Régulation of tolerogenicity and immunogenicity

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Résumé large public

Le système immunitaire constitue un rempart contre les agressions telles que les infections. Sans contrôle par des mécanismes anti-inflammatoires, aussi connus sous le qualificatif tolérogène, le système immunitaire engendrerait une inflammation nocive pour l'organisme et pourrait causer l'apparition de maladies auto-immunes. Le but de ce doctorat est de mieux comprendre ces mécanismes de régulation par l'étude de deux sujets.

Le premier concerne la molécule CD11b et son lien avec un modèle animal de polyarthrite rhumatoïde, l'arthrite induite par le collagène. Dans la plupart des cas, CD11b promeut l'inflammation par divers procédés. Cependant, nous avons remarqué que son absence dans la lignée de souris C57BL/6 rend ces souris sensibles au développement de l'arthrite. Nos résultats indiquent que CD11b prévient cette maladie en bloquant une molécule pro-inflammatoire, l'IL-6, responsable de l'induction de cellules pathogènes pour l'arthrite. Ces découvertes aideront la recherche de nouvelles thérapies capables de moduler le système immunitaire, particulièrement dans le contexte de maladies auto-immunes comme la polyarthrite rhumatoïde.

Le deuxième projet se concentre sur l'étude du co-répresseur NCoR1. La fonction des co-répresseurs est de diminuer l'expression de certains gènes. NCoR1 est classiquement connu pour son rôle dans l'embryogénèse et le développement. Dans le cadre de notre collaboration avec le groupe de S. Raghav, nous avons constaté que NCoR1 bloque l'expression de plusieurs molécules anti-inflammatoires dans un type spécifique de cellules, les cellules dendritiques. Les expériences animales ont confirmé le potentiel anti-inflammatoire de cet effet. Ces découvertes permettront de mieux comprendre les différents acteurs de la réponse immunitaire afin de compléter les traitements actuellement disponibles.

Résumé

La réponse immunitaire résulte d'un équilibre dynamique et perpétuel entre l'immunité et la tolérance immunitaire. En cas de perte de régulation, le système immunitaire peut engendrer des dommages considérables à l'organisme et mener à des maladies auto-immunes telles la polyarthrite rhumatoïde. Par conséquent, une multitude de facteurs sont impliqué dans les processus de régulation. Certains ont même un double rôle avec des fonctions pro-inflammatoires et tolérogènes selon le contexte immunitaire. Ceci est le cas de l'intégrine CD11b. Bien qu'elle soit plus connue pour sa promotion de l'inflammation, son absence est associée à une augmentation des lymphocytes Th17. La polyarthrite rhumatoïde et son modèle murin de référence, l'arthrite induite par le collagène (CIA), étant également associées aux Th17, nous avons décidé d'investiguer le rôle de CD11b dans l'induction de la CIA. Pour cela, nous avons utilisé des souris CD11b$^{-/-}$ à fond génétique C57BL/6 résistant à la CIA, et les avons comparées aux souris C57BL/6 de type sauvage (WT).

Les souris CD11b$^{-/-}$ étaient sensibles à la CIA, contrairement aux souris WT. La caractérisation immunitaire a montré une augmentation de Th17 avec diminution concomitante de lymphocytes T régulateurs (Treg) dans les souris CD11b$^{-/-}$ immunisées. L'interleukine 6 (IL-6) est un facteur de différenciation important pour les Th17 au détriment des Treg. L'analyse sérique de l'IL-6 a indiqué une augmentation de la concentration d'IL-6 dans les souris CD11b$^{-/-}$ immunisées en comparaison aux souris WT immunisées. Les cocultures de cellules dendritiques (DC) ex vivo obtenues de souris CD11b$^{-/-}$ ou WT avec des lymphocytes T naïfs ont montré que les DC CD11b$^{-/-}$ provoquent une élévation des Th17 via l'IL-6 et qu'elles pourraient être responsables de l'arthrite. De plus, le traitement d'anti-IL-6R a démontré que la signalisation de l'IL-6 est non seulement nécessaire pour la polarisation (priming) des lymphocytes mais aussi pendant la phase effectrice de la maladie. En outre, nous avons constaté

que le transfert de DC CD11b^{+} pouvait réduire le score clinique de l'arthrite. Nous avons créé une nouvelle lignée de cellules dendritiques exprimant le CD11b avec invalidation génétique du CD11b. Cela permettra de plus amples études sur ce sujet.

Le CD11b est un bon exemple pour montrer la complexité de la régulation immunitaire. Comme mentionné, de nombreux facteurs sont impliqués dans la tolérance. Il est donc naturel de penser que des co-répresseurs prennent part à ce processus. Nous avons remarqué que le co-répresseur 1 de récepteur nucléaire (NCoR1), dont le rôle dans le développement et la maturation est essentiel, réprime la transcription génétique de différentes molécules tolérogènes dans les cellules dendritiques, surtout après leur activation. Pour ce projet nous avons débuté une collaboration avec le groupe du Dr. Sunil Raghav. Son groupe a effectué des knockdowns lentiviraux sur notre lignée de cellules dendritiques CD8 caractérisée en détail. Les cocultures comprenant ces cellules et des lymphocytes T naïfs a induit une augmentation du nombre de Treg. Afin de le confirmer, nous avons développé une lignée de souris avec invalidation génétique de NCoR1 spécifique aux DC (souris N1). Les DC de ces souris ont synthétisé plus d'IDO1, IL-10 et d'IL-27 que leur équivalent de type sauvage après leur activation. De plus, les immunisations d'ovalbumine (OVA) ont généré une différenciation de Treg plus élevée en comparaison aux souris WT. Les répercussions de ces résultats dans le contexte d'infections ont été testées par trois modèles d'infection. Ceux-ci ont tous provoqué une augmentation relative de Treg. Par ailleurs, nous avons constaté une augmentation de la morbidité dans le cas de l'infection par leishmania et par helminthes.

Pour conclure, ces deux projets prennent toute leur importance pour mieux comprendre les défis posés par la régulation immunitaire. Les connaissances acquises permettront de développer de nouveaux traitements qui moduleront le système immunitaire dans différents contextes telles que les maladies auto-immunes comme la polyarthrite rhumatoïde.

Abstract

The immune system stands on a continual balance between immunity and immune tolerance. Immune response lacking regulation generates severe damage to the organism and can lead to autoimmune diseases such as rheumatoid arthritis. Consequently, many factors need to be implicated to regulate the immune response. Some of them even have a dual role by exerting pro-inflammatory as well as tolerogenic functions according to the immune context. This is the case for the integrin CD11b. Although it is usually known to be pro-inflammatory, its deficiency is associated with an increase of the pro-inflammatory T helper 17 cells (Th17). As rheumatoid arthritis and its gold standard model, the collagen-induced arthritis (CIA), are also associated with Th17, we decided to investigate whether CD11b has a role in CIA induction. For this, we used $CD11b^{-/-}$ mice in a CIA-resistant C57BL/6 background and compared them to C57BL/6 wild type (WT) mice.

$CD11b^{-/-}$ mice were susceptible to CIA, in contrast with WT mice. Immune characterisation showed an increase of Th17 with a concomitant decrease of regulatory T-cells (Tregs) in immunised $CD11b^{-/-}$ mice. Interleukin 6 (IL-6) is an important differentiating factor between Th17 and Treg differentiation. Seric analysis of IL-6 resulted in an IL-6 increase in immunised $CD11b^{-/-}$ mice compared to immunised WT mice. Cocultures of freshly isolated dendritic cells (DCs) from $CD11b^{-/-}$ or WT mice with naïve T-cells indicated that $CD11b^{-/-}$ DCs induce a Th17 increase via IL-6 and could be responsible for the arthritic development. Furthermore, anti-IL-6R treatment demonstrated that IL-6 signalling is crucial not only during the priming but also during the effector phase. Furthermore, we found that adoptive transfer of $CD11b^{+}$ DCs could reduce the disease score. We engineered CD11b knockout (KO) DCs from our CD11b-expressing dendritic cell line which will allow further studies for this project.

CD11b is a good example to show the complexity of immune regulation. As said many factors are involved. It is reasonable to think that co-repressors take part in this process. We have shown that the nuclear receptor co-repressor 1 (NCoR1), an essential co-repressor for development and maturation, represses many tolerogenic molecules in dendritic cells, especially after their activation. We started a collaboration with the group of Dr. Sunil Raghav that performed lentiviral knockdowns on our well-characterised $CD8^+$ dendritic cell line. Cocultures of this cell line with naïve T-cells resulted in increased numbers of Tregs. To confirm these findings, we developed a mouse strain with a DC-specific KO for NCoR1 (N1 mice). KO DCs produced tolerogenic markers such as IDO1, IL-10 and IL-27 upon activation. Moreover, ovalbumin (OVA) immunisations induced an increased differentiation of Tregs in N1 mice compared to WT mice. Implications in infections were tested through three different infection models that all ended with a relative increase in Treg numbers. In addition, increase in disease burden was also found in helminth and leishmania infections.

In conclusion, these two projects are of great importance to better understand immune regulation. It will enable to develop new treatments that will modulate the immune system in different contexts such as autoimmune diseases like rheumatoid arthritis.

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Introduction

Immune system

Organisms suffer relentlessly from different aggressions such as traumas, infections or tumours. This is why the immune system is crucial not only for humans but for all kind of beings. Damage activates this system, which results in inflammation. Although inflammation helps getting rid of the harming agent, it can be deleterious for the body as well. Consequently, the immune system comprises also of regulatory mechanisms that dampen the immune response and promote tissue repair.

The immune system has many players that are usually divided into two categories: the innate immune system and the adaptive immune system.

Innate immune system

The innate immune system constitutes the first line of defence against pathogens. The epithelia forms an initial barrier to the potential pathogens impeding their entry thanks to tight junctions and antimicrobial molecules such as defensins and cathelicidins. These molecules are secreted in the different mucosae of the body. The second line is represented by the innate inflammatory cells, especially monocytes, macrophages, granulocytes and dendritic cells (DCs). These cells are able to sense, internalise and destroy the pathogens. Some of these cells can present parts of the ingested microorganisms to the adaptive immune system in order to mount a more efficient immune response. This is further discussed below. Innate cells can secrete cytokines which are pro- or anti-inflammatory molecules that shape the immune response in order to

better get rid of the pathogen. These cells possess Pattern Recognition Receptors (PRRs) which sense Pathogen Associated Molecular Patterns (PAMPs), from microbes, or Danger Associated Molecular Patterns (DAMPs) from injured or stressed cells. PRR diversity is great, there are Toll-like receptors (TLRs), NOD-like receptors (NLR), C-type lectin receptors (CLR), etc. This diversity enables induction of appropriate immune responses to sensed pathogens. According to the activated PRR, the innate cell undergoes a specific activation which will lead to definite cytokine expressions. Cytokines induce different T-cell differentiations and, by extension, different immune responses. This is discussed later. Furthermore, PRR activation also helps intracellular killing, cytokine and antimicrobial molecule production.

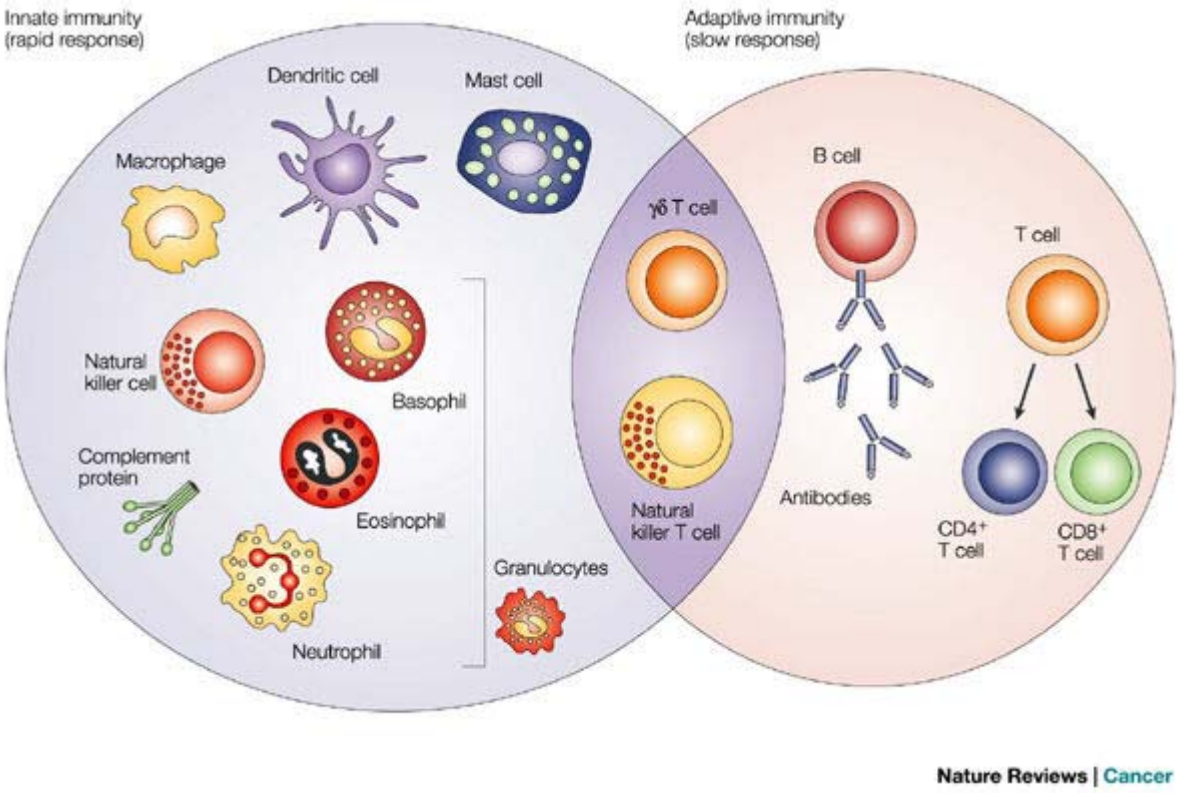


Figure 1 ***Main cells composing the immune system***
The innate and adaptive immune systems are the two components of the immune response. Many cells belong to the innate system in addition to the complement whereas the adaptive immune system consists of lymphocytes and their products. Taken from (1).

Cells belonging to the innate immune system can be regulatory as well, also known as tolerogenic. For example, innate lymphoid cells and DCs are important for tolerance maintenance of the intestinal flora. Other cells such as MDSC and macrophages are tolerogenic in certain circumstances. This is described in the tolerance chapter.

However, the innate immune system is not only constituted by cells. Indeed, many different soluble molecules are involved as well. This is especially the case for the complement system (2).

Complement system

The complement system belongs to the innate immune system. It is composed by seric and cell surface proteins, mostly produced by the liver. Activation of this system induces inflammation through release of inflammatory mediators and sequential enzyme cascades. The complement cascade leads to pathogen recognition, opsonisation and cell lysis. There are three pathways to activate the complement system: the classical, the alternative or the lectin pathway. The classical pathway depends on immune complexes formed by the antigen and its specific antibody, that trigger the first protein of the cascade. The alternative pathway is triggered by failure to inactivate C3b which then induces the complement cascade. Lastly, the lectin pathway is initiated by the binding between pentameric collectins and specific carbohydrate patterns that are uncommon in the host (3).

Macrophages

Macrophages are the most plastic cells of the haematopoietic system. Their main role is to maintain homeostasis and tissue integrity although they are also important for phagocytosis, degradation and mobility. They are capable of antigen presentation but less efficiently than dendritic cells. Moreover, macrophages phagocyte apoptotic cells and red blood cell nuclei which makes them essential for erythropoiesis. In the case of macrophage deficiency, osteopetrosis occurs. They also regulate stem cells in the intestines (4)

Macrophages stem from different precursors. Tissue-resident macrophages usually derive from embryonic precursors that seeded the tissues before birth. However, some monocytes, which also play a role in inflammation, can also differentiate into macrophages as well as dendritic cells (5).

There are three main subsets although there is a wide diversity of macrophages. Firstly, in a pro-inflammatory context, macrophage TLR activation in presence of Th1-secreted IFNγ leads to classically activated macrophages, also called M1. They secrete pro-inflammatory cytokines and initiate defence mechanisms against pathogens such as oxidative processes. Secondly, macrophages can differentiate into alternatively activated macrophages or M2 in presence of IL-4 and IL-13, usually secreted in a Th2 response. M2 functions involve fibrosis promotion, healing, insulin resistance inhibition and immune regulatory functions. These cells and the last macrophage subset, Mregs, are important to control exaggerated inflammation and suppress autoimmune processes. While Mregs are immune suppressors and are induced by Treg-mediated secretion of IL-10, M2 have a role in the inactivation of the immune response in tumours. Mregs are discussed in detail in the tolerance chapter. Interestingly, macrophages can switch from one state to another, from pro-inflammatory to anti-inflammatory (4).

Dendritic cells

Dendritic cells (DCs) are key players of the immune system. They are able to initiate and orientate different kinds of responses depending on the pathogen and the environment they sense (2). These cells are generally located in the periphery and patrol in an immature state, which is rather tolerogenic and endocytotic (6). Once they sense PAMPs or DAMPs, their phenotype change and they become pro-inflammatory. Endocytosis is decreased and they increase their capacity to present antigens to lymphocytes including their costimulatory molecules (2, 6). They are called professional antigen-presenting cells (APCs). When activating T-cells through MHC and costimulatory molecules, they are able to induce the most appropriate type of response by secreting different cocktails of cytokines (7, 8). This ability makes them very important for immune response and tolerance.

Many subsets have been described (9) and there are different ways of dividing them. Generally, they can be divided into two main categories: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDCs are specialised in antiviral response. Their major feature is abundant secretion of interferon type I. They can, nevertheless, present antigens and modulate the immune response as well (10). cDCs, on the other hand, are the classical APCs. They express high levels of CD11c and MHC class II. cDCs are distinguished from the other cells by these two markers and the absence of T-cell, B-cell and macrophage lineage markers (11). These cells constitute a very heterogeneous group, but we will focus on two major subsets.

The first one is the $IRF8^+$ DCs, which are also known in the spleen as $CD8^+$ DCs and in the skin as $CD103^+$ DCs (12). These DCs received the new denomination cDC1. They depend on the transcription factors IRF8 and BATF3 amongst others (12, 13). In the periphery, these cells are $CD8^-$ $CD103^+$ or $CD24^+$. Moreover, they express the chemokine receptor XCR1. They have the ability to cross-present antigens to $CD8^+$ T-cells. Also good inducers of type 1 response

because they have the capacity to secrete IL-12 (14). They respond to different TLRs, mainly TLR3 and TLR9 that are activated by polyIC and CpG respectively (15). In the spleen, this subset represent around 20% of the conventional dendritic population (11).

The second group is composed of the $IRF4^+$ DCs, which is mainly composed of $CD11b^+$ cells, now also called cDC2. CD11b is a subunit of the heterodimeric integrin Mac-1, which is discussed later. This molecule is very important for many inflammatory processes, including leucocyte migration, adhesion, activation and, interestingly, tolerance induction (16, 17). These cells express many TLRs including TLR4 and TLR9, stimulated respectively by LPS and CpG (15). SIRPα (CD172α) and CD4 are two other surface markers for this population although CD4-negative cDC2s are abundant too. $CD11b^+$ DC subset is the largest population of splenic dendritic cells. They interact preferably with $CD4^+$ T-cells (9). Two other transcription factors further divide them: the $KLF4^+$ population and the $NOTCH2^+$ population. $KLF4^+$ DCs are important for type 2 response whereas $NOTCH2^+$ DCs induce type 3 response (13). Importantly, $CD11b^+$ DCs do not rely on BATF3 for their development.

Adaptive immune system

The counterpart of the innate system is the adaptive immune system. It takes place when an infection overcomes the innate immune system. Only vertebrates have this system which has two important features: specificity and memory. The cells composing it are lymphocytes. T lymphocytes, also called T-cells, engender the cellular response whereas B lymphocytes, or B-cells, generate the humoral response. Lymphocytes have a common precursor present in the bone marrow where the first differentiations occur. However, T-cell precursors migrate then into the thymus to further mature into naïve T-cells, while B-cells remain in the bone marrow until complete maturation into naïve B-cells.

Although these cells express TLRs as well (18), their key receptor is the T-cell receptor (TCR) or B-cell receptor (BCR) for T-cells and B-cells respectively. They recognise an enormous variety of specific antigens thanks to a process called the N(D)J recombination, enabled by the recombination activating gene (RAG).

Once mature, cells migrate into secondary lymphoid organs where they interact with antigen-presenting cells (APCs), which are mainly secondary tissue resident dendritic cells or inflammatory immigrated DCs. Due to the very low number of specific cells, it would be extremely difficult for a lymphocyte to encounter its cognate antigen without concentrating them in a specific organ (in this case the secondary lymphoid organ) and bringing the antigens in there. When their specific antigen is recognised, lymphocytes are activated, proliferate and differentiate into effector cells.

This process is long. Indeed, it takes several days (two weeks for antibodies, one week for T-cells). However, upon reencounter with a pathogen, memory lymphocytes will respond much faster and more efficiently. These lymphocytes stem from previous infections that instead of undergoing apoptosis, they differentiated into memory cells. They are responsible for the immune response against a recurrent infection (19). This concept is the base for vaccines.

B-cells

Humoral response protects extracellular spaces. It comprises B-cells, plasma cells and their antibodies, also called immunoglobulins. B-cells are able to sense a specific antigen through their B-cell receptor (BCR). B-cell activation can then either be dependent or independent on T-cells. Most of B-cell activation is T-cell dependent. In this case, B-cells recognise an antigen, internalise it and present it to activated cognate helper T-cells through their MHC-II molecule.

T-cells deliver a second signal (CD40L/CD40 interaction) and secrete cytokines that activate B-cells. IL-4 is the main cytokine that activates B-cells. It is supplied to B-cells by follicular T-cells (Tfh) or Th2. Later, IL-5 and IL-6 are produced to help B-cells differentiate into plasma cells. Initial interaction between B-cells and T-cells occur at the border between the T-cell and B-cell areas. The interaction continues then in the follicle where they migrate and develop germinal centres. There, B-cells undergo somatic hypermutation and immunoglobulin (Ig)-class switching. Somatic hypermutation increases antigen specificity for the same pathogen and occurs in the variable region (antigen-binding region) of the immunoglobulin. Ig-class switching permits different functions of the immunoglobulin, defined by its heavy chain. This time it is the constant region that is modified.

An activated B-cell can either become a memory B-cell that can be rapidly be reactivated in case of antigen reencounter, or differentiate into a plasma cell. Memory B-cells secrete very low amounts of immunoglobulins and have already undergone somatic hypermutation and Ig-class switching. Plasma cells are specialised in immunoglobulin secretion. They are mature cells that do not differentiate anymore. Some remain in the follicle but most of them relocate into the bone marrow or sites of inflammation.

In few cases, B-cells are also activated without T-cell help. In this situation, antigens, called Thymus-independent antigens, are nonproteins molecules. They can act as B-cell mitogens like LPS or be repetitive antigens, which can activate mature B-cells and induce IgM in addition to some IgG production. They usually cannot induce somatic hypermutation nor memory B-cells. Few cells can undergo the Ig-class switching to produce IgG.

There are five main classes of immunoglobulins. These classes determine their function. The different classes are: IgM, IgD, IgG, IgA and IgE. One more class of antibodies is present in neonates but disappears few months after birth (IgF).

The first immunoglobulin produced during the humoral response is the IgM. B-cells have not undergone somatic hypermutation and Ig-class switching before production of this Ig. This is why it has a lower affinity compared to the other Igs. However, they can form pentamers, increasing in this way the overall avidity. Because of the pentamer size, IgM usually remains in the blood and is very efficient in activating the complement cascade. They do not require T-cell help for their development.

IgG, IgA and IgE are produced after somatic hypermutation and Ig-class switching. Consequently, they have a higher affinity for their specific antigen. IgA can form dimers and is principally secreted in secretions especially in the intestinal and respiratory tracts. IgAs are weak osponins but good at neutralising pathogens. IgG has four subclasses. They are the main Igs in the blood and extracellular fluids. In addition, they are excellent opsonins and efficient in activating the complement cascade. IgG1 is associated with a Th2 response while IgG2a is correlated to a Th1 response. Finally, concentration of IgE is very low in the organism. However, it has a very high avidity for their receptors which are mainly expressed on mast cells. When IgE binds their receptor, mast cells rapidly degranulate and induce histamine-mediated reactions (2).

T-cells

As mentioned before, T-cell precursors leave the bone marrow to mature in the thymus. At this stage they do not express the T-cell receptor (TCR) co-receptors CD4 and CD8. T-cells first undergo a positive selection, in which the TCR must interact with moderate affinity with the self-MHC (Major Histocompatibility Complex) molecules. Cells are double positive for CD4 and CD8 during the positive selection. If cells recognise MHC class I, they will be $CD8^+$ T-cells and lose the expression of CD4, and if they interact with the MHC class II molecule, they

mature into $CD4^+$ T-cells and respectively lose CD8 expression. Cells that do not sense MHC molecules are not selected and become apoptotic. It is usually also the case for cells that interact with MHC too strongly, otherwise autoimmune diseases could arise. However, some of these cells have a different fate that will be discussed in the tolerance section.

After the positive selection, surviving cells go through a negative selection. Here, thymic medullary epithelial cells (mTEC) or medullary dendritic cells present self-antigens. This selection is probably mediated via three transcription factors, two of which have been characterised: AIRE and FEZ2. They induce the expression of thousands of self-peripheral genes in mTECs. When a T-cell recognises self-antigens, they are deleted, which prevents autoimmune diseases.

After these two steps, T-cells are mature and called naïve T-cells. They are either $CD8^+$ T-cells (cytotoxic T-cells) or $CD4^+$ T-cells (helper T-cells). Both migrate via the bloodstream to secondary lymphoid organs to encounter their cognate antigen. This concentrates the contact between T-cells and APCs.

The migration is determined by adhesion molecules and chemokines. Selectins induce rolling on the endothelial wall. This enables other firmer interactions such as integrin binding. If chemokines are present, rolling cells can interact with them and activate their integrins. Integrins then change conformation and bind to adhesion molecules such as ICAM. Without this process, T-cells cannot extravasate from the bloodstream and access lymph nodes. T-cells further migrate according to the sensed chemokine gradient until they reached the T-cell zones of the lymphoid organ.

Three signals are needed for T-cells to be activated and start the adaptive immune response. The first signal is the specific interaction between the TCR and the complex cognate antigen/MHC molecule. Costimulation is the second signal. The most known interaction is

between the costimulatory molecule CD28 present on T-cells and the costimulatory molecule CD80 or CD86 present on APCs (DCs). This binding is essential for T-cell activation. If this interaction is absent, T-cells are not activated and in case of signal one occurrence, they become anergic. T-cells, especially regulatory T-cells, can express CTLA4 which is homologous to CD28. However, this molecule transmits an inhibitory signal contrary to CD28 that stops T-cell activation. In addition, CTLA4 can be expressed by DCs and released as micro-vesicles in the surroundings (20) and internalisation of these vesicles by the neighbouring DCs results in suppression of their maturation and thereby reduced activation of Th cells (21). The last or third signal comes from cytokines. They are secreted by APCs and according to the type of cytokine expressed, T-cells differentiate into specialised effector cells that can display their immunogenic abilities. This third signal initiates T-cell activation and proliferation, in particular the cytokine IL-2. Effector T-cells do not require costimulation to act. This process is the priming. If T-cells do not recognise their specific antigen in a secondary lymphoid organ, they recirculate in the blood and re-enter another lymphoid organ.

