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Controlling established immune memory with tolerogenic dendritic cells and encapsulated cell technology

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Controlling established immune memory with tolerogenic dendritic cells and encapsulated cell technology

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présentée à la

Faculté de biologie et de médecine
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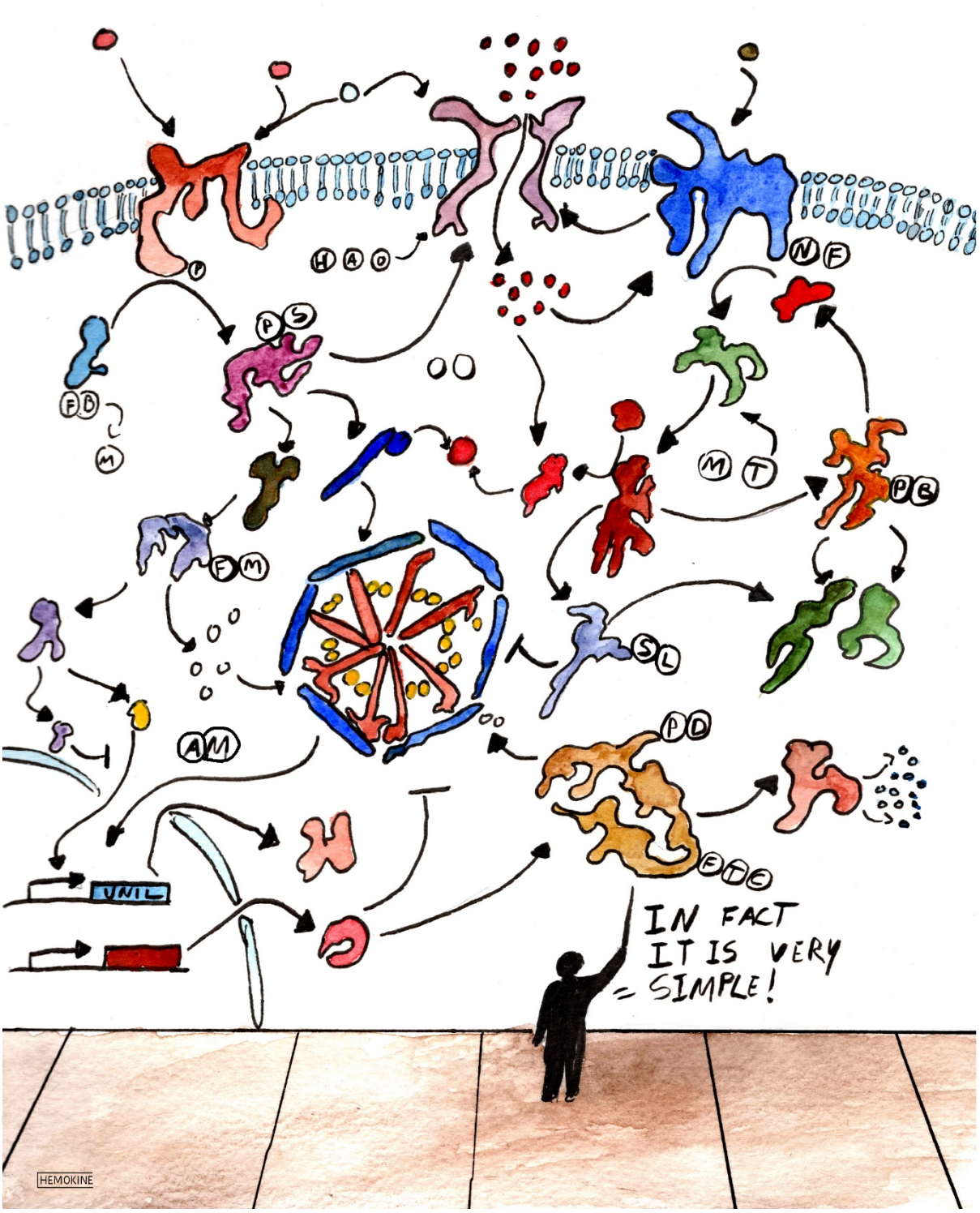

Illustration for the DB Days 2019 poster, Department of Biochemistry, UNIL

RÉSUMÉ GRAND PUBLIC: THÈSE ADRIEN ENGEL

Le contrôle de la mémoire immunitaire par les cellules dendritiques tolérogènes ainsi que par l'encapsulation de cellules dans un implant sous-cutanée

Afin d'attaquer seulement les pathogènes le système immunitaire est extrêmement régulé. En cas de dysfonctionnement les cellules destinées à la protection de l'intégrité de l'organisme se dirigent contre des composants nécessaires au bon fonctionnement du corps et induisent une maladie auto-immunitaire (Gaine de myéline (nerfs) pour la sclérose en plaque, collagène pour la polyarthrite rhumatoïde). Ces attaques peuvent être si fortes qu'elles peuvent causer des handicaps sévères. Elles sont généralement traitées par des drogues immunosuppressives qui peuvent avoir d'importants effets secondaires. Les cellules dendritiques peuvent induire et reprogrammer la mémoire immunitaire spécifique. Elles sont la clef de voûte entre le système immunitaire inné et adaptatif. Elles patrouillent constamment dans la majorité des tissus de l'organisme et présentent aux lymphocytes des composants des corps étrangers (antigènes) rencontrés dans les tissus. Certaines de ces cellules sont tolérogènes et arrivent à maintenir la tolérance en changeant les cellules du système immunitaire adaptif.

