cells expressing kinase-dead Rip2 mutants displayed decreased Rip2 protein levels and impaired inflammation in response to Nod1 and Nod2 stimulation. Similar results were obtained in vivo, suggesting that kinase-dependent Rip2 autophosphorylation might represent an important regulatory mechanism for the induction of Nod1- and Nod2-dependent immune responses. Our data thus challenge the importance of Rip2 kinase in the initiation of adaptive immune responses, but highlight a role for its kinase activity in regulating innate immunity.

Exposure to bacteria and their products, especially early in life, has been associated with decreased incidence of allergy, indicating that environmental factors have a strong impact on shaping the development of immunity or disease. However, the mechanisms underlying these beneficial effects remain poorly understood. As described in the second part of this thesis, we developed a mouse model in which bacterial challenge in the lung suppressed allergic airway inflammation. We found that intranasal administration of *Escherichia coli* could inhibit airway hyperresponsiveness, lung eosinophilia and effector Th2 immune responses in a TLR4 dependent way, but independently of Th1 or regulatory T cells. Moreover, *E.coli*-treated mice displayed impaired dendritic cell maturation and function in the lung, but not in the draining lymph node in the activation of effector Th2
cells. Of note, we also found that *E.coli* treatment induced high frequencies of γδ T cells in the respiratory tract, and further demonstrated that these bacterially-induced cells play an important role in the regulation of airway hyperresponsiveness. Taken together these data reveal a novel pathway of immunoregulation in the airways which has important implications for both basic lung immunology and the development of new therapeutic strategies.
2. Riassunto

Le risposte immunitarie innate e specifiche interagiscono fortemente allo scopo di proteggere l’individuo da agenti patogeni e assicurare reazioni appropriate a sostanze allergiche. Diverse proteine sono implicate nell’attivazione di entrambe le risposte immunitarie. Una di queste è la chinasi Rip2, che fu inizialmente descritta come una proteina molto importante per la trasduzione di segnali inviati dai recettori Toll-like e Nod-like e per l’attivazione dei linfociti T. In questa tesi di dottorato abbiamo studiato il ruolo di Rip2 nel regolare l’attivazione, la proliferazione e la produzione di citochine da parte di linfociti T in risposta a vari stimoli. Inoltre abbiamo analizzato in dettaglio l’importanza dell’attività fosforilatrice di Rip2 nella trasduzione di segnali cellulari indotti dai recettori Nod-like Nod1 e Nod2. I nostri risultati indicano che la chinasi Rip2 non è necessaria per lo sviluppo di risposte immunitarie specifiche in vitro come pure in vivo in risposta ad infezioni causate da LCMV e da *Listeria monocytogenes*. Abbiamo poi dimostrato che l’attività di chinasi di Rip2 è essenziale per mantenere la stabilità della proteina. Infatti, il livello di Rip2 è fortemente ridotto in cellule dendritiche che esprimono una variante di Rip2 in cui l’attività di chinasi è compromessa. Lo stesso risultato è stato confermato in vivo con topi Rip2 knock-in, portandoci alla conclusione che l’autofosforilazione di Rip2 è un meccanismo importante per il controllo di risposte immunitarie indotte dall’attivazione di Nod1 e Nod2. Per questo motivo i nostri risultati mettono in discussione il fatto che Rip2 sia necessaria per l’attivazione di cellule T effettive, e contemporaneamente sottolineano l’importanza della fosforilazione di questa proteina nel controllare le risposte immunitarie innate.

Il diretto contatto con batteri e derivati è stato più volte associato ad una minore probabilità di sviluppare risposte allergiche, attribuendo perciò a determinati fattori ambientali un ruolo considerevole nell’influenzare il sistema immunitario. Tuttavia i meccanismi responsabili di questi effetti benefici rimangono fino ad oggi senza una convincente spiegazione. Come descritto nella seconda parte di questa tesi, abbiamo sviluppato un modello in cui la somministrazione per via intranasale di batteri porta ad un’abolizione di successive risposte allergiche. I topi trattati con *Escherichia coli* mostrano minori sintomi allergici quali iperreattività bronchiale, infiltrazione di eosinofili nei polmoni e attivazione di linfociti T helper 2. Come abbiamo potuto appurare, queste
diminuzioni dipendono dalla stimolazione del recettore TLR4, ma non dall’aumentata attivazione di cellule effettrici T helper 1 o cellule T regolatorie. Abbiamo poi constatato che in seguito alla somministrazione dei batteri, lo stato di maturazione e la funzionalità delle cellule dendritiche residenti nei polmoni, ma non di quelle dei linfonodi drenanti, è ridotta in maniera significativa. Inoltre, abbiamo osservato che l’inalazione di batteri causa nelle vie respiratorie un afflusso di linfociti T γδ, che sembrano avere un ruolo molto importante nel controllo dell’iperreattività bronchiale. In conclusione, i nostri risultati svelano un nuovo meccanismo regolatorio che ha implicazioni importanti non solo per le risposte immunitarie dei polmoni in generale, ma anche per lo sviluppo di nuove terapie.
3. General introduction

3.1 The immune system

The environment to which every individual is exposed bears foreign invaders that represent a constant threat to the organism’s health. The immune system is a remarkably efficient defense system that has evolved to recognize these invaders, and protect the host from the development of diseases. In addition, the immune system has the capacity to respond not only to foreign agents, but also to cellular stress or cancer. The importance of the immune system becomes evident when some of its components are missing or impaired in their function, leading to chronic diseases or autoimmunity. The immune system can be divided into two different arms, which together ensure a high degree of protection: the first component is innate immunity, which represent the first line of defense against infections; the second component is the adaptive immune response which is characterized by a high degree of specificity and immunological memory.

3.2 Innate immunity

The anatomical barriers of the skin and the mucosa represent the very first line of defense, whereby low pH, secretion of antimicrobial substances as well as mucous production act to trap potential invaders and prevent their entry into the host. However if a pathogen crosses these barriers, other effector molecules and cells must come into play. Phagocytic cells such as neutrophils and macrophages play an important role in the uptake of pathogens, and in the induction of inflammatory responses; the serum proteins of the complement system can facilitate phagocytosis and elimination of these invaders. Pathogens express pathogen-associated molecular patterns (PAMPs) that are recognized by receptors on innate immune cells called pattern-recognition receptors (PRRs). Binding of PAMPs to these receptors will induce a signaling cascade inside the cell that will eventually lead to the transcription of several inflammatory genes. Activation of PRRs is
crucial for the initiation of innate immunity, which strongly influences ensuing adaptive immune responses.

3.2.1 Toll-like receptors, RIG-I-like proteins, and Nod-like receptors

The most prominent and extensively studied family of PRRs consists of the Toll-like receptors (TLRs), which were first described in *Drosophila melanogaster*. Toll-like receptors are transmembrane receptors expressed on the cell surface or within endosomes, and recognize components of bacteria, viruses, fungi and protozoan via Leucine-rich repeats (LRRs). In particular TLR2 and 4 recognize the bacterial cell wall components peptidoglycan and lipopolysaccharide (LPS), respectively; TLR5 binds flagellin; TLR9 bacterial and viral DNA; TLR3 and 7 recognize viral double-stranded and single-stranded RNA, respectively (1). Upon binding of microbial products to TLRs, a signaling cascade is initiated inside the cell that culminates in the activation of inflammatory genes and development of appropriate immune responses to fight the invader. Briefly, two major signaling pathways have been described: the first one relies on the intracellular adaptor molecule MyD88 and leads to the activation of NF-κB as well as MAP kinases, whereas the TRIF-dependent signaling pathway culminates with the activation of type I interferons, and plays an important role mainly in the course of viral infections (2).

Another group of PRRs playing a role during viral infections consists of the recently discovered RIG-I-like proteins (RLRs), RIG-I and MDA5 (3). RIG-I-like proteins are located in the cytoplasm and recognize double-stranded RNA. Similarly to the anti-viral TLR3, 7 and 9, RLRs activate NF-κB and IRF3 and induce the production of type 1 interferons; however in contrast to TLRs, they are expressed by all cell types. It is therefore likely that TLRs and RIG-I-like proteins act together to ensure a high degree of protection in the course of viral infections (4).

The Nod-like receptors (NLRs) are the components of a third family of PRRs. NLRs are cytosolic proteins composed of an effector CARD-, Pyrin or BIR domain, which mediates binding to other proteins containing the same domain; a nucleotide binding domain, important for self-oligomerization; and Leucine-rich repeats, which like TLRs might be involved in microbial recognition (5). NLRs have been linked to sensing of
microbial components in the cytosol and the induction of inflammatory responses; Nod1 and Nod2 (discussed later) have been shown to activate NF-κB and MAP kinase, whilst Ipaf, NALP1 and NALP3 are responsible for caspase-1 activation and IL-1β secretion (6). In particular the latter molecules have been shown to oligomerize and recruit other adaptor molecules in a signaling complex called the ‘inflammasome’ (7). Assembly of this complex in turn leads to the recruitment and activation of caspase-1, which processes pro-IL-1β and pro-IL-18 into the effector inflammatory cytokines IL-1β and IL-18 (8). Importantly, production of pro-IL-1β and pro-IL-18 typically requires TLR stimulation, highlighting the interplay between the different PRRs in the induction of inflammation (4). The Ipaf inflammasome is activated by intracellular pathogens such as *Salmonella typhimurium*, whereas assembly of the NALP3 inflammasome is stimulated by microbial products in combination with ATP, bacterial RNA, as well as endogenous molecules such as monosodium urate (9). However, the exact mechanisms through which these NLRs sense their activating compounds still remain to be elucidated.

### 3.2.2 Nod1 and Nod2

Unlike the inflammasome components, the ligands for the CARD-containing NLRs Nod1 and Nod2 are known. Nod1 recognizes the dipeptide γ-D-glutamyl-meso-diaminopimelic acid (*meso*-DAP), expressed by most Gram-negative bacteria and some Gram-positive bacteria such as *Listeria monocytogenes*; Nod2 senses muramyl dipeptide (MDP), which is a common component of all bacterial cell walls (10). In mice Nod1 has been shown to play a role in host defense against invasive pathogens such as *Helicobacter pylori* and *Escherichia coli* (11, 12), and Nod2 deficient animals were susceptible to oral infection with *Listeria monocytogenes*, but could control bacterial growth upon intravenous infection. These results indicate that Nod1 and Nod2 play an important role in conferring protection against bacterial infections. It could be further speculated that TLR signaling may be sufficient for the induction of inflammation, and Nod1 and Nod2 might come into play when the role of TLRs is bypassed or less pronounced (9). In support of this hypothesis a recent publication by Kim et al. highlights the importance of Nod1- and
3. General introduction

Nod2-mediated inflammation following infection with *Listeria* in mice previously tolerized to TLR signaling (13).

The importance of Nod1 and Nod2 at mucosal surfaces is further underlined by the outcome of mutations in their genetic sequence, which are associated with higher susceptibility to inflammatory bowel diseases (14-16). The mechanisms underlying this process are not clear, but the increased inflammation observed in Crohn’s disease patients bearing mutations in Nod2 might correlate with inappropriate responses to commensal bacteria in the intestine (17-19). In addition to the intestinal diseases, mutations in Nod1 and Nod2 gene sequences have also been associated with the incidence of allergy and asthma (20-23). Interestingly, the so called ‘hygiene hypothesis’ (discussed later) highlights the beneficial effects bacterial exposure might have in preventing the development of allergic and asthmatic responses. It is therefore tempting to speculate that an altered NLR signaling due to gene mutations might lead to the disappearance of the inhibitory effects caused by the bacteria. Of note, we initially developed our model of asthma modulation upon bacterial administration according to this hypothesis, and sought to test it in mice deficient in Nod1 and Nod2 signaling.

As previously mentioned, stimulation of Nod1 and Nod2 leads to NF-κB as well as MAP kinase activation, and this process has been shown to be entirely dependent on the Rip2 kinase (24).

3.2.3 **Rip2**

Rip2, also known as RICK or CARDIAK, is a widely expressed serine/threonine kinase composed of an N-terminal kinase domain, an intermediate domain and a C-terminal CARD-domain, also expressed by Nod1 and Nod2. Initial studies indicated Rip2 was a kinase involved in NF-κB activation as well as apoptosis (25-27). Subsequent publications suggested a role for Rip2 in mediating signaling downstream of TLRs, the T cell receptor, as well as Nod1 and Nod2 (28-31). In particular, macrophages deficient in Rip2 showed impaired ability in secreting inflammatory cytokines in response to TLR2 and TLR4 signaling, and T cells from Rip2 knock out mice displayed impaired proliferative capacity in response to different stimuli. Recently however, we and others
failed to reproduce these results, as no defect in T cell activation and cytokine production could be observed in vitro and in vivo (32-34). Moreover, other investigations further challenged the role of Rip2 in mediating TLR signaling, rather attributing it to Nod1 and Nod2 contaminants in the ligand preparations (24). Indeed, it is now established that induction of Nod1- and Nod2-mediated inflammation strictly depends on Rip2 (19, 24, 35).

Figure 1. Rip2 in the signaling pathways downstream of Nod1 and Nod2 (Strober, Nat Rev Immunol, 2006)

Binding of meso-DAP and MDP leads to oligomerization of Nod1 and Nod2, respectively, and the subsequent binding of Rip2 through their respective CARD domains (36). This induces the activation of Rip2, and culminates with the activation of NF-κB and MAP kinases (Fig.1, 19, 37, 38). Recently the signaling pathways underlying this process have started to be elucidated; activation of Rip2 requires its ubiquitinylation, which has been shown to be essential for NF-κB activation (39, 40). This subsequently induces the recruitment and activation of TAK1 (41, 42), which has been previously implicated in NF-κB as well as MAP kinase activation (2, 43). The next step is the interaction of Rip2 and TAK1 with the IKK complex and the ubiquitinylation of IKKγ, also known as NEMO, a
process that leads to the phosphorylation and degradation of the NF-κB-inhibitor IκB-α (44, 45). NF-κB can thus translocate to the nucleus and initiate the transcription of inflammatory genes. Of note, two recent reports suggest the negative regulation of Rip2-dependent NF-κB activation by the ubiquitin-editing enzyme A20, as well as caspase-12 (46, 47). In contrast to NF-κB, the mechanisms leading to MAP kinase activation via Nod1/Nod2 signaling and Rip2 activation are not clarified, but might involve the adaptor protein CARD9 (48).

The role of Rip2 kinase activity in mediating signaling downstream of Nod1 and Nod2 is still controversial and poorly understood. Several investigations suggested that Rip2 might rather act as an adaptor molecule, rendering its kinase activity dispensable to exert its function (19, 30, 39, 44). However, most of the experiments leading to this conclusion were performed in vitro with overexpression systems, which may give raise to artifacts. Moreover, in line with a recent report (42), we could show that mutations rendering the kinase domain of Rip2 inactive lead to instability of the protein and consequently to impaired Nod1 and Nod2-dependent signaling and inflammation. These results suggest a role for Rip2 autophosphorylation as an important regulatory mechanism ensuring protein stability and functionality.

### 3.3 Adaptive immunity

Adaptive immune responses represent the second arm of defense of an organism against infection. In contrast to innate immune responses, adaptive responses are not immediate, and need more time to develop (normally 5 to 6 days). However this delay is compensated for by a high degree of specificity and diversity: B and T cells, the effector cells of adaptive immunity, have rearranged their receptors to ensure optimal recognition of the most diverse antigens and to induce a highly specific immune response to protect the host. Moreover, the first encounter with a certain antigen generates immunological memory which will induce a significantly faster and stronger response upon a second challenge with the same antigen. It is important to mention, as it is also described in this thesis, that adaptive immune responses are extensively influenced by innate immune responses, and vice versa.
As mentioned above, effector cells of the adaptive immune system include B and T cells. B cells are generated in the bone marrow and their specific receptor consists of a membrane-bound antibody molecule. An antigen binding directly to this antibody on the surface induces activation of the B cell, which can eventually differentiate into a memory B cell or an effector, antibody-secreting B cell, called plasma cell. Antibodies play a crucial role in protecting the host during viral, bacterial and parasitic infections; antibody-coated bacteria and helminths can readily be eliminated by the host, and virus-neutralizing antibodies prevent infection of host cells.

In contrast to B cells, T cells cannot recognize the antigen directly, which instead needs to be processed into smaller peptides that bind to MHC molecules expressed on the surface of professional antigen presenting cells (APCs) such as dendritic cells, or alternatively by infected cells. The T cell receptor will then bind this peptide-MHC complex and lead to T cell activation.

T cell precursors are generated in the bone marrow and subsequently migrate to the thymus, where they undergo selection steps to become functional T cells. In the thymus, T cells develop into common αβ T cells (97-99%) or γδ T cells.

### 3.3.1 γδ T cells

This latter T cell subset, discussed in the last part of this thesis, does not undergo positive and negative selection, and leaves the thymus to mainly colonize epithelial and mucosal surfaces such as the skin, the lung and the gut. The exact function of γδ T cells remains to be elucidated, but they have been shown to regulate immune responses by providing a first line of defense against certain bacterial infections, or by promoting tissue repair (49). Interestingly, in contrast to αβ T cells, γδ T cells display a low degree of specificity: in humans they have been shown to recognize low-molecular-mass pathogen-derived products, nucleotides derivatives, as well as self-antigens upregulated by infected or transformed cells such as MHC class I polypeptide-related sequence A (MICA), a class IB MHC protein expressed by stressed epithelial cells (50). For the poor similarity with αβ T cells, the lack of specificity and their localization in non-lymphoid tissues, γδ T cells have in fact been assigned rather to the innate arm of immune responses.
3. General introduction

3.3.2 αβ T cells

In the thymus, the majority of T cell precursors will develop into αβ T cells, which can be further divided into two cell subsets, CD4- and CD8-expressing T cells. CD8 T cells, also known as cytotoxic T lymphocytes (CTLs), are activated by antigens presented in the context of MHC class I molecules, which is generally expressed by all cell types. For antigen recognition by CD8 T cells, target cells must be infected with the pathogen and processed peptides endogenously loaded on MHC class I. Alternatively, cross-presentation of exogenous antigens to CD8 T cells by antigen presenting cells can also occur. Cytotoxic CD8 T cells eventually recognize infected cells or tumor cells, and eliminate them by releasing cytotoxic mediators such as perforin and granzyme, or by inducing apoptosis via the Fas-FasL pathway.

CD4 T cells recognize antigens presented by APCs on MHC class-II molecules, and play a crucial role in orchestrating immune responses through secretion of effector cytokines (discussed later). After binding of the T cell receptor on the peptide-MHC complex, a signaling cascade is initiated that eventually culminates in the nuclear translocation of NF-κB, and the subsequent transcription of genes important for T cell activation and function. Shortly after antigen recognition by the T cell receptor, specific residues in the intracellular domain of the TCR-accessory molecules CD3 and CD28 become phosphorylated, leading to the activation of phospholipase Cγ-1 and the release of second messengers from the plasma membrane. These activate protein kinase C-θ (PKC-θ), which in turn leads to the activation of CARMA-1. Of note, mice deficient in PKC-θ display impaired development of Th2 but not Th1 responses, highlighting the importance of an intact signaling machinery for the induction of specific effector functions in T cells (51). Upon activation of CARMA-1, the CARMA-1 signalosome is assembled; this signaling complex consists of Bcl-10 (which associates with CARMA-1 via CARD-domain), MALT1, TRAF2, TRAF6, TAK1 and IKK-γ. Assembly of this signaling machinery culminates with the activation of IκB and JNK, and of their downstream effector molecules NF-κB and AP-1, respectively (52). Interestingly, molecules such as TRAF2 and TRAF6 also play a role in innate immunity by mediating signaling downstream of Toll-like-, IL-1- as well as TNF-receptors (2). As previously mentioned, Rip2 was also suggested to mediate signaling during both innate and adaptive immune responses via the association with TLRs or Nod1/Nod2 and Bcl-10, respectively (28, 29,
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However, results discussed in this thesis as well as in recent publications question some of these findings (24, 33, 34).