Effector $CD8^+$ T-cells, also called cytotoxic T lymphocytes (CTL), are important to fight intracellular pathogens, in particular viruses. They can kill infected cells that they recognise through their TCR. Indeed, infected cells present viral peptides to $CD8^+$ T-cells through their MHC-I molecules. $CD4^+$ T-cells are usually required to initiate the $CD8^+$ T-cell response by further activating DCs thanks to their CD40 expression. Moreover, $CD4^+$ T-cells secrete IL-2 which further helps CTLs to get activated. When DCs are fully activated they can deliver enough costimulatory signal to $CD8^+$ T-cells for their differentiation into effector cells. CTLs induce apoptosis by release of perforin and granzymes contained in their granules. Perforin generates pores in target cells through which granzymes can enter and induce apoptosis. Furthermore, CTLs induce cell death by expressing FasL that binds to Fas expressed on target

cells. They also have the ability to secrete different cytokines that enhance the immune response.

$CD4^+$ T-cells, called helper T-cells (Th), are the main supporters of the immune response. They differentiate into different subsets according to the immune response needed to fight the infectious pathogen. As said earlier, the cytokine milieu, mostly secreted by DCs, will determine T-cell differentiation. This is the reason why DCs are said to be key inducers and shapers of the immune response.

Type 1 immune response is characterised by the presence and induction of Th1. Induced by IL-12 and IFNγ, they secrete in turn IFNγ and IL-2. They also promote macrophage activation and killing through cytokines and CD40L. Furthermore, they help antibody production against extracellular bacteria. The associated antibody is the IgG2a. Th1 are especially important for intracellular infections such as mycobacteria, leishmania and viruses. Th1 are characterised by the transcription factor Tbet.

Type 2 response is dependent on Th2. These cells differentiate upon IL-4 stimulation that induce expression of the transcription factor GATA3. Th2 produce IL-4, IL-5 and IL-13. They activate eosinophils, basophils and mast cells. This response confers protection against parasites, especially helminths. They also induce the Ig-class switching of B-cell to produce IgE. Th2 were classically known to be key players in the humoral response by activating B-cells. However, it appears that another subset, discussed later, is responsible for that. Th2 are also involved in allergic response.

The third type of immune response, type 3, is the mediated by Th17. They are characterised by the transcription factor RORγT and the cytokine IL-17. Th17 differentiate when TGFβ and IL-6 are present in the milieu (2). They promote acute inflammation and help protect against fungi and extracellular bacteria, mostly by inducing a neutrophilic response. In addition, they help

protect against certain intracellular pathogens (22). They were also found to be involved in many autoimmune diseases as mentioned later.

Follicular T-cells, Tfh provide B-cells help to activate and create a humoral response. They are involved in Ig-class switching and affinity maturation. Molecules responsible for their induction are not well-known although IL-6 seems to be required for their development.

Regulatory T-cells (Tregs) is the last main $CD4^+$ T-cell subset. They are immunosuppressive cells and are mainly induced by TGFβ. Better explanation will be held in the tolerance section. Interestingly, IL-6 determines whether a naïve T-cell differentiate into a Treg or a Th17. As Th17 are often involved in autoimmune diseases that can be prevented or treated by Tregs, IL-6 has a key role in the balance between tolerance and immune response (23).

Other minor $CD4^+$ subsets have been discovered and are being characterised. However, due to the very scant current knowledge, they will not be discussed here.

As B-cells, T-cells that were activated during an immune response can differentiate into memory T-cells that will respond much faster and more efficiently upon reencounter. This mechanism confers protection against pathogens against which an immune response already occurred.

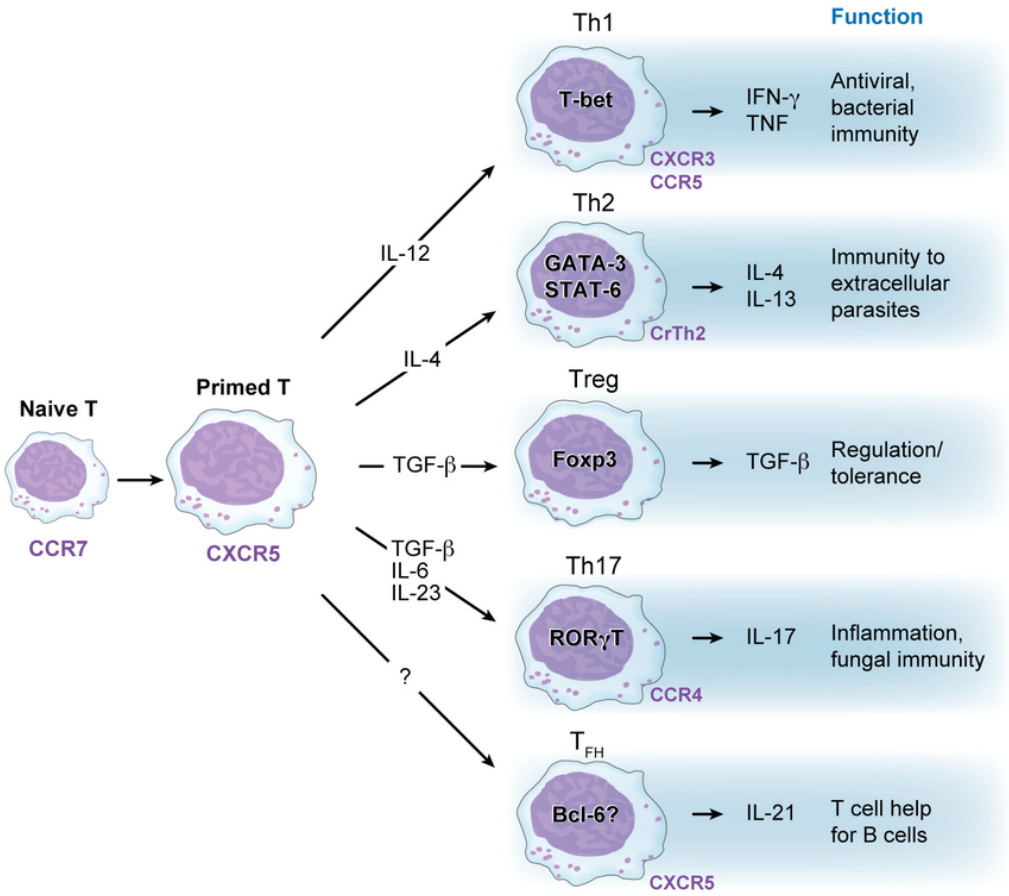


Figure 2 ***T-cell subsets***
$CD4^+$ T-cell differentiations and functions. T-cell subsets are defined by sensed cytokines. Taken from (24).

Immune tolerance

The immune system consists of a delicate balance between protection against pathogens or tumour cells and prevention of autoimmune diseases. Indeed, if immunogenicity is too strong, auto-inflammatory processes can occur. The ensemble of the immune checkpoints to prevent them is called immune tolerance. It is classically divided in two: the central tolerance and the peripheral tolerance.

Central tolerance

Lymphocytes must undergo a strict selection, in the thymus for T-cells, or in the bone marrow for B-cells. Indeed, in the thymus, there is first a positive selection to choose only cells that react to self-MHC molecules. A negative selection then takes place, in which cells that have reacted too strongly during the first selection or cells that react to self-peptides presented by DCs or mTEC are either deleted or edited (25, 26). Edition is the process by which the auto-reactive lymphocyte rearranges its receptor to change its specificity. This happens in B- and T-cells. Sometimes, auto-reactive T-cells can be differentiated into Treg cells instead of undergoing apoptosis. This is the clonal diversion. The genes AIRE and FEZ2 allow to present many self-antigens that would otherwise not be present in the thymus. This widens the range of self-peptides tested. These processes are defined as the central tolerance.

Peripheral tolerance

The central tolerance is not enough to prevent auto-reactive reactions of immune cells. Other processes are needed to ensure a good balance of the immune system. These are called peripheral tolerance. One of the mechanisms to impede autoimmune reaction is the ignorance. Naïve T-cells have no access to non-lymphoid tissue. They need expression of adhesion molecules that are only produced in case of activation. This keeps naïve T-cells in the lymph, lymphoid tissues and bloodstream. Furthermore, tissues need to be inflamed in order to express appropriate ligands, attract activated T-cells and enable their extravasation.

Another second mechanism is the anergy. When a reactive T-cell encounters an antigen-loaded APC that does not express costimulatory signals, T-cells become anergic. This effect is far more efficient if the APCs, such as DCs, express the surface molecules PD-1 and CTLA-4. As a result they cannot respond anymore and become part of the self-tolerance and T-cell homeostasis (27).

The most famous example of peripheral tolerance is probably the immune suppression by regulatory T-cells, already mentioned in the T-cell section. Tregs can suppress other autoreactive lymphocytes even though their antigen specificity is different, as long as the antigen is present in the same tissue. A hallmark of Tregs is the expression of the transcription factor FoxP3. It is not only a marker for these cells but also has functional implications. Indeed, its mutation induces many autoimmune diseases (28) however, there are a few Treg subtypes that are FoxP3 negative, such as IL-35^{+} Tregs or IL-10^{+} Tregs also called Tr-1.

Tregs are usually divided into two main groups, the natural Tregs and the induced Tregs (iTregs).

Auto-reactive T-cells that escaped from the thymic negative selection deletion can differentiate into natural Treg. They express CD4, CD25, CD62L and CTLA-4, representing 10 to 15% of CD4^{+} T-cells. Their function is to inhibit other self-reactive T-cells differentiate into effector

cells and to stop the function of effector cells. They act through contact-dependent manner, in which their CLTA-4 competes with effector T-cells for B7 from APC. By secreting IL-10 and TGFβ, whose functions are discussed below, they also induce tolerance.

Tregs can also be induced in the periphery when they recognise an antigen presented on an immature or tolerogenic DC (tol-DC). The APCs secrete TGFβ which, in absence of IL-6 or other proinflammatory factors, polarise naïve T-cells into induced Treg (iTregs). These cells are crucial for oral or commensal microbiota tolerance. As natural Tregs, iTregs produce TGFβ and IL-10, which suppress the immune response. Recently, many iTreg subsets have been discovered and characterised. They are a source of controversy among researchers; however, some of these subsets can be mentioned. As said, most of them are $FoxP3^+$ Tregs. Yet, there are two specific subsets which do not express this transcription factor. The first one is the IL-10 producing Tregs (Tr-1) that appears to be crucial in maintenance of tolerance in the gut (29). The second one is a potent inhibitor of the immune response and is currently gaining much interest. The $IL35^+$ Treg subset. It was found that injections of these cells could rescue the autoimmune phenotype of $FoxP3^{-/-}$ mice as well as protect mice from a EAE (an autoimmune model for multiple sclerosis) (30). Tissue-residing Tregs are other subsets of regulatory T-cells, which further reflects the variety of Tregs. Transcriptional programs in these cells often differ from lymphoid Treg programs (31). The cytokine IL-2 is necessary for maintaining mature Treg identity (31).

T-cells are not the only regulatory cells and immune checkpoints to prevent autoreactive lymphocytes are also present in B-cell maturation and activation. As in T-cells, mechanisms for B-cell peripheral tolerance comprise induction of anergy, antigen receptor desensitisation or tolerance to antigens that co-engage sialic acid-binding immunoglobulin-like lectin (Siglec) inhibitory receptors (25). This happens once B-cells have already left the bone marrow.

Moreover, it is reversible as some pathogens have very similar epitopes to self-antigens. Breg (regulatory B-cells) also have a role in regulating immune response and inducing peripheral tolerance through secretion of IL-10, IL-35 and TGFβ (32). Production of the immunoglobulin IgG4 produced by B-cells could also be considered as a participant to tolerance because of its blocking antibody action (33).

In addition, two subtypes of macrophages can be considered anti-inflammatory. Firstly, M2 macrophages are important for resolution of inflammation. Above all, regulatory macrophages (Mregs) are tolerogenic. They can secrete IL-10 and were shown to inhibit effector T-cells via iNOS. The rest of their suppressive function is mediated through IDO and contact dependent T-cell deletion (34).

A bone marrow-derived population, called myeloid-derived suppressor cells (MDSC), is an additional tolerogenic cell subset. These cells can induce tolerance to alloantigen. They produce the tolerogenic cytokines IL-10 and TGFβ and probably promote Treg induction (34).

Moreover, mesenchymal stromal cells also have the potential to inhibit the immune response. They can act on many different cells. These cells inhibit dendritic cell differentiation, T-cell proliferation and production of IFNγ and IL-2. In addition, they promote Treg differentiation (34).

Last but not least, tolerogenic DCs (tol-DCs) are also essential inducers of tolerance. These cells are discussed later.

Tolerogenic molecules

TGFβ has been known for many years now. It has three family members, TGFβ1, 2 and 3. They are very similar although TGFβ1 is predominant in mice. It is first produced as a latent complex that cannot signal. This complex is stored in the extracellular matrix until peptidases or integrins activate it into active TGFβ. The main sources of this anti-inflammatory cytokine are T-cells (effector and regulatory T-cells). However, many other cells can produce it. Its main function is to dampen the inflammatory response by blocking T-cell production of cytokines, T-cell division (through IL-2 suppression) (35), and killing capacities. It suppresses the transcription factors Tbet and GATA3 (36) and can also regulate $CD8^+$ T-cells. Interestingly, it induces alone Treg and provides a survival signal for these cells. However, in the concomitant presence of IL-6, naïve T-cells differentiate into Th17, which are pro-inflammatory (37). Furthermore, it acts on DCs to keep them in an immature state (38). Finally, in case of TGFβ deficiency, a multiple organ inflammation arises and can induce embryonic death.

IL-10 is a pleiotropic cytokine. It is produced by many different cells, including dendritic cells, macrophages, neutrophils, NK cells, B- and T-cells. IL-10 has a wide range of target cells. It signals through Jak1 and STAT3. IL-10 main function is immune regulation. It inhibits antigen presentation, costimulation on APCs (39) and inflammatory cytokine production such as IL-12, TNFα and IL-5. DC differentiation is also impaired by IL-10. It arrests T-cell proliferation through IL-2 decrease (40). As proof for its key role in immune tolerance, IL-10 deficiency in mice leads to lethal colitis. Indeed, IL-10 is needed to maintain intestine flora tolerance. It promotes natural Tregs, and antigen presentation by APCs in presence of IL-10 produces $FoxP3^-$ $IL\text{-}10^+$ Tregs, also called Tr-1. These cells are vital for intestine flora tolerance (41). Another example of IL-10-mediated tolerance is the persistence of the leishmania parasites in case of IL-10 secretion (39).

However, IL-10 has some pro-inflammatory roles as well. It is involved in $CD8^+$ T-cell response, including their proliferation and cytotoxic functions. This is particularly true in tumour contexts (42). Moreover, IL-10 is a survival signal for B-cells and helps them mature into plasma cells.

On the other hand, IL-35 is a relatively newly discovered cytokine. It is composed of two chains, IL-12p35 and EBI3. It binds to T-cells and signals through STAT1 and 4 or binds to B-cells and then signals via STAT1 and 3. IL-35 is usually produced by natural Tregs. Its main function is to suppress T-cell proliferation and induce $IL35^+$ $FoxP3^-$ Tregs (30). IL-35 also promotes IL-10^+ Breg differentiation and protect against autoimmune diseases (43). Indeed, it was found to prevent collagen-induced arthritis (CIA) and generate IL-10^+ Tregs (44). It was discovered that IL-35 producing DCs decrease EAE even after induction of the disease and augment tumour growth (45).

In addition to cytokines, there are other tolerogenic molecules. One of them is the Indoleamine 2, 3-dioxygenase (IDO). It is an intracellular enzyme that degrades tryptophan (an essential amino acid). The reaction generates kynurenines, important metabolites for tolerance. Indeed, L-kynurenine activates ahr that induces Treg differentiation and inhibits T-cell proliferation (46, 47)

There are two IDOs: IDO1 and IDO2. IDO1 is ubiquitous whereas IDO2 is only present in some human DCs. IDO2s role is unclear and this enzyme, contrary to IDO1 is not inducible by virus or IFNγ. IDO1 is produced by macrophages, monocytes and DCs. Its expression is not constitutive. Indeed, IDO1 is induced by TLRs, especially 3 and 4 in DCs, TNFα, TGFβ, IFNβ and IFNγ among other pathways. IDO1 is important for the control of some infectious agents. Nevertheless, its main action is to promote immune suppression. In presence of TGFβ, it promotes generation and maintenance of Tregs. DCs expressing IDO1 suppress immune T-cells. IDO1 is also associated with Th1 and Th17 inhibition (48). This enzyme is involved in

cytotoxic function decrease in $CD8^+$ T-cells. IDO1 is partly responsible for HIV evasion mechanism.

It was found that cholera toxin B vaccines could help treat autoimmune diseases like uveitis. One mechanism involved is DC inhibition of maturation. This is probably because high upregulation of IDO1 could be measured in DCs, which decreases autoimmune diseases, such as collagen-induced arthritis (48, 49).

Another cytokine commonly considered as anti-inflammatory is the IL-27. It consists of two chains: ebi3 and p28. IL-27 receptor is the combination of gp130, shared with the IL-6 receptor and IL27R. This cytokine is mostly produced by APCs upon TLR activation. It signals via Jak/STAT. IL-27s role is controversial. It has some pro-inflammatory features such as Th1 promotion but acts otherwise as a tolerogenic molecule. Indeed, it is usually associated with Th17 inhibition and IL-10 producing $FoxP3^-$ Tregs induction. Th17 are blocked through STAT1 and not through competition with IL-6 for the gp130 subunit (50).

As said above, there is still controversy about the role of IL-27. Some studies support the fact that IL-27 limits Th1 and Th2. It was found that this cytokine suppresses Th1 in EAE, as well as Th2 and Th17. In this study, IL-27 signalling in DCs suppressed EAE and induced CD39 in DCs, which cleaves extracellular ATP and diminished nucleotide dependent activation of NLRP3 inflammasome (51). They also reported that IL-27 decreases MHC-II and costimulation in DCs and increased IL-10, TGFβ and IDO production. Moreover, it was also suggested that IL-27 inhibits $FoxP3^+$ T-cells further increasing the complexity of IL-27 function. However, it was found that depending on the immune context, $FoxP3^+$ T-cells can also be promoted by IL-27. The examples of Th1 and $FoxP3^+$ Tregs demonstrate the importance of the inflammatory milieu in IL-27 signalling. IL-27 was also reported to decrease CIA severity (52).

First project: The role CD11b in collagen-induced arthritis

Autoimmune diseases

Tolerance is crucial to prevent development of autoimmune diseases. However, sometimes regulatory mechanisms are overcome and these diseases can occur. Autoreactive pro-inflammatory T-cells react against self-antigens and initiate an immune response as if they were reacting against pathogens resulting in a sterile inflammation. In this context, B-cells and plasma cells can produce auto-antibodies as well further augmenting the response.

Surprisingly, although some autoimmune diseases were classically thought to be associated with an autoreactive Th1 response, increasing knowledge indicates that the pro-inflammatory Th17 could be responsible for their occurrence (53-56). One of the most prevalent diseases among them is the rheumatoid arthritis (57).

Rheumatoid arthritis

One of the most common autoimmune diseases is the rheumatoid arthritis (RA) (57). 1% of the world population suffers from it. As many autoimmune diseases, it affects mostly women. It usually develops between age of 30 and 60. As many autoimmune disorders, its specific cause is unknown. Concordance rates among monozygotic twins is 15 to 30%, whereas among dizygotic twins it is only 5%, which indicates that genetic factors are involved in its pathogenesis (58). One established candidate gene for this association is the HLA-DRB1 which confers susceptibility to the disease. Other HLA genes were correlated to the development of RA as well. Moreover, association of different genes further increases the risk and could give a first hint about arthritis severity. Similarly to many diseases, it is most probably the interaction

between genes and environment that promotes RA. Combination of a high risk HLA with smoking, for example, enhances the odds of having arthritis significantly-(59). Infectious agents are another possible source for disease induction. Indeed, Epstein-Barr virus (EBV) and *P. gingivalis*, a virus known to induce different diseases such as cancers, has also been associated with RA. Although the reason is unknown, some hypothesise molecular mimicry and immune complex formation (60). Many actors play a role in its pathogenesis. Pro-inflammatory Th1 and especially Th17 are responsible for a cellular autoreactive response. The autoimmune process is further supported by auto-antibodies that can form immune complexes and activate the complement cascade. Two specific auto-antibodies for the rheumatoid arthritis are auto-antibodies against citrinullated self-proteins and the rheumatoid factor. They can predate the disease by up to 10 years (61). Figure 3 portrays the different players of this inflammatory process.

RA is characterised by joint inflammatory infiltration, autoantibody production, synovitis, cartilage destruction and bone erosion. It leads to severe pain and handicap in daily life. In addition to the local inflammation, systemic manifestation such as osteopenia, muscle weakness, ocular inflammation, lung involvement, cardiac disease, etc. can also occur and increase the disease burden. It is a chronic disease whose treatments are either symptomatic such as NSAR or strong immunosuppressive drugs, like abatacept (CTLA4-Fc), anti-TNFα, anti-IL-1β or anti-IL-6R.

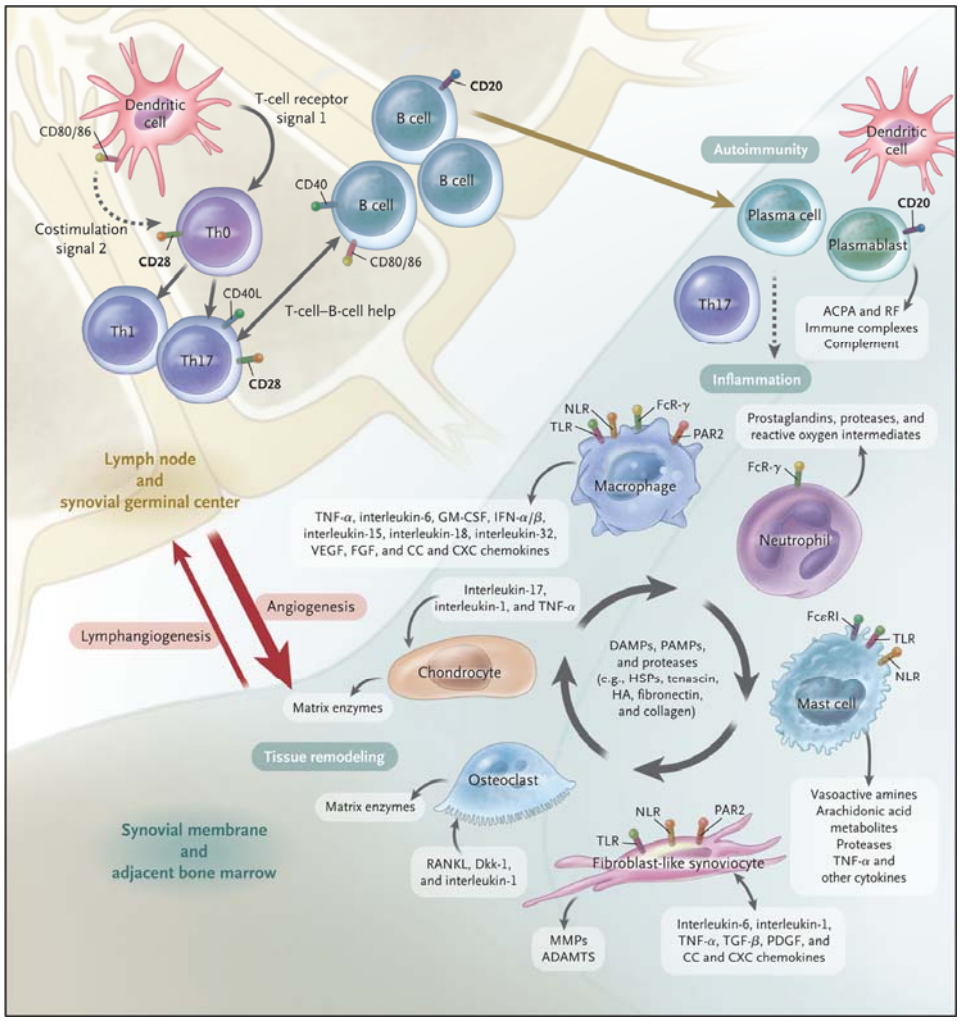


Figure 3 ***Pathogenesis of RA***
Cellular players and mediators of the rheumatoid arthritis. Taken from (57).

Models of RA

Here are the reasons why many models for rheumatoid arthritis have been developed. The most studied one is the collagen-induced arthritis (CIA), discussed later. A second model is the collagen-antibody-induced arthritis. It enables to induce 100% of mice irrespective of their MHC class II. In this model, serum collected from immunised mice is transferred in non-immunised mice. The latter developed arthritis within 48 hours. This model is used to study the humoral response of RA.

Another model is the zymosan-induced arthritis. This resides in injecting zymosan intra-articularly in the knees. This induces a localised proliferative inflammatory arthritis.

RA is also explored through transgenic mice that spontaneously develop arthritis. One of them is an overexpressing TNFα mouse line. It results in a chronic disease, unlike the CIA. The SKG mice are also transgenic. ZAP-70 is mutated in these mice which results in an altered T-cell selection and arthritis. According to Hirota et al., arthritis is dependent on Th17 generation in this model (62). This further supports the pathogenic role of this subset in RA.

Collagen-induced arthritis

CIA consists of two immunisations with collagen II. As previously said, it is the gold standard model for rheumatoid arthritis. Indeed, several CIA features are found in RA, such as mononuclear cell infiltration, synovial hyperplasia and cartilage degradation. Moreover, susceptibility is defined by the MHC class II haplotype, as in RA. H-2^q (DBA/1 mice) or H-2^r MHC haplotypes make mice susceptible to the disease, therefore the C57BL/6 strain of mice (H-2^b) is considered resistant even though this remains controversial among researchers (61, 63, 64).

CIA is a systemic disease mediated by a combined response of B- and T-cells, which reflects RA physiopathology (65). Proinflammatory cytokines such as TNF-α, IL-1, and IL-6 also play a role in CIA inflammation, although antibodies specific for CII are needed for complete development of the disease (66). Nevertheless, there are some differences between CIA and RA. Firstly, there is no rheumatoid factor in CIA, nor is there sex difference in CIA induction. Secondly, in many studies, CIA resulted in a monophasic disease (67), although not present in our case.

IL-6 and its signalling pathways

IL-6 is a pleiotropic cytokine. Its signalling pathway can be triggered by two forms of the same receptor: membrane IL-6 receptor (IL-6R) on target cells or soluble IL-6R in the serum. IL-6R interacts with gp130 which is important for the signal transduction. gp130 is part of IL-35 and the IL-27 receptor, two regulatory cytokines discussed in the chapter concerning tolerance. It is ubiquitous. Thus, IL-6 could influence many different cells through interaction with its soluble receptor (sIL-6R). However, soluble gp130 is also present in the blood and prevents sIL-6R signalling through membrane gp130. Signalling through sIL-6R is called trans-signalling (68). IL-6 signalling can be blocked by anti-IL-6 or anti-IL-6R neutralising antibodies (69). Its pathway involves Jak/STAT and MEK/ERK. As seen before, it is crucial to induce Th17 lymphocytes and consequently decrease Treg population via STAT3. It also favours Tfh and B-cell maturation and activation as well as T-cell survival and proliferation. IL-6 also shifts monocyte differentiation into DC to differentiation into macrophages (69).

IL-6 also acts as an acute phase protein inducing fever and acute phase inflammatory products secreted by the liver. It promotes angiogenesis and influences neutrophil trafficking. Its blockade diminishes neutrophilia in arthritis (68).

In addition, IL-6 also has anti-inflammatory functions such as epithelial regeneration. Indeed, it was found that IL-6 deficiency in a dextran sulfate sodium colitis model increased the inflammatory score. According to some researchers, IL-6 pro-inflammatory functions would rather be induced by the trans-signalling whereas the cis-signalling would be involved in the anti-inflammatory properties of IL-6 (70).

But most relevantly for this project, IL-6 presence was also found in autoimmune diseases, and in particular RA.