L'étude présentée ici se focalise sur l'ingénierie de ces cellules dendritiques pour les aider à reprogrammer spécifiquement la mémoire immunitaire responsables de la maladie. Nous avons utilisé un modèle de cellules dendritique appelé « Mutu » précédemment présenté qui permet de les manipuler plus facilement. Par des moyens de bio-ingénieries nous avons induit la sécrétion constante d'une cytokine tolérogène : l'interleukine 27 (ou IL-27). Ces cellules appelées « CDs IL-27 » se sont révélées être de puissants inhibiteurs de la prolifération des lymphocytes T *in vitro*. Cette nouvelle thérapie cellulaire a été testée dans un modèle animal d'hypersensibilité induite où les souris sont immunisées contre une protéine étrangère puis restimulées au niveau de la patte quelques semaines plus tard. Nous avons vu que les CDs IL-27 spécifiquement pulsées contre cette même protéine sont capables de changer la mémoire immunitaire et ainsi limiter fortement l'inflammation au niveau de la patte. Ces cellules ont révélé des propriétés thérapeutiques dans un autre modèle de souris qui se rapproche plus de la sclérose en plaque chez l'homme. L'injection de CDs IL-27 induit une baisse de la gravité des symptômes (une paralysie partielle ou total au niveau de l'arrière train).

Dans un second temps nous avons étudié le potentiel d'un implant sous-cutané possédant des cellules encapsulées qui ont été génétiquement modifié pour sécréter de façon stable et systémique l'IL-27 dans toute la souris. Cet implant s'est révélé être un puissant modulateur de l'inflammation dans le modèle animal le l'hypersensibilité induite.

Notre étude a montré deux méthodes (implants sous cutanés et les CDs IL-27) qui peuvent induire la tolérance dans un environnement inflammatoire. Ainsi l'IL-27, les cellules CDs tolérogènes, ainsi que la sécrétion systémique de cytokines tolérogènes sont des outils prometteurs pour une prochaine thérapie contre les maladies auto-immunes comme la sclérose en plaque ou la polyarthrite rhumatoïde.

RESUME

Les maladies auto-immunitaires sont la conséquence d'une réponse disproportionnée contre un antigène du soi. Aujourd'hui les stratégies thérapeutiques se basent sur des immunosuppresseurs qui sont associés a des effets secondaires importants. Traiter uniquement les cellules mémoires qui induisent une immunité contre les antigènes du soi est possible en vaccinant les souris avec des cytokines tolérogéniques pendant un court moment. Nous avons développé deux approches *in vivo* pour changer la mémoire immunitaire spécifique.

Dans la première approche nous avons utilisé les cellules dendritiques (CDs) tolérogéniques. Ces cellules présentent naturellement des antigènes aux cellules T et peuvent aussi former des cellules T régulatrices, gardiennes de la tolérance. Nous avons généré une lignée cellulaire de cellules dendritiques CD8α+ capable de sécréter une large quantité d'IL-27. Cette cytokine tolérogène a été choisie car elle a été récemment décrite pour induire fortement des lymphocytes T régulateurs Tr1. Ces cellules dendritiques secrétant de l'IL-27 que nous avons appelé « CDs IL-27 » ont un phénotype particulier, elles ont une expression moins élevée du complexe majeur d'histocompatibilité (CMH-II) ainsi que des protéines de co-stimulation. Ces cellules inhibent fortement la prolifération des lymphocytes T CD4. Chez la souris, nous avons conduit une expérience d'hypersensibilité induite sur la souris que nous avons immunisée contre une protéine étrangère puis restimulée au niveau de la patte quelques semaines plus tard. Nous avons vu que les CDs IL-27 spécifiquement pulsées contre cette même protéine sont capables de changer la mémoire immunitaire préétablie et ainsi limiter l'inflammation au niveau de la patte. Ces cellules ont aussi révélé un puissant potentiel thérapeutique dans un modèle de la sclérose en plaque chez la souris. Les CDs IL-27 pulsées avec le peptide de myéline responsable de la maladie ont révélé que la plupart des souris ainsi traitées ont montré une plus grande guérison avec des souris traitées après apparence des premiers symptômes.