### 3.3.3. T helper cell subsets

Following T cell activation in response to pathogens and non-infectious antigens, naïve CD4 T cells can differentiate into four major effector T cell subsets with the aim to efficiently protect the host (Fig. 2). These subsets consist of effector Th1, Th2, Th17 as well as regulatory T cells, and the differentiation process is regulated by the presence of PAMPs, the antigen dose, the upregulation of costimulatory molecules and most importantly the cytokine milieu (53).

![Figure 2. CD4 T cell subsets (Marsland, Trends in Immunology, 2008)](image)

Th1 cells develop in response to viral and bacterial infections and are characterized by the production of IFN-γ and TNF-α, which induce host protection by activating macrophages and cytotoxic T cells. Differentiation of naïve CD4 T cells into effector cells is mainly induced by IL-12, however strong TCR stimulation and TLR activation have also been shown to induce IFN-γ producing cells, even in the absence of this cytokine (54, 55).
After activation of CD4 T cells in the presence of Th1-favoring conditions, the master regulator of this cell subset, the transcription factor T-bet, is activated and IFN-γ production induced (56). Inflammatory cytokines such as IL-18, IL-27 as well as IFN-γ itself can also induce IFN-γ production and therefore play an important role in Th1-dependent immune responses (54, 57). Importantly, Th1 can counteract Th2 immune responses via T-bet-dependent suppression of IL-4 secretion.

In contrast to the Th1 cell subset, Th2 cells are preferentially induced by low antigen doses or weak TCR stimulation, and their development is not directly linked to the presence of PAMPs (53, 58), although in some models induction of Th2 responses by low amounts of TLR ligands has been observed (59). However, most importantly Th2 cell development depends on IL-4, as mice deficient for this cytokine also lack Th2 effector cell subsets (60); IL-4 leads to Th2 differentiation by activating the transcription factors STAT6 and GATA-3 (61). Th2 cells secrete IL-4, IL-5, IL-13, IL-9 as well as GM-CSF, and play a crucial role in conferring protection against intestinal helminth infections. Th2 effector cytokines act on mast cells, epithelial cells, goblet cells, eosinophils and B cells to stimulate mucus production, smooth muscle contraction, IgE production and eventually expel the worm (62). In addition, Th2 cells are known be the major player in the pathogenesis of allergic diseases including asthma (discussed below). The early source of IL-4 responsible for Th2 cell development has long been questioned, as nearly all cells have been proposed as possible producers; interestingly, a recent publication by Sokol et al. suggests that basophils can be directly activated by proteolytic allergens to secrete IL-4 and induce Th2 cell differentiation (63).

The remaining CD4 T cell subsets, Th17 and inducible regulatory T cells, share the cytokine TGF-β as a crucial initiator of their differentiation. However, Th17 cells further require the presence of IL-6, and their development can be induced by TLR stimulation as well as high antigen doses; in contrast inducible Tregs are promoted by weak TCR signaling and poor co-stimulation (53, 64, 65). Retinoic acid has been shown to regulate the decision process in favor of inducible Tregs by inducing FoxP3 and suppressing Th17 development (66). Th17 cells are generally pro-inflammatory, secrete IL-17 (also known as IL-17A), IL-17F as well as IL-22, and have been involved in conferring protection against bacterial infections, but also in promoting autoimmune diseases (67, 68). STAT3 and the lineage-specific transcription factor ROR-γt have been identified as important
regulators of Th17 differentiation, and IL-23 has been shown to enhance cytokine production by effector Th17 cells (67).

As mentioned before, inducible Tregs can be generated from naïve T cells in the presence of TGF-β, retinoic acid as well as IL-2, and are characterized by the expression of the lineage-specific transcription factor FoxP3 (69). Their major role is to induce peripheral tolerance and to prevent excessive inflammation in the course of immune responses (70). Tregs secrete the inhibitory cytokines IL-10 and TGF-β, which can directly inhibit effector T cell function; however they have also been shown to exert their suppressive function via direct cell-contact or via CTLA-4-dependent down-regulation of dendritic cell maturation and function (71). Of note, inducible allergen-specific, IL-10-producing regulatory T cells have been shown to play an important role in the regulation of asthma (72), and their malfunction might lead to the aberrant Th2 responses typical of allergic individuals (discussed later).

3.3.4. Asthma

Asthma is a chronic allergic disease characterized by an aberrant immune response to normally harmless antigens (73, 74) that eventually cause wheezing, coughing, tightness of the chest and difficulty in breathing (75-77). The T helper 2 cell subset is the major player in the induction of classical disease features through the secretion of various cytokines (Fig. 3, 77). In particular, Th2 cells secrete IL-5 and GM-CSF that promote recruitment and survival of eosinophils and dendritic cells, whilst IL-4 and IL-13 induce upregulation of adhesion molecules to enhance cellular infiltration as well as goblet cell hyperplasia and consequently mucus production (78). The latter cytokines additionally induce B-cell isotype switching and IgE production (79). IL-9 is an important factor for mast cell development and proliferation, and together with IL-13 is responsible for enhanced smooth muscle cell activity and airway hyperresponsiveness (80, 81). Moreover, effector Th2 cytokine secretion leads to structural changes of the epithelium including collagen deposition, airway wall remodeling and tissue damage, which eventually cause hyperresponsiveness also to non-specific stimuli such as pollutants, physical stimuli and cold air (75, 82).
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Figure 3. Th2 cells induce classical asthma features such as airway eosinophilia, goblet cell hyperplasia, tissue remodeling and airway hyperresponsiveness (Hammad, Nat Rev Immunol, 2008).

In the lung, dendritic cells are organized in a dense network allowing optimal uptake of airborne particles (83). Upon antigen encounter, they readily migrate to the draining lymph node, where they activate specific recirculating naïve as well as central memory T cells to become effector T cells and migrate to the site of inflammation (Fig. 4, 84, 85). However, experiments with conditional dendritic cell deficient mice have shown that DCs also play a crucial role in the restimulation of previously activated Th2 cells and the generation of fully competent cytokine-producing effector cells in inflamed tissues (86).
3. General introduction

Figure 4. In asthmatic responses, DCs are crucial for antigen uptake and transport, T cell activation in the draining LN and restimulation in the periphery (Lambrecht, Nat Rev Immunol, 2003).

Under non-pathogenic conditions, exposure to harmless protein antigens in the lung leads to the induction of tolerance. Dendritic cells transport this antigen to the draining LN and initiate rapid T cell proliferation, however they fail to induce effector function and cytokine production by the same T cells (87, 88). The mechanisms underlying the generation of tolerance are controversial and might involve the activation state of the dendritic cells, the migration and antigen presentation by different DC subsets, as well as IL-10 production and regulatory T cell induction (89-93). As previously mentioned, asthma develops in response to harmless antigens, indicating that breaking of tolerance must occur. This was shown to take place when the allergen was administered together with low doses of a PAMP such as LPS (59). Contamination of allergens with TLR and NLR ligands such as LPS or peptidoglycan could thus lead to dendritic cell activation and represent a common mechanism for the initiation of an allergic immune response (82, 94). Moreover several allergens have been shown to induce dendritic cell maturation and to stimulate epithelial cells via their intrinsic proteolytic activity, a process that can also overcome the development of tolerance (95-97).
The incidence of asthma in individuals has a strong genetic component, however it is well recognized that environmental factors significantly contribute to disease development (73). Several asthma susceptibility genes have been described: these include genes involved in the induction of innate immunity such as TLR4 or Nod1 (21, 98), genes which are crucial for Th2 cell differentiation such as IL-4 and IL-13 (99, 100), and genes associated with epithelial cell homeostasis, airway structure and lung function such as CCL11, filaggrin, TNF or ADAM33 (101, 102). The association between asthma incidence and mutations in these genes highlights the importance of an intact epithelial barrier and lung homeostasis in protecting against airborne invaders, and the role Th2 immune responses play in regulating disease. Furthermore the involvement of pattern-recognition receptors emphasizes the influence environmental agents have in the pathogenesis of asthma. Accordingly, the contribution of certain gene polymorphisms to disease development might strongly depend on the environment the affected individual is exposed to.

Notably, the incidence of asthma has experienced a dramatic increase over the last 20 years in developed countries, providing additional evidence that genetic factors alone cannot be responsible for disease manifestation. These observations led to the development of the ‘hygiene hypothesis’, which postulates that the decrease of viral and bacterial infections in western countries due to vaccination, antibiotics and overall better hygienic conditions has led to the loss of protective mechanisms that could inhibit development of allergic diseases and asthma (73, 103). The original idea implied that the induction of Th1 immunity by the different pathogens could counteract Th2-dependent asthmatic responses (104). However, this model could not explain the increase of other inflammatory disorders such as Crohn’s disease or diabetes, nor the absence of asthma in individuals infected with Th2-inducing parasites (105). At present the general belief is that infections with different pathogens can modulate the outcome of asthma by inducing both Th1 as well as T regulatory immune responses; a fine balance between Th1, Tregs and Th2 cell subsets might eventually act to inhibit the onset of allergic diseases (106-108). Interestingly, allergic individuals have more recirculating Th2 cells but display lower frequencies of allergen-specific, IL-10-producing regulatory T cells when compared to healthy subjects, highlighting the importance of the latter cell subset in regulating allergic immune responses (72, 109).
3.4 References


3. General introduction


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4. Results

4.1 Effective T cell immune responses in the absence of the serine/threonine kinase RIP2

Chiara Nembrini¹, Regina Reissmann¹, Manfred Kopf¹, and Benjamin J. Marsland¹

¹Institute of Integrative Biology, Molecular Biomedicine, ETH Zürich, Switzerland

Address correspondence to: Ben Marsland, Molecular Biomedicine, Swiss Federal Institute of Technology, Wagistrasse 27, 8952 Schlieren, Switzerland
EMAIL: marsland@env.ethz.ch, TEL: 0041 1 633 6470
4.1.1 Abstract

The serine/threonine kinase RIP2 has been reported to be essential for Nod1 and Nod2 mediated cell activation, and has been suggested to play a role in the signaling cascade downstream of the T cell receptor. We sought to ascertain the exact role of RIP2 in T helper cell differentiation and CD8+ T cell effector function in vivo and in vitro. In contrast to previous reports, we found that RIP2 deficient T cells did not exhibit impaired proliferation upon TCR engagement in vitro, and differentiation to cytokine producing Th1 or Th2 cells was normal in the absence of RIP2. These results were confirmed in vivo, as wild type and RIP2 deficient virus-specific CD8+ T cells expanded comparably in mice after LCMV infection. Wild type and RIP2 deficient CD4+ and CD8+ T cells from infected mice also showed similar proliferation and cytokine production when restimulated with full or partial agonist peptides ex vivo. Furthermore, no significant difference in adaptive T cell responses could be observed between wild type and RIP2 deficient mice after Listeria monocytogenes infection. Thus contrary to early reports, our data shows that RIP2 is not an essential component of the TCR signaling machinery.
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4.1.2 Introduction

Protective immune responses against invading pathogens are typically driven by an early innate immune response followed by a more specific T and B cell mediated adaptive immune response. Naïve T cell activation is initiated upon recognition of antigen in the context of MHC by the T cell receptor (TCR). Once the TCR is triggered, signaling machinery is assembled that leads to the activation of NF-κB; a transcription factor controlling the expression of many genes required for the effector function, proliferation and survival of the T cell. Specifically, TCR engagement leads to Protein Kinase C-θ activation (1), followed by the sequential assembly of a complex formed by the molecules CARMA1, Bcl10 and MALT1 (2). This complex is involved in the activation of the TRAF molecules TRAF2 and TRAF6, leading to the recruitment of the IKK complex, which regulates NF-κB nuclear translocation by phosphorylating and activating the NF-κB inhibitor, IκB (3). It has previously been described that TRAF6 directly ubiquitylates the IKKγ subunit (4), whereas the kinase TAK1 has been shown to phosphorylate IKK (5), thereby leading to activation of the IKK complex and ultimately NF-κB.

Receptor-interacting protein 2 (RIP2/ RICK) is a widely expressed serine/threonine kinase which has recently been shown to be a key effector molecule mediating signaling downstream of the Nod-like receptors, Nod1 and Nod2 (6). Although RIP2 has previously been implicated in inducing inflammation after Toll-like receptor stimulation, recent data indicate that it is not required for this process (6-9). RIP2 has also been reported to play a central role in TCR signaling; specifically, RIP2 deficient T cells were shown to have impaired proliferative capacity when stimulated with anti-CD3 antibodies in vitro (7, 8, 10). An association between RIP2 and several molecules involved in TCR signaling has also been suggested in some reports. In particular, RIP2 has been shown to co-precipitate with Bcl10 following TCR stimulation and to mediate its phosphorylation (10). In addition, several in vitro studies suggest that RIP2 can associate with TRAF6 (11, 12), IKKγ/NEMO (13, 14), as well as TAK1 (15, 16). RIP2 appears therefore to be an important component of the signaling machinery assembled after TCR engagement and its absence has profound implications for T cell function.

Many studies have produced conflicting results when comparing in vitro and in vivo findings. In the absence of the serine/threonine kinase PKC-θ for example, T cells exhibit an anergic phenotype in vitro, and in vivo Th2 immune responses are impaired. In
comparison, upon activation in an in vivo Th1/CTL inflammatory setting these cells exhibit normal effector function and survival (17-20). Thus, it is important to rigorously study T cell responses in vitro and in vivo before drawing firm conclusions. Our current study aimed to clarify the extent to which RIP2 played a role in inducing T cell effector function in vitro and in vivo. In contrast to early reports, our results show that in vitro T cell proliferation as well as T helper cell differentiation to Th1 or Th2 cell subsets was unaffected by the absence of RIP2. Moreover, in vivo effector functions of RIP2 deficient CD4 and CD8 T cells upon specific peptide challenge, infection with Listeria monocytogenes or lymphocytic choriomeningitis virus was comparable to control wild-type T cells in all conditions tested. Taken together, RIP2 signaling appears to play a redundant role in the development of in vitro and in vivo T cell immune responses.
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4.1.3 Materials and Methods

Mice

C57BL/6 and BALB/c wild-type mice were obtained from Charles River Breeding Laboratories. RIP2 deficient mice were kindly provided by Prof. R. Flavell (Yale University, New Haven, USA). SMARTA-2, lymphocytic choriomeningitis virus (LCMV) GP13 TCR transgenic mice (GLNGPDIYKGVYQFCSV) have been described (18). Both mouse strains are backcrossed to C57BL/6 background for >8 generations. Mice were maintained specific pathogen-free at BioSupport, Zürich in isolated ventilated cages. Animal used in experiments were between 8 and 10 weeks of age. All experiments were performed with permission from the Zürich Animal Ethics Committee.

Pathogens, reagents

LCMV isolate WE was originally obtained from Prof. R. M. Zinkernagel (University Hospital, Zurich) and propagated on L929 cells. Listeria monocytogenes was kindly provided by Dr. Maries van den Broek (University Hospital, Zurich). Anti-CD3 and anti-CD28 antibody were purchased from eBioscience. LCMV glycoprotein peptides GP13, GP33, and GP33-V4Y (21, 22) were purchased from NeoMPS. Phosphorothioate-modified CpG containing oligonucleotide was synthesized by Microsynth. The following oligonucleotide sequence was used 1668pt (5'-TCC ATG ACG TTC CTGAAT AAT-3).

Proliferation assay

CD4^+ T cells were purified from splenic single-cell suspensions by magnetic separation (MACS, Miltenyi Biotech) and incubated with anti-CD3 antibody or dendritic cells and the specific antigen at different concentrations. Cells were cultured for 72 h at 37°C with ^3H-Thymidine added (1 μCi/well) for the last 12 h. Total ^3H-Thymidine incorporation was measured as an indicator of cell proliferation. Alternatively, purified T cells were incubated with a final concentration of 2.5 μM CFSE (Molecular Probes) for 7 min, followed by extensive washing in media before culture and subsequent analysis of proliferation by CFSE dilution with flow cytometry.

T cell-DC coculture

CD4^+ and CD8^+ T cells were isolated from the spleens of transgenic SMARTA2
mice or LCMV infected mice by MACS bead separation. Splenic CD11c+ dendritic cells from naïve mice were also isolated by magnetic separation. Isolated T cells (6.5-10x10^4 cells/well) and DCs (1.4-2x10^4 cells/well) were cultured in 96-well plates in the presence of GP13, GP33 or GP33-V4Y peptide at the indicated concentrations. Proliferation was assessed on day 3 as described in the previous section. Additionally, cells were activated on day 3 in the presence of PMA and ionomycin for 4 h (with the addition of Brefeldin A for the last 2 h). IFN-γ and IL-4 production was determined by intracellular cytokine staining and flow cytometry.

**Staining of LCMV-GP33 specific CD8+ T cells**

Blood was collected in heparin containing tubes. After red blood cell lysis, cells were incubated with 10 µg/ml GP33 PE-conjugated MHC class I tetramers at 4 °C for 45 min. APC-labeled anti-CD8 mAb (eBioscience) was subsequently added for 20 min at 4 °C. Cells were washed and analyzed by flow cytometry.

**Intracellular cytokine staining and FACS analysis**

After stimulation with PMA/ionomycin, cells were washed with PBS/0.1% BSA and surface stained with FITC- or PercP-labeled anti-CD4 or PE-labeled anti-CD8 mAb (eBioscience). Next, cells were washed with PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were incubated in permeabilization buffer (0.5% saponin/PBS/0.5% BSA) containing PE-labeled anti-IL-4, APC-labeled anti-IFN-γ or FITC-labeled anti-TNF-α mAb (eBioscience) for 30 min at room temperature. After washing in permeabilization buffer, cells were resuspended in PBS/0.1% BSA and analyzed by flow cytometry (FACS Calibur; BD Biosciences) and FlowJo software (Tree Star).

**Adoptive transfer experiment**

TCR transgenic CD4+ T cells were purified by magnetic separation and incubated with a final concentration of 2.5 µM CFSE (Molecular Probes) for 7 min, followed by extensive washing in medium before transfer. Labeled T cells (5x10^6) were resuspended in 200 µl of PBS and injected into the tail vein of C57BL/6 mice. After 24 h, recipients were immunized s.c. in the hind-leg flank with 5 µg GP13 together with 10 nmol CpG. On day 3 after immunization, cells from inguinal lymph nodes were restimulated with 1 µM GP13
peptide for 6 h at 37°C in IMDM. For the final 3 h, 10 µg/ml Brefeldin A was added. Thereafter, cells were stained and analyzed by flow cytometry as described previously.

**LCMV infection**

Mice were injected in the tail vein with 250 pfu LCMV. On day 7 and 13 mice were bled and percentage of LCMV-GP33 specific CD8\(^+\) T cells determined by tetramer staining as described before. On day 13, mice were sacrificed and spleens removed to isolate CD4\(^+\) and CD8\(^+\) T cells. Cells were stimulated in vitro for 3 days with freshly isolated CD11c\(^+\) DCs and specific peptide, and proliferation as well as cytokine production analyzed as described.

**Listeria monocytogenes infection**

Frozen glycerol stocks of *L. monocytogenes* were grown overnight at 37 °C in Brain-Heart-Infusion medium and bacterial density was measured by specrophotometry at 600 nm. 2x10\(^3\) colony forming unit (cfu) were injected in the tail vein of C57BL/6 and RIP2 deficient mice. On day 6 after infection, spleens were processed and cells restimulated for 4 h in the presence of PMA/ionomycin or Heat-Killed *L. monocytogenes*. Cytokine production by CD4\(^+\) and CD8\(^+\) T cells was analyzed by intracellular cytokine staining and flow cytometry.

**Acknowledgements**

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4. Results

4.1.4 Results

RIP2 deficient CD4 T cells proliferate normally in response to polyclonal stimulation.