CD11b

The CD11b protein is the α_M subunit of the Mac-1 integrin (CD11b/CD18, Mac-1, CR3 or $\alpha_M\beta_2$) which is a member of the beta 2 integrin family of adhesion receptors. As mentioned in the DC description, CD11b is expressed on a DC subset. However, Mac-1 is also expressed on other cells, mainly myeloid cells such as monocytes, macrophages, granulocytes, and natural killer cells. As integrin, it plays a role in differentiation, cell migration, immune response and tissue architecture. CD11b has over 40 reported ligands which could partly explain the many different functions of this molecule. To mention a few, ECM proteins, ICAM-1, GPIbα, JAM-3, elastase, myeloperoxidase, plasminogen, CD40L, HMGB1 are some of Mac-1 ligands.

This molecule is involved in leucocyte adhesion, phagocytosis, activation, migration, diapedeses and degranulation. It can sense alarmins such as defensins and HMGB 1 which activates monocytes or granulocytes and promote their migration. However, neutrophil cationic proteins can also act that way (16, 71). Furthermore, Mac-1 promotes adherence to endothelial cells and microorganisms.

Although, CD11b integrin is classically associated with inflammatory responses (72-74), several studies have demonstrated that absence of CD11b/CD18 is implicated in the development of several inflammatory diseases (75, 76). For example, its absence or mutation has been linked to autoimmune processes such as B-cell hyperproliferative response, lupus and nephritis (77, 78). Lastly, CD11b deficiency was also associated with increased numbers of Th17 and breach of oral tolerance (17). However, the mechanisms by which this integrin controls these diseases are not well known. It was recently reported the Treg/neutrophil interaction through ICAM-1/CD11b induced IL-10-producing neutrophils (79). Moreover, Cao et al. stated that CD11b dampened inflammatory responses through MyD88 and TRIF degradation via syk and src (80). According to them, $CD11b^{-/-}$ macrophages secrete more

TNFα, IFNβ and IL-6 after LPS or CpG stimulation. They found that CD11b deletion increases TNFα and decreases IL-10 resulting in exacerbated inflammation in a murine colitis model (81).

Aims

As mentioned, Th17 are crucial for RA development. In addition CD11b dampens IL-6 secretion of macrophages, which probably decreases Th17 differentiation given that IL-6 is a central factor for Th17. Consequently, CD11b could be implicated in RA prevention and its most studied model, the CIA.

In our first project, we aimed to investigate the role of CD11b in CIA pathogenesis. To do so, we decided to induce CIA in a resistant mouse strain in order to assess if absence of CD11b alone could enable development of arthritis. $CD11b^{-/-}$ C57BL/6 mice were used and compared to their WT counterparts. We then aimed to study the immune response, and more particularly the cellular response, including Th17 cells. Better understanding of the CIA pathophysiology facilitates development of new therapies for RA. According to our results, two different kinds of treatment were tested in our model. Finally, we developed a CD11b KO cell line that enables researchers to further investigate CD11b intracellular signalling without the usual ex vivo cell limitations.

Second project: The role of NCoR1 in DC activation

Tolerogenic DCs

The example of RA and other autoimmune diseases points out the importance of finding new treatments that could ideally cure inflammatory diseases. Acquiring more knowledge on tolerance such as in our CD11b project, enables us to find new ways to induce tolerance. A promising direction is the generation of tolerogenic dendritic cells (tol-DCs). Tol-DCs are naturally present in the body and prevent development of autoimmune processes by promoting tolerance through various mechanisms (82). They were classically believed to be immature DCs which display many tolerogenic features such as low costimulatory molecule and MHC molecule expression. In the steady state, immature DCs are not activated and can present low doses of antigen instead of maturing and migrating to secondary lymphoid organs and initiate the immune response. Without a strong costimulatory signal, T-cells become hyporesponsive or deleted (83). However, immature DCs are not the only tolerogenic DCs. Mature DCs can also promote tolerance, for example by depleting autoreactive cells in the thymus during the negative selection (83). In the periphery, tol-DCs not only delete autoreactive T-cells but also induce Tregs and Bregs (84). Another mechanism of tolerance induction by tol-DCs, apart from low costimulation during antigen presentation, is the production of tolerogenic molecules such as IL-10, TGFβ and IDO1 (82). Tol-DCs can express PDL1 that interacts with PD1 on T-cells. This promotes cell death in effector T-cells and inhibits apoptosis in Tregs. Tregs can in turn modulate DC function by blocking their differentiation. In addition to conventional DCs, pDCs have a tolerogenic subset too.

All this encourages development of tol-DC in order to treat autoimmune diseases or prevent graft rejection in transplantation. A subset of tol-DC could be induced in vitro by mimicking

the microenvironment of secondary lymphoid organs in presence of TGFβ. It resulted in mature tol-DCs which secrete high amounts of IL-10 and low amounts of IL-12 (84).

In conclusion, engineering tol-DCs is of great relevance to prevent or treat autoimmune diseases. Our lab already generated a $CD8^+$ DC line constitutively producing IL-35. Adoptive transfer of these DCs could suppress EAE even after induction of encephalitogenic T-cells (45).

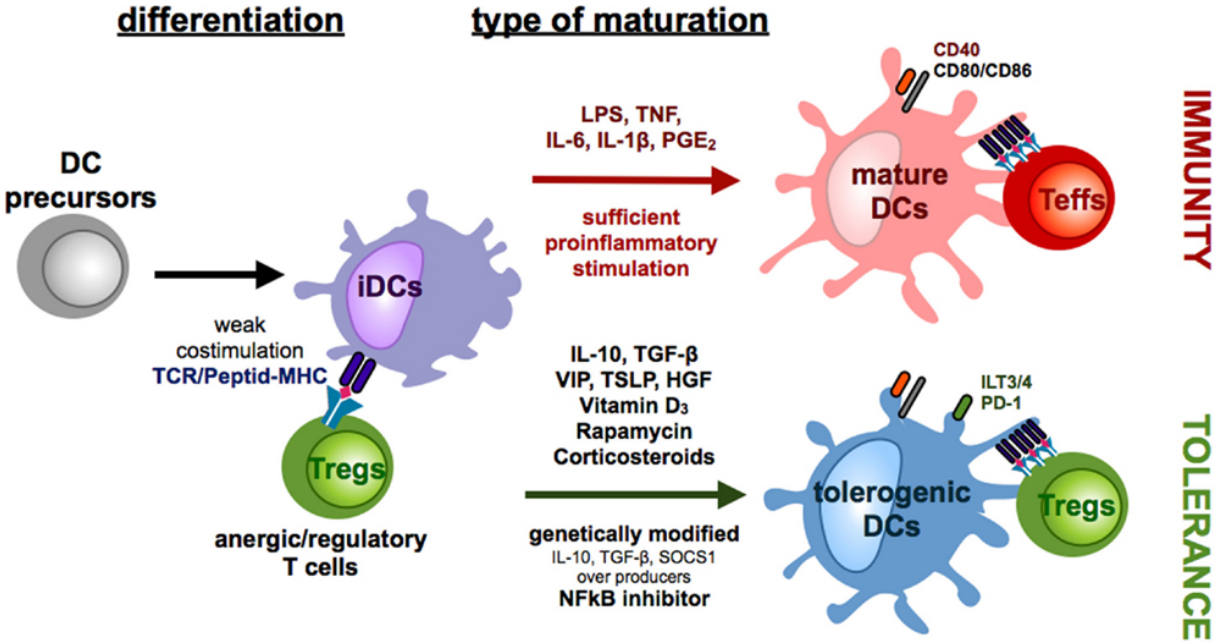


Figure 4 ***DC differential maturation***
According to their milieu, DCs can either mature into pro-inflammatory DCs or tol-DCs. Taken from (85).

DC regulation at the transcriptional level

DCs receive multiple stimulatory cues to generate signal-specific responses. It appears improbable that transcription factors alone can integrate all regulatory signals and ensure the appropriate transcriptional response. One of the well characterised mechanisms of how nuclear receptors regulate transcriptional responses is through the recruitment of transcriptional co-

regulators (co-repressors or co-activators) (86-88). Nuclear receptors are classically known to control their target genes by switching from co-repressor recruitment to co-activator recruitment upon binding of agonist ligands. However, there are other co-repressor models. An interesting model is the combinatorial model, in which co-repressors and co-activators work closely together in order to have a selective and stable gene activation and repression (89).

Recent reports show that treatment of DCs with high affinity nuclear receptor ligands such as rosiglitazone, vitamin-D and retinoic acid modulates the expression of their costimulatory molecules and cytokines. Thereby it disturbs their functional responses (90-92). Vitamin-D and dexamethasone treatment generates regulatory or tolerogenic DCs by suppressing the expression of the activation/costimulation markers CD80, CD86 and enhancing the expression of the anti-inflammatory cytokine IL-10 (93). This suggests the involvement of nuclear receptor co-repressors such as NCoR1 and its homologue SMRT in controlling DC functions, as they were the first ones identified in complexes with unliganded nuclear receptors such as PPARγ, TR, RXRγ and ER (94-97).

NCoR1

Nuclear receptor co-repressor 1 (NCoR1) is a co-repressor which forms complexes and binds enhancers in order to silence gene transcription (98). Among other mechanisms, NCoR1 can repress gene transcription by recruitment of histone deacetylases (HDACs), resulting in closure of chromatin structure and gene repression (89). NCoR1 was first described in embryonic development and associated with different diseases, such as cancer and thyroid hormone resistance (89). NCoR1s main roles are cell fate determination, cell differentiation, and lineage progression. Indeed, its mutation results in embryonic death by cardiac defects. Moreover,

NCoR1 is involved in cell proliferation, muscle differentiation, adipose tissue regulation and neural stem cell maintenance (94).

However, in the last few years, their implication in inflammatory processes has received growing interest in research. Different macrophage models were used to assess NCoR1 function in these cells. Ghisletti et al. found that macrophages need to be de-repressed from NCoR1 in order to get activated (99). They revealled that NCoR1 also interacts with non-NR TFs such as NFκB and AP1 that are reported to play a crucial role in controlling macrophage responses (96, 99). Analysis of recently published NCoR1 ChIP-seq data in macrophages revealed that NCoR1 also binds to genes that are accountable for antigen recognition, costimulation and T-cell polarisation in dendritic cells. Unexpectedly, NCoR1 functions in DCs were scantly investigated, which is even more surprising if we consider the fact that DCs are regarded, as said earlier, to be key inducers of the immune response and tolerance.

Aims

Macrophages studies clearly indicate an important role of NCoR1 in their activation. To further this investigation, the relationship between DCs and NCoR1 should be explored as well. DCs have the ability to shape the adaptive immune response. This is of great relevance in a clinical context to artificially modulate the immune response according to the disease. NCoR1 interaction with unliganded nuclear receptors that are influenced by immunomodulatory factors such as dexamethasone and vitamin D supports the importance of this study.

Thus, in this second project, we intended to characterise the role of NCoR1 in DCs. According to obtained results, we investigated the relevance of targeting NCoR1 to modulate a specific

immune response. This could enable development of potential therapies for immune disorders, such as DC vaccination, transplantation and autoimmunity.

Unfortunately, ex vivo DCs are extremely fragile and undergo rapid apoptosis in vitro. Therefore, most research on DCs is performed on bone marrow derived DCs, which are nevertheless, slightly different from conventional DCs. Their in vitro instability makes treatments such as lentiviral knockdowns impossible. This is why a well-characterised $CD8^+$ dendritic cell line that was developed in our lab by previous researchers was used for this research (100). The similarity of this cell line with WT murine DCs enables robust analogy between the DC line results and the expected results on ex vivo DCs. In this project, we collaborated with the group of Dr. Sunil K. Raghav who performed an NCoR1 lentiviral KD on our DC line. To test the role of NCoR1 in vivo, we developed a mouse strain with a DC-specific conditional KO for NCoR1.

Results

First project: The role of the integrin CD11b in collagen-induced arthritis (CIA)

CIA is the gold standard model to study RA that is a relative frequent autoimmune disease. RA involves many disabling symptoms and can lead to lethal comorbidities such as coronary disease. A better knowledge of RA physiopathology can help us find new therapeutic targets and modulate the disease. We found that CD11b has a crucial role in protecting CIA-resistant mice against arthritis as CD11b deficient mice become susceptible to the disease.

Induction of the disease

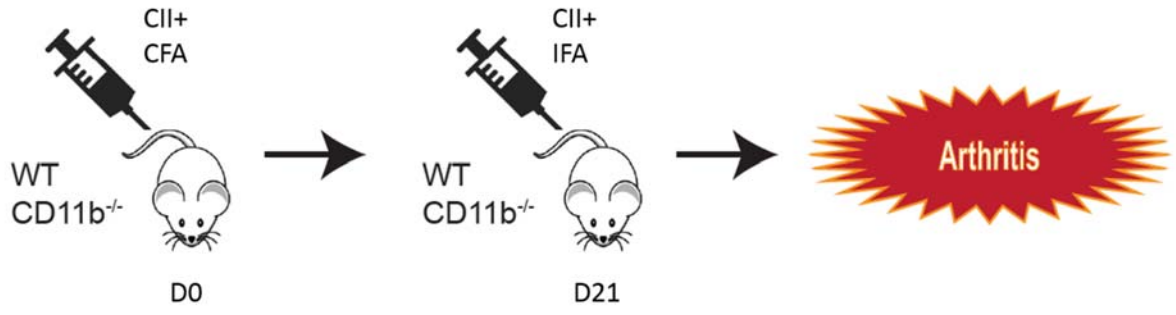


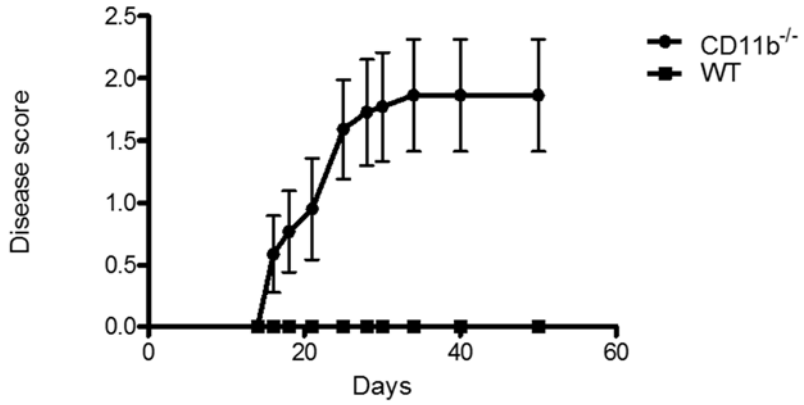
Figure 5 ***Collagen-induced arthritis induction***
Collagen-induced arthritis is induced by a first immunisation at day 0 of collagen II in CFA and a booster at day 21 in IFA. Immunisations were performed on WT and CD11b$^{-/-}$ mice. Mice were monitored until development of arthritis.

As mentioned in the introduction, C57BL/6 mice are naturally resistant to CIA although it can be induced in mice strains with other HLA haplotypes. To evaluate the requirement of the integrin CD11b for the development of CIA, the standard protocol for CIA induction was

applied in C57BL/6 background for WT and CD11b$^{-/-}$ mice. The standard protocol includes two collagen II immunisations as described in Figure 5.

The onset and progression of arthritis was assessed using a clinical score, described in the materials and methods, based on erythema and oedema of affected joints. Mice were examined for 50 days after the second immunisation.

Arthritis scores were only observed in CD11b$^{-/-}$ mice with the peak of disease from day 28 to day 50 (Figure 6). Sixty five percent of CD11b$^{-/-}$ mice developed arthritis with a mean onset on Day 25 after initial immunisation. Interestingly, around 20% of the CD11b$^{-/-}$ mice had developed arthritis before the second immunisation of CII-CFA.



	Median days of onset	CIA incidence (%)
Wild type	0	0
CD11b$^{-/-}$	25	65

Figure 6 ***Arthritis score and incidence***
Clinical scores and incidence of arthritis in WT and CD11b$^{-/-}$ mice. Results shown are pooled from three independent experiments (n = 20 mice/group). Data are means ± SEM. Results shown are pooled from two separate experiments (n = 8 mice/group). *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

Figure 7 shows macroscopic and microscopic comparisons between immunised WT and CD11b$^{-/-}$ mice. On the histology results, a normal architecture of paw and knee joints can be observed on WT mice. On the opposite, CD11b$^{-/-}$ mice display severe inflammation with macroscopic swelling and redness. Histological analysis demonstrates synovitis, cartilage destruction, architectural changes of joints, and a massive lymphocytic infiltration.

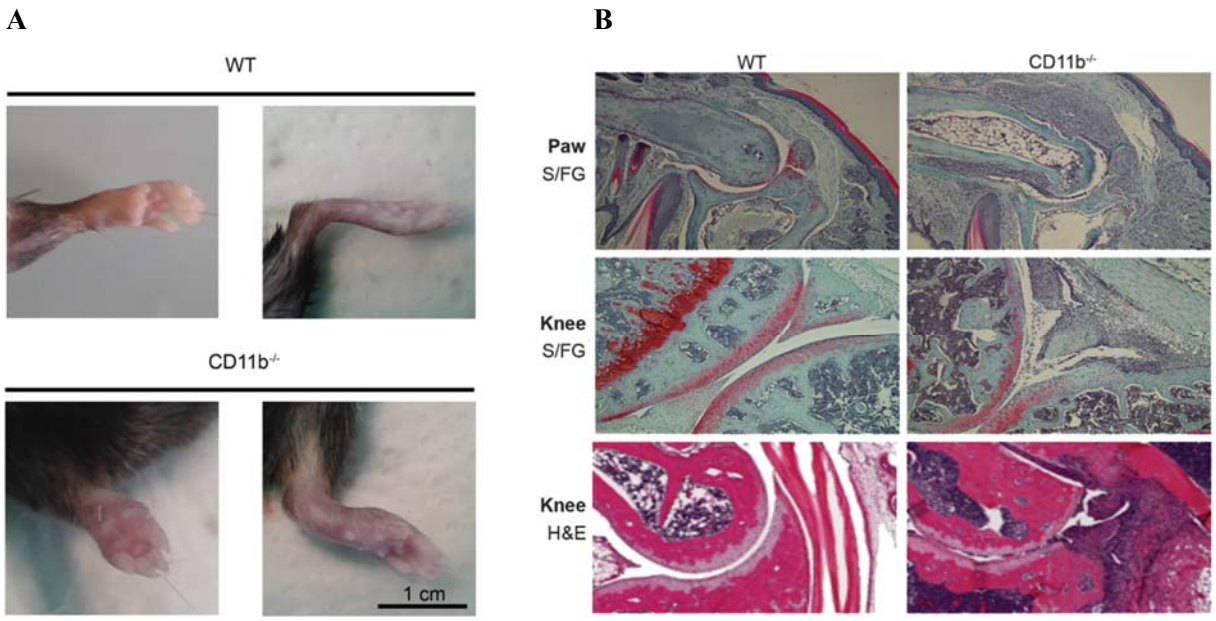


Figure 7 ***Macro- and microscopic representation of CIA in immunised mice***
A) Representative photographs from hind limb and paws on day 30 showing severe arthritis in CD11b$^{-/-}$ mice compared to WT mice. Images are representative of five independent experiments. B) Histological examination of the joints from WT and CD11b$^{-/-}$ mice on day 40. Paraffin-embedded hind paw and knee sections were stained with H&E or S/FG. Original magnification ×100 for H&E and S/FG. Images are representative of three independent experiments.

CIA immune response in CD11b$^{-/-}$ mice

T-cell response: Th17/Treg balance is shifted towards Th17 in immunised CD11b$^{-/-}$ mice

Populations of adaptive immune cells were characterised in the steady state. No significant difference was observed between CD11b$^{-/-}$and WT mice. However, flow cytometric analysis of helper T-cell populations twenty days after the second immunisation revealed higher

percentages of IL-17^{+} CD4^{+} T-cells in immunised CD11b$^{-/-}$ mice compared to WT mice (Figure 8A). Cells were harvested from the inguinal lymph nodes, in this case the draining lymph nodes. They were then restimulated in vitro with PMA/ionomycin for 6 hours. The amount of IL-17 in the culture supernatants from PMA/ionomycin-stimulated inguinal lymph node cells of immunised CD11b$^{-/-}$ mice was significantly higher than that of WT mice (15-fold). In order to further validate the importance of IL-17 in our model, we assessed levels of IL-17 in the joint lysates of immunised mice. We again found higher levels in CD11b$^{-/-}$ mice compared to WT mice (Figure 8B).

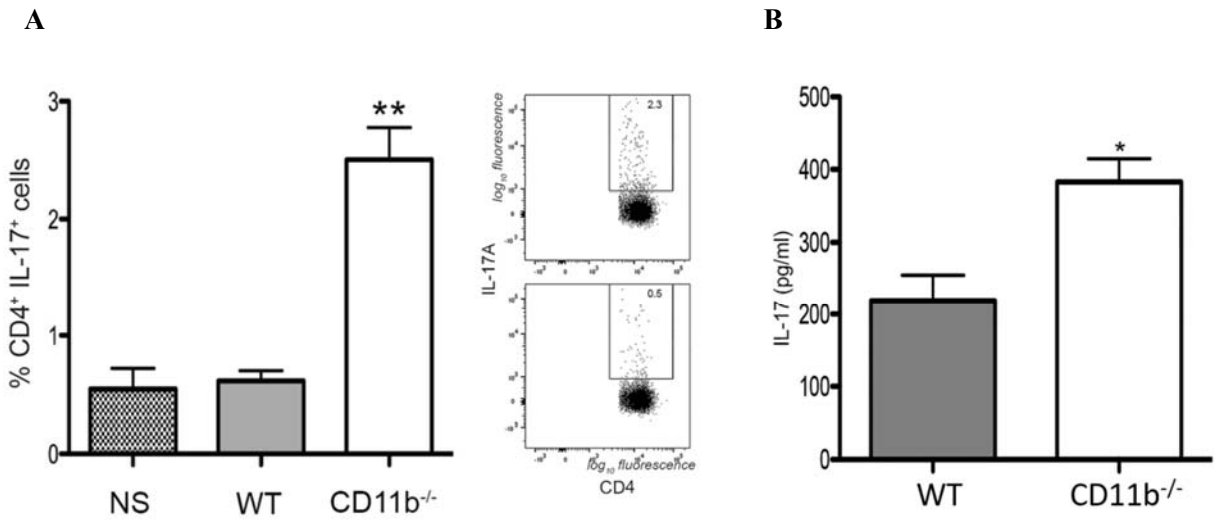


Figure 8 ***Th17 are increased in immunised CD11b$^{-/-}$ mice***
A) Percentage of IL-17 positive cells in CD4^{+} T-cell populations of inguinal lymph nodes from non immunised (NS), immunised WT and CD11b$^{-/-}$ mice on day 20 after the second immunisation. IL-17 presence was determined in inguinal lymph node single cell suspensions by intracellular staining after ex vivo stimulation with PMA/ionomycin in the presence of brefeldin A for 5 h. B) Assessment by ELISA of IL-17 levels in the joint cell lysates of immunised mice. A+B) Data are shown as mean ± SEM (n = 6 mice/group) and are representative for two independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

We next analysed the regulatory pendent of Th17, the Tregs, known to counterbalance pro-inflammatory cells and autoimmune diseases. We found that immunised CD11b$^{-/-}$ mice developed fewer numbers of FoxP3^{+} T- cells in their draining lymph nodes than in their equivalent WT mice (Figure 9A).

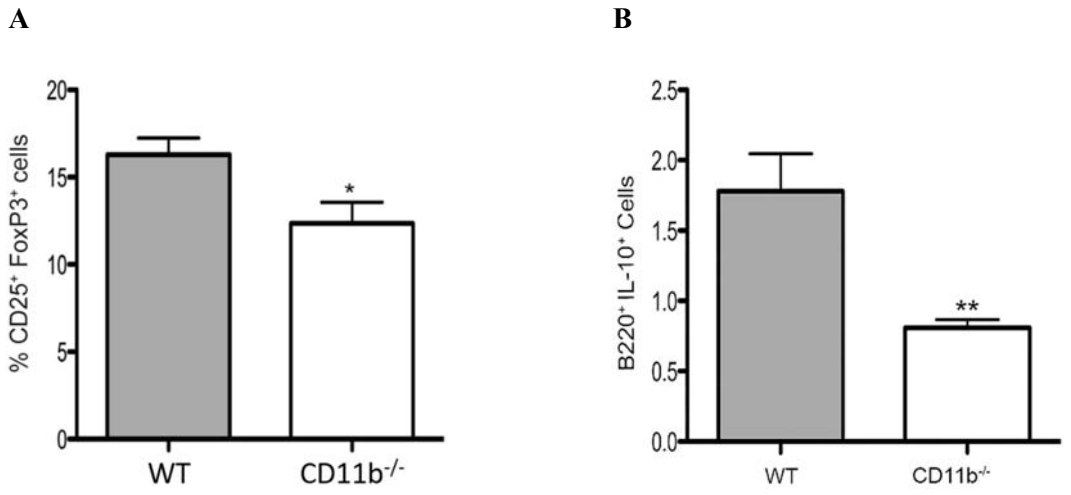


Figure 9 ***Tolerogenic lymphocytes are decreased in immunised CD11b$^{-/-}$ mice***
A) Percentages of CD25^{+} Foxp3^{+} cells in CD4^{+} T-cells from inguinal lymph nodes of -immunised WT and CD11b$^{-/-}$ mice on day 30 after immunisation. B) Percentage of IL-10 positive cells in B220^{+} cells from inguinal lymph node of immunised WT and CD11b$^{-/-}$ mice on day 30 after immunisation. IL- 10 presence was examined by intracellular staining after ex vivo stimulation with LPS in the presence of brefeldin A for 6h. A+B) Data are shown as mean ± SEM (n = 6 mice/group) and are representative for two independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

Taken together, these results give a first hint that CIA results from an imbalance in which Th17 response is disproportionally increased compared to the Treg response.

IL-10-producing B cells, another regulatory lymphocyte also called Bregs, can modulate autoimmune diseases by affecting the frequency and equilibrium of both Treg and Th17 cells (101). As this balance is disturbed in our case, the proportions of Bregs were analysed as well. 6h-LPS-stimulated splenocytes revealed a significant reduction of IL-10-producing B cells in immunised CD11b$^{-/-}$ mice compared to the controls (Figure 9B).

IL-6 a key mediator of CIA

As mentioned in the introduction, IL-6 is a potent inducer of Th17 and is a key factor of the balance between Th17 and Treg. It probably has an important role in the pathogenesis of RA as well (102, 103). To explore the role of IL-6 in CIA, serum IL-6 levels were measured and correlated with disease occurrence. We found that sick CD11b$^{-/-}$ mice had significantly higher serum IL-6 levels than WT controls (Figure 10A).