Dans un second temps nous avons étudié le potentiel d'un implant sous cutané possédant des cellules encapsulées génétiquement modifiées pour sécréter de façon stable l'IL-27 de façon systémique dans toute la souris. Dans le modèle de l'hypersensibilité induite les souris possédant l'implant capable de sécréter l'IL-27 ont révélé une inflammation fortement réduite après ré-administration de l'antigène dans la patte.

Notre étude a établi deux méthodes basées sur l'IL-27 qui se sont révélées efficaces pour induire la tolérance. Cette étude démontre que l'IL-27, les cellules CDs tolérogènes ainsi que la sécrétion systémique de cytokines tolérogènes sont des outils prometteurs d'une prochaine thérapie cellulaire destinée à changer la mémoire immunitaire spécifique.

ABSTRACT

Autoimmune diseases are the consequence of an aberrant response against self-antigen. Currently, therapeutic strategies are based on immune modulators that reduce immunity, with many associated side effects. Treating autoimmune diseases by changing only self-antigen-specific memory T cells represents a promising approach that can be achieved by vaccinating mice under short-term tolerogenic cytokine treatment. We have developed two *in vivo* approaches to change the immune-specific memory.

For the first approach, we used tolerogenic dendritic cells (DCs). These cells naturally present antigens to T cells and also can induce regulatory lymphocytes to establish antigen-specific immune tolerance. We generated a $CD8\alpha^{+}$ DC line constitutively secreting the tolerogenic cytokine IL-27. We chose IL-27 because it was recently shown to induce a strong type 1 regulatory T cell (Tr1) response. A tolerogenic phenotype was characterized for this DC line, comprising downregulation of major histocompatibility complex (MHC) class II and co-stimulatory molecules. The cells were also capable of inhibiting the antigen-specific proliferation of T cells *in vitro*. In a delayed-type hypersensitivity response (DTH) assay, we found that IL-27 DCs were also able to reduce memory restimulation, probably through induction of anergy.

Vaccination of mice with ovalbumin (OVA)-pulsed IL-27 DCs strongly decreased footpad swelling after OVA challenge in the DTH response by inducing antigen-specific IL-10 secretion in the draining lymph nodes in a recall assay. Furthermore, the IL-27 DCs were able to treat the symptoms in the multiple sclerosis mouse model experimental autoimmune encephalomyelitis (EAE). When IL-27 DCs (previously pulsed with the antigen responsible of the disease (Myelin Oligodendrocyte Glycoprotein-35-55 MOG)) and activated with CpG) were injected immediately after the onset of EAE symptoms, they were able to significantly change its evolution, reducing EAE disease scores.

The second approach was based on a subcutaneous cellular implant containing genetically engineered cells that continuously secreted high amounts of IL-27. The implants were inserted in the back of mice and the cytokine was released systemically for weeks. In a DTH assay, the mice with IL-27-secreting implants showed decreased local inflammation and reduced T helper 1 (Th1) response after challenge compared with mice with control implants.

These findings suggest two new possible therapies in which IL-27 contributes to immune tolerance. This study shows that IL-27-transduced DCs and systemic secretion of IL-27 by a subcutaneous cellular implant are promising immunotherapeutic approaches to change established immune memory.

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INTRODUCTION

1. THE INNATE IMMUNE SYSTEM

The innate immune system is generally recognized as the first line of defense. It appeared very early in evolution, and it is shared with all multicellular organisms, including plants, insects, and vertebrates. Most pathogens are physically blocked by a physical barrier (skin, mucus). The skin, for example, is made of several stratified layers of epithelial cells bound together by different cellular junctions.

Once pathogens pass through the skin, they are recognized and phagocytized by specialized cells from the innate immune system. Those cells are, most of the time, bone marrow-derived and have different specializations[1]. They include several types of dendritic cells that will be defined in another chapter (cDC1, cDC2, pDCs, Langerhans cells), monocytes differentiating into DCs and macrophages, and granulocytes[2].

Together, these cell types can identify and destroy most microbes shortly after infection. Identification is based on characteristic motifs of the pathogens called pattern recognition receptors (PRRs; more than 100 PRRs have been identified). Innate immune cells express PRRs, and they are also present in serum. A broad diversity of pathogens-bacteria, viruses, fungi, and unicellular parasites—can be identified by PRRs in the innate immune system[3]. to generate an appropriate response against infecting pathogens. PRRs can identify the vast majority of PAMPs. Equipped with this, each innate lymphocyte can recognize most pathogens. For example, they can recognize the lipopolysaccharide (LPS) that covers the membrane of gram-negative bacteria or the unmethylated CpG DNA motifs (CpG) and double-stranded RNA of viral origin[1]. One group of such receptors are the Toll-like receptors (TLRs). Upon activation, TLRs recruit adapter proteins in the cytosol, leading to a chain reaction that results in an inflammatory response (Figure 1). For example, TLR4 can recognize LPS, TLR3 senses double-strand RNA (of viral origin), and TLR9 senses unmethylated CpG sequences in DNA molecules[4]. When activated, innate cells can alert neighboring cells by secreting cytokines and chemokines. Other receptors of the innate system are danger-associated receptors such as the inflammasomes [nucleotide-binding and oligomerization domain (NOD)-like receptors, NLRs], cytoplasmic receptors [Rig-I-like receptors, (RLRs), cyclic GMP-AMP synthase (cGAS), stimulator of interferon genes (STING)], surface receptors (scavenger receptors, C-type lectin receptors, Fc receptors, complement receptors), and soluble receptors (complement, acute phase proteins)[1,3].