RIP2 has been proposed to be an important effector molecule that mediates signaling downstream of the T cell receptor. In particular, RIP2 deficient T cells were shown to have decreased proliferative capacity when compared to wild type controls (7, 8, 10). We sought to investigate more closely the role of RIP2 in the induction of T cell proliferation in response to various stimuli. Accordingly, we purified splenic CD4+ T cells from wild type and RIP2 deficient mice and stimulated them with different concentrations of anti-CD3 antibody, either plate-bound (Fig. 1A) or soluble with the addition of anti-CD28 antibody (Fig. 1B). After 3 days, the proliferation of these cells was assessed by measuring $^3$H-labeled Thymidine incorporation. In contrast to previous reports, we did not observe any defect in T cell proliferation in the absence of RIP2, irrespective of the concentration of anti-CD3. To further test these results, we stimulated purified CD4+ T cells with different concentrations of the mitogen Concanavalin A (ConA) for 3 days. Again, RIP2 deficient CD4+ T cells displayed normal $^3$H-Thymidine incorporation when compared to wild type controls (Fig. 1C). Both wild-type and RIP2-deficient CD4+ T cells exhibited comparable degrees of activation induced cell death at high concentrations of anti-CD3 and ConA (Fig. 1B and C).

We have previously shown that induction of T cell effector function is dependent on the strength and quality of the stimulatory signal (23). As stimulation with anti-CD3 antibody or ConA leads to strong activation of naïve lymphocytes independent of antigen specificity, we speculated that the RIP2 kinase might play a role in mediating T cell activation and proliferation in response to weaker TCR signals. To test this hypothesis, we performed a mixed lymphocyte reaction (MLR) in which the TCR weakly interacts with allogeneic MHC class II molecules, thereby inducing T cell activation and proliferation. We isolated CD4+ T cells from wild type C57BL/6 and RIP2 deficient mice (also on a C57BL/6 background) and incubated them with splenic CD11c+ dendritic cells from a BALB/c mouse (Fig. 1D). $^3$H-Thymidine incorporation was measured after 5 days of culture. Again, we did not detect any differences between wild type and RIP2 deficient CD4+ T cells, nor were any defects found in CD8+ T cells stimulated under similar conditions (data not shown). Taken together, these results suggest that RIP2 is not required for the induction of T cell proliferation after TCR stimulation.
4. Results

Figure 1. In vitro TCR engagement induces comparable proliferation of wild type and RIP2 deficient CD4+ T cells. Splenic CD4+ T cells were isolated from C57BL/6 and RIP2 deficient mice and stimulated with serial dilutions of anti-CD3 mAb, either plate-bound (A) or soluble with the addition of 2.5 µg/ml of anti-CD28 mAb (B). (C) CD4+ T cells from wild type and RIP2 deficient mice were cultured for 3 days in the presence of Concanavalin A at the indicated concentrations. (D) Splenic CD11c+ DCs were isolated from a naïve BALB/c mouse and cultured with wild type and RIP2 deficient CD4+ T cells (C57BL/6 background) at the indicated ratios for 5 days. Proliferation was assessed by 3H-Thymidine incorporation. Results represent counts per minute (c.p.m.) of CD4+ T cells from 3 different mice per group and are presented as mean ± SD.

RIP2 is not required for T cell differentiation to T helper 1 or T helper 2 cell subsets.

Our results indicated that RIP2 was not necessary for inducing polyclonal T cell proliferation; however, a role for this kinase in mediating proliferation and differentiation of T cells after specific antigen stimulation has yet to be investigated. We used a TCR transgenic T cell-DC coculture system in which CD4+ T cells can be specifically activated and differentiated in vitro to Th1 or Th2 cell subsets by varying the concentration of antigen (24). To this end, RIP2 deficient mice were crossed onto the TCR-transgenic background, SMARTA2 (25). CD4+ T cells were then isolated from spleens of these RIP2-
/-xSMARTA2 mice as well as RIP2+/+xSMARTA2 mice and cultured with different concentrations of the specific peptide GP13 presented on splenic CD11c+ DCs. Proliferation was assessed after 3 days by CFSE dilution and FACS analysis (Fig. 2A) as well as 3H-Thymidine incorporation (Fig. 2B). CD4+ T cells from RIP2 deficient transgenic mice underwent the same number of divisions as their wild type counterparts at all antigen concentrations tested (Fig. 2A). Moreover, 3H-Thymidine incorporation by proliferating RIP2 deficient SMARTA2 cells was comparable to SMARTA2 cells (Fig 2B).

In this co-culture system, high antigen concentration leads to Th1 differentiation, whereas low antigen concentrations favor Th2 differentiation. To investigate whether RIP2 kinase is involved in T helper cell differentiation, we cultured splenic wild type or RIP2 deficient CD4+ SMARTA2 T cells with dendritic cells and either 1000 nM or 0.3 nM GP13 peptide to induce Th1 and Th2 cells, respectively. After 3 days, cells were restimulated with PMA/ionomycin and intracellular cytokine staining for IFN-γ and IL-4 was performed. As shown in Fig. 2C, the percentage of IFN-γ or IL-4 producing cells was comparable between RIP2 competent and RIP2 deficient T cells, indicating that this kinase does not play a role in inducing CD4+ T cell differentiation to the Th1 or Th2 cell subsets in vitro.
4. Results

Figure 2. RIP2 deficient transgenic CD4$^+$ T cells proliferate and differentiate to Th1 and Th2 effector cells in vitro. (A) CD4$^+$ T cells were isolated from SMARTA2 and RIP2-/-xSMARTA2 mice, CFSE labeled and cultured with CD11c$^+$ DCs and GP13 peptide at the indicated concentrations. Proliferation was determined after 3 days by CFSE dilution and FACS analysis. (B) CD4$^+$ T cells were isolated and cultured as described in (A). Proliferation was assessed by $^3$H-Thymidine incorporation after 3 days. Results represent counts per minute of CD4$^+$ T cells and are presented as mean ± SD. (C) SMARTA2 and RIP2-/-xSMARTA2 CD4$^+$ T cells were cultured with CD11c$^+$ DCs and GP13 peptide at 1000 nM or 0.3 nM. After 3 days, cells were restimulated in the presence of PMA/ionomycin for 4 h and the proportion of IFN-γ and IL-4 producing cells was determined by intracellular cytokine staining and flow cytometry. Values represent percentage of positive cells in each quadrant.

RIP2 deficient CD4$^+$ transgenic T cells display normal in vivo proliferation and IFN-γ production.

Our data suggests that in vitro T cell proliferation and differentiation to cytokine producing effector T cells is normal in the absence of RIP2. To verify these results in vivo, we isolated CD4$^+$ T cells from SMARTA2 and RIP2-/-xSMARTA2 mice, labeled them with CFSE and injected them i.v. into wild type C57BL/6 mice. On day 1 after transfer,
mice were immunized s.c. with GP13 peptide together with the Toll-like receptor ligand CpG as an adjuvant. Three days after immunization, cells were isolated from the draining lymph node and specifically restimulated with GP13 peptide. Irrespective of the absence of RIP2, T cells proliferated and similar numbers of wild type and RIP2 knock out transferred cells could be found in each cycle (Fig. 3A). Moreover, IFN-γ production, detected only by the cells that underwent at least 4 divisions was comparable between the groups (Fig. 1B). Thus, we conclude that RIP2 is not required for \textit{in vivo} T cell proliferation and cytokine production.

**Figure 3. RIP2 is not required for CD4$^+$ T cell proliferation and IFN-γ production \textit{in vivo}.** (A) CD4$^+$ T cells were isolated from SMARTA2 and RIP2/-xSMARTA2 transgenic splenocytes, CFSE labeled and injected i.v. into naïve C57BL/6 mice. After 24 h, mice were immunized s.c. in the hind-leg flank with 5 µg of GP13 peptide mixed with 10 nmol CpG in PBS. On day 3 after immunization, cells were isolated from inguinal lymph nodes and proliferation was determined by CFSE dilution and flow cytometry. In the left panel, total number of cells in each division cycle is indicated. Data are represented as mean ± SD (3 mice per group). (B) Lymph node cells were restimulated for 6 h with 1000 nM GP13 peptide and the proportion of IFN-γ producing cells were determined by intracellular cytokine staining and flow cytometry. Values in FACS plots represent the percentage of cells detected in the indicated gates. Representative data from one mouse per group are shown.
RIP2 is superfluous for the induction, proliferation and cytokine production of LCMV-specific CD4$^+$ and CD8$^+$ T cells.

The results described so far revealed that CD4$^+$ T cells did not require RIP2 signaling to be activated by their specific antigen, both in vitro and in vivo. We sought to further investigate whether in vivo CD4$^+$ and CD8$^+$ effector T cell responses could be induced in response to lymphocytic choriomeningitis virus (LCMV) in the absence of RIP2. Accordingly, we infected wild type and RIP2 deficient mice with 250 pfu LCMV, and expansion of GP33-specific CD8$^+$ T cells was measured in the blood on day 7 and 13 after infection by tetramer staining and flow cytometry (Fig. 4A). No significant difference could be observed in the percentage of GP33-specific CD8$^+$ T cells between the two groups, suggesting that in vivo antiviral effector T cell expansion is not affected by RIP2 deficiency. On day 13 post infection total splenic CD4$^+$ and CD8$^+$ T cells were isolated from infected wild type and RIP2 deficient mice and restimulated ex vivo for 3 days with CD11c$^+$ DCs and specific peptide. T cell proliferation was then assessed by $^3$H-Thymidine incorporation. As shown in Fig. 4B and C, RIP2 deficient CD4$^+$ and CD8$^+$ T cells did not show impaired proliferation in response to any of the antigen concentrations tested. We then altered the quality of TCR signal by stimulating the cells with the partial agonist peptide GP33-V4Y. As expected, CD8$^+$ T cell proliferation was substantially reduced, however it did not influence the requirements for RIP2, as $^3$H-Thymidine incorporation by RIP2 deficient CD8$^+$ T cells was similar to wild type controls (Fig. 4D). Effector function was similarly unimpaired as total number of CD4$^+$ and CD8$^+$ IFN-γ producing T cells after 3 days of culture with DCs and GP13 or GP33, respectively, was comparable between wild type and RIP2 deficient mice (Fig. 4E).
Figure 4. RIP2 deficient mice mount normal effector T cell responses during LCMV infection. Wild type and RIP2 deficient mice were infected i.v. with 250 pfu LCMV. (A) On day 7 and 13 after infection, frequencies of LCMV-GP33 specific CD8\(^+\) T cells in the blood was determined by tetramer staining and flow cytometry. (B), (C) and (D) CD4\(^+\) and CD8\(^+\) T cells were isolated from the spleen of infected mice on day 13 and incubated with freshly purified naïve CD11c\(^+\) DCs and the indicated concentration of GP13, GP33 and GP33-V4Y peptides, respectively. Proliferation was assessed by \(^3\)H-Thymidine incorporation after 3 days of culture. Results represent counts per minute of CD4\(^+\) (A) or CD8\(^+\) T cells (B and C) from 3 different mice per group and are presented as mean ± SD. (E) CD4\(^+\) and CD8\(^+\) T cells from infected wild type and RIP2 deficient mice were isolated on day 13 and cultured with CD11c\(^+\) DCs and 100 nM of GP13 or GP33 peptide, respectively. After 3 days cells were restimulated in the presence of PMA/ionomycin for 4 h and total IFN-γ producing cell numbers were determined by intracellular cytokine staining and flow cytometry. Data are represented as mean ± SD of 3 mice per group.
RIP2 deficient mice develop normal effector T cell responses after Listeria monocytogenes infection.

RIP2 signaling has been shown to be involved in the induction of innate immunity against the gram-positive bacterium *Listeria monocytogenes* (7, 26, 27). To investigate whether RIP2 was similarly important for the generation of T cell responses against this pathogen in vivo, we infected wild type and RIP2 deficient mice with 2x10^3 cfu *L. monocytogenes*. After 6 days splenocytes from infected mice were isolated and restimulated with PMA/ionomycin or heat-killed *L. monocytogenes*. Inflammatory cytokine production by CD4^+^ and CD8^+^ T cells was assessed by intracellular cytokine staining and flow cytometry. Total T cell numbers in the spleen were comparable between wild type and RIP2 deficient mice (Fig. 5A), and no significant difference in IFN-γ producing cells, both CD4^+^ and CD8^+^, could be observed between the groups (Fig. 5B and C). Similarly, the percentage of TNF-α producing CD8^+^ T cells was also comparable between wild type and RIP2 deficient splenocytes (Fig. 5D and data not shown).
Figure 5. The absence of RIP2 does not influence T cell responses induced upon infection with *Listeria monocytogenes*. RIP2 competent and deficient mice were infected i.v. with $2 \times 10^3$ cfu *L. monocytogenes*. On day 6 after infection, mice were sacrificed and splenocytes restimulated for 4 h with PMA/ionomycin or heat-killed *Listeria* at a cell:bacterium ratio of 1:10. (A) Splenocytes were counted and total CD4$^+$ and CD8$^+$ T cells numbers determined by surface staining and flow cytometry. Percentage of (B) CD4$^+$ and (C) CD8$^+$ IFN-$\gamma$ producing T cells was determined by intracellular cytokine staining and FACS analysis. Data are represented as mean ± SD of 3 to 4 mice per group. (D) CD8$^+$ T cells from the spleen of infected mice were restimulated in the presence of PMA/ionomycin and IFN-$\gamma$ as well as TNF-$\alpha$ production was analyzed by intracellular cytokine staining and flow cytometry. Values represent percentage of positive cells in each quadrant.
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4.1.5 Discussion

Upon recognition of specific-antigen in the context of MHC, a signaling cascade downstream of the T cell receptor is initiated leading to a range of events including cell activation, proliferation, differentiation and apoptosis. Several in vitro reports indicate that RIP2 might be an important component of this signaling machinery, as RIP2 deficient T cells showed impaired activation and proliferation after TCR engagement (7, 8, 10). Notably however, one report by Lu et al. alluded to a different RIP2 knock out mouse that did not exhibit a defect in T cell function (9) and similar results are also presented in a more recent publication (28). We have utilized a series of in vitro and in vivo systems to establish what role the RIP2 kinase plays in mediating signaling downstream of the TCR and thus in inducing effector T cell responses. In contrast to previous reports, we did not observe any defect in T cell proliferation when cells were polyclonally activated with anti-CD3 mAb or the mitogen Concanavalin A. Further in vitro studies where transgenic T cells were cocultured together with DCs and specific antigen similarly showed no role for RIP2 in T cell proliferation. To investigate the role of RIP2 in mediating T cell effector function, we assessed the capacity of RIP2 deficient CD4+ T cells to differentiate into distinct cytokine producing T helper cell subsets. Accordingly, we cultured wild type and RIP2 deficient CD4+ SMARTA2 transgenic T cells with DCs and high or low concentrations of their cognate antigen GP13, to induce IFN-γ-producing Th1 or IL-4-producing Th2 cells, respectively (19). After 3 days of culture, we did not observe any significant difference in IFN-γ or IL-4 production between wild type and RIP2 deficient CD4+ T cells. Thus, we conclude that RIP2 is not required for in vitro differentiation to Th1 or Th2 cell subsets.

In prior studies looking at the serine/threonine kinase PKC-θ, we have reported that the kinase is differentially required dependent upon the in vitro or in vivo system utilized (18, 19). Accordingly, to verify our initial in vitro findings showing RIP2 was not required for T cell proliferation or differentiation, we used a series of in vivo T cell effector function models. Wild type or RIP2 deficient CD4+ transgenic T cells were transferred into naive recipient mice that were subsequently immunized with the specific antigen, GP13. Activation of the transferred transgenic T cells by GP13 led to proliferation and IFN-γ production, however no difference could be observed between wild type and RIP2 deficient CD4+ T cells. The role of RIP2 in inducing effector T cell responses in vivo was
further investigated by infecting wild type and RIP2 deficient mice with LCMV. We analyzed the anti-viral response by assessing the expansion of virus-specific CD8$^+$ T cells \textit{in vivo} and the proliferation and IFN-\(\gamma\) production of CD4$^+$ and CD8$^+$ T cells after \textit{ex vivo} antigen-specific restimulation. Frequencies of GP33-tetramer positive CD8$^+$ T cells was comparable between wild type and RIP2 deficient mice, and proliferation as well as cytokine production was normal even upon stimulation with a weak agonist peptide, in the absence of RIP2. In a final investigation into T cell function \textit{in vivo} we infected mice with \textit{L. monocytogenes} and similarly found no apparent defect in T cell expansion or effector function.

As RIP2 was originally associated with playing a role in the induction of apoptosis (11, 29), we investigated T cell apoptosis both \textit{ex vivo} and Fas-mediated \textit{in vitro} and found no role for RIP2 (data not shown). Given the broad expression of RIP2, it is still likely that RIP2 could mediate apoptosis in other cell types, but further \textit{in vivo} studies are required to clarify this point. A recent study also questioned results obtained from early reports concerning RIP2 deficient mice, specifically showing that whilst RIP2 clearly played a role in NLR signaling, it did not play a role in TLR signaling (6). It remains difficult to reconcile the different reports of RIP2 functionality; however, possibilities include the use of mice on mixed genetic backgrounds and TLR-ligand preparations that may have contained NLR-ligand contaminants.

In this report we analyzed the role of RIP2 signaling in T cells in a range of \textit{in vitro} and \textit{in vivo} models. In contrast to early reports, we conclude that RIP2 is dispensable for the induction of T cell proliferation and cytokine production after \textit{in vitro} polyclonal or antigen-specific stimulation; moreover, \textit{in vivo} effector T cell responses upon viral and bacterial infections are not affected by the absence of RIP2. Thus, whilst the RIP2 serine/threonine kinase plays a role in innate immune responses, it is superfluous for T cell proliferation and effector function.
4. Results

4.1.6 References


4.2 The kinase activity of Rip2 determines its stability and consequently Nod1- and Nod2-mediated immune responses

Chiara Nembrini¹, Jan Kisielow¹, Abdijapar T. Shamshiev¹, Luigi Tortola¹, Anthony J. Coyle², Manfred Kopf¹ and Benjamin J. Marsland¹

¹ Institute of Integrative Biology, Molecular Biomedicine, ETH Zürich, Switzerland
² Inflammation Division, Millennium Pharmaceuticals, Incorporated, Cambridge, MA

Address correspondence to: Ben Marsland, Molecular Biomedicine, Swiss Federal Institute of Technology, Wagistrasse 27, 8952 Schlieren, Switzerland
EMAIL: marsland@env.ethz.ch, TEL: 0041 1 633 6470
4.2.1 Abstract

Rip2 (RICK, CARD3) has been identified as a key effector molecule downstream of the pattern recognition receptors, Nod1 and Nod2; however, its mechanism of action remains to be elucidated. In particular, it is unclear whether its kinase activity is required for signaling or for maintaining protein stability. We have investigated the expression level of different retrovirally expressed kinase-dead Rip2 mutants, as well as the requirement of Rip2 kinase activity in the signaling events that follow Nod1 and Nod2 stimulation. We show that in primary cells expressing kinase-inactive Rip2, protein levels were severely compromised, and stability could not be reconstituted by the addition of a phospho-mimetic mutation in its autophosphorylation site. Moreover inflammatory cytokine production in response to Nod1 and Nod2 ligands was abrogated both in vitro and in vivo in the absence of Rip2 kinase activity. Our results highlight the central role Rip2 kinase activity plays in conferring stability to the protein and thus in the induction of Nod1 and Nod2-mediated innate immune responses.
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4.2.2 Introduction

A key step in the initiation of effector immune responses is the recognition of highly conserved molecules expressed by microbial pathogens. The immune system has developed specific receptors that sense these so-called pathogen-associated molecular patterns (PAMPs) and initiate appropriate immune responses. One key family of pattern recognition receptors is the Nod-like receptor family (NLR) (1-3), of which two members, Nod1 and Nod2, have been implicated in the recognition of bacterial peptidoglycan derivatives released into the cytosol upon bacterial infection (4-6). Several studies have shown that Nod1 plays a role in host defense against invasive pathogens such as *Helicobacter pylori* and *Escherichia coli* (7, 8); and Nod2 mutations have been associated with a higher incidence of Crohn’s disease (9, 10), thus highlighting these NLRs as important regulators of inflammatory immune responses.