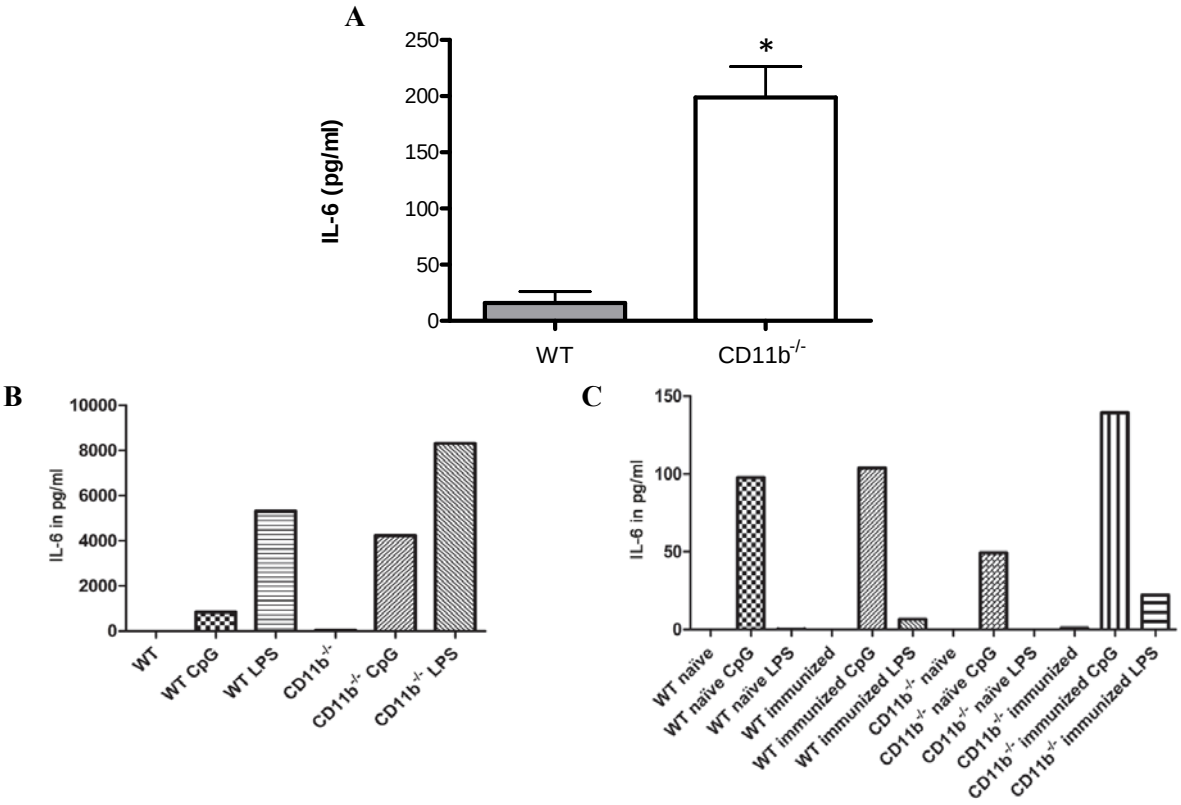


Figure 10 ***IL-6 is increased in immunised CD11b$^{-/-}$ mice***

A) Concentr ations of IL-6 in serum from immunised mice quantified by ELISA. Data are shown as means ± SEM n = 5 mice/group and are representative of three independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests B) IL-6 concentration in peritoneal macrophage supernatant after 15 hour treatment with CpG, LPS or no treatment. Macrophages were isolated from immunised CD11b$^{-/-}$ and WT mice. C) IL-6 concentration in MACS purified DC supernatant after 15 hours in presence or absence of CpG or LPS. DCs were isolated from naïve and immunised mice. B+C) Data are shown as means and are representative of two independent experiments.

Supernatants from magnetically enriched DCs or freshly isolated peritoneal macrophages indicated that both types of cells could be responsible for this increase (Figure 10B+C).

IL-6 signals through IL-6 receptor (IL-6R), which is either a membrane protein or a soluble factor present in the serum. Soluble IL-6R (sIL-6R) was measured in the serum of naïve and immunised mice. Although concentrations were comparable between WT and CD11b$^{-/-}$ mice, we found a significant increase in immunised mice (Figure 11).

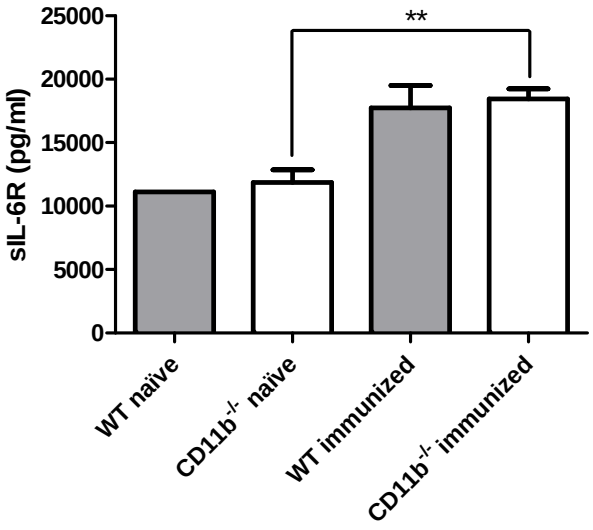


Figure 11 ***Soluble IL-6R concentration is increased in immunised mice***
Concentration of soluble IL-6R in serum of immunised and naïve mice. IL-6 levels were quantified by ELISA. Data are means ± SEM n = 5 mice/group. Data are shown as mean ± SEM (n = 6 mice/group) and are representative for two independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

Acute phase cytokines

Regardless of the Th17 – Treg balance, other acute phase cytokines were described as important for rheumatoid arthritis in humans (104). Indeed, therapies targeting TNFα or IL-1β are already available to treat rheumatoid arthritis. However, when tested in our model, no significant difference could be noted in IL-1 and TNFα levels (Figure 12).

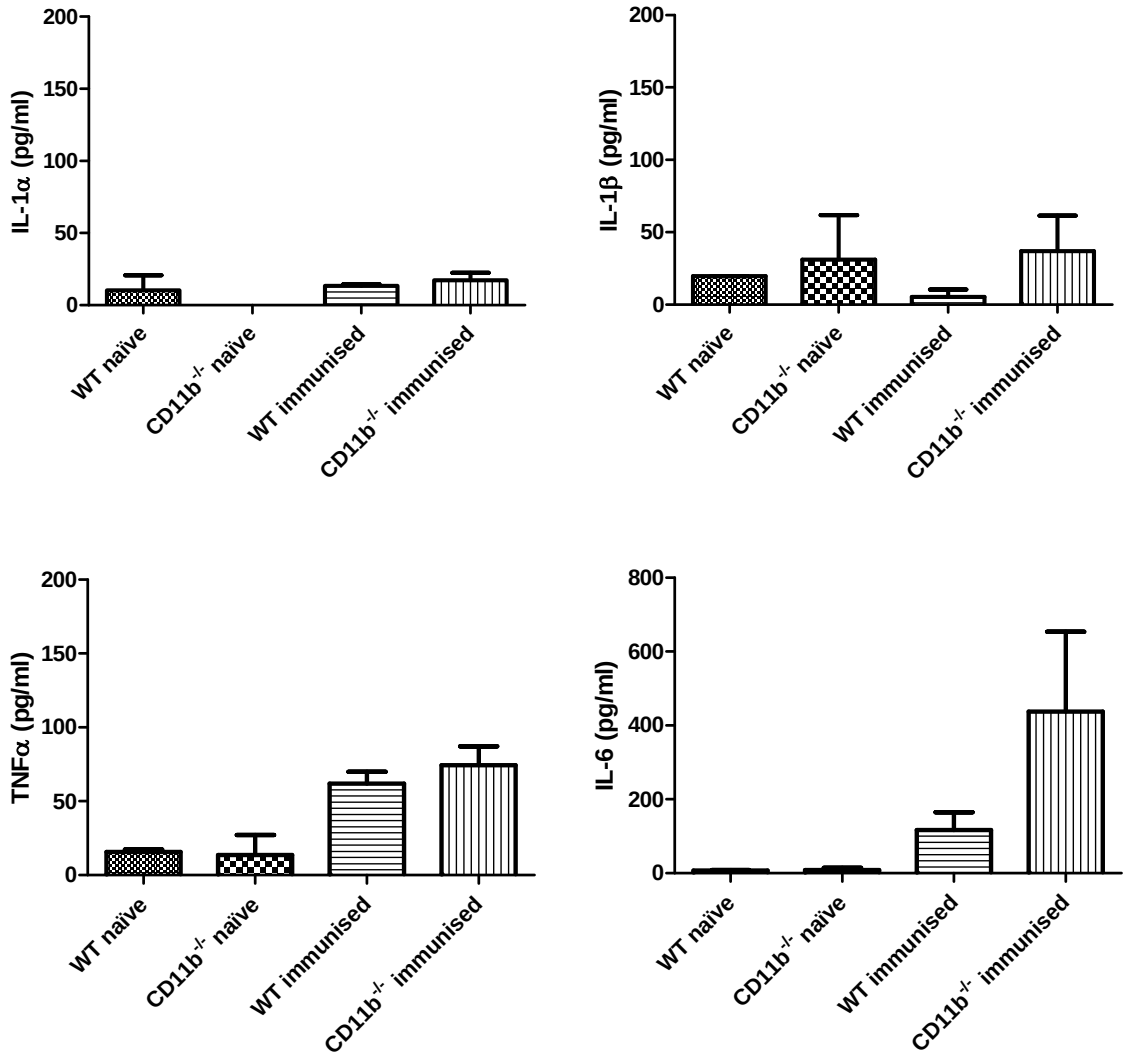


Figure 12 ***Serum concentration of acute phage cytokines***
Concentration of RA relevant cytokines in serum of immunised and naïve mice. Cytokine levels were quantified by LEGENDplex. Data are shown as mean ± SEM (n = 3 mice/group).

CD11b$^{-/-}$ DCs induce Th17 and CIA via IL-6

We next investigated the consequence of blocking IL-6 signalling by using a blocking antibody against IL-6R, an anti-IL-6R mAb (15A7). We tested its action in vitro, on Th17 differentiation. Naïve CD4^{+} T-cells were cocultured with freshly isolated DCs from either WT or CD11b$^{-/-}$

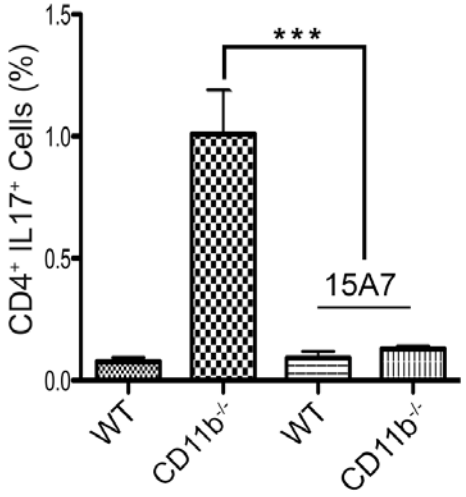


Figure 13 ***Effect of anti-IL-6R in vitro***
Percentage of IL-17 positive cells in $CD4^+$ T-cells from cocultures with ex vivo DCs. DCs were isolated from untreated WT and $CD11b^{-/-}$ mice and cultured with $CD4^+$ T-cells, stimulated with anti-CD3 and TGF-β in the presence or absence of anti-IL-6R mAb 15A7. After 4 days, IL-17 presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h. Data are shown as mean ± SEM (n = 8-9) and are pooled from three independent experiments. *p<0.05, **p<0.01, ***p<0.001; Mann–Whitney U tests.

mice. To enable Th17 differentiation, TGFβ was added to the cocultures. As shown in Figure 13, $CD11b^{-/-}$ DCs induced more Th17 than WT DCs. The addition of IL-6R neutralising mAbs potently inhibited Th17 differentiation. This confirms that the disease is probably caused by DCs through IL-6-mediated Th17 induction.

To evaluate the action of the anti-IL-6R mAb (15A7) on disease severity in vivo, both 15A7 and IgG isotype-matched control antibodies were administrated intraperitoneally into $CD11b^{-/-}$ mice twice a week, starting from the time of the second immunisation. The 15A7 treatment showed a significant decrease of disease severity in comparison to IgG isotype-matched control (Figure 14A).

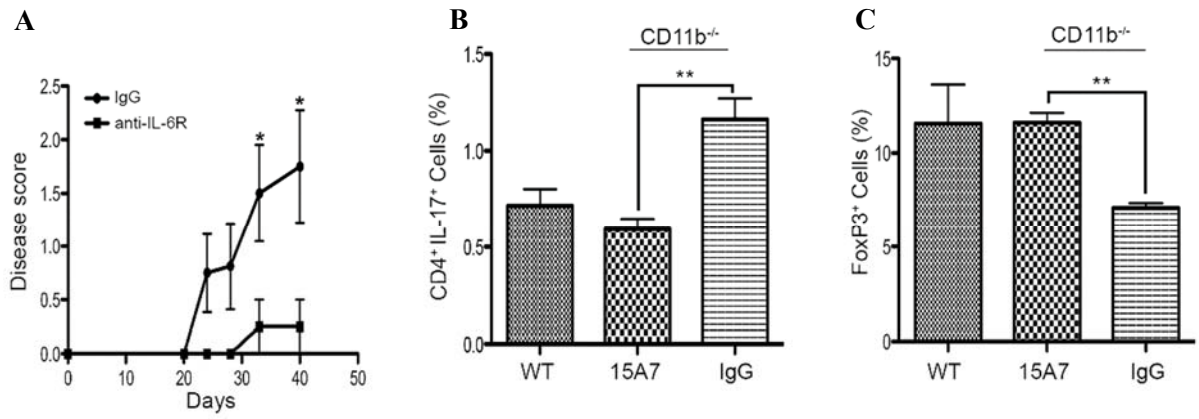


Figure 14 ***In vivo effect of anti-IL-6R on CIA***
A) Clinical scores of arthritis in CD11b$^{-/-}$ mice treated with anti-IL-6R or isotype-matched control monoclonal antibodies. B+C) Percentage of IL-17 positive (B) and FoxP3 positive (C) cells in CD4^{+} T-cells isolated from inguinal lymph nodes of mice treated with anti-IL-6R or isotype-matched control antibodies. A-C) Data are shown as mean ± SEM (n = 9 mice/group) and are pooled from two independent experiments. *p<0.05, **p<0.01, ***p<0.001; Mann–Whitney U tests.

Twenty days after the second CII immunisation, inguinal lymph nodes were harvested and analysed by flow cytometry to profile CD4^{+} T-cells. Figure 14B shows significant reduction in Th17 in mice treated with 15A7 compared to isotype-matched control IgG treated mice. Intriguingly, the percentage of Treg became comparable to WT mice after 15A7 treatment (Figure 14C).

CD11b^{+} DC line

Our lab engineered a novel CD11b^{+} dendritic cell line stable for in vitro cultures and capable of undergoing various manipulations such as lentiviral-CRISPR infections. It was derived from the same genetic background as the CD8^{+} DC line mentioned in the introduction, however it is additionally KO for BATF3. Oncogene-positive transgenic mice CD8^{+} DC tumours. However, if BATF3 (a necessary transcription factor for CD8^{+} DC maturation) is invalidated, mice generate CD11b^{+} DC tumours (an article describing this line is currently in preparation).

CD11b^{+} DCs reduce severity of CIA in CD11b$^{-/-}$ mice

Firstly, we demonstrated the potential of our CD11b^{+} DC line to exert immune regulatory effects in vivo and ameliorate CIA as previously described in the literature (105-108). The advantage of this line is to have pure cells, thus excluding the potential effect of contaminating cells. As reported in Figure 15, CD11b$^{-/-}$ mice treated with CD11b^{+} DCs showed significantly reduced disease severity compared with the untreated mice.

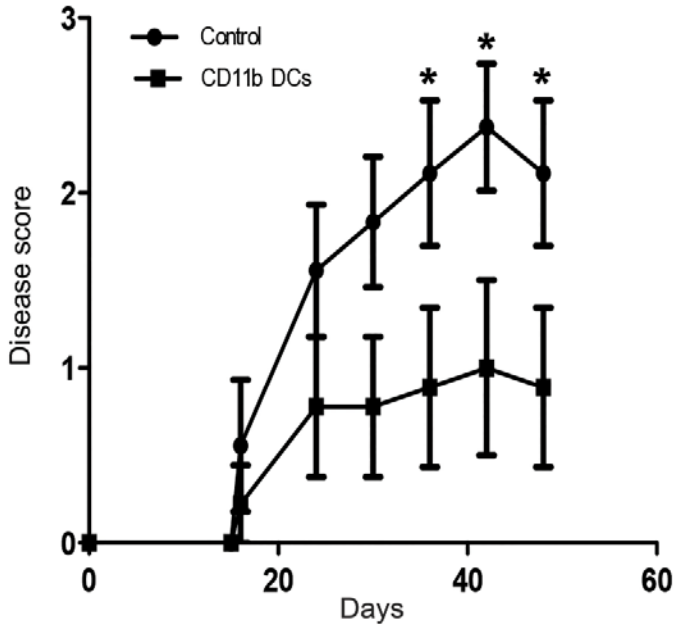


Figure 15 ***Adoptive transfer of CD11b^{+} DCs ameliorates disease score***
Clinical score in immunised CD11b$^{-/-}$ mice after CD11b^{+} DC transfer. CD11b^{+} DCs were used for adoptive transfer intraperitoneally into CD11b$^{-/-}$ mice 1 day before the second immunisation. The severity of arthritis was compared between control and DC transferred CD11b$^{-/-}$ mice. Data shown are means ± SEM (n = 16 mice/group) and are pooled from two separate experiments.

CD11b KO DC line generation

Secondly, this cell line was used to create a CD11b KO line in order to facilitate and further investigate the role of CD11b in DCs. We developed a specific plasmid for a CRISPR KO of CD11b, which was used on HEK cells to generate lentiviruses (Figure 16). These lentiviruses contain a puromycin-resistant gene that was used in order to deplete as much as possible untransduced cells.

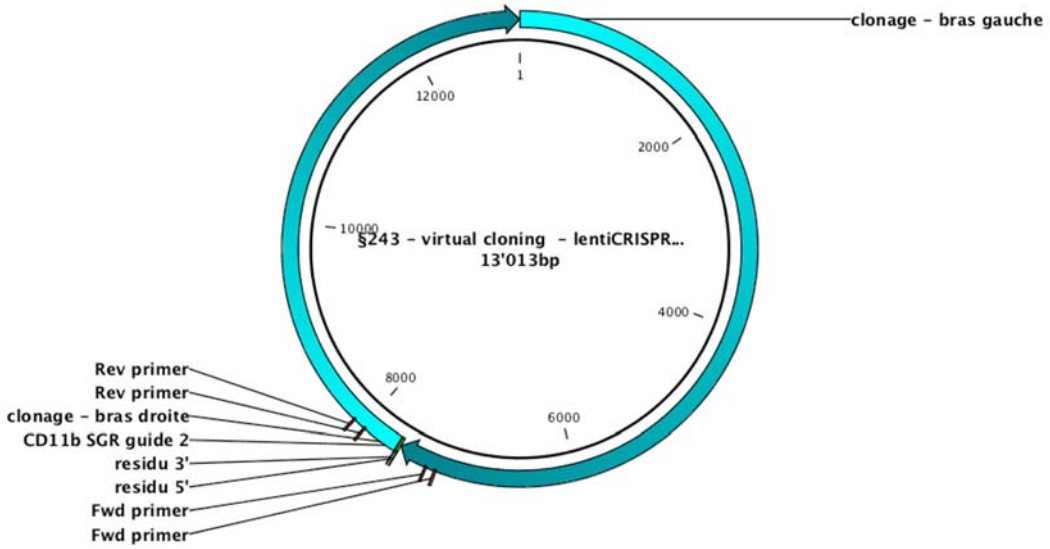


Figure 16 ***Plasmid outline***
Data generated by C. Lavanchy.

$CD11b^+$ DCs were then infected with different dilutions of the virus and treated with puromycin. Cells were sorted by flow cytometry into two categories, $CD11b^+$ and CD11b KO cells. Figure 17 shows the percentage of CD11b KO cells in ones treated with lentivirus and selected with puromycin. More than 60% were found to be CD11b KO dendritic cells.

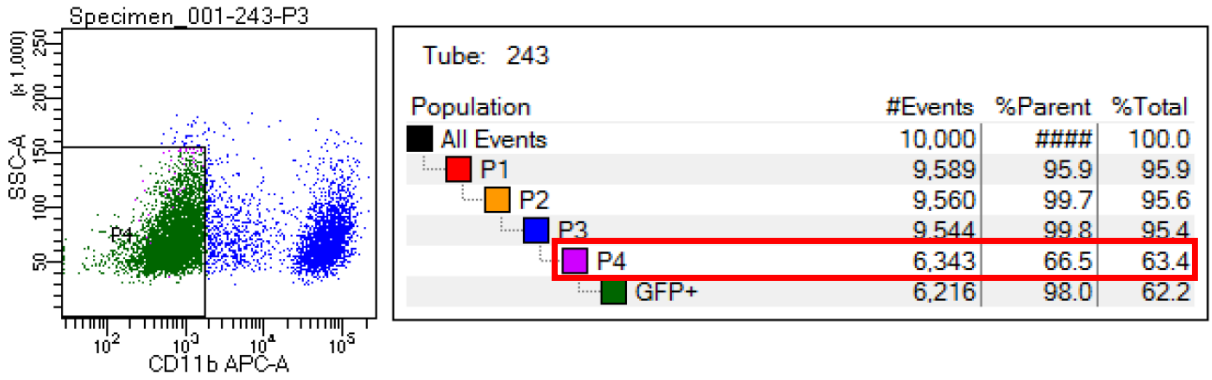


Figure 17 ***CD11b$^+$ DCs lentivirus-transduced and puromycin-selected***
Dot plot showing CD11b KO DCs sorted by flow cytometry. Cells were transduced with a lentiCRISPR-virus and selected by puromycin.

CD11b KO DCs produce more IL-6 than CD11b$^+$ DCs

In addition to our results, it has also been reported that CD11b on DCs inhibits IL-6 production induced by TLR stimuli (109). We confirmed this in our CD11b KO DC line through LPS or CpG stimulation of our DCs. IL-6 was then measured in the supernatant by ELISA. Figure 18 shows an increase in IL-6 secretion in CD11b KO DCs, as expected.

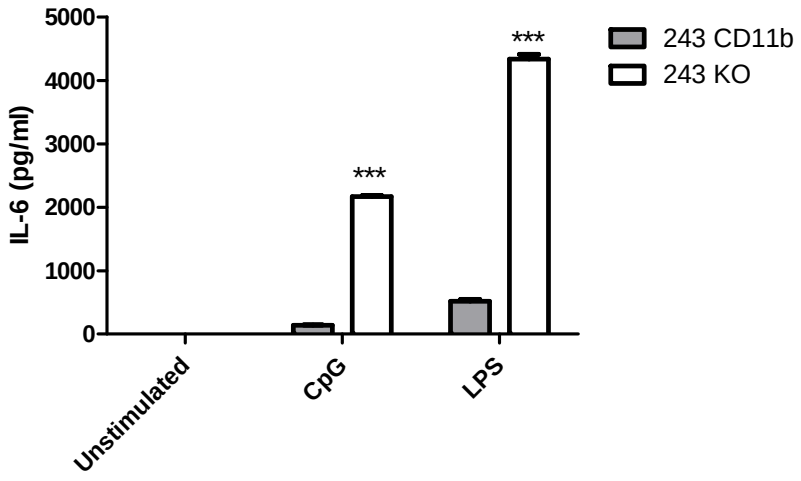


Figure 18 ***CD11b KO DCs secrete more IL-6 than their control***
IL-6 concentration in DC supernatant after 16 hour treatment with CpG, LPS or no treatment. IL-6 levels were quantified by ELISA. Data are shown as means ± SEM (n = 4/condition) and are representative of two independent experiments. 243 CD11b represent CD11b$^+$ control DCs and 243 KO represents CD11b KO DCs. *p<0.05, **p<0.01, ***p<0.001; Unpaired t-tests.

CD11b KO DCs induce more Th17 than CD11b$^+$ DCs

As mentioned above, this line enables us to work with pure cells, without having effects from potential contaminating cells. Thus, we performed another coculture experiment in which the DC lines CD11b KO versus CD11b$^+$ DCs were cocultured with naïve CD4$^+$ ovalbumin-specific T-cells (OT-II cells). Our CD11b KO DC line could reproduce an increased induction of Th17 compared to the CD11b$^+$ DCs (Figure 19A). ELISA from supernatant of the cocultures further confirmed the increased Th17 response by denoting higher IL-17 levels in CD11b KO DC conditions (Figure 19C). The cocultures demonstrated once more the shifted balance between Th17 and Treg resulting from the absence of CD11b with diminished percentages of Tregs in CD11b KO conditions (Figure 19B).

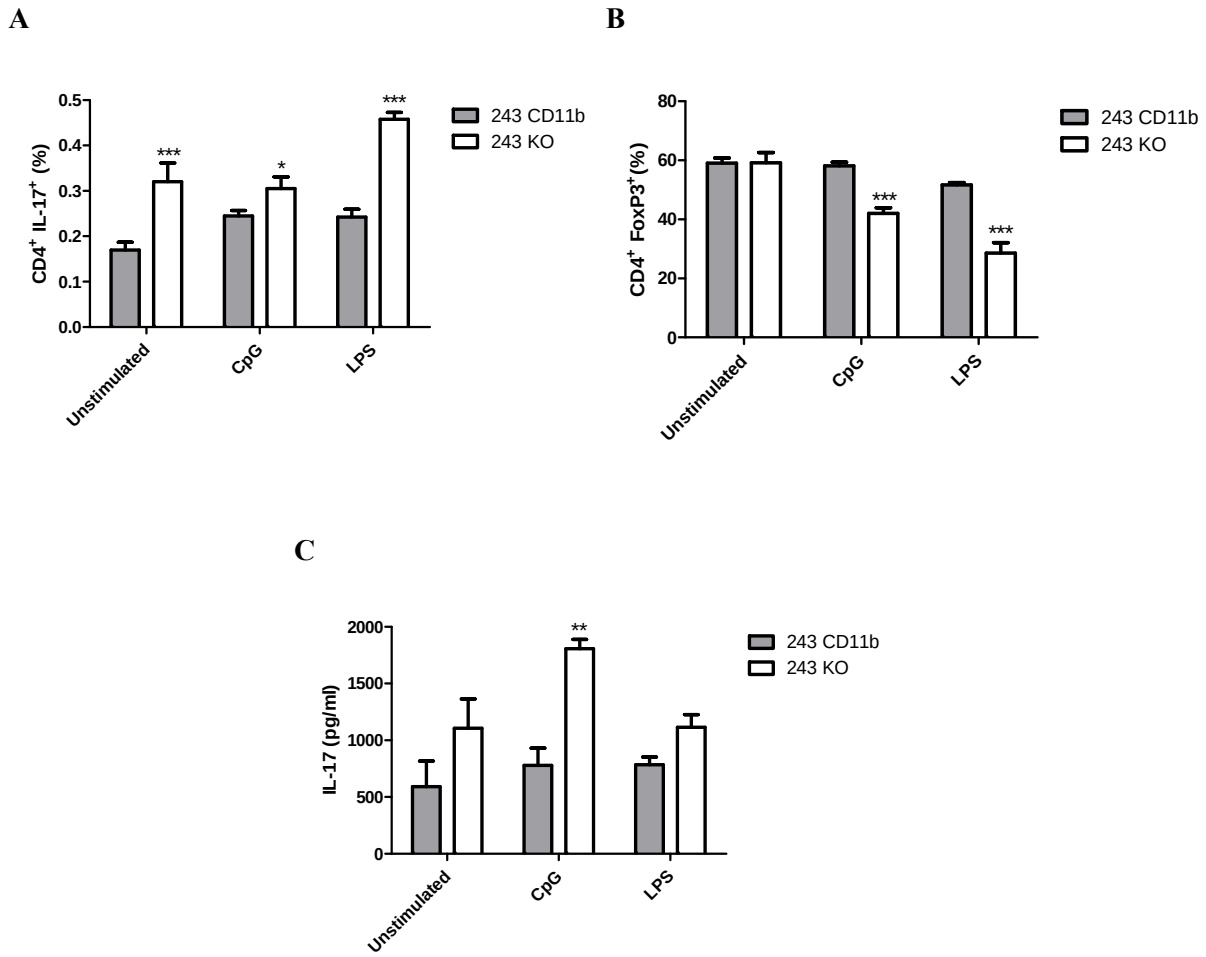


Figure 19 ***CD11b KO DCs induce more Th17 than their controls***
A+B) Percentage of IL-17 positive (A) or FoxP3 positive (B) cells in $CD4^+$ T-cells from cocultures with DC lines. OVA-specific $CD4^+$ T-cells were isolated from OT-II mice and cultured with DCs with OT-II peptide ($OVA_{323-339}$), IL-2 and TGF-β in the presence or absence of CpG or LPS. After 4 days, IL-17 presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h. C) IL-17 concentration in supernatant from above mentioned cocultures quantified by ELISA. A-C) Data are shown as mean ± SEM (n = 4) and are representative for two independent experiments. *p<0.05, **p<0.01, ***p<0.001; Unpaired t-tests.