Rip2, also called CARD3, RICK or CARDIAK, is a serine/threonine kinase, which was implicated in the induction of NF-κB activation and apoptosis (11-13). Rip2 has been described to be critical for responses against TLR ligands such as LPS (14, 15), although findings from recent studies did not support this conclusion (16). Rip2 contains a caspase-recruitment domain (CARD), which mediates interaction with other CARD-containing proteins such as Nod1 and Nod2, in addition to an N-terminal kinase domain and an intermediate domain. Nod1 and Nod2 associate with Rip2 upon peptidoglycan ligation (17) leading to downstream signaling events which culminate in NF-κB and MAP kinase activation (15, 18-20). Although the exact mechanism still remains unclear, recent reports have suggested the MAPKKK family member TAK1 provides the link between Rip2 and NF-κB activation upon Nod1 and Nod2 stimulation (21-23). The role of Rip2, in particular its kinase activity, in NLR signaling remains unclear. In vitro investigations have suggested that Rip2 kinase activity may be dispensable for mediating immune responses initiated by NLR-ligands (21, 24, 25); however, the use of protein overexpression in cell lines have left this question unresolved. In addition, disruption of Rip2 kinase activity has been previously associated with a loss in protein stability (23).

In the current investigation we sought to elucidate the role of Rip2 kinase activity in mediating inflammatory signals upon NLR stimulation in vitro as well as in vivo. To this end, we utilized both Rip2 knock-out (15) and Rip2 kinase-dead knock-in mice (24) and retrovirally reconstituted Rip2 deficient cells with different kinase-inactive mutants.
We show here that in the absence of intact kinase activity Rip2 protein is not stable, and that insertion of a phospho-mimetic mutation is not sufficient to restore stability. As a consequence, signaling downstream of Nod1 and Nod2 and inflammatory cytokine production is impaired both in vivo and in vitro. Our results highlight Rip2 kinase activity as a central regulator of protein stability and consequently innate immune responses triggered by Nod1 and Nod2 ligands.
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4.2.3 Materials and Methods

Mice

C57BL/6 wild-type mice were obtained from Charles River Breeding Laboratories. Rip2 deficient mice were kindly provided by Prof. R. Flavell (Yale University, New Haven, USA), Rip2 K47A mice (24) were backcrossed >7 times onto a C57BL/6 background and bred in the BioSupport animal facility. Mice were maintained specific pathogen-free at BioSupport, Zürich in isolated ventilated cages. Animal used in experiments were between 8 and 10 weeks of age. All experiments were performed with permission from the Zürich Animal Ethics Committee.

Reagents

Cell culture LPS from *Escherichia coli* 0111:B4 was purchased from Sigma-Aldrich. MDP and ultrapure LPS from *Escherichia coli* 0111:B4 for in vivo injection were purchased from Invivogen. The synthetic Nod1 ligand FK565 (26) was supplied by Astellas Pharma Inc. (Osaka, Japan).

Stimulation of BM-DCs and BM-DMs.

Bone marrow derived dendritic cells (BM-DCs) were generated from bone marrow cells cultured for 10 days in complete RPMI medium supplemented with GM-CSF. At day 10, cells were incubated with LPS, MDP or FK565. For intracellular cytokine staining, cells were activated for 6 h, and 10 µg/ml brefeldin A was added to the cultures for the final 3 h. Alternatively, DCs were activated overnight and supernatant was collected for ELISA. Bone marrow derived macrophages (BM-DMs) were prepared from bone marrow cells cultured for 7 days in complete RPMI medium supplemented with 10% L929 supernatant containing macrophage-stimulating factor.

Cytokine and chemokine detection.

For detection of intracellular cytokine production, BM-DCs were stained with biotin-conjugated anti-CD11c mAb (BD Biosciences), PercP-labeled streptavidin (BD Bioscience), and after fixation with FITC-labelled anti-TNF-α mAb, PE-labelled anti-IL-6 mAb and APC-labeled anti-IL-12p40 mAb (all eBioscience). Cells were washed and analysed by flow cytometry (FACSCalibur®; Becton Dickinson) and FlowJo software.
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(Tree Star, Inc.). For cytokine detection in supernatants from activated BM-DCs, alkaline-phosphatase-dependent ELISA was performed. Anti-IL-6 and anti-IL-12p40 mAb were purchased from eBioscience. For chemokine detection, total RNA was isolated from activated BM-DMs and subjected to reverse transcription using SuperScript III RT (Invitrogen). Quantitative real-time RT-PCR was performed using Brilliant SYBR Green (Stratagene) on an iCycler (Bio-Rad Laboratories). Expression was normalized to the housekeeping gene GAPDH.

**Endotoxic shock.**

Naïve C57BL/6, Rip2 knock-out and Rip2 K47A knock-in mice were primed by intraperitoneal injection of MDP (500 µg) and challenged by intraperitoneal LPS injection (250 µg) 24 hours after priming and monitored for general condition and survival. Blood samples were collected in EDTA before MDP immunization and 1.5 h after LPS challenge. Serum was analyzed for cytokine levels by ELISA.

**Western Blot**

Monoclonal antibody against Rip2 (clone Nick-1) was purchased from Santa Cruz Biotechnology. Antibodies against the phosphorylated form of p38, ERK, JNK and IκB-α were purchased from Cell Signaling. Polyclonal antibody against β-actin was purchased from Santa Cruz Biotechnology. Activated BM-DMs were lysed in Triton-X buffer containing protease inhibitors. Lysates were loaded on SDS-PAGE gels, transferred to nitrocellulose membranes and immunoblotted with the primary Abs followed by incubation with HPRT-conjugated secondary Abs (Southern Biotechnology). Proteins were detected by ECL.

**Retroviral mediated gene complementation in bone marrow cells.**

cDNA encoding wild type or mutated forms of Rip2 were cloned into pMY-IRES-GFP retroviral vectors (27), which were then used to transfect Phoenix packaging cells (28). Bone marrow cells were isolated from Rip2-deficient mice previously injected with 5’Fluorouracil (29), and infected with supernatant from the retrovirally transfected packaging cell line. Infected stem cell-enriched bone marrow cells were then cultured in the presence of IL-6, IL-3 and stem cell factor (SCF). After 6 days of culture infected, GFP-expressing cells were sorted using a FACS Vantage SE (Becton Dickinson).
Acknowledgements

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4.2.4 Results and Discussion

**Rip2 kinase activity is required for inflammatory cytokine production upon Nod1 and Nod2 stimulation.**

Rip2 has been reported to regulate downstream signaling of NLRs; however, the importance of its kinase activity remains unclear. We sought to establish the requirements for Rip2 and its kinase activity in the signaling events following Nod1 and Nod2 stimulation in vitro. Accordingly, we generated bone marrow-derived dendritic cells (BM-DCs) from naive C57BL/6, Rip2-deficient mice and Rip2 kinase-inactive knock-in mice (Rip2 K47A). In the latter, the residue crucial for ATP-binding, lysine 47, is mutated to alanine. Wild type, Rip2 knock-out and knock-in dendritic cells were then stimulated with the Nod1 and Nod2 ligands, FK565 and muramyl dipeptide (MDP), respectively. Inflammatory cytokine production by the activated BM-DCs was determined after 6 hours by intracellular staining and flow-cytometry (Fig. 1A) or after 24 hours in culture supernatant by ELISA (Fig. 1B, left panel). Although MDP and FK565 alone poorly induced cytokine secretion, cooperative induction of IL-6, IL-12p40 and TNF-α was observed in wild-type cells when these ligands were administered together with LPS. Cells from Rip2-deficient mice did not respond to MDP or FK565 alone, nor in combination with LPS, confirming the importance of Rip2 in Nod1 and Nod2 responses. Notably, Rip2 kinase-inactive BM-DCs exhibited a similarly impaired cytokine response upon stimulation with MDP and FK565, indicating that an intact kinase activity was required for Nod1 and Nod2 induced cytokine responses.

In addition to inflammatory cytokines, microbial products also induce chemokine secretion. In particular, stimulation with a synthetic Nod1 ligand has been shown to induce chemokines such as KC (also known as CXCL1) in vitro and in vivo (30, 31). Our aim was to determine whether Rip2 kinase activity was involved in chemokine production upon NLR stimulation. Accordingly, we generated bone marrow-derived macrophages from C57BL/6, Rip2 knock-out and Rip2 knock-in mice and stimulated them with MDP and FK565 alone or together with LPS. After 6 h, total RNA was isolated, reverse-transcribed, and KC mRNA expression was determined by quantitative real-time PCR analysis (Fig. 1B, right panel). There was a 10- to 30-fold increase in KC mRNA expression when wild-type cells were stimulated with FK565 or MDP together with LPS. Comparatively, stimulation of both Rip2 deficient and kinase-dead cells did not lead to KC mRNA
transcription. These results are in line with the cytokine data and highlight a role for Rip2 kinase activity in inflammatory responses following stimulation with bacterial products.

To test our in vitro results, we investigated whether Rip2 kinase activity was also required to mediate NLR signaling in vivo. It has previously been shown that MDP administration renders mice more susceptible to LPS-induced endotoxic shock (32). Accordingly, we sensitized wild type, Rip2 knock-out and Rip2 kinase-inactive mice with MDP, and subsequently challenged with LPS. Inflammatory cytokine levels in the serum were measured 1.5 hours after LPS administration (Fig. 1C). A strong synergy between MDP and LPS was evident in wild-type mice as shown by significantly higher levels of IL-6 and IL-12p40 as compared to stimulation with single ligands; however, this synergy was not observed in the serum of Rip2 kinase-inactive or knock-out mice, where only the effect of LPS was detected. Thus, also in vivo the absence of Rip2 kinase activity leads to impaired inflammatory cytokine production upon MDP stimulation. Notably, injection of ultrapure LPS alone induced similar levels of IL-6 and IL-12 in all groups of mice, excluding a role for Rip2 in mediating TLR-4 signaling in vivo.

The cytokine storm induced by LPS during an endotoxic shock can lead to organ dysfunction and eventually death (33). We therefore monitored survival of mice injected with MDP or PBS and challenged with LPS (Fig. 2D). Wild type mice sensitized with PBS (right panel) showed a better survival rate than their MDP-immunized counterparts, which all succumbed to LPS challenge by day 4 (left panel). Conversely, we did not observe any significant difference in survival between Rip2 knock-out or knock-in mice sensitized with either MDP or PBS, indicating that no immune response to this Nod2 ligand took place in the absence of an intact Rip2 kinase domain.
Figure 1. Inflammatory responses following Nod1 and Nod2 stimulation are abrogated in the absence of Rip2 kinase activity. (A) BM-DCs were generated from C57BL/6, Rip2 knock out and Rip2 K47A knock in mice and stimulated with 25 µg/ml MDP or 3 µg/ml FK565 alone or with the addition of 10 ng/ml LPS. As a control, cells were also activated with 10 ng/ml LPS alone. After 6 hours, TNF-α and IL-12p40 production was assessed by intracellular cytokine staining and flow cytometry. Numbers in FACS plots
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represent percentage of positive cells in each quadrant. Data are representative of 3-4 repeat experiments. (B) BM-DCs and BM-DMs were stimulated with 25 µg/ml MDP or 3 µg/ml FK565 alone or with the addition of 10 ng/ml LPS. Left panel: IL-6 production was measured in the supernatant by ELISA after 24 h. Results represent cytokine production by BM-DCs from 3 different mice per group and are presented as mean ± SD. Right panel: total RNA was isolated from BM-DMs after 6 h and KC expression analyzed by quantitative real-time PCR. Results are represented as fold increase in chemokine expression compared to non-stimulated controls. The experiment was repeated 2-3 times. (C) C57BL/6, Rip2 knock out and Rip2 K47A mice were primed i.p. with 500 µg MDP and challenged with ultrapure LPS (250 µg). Alternatively, mice were primed with PBS. Serum was collected from naïve animals and 1.5 h after LPS challenge; IL-6 and IL-12p40 levels were measured by ELISA. *, P < 0.05; statistically significant differences. (D) Survival of wild type, Rip2 knock out and Rip2 K47A mice after LPS challenge. The experiment was repeated twice with 8 mice per group.

Rip2 kinase activity is required to ensure protein stability and efficient signaling downstream of Nod2.

Our results indicate that Rip2 deficiency and kinase-inactivation both lead to abrogated signaling following NLR stimulation. Notably, different reports showed that a mutation in the Rip2 kinase residue, lysine 47, leads to a decrease in protein expression (23, 24). Data from Lu et al. suggested that splenocytes isolated from Rip2 kinase-dead knock-in mice expressed decreased levels of Rip2 as compared to wild type splenocytes, although expression could be enhanced by stimulating the cells with LPS. To further investigate the role of Rip2 kinase activity on the stability of the protein, we assessed Rip2 expression under steady state conditions or after stimulation with LPS in bone marrow-derived macrophages. Accordingly, wild type, Rip2 knock-in or Rip2 knock-out cells were stimulated with LPS for 6 and 24 h and protein expression was analyzed by Western blot. In line with previous reports, we found that protein levels of the kinase-dead Rip2 mutant were lower in BM-DMs as compared to its wild type counterparts (Fig. 2A). Moreover, we found that activation with LPS was not sufficient to restore Rip2 expression in knock-in cells. This indicates that K47A mutation renders the Rip2 protein unstable; yet the possible causes for this instability remain unknown.
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Figure 2. Kinase inactivation affects Rip2 protein stability and phosphorylation of effector signaling molecules downstream of Nod2. (A) BM-DMs were generated from C57BL/6, Rip2 K47A knock in and Rip2 knock out mice and stimulated with 100 ng/ml LPS. Total cell lysates were prepared and blotted against Rip2 and β-actin. (B) Retrovirally infected bone marrow cells expressing wild type, K47R or K47R-S176E Rip2 protein were lysed and blotted against Rip2 and β-actin. (C) BM-DMs were generated as in (A) and stimulated with 25 µg/ml MDP. Cell lysates were blotted with antibodies against the phosphorylated forms of p38, ERK, JNK and IκB-α. The same samples were blotted with polyclonal antibodies against β-actin. Proteins were detected by ECL.

Replacement of a lysine with the uncharged and hydrophobic amino acid alanine in the K47A kinase-dead mutation could potentially lead to misfolding of the Rip2 protein. To circumvent this problem, we generated a new mutation in which lysine 47 is replaced by the more similar amino acid arginine. Wild type and K47R Rip2 mutant gene sequences were cloned into retroviral pMY-IRESGFP vectors, and the resulting retroviruses were used to infect Rip2-deficient bone-marrow cells. GFP positive cells expressing wild type or K47R mutant Rip2 were FACS sorted, lysed and the level of Rip2 protein expression was analyzed by Western blot. As shown in Figure 2B, protein expression of the Rip2 K47R
mutant was reduced as compared to the retrovirally expressed wild type Rip2, suggesting that protein instability is not due to a charge difference.

As Rip2 has been shown to undergo autophosphorylation only in the presence of an intact kinase activity (34), it can be speculated that this is an important mechanism by which the protein is stabilized. Serine 176 has been mapped as a Rip2 autophosphorylation site, and mutation of this amino acid influences kinase activity (34). We sought to generate a stable kinase-inactive Rip2 mutant by mimicking autophosphorylation; for this purpose serine 176 was replaced by the negatively charged phospho-mimetic glutamic acid. We cloned the S176E mutation into our retroviral plasmid expressing the Rip2 mutant K47R and subsequently infected Rip2 deficient bone marrow cells. Expression of the K47R-S176E Rip2 double mutant was compared to wild type and K47R Rip2 protein expression. We found that introduction of the S176E phospho-mimetic mutation did not lead to a stable kinase-inactive mutant, as protein expression was comparable to the Rip2 K47R mutant (Fig. 2B). However, it remains possible that other serine amino acids, such as in the intermediate or CARD domain are also involved in autophosphorylation and thus might support protein stability.

Our results indicate that Rip2 kinase activity is required to preserve inflammatory immune responses following Nod1 and Nod2 stimulation. We speculate that Rip2 kinase activity primarily functions to stabilize Rip2 expression levels such that it can participate in the signaling cascade downstream of Nod1 and Nod2. Nevertheless, a role for Rip2 kinase activity in directly phosphorylating downstream effector molecules in vivo remains to be elucidated and cannot be excluded. Rip2 has previously been shown to mediate phosphorylation of different signaling molecules in vitro (19, 35), as well as to induce IKK-γ/NEMO ubiquitinylation, thereby controlling NF-κB activation (25). Moreover, recent reports describe TAK1 kinase as the crucial effector molecule downstream of Rip2 in NLR-signaling (21, 22), and Rip2 overexpression has been shown to mediate TAK1 phosphorylation and activation (23). Hasegawa et al. could additionally show that Rip2 polyubiquitinylation is required for interaction with TAK1 and subsequent NF-κB activation. Of note, polyubiquitinylation was independent of Rip2 kinase activity and a weak level of NF-κB activation could be observed in cells expressing a kinase-dead Rip2 variant. However, expression of this kinase-inactive mutant was also impaired, limiting possible conclusions regarding Rip2 functionality. Moreover NF-κB activation was achieved by inducing protein overexpression and oligomerization, and not by activating
endogenous proteins with Nod1 and Nod2 ligands. We activated wild type, Rip2 knock-in and Rip2 knock-out BM-DMs with MDP and analyzed phosphorylation of different signaling molecules, including NF-κB-inhibitor IκB-α. The results shown in Figure 2C indicate that under more physiological conditions, NF-κB and MAP kinase activation did not take place in the absence of Rip2 kinase activity.

Taken together, our data show that the serine/threonine kinase, Rip2, is a central regulator of NLR-induced immune responses. The kinase activity of Rip2 is necessary to preserve protein stability and is thus critical for efficient transmission of Nod1 and Nod2 signals. Mutations in Nod1 and Nod2 genes have been shown to correlate with onset of inflammatory disorders such as inflammatory bowel disease (IBD) (9, 10, 36). Interestingly, Rip2 kinase was shown to be activated during experimentally induced ulcerative colitis, and blockage of Rip2 kinase activity by the synthetic compound SB203580 could inhibit NF-κB activation, inflammatory cytokine production and consequently disease in a mouse model of IBD (36). Thus, regulation of Rip2 kinase activity may still prove to be a valid therapeutic strategy for the treatment of diseases linked to Nod1 and Nod2.
4.2.5 References


4.3 Inhalation of bacteria suppresses ensuing Th2 effector responses by long-term modulation of lung dendritic cell function.

Chiara Nembrini¹, Regina Reissmann¹, Manfred Kopf¹, and Benjamin J. Marsland¹

¹ Institute of Integrative Biology, Molecular Biomedicine, ETH Zürich, Switzerland

Address correspondence to: Ben Marsland, Molecular Biomedicine, Swiss Federal Institute of Technology, Wagistrasse 27, 8952 Schlieren, Switzerland
EMAIL: marsland@env.ethz.ch, TEL: 0041 1 633 6470
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4.3.1 Abstract

Airborne microbial products have been reported to promote immune responses that suppress asthma, yet how these beneficial effects take place remains controversial and poorly understood. As lung dendritic cells are known to be crucial for the induction and maintenance of asthma, we focused our attention on the role bacteria may play in influencing their function in situ. We found that pulmonary exposure with the bacterium *Escherichia coli*, led to a suppression of Th2 responses in a murine model of ovalbumin (ova)-induced asthma. This inhibition was characterized by reduced airway hyperresponsiveness, decreased eosinophilia and cytokine production by T cells in the lung. Surprisingly, this immune modulation was not mediated by the induction of Th1 responses nor regulatory T cells, but was dependent on TLR-4 signaling. Furthermore, DC migration to the draining lymph nodes and subsequent activation of T cells was unaffected by prior exposure to *E.coli*. In the lung of non-treated mice, ovalbumin was primarily presented by airway CD11b+ CD11c+ DCs expressing high levels of MHC class II molecules. Conversely, in the lung of *E.coli*-treated mice DCs displayed a less activated phenotype and impaired antigen presentation capacity. Consequently, in situ Th2 cytokine production by ova-specific effector T cells recruited to the airways was significantly reduced. This immunosuppression could not be overcome by the local administration of activated dendritic cells. Taken together, these data suggest a novel immunoregulatory mechanism, in which bacterial exposure can alter dendritic cell function and the lung environment to suppress local Th2 responses.
4.3.2 Introduction

Mucosal tissues, such as the lung, are constantly exposed to both innocuous and pathogenic environmental antigens. Cells situated in close proximity with these surfaces play a central role in orchestrating immune responses upon antigen encounter, deciding between induction of tolerance or initiation of an effector immune response. This process needs to be tightly regulated, as disruption of homeostasis can lead to the development of detrimental immune responses such as allergy and asthma. Asthma is a chronic inflammatory response of the respiratory tract to normally harmless environmental antigens, characterized by eosinophilia, goblet cell hyperplasia, bronchial hyperresponsiveness and airway tissue remodeling (1). Dendritic cells (DCs) play a crucial role in the initiation of an asthma response (2); following antigen uptake, they migrate to the draining lymph node, where they drive effector Th2 cell development (2, 3). Most importantly DCs have also been shown to be required not only for the initiation of asthma, but also for the maintenance of inflammatory Th2 responses in the lung (4). Regulation of dendritic cell function has therefore been proposed as a possible vaccine target for the suppression of asthma (5).

Immune responses to airborne pathogens such as viruses and bacteria have been shown to influence the development of allergic responses in the respiratory tract (6-10); in some cases suppression of asthma was observed (11, 12) whilst in others it was exacerbated (13). In mice, treatment with microbial products such as endotoxins (14, 15), CpG (16, 17) or other Toll-like receptor ligands (18, 19) could inhibit the classical features of asthma such as eosinophilia, IL-5 production and airway hyperresponsiveness. Moreover administration of pathogen-derived products in human clinical trials has shown positive results in the treatment of atopic diseases such as allergic rhinitis (20). Microbial products have been proposed to drive suppression of asthma via the induction of counteracting Th1 or CD8 T cells as well as T regulatory cell responses (11, 21-23). However, the exact mechanisms underlying this immune modulation in the lung remain controversial and poorly understood (16, 24-26). As DCs are known to play a crucial role in inducing immune responses to both harmless antigens such as allergens as well as pathogenic invaders, it is plausible that regulation of DC function by microbial agents in the airways could influence the outcome of allergic responses and asthma.
In the current study we sought to analyze the effect of microbial exposure in the lung on the subsequent development of an asthma response, and in particular investigate the role of DCs in regulating this process. In our model, pulmonary exposure with the bacterium *Escherichia coli* prior to asthma induction led to the suppression of subsequent Th2 responses. This inhibition was characterized by reduced airway hyperresponsiveness, decreased eosinophilia and cytokine production by T cells in the lung. We show that *E.coli* treatment influenced local DC function in the lung, such that these cells failed to fully mature and present antigen to effector T cells. Accordingly, such antigen-specific T cells that were recruited to the inflamed airways displayed impaired cytokine production *in situ*. The suppressive effect caused by the bacteria was TLR-4-dependent, long lasting, and surprisingly not mediated by the induction of Th1 responses nor regulatory T cells. Our data highlight the impact modulation of the lung environment can have on the outcome of subsequent immune responses, and provide a novel mechanism by which environmental exposure to bacteria can alter lung DC function.
4.3.3 Materials and Methods

Mice and pathogens

Mice were maintained specific pathogen-free at BioSupport, Zürich in isolated ventilated cages. C57BL/6 and Balb/c wild-type mice were obtained from Charles River Breeding Laboratories. All gene-deficient mice described in this report were bred at Biosupport. OT-II ova-transgenic mice were kindly provided by Dr. Federica Sallusto (IRB, Bellinzona, Switzerland). Animal used in experiments were between 8 and 12 weeks of age. All experiments were performed with permission from the Zürich Animal Ethics Committee.

Glycerol stocks of *Escherichia coli* DH5αpDS (DS Red) were grown overnight in LB medium supplemented with 100 µg/ml Ampicillin. OD$_{600}$ was measured in the morning to determine concentration of the bacteria. $10^7$ CFU *E.coli* were resuspended in 50 µL PBS and administered intranasally.

Ova-induced airway inflammation

Mice were immunized by intraperitoneal injection with 100 µg ovalbumin (grade V; Sigma-Aldrich) in 200 µl of alum adjuvant (SERVA; Electrophoresis GmbH). 7 days later, mice were challenged by intranasal (i.n.) inoculation with 100 µg ova in 50 µl PBS. *E.coli* were administered 3 to 21 days prior to immunization. FITC-labeled ovalbumin (Molecular Probes) was administered at a concentration of 2 mg/mL.

Measurement of Airway Responsiveness.

On day 3 after i.n. challenge with ova, mice were placed in individual, unrestrained whole body plethysmograph chambers (Buxco Electronics). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methacholine chloride (MetCh). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh (Aldrich Chemie) and monitoring the breathing pattern for 5 min after initiation of aerosol dose.

Collection and analysis of bronchoalveolar lavage (BAL) and lung cells.
Mice were sacrificed, the trachea was cannulated and BAL performed by flushing the airways three times with 1 ml PBS. Total BAL cells were counted using a Coulter Counter (IG Instruments) and spun onto glass slides using a Cytospin 2 (Shandon Southern Products, Ltd.). Cells were then stained with Diff Quick staining set (Siemens-Dade Behring) and, once dried, embedded in Eukit solution (O. Kindler GmbH & Co.) under glass coverslips. Percentages of eosinophils, macrophages, lymphocytes and neutrophils were determined microscopically by counting 200 cells/sample using standard morphological and cytochemical criteria. Alternatively, BAL cells were analyzed by flow cytometry. Lung was perfused with 10 mL PBS and digested in medium supplemented with 2 mg/ml Collagenase IV. Afterward, a 30% Percoll gradient was applied to the cells to isolate lung leukocytes. Total cell numbers were determined with a Coulter Counter and analysis performed by flow cytometry.

**Lung histology**

Mice were sacrificed, the trachea was cannulated and the lung flushed with 1 mL 4% neutral buffered formalin. The lung was then removed, collected in formalin and processed for Hematoxylin and Eosin (H&E) and Periodic acid-Schiff (PAS) staining. Histological section were evaluatated according to general inflammation, eosinophilia and goblet cell hyperplasia.

**Antibodies and FACS analysis.**

Surface staining was carried out in PBS/0.1% BSA. Monoclonal Ab recognizing CD4, CD25, and CD11c were purchased from eBioscience; anti-CD11b, -CD40, -I-A\(^b\) mAb were purchased from BD Pharmingen. For intracellular cytokine staining, cells were stimulated with PMA/ionomycin for 4 h, with the addition of Monensin (Sigma-Aldrich) to retain cytokines in the cytoplasm. After surface stain, cells were washed with PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were incubated in permeabilization buffer (0.5% saponin/PBS/0.5% BSA) containing anti-IL-4, -IFN-\(\gamma\), FoxP3 (all eBioscience) or anti-IL-5 mAb (BD Pharmingen) for 30 min at room temperature. After washing, cells were resuspended in PBS/0.1% BSA and analyzed by flow cytometry (FACS Calibur; BD Biosciences) and FlowJo software (Tree Star). Sorting of lung cell populations was performed on a FACS Aria (BD Biosciences).
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**Proliferation assay and transfer of Th2 cells**

CD4 T cells were purified from draining LN or splenic single-cell suspensions by magnetic separation (MACS, Milteny Biotech). For proliferation assay, cells were cultured for 72 h at 37°C with $^3$H-Thymidine (1 μCi/well) added for the last 12 h, and total $^3$H-Thymidine incorporation was measured. For Th2 cell differentiation, ova-transgenic CD4 T cells from OT-II mice were cultured for 3 days at 37°C in the presence of freshly isolated splenic CD11c+ DCs, 5 μg/mL ova-peptide$^{323-339}$ (EMC microcollections), 10 ng/mL rIL-4 (BD Pharmingen) and 10 μg/ml anti-IFN-$\gamma$ antibody (clone XMG1.2). Th2 cells were tested for IL-4 and IL-5 production by intracellular staining and flow cytometry. 3x10^6 cells were injected intravenously into CD45.1+ recipient mice; on the same day, and for the following 3 days, mice were challenged with 100 μg ova in PBS.

**ELISA**

Supernatant from BAL was isolated, and cytokine concentrations were determined. Capture anti-IL-5 antibody and biotin-conjugated anti-IL-5 detection antibody were purchased from BD Pharmingen. Alkaline phosphatase-labeled streptavidin (Southern Biotechnology) and substrate p-nitrophenyl phosphate (Sigma-Aldrich) were used. OD was determined at 405 nm using a SpectraMax spectrophotometer (Bucher Biotech). For IL-13 detection, IL-13 ELISA Ready-SET-go! (eBioscience) was used. Reaction was stopped with 2N H$_2$SO$_4$, and OD determined at 470 nm.

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4.3.3 Results

**Administration of E.coli to the lung leads to long-term suppression of allergic airway inflammation.**

Immune responses to airborne pathogens have been shown to influence the development of allergy in the lung. Several investigations could determine a correlation between exposure to microbes and their products, especially early in life, and suppression of asthmatic responses (27, 28). However, the mechanisms underlying this inhibition are yet to be fully elucidated, and depending on the experimental model, exacerbation of the allergic disease has also been observed (13, 26, 29, 30). In this study we sought to investigate the influence of *Escherichia coli* exposure in the lung on the development of allergic airway inflammation (AAI) by treating the mice with bacteria prior to allergic disease induction. This particular bacterial strain could not replicate in the lung and was cleared by day 3 after administration (data not shown).

As a standard protocol, wild type C57BL/6 mice were treated with $10^7$ colony forming units (CFU) of *E.coli*, which were cleared from the lung within 2 days (data not shown). Three days later mice were sensitized by intraperitoneal injection of the protein antigen ovalbumin (ova) in alum. Mice were subsequently challenged intranasally with ova on day 7 after immunization. After 3 days, i.e. 2 weeks after *E.coli* treatment, airway hyperresponsiveness (AHR) was measured in a whole body plethysmograph. Mice treated with the bacteria (*E.coli* treated) were compared to control non-treated mice (ova only, Figure 1A). As expected, mice sensitized with ova in alum and challenged intranasally with ova in PBS developed airway hyperreactivity upon methacholine inhalation as indicated by the high PenH value. Non-treated, naïve mice did not respond to the increasing dose of MetCh. Notably, a response comparable to naïve mice was observed in *E.coli*-treated mice, which did not display any increase in PenH upon stimulation with methacholine, indicating that *E.coli* treatment was able to suppress AHR.
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Figure 1: Intranasal *E. coli* treatment prior to induction of allergic airway inflammation suppresses cardinal disease features. C57BL/6 mice were treated with $10^7$ colony forming units (CFU) *E. coli* i.n. and 3 days later immunized i.p. with ova in alum. Seven days later, mice were challenged i.n. with ova in PBS. Alternatively, mice were immunized and challenged with ova without prior inhalation of bacteria (ova only). (A) On day 3 after i.n. challenge, mice were exposed to increasing doses of MetCh and airway hyperresponsiveness was determined in a full body unrestrained plethysmograph. (B) On day 4 after i.n. challenge, bronchoalveolar lavage (BAL) was isolated and total cell numbers were determined with a Coulter Counter. Proportions of eosinophils, macrophages and lymphocytes were determined by differential cell counts (C), and total eosinophil numbers calculated accordingly. (D) Lungs were collected in formalin solution on day 4 after challenge with ova; histological lung sections show cellular infiltration as determined by H&E staining and goblet cell hyperplasia (insert, 100x magnification) as determined by Alcian Blue and PAS staining. (E) On day 4 after challenge, BAL CD4 T cells were restimulated with PMA/ionomycin and
4. Results
cytokine production determined by intracellular staining and flow cytometry (left panel). Values in FACS plots represent the percentage of cells detected in each gate. Representative data from one mouse per group are shown. Total CD4 numbers were determined by flow cytometry (right panel). C57BL/6 mice were treated with *E.coli* 3 weeks prior to i.p. immunization with ova in alum: airway hyperresponsiveness (F), eosinophil numbers (G) and proportion of IL-4- or IL-5-producing CD4 T cells (H) were determined as described in (A-C) and (E). Results represent values from 3-4 mice per group and are presented as mean ± SD. *, P < 0.05; statistically significant differences. Experiments were repeated ≥ 2 times.

During an acute AAI response, increased cell infiltration and eosinophil recruitment to the airways can be observed. To further analyze the role of bacteria in modulating an ova-dependent allergic response, *E.coli* - and non-treated mice sensitized with ova were sacrificed on day 4 after challenge and cellular infiltrates in the bronchoalveolar lavage determined. Mice treated with *E.coli* prior to asthma induction had significantly less cells in the airways when compared to control asthmatic mice (Fig. 1B, left panel). The latter had high frequencies of eosinophils in the BAL (Fig. 1B and C), in contrast to the mice treated with the bacteria, whose airway cellular infiltrates mostly consisted of monocytes/macrophages as determined by differential cell counts (Fig. 1C). These results are in line with the AHR data and show that administration of *E.coli* has a dramatic influence upon the development of AAI.

To further study the observed suppression of AAI features upon *E.coli* treatment, we performed histological analysis of the lung. Mice were treated with *E.coli* prior to asthma induction and compared to non-treated mice as described in the previous section. On day 3 after ova-challenge lungs were collected into formalin, and tissue sections analyzed after H&E and PAS staining. In addition to the expected decrease in eosinophilia, histological sections also show reduced goblet-cell hyperplasia in *E.coli* treated mice, confirming that bacterial administration leads to inhibition of the typical features of allergic airway hyperresponsiveness (Fig. 1D). Treatment with 10^7 CFU *E.coli* induced elevated airway neutrophilia, which peaked at 24 h and resolved by day 3 after challenge (data not shown). Notably, no changes in lung structure due to intranasal *E.coli* administration were observed (Fig. 1D and data not shown).

Asthma is a Th2 lymphocyte-mediated inflammatory disease; Th2 effector cells secrete cytokines such as IL-5 and IL-4, which mediate eosinophil recruitment and survival as well as bronchial hyperresponsiveness (31). We sought therefore to investigate whether the observed suppression of AAI features by *E.coli* administration correlated with impaired
Th2 responses. Accordingly, CD4 T cells isolated 4 days after challenge from the airways of allergic as well as \textit{E. coli}-treated mice were restimulated in vitro and Th2 cytokine production was analyzed by flow cytometry. As shown in Fig. 1E, IL-4 and IL-5 production was significantly reduced in \textit{E. coli}-treated mice as compared to non-treated, ova-sensitized mice. Moreover, recruitment of CD4 T cells was also reduced (Fig. 1E, right panel).

The results described so far were obtained by sensitizing the mice with ova three days after intranasal administration of \textit{E. coli}. To further characterize the duration of disease inhibition, we analyzed whether bacteria administration one month before challenge with ova was still modulating the outcome of the AAI response. As shown in Fig. 1F, non-treated, ova-sensitized mice developed airway hyperresponsiveness in response to increasing doses of methacholine, and the PenH value for these mice was significantly higher when compared not only to naïve mice but also to \textit{E. coli}-treated mice. These data mirror the results with mice treated with \textit{E. coli} only 3 days prior to ova sensitization. To further confirm the suppressive effect of the bacteria, we analyzed eosinophilia as well as Th2-cytokine production by CD4 T cells in the airway of mice treated with \textit{E. coli} one month earlier. In line with the AHR results, prior \textit{E. coli} administration led to reduced frequencies of eosinophils and cytokine-producing CD4 T cells in the BAL as compared to non-treated, ova-sensitized mice (Fig. 1G and H). Taken together our results indicate that \textit{E. coli} treatment induces a long-lasting suppressive effect, which inhibits the development of ensuing allergic airway inflammation.

**\textit{E. coli} mediated suppression is TLR-4 dependent.**

The recognition of pathogen-associated molecular patterns (PAMPs) by pattern-recognition receptors such as Toll-like or Nod-like receptors is a critical step in the induction of immune responses against bacteria and other invaders (32, 33). Moreover stimulation with different TLR ligands and the ensuing inflammation have been shown to influence the outcome of an allergic response (7, 25). We sought therefore to investigate the requirements for TLR signaling in our model of AAI suppression by \textit{E. coli} administration; as activation of TLR-4 by gram-negative bacteria such as \textit{E. coli} has been extensively described (34), we first focused our attention on the role of this receptor.
Accordingly, wild type and TLR-4 deficient mice were treated or not with *E.coli* i.n., followed by ova-sensitization and challenge. Of note, due to the background of the gene-deficient mice, these experiments were performed with wild type Balb/c mice, whereby *E.coli*-mediated AAI suppression was as efficient as in C57BL/6 mice (data not shown). Eosinophil infiltration in the BAL was determined by differential cell counts on day 4 after challenge. As expected, wild type *E.coli*-treated mice displayed lower eosinophil frequencies as compared to their non-treated counterparts. However, this reduction was not observed in TLR-4 deficient mice, as both non- and *E.coli*-treated mice had high percentages of eosinophils in the BAL (Fig. 2A). Furthermore, in contrast to the wild type situation, total cell infiltration in the airways of TLR-4 deficient mice was not influenced by administration of the bacteria (Fig. 2A, right). Given that no difference in eosinophilia was observed in TLR-4 deficient mice after *E.coli* treatment, we analyzed whether IL-5 production by CD4 T cells was unimpaired. Indeed, non- and *E.coli*-treated TLR-4 knock out mice had comparable frequencies of IL-5 producing CD4 T cells in the airways, whereas bacteria administration in wild type mice again led to significant inhibition of IL-5 production by these cells (Fig. 2B). Our data show that eosinophilia and Th2 cytokine production are not impaired in TLR-4 deficient mice upon administration of the bacteria, and thus suggest that in the lung *E.coli* induces TLR-4-dependent immune responses which can eventually lead to AAI suppression.

In addition to TLRs, we focused our attention on Nod-like receptors, as these are known to recognize cell wall-components of several bacteria including *E.coli*, and to subsequently induce an inflammatory response (35). Interestingly, mutations in the *nod1* gene have been associated with incidence of asthma in humans (36). To investigate the role of Nod1 and Nod2 in our model, we carried out *E.coli* treatment and ova sensitization in mice lacking the kinase Rip2, which has been shown to be crucial for the signaling downstream of these receptors (37). As shown in Fig. 2C, *E.coli* treatment could inhibit eosinophil influx in the airways of both wild type and Rip2 deficient mice, suggesting that in this case Nod1 and Nod2-dependent inflammatory pathways are not major players in the inhibition of allergic airway inflammation.
T helper 1 and T regulatory responses do not play a role in modulation of allergic airway inflammation by *E.coli*.