Could CD11b KO DCs alone induce CIA?

As $CD11b^{-/-}$ DCs seem to be responsible for the IL-6 increase and Th17 induction, collagen II-pulsed CD11b KO DCs were adoptively transferred in WT mice to assess whether CD11b KO DCs alone were sufficient to induce the disease. In this sole experiment, no disease could be induced in WT mice, neither with $CD11b^{+}$ nor with CD11b KO DCs.

Src is a mediator of CD11b

According to Cao et al., src is the key mediator of CD11b inhibition of pro-inflammatory cytokine production such as TNFα and IL-6 (80, 81). We tried to reproduce these results first in freshly isolated DCs from $CD11b^{-/-}$ and WT mice. DCs were sorted by magnetic cell separation and treated with the same src inhibitor as Cao et al. used in some experiments. They were then stimulated with CpG or LPS for 16 hours. Unfortunately, we had very contradictory results indicating a possible contamination. Furthermore, the purity check indicated that many contaminating cells had remained in our experiment.

Secondly, in order to avoid any effect from contaminating cells, we used our newly generated CD11b KO DC line. However, no difference could be observed in IL-6 levels between src treated and untreated DCs.

Second project: The role of NCoR1 in DC activation

As the previous project shows, tolerogenic DCs such as $CD11b^+$ DCs could be very important tools to treat a wide variety of inflammatory diseases or simply to modulate the immune response. The same DCs can be proinflammatory or tolerogenic depending on their actual state. How tolerogenicity is regulated remains poorly understood, although a better comprehension of this regulation is crucial to develop new treatments in immunology. My second project focuses on a co-repressor factor, NCoR1, commonly known to be involved in maturation, and its role in DC activation. We found that this factor is important to regulate the tolerogenic program in DCs, more particularly in $CD8^+$ DCs. This project stems from a collaboration with the group of Dr. Sunil Raghav. My part in this project concerned the development and testing of in vivo and in vitro biological readouts to confirm the tolerogenic state of the DCs after NCoR1 KD or KO.

NCoR1 knockdown DCs induce tolerogenicity

Our collaborating group, the group of Dr. Sunil K. Raghav, performed lentiviral Knockdowns (KD) for NCoR1 in a well characterised $CD8^+$ DC line that our group derived (100). The NCoR1 KD remained stable with an 85% reduction of NCoR1 expression. They found that these KD DCs display tolerogenic features after activation. Figure 20 shows an increase in many tolerogenic molecules, including PDL1, IDO1, IL-10 and IL-27 after CpG stimulation, a TLR9 agonist expressed on $CD8^+$ DCs. These results were confirmed at the protein level by flow cytometric analysis of IL-10, IL-27, IDO1, CTLA4, IL-6, IL-12p40 and PDL1. They then performed a large genomic analysis using RNA seq and ChIP seq and showed that two genetic programs are preferentially touched by NCoR1 KD: anti-viral response and immune tolerance,

which confirms the preliminary results. The large majority of the differentially expressed genes in KD cells were directly regulated by NCoR1 expression. Hence, NCoR1 is a direct inhibitor of various tolerogenic genes in DCs.

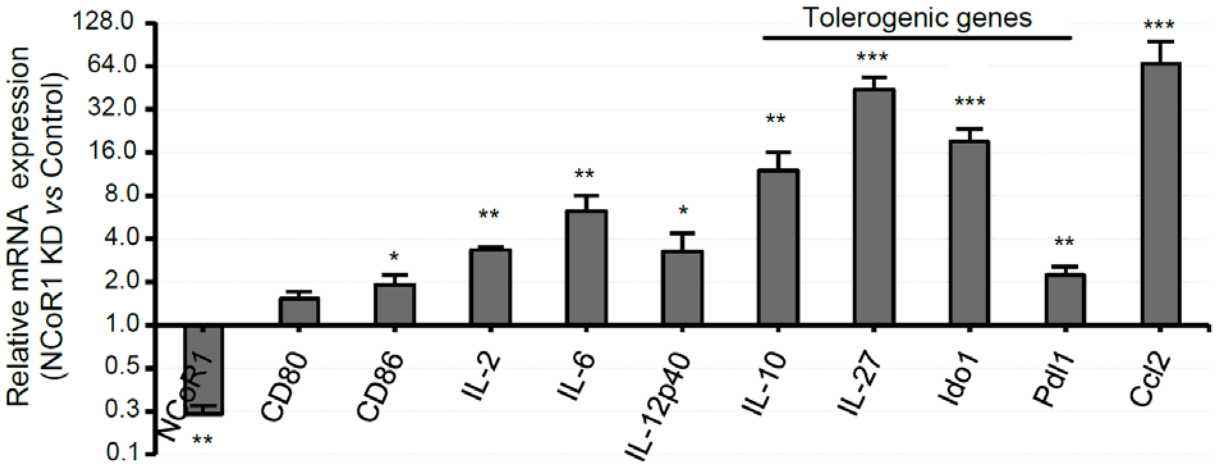


Figure 20 ***NCoR1 KD DCs show tolerogenic features***
Relative expression of selected genes from NCoR1 KD DCs to control empty vector DCs. Cells were stimulated with CpG for 6 hours before RNA extraction. Data kindly provided by the group of Sunil K. Raghav.

Moreover, their flow cytometric analysis of KD cells versus empty vector cells revealed that the costimulatory molecules CD80, CD86, CD40 and MHC-I and -II were more expressed in the KD DCs after CpG activation.

Conventional DCs from N1 mice present tolerogenic features

To validate these findings in vivo, DC-specific conditional KO mice (N1) for NCoR1 in C57BL/6J background were developed in our lab. They were obtained by crossing transgenic mice carrying a Cre-recombinase dependent on the minimal promoter of CD11c, which is almost exclusively expressed on DCs, with floxed NCoR1 mice.

T-cell repartition in N1 mice do not present evident changes

Genotyping PCR confirmed that NCoR1 ablation was nearly 100% in FACS-sorted $CD8^+$ and $CD11b^+$ DCs from these mice (Figure 21A). Basic characterisation of these mice, compared to WT mice, showed similar T-cell subpopulations (Figure 21B).

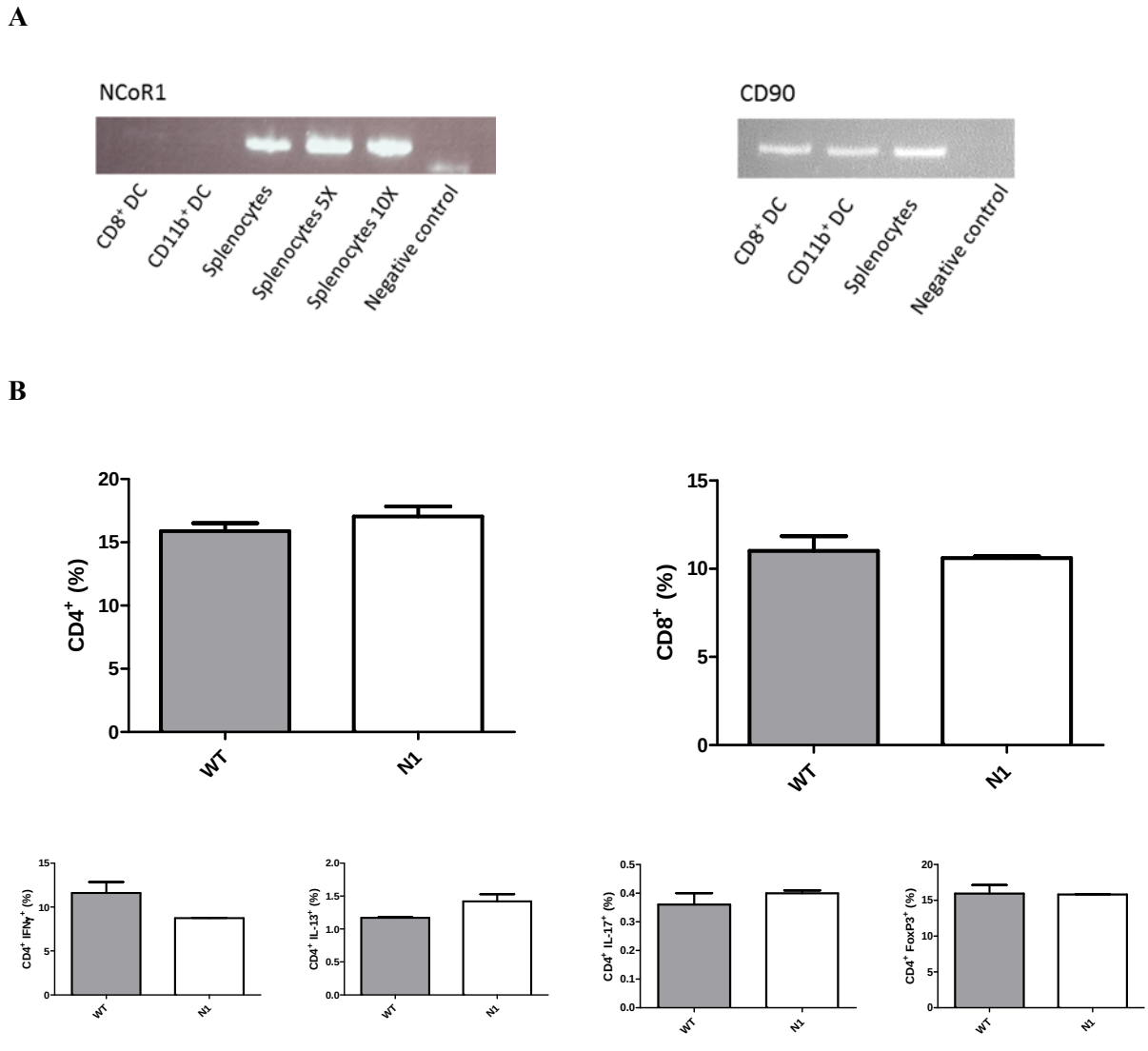


Figure 21 ***N1 mice basal T-cell immune system***
A) Agarose gel picture showing PCR product for the gene NCoR1 and the control gene CD90 in N1 mice. The PCR for was performed on DNA extracted from 4 x 10^5 $CD8^+$ or $CD11b^+$ FACS-sorted DCs or splenocytes isolated from N1 mice. Splenocytes 5X represents DNA from 2 x 10^6 and Splenocytes 10X from 4 x 10^6 splenocytes. Negative control represents PCR without template DNA. B) Percentages of $CD4^+$ and $CD8^+$ T-cell populations in total splenocytes at basal state in N1 versus WT mice. C) Percentages of $CD4^+$ T-cell subsets at basal state in N1 versus WT mice. Data are shown as mean ± SEM (n = 3).

DC characterisation of N1 mice

Interestingly, DC subset analysis indicated higher percentages of $CD8^+$ DCs and concomitant decreased percentages of $CD11b^+$ DCs in N1 mice (Figure 22). CpG stimulation did not affect these changes. Proportions of cDCs and pDCs were conserved with and without CpG.

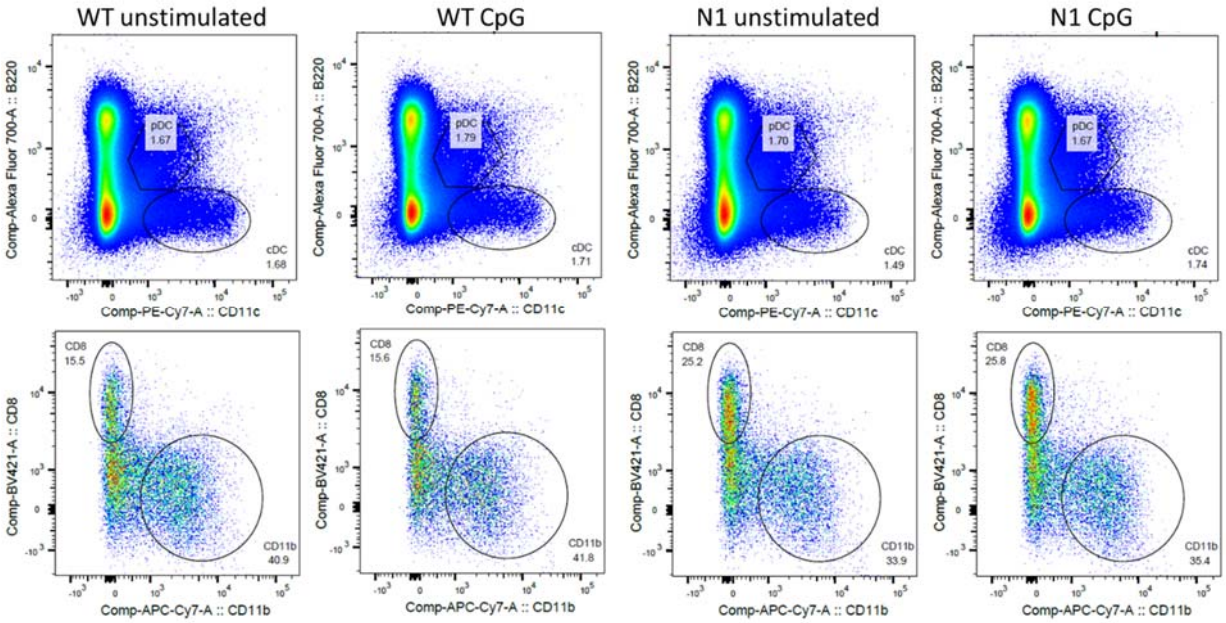


Figure 22 ***N1 mice have a higher proportion of $CD8^+$ DCs and smaller population of $CD11b^+$ DCs***
Dot plots showing the percentage of pDCs, cDCs and further gated $CD8^+$ and $CD11b^+$ DCs from N1 and WT mice. $CD8^+$ and $CD11^+$ DC population stem from the cDC population. Mice were either injected with 50 µg CpG or not injected.

As shown in Figure 23A, NCoR1 KO $CD8^+$ DCs express slightly less CD80 and more MHC class II molecules than WT $CD8^+$ DCs. They display similar expression of the costimulatory molecules CD86 and CD40. Parallel expression patterns could be observed on $CD11b^+$ DCs, although the differences were lower than in $CD8^+$ DCs (Figure 23B).

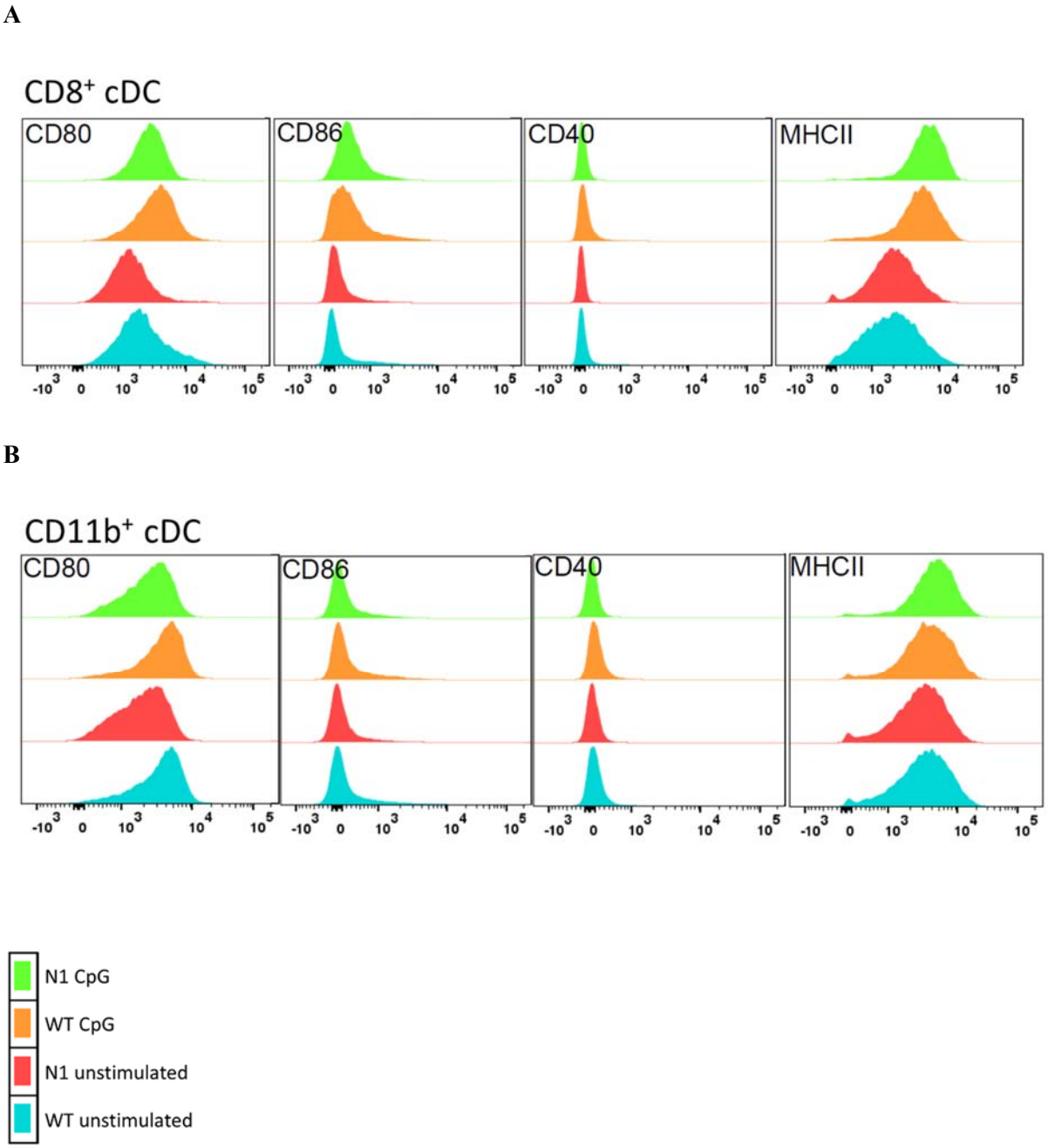


Figure 23 ***Costimulatory molecule expression is similar in N1 and WT DCs***
A+B) MFI from flow cytometric analysis of surface activation markers CD80, CD86, CD40 and MHC-II in $CD8^+$ DCs (A) and $CD11b^+$ DCs (B). DCs were gated from N1 versus WT mouse spleens activated in vitro for 6h with CpG or left unstimulated (n = 6 per group).

Conventional DCs from N1 mice present tolerogenic features

We next analysed expressions of tolerogenic factors by flow cytometry in N1 and WT mice. To obtain sufficient numbers of DCs, we treated the mice with serum from FLT3L transgenic mice for eight to ten consecutive days. Splenocytes were then isolated and stimulated with or without

CpG for 6 hours. Percentages of $CD8^+$ DCs were further increased after FLT3L treatment in N1 as well as in WT mice. However, absolute numbers of pDC $CD11c^{intermediate}$ $B220^+$), $CD8^+$ and $CD11b^+$ cDC ($CD11c^{high}$ $B220^-$) were both increased with FLT3L (figure not shown).

We found that in $CD8^+$ DCs, NCoR1 ablation significantly enhanced the expression of IDO1 and IL-27 after 6 hours of stimulation CpG whereas IL-10 and IL-6 showed a marginal but insignificant increase (Figure 24A). Similarly, we found that $CD11b^+$ DCs from N1 mice showed a significant increase in IDO1 and IL-27 expression after 6 hours of CpG stimulation. IL-10 and IL-6 exhibited an increasing trend yet without reaching significance (Figure 24B).

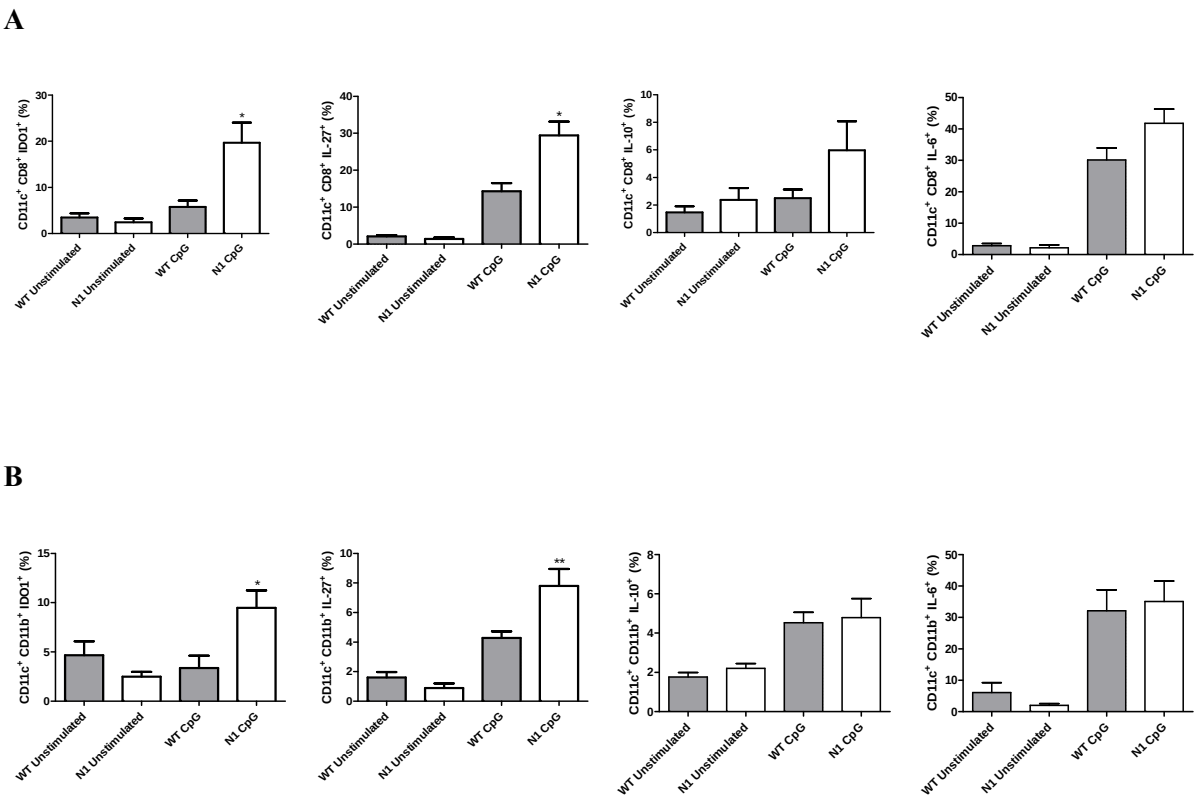


Figure 24 ***N1 DCs produce tolerogenic factors***
A+B) Percentage of positive cells for IDO1, IL-27, IL-10 or IL-6 in $CD8^+$ (A) or $CD11b^+$ (B) DCs from N1 versus WT mice. Data are shown as mean ± SEM (n = 6 mice/group) and are pooled from two independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests

These observations reflect what was found in NCoR1 KD cells, suggesting that NCoR1 depletion induces tolerogenicity in cDCs.

NCoR1 KD/KO DCs induce higher numbers of Tregs

In vitro: cocultures

To further test tolerogenicity in the KD $CD8^+$ DCs, we assessed Treg induction after coculturing KD DCs with naïve $CD4^+$ OVA-specific T-cells (OT-II cells). Cocultures were performed in the presence of the peptide with or without CpG. T-cell profiling through flow cytometry showed an increased $FoxP3^+$ T-cell population in the KD + CpG condition (
Figure 25A). Interestingly, T-cells cocultured with KD DCs proliferated more than the controls (
Figure 25C). Furthermore, the Th1 polarisation markers Tbet and IFNγ were significantly reduced (
Figure 25B), whereas Th2 polarization marker GATA3 showed an increase.

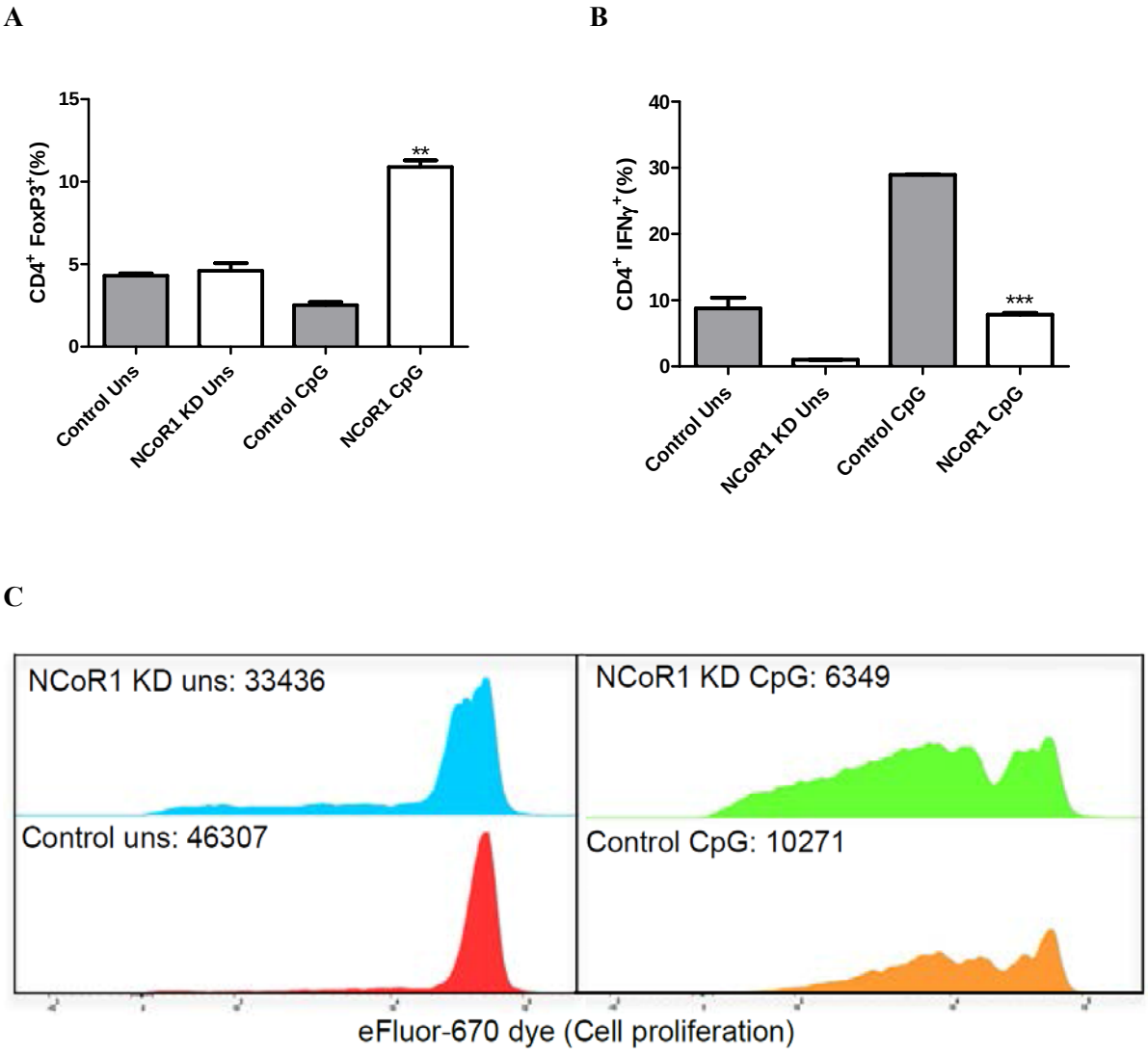


Figure 25 ***NCoR1 KD DCs induce a Treg differentiation***

A+B) Percentage of FoxP3+ (A) or IFNγ positive (B) cells in $CD4^+$ T-cells from cocultures with NCoR1 KD DCs or their controls. C) MFI plots showing T-cell proliferation represented by dilutions of the proliferation dye $eFluor^{670}$. A-C) OVA-specific $CD4^+$ T-cells were isolated from OT-II mice and cultured with DCs and OT-II peptide ($OVA_{323-339}$) in the presence or absence of CpG. After 4 days, IFNγ presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h whereas FoxP3 did not need restimulation before intracellular staining. Data are shown as mean ± SEM (n = 3 mice/group) and are representative for two independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

In vivo: vaccination

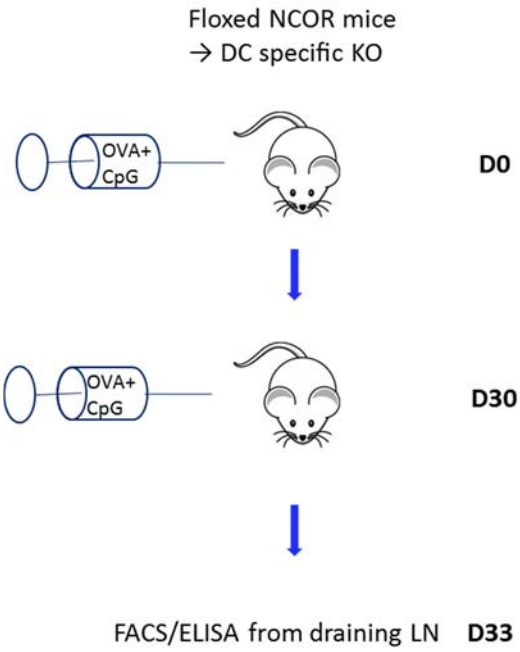


Figure 26 ***Outline of OVA immunisation***
N1 or WT mice were vaccinated subcutaneously at day 0 and at day 30 with ovalbumin in CpG and PBS. At day 33, draining lymph nodes (inguinal lymph nodes) were harvested for flow cytometric analysis.