So far we could show that intranasal administration of bacteria considerably inhibited the development of allergic airway inflammation, yet the mechanisms underlying this process still remain to be clarified. To further investigate how *E.coli* treatment could modulate AAI we assessed the role of T helper 1 responses, as Th1 effector cells have been shown to be induced by bacteria and their products (38), and to exert a suppressive function on Th2 immune responses (39). To this end, we first measured IFN-γ secretion by CD4 T cells in the airways of non- and *E.coli*-treated mice on day 4 after challenge. We did not observe increased frequencies of IFN-γ-producing BAL CD4 T cells in mice...
treated with *E. coli* (Fig. 3A). To further assess the role of Th1 effector cells and IFN-γ, we repeated *E. coli*-treatment and AAI induction in IFN-γ-deficient mice. Airway hyperresponsiveness was measured on day 3 after ova challenge, and eosinophilia and Th2 cytokine production were analyzed the following day. As shown in Fig. 3B, even in the absence of IFN-γ, mice treated with *E. coli* showed a response to methacholine which was comparable to naïve controls. As expected, non-treated, ova-sensitized IFN-γ knock-out mice exhibited a very strong response to the bronchoconstrictor. Notably *E. coli*-treatment could inhibit eosinophil recruitment in IFN-γ deficient mice (Fig. 3C) and IL-4 and IL-5 production by BAL CD4 T cells was also impaired (data not shown), mimicking the disease outcome observed in wild type mice (Fig. 1). These results suggest that the administration of bacteria does not induce a switch to a Th1 environment in the lung, and that IFN-γ production by CD4 T cells or other cell types such as CD8 T or NK cells is not necessary to mediate AAI suppression in this model.

Upon intranasal *E. coli* administration, we observed a long-term suppression of Th2-mediated allergic responses, which is independent of Th1 immunity. To further investigate the possible mechanisms leading to inhibition of AAI, we analyzed the role of regulatory T cells (T regs), as these cells exert their modulatory function in a wide range of immune responses, including allergy (40-42). Of note, bacterial components in the lung have been described to induce regulatory T cell responses capable of suppressing eosinophil recruitment to the airways (22, 23). We analyzed the frequencies of regulatory T cells in the lung of *E. coli*- and non-treated mice according to FoxP3 and CD25 expression by CD4 T cells. As shown in Fig. 3D, the proportion of FoxP3+CD25+ CD4 T cells was decreased in *E. coli*-treated mice as compared to allergic mice. This result was reflected in the total regulatory T cell number (Fig. 3E). T regs were present at higher frequencies in the lung of allergic mice as compared to naïve mice; in contrast, this difference was not as pronounced in *E. coli*-treated mice, which displayed lower regulatory T cell numbers as compared to non-treated, ova-sensitized mice. These results are in line with the observed decrease in inflammation of *E. coli*-treated mice following AAI induction.
Figure 3: Inhibition of the development of AA1 is independent of Th1 and regulatory T cell responses.

(A) Non-treated and *E. coli* treated C57BL/6 mice were immunized and challenged with ova. On day 4 after i.n. challenge, BAL cells were isolated and proportion of IFN-γ-producing CD4 T cells determined after restimulation and intracellular staining. (B) IFN-γ deficient mice were treated as described in (A); airway responsiveness to increasing doses of MetCh was measured in a full body unrestrained plethysmograph on day 3 after i.n. ova challenge, and 24 h later eosinophil numbers in the BAL were determined (C). (D) Proportion of CD4+ CD25+ FoxP3+ regulatory T cells in the lungs of non-treated and *E. coli*-treated C57BL/6 mice was determined by flow cytometry on day 4 after challenge with ova. Values in histograms represent the percentage of cells detected in the indicated gate. Representative data from one mouse per group are shown. (E) Total T reg numbers in the lungs were calculated after determining total lung cell numbers with a Coulter Counter. (F) IL-10 deficient mice were treated as described in (A). Eosinophil numbers in the BAL were determined on day 4 after i.n. ova challenge by differential cell counts. Results represent values from 3-4 mice per group and are presented as mean ± SD. *, P < 0.05; statistically significant differences. Experiments were repeated 2-3 times.
In our model, bacterial administration did not seem to induce enhanced regulatory T cell responses. However, even though *E.coli*-treatment did not affect T regs frequencies in the lung, it could still have an influence on their function. As IL-10 has been recognized as an important effector cytokine in regulatory T cell immune responses (42, 43), we analyzed its role in inducing AAI suppression following *E.coli* administration. Accordingly, IL-10 deficient mice were treated with $10^7$ CFU *E.coli* followed by immunization and challenge with ova. Analysis of allergic inflammation was performed as previously described. As shown in Fig. 3F, eosinophil numbers in BAL of *E.coli*-treated IL-10 deficient mice were significantly reduced when compared to the non-treated counterparts. Airway hyperresponsiveness and cytokine production were similarly impaired (data not shown). Our data indicate that suppression of AAI by intranasal *E.coli* administration takes place independently of IL-10, further excluding a major role of regulatory T cell responses in the inhibition of this process.

**E.coli**-treated mice display normal dendritic cell migration and CD4 T cell priming in the lung draining lymph node.

Dendritic cells take up antigen in the airways and rapidly transport it to the lung draining lymph node (dLN) where presentation to specific T cells can occur (44). An intact DC migratory capacity is therefore crucial for the induction of immune responses of the respiratory tract (45, 46). We sought to investigate whether *E.coli* administration might influence DC migration and antigen transport to the draining LN in the course of an allergic response. First, total DC number was determined in the mediastinal LN of non- and *E.coli*-treated mice 24 and 48 h after ova-challenge. Migrating DCs were identified according to the expression of high levels of CD11c and CD11b (47, 48). An increased influx of dendritic cells to the draining LN was observed 24 h after challenge, followed by a reduction in numbers by 48 h (Fig. 4A). Notably, total DC numbers were comparable between non-treated asthmatic mice and mice treated with *E.coli* prior to AAI induction, suggesting that administration of the bacteria did not affect dendritic cell migration to the dLN.

We then more closely analyzed antigen transport to the draining LN by challenging the mice with FITC-conjugated ova. Antigen-bearing FITC+ cells were detected in the
mediastinal LN 24 h after challenge, and again comparable cell numbers could be observed between non- and E.coli-treated mice (Fig. 4B). Moreover, no significant impairment in antigen uptake and transport by CD11b\text{high}\text{CD11c}\text{high} DCs could be observed in E.coli-treated mice (Fig. 4C), and expression of activation markers such as MHC class II and CD40 was also normal when compared to non-treated allergic mice (Fig. 4D). These results indicate that intranasal administration of the bacteria does not alter migratory capacity and maturation of antigen-bearing dendritic cells in the lung draining LN, and suggest that antigen presentation to ova-specific T cells and their activation might occur normally in E.coli-treated mice.
Figure 4: Dendritic cell migration to the draining lymph node and CD4 T cell priming are not impaired by administration of the bacteria. Non-treated and E.coli-treated C57BL/6 mice were immunized with ova and challenged i.n. with FITC-labeled ova 7 days later. After 24 and 48 h, single cell suspensions of lung draining lymph nodes were prepared. (A) Total dendritic cell numbers in the draining lymph node were determined by flow cytometry according to CD11c and CD11b expression. (B) Total FITC+ LN cell number was determined by flow cytometry at 24 h after challenge (C) Total FITC+ DC numbers were determined as described in (A). (D) MHC class II and CD40 expression on LN CD11c+ CD11b+ dendritic cells was determined by flow cytometry 24 h after challenge. (E) On day 4 after i.n. challenge with ova, CD4 T cells were purified from the draining LN of non-treated and E.coli-treated mice and cultured with splenic DCs and different concentrations of ova protein. Proliferation was determined after 3 days by ³H-Thymidine incorporation. Alternatively, IL-5 and IFN-γ secretion in culture supernatant was determined on day 3 by ELISA (F). Results represent values from 3-4 mice per group and are presented as mean ± SD. Experiments were repeated 2-3 times.
To further test this hypothesis, we analyzed the proliferative capacity of CD4 T cells in the draining LN of non-treated and *E.coli*-treated mice. Accordingly, CD4 T cells were MACS-sorted from LN cell suspensions and cultured with freshly isolated splenic DCs and different concentrations of ova; proliferation was assessed after 3 days by \(^3\)H-thymidine incorporation. As shown in Fig. 4E, CD4 T cells isolated from *E.coli*-treated mice were able to proliferate to a similar extent as compared to T cells from non-treated ova-sensitized mice. As an additional measurement of T cell effector function, IL-5 and IFN-\(\gamma\) production by LN CD4 T cells was measured in the supernatant of a 3-day culture with DCs and 200 \(\mu\)M ova. Cytokine production by CD4 T cells was unaffected in *E.coli*-treated mice as compared to non-treated, allergic mice (Fig. 4F). Taken together our results suggest that administration of the bacteria does not lead to AAI suppression via the inhibition of T cell priming and activation in the lung draining LN.

**Intranasal *E.coli* administration alters dendritic cell function in the lung.**

In the periphery, DCs play a crucial role in the recognition of microbes and the initiation of immune responses through antigen presentation to T cells (49). Of note, a role for DCs in antigen presentation to T cells in non-lymphoid tissue has also been described (50); in particular it could be shown that in the lung, stimulation of memory T cells by local DCs is crucial for the establishment and maintenance of an asthmatic response (4, 51, 52). In an attempt to investigate the mechanisms underlying AAI suppression in our model, we hypothesized that *E.coli* administration could negatively influence DC function in the lung tissue as opposed to the draining LN, where antigen uptake and T cell priming seemed to be normal following exposure to the bacteria. To test this assumption we first analyzed antigen uptake by DCs in the lung by challenging control non-treated as well as *E.coli*-treated mice with ova-FITC. Lungs were isolated 24 h after challenge and FITC+ cells analyzed by flow cytometry (Fig. 5A); total FITC+ cell numbers were comparable between *E.coli-* and non-treated mice (data not shown). We further characterized FITC+ cells according to their expression of the surface molecules CD11b and CD11c, which are expressed at different levels by antigen presenting cells in the lung (48, 53). As shown in Fig. 5A, in non-treated mice the largest proportion of antigen-loaded cells consisted of CD11b+ CD11c+ cells, which in the lung correspond to DCs with high antigen uptake and
presentation capacity (54). In contrast, in the lung of *E. coli* treated mice, only a small fraction of DCs was able to take up the antigen, which was instead mostly present in the CD11c+ CD11b<sup>inter</sup> cell population (Fig. 5A). We further confirmed this result by analyzing total ova-FITC+ cell numbers; *E. coli*-treated mice had significantly decreased numbers of FITC+ DCs when compared to the non-treated, allergic counterparts (Fig. 5B, left). Conversely, a higher number of antigen-loaded CD11c+ CD11b<sup>inter</sup> cells could be observed after bacterial administration (Fig. 5B, right).

Our results suggest that upon *E. coli* administration, lower frequencies of DCs were taking up the antigen upon intranasal challenge. We sought therefore to clarify whether treatment with the bacteria would alter lung DC function. We first analyzed expression of activation markers on the surface of lung DCs as well as CD11c+ CD11b<sup>inter</sup> cells following ovalbumin challenge. In line with previous reports, CD11c+ CD11b+ DCs of non-treated, allergic mice displayed high surface expression levels of MHC class II; however DCs isolated from *E. coli*-treated mice expressed significantly lower levels of this activation marker (Fig. 5C, left panel). In contrast to dendritic cells, CD11c+ CD11b<sup>inter</sup> cells only expressed intermediate levels of MHC class II, and no difference could be observed between the two mouse groups (Fig. 5C, right panel). These data suggest that in mice treated with the bacteria antigen uptake and presentation by dendritic cells at the site of inflammation might be impaired, and that CD11c+ CD11b<sup>inter</sup> cells might not be as effective antigen presenting cells as DCs.
4. Results

Figure 5: Lung dendritic cells display impaired maturation and effector function following inhalation of *E. coli*. Non-treated and *E. coli*-treated C57BL/6 mice were immunized with ova and challenged i.n. with FITC-labeled ova 7 days later. After 24 and 48 h, mice were sacrificed and lung cell isolated. (A) Uptake of ova-FITC by different CD11b- and CD11c-expressing lung cell populations was determined by flow cytometry 24 h after i.n. challenge. Values in FACS plots represent the percentage of cells detected in the indicated gates. Representative data from one mouse per group are shown. (B) Total numbers of FITC+ cells of the indicated populations were determined. (C) Expression of MHC class II by the indicated lung cell
populations was assessed by flow cytometry. (D) CD11c+CD11b+ and CD11c+CD11b\textsuperscript{int} cells were FACS-sorted from the lung of non-treated and \textit{E. coli}-treated mice 24 h after i.n. ova challenge, and cultured in vitro with ova-specific CD4 T cell isolated from transgenic OT-II mice. Proliferation was assessed after 3 days by \textsuperscript{3}H-Thymidine incorporation. Alternatively, CD4 T cells were restimulated with PMA/ionomycin on day 3 and IFN-\(\gamma\) production determined by intracellular staining and flow cytometry (E). (F) Total lung CD11c+CD11b+ and CD11c+CD11b\textsuperscript{int} numbers were determined in non-treated and \textit{E. coli}-treated mice by flow cytometry. Results represent values from 3-4 mice per group and are presented as mean ± SD. *, \(P < 0.05\); statistically significant differences. Experiments were repeated 2-3 times.

To further elucidate these findings, we studied the capacity of the different lung antigen presenting cells (APCs) in presenting antigen to naïve ova-specific T cells \textit{in vitro}. Accordingly, 24 h after ovalbumin challenge CD11c+ CD11b+ DCs as well as CD11c+ CD11b\textsuperscript{int} cells were FACS sorted from lung cells of non-treated and \textit{E. coli}-treated mice and cultured with CD4 T cells isolated from transgenic OT-II mice. Three days later, proliferation of ova-specific cells was assessed by \textsuperscript{3}H-Thymidine incorporation, and cytokine production analyzed by intracellular staining and flow cytometry. Proliferation of CD4 T cells cultured with DCs from \textit{E. coli}-treated mice (\textit{E. coli}-DCs) was drastically reduced when compared to the non-treated allergic controls (Fig. 5D, left). We could further demonstrate that CD11c+ CD11b\textsuperscript{int} cells isolated from non-treated mice were less capable of inducing T cell expansion than CD11b+ CD11b+ DCs, and that this population could also be negatively regulated by treatment with the bacteria (Fig. 5D, right). In line with the impairment in proliferation, lower proportions of CD4 T cells were producing IFN-\(\gamma\) when stimulated by \textit{E. coli}-DCs compared to control DCs (Fig. 5E), whereas CD11c+ CD11b\textsuperscript{int} cells from both non-treated and \textit{E. coli}-treated mice poorly induced IFN-\(\gamma\) production.

Considering the influence of \textit{E. coli} administration on lung DC maturation and function, we sought to investigate whether treatment with the bacteria would also have an impact on the frequencies of the different APCs following ova challenge. Of note, in the immediate time after antigen inhalation a significant influx of DCs into the airways can be observed, which is supported by an increase in precursors cells in the bone marrow (55). We isolated lung cells from non-treated as well as \textit{E. coli}-treated mice 24 and 48 h after challenge, and APC frequencies were determined by flow cytometry. As already described, we could observe a rapid increase in CD11c+ CD11b+ DC numbers in the lung, yet no
significant difference between non- and *E.coli*-treated mice could be detected (Fig. 5F, left panel), suggesting that administration of the bacteria was not influencing DC recruitment into the airways. In contrast to DCs, frequencies of CD11c+ CD11bint cells did not significantly change following intranasal challenge, and only a small, non-significant, difference in total cell numbers in *E.coli*-treated mice could be observed (Fig. 5F, right panel). Taken together our data show that intranasal administration of bacteria alters DC function in the lung without affecting their recruitment.

**Cytokine production by effector Th2 cells in the lung is impaired in *E.coli*-treated mice.**

At the peak of an asthma response, the airways of mice previously treated with *E.coli* displayed reduced total numbers of CD4 T cells along with impaired Th2 cytokine production; however so far no conclusion could be drawn on the specificity of these cells. We therefore sought to analyze in more detail whether *E.coli*-treatment could affect migration of antigen-specific effector Th2 cells to the site of inflammation and *in situ* cytokine production. Thus, ova-specific CD4 T cells from OT-II transgenic mice were differentiated in vitro to Th2 effector cells, and subsequently injected intravenously into mice previously treated with *E.coli* or non-treated mice as a control (4). Mice were then challenged intranasally with ova, and number of transferred cells was determined in different tissue compartments. We observed a recruitment of transferred cells into the airways upon intranasal challenge. Surprisingly, a similar number of CD45.2+ T cells could be detected in the BAL, lung and draining LN of non-treated as well as *E.coli*-treated mice, suggesting that administration of the bacteria did not impair effector T cell recruitment to the site of inflammation (Fig. 6A).
Figure 6: Th2 cells recruited to the lung of E.coli-treated mice display impaired cytokine production. CD45.2+ CD4 T cells isolated from the spleen of ova-transgenic OT-II mice were polarized to Th2 cells in vitro and adoptively transferred into CD45.1+ C57BL/6 mice previously treated with E.coli or into non-treated mice. On the same day, and for the following 3 days, adoptively transferred mice were challenged i.n. with ova. 24 h after the last challenge, BAL, lung and draining LN were isolated. (A) Total CD45.2+ cell numbers were determined by flow cytometry in the BAL (left panel), lung and dLN (right panel). Total cell numbers in the BAL were determined with a Coulter Counter (B), and eosinophil proportion assessed by differential cell counts (C). (D) Cytokine production by transferred lung CD45.2+ was determined after in vitro restimulation with PMA/ionomycin and intracellular staining. Representative data from one mouse per group are shown. Alternatively, IL-13 and IL-5 secretion in BAL fluid was determined by ELISA (E). Results represent values from 3 mice per group and are presented as mean ± SD. *, P < 0.05; statistically significant differences.
It has previously been shown that transfer of Th2 cells followed by intranasal antigen exposure can induce asthma features such as airway hyperresponsiveness and eosinophilia (56). We therefore investigated the immune response in the lung of adoptively transferred *E. coli*- and non-treated mice following challenge with ovalbumin. Accordingly, total BAL cell numbers as well as the proportion of eosinophils were determined. As shown in Fig. 6B, intranasal challenge led to an increase in cell infiltration into the airways, whereby no difference in numbers could be observed between non-treated and *E. coli*-treated mice. However, mice treated with the bacteria prior to Th2 cell transfer displayed significantly lower eosinophil frequencies in the BAL, indicating that prior *E. coli*-treatment was able to impair the effector function of polarized Th2 cells in the lung (Fig. 6C).

In this case, already primed T cells were transferred, which led us to the hypothesis that administration of the bacteria may alter the induction of T cell effector function at the site of inflammation. To further clarify our findings we therefore analyzed Th2 effector cytokine production by transferred cells in the airways after challenge. Accordingly, lung cells were restimulated *in vitro* and intracellular cytokine production analyzed by flow cytometry (Fig. 6D). Notably, we could observe a reduction in frequencies of IL-4- and IL-5-producing transferred T cells in the lung of *E. coli*-treated mice as compared to non-treated mice challenged with ovalbumin. This result was further confirmed by measuring cytokine secretion in the BAL fluid of these mice by ELISA. Accordingly, IL-13 and IL-5 could be detected in the airways of non-treated, ova-challenged mice, but not in *E. coli*-treated or non-challenged control mice (Fig. 6E), indicating that effector cytokine production by previously primed, antigen-specific T cells in the lung was impaired in mice treated with the bacteria.

Taken together, our results indicate that *E. coli*-treatment leads to an impairment of Th2 effector functions in the lung, which eventually results in the suppression of classical AAI features such as airway eosinophilia. The inhibition of lung memory T cell activation in *E. coli*-treated mice correlates with the observed alteration in dendritic cell function; however, it remains possible that other inhibitory mechanisms could act directly on T cells to suppress the development of asthma.
4. Results

4.3.4 Discussion

In the present investigation, we sought to analyze the role of bacterial inhalation in regulating ensuing immune responses in the respiratory tract; specifically, we studied the outcome of allergic airway inflammation induced after intranasal infection with the extracellular bacteria *Escherichia coli*. We chose this particular bacterial strain because it induces a strong inflammation but is promptly cleared from the lung (data not shown), allowing us to distinguish the immune response elicited against the microbial products and the subsequent allergic response. We could show that intranasal administration of *E. coli* prior to AAI induction lead to the abrogation of airway hyperresponsiveness, Th2 cell effector function and lung eosinophilia.

Development of an asthmatic response involves antigen uptake by airway DCs and their migration to the draining LN (44, 57). In the lymphoid tissue, DCs present the antigen to specific naïve and memory T cells, leading to the generation of effector Th2 cells (58), which then migrate to the site of inflammation (2). In the inflamed lung, Th2 cells secrete IL-4, IL-13, IL-5 to induce the recruitment of eosinophils, goblet cell hyperplasia and changes in epithelial and smooth muscle cells leading to airway remodeling as well as tissue damage (51). In the recent past it became evident that Th2 cells require further activation by dendritic cells in the inflamed lung to efficiently produce inflammatory cytokines (31). Using CD11c-DTR transgenic mice, Van Rijt et al. could show that already primed Th2 cells failed to produce cytokines in the absence of lung DCs and that local depletion of these cells during already established airway inflammation inhibited further infiltration of eosinophils and goblet cell hyperplasia. Due to the critical role played by DCs in the sensitization phase as well as in the maintenance of ongoing allergic inflammation, we sought to investigate whether intranasal *E. coli* administration could modulate DC function, and thus the development of AAI. We could demonstrate that following challenge with ova, DCs readily transported the antigen to the draining LN in both non-treated as well as *E. coli*-treated mice. Moreover, in this compartment no difference in DC maturation or activation of specific T cells could be observed between the two groups of mice, which was indicative of normal DC function.

However, we observed a dramatic reduction of Th2 cytokine production by CD4 T cells in the BAL and lung of *E. coli*-treated mice, suggesting that activation of these cells was not taking place. We therefore speculated that DC function in the lung as opposed to
the LN might be affected. Indeed, we could show that following intranasal challenge with FITC-labeled ova, CD11b+ CD11c+ DCs from the lung of E. coli-treated mice had taken up significantly less antigen when compared to non-treated, allergic mice. Moreover, although total DC numbers were comparable between non-treated and E. coli-treated mice, the same DCs expressed lower levels of MHC class II molecules, indicating that bacterial administration could influence the maturation of DCs and their presentation capacity without affecting their survival or recruitment to the inflamed lung. We further confirmed these results by sorting this DC population from the lungs of non-treated and E. coli-treated mice. DCs from non-treated, allergic mice could efficiently present ova-protein to transgenic CD4 T cells, inducing proliferation and cytokine production. In contrast, this process was drastically inhibited when using E. coli-DCs as APCs. The lung CD11b+ CD11c+ MHC$_{\text{high}}$ DC population has been described to mediate in situ antigen presentation (48, 53). Moreover, upon intranasal challenge with the Leishmania major-derived LACK peptide, the same population was described as the most efficient in driving T cell proliferation (54). We can therefore speculate that alteration of lung DC maturation and effector function might be a crucial mechanism for the inhibition of effector CD4 T cell activation and consequently AAI upon intranasal administration of the bacteria.

Interestingly, most of the antigen administered to the airways of E. coli-treated mice was taken up by another cell population, which expressed high CD11c levels but only intermediate levels of MHC class II and CD11b. We sorted these CD11c+ cells from the lung of non-treated as well as E. coli-treated mice and again analyzed their presentation capacity in an in vitro culture with CD4 transgenic T cells. Our results indicate that this lung APC population is less efficient than CD11b+ CD11c+ DCs in activating naïve T cells in vitro (54, 59). Interestingly, Matthews et al. describe a resident lung CD11c+ CD11b- population capable of retaining an intranasally administered antigen over a longer period of time, and expressing characteristics of both macrophages and DCs. In the airways, macrophages have been shown to express CD11c, to have poor antigen presentation capacity and to suppress lung DC function (60-63). Further work will be required to characterize the CD11c+ cell population responsible for antigen uptake in the lung of E. coli-treated mice; however we can conclude that upon administration of the bacteria, antigen presentation by less activated and functional CD11b+ CD11c+ DCs as well as less efficient CD11c+ APC might eventually lead to the observed impairment in effector T cell activation. This was further confirmed by transferring already differentiated,
cytokine-producing ova-specific Th2 cells into non-treated and *E.coli*-treated mice. After intranasal challenge with ova, transferred cells were recruited in similar numbers to the lung of both groups of mice. Cytokine production by these Th2 cells was however significantly inhibited in *E.coli*-treated mice when compared to the non-treated counterparts. These results indicate that following bacterial challenge, restimulation of recruited Th2 cells by lung APC to elicit full effector T cell function is impaired, leading to decreased downstream pathways such as airway eosinophil infiltration.

It is important to mention that at the peak of the asthmatic response, 4 days after ova-challenge, we observed lower frequencies of CD4 T cells in the airways of *E.coli*-treated mice. Of note, during allergic airway inflammation DCs also play an important role in the secretion of Th2-attracting chemokines such as CCL17 and CCL22 (64, 65). Considering the impaired lung DC function observed following *E.coli*-administration, we could hypothesize that the decrease in airway inflammation might also derive from an impairment in Th2 cell recruitment. However, as previously mentioned, when we transferred already activated Th2 cells, we could detect the same number of specific CD4 T cells in the airways of mice receiving the bacteria as well as non-treated mice, suggesting that inhibition of effector cell recruitment is not critical for the suppression of asthma in *E.coli*-treated mice.

We found that intact TLR4 signaling was a crucial factor for the inhibition of asthma in our model; TLR4 deficient mice treated with the bacteria displayed the same extent of airway inflammation as non-treated, ova-sensitized mice. These results indicate that LPS-dependent innate immune responses triggered by *E.coli* in the lung are essential in modulating asthma suppression. Of note, a role for endotoxin in the negative regulation of allergic responses has been postulated, although depending on the dose and timing of the exposure, the opposite effect could also be observed (7, 14, 29, 66-68). Moreover, TLR4 has also been listed as a susceptibility gene in the development of asthma (69). Interestingly, inhibition of asthma development via environmental exposure to endotoxin was associated with increased Th1 cell-mediated responses (25, 66), and the same result was observed when mice were intranasally treated with high doses of LPS (70). However in our model we could show that *E.coli* administration did not induce a shift to a Th1 immune response, and suppression of allergic inflammation was independent of IFN-γ. We therefore speculate that the inhibitory effect might not be due only to LPS-mediated TLR4 stimulation; instead, a synergistic effect of different *E.coli*-derived TLR ligands such as...
MDP or CpG is plausible. Notably these ligands have been previously shown to act synergistically in the induction of inflammatory responses (71, 73). Nevertheless, the importance of LPS and TLR4 signaling in our model remains clear, and the experiments performed in Rip2 deficient mice indicate that other pathways might only play a secondary role. In this respect, it remains to be elucidated how TLR4 signaling could influence the outcome of asthma independently of Th1 responses. In particular, it is unclear whether TLR4 stimulation directly on DCs could lead to the observed changes in this cell population in the lung. Alternatively, TLR4 has also been shown to be expressed by human airways epithelial cells (74), and stimulation of these cells by LPS might modify the lung environment to mediate the inhibitory effect. Ongoing experiments involving wild type and TLR4 deficient bone marrow chimeras will help us clarify this issue.

As mentioned above, at present it is still unclear what might influence DC function in the lung. In view of the timing of bacterial administration and asthma induction in our model, the limited life-span of lung CD11b+ CD11b+ DCs (62), and the constant recruitment of new DCs from the blood to the airways during inflammation (55), we consider a direct regulation of DC function by E.coli unlikely to be the only effect in mediating asthma suppression. Of note, we attempted to reconstitute lung DC function and thus the induction of AAI in E.coli treated mice by challenging mice with fully competent, ova-pulsed bone marrow-derived DCs instead of ova-protein alone. In line with previous reports, BM-DC administration induced AAI features in sensitized mice (4), but failed to do so in mice previously treated with the bacteria (data not shown). These results could indicate that the lung environment exhibits a suppressive effect upon DCs that might remain constant following E.coli administration or become re-activated at the time of antigen challenge. Such a phenomenon would further explain the long-lasting suppression of allergic airway inflammation following treatment with the bacteria.

Considering that antigen was differentially distributed between lung APC populations in non-treated and E.coli-treated mice, we can speculate that in the latter, pulmonary macrophages engulf the administered antigen and then suppress lung DC function, whilst in non-treated mice, the presence of airway inflammation might overcome the suppressive effect by the same macrophage population (60, 63). Of note, turnover of pulmonary macrophages has been shown to be of several days (59, 62), indicating that these cells might exert their inhibitory function over a longer period of time. In addition to the possible effect mediated by this APC population, a recent review by Hammad and
Lambrecht highlights the role of lung epithelial cells in regulating DC function and airway inflammation (31). In particular it is discussed how an airborne antigen can activate lung epithelial cells to produce important factors such as the cytokines TSLP and GM-CSF, which can specifically induce maturation of DCs for the priming of Th2 immune responses (75, 76). Moreover epithelial cells have also been shown to secrete cytokines such as IL-25, which directly acts on Th2 cells to enhance their effector function (77). Another important interaction between epithelial cells and antigen presenting cells in the airways occurs at the level of CD200 and CD200R. Regulation of CD200-CD200R interaction has been shown to prevent excessive inflammation following influenza virus infection, but might also play a role in regulating lung homeostasis during other lung immune responses (78, 79). Considering these recent findings we could speculate that intranasal E.coli administration might influence epithelial cell function to alter lung DC as well as T cell function in a way that allergic airway responses can no longer develop. In this respect it would be of interest to investigate whether the same bacterial treatment might also inhibit the development of other, Th2-independent immune responses of the lung such as the already mentioned influenza virus infection.

The role of environmental exposure to microbes and their products in regulating allergic immune response in the respiratory tract has been described (7, 10, 17, 25, 77). The ‘hygiene hypothesis’ provides a correlation between the dramatic increase in the incidence of asthma over the past few years and a decrease in viral and bacterial infections due to vaccination and better hygienic conditions (8). The mechanisms underlying this theory and the regulation of asthma by pathogens and their products are complex and still poorly understood; however, a fine balance between effector Th1 and regulatory T cells in inhibiting Th2-dependent allergic responses has been postulated (27, 80). Surprisingly, we describe suppression of allergic airway inflammation following intranasal bacterial administration that does not depend on a switch to Th1 responses, nor on the induction of elevated regulatory T cell frequencies and IL-10 production. In conclusion we therefore propose a novel mechanism for the inhibition of AAI that acts to significantly alter lung DC function and consequently hinders the establishment of Th2-dependent disease features. Taken together our findings not only highlight once more the role of dendritic cells in orchestrating allergic airway inflammation, but also add another level of complexity on the role microbes and derivatives can have in modifying lung homeostasis and influencing immune responses in the respiratory tract.
4. Results

4.3.5 References


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dendritic cells is supported by an increase in CD31(hi)Ly-6C(neg) bone marrow precursors in a mouse model of asthma. Blood 100:3663-3671.


4. Results


4.4 Bacterially-induced γδ T cells provide long-term protection against allergic airway hyperresponsiveness.

Chiara Nembrini¹, Jan Kisielow¹, Manfred Kopf¹, and Benjamin J. Marsland¹

¹ Institute of Integrative Biology, Molecular Biomedicine, ETH Zürich, Switzerland

Address correspondence to: Ben Marsland, Molecular Biomedicine, Swiss Federal Institute of Technology, Wagistrasse 27, 8952 Schlieren, Switzerland
EMAIL: marsland@env.ethz.ch, TEL: 0041 1 633 6470
4.4.1 Abstract

γδ T cells are a lymphocyte subset characterized by limited specificity and preferential localization at epithelial and mucosal surfaces. Although the exact function of γδ T cells still remain to be elucidated, these lymphocytes are thought to contribute to a first line of defense against pathogenic as well as non-pathogenic agents, and to thereby regulate immune responses as well as tissue homeostasis. In the present report, we show that high numbers of IL-17-producing γδ T cells were recruited to the respiratory tract upon intranasal challenge with *Escherichia coli*, and that this lymphocyte population resided in the lung for several weeks. We further demonstrate that *E.coli*-administration prior to allergen sensitization was able to suppress the development of eosinophilia, Th2 effector function and airway hyperresponsiveness (AHR) in a mouse model of asthma. The suppression of AHR was dependent on the presence of γδ T cells, as mice lacking this lymphocyte subset still developed enhanced airway hyperreactivity following *E.coli*-treatment and allergic airway inflammation (AAI) induction. Our findings highlight the important role γδ T cells play in regulating lung function in response to inflammation and immunity.
4. Results

4.4.2 Introduction

In the lung, numerous lymphocyte subsets are involved in the development of immune responses against airborne pathogens and allergens. The role of the classical αβ T cells in conferring protection against viruses and bacteria has been extensively described (1-4). During allergic responses such as AAI, effector T helper 2 cells migrate to the lung, where they produce inflammatory cytokines that drive the classical features of AAI such as AHR, eosinophilia, goblet cell hyperplasia and tissue remodeling (5). In contrast to αβ T cells, the role of γδ T lymphocytes in regulating immunity of the respiratory tract remains controversial or poorly understood.

γδ T cells are known to undergo VDJ-recombination of the TCR, but their specificity remains limited to few so-called “self” antigens such as the stress-induced MHC class Ib molecules T10/22 (6). γδ T cells are preferentially located in peripheral tissues, where they increase in number in response to infectious as well as non-pathogenic stimuli (7). These characteristics lead to the general assumption that γδ T cells are part of the innate immune system (8, 9) and might be important in the regulation of immune responses and normal tissue homeostasis (7). Of note, γδ T lymphocytes have been shown to produce keratinocyte growth factors (10), and to protect from epithelial cell damage (11), underlining a potential role for these cells in mediating tissue repair (12, 13).

In the lung, recruitment of γδ T cells has been observed during immune responses to several bacteria including Mycobacteria tuberculosis and Streptococcus pneumoniae (14-17). γδ T cells have additionally been shown to play a protective role against lung infection with Nocardia asteroides (11); however, overall mice lacking γδ T lymphocytes do not show dramatic impairments in mounting immune responses to pathogens (18). γδ T cells have been associated with allergic immune responses of the lung such as asthma; in particular, they have been shown to influence the development of airway hyperresponsiveness (19, 20). However, both beneficial as well as detrimental effects of γδ T cells on AHR have been described, depending on the experimental procedure used and the cell subset considered. γδ T cells expressing the Vγ1 chain have been shown to promote airway hyperreactivity, whereas Vγ4-expressing cells appear to be negative regulators of AHR (21, 22). These contrasting findings and the presence of different γδ T
cell subsets in the lung emphasize the diverse functions γδ T cells might have in influencing the lung environment.

Recently, we described a mouse model for the modulation of asthma dependent on the intranasal administration of the bacteria *Escherichia coli*. Treatment with *E.coli* led to suppression of ovalbumin-induced Th2 responses with impaired airway hyperresponsiveness and eosinophilia. In the present investigation, we sought to analyze the role of γδ T cells in regulating this process. We show that upon intranasal *E.coli* administration, considerable numbers of IL-17-producing γδ T cells are recruited to the airways, where they remain active for up to 30 days following challenge. Moreover these cells are present at higher frequencies in the lung of *E.coli*-treated mice sensitized and challenged with ovalbumin, and contribute to the suppression of airway hyperresponsiveness but not other asthma features. Our results reveal that γδ T cells might play an important role in the immunomodulation of allergic responses following prior exposure to bacteria in the lung.
4.4.3 Materials and Methods

Mice and pathogens

C57BL/6 wild-type mice were obtained from Charles River Breeding Laboratories. TCR-δ deficient mice were kindly provided by Prof. Rob MacDonald (Ludwig Institute for Cancer Research, Lausanne, Switzerland). Mice were maintained specific pathogen-free at BioSupport, Zürich in isolated ventilated cages. Animal used in experiments were between 8 and 12 weeks of age. All experiments were performed with permission from the Zürich Animal Ethics Committee.

Glycerol stocks of *Escherichia coli* DH5αpDS (DS Red) were grown overnight in LB medium supplemented with 100 µg/ml Ampicillin. OD$_{600}$ was measured in the morning to determine concentration of the bacteria. 10$^7$ CFU *E.coli* were resuspended in 50 µL PBS and administered intranasally. For determination of bacterial load, lung cells were isolated and plated on agar plates supplemented with Ampicillin.

Ova-induced airway inflammation

Mice were immunized by intraperitoneal injection with 100 µg ovalbumin (grade V; Sigma-Aldrich) in 200 µl of alum adjuvant (SERVA; Electrophoresis GmbH). 7 d later, mice were challenged by intranasal (i.n.) inoculation with 100 µg ova in 50 µl PBS. *E.coli* were administered 3 to 5 days prior to immunization.

Collection and Analysis of Bronchoalveolar Lavage (BAL) Cells.

Mice were sacrificed, the trachea was cannulated and BAL performed by flushing the airways three times with 1 ml PBS. Total BAL cells were counted using a Coulter Counter (IG Instruments) and spun onto glass slides using a Cytospin 2 (Shandon Southern Products, Ltd.). Cells were then stained with Diff Quick staining set (Siemens-Dade Behring) and, once dried, embedded in Eukit solution (O. Kindler GmbH & Co.) under glass coverslips. Percentages of neutrophils and eosinophils were determined microscopically by counting 200 cells/sample using standard morphological and cytochemical criteria. Alternatively, BAL cells were analyzed by flow cytometry.

FACS analysis

BAL cells were analyzed for surface expression of γδ-T cell receptor, Vγ4-chain,
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αβ-TCR, or CD4 by staining with Biotin-conjugated anti-γδ TCR mAb (GL-3, BD Pharmingen) followed by streptavidin-APC, FITC-labeled anti-Vγ4 mAb (BD Pharmingen), PE-labeled anti-TCRβ mAb (eBioscience), or PercP-labeled CD4 (BD Pharmingen) in PBS/0.1% BSA. For intracellular cytokine staining, cells were stimulated with PMA/ionomycin for 4 h, with the addition of Monensin (Sigma-Aldrich) to retain cytokines in the cytoplasm. After surface stain, cells were washed with PBS and fixed with 2% paraformaldehyde for 20 min at room temperature. Fixed cells were incubated in permeabilization buffer (0.5% saponin/PBS/0.5% BSA) containing Alexa-Fluor 647-conjugated anti-IL-17A mAb, FITC-labeled anti-IFN-γ mAb (both eBioscience) or APC-labeled anti-IL-5 (BD Pharmingen) for 30 min at room temperature. After washing, cells were resuspended in PBS/0.1% BSA and analyzed by flow cytometry (FACS Calibur; BD Biosciences) and FlowJo software (Tree Star).

**Measurement of Airway Responsiveness.**

On day 3 after i.n. challenge with ova, mice were placed in individual, unrestrained whole body plethysmograph chambers (Buxco Electronics). Airway responsiveness was assessed in mice by inducing airflow obstruction with aerosolized methacholine chloride (MetCh). Pulmonary airflow obstruction was assessed by measuring PenH using BioSystem XA software (Buxco Electronics). Measurements of MetCh responsiveness were obtained by exposing mice for 3 min to incremental doses of aerosolized MetCh (Aldrich Chemie) and monitoring the breathing pattern for 5 min after initiation of aerosol dose.
4.4.4 Results and Discussion

**Intranasal E.coli administration induces high frequencies of IL-17-producing γδ T cells in the lung.**

In contrast to the classical αβ T lymphocytes, γδ T cells are preferentially found in non-lymphoid tissues such as skin and lung, where they might provide a first line of defense against invading agents (18). Under steady-state conditions, their relative number is small; however, high frequencies of γδ T cells can be induced in the periphery upon infection or inflammation. In the lung this process takes place following infection with several bacteria such as *Mycobacterium tuberculosis* and *Streptococcus pneumoniae* (16, 23). We observed a suppression of allergic airway inflammation following intranasal administration of the extracellular bacteria *Escherichia coli*, and sought to investigate whether γδ T cells played a role in this phenomenon.