To access the NCoR1 KO in DCs in vivo on T-cell differentiation, we vaccinated N1 and WT mice with OVA and CpG at day 0 followed by a booster immunisation at day 30 as in Figure 26.

Three days after the booster injection, draining lymph nodes were harvested and T-cells were profiled. Effector T helper lymphocytes were gated as $CD4^+CD44^+$. We found that N1 mice had significantly higher percentages of $FoxP3^+$ cells than WT mice (Figure 27). This further supports the hypothesis that NCoR1 KO/KD DCs are tolerogenic and capable to induce more Tregs. Other T-cell populations did not show significant difference between N1 and WT mice.

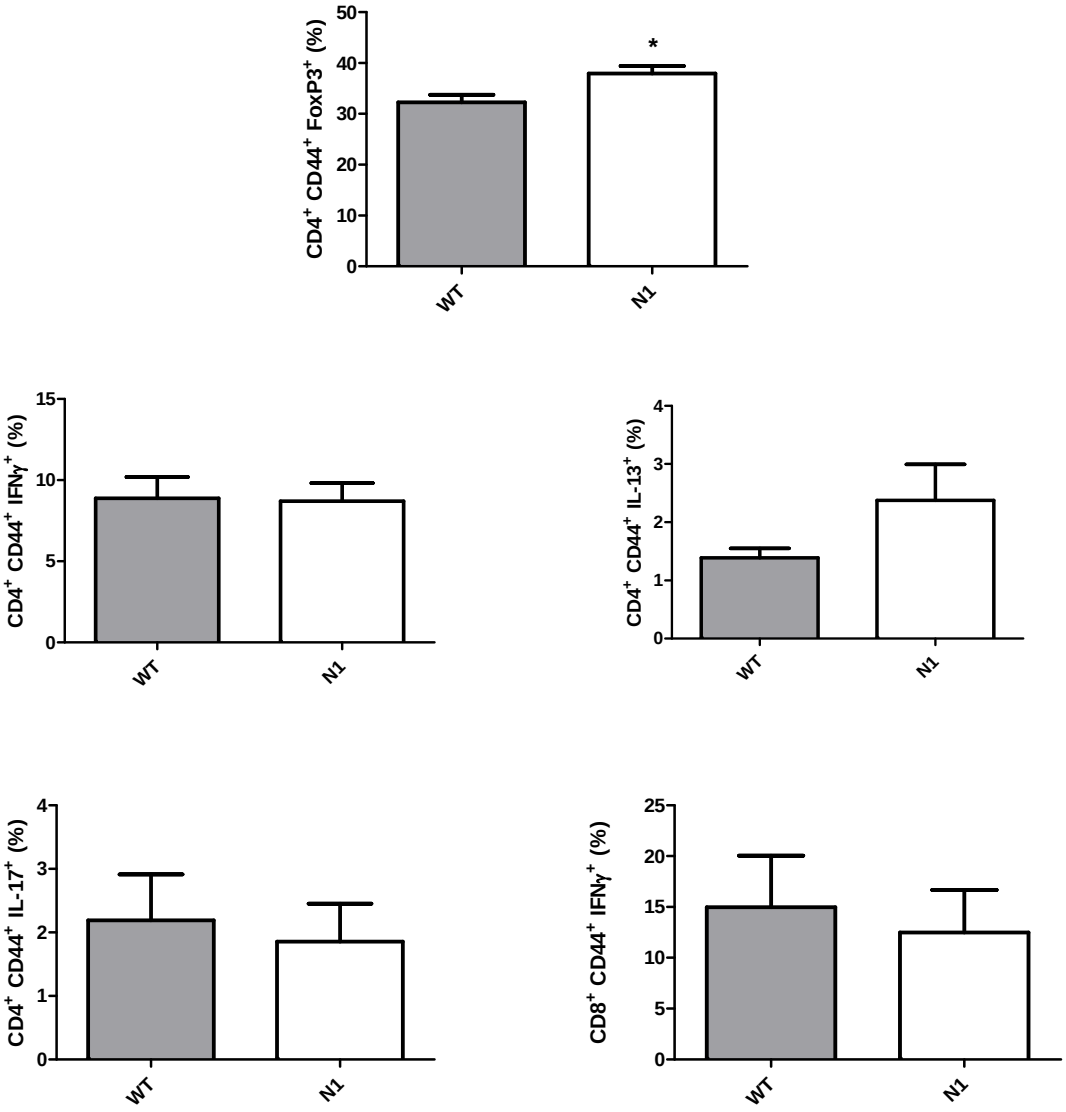


Figure 27 ***N1 mice have increased numbers of Treg after OVA immunisation in CpG***
Percentage of FoxP3, IFNγ, IL-13 or IL-17 positive cells in $CD4^+$ $CD44^+$ T-cells and IFNγ positive cells in $CD8^+$ $CD44^+$ T-cells. Cells were isolated from immunised mice, three days after the booster injection. Cytokine presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h whereas FoxP3 did not need restimulation before intracellular staining. Data are shown as mean ± SEM (n = 15 mice/group) and are pooled from 5 independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

A second experiment was carried out to demonstrate DC capacity to induce Treg. OT-II cells were adoptively transferred in N1 or WT mice at day 0. Mice were then vaccinated at day 1 and inguinal lymph nodes harvested for the same analysis as in the OVA immunisation experiment. Tregs could unfortunately not be induced properly with our experimental setup.

Infection models in N1 mice

A tolerogenic response can have important repercussions in infections. That is why our collaborative group (group Sunil K. Raghav) challenged $CD8^+$ NCoR1 KD DCs with different bacteria to evaluate the DC response to microorganisms instead of a simple stimulus such as CpG. They found that *mycobacterium smegmatis*, a commonly used model to study *m. tuberculosis*, induced an increased secretion of IL-10 in KD DCs.

Mycobacterium smegmatis induce more Tregs in N1 mice

To understand implications of these results in vivo, we infected N1 and WT mice with *m. smegmatis* in the right footpad and then assessed the T-cell response in the draining lymph nodes. Mice were injected at day 0 and day 14. Three days after the second injection, we found an increased $FoxP3^+$ effector T-cell population in the popliteal lymph nodes of N1 mice (Figure 28). Interestingly, higher numbers of $IL\text{-}13^+$ helper T cells were also identified in infected N1 mice.

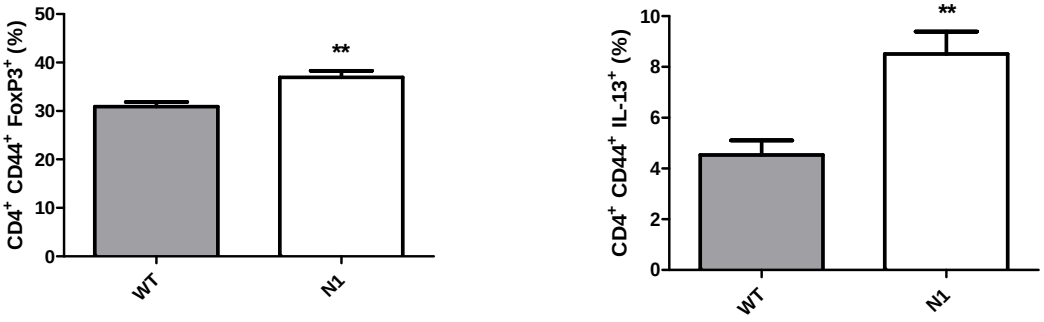


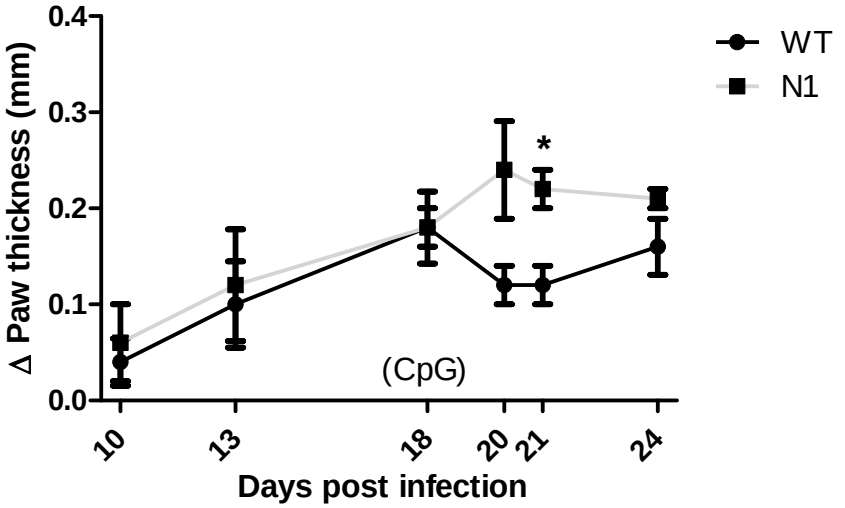
Figure 28 ***Increased Treg and Th2 differentiation in N1 mice after m.smegmatis infection***
Percentage of FoxP3 or IL-13 positive cells in $CD4^+$ $CD44^+$ T-cells. Cells were isolated from m. smegmatis infected mice three days after the second infection. IL-13 presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h. Data are shown as mean ± SEM (n = 8 mice/group) and are pooled from 2 independent experiments. *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

We did not find any significant difference in the IFNγ, IL-17 and IL-10 expressing effector T-cells. Bacterial load was assessed by smearing lysates from the infected footpads but N1 and WT mice had similar loads of bacteria.

Leishmania major causes increased lesions in N1 mice

We next developed a *leishmania major* (*L. major)* parasitic infection model in our mice. After subcutaneous injections of parasites in the right footpad of N1 and WT mice, lesions were monitored before and after the CpG challenge by measuring the difference in thickness between the infected and healthy paw. As cross-presenting $CD8^+$ DCs are essential to develop immune protection against *L. major* between 17-19 days (110), mice were administered with CpG IP at day 18 post-infection. Three days after CpG injection (day 21), we observed a significant increase in lesion size of N1 mice compared to WT mice (Figure 29A).

A



B

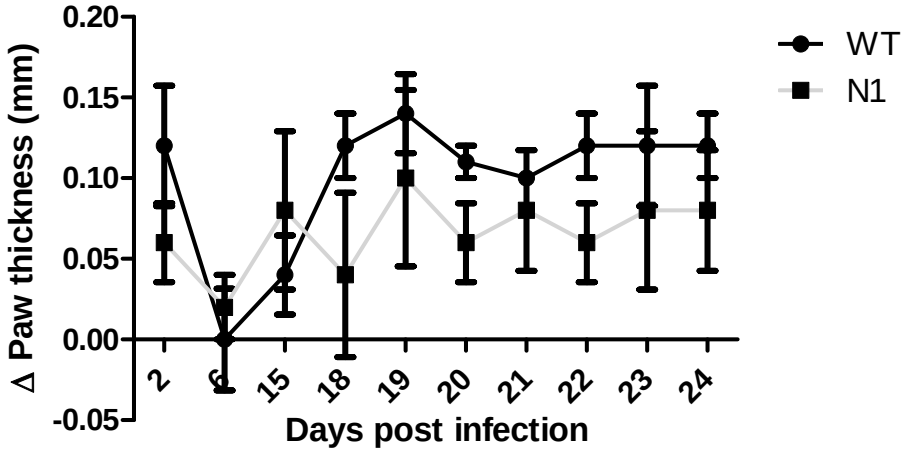


Figure 29 **Leishmania major *transiently induce increased lesions in N1 mice***
Paw thickness difference between infected and healthy paw representing leishmanial lesion caused by its infection. A) 50 µg CpG was injected intraperitoneally in each mouse at day 18. Mice were monitored until day 14. B) Mice were not treated with CpG. Data are shown as mean ± SEM (n = 5 mice/group). *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

The difference disappeared a few days after the CpG injection. CpG-untreated N1 mice did not show any significant increase (Figure 29B). Figure 30 displays the flow cytometric profiling of T-cells from the draining lymph nodes of infected mice at day 24 post infection. As expected, CpG-treated N1 mice developed more FoxP3^{+} T-cells than their WT counterparts. Nevertheless, this was not the case for untreated mice. Surprisingly, CpG-treated N1 mice also

demonstrated higher percentages of IFNγ$^+$ T-cells and fewer IL-13$^+$ T-cells, contrary to what was found in the *m. smegmatis* model. Absolute numbers of lymph node cells were comparable or slightly higher in N1, which confirms the increased numbers of Tregs and Th1.

Finally, parasite load was estimated by qPCR in infected footpads. Unfortunately, due to very scant parasitic RNA, obtained results which indicated similar numbers between CpG-treated N1 and WT mice, could not be properly validated. A limiting dilution assay was performed to assess parasite load in footpads of CpG-untreated mice. No significant difference was observed.

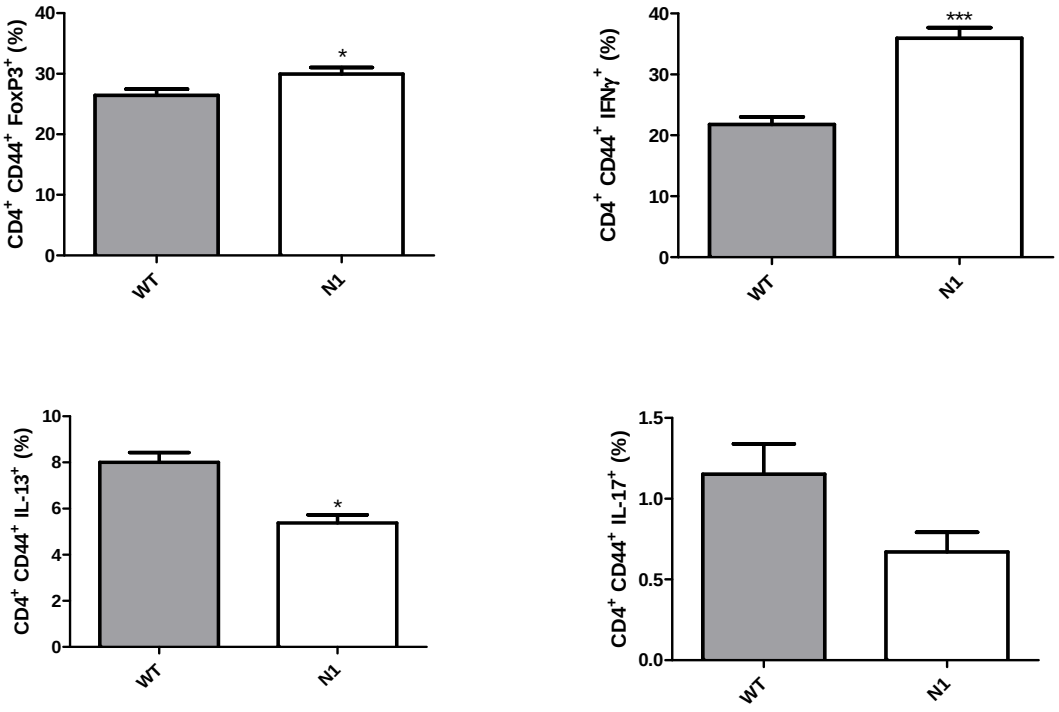


Figure 30 ***T-cell response in draining lymph nodes of leishmania infected mice***
Percentage of FoxP3, IFNγ, IL-13 or IL-17 positive cells in CD4$^+$ CD44$^+$ T-cells. Cells were isolated from the draining lymph node of leishmanial-infected mice at day 24 after infection. Cytokine presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h whereas FoxP3 did not need restimulation before intracellular staining. Data are shown as mean ± SEM (n = 5 mice/group). *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

Heligmosomoides polygyrus produces increased number of eggs in N1 mice

In order to better understand and characterise the immune response induced by NCoR1 KO DCs, we tested a last infection in our mice. Our collaborating group had tested the effect of NCoR1 KD DC transfers in WT mice after infecting them with helminths (*Heligmosomoides polygyrus)*. Interestingly, they had found that the worm burden strongly increases after DC transfer. Therefore, we used the same parasite to test it in our N1 mice. *Heligmosomoides polygyrus,* triggers a type 2 immune response (Th2-mediated immune response). Consequently, this analysis further completed immune characterisation of N1 mice. N1 and WT mice were infected orally with 200 larvae per mouse. In order to induce a possible difference as in our *l. major* model, mice were injected IP with CpG at day 11 post infection. Infection burden was assessed by counting faecal eggs of each mouse, starting at day 10.

We counted higher numbers of helminth eggs at day 17 in the faeces of N1 mice (Figure 31A). The intestinal worm counts showed an insignificant but increasing trend in these mice as well (Figure 31B).

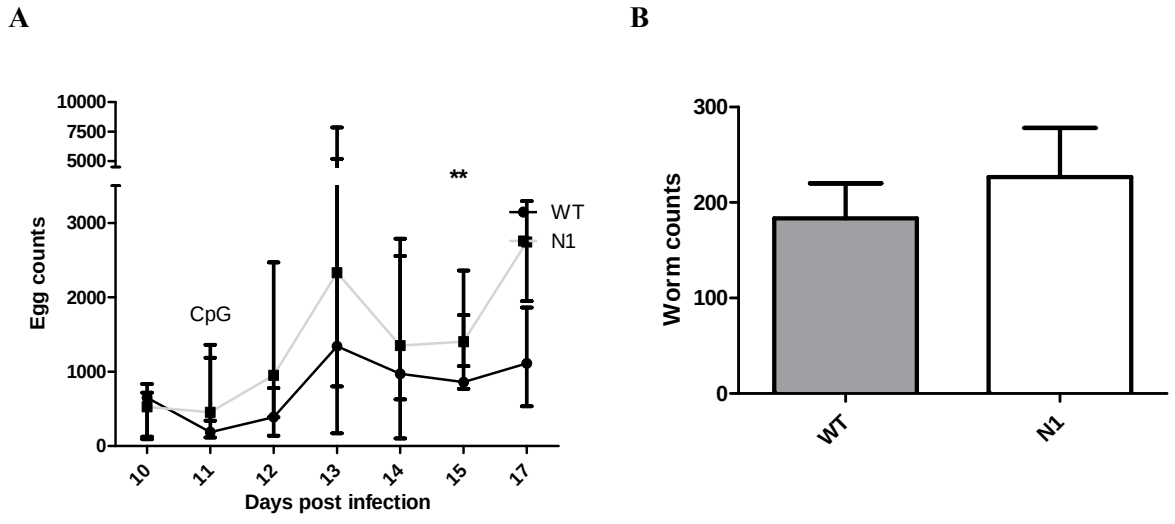


Figure 31 ***Increased helminth infection in N1 mice***
A) Egg counts in N1 versus WT mice. Eggs were counted in faeces each day starting at day 10 after infection. CpG was injected at day 11 in each mouse. B) Worm counts in small intestines of WT and N1 mice. Intestines were left in PFA 8% for two days before counting worms under a stereomicroscope. Data are shown as mean ± SEM (n = 5 mice/group). *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

In addition, the effector T-cell profiling from mesenteric lymph nodes of infected animals also showed an increased FoxP3$^+$ population in N1 mice (Figure 32).

IL-13$^+$ T-cells were found to be decreased in N1 mice. Analysis of IFNγ^+ and IL-17$^+$ T-cell populations did not show a significant difference.

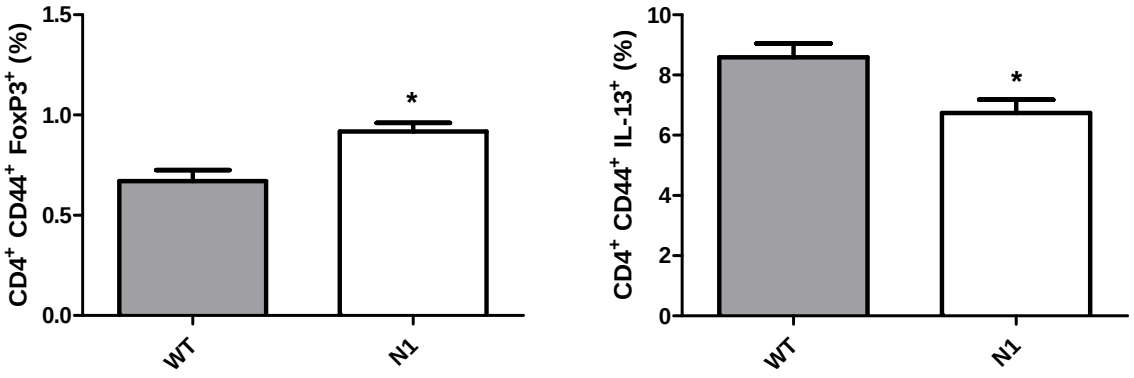


Figure 32 ***T-cell response in helminth infected mice***
Percentage of FoxP3 or IL-13 positive cells in CD4$^+$ CD44$^+$ T-cells. Cells were isolated from mesenteric lymph nodes of helminth-infected mice at day 17 after infection. IL-13 presence in T-cells was determined by stimulation with PMA/ionomycin in the presence of brefeldin A for 5h. Data are shown as mean ± SEM (n = 5 mice/group). *p<0.05; **p < 0.01; ***p < 0.001; Mann-Whitney U tests.

N1 mice in a model of murine rectal cancer and EAE

Tolerogenicity is of great importance not only in infections but also in autoimmune diseases and cancer. Indeed, autoimmune processes are blocked by tolerogenic cells and molecules whereas cancer thrives when the immune response is dampened. Therefore, we tested a cancer and an autoimmune disease model in our N1 versus WT mice.

N1 mice do not develop bigger CMT93 tumours

To test cancer development in our mice, we used cancer cells from mouse rectum carcinoma (CMT-93 cells) and injected them in each mouse right flank. They were then monitored every 3 to 4 days to assess tumour growth. At day 30, mice were sacrificed and the T-cell profiled. Although we expected higher tumour volumes in N1 mice, they were similar in both mouse lines (N1 and WT) and no significant increase in the Treg population was noted. The experiment was repeated and CpG was injected every 4 days IP in order to induce IL-10 secretion and possibly a tolerogenic phenotype. However, results did not differ from the first experiment (Figure 33).

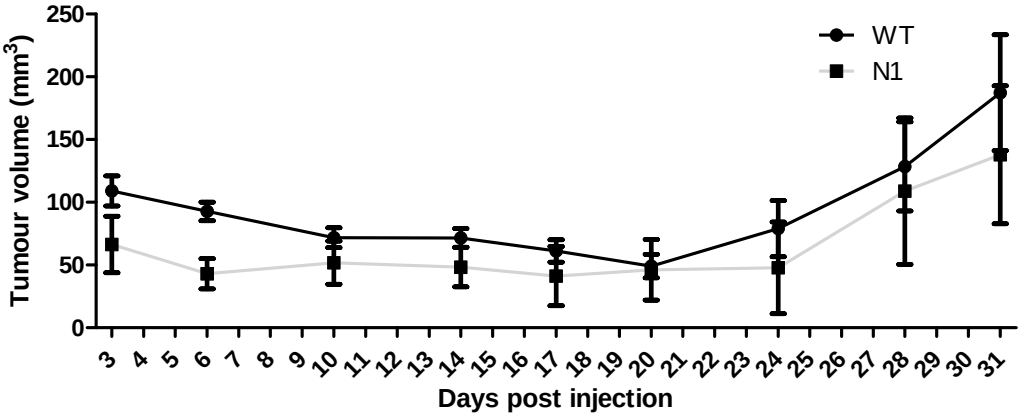


Figure 33 ***No significant difference in tumour size between N1 and WT mice***
Tumour volume measured on mice injected subcutaneously with CMT-93 tumour cells. Mice were injected with CpG every 4 days intraperitoneally. Data are shown as mean ± SEM (n = 5 mice/group) and are representative for 2 experiments.

Experimental autoimmune encephalomyelitis (EAE) scores are similar in N1 and WT mice

Two EAE protocols were tested in order to assess an autoimmune model in our mice. We first performed a passive EAE with immunisation of MOG in CFA and pertussis toxin injections in N1 and WT mice. Draining lymph nodes were then harvested and restimulated in vitro for three days. Finally, cells were reinjected into WT mice which were monitored and scored for four weeks. All mice developed severe EAE, without difference between the two groups.

The second experiment consisted of active EAEs and comprised only the first step with MOG immunisation and pertussis toxin injections. Animals were observed until development of EAE. They were then scored as for the passive EAE. Unfortunately, disease was induced in only 50% of mice and no difference could be noticed between N1 and WT mice. Figure 34 shows the percentage of weight loss due to the disease at different time points.

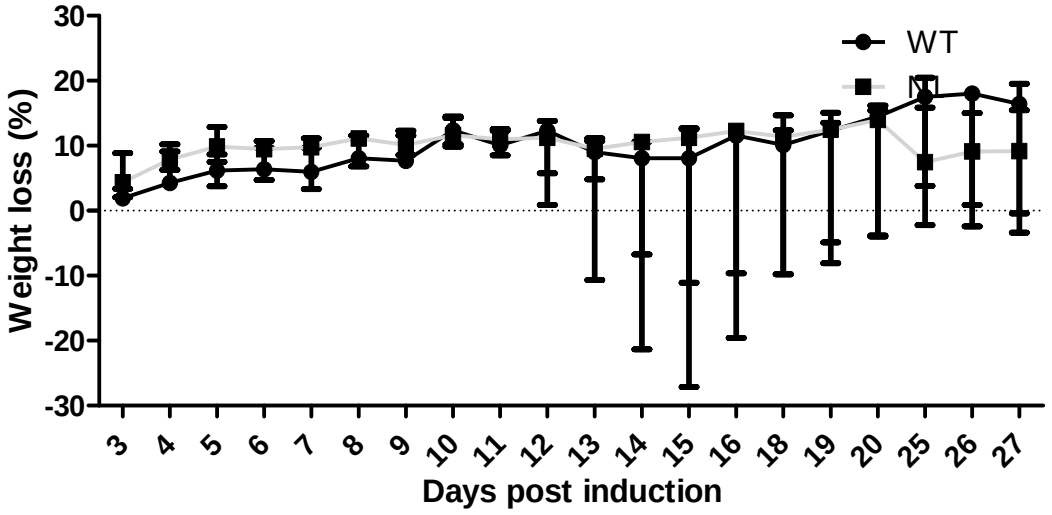


Figure 34 ***EAE does not differ in N1 from WT mice***
Weight loss representing EAE severity in N1 and WT mice after induction of active EAE. Data are shown as mean ± SEM (n = 5 mice/group) and are representative for two experiments.

Discussion

The role of CD11b in CIA

Immune balance is critical to prevent autoimmune diseases and infections. That is why many factors are implicated. Some even have dual roles. CD11b is one of them. While it is classically known to be pro-inflammatory (73, 74), our work demonstrated that its presence is crucial to prevent development of CIA in C57BL/6 mice.

CD11b renders C57BL/6 mice susceptible to CIA

It is common knowledge that H-2^b mice do not develop CIA (64) although some authors reported a reduced but present susceptibility to CIA in C57BL/6 mice (111). In our study, all WT mice remained resistant to the disease although increasing mycobacterial load and collagen concentrations were tested as well. Probably, increasing number of collagen II immunisations could break C57BL/6 tolerance and allow arthritis development but this adapted protocol is not permitted according to the Swiss Federal and Cantonal Veterinary authorities. On the contrary, CIA could be induced in most of $CD11b^{-/-}$ mice, meaning that CD11b KO confers CIA susceptibility to H-2^b mice. Furthermore, clinical scores and histology indicates severe arthritis in immunised $CD11b^{-/-}$ mice. As a consequence, CD11b is probably a potent immune checkpoint to impede autoimmune processes.

CIA is mediated by Th17 in immunised CD11b$^{-/-}$ mice

Th17 are associated with different autoimmune diseases, including RA (55, 57). The hallmark of these cells is IL-17 secretion, which has many deleterious roles in RA. Indeed, it promotes synovial inflammation via synoviocyte, fibroblast and endothelial cell activation. It upregulates CCL20 which is important for T-cell chemotaxis. Furthermore, it acts synergistically with TNFα to activate the proteins C3 and factor H of the alternative complement pathway, known to be upregulated in RA. It promotes cartilage destruction through different mechanisms. Firstly, by recruiting inflammatory cells to the affected joints, and secondly through chondrocyte dysregulation and proteoglycan loss. In addition, their positive effect on osteoclastogenesis provokes bone resorption (112, 113). Prevention of CIA development in case of IL-17 deficiency in mice demonstrates IL-17s crucial role in arthritis.

The Th17 increase found in immunised CD11b$^{-/-}$ mice correlates with the key role of IL-17 in CIA physiopathology reported by previous research (114, 115), as mentioned above. Murphy et al. showed that C57BL/6 resistant mice deficient for IL-12 become susceptible to CIA and hold higher amounts of IL-17 producing T-cells (116). This further points out the pathogenic role of Th17 rather than the role of Th1.

Th17 increase was accompanied by a Treg concomitant decrease, indicating that CD11b promotes Treg and inhibits Th17 differentiation. This imbalance between Th17 and Treg is even more interesting since Treg and Th17 share many features although they have opposite functions. These T-cells can switch from one differentiation to another and they even share transcription factors (23, 117, 118). They are both induced by TGFβ. The differentiating factor between these two subsets is the IL-6, as mentioned in the introduction.

IL-6 a key mediator for CIA

IL-6 is crucial in our model. Its increase after collagen II immunisation in CD11b$^{-/-}$ mice explain the increase in arthritogenic Th17 cells and its blockade significantly decreased CIA incidence and severity in immunised CD11b$^{-/-}$ mice. Our results mirror IL-6 implication in RA, in which IL-6 concentration is usually elevated. The monoclonal antibody anti-IL-6R (tolicizumab) used to treat RA is an undeniable proof that IL-6 has a central role in RA pathology. This cytokine is important for B-cell differentiation into plasma cells in addition to act as a pro-survival factor for plasmablasts. It is also involved in Tfh differentiation, as mentioned in the introduction. These two effects enhance the B-cell response and promote auto-antibody generation like ACPA. IL-6, as IL-17, upregulates RANKL and favours osteoclastogenesis (119). It is a central molecule to promote inflammation, including in the joint where it can promote angiogenesis through VEGF, induce vascular leakage and recruit inflammatory cells via activation of endothelial cells (120). Finally, its most relevant role in our project is the induction of Th17 at the expense of Tregs.

As IL-6 is critical for Th17 induction (102), blocking IL-6 pathway through anti-IL-6R should be done in particular during the first immunisation and its inhibition at time of restimulation, as in our experiment, should not influence development of the disease. Indeed, at that time point, the Treg/Th17 balance should already be established. However, it seems that IL-6 signalling is needed even at time of restimulation and after. Ex vivo analysis of the draining lymph nodes from immunised mice show that IL-6 probably acts as a pro-survival factor for Th17 cells and induces Treg population contraction.

Nonetheless, it should be mentioned that there are other factors that promote Th17 in an IL-6-independent mechanism. Prostaglandins, in particular PGE2, increase the Th17-specific transcription factor RORγT and decrease Th1 transcription factor Tbet (121). It upregulates IL-

23 receptor and PGI2 induce IL-17-producing γδT-cells (122, 123). Although our analysis focused on IL-6, prostaglandins remain good candidates for Th17 induction and possibly CIA development. Next experiments should take these molecules into consideration.

IL-6 signalling

IL-6 signalling pathway can be triggered by two forms of the same receptor: membrane IL-6 receptor (IL-6R) on target cells (IL-6 cis signalling) or soluble IL-6R in the serum (IL-6 trans signalling) (68). Because of this soluble receptor, CIA could also have been induced because of increase of sIL-6R in immunised $CD11b^{-/-}$ mice. Although immunised mice produced significantly higher amounts of sIL-6R, no difference was observed between WT and $CD11b^{-/-}$ mice, which refutes this hypothesis. sIL-6R may be needed but is not sufficient to induce arthritis in immunised mice. IL-6 increase is essential to promote Th17 differentiation and develop CIA.

DC and macrophages are sources for IL-6 and potentially responsible for arthritis development

As said, IL-6 is part of the effector phase of autoimmune arthritis. Clinical trials have shown that blocking IL-6 pathway with an anti–IL-6R antibody (tocilizumab) treatment is very efficient in established RA in human patients (124, 125). Nevertheless, it remains unclear how the production of IL-6 is controlled at molecular and cellular levels although it is known that many different cells can secrete IL-6. These cells could, consequently, all be responsible for arthritis. Our coculture results give a first hint that $CD4^{+}$ $CD11b^{-/-}$ DCs are good candidates for

disease induction. They were able to reproduce in vitro the Th17/Treg imbalance towards Th17 with concomitant decrease of Tregs. In addition, IL-6 quantification in ex vivo DC and macrophage supernatants show that not only DCs but also macrophages could be responsible for IL-6 increased secretion. Nevertheless, working with ex vivo DCs is very challenging. They are easily activated and do not last long in vitro. Our newly generated CD11b KO DC line takes much relevance in this context. First characterisation confirmed increased IL-6 secretion in CD11b KO cells compared to their $CD11b^{+}$ controls. In addition, cocultures of naïve $CD4^{+}$ T-cells also presented increased Th17 and decreased Treg differentiation in CD11b KO conditions.

To evaluate whether $CD11b^{-/-}$ DCs were indeed responsible and sufficient to induce CIA, we decided to perform DC adoptive transfers. As large numbers of ex vivo DCs are very fragile, survive very little time and necessitate very high numbers of mice, we chose to inject only CD11b KO DCs from our DC line. CIA was not induced after DC injections in WT mice possibly because DCs are not sufficient for disease development. However, this pilot experiment needs further adjustments and to be replicated to exclude this hypothesis. Firstly, a depleting antibody against $CD8^{+}$ T-cells could be used in order to delay DC rejection. It would prolong time for antigen presentation. Secondly, numbers of DCs could be increased and the injection site changed. Finally, cells were not stimulated before injection which could impede immune response. Indeed, as mentioned in the introduction, DCs need to mature via PRR activation in order to mount an immune response. This is why the experiment should be repeated with DC stimulation prior to adoptive transfer.

Unfortunately, due to lack of time, these experiments could not be performed until now.

CD11b inhibition of IL-6

Besides its key role in cell migration and adhesion, increasing evidence supports the notion that CD11b is also involved in the suppression of proinflammatory cytokines and in tolerance maintenance. Behrens et al. have shown that following CD11b ligation, DCs are less immunogenic (109). In addition, it has been reported that CD11b deficiency resulted in increased inflammatory cytokine production by DCs both in vitro and in vivo upon TLR activation. Han et al. have demonstrated that after TLR stimulation, CD11b is activated and promotes Cbl-b-mediated degradation of MyD88 and TRIF through recruitment of src and syk in macrophages. This results in inhibition of TLR-mediated responses which leads to the decrease of TNFα and IL-6 production (80). The same group further demonstrated that CD11b deficiency severely increased DSS-induced colitis by promoting IL-10 production and inhibiting TNFα secretion by bone marrow-derived DCs and peritoneal macrophages (81). The implicated pathway involves activation of src-Akt. In our case a src inhibitor should block src-mediated inhibition of IL-6 in $CD11b^+$ DC so that IL-6 production in these cells reach IL-6 production in CD11b KO DCs. Because of the many available src inhibitors, we chose to use the same src inhibitor as Han et al. Unfortunately, we could not find IL-6 increases after treating cells with a src inhibitor, neither in freshly isolated ex vivo macrophages and DCs nor in our DC lines. Possibly, the protocol for PP1 treatment could differ from Han et al. Different concentrations of inhibitors should be tested as a highly diluted concentration would not affect the cells and highly concentrated, the product could be toxic. Because of great src variability, another inhibitor should also be tested before excluding this pathway.

CIA is also a humoral disease

In addition to pathogenic T-cell responses, patients suffering from RA produce auto-antibodies such as the rheumatoid factor and the anti–citrullinated protein antibody (ACPA). These can form immune complexes and trigger the complement cascade that further increases inflammation (57). Our group found that arthritic CD11b$^{-/-}$ mice had significantly higher levels of seric collagen-specific IgG1 than WT mice. Whereas development of CIA can be caused by collagen-specific IgG2a (126), others suggest that other isotypes might be of importance in the induction of arthritis. In support to our findings, Maccioni et al showed that IgG1 was the dominant isotype in the K/BxN mouse model (127). In accordance with the role of Th17 induced IgG1 class switch, Hickman-Brecks et al. have reported that they are able to induce IgG1 dominant responses in the Th17 polarized KRN7T-bet$^{-/-}$ adoptive transfer model of arthritis (128).

Analysis of Tfh cells would help further characterisation of the humoral response. Interestingly, a new CD4^{+} T-cell, Tph, was discovered in RA patients which appears to be important for pathogenic activation of autoreactive B-cells in inflamed non-lymphoid sites. Global transcriptomics differentiate this subset from Tfh. They also express unique chemokine receptors to promote migration to the inflamed sites (129). For future experiments, Tph should be sought and analysed, especially in joints but also in draining lymph nodes.

CD11b^{+} DCs treatment

Previous studies have shown an efficient treatment of established murine CIA by adoptive transfer of in vitro modified DCs (106, 108, 130). In agreement with these observations, our results demonstrated that our newly developed CD11b^{+} DC line is able to inhibit the

development of arthritis. However, further studies are required to better understand how these $CD11b^+$ DCs promote tolerance after the first immunisation in $CD11b^{-/-}$ mice.

CD11b a key molecule to prevent autoimmune diseases

Altogether, the results point toward a role of CD11b to prevent autoimmunity. As previously mentioned, CD11b alteration or deficiency has been shown to promote SLE and other inflammatory diseases (75, 131). Autoimmune diseases represent an important health issue in developed countries and optimal treatments remain difficult to find. It is crucial to find new targets for therapies. In this context, CD11b seems to be a promising target. Adoptive transfer of tolerogenic $CD11b^+$ cells could be considered as it is performed with Treg cells (132). Gene therapy with CD11b induction is another possible alternative.

Outlook

Further understanding of the mechanisms underlying the link between CD11b and tolerance can be obtained thanks to our newly generated CD11b KO DC line. Future experiments should comprise a detailed analysis of the link between CD11b and IL-6 inhibition in DCs. This will open many new possible targets to dampen or augment immune response. Another angle could be explored by assessing whether CD11b also have an influence on prostaglandins as they are Th17 inducers as well. More work should also be done to confirm whether DCs are sufficient to induce CIA, or whether other cells are needed or responsible for arthritis development.

To conclude, this project identified a new connection between CD11b, IL-6, and arthritis (Figure 35). The results extend the crucial role of CD11b in the regulation of the immune system

from previous studies. The finding that resistant H-2^b mice become susceptible to CIA after CD11b loss opens the possibility for a parallel role in human autoimmune susceptible patients. Therefore, therapeutic strategies targeting CD11b and its anti-inflammatory functions could potentially be novel therapies to treat patients suffering from autoimmune diseases.

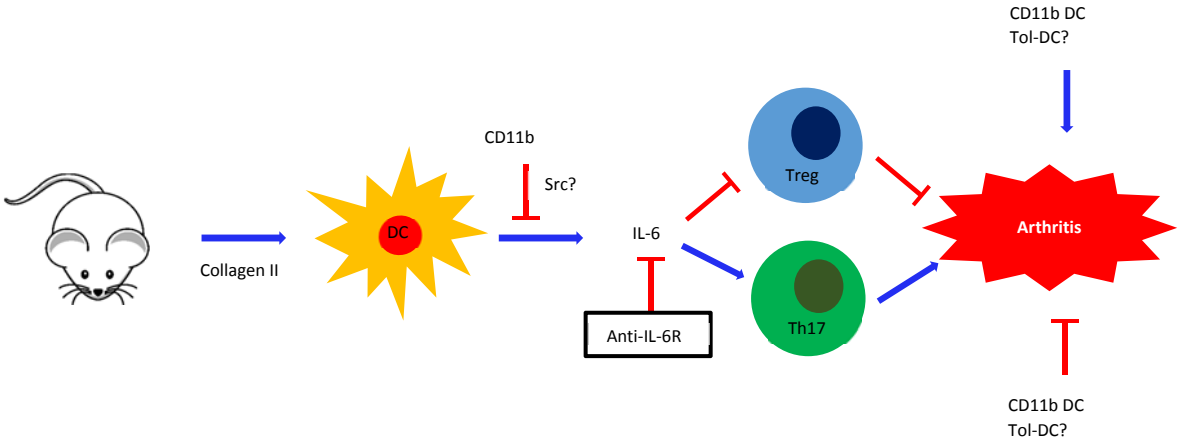


Figure 35 ***Diagram of the first project***
In case of CD11b deficiency, collagen II immunisation activates DCs which in turn produce IL-6. IL-6 shift the balance towards Th17 differentiation. This induces arthritis development. Blocking IL-6 pathway with as anti-IL-6R antibody blocks this shift and prevents arthritis. Adoptive transfer of $CD11b^+$ DC also dampens arthritis.

The role of NCoR1 in dendritic cell activation

Our CD11b project demonstrated the importance of tolerance and being able to induce it in the case of autoimmune processes. Adoptive transfer of $CD11b^+$ DCs showed that DCs can induce tolerance and reduce disease score. Our NCoR1 project investigated the tolerogenicity induced by knocking down the co-repressor NCoR1 in dendritic cells.

NCoR1 regulate the DC tolerogenic program

Our results reveal that NCoR1 acts as a master repressor of the tolerogenic program in DCs. Its KD renders $CD8\alpha^+$ DCs tolerogenic upon activation by TLR ligands or microbes. Preliminary results from our collaborating group showed upregulation in NCoR1 KD DCs.

The upregulated genes found in the NCoR1 KD DC line after CpG stimulation IL-10, PDL1, IL-27, CTLA4 and IDO1 are reported to induce strong immune suppressive responses (85, 133-135). Although upregulation was less substantial in freshly isolated KO DCs, a parallel increase in expression was detected for IL-10, IL-27 and IDO1. These molecules all promote, according to the context, tolerance (see introduction). They are linked together through different pathways. For instance, CTLA4, which inhibits T-cells, can induce IL-10 and IDO1 genes upon ligation with B7 in DCs (136-138). IL-10 in turn can induce many genes, like PDL1, through STAT3 signalling (139). Interestingly, STAT3 and STAT1 are activated by IL-27 and IL-12 and induce expression of IDO1, PDL1 and IL-10 (140). The STAT pathway was consequently checked in KD DCs by the S. Raghav group and both STATs were found to be activated in NCoR1 KD DCs. This should shift the immune balance towards an increase of Tregs and concomitant decrease of Th17 (141). Furthermore, studies suggest that STAT3 is also

phosphorylated via IL-6 and IL-10 signalling in immune cells (142), however, it appears unlikely that increased IL-6 is responsible for this STAT3 activation in NCoR1 KD cells. Indeed, the S. Raghav group reported that *IL-6rα* expression was found to be significantly decreased after CpG stimulation with a concomitant increase in *Socs3* (143). Nevertheless, IL-6 was also found to be upregulated. Although most functions of this cytokine are pro-inflammatory, some claim that it displays tolerogenic features as well (70, 144). For instance, Yan and al. showed that IL-6 is needed for B-cell editing. They found that when IL-6 signalling is blocked autoantibody titers increase (145).

Type-I IFN signalling is also suggested to stimulate IL-10 and suppress IL-12 and IL-23 cytokines in DCs (146). Interestingly, *Ifnβ1* and its signalling intermediate *Stat2* were found to be significantly increased in CpG stimulated NCoR1 KD DCs by the S. Raghav group. IFNβ1 signalling also activates IRF3/IRF7 TFs that represses IL-12 by binding to its proximal promoter (147).

Taken together, these points clearly indicate that NCoR1 KD cells have tolerogenic potential. Characterisation of NCoR1 KO DCs freshly isolated from N1 mice supports this assumption.

Moreover, RNA seq and ChIP seq analysis performed by the group of S. Raghav indicate that NCoR1 directly represses these tolerogenic genes. Once NCoR1 is knocked down these genes can consequently be expressed.

NCoR1 blockade in DCs induce Treg differentiation

Some surface markers and secreted factors that showed slight increase after NCoR1 KD are often considered inflammatory. Nevertheless, costimulation and antigen-presentation by MHC class II is required for Treg generation (148). Therefore, an increase of costimulatory molecules can contribute to Treg differentiation. As Tregs express CD25 (IL-2Rα), increased IL-2 secreted by NCoR1 KD DCs could induce Treg proliferation and clonal expansion and further contribute to tolerogenicity. A second cytokine, IL-12p35, usually known for its pro-inflammatory functions, showed slight upregulation in the RNA analysis performed by the group of S. Raghav. This cytokine can also pair with EBI3, also upregulated upon NCoR1 KD, to form IL-35. As IL-35 is an inducer of Tregs, this is an additional point stating that NCoR1 blockade induces Treg differentiation.

The major function of a tolerogenic DC is to establish tolerance, especially through induction of Tregs. As NCoR1 KD/KO DCs display many different tolerogenic factors and express slightly increased presentation molecules, they should induce increased numbers of regulatory T-cells. Coculture and OVA immunisation experiments confirmed this hypothesis, showing that NCoR1 blockade in DCs promote tolerance during the immune response. Paradoxically, the proliferation dye in T-cells demonstrated an increased proliferation in $CD4^+$ T-cells after coculturing them with DCs. However, this increase could be an increase of Treg cells rather than effector T-cells. Further analysis of T-cell subpopulations should be performed in order to confirm this.

In vivo implication in infectious models

DC tolerogenicity and Treg induction can diminish immune defence against pathogens (149, 150). Nonetheless, some reports also state a protective role of Tregs in infections by preserving low amounts of pathogens, which maintains a protective immunity (151). In order to understand the physiological implications of NCoR1 depletion in DCs during infections, three models, a mycobacterial and two parasitic models, were used on our N1 mice. Expectedly, all models developed increased Treg differentiation, although $CD4^+$ T-cell subset characterisation showed different responses according to the infecting agent.

Mycobacterium smegmatis was chosen for its great potential to induce IL-10 production in NCoR1 KD DCs, tested by the group S. Raghav. As Tregs dampen the immune response, and consequently, effector T-cell differentiation, an increase in mycobacterial load was expected. Although Treg were found to be increased in N1 mice, no significant difference could be observed in mycobacterial load between N1 and WT mice. Th1 subpopulations were similar in both mouse groups, which could explain the absence of significant difference in mycobacterial load. Indeed, Th1 are extremely important to fight intracellular pathogens and help macrophage immunity against mycobacteria (152, 153). It should be noted, nevertheless, that this experiment is missing kinetic measures. In order to ensure the lack of difference, T-cell subpopulations and mycobacterial loads should be assessed at other time points. Surprisingly, Th2 were significantly increased in response to *m. smegmatis* infection. Unfortunately, a quick review of the literature showed a surprising absence of publications on the relationship between Th2 and *m. smegmatis*.

On the contrary, a Th2 response is clearly associated with the defence against helminths, and in particular *Heligmosomoides polygyrus* (154). Opposed to our *m. smegmatis* model, N1 mice infected with helminths displayed decreased numbers of Th2 compared to WT mice. This was

reflected in a significant increase of secreted eggs at day 17. Additionally, a concomitant increase in Tregs was observed at time of sacrifice which could further explain the increase in egg counts. Indeed increased Treg numbers in helminth infection leads to retention of worms in intestines (155). While an increase in worm counts was measured in the small intestine of N1 mice, no significance could be reached. The egg increase could be associated with an increase in helminth infectability rather than with an increase in worm numbers.

Last but not least, N1 mice were challenged with footpad infection of *leishmania major*. IL-10 and Treg are known to increase disease burden and enable leishmania chronic persistence in infected animals (150, 156). However, Treg role in leishmania infection remains controversial. Tregs are important for protective immunity by maintaining small amounts of parasites, but they can also be associated with chronic lesions (151, 157). The increased lesions observed in N1 mice after *l. major* infection could be explained at least partly by the increased numbers of Tregs observed in the draining lymph node and a higher IL-10 production from the NCoR1 DCs (158). Paradoxically, an important increase of Th1 and a discrete decrease in Th2 was present in infected N1 mice. Th1 response is classically associated with leishmania resistance whereas Th2 response promotes leishmania susceptibility and persistence (159). However, an increased Th1 response could also generate an exaggerated immune response that transiently augments leishmania lesions in N1 mice. It should also be stated that CpG is necessary to generate a difference between N1 and WT mice.

An autoimmune model: EAE

Tolerogenic DCs are important tools to prevent and even treat autoimmune diseases. Adoptive transfer of $CD11b^+$ DCs in immunised $CD11b^{-/-}$ mice demonstrated the important potential of DCs to fight autoimmune processes. Our group engineered a potent tolerogenic DC line that prolongs graft survival and decreases EAE severity (45). In this context, N1 mice represent excellent tools to study the potential of NCoR1 depletion in DCs to treat autoimmune diseases. Disappointingly, neither passive nor active EAE could be influenced by NCoR1 KO DCs. Possibly, the autoimmune process in EAE is too strong to be counterbalanced by the tolerogenic effect of NCoR1 depletion in DCs. Other autoimmune models should be tested. NCoR1 KD DCs have higher tolerogenic properties. They could be used in EAE models in WT mice as well to see whether they could prevent EAE development.

NCoR1 promotes development of $CD8^+$ DC over $CD11b^+$ DC

NCoR1 is mostly known for its crucial role in cell maturation (89). In DC, apart from its role on tolerogenicity, our results indicated that NCoR1 favours $CD8^+$ DC differentiation over $CD11b^+$ DC differentiation. NCoR1 is probably involved in DC differentiation during development. As no link between NCoR1 and DC differentiation has yet been described, further investigation should be pursued to explore DC physiology and development. This understanding could help us target more specific DC subset and induce preferentially, to our convenience, one subset over another.

Outlook

Our work demonstrated that genetic blockade of NCoR1 generates a tolerogenic response characterised by Treg induction and increased infection burden. This way, NCoR1 acts as a master regulator of the tolerogenic program that remains repressed in DCs after CpG or microbial challenge. When NCoR1 is removed, tolerogenic genes can be activated and expressed after CpG or microbial stimulation (Figure 36). Our infection models confirmed the importance of NCoR1 repression in DCs to mount efficient immune response and decrease infection burden. Although our autoimmune model was not conclusive, NCoR1 blockade in DCs should still be considered as potential therapy for inflammatory processes. Association with other tolerogenic strategies could create a synergistic effect to induce tolerance.

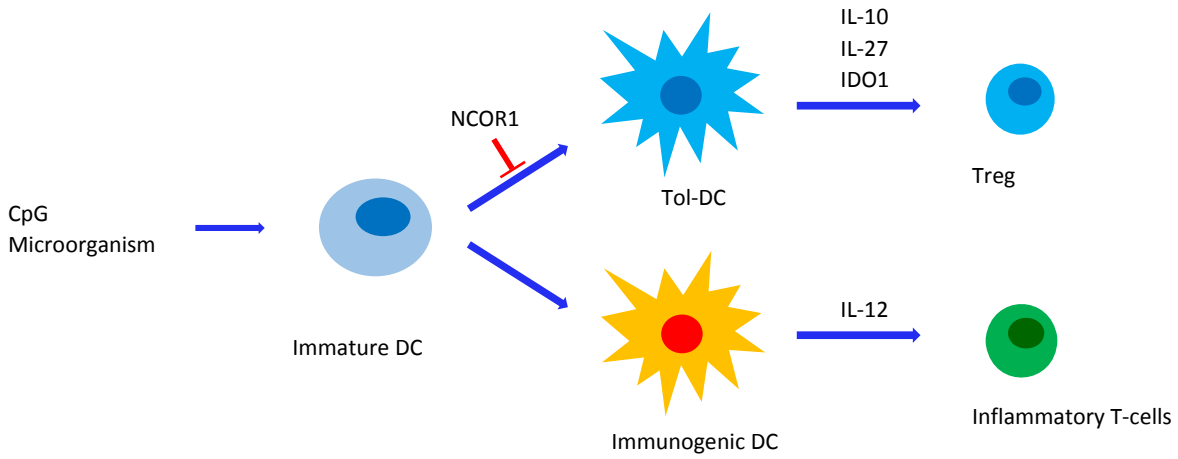


Figure 36 ***Diagram of the second project***
NCoR1 prevents activation of the tolerogenic program after DC stimulation. In case of NCoR1 inactivation, DCs become tolerogenic upon activation and induce an increased Treg differentiation.

Methods

General methods

Culture conditions

All cells were handled and prepared on a class 2 laminar flow bench under sterile conditions. 293T HEK, CMT-93 cells and macrophages were cultured in DMEM + Glutamax with 10% FCS and 1% Penicillin/Streptomycin at 37°C in a 5% CO_2 atmosphere. Before cells reached 90% confluency, 293T HEK cells were mechanically detached, centrifuged and split in new flasks. CMT-93 tumour cell medium was aspirated and cells washed two times with PBS. Cells were then detached by addition of 0.25% Trypsin. After 3 minutes of incubation at 37°C, cells were collected in 10 ml medium and 320 g centrifuged for 5 minutes. The cells were then split in fresh medium.

Dendritic cell lines were cultured in complete IMDM + Glutamax (with 10% FCS, 10 mM HEPES, 50 μM β-Mercaptoethanol, 0.075 % Sodium Bicarbonate, 50 U/ml penicillin and 10 μg/ml streptomycin) at 37°C in a 5% CO_2 atmosphere. Cells were split when reaching 90% confluency. DCs were collected by incubation in cell dissociation buffer (10 mM HEPES and 5 mM EDTA in PBS) and centrifuged for 5 minutes at 320 g and resuspended before splitting them in fresh medium.

Flow cytometry analysis

Staining was performed on single cell suspensions from draining lymph nodes or spleens. In case of intracellular cytokine staining cells were restimulated in vitro at 37°C in complete medium before the surface staining. Restimulation for T-cells consisted of a 4 to 5 hour incubation with phorbol myristate acetate (PMA, 10 ng/ml), ionomycin (500 ng/ml) and brefeldin A (10 µg/ml). B-cell restimulation included a 6 hour incubation with LPS (200 ng/ml) and brefeldin A (10 µg/ml) while DC restimulation was a 2 hour incubation with CpG (1µM) followed by a 6 hour incubation with brefeldin A (10 µg/ml).