In our model, inhibition of AAI was obtained by challenging mice i.n. with *E.coli* prior to induction of the allergic disease. First, we analyzed the extent of inflammation induced by this *E.coli* dose by monitoring neutrophil infiltration to the airways, as these cells are known provide the first line of defense against bacterial insults in the lung (24). Accordingly, *E.coli* were administered to wild type C57BL/6 mice and neutrophil influx in the bronchoalveolar lavage (BAL) was determined in the following days by differential cell counts. As shown in Fig. 1A, a strong influx of neutrophils into the airways could be observed, which peaked at 24 h after challenge and resolved by day 6. As a result of neutrophil infiltration, *E.coli* were progressively cleared from the lung of infected mice and were no longer detected on day 6 after administration of the bacteria (Fig. 1B). These results indicate that *E.coli* induce a short-lived, innate immune response which prevents bacterial replication.
4. Results

Figure 1: γδ T cells are recruited to the lung upon intranasal challenge with E.coli. C57BL/6 mice were treated i.n. with \(10^7\) colony forming units (CFU) of E.coli. (A) Neutrophil numbers in the bronchoalveolar lavage (BAL) were determined by differential cell counts at the indicated time points. (B) Lung cells were isolated and distributed on agar plates, and CFUs determined after overnight incubation at 37°C. (C) γδ T cell numbers in the BAL and in the lung were measured on the indicated days by surface staining and flow cytometry. (D) Proportions of Vγ4+ γδ T cells in the lung were determined on day 13 after challenge with the bacteria. (E) Cytokine production by γδ T cells was assessed by intracellular staining and flow cytometry on day 13 after E.coli challenge. Values in FACS plots represent the percentage of cells detected in each gate. Representative data from one mouse per group are shown. (F) IL-17 production by γδ T cells was determined as in (E) at different time points. Results represent values from 3 mice per group and are presented as mean ± SD. Experiments were repeated twice.
Next, we followed the appearance of γδ T cells in the BAL and lung of E.coli-treated mice by flow cytometry. In line with previous reports (6), we detected approximately $10^4$-$10^5$ γδ T cells in the lung parenchyma of naïve mice (day 0), whereas only few lymphocytes were present in the BAL (Fig. 1C). However, a rapid increase in lung γδ T cell numbers could be observed already by day 4, with a peak on day 6 following challenge with the bacteria. The extent of γδ T cell infiltration was even more dramatic in the bronchoalveolar lavage, where a 100-fold increase in lymphocyte numbers could be observed by day 6 after E.coli administration. γδ T cell frequencies remained elevated for at least 30 days following intranasal challenge with the bacteria (Fig. 1C), indicating that in the respiratory tract E.coli could induce a significant γδ T cell response which persisted long after bacterial clearance from the lung tissue.

In steady-state conditions, three main γδ T cell subsets can be detected according to the expression of the Vγ4, Vγ1 or Vγ6 chain. As γδ T cells bearing the Vγ4 chain are the most represented subset in the lung (25), we sought to analyze expression of Vγ4 following challenge with the bacteria. Our results show that upon E.coli administration the proportion of Vγ4+ γδ T cells decreases as compared to naïve mice, indicating the enrichment of another cell subset (Fig. 1D). Additional analysis will be required to further characterize γδ T cell subsets induced upon exposure to the bacteria.

Next, we sought to analyze whether E.coli-induced γδ T cells were able to produce inflammatory cytokines. In particular, γδ T cells have been shown to produce IL-17 in the course of bacterial infections (26-28), yet production of other cytokines such as IFN-γ and IL-4 has also been observed (29). Accordingly, we isolated BAL cells from E.coli-treated mice at different time-points and restimulated them in vitro. Cytokine production was assessed by intracellular staining and flow cytometry. In line with previous reports, high frequencies of IL-17-producing γδ T cells could be detected, whilst IFN-γ production by the same cells was minimal (Fig. 1E). Of note, production of IL-17 was maintained at high levels for at least 30 days (Fig. 1F). Whether IL-17 secretion by γδ T cells plays a crucial role in conferring protection against bacterial infections still remain to be elucidated (30). Nevertheless, recent reports indicate that γδ T cell-derived IL-17 might be important for the induction of optimal immune responses following Mycobacterium bovis-BCG or Listeria monocytogenes infection (26, 31).
Taken together these data describe the induction of an IL-17-producing γδ T cell population in the respiratory tract following intranasal challenge with E.coli. In the lung, γδ T cells have been suggested to provide a first line of defense during several bacterial infections (11, 32), and E.coli infection in the peritoneum has been shown to rely on γδ T cells to induce neutrophil infiltration and to limit bacterial growth (28, 33). In response to E.coli inhalation, we observe a rapid influx of neutrophils to the airways, which precede the enhanced infiltration of γδ T cells. Clearance of the bacteria from the lung tissue also occurs prior to γδ T cell induction, leading us to the speculation that in this model, γδ T lymphocytes might not play a crucial role in mediating protection against E.coli. We can further hypothesize that these cells are induced in the lung in response to the strong inflammation caused by the bacteria and might rather be important in regulating tissue homeostasis and limiting tissue damage.

**E.coli-induced γδ T cells can protect from airway hyperresponsiveness during asthma.**

After intranasal treatment with E.coli, a large population of γδ T cells could be detected in the BAL and lung parenchyma (Fig. 1C). We sought to investigate whether increased lung frequencies of this lymphocyte subset might have an effect on the development of AAI, which was induced in mice by intraperitoneal injection of ova followed by intranasal challenge with the same antigen. Accordingly, we first analyzed by flow cytometry proportions of γδ T cell in the airways of asthmatic mice 4 days after challenge with ova, and compared the results with mice treated with E.coli prior to disease induction. As shown in Fig. 2A, we observed a significantly higher proportion of γδ T cells in the BAL of E.coli- and ova-treated mice as compared to mice treated with ova only. Of note, the increase in γδ T lymphocyte frequencies in mice treated with the bacteria was accompanied by a decrease in classical αβ T cells at the peak of the asthma response. We then measured total cell numbers of γδ T cells in the BAL of ova-treated, E.coli/ova-treated and naïve mice. In line with previous reports (22, 34), ova administration alone could induce higher γδ T cell numbers in the airways of challenged mice when compared to their naïve counterparts (Fig. 2B). However, this increase was even more significant in mice treated with E.coli prior to AAI induction.
In a previous study, we found that *E. coli* administration prior to ova sensitization led to inhibition of classical AAI features such as Th2 cytokine production, eosinophil infiltration and AHR. Considering the elevated γδ T cell numbers in the airways of *E. coli*-treated mice, we sought to investigate whether these cells were playing a direct role in mediating suppression of asthma. Accordingly, wild type and TCR-δ deficient mice were treated with *E. coli* 3 days prior to immunization with ova, followed by i.n. challenge after an additional 7 days. On day 4 after challenge with ova, eosinophil infiltration in the BAL of *E. coli*-treated mice was determined by differential cell counts and compared to mice sensitized with ova only. As previously described, administration of bacteria led to a dramatic decrease in eosinophil numbers in wild type mice (Fig. 2C, left). A similar result was observed in mice lacking γδ T cells, where eosinophil numbers were significantly reduced in *E. coli*-treated mice when compared to asthmatic control mice (Fig. 2C, right). Of note, TCR-δ deficient mice displayed lower eosinophil frequencies in the airway upon classical ova-dependent sensitization and challenge when compared to wild type counterparts. This result is in line with previous reports suggesting that γδ T cells might play a role in the induction of normal allergic airway responses including eosinophil recruitment to the lung (19, 20).

To further investigate the role of γδ T cells in regulating asthma responses, we analyzed Th2 cytokine production in wild type and TCR-δ knock out mice previously treated with *E. coli* as well as mice sensitized with ova alone. BAL cells were isolated 4 days after i.n. ova-challenge and IL-5 production by CD4 T cells assessed after *in vitro* restimulation by intracellular staining and flow cytometry. As shown in Fig. 2D, both wild type as well as TCR-δ deficient mice treated with ova alone displayed elevated frequencies of IL-5-producing CD4 T cells in the BAL, indicating that even in the absence of γδ T cells mice could still mount normal Th2 responses. Of note, development of IL-5-producing CD4 T cells was impaired in mice treated with *E. coli* prior to asthma induction, however no difference could be observed between wild type and γδ T cell deficient mice.

The cardinal feature of allergic airway inflammation is the development of airway hyperresponsiveness (35). Of note, several reports have postulated a role for γδ T cells in regulating AHR (19, 20, 22, 36); however the diversity of lung γδ T lymphocyte subsets as well as the usage of different experimental procedures has led to disparate and controversial results. In particular, mice genetically lacking γδ T cells were shown to have increased AHR(19, 20), whilst studies involving γδ T cell depletion by antibody
administration described both beneficial as well as detrimental effect on AHR, depending on the timing of Ab-treatment and the cell subset involved (21).

We sought to analyze the role of γδ T cells in modulating AHR in our model of E.coli administration and AAI induction. To this purpose wild type and TCR-δ deficient mice were immunized and challenged with ova with or without being previously treated with the bacteria. The development of airway hyperreactivity to increasing doses of the bronchoconstrictor methacholine was measured on day 3 after intranasal ova-challenge in a whole body unrestrained plethysmograph. As expected, wild type mice sensitized with ovalbumin developed AHR in response to methacholine, whilst mice previously treated with E.coli exhibited no sign of airway hyperresponsiveness (Fig. 2E, left panel). Notably, the inhibition of AHR caused by treatment with the bacteria could no longer be observed in TCR-δ deficient mice. In the absence of γδ T cells, both non-treated as well as E.coli-treated, ova-sensitized mice developed AHR in response to methacholine to a similar extent as their wild type, allergic counterparts (Fig 2E, right panel), indicating that γδ T cell infiltration plays a critical role in downregulating this important feature of asthma.
4. Results

Figure 2: *E. coli* inhalation does not suppress allergic airway hyperresponsiveness in γδ T cell deficient mice. (A) C57BL/6 mice were treated with 10⁷ CFU *E. coli* and immunized i.p. 3 days later with ova in alum. Seven days later, mice were challenged i.n. with ova in PBS. Alternatively, mice were sensitized and challenged with ova without prior administration of the bacteria (ova only). On day 4 after i.n. challenge with ova, BAL cells were isolated and proportions of αβ and γδ T cells determined by surface staining and flow cytometry. Representative data from one mouse per group are shown. (B) Total γδ T cell numbers in the BAL of non-treated, *E. coli*-treated and naïve mice were calculated according to FACS results. (C) C57BL/6 and TCRδ deficient mice were treated as described in (A), and eosinophil numbers in the BAL were determined 4 days after ova challenge. (D) Additionally, BAL cells were restimulated in vitro with PMA/ionomycin, and proportion of IL-5-producing CD4 T cells was assessed by intracellular staining and flow cytometry. (E) On day 3 after i.n. challenge with ova, non-treated and *E. coli*-treated C57BL/6 and TCRδ mice were exposed to increasing doses of MetCh, and airway hyperresponsiveness was determined in a full body unrestrained plethysmograph. Results represent values from 3-4 mice per group and are presented as mean ± SD. *, P < 0.05; statistically significant differences. Experiments were repeated 2-3 times.
Taken together, our data show that intranasal *E. coli* administration leads to the infiltration of a lung γδ T cell population which can protect mice from developing allergic airway hyperresponsiveness. Notably, antigen-induced $\gamma \delta$ T cells have been described to be involved in the inhibition of AHR (21, 37), yet as shown in Fig. 1D we did not observe an enrichment of this subset following exposure to the bacteria, suggesting that another γδ T cell subset might be more relevant in modulating AHR. In contrast to previous reports (19–21), we observed only minor differences in the development of a normal ova-dependent response between wild type and TCR-δ deficient mice, and could not detect any impairment or increase in AHR in the absence of γδ T cells. Instead, we could show that upon treatment with the bacteria, deficiency in γδ T cells led to restoration of AHR, without affecting eosinophil infiltration or Th2 effector function. Of note, this result confirms that AHR and cellular inflammation in the lung can develop independently (35).

A role for γδ T cells in regulating tissue homeostasis as well as in preventing excessive damage derived from immune responses and inflammation has been postulated (7, 38); in particular γδ T lymphocytes have been shown to directly recognize stress-induced MHC molecules (39), to induce tissue repair by producing growth factors (40) and to facilitate the elimination of bacteria as well as necrotic cells (11). In our model, intranasal administration of *E. coli* did not cause major changes in lung structure (data not shown). Yet, we can hypothesize that in the absence of γδ T cells, the immune response against the bacteria might lead to physiological changes and remodeling of lung tissue, and eventually to AHR. Further work will be required to elucidate in which manner γδ T cells act to prevent these changes; of note, experiments with IL-17 and IFN-γ deficient mice suggest that these cytokines are not involved (data not shown). In conclusion we demonstrate a role for bacterially-induced γδ T cells in regulating lung functions to subsequent unrelated inflammation, and in protecting against the development of airway hyperresponsiveness, thus highlighting the relevance of this lymphocyte subset in modulating immune responses at mucosal surfaces.
4. Results

4.4.5 References

4. Results


of gamma delta T cell subsets in regulating airway responsiveness: V gamma 1+ cells, but not V gamma 4+ cells, promote airway hyperreactivity, Th2 cytokines, and airway inflammation. *J Immunol* 172:2894-2902.


5. Concluding remarks

The two primary aims of this thesis were to investigate the role of Rip2 kinase in mediating signaling downstream of innate and adaptive immune receptors, and to study the influence of bacterial infection in the lung on the development of allergic airway responses in mice. These two project yielded several novel discoveries.

With respect to Rip2, our results show that in contrast to the prevailing paradigm, the absence of Rip2 kinase in T cells did not influence their proliferative capacity and effector function, both in vitro and in vivo. However, Rip2 did play a key role in the induction of inflammation in response to Nod1 and Nod2 stimulation, highlighting the importance of this kinase in innate immune responses to bacterial products. Following on from this finding, we sought to further investigate whether the kinase activity of Rip2 was required to mediate signaling downstream of NLRs by directly phosphorylating other effector molecules, or whether Rip2 was rather acting as an adaptor protein as suggested by previous in vitro studies. We found that the kinase activity of Rip2 was primarily involved in ensuring protein stability, and accordingly in preserving signaling downstream of Nod1 and Nod2. We speculate that Rip2 requires autophosphorylation to be stable, however the mechanisms underlying this process remain to be determined. We attempted to restore Rip2 protein stability in the absence of kinase activity by introducing a phosphomimetic mutation at a known autophosphorylation site. Even under this condition, protein levels of kinase-dead Rip2 mutants were significantly decreased as compared to wild type Rip2; however, we cannot exclude that other mutations might indeed lead to the stabilization of the kinase. Taken together our results provide new evidence that in primary cells Rip2 kinase activity is critical to ensure adequate protein levels and thus efficient signaling downstream of Nod1 and Nod2.

In the second part of this thesis we analyzed the interplay between innate and adaptive immune responses in a mouse model of allergic airway inflammation. Our results show that exposure to E.coli prior to the induction of allergic airway inflammation led to impaired dendritic cell function and effector Th2 cell responses in the lung. Consequently, mice treated with the bacteria displayed significantly decreased eosinophilia and airway hyperresponsiveness. Notably, the activation and function of dendritic cells, as well as the priming of specific T cells was normal in the draining lymph node, indicating that the
**5. Concluding remarks**

*E.coli*-mediated inhibitory effect was restricted to the site of inflammation. In contrary to expectations, Th1 and regulatory T cell responses did not play a role in mediating the suppression of allergy in the lung; however *E.coli*-induced γδ T cells were involved in modulating airway hyperresponsiveness. Moreover, we observed that TLR4 signaling was essential for the inhibition of all features of AAI. As both dendritic cells and epithelial cells in the lung express TLR4, it is of importance to determine whether *E.coli* administration acts to alter airway dendritic cell function, or whether a more profound change involving non-immune cells is induced. To this end, bone marrow chimaeras will be generated in which TLR4 is expressed by either immune cells or stromal cells. Additionally, a comprehensive analysis of the surface molecules, as well as chemokines and cytokines expressed by epithelial cells in the lung following *E.coli* treatment will help us clarify our findings on suppression of AAI. In conclusion, the results presented in this thesis reveal a novel pathway by which bacterial infections can shape allergic responses in the lung, and might suggest new strategies for the treatment of allergic airway diseases.
6. Appendix

6.1 Abbreviations

AAI  Allergic airway inflammation
AHR  Airway hyperresponsiveness
APC  Antigen presenting cell
BM-DC / DM  Bone-marrow derived dendritic cell / macrophage
BAL  Bronchoalveolar lavage
BALF  Bronchoalveolar lavage fluid
CARD  Caspase recruitment domain
CD  Cluster of differentiation
c.p.m.  Counts per minute
CTL  Cytotoxic T lymphocyte
DC  Dendritic cell
E.coli  Escherichia coli
ELISA  Enzyme-linked immunosorbent assay
FACS  Fluorescence-activated cell-sorting
FITC  Fluorescein isothiocyanate
FK565  Synthetic Nod1 ligand
FoxP3  Forkhead box P3
GM-CSF  Granulocyte-macrophage colony stimulating factor
IFN-γ  Interferon-γ
IL  Interleukin
i.n.  Intranasal
i.p.  Intraperitoneal
i.v.  Intravenous
LCMV  Lymphocytic choriomeningitis virus
LN  Lymph node
LPS  Lipopolysaccharide
LRR  Leucine-rich repeat
KO  Knock out
MDP  Muramyl dipetide
_Meso-DAP_  γ-D-glutamyl-*meso*-diaminopimelic acid
MetCh  Methacholine
MHC  Major histocompatibility complex
MLR  Mixed lymphocyte reaction
MyD88  Myeloid differentiation primary response gene 88
NF-κB  Nuclear factor kappa B
NLR  Nod-like receptor
Nod1/2  Nucleotide-binding oligomerization domain 1/2
Ova  Ovalbumin
PAMP  Pathogen-associated molecular pattern
PenH  Dimensionless parameter (enhanced pause) for airway resistance
PMA  Phorbol myristate acetate
PRR  Pattern recognition receptor
Rip2  Receptor-interacting protein 2
RLR  RIG-like protein
SD  Standard deviation
TAK1  Transforming growth factor beta-activated kinase 1
TCR  T cell receptor
Th1  T helper 1
Th2  T helper 2
Th17  T helper 17
TGF  Transforming growth factor
TLR  Toll-like receptor
TRAF  TNF receptor-associated factor
Treg  Regulatory T cell
TRIF  TIR domain-containing adapter-inducing interferon-β
TSLP  Thymic stromal lymphopoietin
WT  Wild type
6.2 Curriculum vitae

Generalities

Name: Chiara Nembrini  
Nationality: Swiss  
Date of birth: August 7, 1980 in Bellinzona (TI)  
Address: Chemin de Pierreval 6, 1007 Lausanne  
E-mail: chiara.nembrini@epfl.ch

Currently  
Post-Doctoral fellow in the Laboratory for Regenerative Medicine and Pharmacobiology, Integrative Biosciences Institute, EPFL, Lausanne

Education

PhD in Natural Sciences, in the Laboratory of Molecular Biomedicine, Institute of Integrative Biology, ETH Zürich  
under the supervision of Dr. Benjamin J. Marsland and Prof. Dr. Manfred Kopf

March 2004  
Degree of Master of Science, ETHZ, Zürich  
Willi-Studer-Award for the best diploma of the year

Oct 1999 - March 2004  
Biochemistry and Molecular Biology Studies at the ETHZ, Zürich  
Diploma thesis at the Institute of Experimental Immunology, University Hospital, Zürich; “Relationship between adaptive immunity in maternal milk and neonatal immune responses to helminth parasites” under the supervision of Prof. Dr. Nicola Harris

Sep 1995 - June 1999  
Maturità tipo B, Liceo Cantonale di Bellinzona
Publications


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