Before staining the cells, samples were washed once with FACS buffer (2 mM EDTA, 2% FCS in PBS). Cells were stained for surface molecules during 30 minutes at 4°C in the dark. Staining solutions contained 50% 24G2 Fc blocking antibody and 50% FACS buffer. Samples were then washed twice with FACS buffer. For FoxP3 intracellular staining, cells were fixed and permeabilised with FoxP3 Fixation/Permeabilisation solution (ebioscience) during 1 hour at 4°C. For cytokine staining, cells were fixed in paraformaldehyde (PFA) 2% for 15 minutes at room temperature. Fixation was followed by two washes with a permeabilisation buffer (ebioscience) and intracellular staining at room temperature for 30 minutes. Staining antibodies were diluted in a permeabilisation buffer. Samples were examined in a LSRII flow cytometer, and raw data was analysed on the computer program FlowJo X.

RNA extraction and qPCR

RNA isolation for qPCR was extracted by addition of 500 µl Trizol on cells. Cell lysate was precipitated by addition of 100µl chloroform. Lysate was centrifuged for 15 minutes at $15x10^3$ g and 250µl of isopropyl alcohol was added to the aqueous phase. Precipitate was pelleted by centrifugation for 10 minutes at the same speed and washed by addition of 500 µl 75% ethanol. The ethanol was completely removed after centrifugation and pellet resuspended in 30 µl of RNA secure. RNA was incubated at 60°C for 15 minutes and stored at -70°C. 2 µl (200 ng/µl) random nonamer primers were added to10µl of RNA and denatured for 5 minutes at 70°C. Primers were allowed to align at 4°C for 10 minutes. A 4 µl of 5x RT buffer, 1 µl of 10 µM dNTP, 1 µl of 0.5µg/µl BSA, 2 µl 100mM DTT and 0.5 RNASin mixture was pre-incubated for 10 minutes at room temperature. 0.5 µl Superscript reverse transcriptase was added and the reaction incubated for 50 minutes at 42°C. The Enzyme was inactivated at 70°C for 5 minutes and the remaining RNA digested with 1 µl RNAse H. The Reaction was purified using a Qiaquick PCR purification kit following the manufacturer's instructions. 2 µl of the resulting purified cDNA was mixed with 0.25 µl of each primer (10µM) and 2,5µl SYBR Fast Green qPCR Master mix. Reactions were run in technical triplicates on a LightCycler480 rtPCR machine (Roche). A standard protocol was used for most of the analysed genes: Initial denaturation for 5 minutes at 95°C. 45 cycles of amplification: Denaturation for 10 seconds at 95°, primer annealing for 10 seconds at 60°C, expansion at 72°C for 10 seconds and signal acquisition at 78°C. Followed by acquisition of a melting curve starting at 65°C (60 seconds) and increasing the temperature to 97°C at a rate of 0.11°C/s with 5 signal acquisitions per second. Relative expression for each gene of interest was calculated using the delta-delta CT calculation and represented as expression normalised to β-actin.

First project

Cytokine detection

IL-17 in arthritic joints

Hind paws were harvested and homogenised in a tissue grinder in PBS. Lysates were centrifuged at 4000 g for 5 minutes at 4 °C two times in order to remove joint debris. Supernatant was then stored at -80°C before until assessment of IL-17 by Enzyme Linked Immunosorbent Assay (ELISA). The ELISA kit was purchased at eBioscence and the procedure performed according to the manufacturer's instructions. Results were read using a 450 nm microplate reader (Ledetect 96).

Serum preparation and cytokine detection in immunised mice

Peripheral blood was isolated from mice by cardiac puncture. Samples were incubated for 1 hour at room temperature and centrifuged at full speed for 10 minutes at 4°C. Serum was collected and frozen at -80°C until ELISA analysis of IL-6 (eBioscence), sIL-6R (sigma) and LEGENDplex (biolegend) analysis of IL-6, TNFα, IL-1α and IL-1β. All procedures were performed according to manufacturer's instructions. ELISA results were read by a 450 nm microplate reader (Ledetect 96). LEGENDplex results were obtained on a LSRII cytometer and analysed with the software provided by LEGENDplex.

DC and macrophage isolation for cytokine analysis

Ex vivo DCs were obtained from CD11b$^{-/-}$ or WT spleens digested for 20 min at 37°C with collagenase D (1 mg/ml) and DNAse I (40 µg/ml). CD4^{+} DCs were isolated through magnetic cell separation (MACS CD4 DC isolation kit, Miltenyi). Peritoneal macrophages were acquired by injecting 5 ml ice cold PBS into the peritoneal cavity, massaging it and recollecting the fluids. Macrophages were then centrifuged at 300 g for 5 minutes, counted and plated. Once attached, wells were washed with PBS to remove remaining B-cells.

Ex vivo DCs, peritoneal macrophages and CD11b KO DCs from our transduced DC line as well as their CD11b^{+} controls were plated at same numbers, i.e. 10^5 cells per well in a 48-well-plate. Cells were stimulated with or without CpG (1 µmol/l) or LPS (200 ng/ml) for 16 hours in either complete RPMI (macrophages) or complete IMDM (DCs). After collection, supernatants were frozen at -80°C until IL-6 analysis through ELISA (eBioscience).

SRC inhibition

Ex vivo DCs and macrophages were obtained as described above. Same numbers of ex vivo cells and DC lines (10^5) were plated in a 48-well-plate. Cells were treated with a src inhibitor (PP1) at 10 µM and incubated for 1 hour at 37°C. Wells were then washed and stimulated with the same stimuli as in the previous section. After collection, supernatants were frozen at -80°C until IL-6 analysis through ELISA (eBioscience).

Cocultures

Ex vivo cells

CD11b$^{-/-}$ and WT DCs were obtained by standard collagenase D digestion of spleens and magnetic cell separation (MACS CD4^{+} isolation kit, Miltenyi). 10^4 DCs were plated in round bottom 96-well-plates and pulsed with the specific OT-II peptide (MHC-II-restricted OVA$_{(323-339)}$ peptide, ISQAVHAAHAEINEAGR). 10^5 magnetically purified CD4^{+} T-cells were cocultured with DCs, anti-CD3 Abs (145.2C.11, homemade), TGFβ (*conc*) and IL-2 *(conc)* in complete RPMI. In addition, cells were treated with anti-IL-6R antibody *(réf)* or its control, IgG (20 µg/mL). Four days later, supernatants were collected and stored at -80°C for IL-17 measurement. Cells were restimulated with PMA and ionomycin. CD4^{+} T-cells expressing intracellular IL-17 and FoxP3 were quantitated by flow cytometry.

CD11b KO DC line

10^4 CD11b KO DCs or their CD11b^{+} counterparts were plated in round bottom 96-well-plates. DCs were pulsed with the specific OT-II peptide (MHC-II-restricted OVA$_{(323-339)}$ peptide, ISQAVHAAHAEINEAGR) for 4 hours with or without CpG or LPS stimulation. DCs were washed before adjunction of 10^5 T-cells in complete RPMI. CD4^{+} T-cells were magnetically purified from OT-II mice (EasySep™ Mouse CD4+ T Cell Isolation Kit) and cocultured with the DCs in the presence of IL-2 *(concentration)* and TGFβ *(concentration)*. After four days, supernatants were collected and stored at -80°C for IL-17 measurement. T-cells were restimulated with PMA as well as ionomycin and profiled by flow cytometry.

CD11b KO DC line generation

Plasmid generation

Three specific DNA sequences for CD11b were designed and inserted in a plasmid lentiCRISPR v2. After testing the different sequences, we chose the plasmid N° 243 with the DNA sequence CACCGGCATTCTCTTGGAAGGTCATGTTT. Plasmids were engineered and produced in our lab with the help of Christine Lavanchy.

Transfection

Lentiviral transfection was performed on 293T HEK cells. 10^6 HEK cells were plated in a 75 cm^2 size flask. Plasmid solution was prepared at room temperature by mixing the plasmids 243 (lenti CD11b CRISPR construct, 40 µg), psPAX (30 µg), and pVSV (20 µg) in 3 ml serum free OPTIMEM supplemented with 90 µg PEI. The plasmid solution was vortexed, incubated for 15 minutes at room temperature before being completed with 12 ml of 293T HEK cell medium. 293T HEK supernatant was replaced by the plasmid solution and cells were incubated overnight at 37°C. Supernatant was replaced this time with OPTIMEM supplemented with 1% FCS. Cells were incubated 24 hours before collecting the supernatant containing lentiviruses. Supernatant was filtered using a 0.45µm PVDF filter (Millex) and stored at 4°C. 293T HEK cells were incubated once more with OPTIMEM supplemented with 1% FCS for 24 hours before repeating the same filtration.

Lentiviruses were concentrated by adding 5.7 ml PBS, 1.3 ml NaCl 5M and 8 ml PEG 50% and incubating them overnight at 4°C on a rotating wheel before centrifuging the viral solution at

4000 RPM for 1 hour at 4°C. Virus pellet were resuspended in 200 µl PBS, aliquoted at 50 µl and stored at -80°C.

Transduction

$1,8*10^5$ DCs from the $CD11b^+$ DC line were plated in each well of a 24-well-plate at day 0 and incubated overnight. The medium was removed and replaced with 500 µl of complete IMDM containing 7 µg/ml polybrene and 50 µl of viral particles at day 1. The medium was replaced with normal DC medium after 17 and 41 hours. At day 3 cells were treated with 1 µg/ml puromycin in DC medium for three days. Once cell recovered and expanded, transduction efficiency was checked and cell sorted by flow cytometry. CD11b KO DCs and $CD11b^+$ DCs were put back in culture for in vitro and in vivo experiments.

In vivo experiments

Mice

All mouse strains were in the C57BL/6J background, used at the age of 6–15 weeks and sex-matched. NCoR1 conditional KO mice (N1) were obtained from breedings between NCoR1-lox mice and CD11c-Cre mice. $CD11b^{-/-}$ mice were provided by Prof. Britta Engelhardt (Theodor Kocher Institute, University of Bern). In vivo experimental protocols were approved by the Swiss Federal and Cantonal Veterinary authorities. WT mice were purchased from Envigo.

Collagen-induced arthritis induction

Native chicken type II collagen was dissolved in 10 mM acetic acid overnight at 2 mg/ml. 100µg collagen emulsified in Complete Freund's Adjuvant containing 5 mg/ml mycobacterium tuberculosis was injected intradermally at the base of the tail. Twenty-one days after the first injection a booster injection with 100µg of native chicken collagen was done in Incomplete Freund's Adjuvant intradermally (IFA) at the base of the tail.

Clinical and histological assessment of arthritis

The mice were assessed two times per week for redness and swelling of limbs and a clinical score from 1 to 4 (1: Slight edema and erythema limited to ankle; 2: Slight edema and erythema from the ankle to the tarsal bone; 3: moderate edema and erythema the ankle to the tarsal bone; 4: edema and erythema from the ankle to the entire leg) was given for each limb for up to 50

days. At the end of the experiment, the paws of the mice were taken, fixed, decalcified and embedded with paraffin. Joint sections (5 µm) were stained with Safranin-O-fast green (S/FG) or hematoxylin and eosin (H&E) and evaluated for histological alterations including synovial hyperplasia, pannus formation, cellular infiltration and cartilage erosion. Histological sections were graded independently by two observers unaware of mouse genotype using an established scoring system (from 0: no histological sign to 3: most severe).

CIA immune response

Mice were sacrifice through cervical dislocation or CO_2 euthanasia. Spleen and inguinal lymph nodes were harvested and mashed through a 40 µm cell strainer in order to obtain cell suspensions. Cells were restimulated with PMA (10 ng/ml), ionomycin (500 ng/ml) and brefeldin (10 µg/ml) for T-cell analysis and LPS (200 ng/ml) plus brefeldin A for B-cell analysis in complete RPMI (10% fetal calf serum, β-mercaptoethanol 50 µmol/l, penicillin/streptomycin) at 37°C for 5 hours. After restimulation, the standard protocol for intracellular staining was performed and cells were analysed by flow cytometry.

Anti-IL-6R treatment

CIA was induced as described above in $CD11b^{-/-}$ mice. At time of the second immunisation, mice were treated intraperitoneally with 1 mg anti-IL-6R mAb (15A7) (Bioxell) in 200 µl or with IgG isotype-matched control. Mice were injected twice per week. Twenty days after treatment initiation, mice were sacrificed and inguinal harvested for flow cytometric analysis of T-cells.

Adoptive transfer of CD11b DCs

CD11b DCs from the newly generated CD11b DC line on the C57BL/6 background were injected intraperitoneally ($2x10^6$ cells per mouse) 20 days after the first immunisation. To avoid rejection of $CD11b^+$ DCs via an anti-large T-cell response (160), the CD8 T cells were depleted via two injections with 250µg of anti-CD8a (clone H35) antibody intraperitoneally. The first injection was performed one day before the DC transfer and the second three days later.

Adoptive transfer of CD11b KO DCs

CD11b KO DCs and their control $CD11b^+$ DCs were cultured for 4 hours with collagen II (25 µg/ml) in complete IMDM (10% foetal calf serum, β-mercaptoethanol 50 µmol/l, HEPES 10 mmol/l, penicillin/streptomycin). $3x10^6$ DCs per mouse were injected intraperitoneally at day 0. The same procedure was performed at day 21, however this time with $8x10^6$ DCs per mouse. Mice were monitored for disease development during 5 weeks.

Second project

N1 mouse characterisation

N1 mouse generation

Floxed NCoR1 mice in a C57BL/6 background were obtained from the group of Prof. Auwerx (EPFL). They were crossed with transgenic mice carrying a Cre-recombinase dependent on the minimal promoter of CD11c, which is almost exclusively expressed on DCs. Resulting heterozygous progenies were back-crossed for several generations to obtain pure homozygous $NCoR1^{fl/fl}$ CD11c-Cre^{+} mice, called N1 mice.

N1 mouse genotyping

Ear biopsies were taken from the mice and digested in 600 µl NaOH 50 mM at 95°C for 35 minutes. Basicity was neutralised by adding 60 µl of Tris 1 M. Samples were centrifuged at $15x10^3$ g and 2 µl of the supernatant was used for the DNA template in a standard PCR.

PCR products were loaded on a fluorescent 2% agarose gel and N1 positive mice were selected for experiments and breeding.

NCoR1 KO control

N1 mice were treated with daily injection of FLT3L serum for 9 days before being sacrificed by cervical dislocation or CO_2 euthanasia. Spleens were then harvested and digested with collagenase D (1 mg/ml) and DNAse I (40 µg/ml) for 20 minutes at 37°C. After obtaining single cell suspension, cells were sorted into two subsets through flow cytometry according to the

dendritic cell markers $B220^-$, $CD11c^+$ and $CD8^+$ cells or $B220^-$, $CD11c^+$ and $CD11b^+$ cells. DNA was extracted from 4 x 10^5 FACS-sorted DCs using the DNeasy Blood & Tissue DNA isolation kit (Qiagen) according to the manufacturer's protocol. After NCoR1 gene PCR, samples were loaded on a fluorescent 2% agarose gel to visualise the presence or absence of PCR product.

N1 DC analysis

Mice were sacrificed by cervical dislocation or CO_2 euthanasia. After collection, spleens were digested by collagenase D (1 mg/ml) and DNAse I (40 μg/ml) for 20 minutes at 37°C and passed through a 40 μm cell strainer. Samples were then treated with RBC lysis buffer for 2 minutes and washed with complete medium. Cells were stained and analysed by flow cytometry.

To characterise tolerogenicity in ex vivo DCs, WT and N1 mice were previously treated with 50 μg FLT3L for 9 days. After collagenase D digestion and RBC lysis, cells were either stimulated at 37°C for 2 hours with CpG followed by a 6 hour incubation with brefeldin A, or left without treatment in complete IMDM. Samples were then stained and analysed by flow cytometry

Coculture with NCoR1 KD DCs

10^4 DCs (either NCoR1 KD or control cells) were plated in round bottom 96-well-plates. DCs were pulsed with the specific OT-II peptide (MHC-II-restricted $OVA_{(323-339)}$ peptide, ISQAVHAAHAEINEAGR) for four hours with or without CpG. $CD4^+$ T-cells were magnetically purified from OT-II mice (EasySep™ Mouse CD4+ T Cell Isolation Kit), stained

with eF670 proliferation dye according to manufacturer's instructions and 10^5 cells/well were added to the DCs in complete RPMI. After four days, supernatants were collected and stored at -80°C for IL-17 measurement. T-cells were restimulated with PMA and ionomycin and profiled by flow cytometry.

In vivo experiments

OVA immunisation

Different vaccine formulations were tested for ovalbumin (OVA), however, the most replicable method remained a simple mixture of 50 µg CpG and 10 µg OVA dissolved in PBS. Mice were immunised subcutaneously, at the base of the tail at day 0 and day 30. Inguinal lymph nodes were harvested, processed in order to get cell suspension, restimulated as mentioned above and analysed by flow cytometry for T-cell profiling at day 33.

M. smegmatis

M. smegmatis for the experiment was kindly provided by the group of Prof. McKinney (EPFL, Lausanne). $5*10^6$ active *mycobacterium smegmatis* were injected in the right mice footpads. Fourteen days after the first infection, mice were rechallenged with the same amount of *m. smegmatis* at the same location. Three days after the second injection, popliteal lymph nodes were harvested and cells were analysed through flow cytometry for T-cell profiling. Bacterial load was estimated by colony formation on standard LB agar plates.

Leishmania major

L. major was provided by the group of Tacchini-Cottier (WHO, University of Lausanne). $3*10^6$ *leishmania major* parasites were injected in the right footpad of each mouse. Thickness difference between the right and the left footpad was measured every 4-5 days. Mice were administered intraperitoneally with or without 50 µg CpG at day 18. At the end of the

experiment, footpads and popliteal lymph nodes were harvested. Parasite load was quantified by qPCR on footpads and T-cell profiling was performed on popliteal lymph nodes by flow cytometry.

Heligmosomoides polygyrus

N1 and WT mice were infected with 200 infective L3 larvae of *H. polygyrus* in PBS per mice through oral gavage. At day 11 after infection, mice were treated with intraperitoneal injection of 50 µg CpG-1826. The faeces from treated animals were collected daily to count worm eggs in McMaster chambers. After observing a significant difference in egg count between N1 and WT animals, mice were sacrificed for detailed T-cell profiling of mesenteric lymph nodes by flow cytometry. The intestinal helminth worms were counted from intestines.

Cancer

Mice were injected 50 µg CpG 6 hours before being administered $5*10^6$ CMT-93 cells subcutaneously on their right flanks. CpG injections were repeated on day 3 and day 6. Tumour volume was monitored every three days during one month. At the end of the experiment, draining lymph nodes and tumours were harvested for T-cell profiling.

EAE

Active EAE was obtained by administering subcutaneous injections of 200 µg MOG_{35-55} peptide in CFA (5 mg/ml m. tuberculosis) at day 0 in mouse flanks. In addition mice were injected with 200 ng pertussis toxin and 50 µg CpG intraperitoneally at day 0 and day 2. Mice were then monitored daily for limb and tail paresis/paralysis until full development of the disease.

The protocol for passive EAE includes the same injections, however, draining lymph nodes were harvested at day 12. This includes axillary, brachial and inguinal lymph nodes. After collagenase D digestion and single cell suspension, cells were washed and incubated with 10 µg/ml MOG_{35-55} in RPMI supplemented with 0.5 ng/ml IL-12p70 during three days. WT mice received $12*10^6$ cells intraperitoneally. Disease was monitored for limb and tail paresis/paralysis until full development and its potential recovery.

Statistical Analysis

Unless otherwise stated, statistical analysis was performed applying the nonparametric Mann-Whitney U test, on the computer software Graphpad Prism 5. Differences were considered significant when * $p<0.05$, ** $p\leq0.005$, *** $p\leq0.001$

Materials

Reagents

Product	Supplier	Reference number
2-Mercaptoethanol 50 mM	Gibco	31350-010
Anti-IL-6R	Bioxell	BE0047
Brefeldin A	BioLegend	420601
Cell proliferation dye eFluor 670	eBioscience	65-0840
CFA/IFA	SIGMA	F5881/F5506
Chloroform	SIGMA	C2432
Collagen II	SIGMA	C9301
Collagenase D	Roche	11088866001
CpG 1826	Trilink Technologies	
DNase	Roche	10104159001
Ethanol	Fischer	10048291
Fc blocking antibody 24G2	Home made	
FCS	Pan Biotech	Lot: P140112
Gentamicin 10 mg/ml	Gibco	15710064
HEPES 1M	Gibco	15630-056
IMDM + GlutaMAX	Gibco	31980-022
Ionomycin	Calbiochem	407950
Iso-propanol	Fluka Analytical	59310
LPS	Invivogen	tlrl-eblps
MOG_{35-55}	Protein and Peptide Chemistry Facility, UNIL	
Opti-MEM + GlutaMAX	Gibco	51985-026
OT-II peptide ($OVA_{323-339}$)	Protein and Peptide Chemistry Facility, UNIL	
Ovalbumin	SIGMA	
PBS	MagBil	
PEI	SIGMA	408727
Penicillin/streptomycin	Life Technologies	15070063
PFA	SIGMA	P6148
PMA	Sigma	P8139
Polybrene	SIGMA	H9268
PP1 (src inhibitor)	Selleckchem	S7060
eFluor 670 cell proliferation dye	eBioscience	65-0840
RNA secure	Thermofischer	AM7005
RPMI + GlutaMAX	Gibco	61870-010
Sodium bicarbonate 7.5%	Gibco	25080-60

SYBR Safe DNA Stain	Life Technologies	S33102
TRI Reagent	SIGMA	T9424

Buffers and solutions

Home made solution	Recipe
Antibody solution	50% FACS buffer, 50% Fc blocking antibody 24G2
Cell dissociation buffer	PBS, 10 mM HEPES, 5 mM EDTA
Collagenase D digestion solution	RPMI + GlutaMAX, 2.5% FCS, 10 mM HEPES, 20 µg/ml gentamicin, 40 µg/ml DNase, 1 mg/ml collagenase D
Collection buffer	Complete IMDM, 10% FCS
EasySep buffer	PBS, 2% FCS, 1mM EDTA
FACS buffer (sorting)	PBS, 25 mM HEPES, 1 mM EDTA, 3% FCS
FACS buffer (staining)	PBS, 3% FCS, 5 mM EDTA
MACS buffer	PBS, 2 mM EDTA, 0.5% BSA
Medium, complete IMDM (DC medium)	IMDM + GlutaMAX, 10% FCS, 1% penicillin/streptavidin, 10 mM HEPES, 0.075 % Sodium Bicarbonate, 50 µM 2-Mercaptoethanol
Medium, complete RPMI (T-cell and macrophage medium)	RPMI + GlutaMAX, 10% FCS, 1% penicillin/streptavidin, 50 µM 2-Mercaptoethanol
Red blood cell lysis buffer	H_2O, 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA

Kits

Application	Description	Supplier
Enzyme Linked Immunosorbent Assay (ELISA)	IL-6	eBioscience
	IL-17 (mettre les anciennes références)	eBioscience
	Mouse IL-6 R ELISA Kit	Sigma-Aldrich (RAB0314)
Bead-based immunoassays	LEGENDplex™ Mix and Match System for IL-1α, IL-1β, IL-6 and TNFα	BioLegend (740152, 740157, 740159, 740154, 740371, 740165, 740373)
Magnetic cell sorting	$CD4^+$ dendritic cell isolation kit mouse	Miltenyi (130-091-262)
	EasySep™ Mouse $CD4^+$ T Cell Isolation Kit	Stemcell (19852)
DNA extraction	DNeasy Blood & Tissue Kit	Qiagen (69504)
Intracellular staining	Foxp3 / Transcription Factor Staining Buffer Set	eBioscience (00-5523-00)

FACS antibodies

Antigen	Fluorochrome	Clone	Supplier
B220	$AlexaFluor^{700}$	RA3-6B2	eBioscience
CD11b	APC	M1/70	eBioscience
CD11c	$PECy^7$	N418	eBioscience
CD4	FITC	RM4-5	eBioscience
	PE	RM4-5	eBioscience
CD8α	$eFluor^{450}$	53-6.7	eBioscience
CD25	APC	PC61.5	eBioscience
	$PerCPCy^{5.5}$	PC61.5	eBioscience
CD80	$BrilliantViolet^{421}$	16-10A1	BioLegend
CD86	$AlexaFluor^{700}$	GL-1	BioLegend
GATA3	$eFluor^{660}$	TWAJ	eBioscience
FoxP3	PE	NRRF-30	eBioscience
IDO1	$AlexaFluor^{647}$	2E2/IDO1	BioLegend
IFNγ	PE	XMG1.2	BioLegend
IL-10	FITC	Cat554466	BD pharmingen
	PE	JES5-16E3	eBioscience
IL-13	$eFluor^{450}$	eBio13A	eBioscience
IL-17	APC	eBio17B7	eBioscience
IL-27	PE	MM27-7B1	BioLegend
MHC-II	FITC	M5/114.15.2	eBioscience
Tbet	PE	eBio4B10	eBioscience
TCRβ	$BrilliantViolet^{510}$	H57-597	BioLegend

Primers

Primers were designed using the tool “Primer-BLAST” tool on the NCBI homepage and ordered from Invitrogen

Primer or plasmid	Sequence
CD11b 243 plasmid specific sequence	CACCGGCATTCTCTTGGAAGGTCATGTTT
CD11c-Cre I	AACATGCTTCATCGTCGG
CD11c-Cre II	TTCGGATCATCAGCTACACC
CD90F	CCATCCAGCATGAGTTCAGCC
CD90R	GCATCCAGGATGTGTTCTGA
NCoR1 – 29	GAACTAAGGACAGGAAGGTACAGGG
NCoR1 – 30	AGGTGGAATCACAGAAAGCTGACGC

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Abbreviations

Abbreviation	Meaning
APC	Antigen Presenting Cell
BCR	B-Cell Receptor
CD	Cluster Differentiation
cDC	Conventional Dendritic Cell
CFA	Complete Freud's Adjuvant
CIA	Collagen-Induced Arthritis
CpG	CpG oligodeoxynucleotides
CTL	Cytotoxic T-Lymphocyte
DAMP	Danger Associated Molecular Pattern
DC	Dendritic Cell
DSS	Dextran Sodium Sulfate
EAE	Experimental Autoimmue Encephalitis
FACS	Fluorescence-Activated Cell Sorting
IDO	Indolamin-2,3-Dioxygenase
IFA	Incomplete Freud's Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IP	Intraperitoneal
IV	Intravenous
KD	Knockdown
KO	Knockout
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
MACS	Magnetic-Activated Cell Sorting
mTEC	Medullary Thymic Epithelial Cell
N1	DC-specific conditional KO for NCoR1
NCoR1	Nuclear receptor Co-Repressor 1
OT-II	$CD4^+$ OVA-specific T-cells
OVA	Ovalbumin
PAMP	Pathogen Associated Molecular Pattern
PCR	Polymerase Chain Reaction
pDC	Plasmacytoid Dendritic Cell
PEI	Polyethylenimine
PFA	Paraformaldehyde
pIC	Polyinosinic:polycytidylic acid
PMA	Phorbol Myristate Acetate
PRR	Pattern Recognition Receptor
qPCR	quantitative Polymerase Chain Reaction
RA	Rhumatoid Arthritis
SC	Sub-Cutaneous
sIL6R	Soluble IL-6 Receptor
TCR	T-Cell Receptor

TGFβ	Transforming Growth Factor β
TLR	Toll-Like Receptor
Tol-DC	Tolerogenic Dendritic Cell
Treg	Regulatory T-cell

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