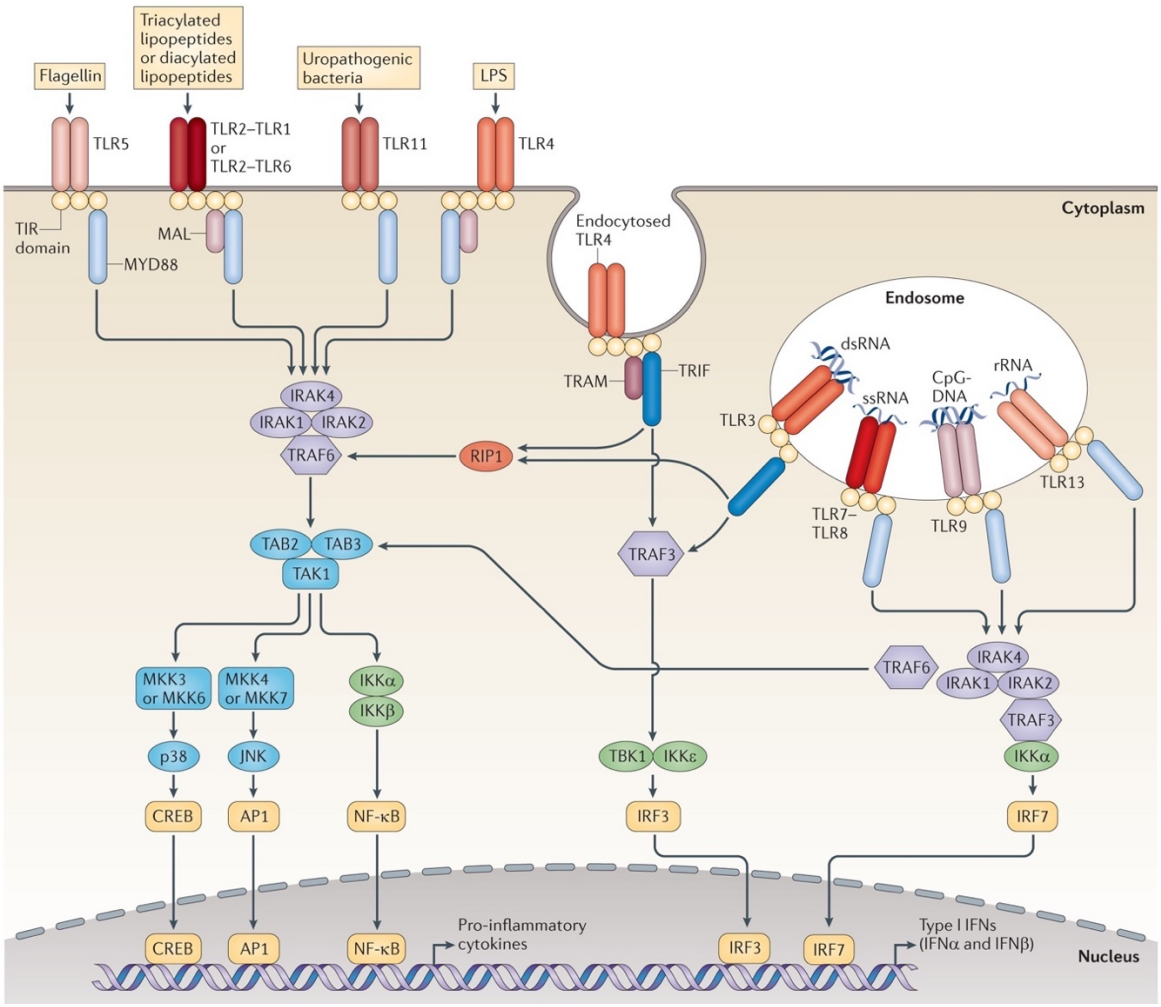


Figure 2: TLRs have different specificity mechanisms and localization. The heterodimer TLR1, TLR2, TLR4, TLR5 and TLR6 are localize on the cell surface, and TLR3, TLR4 TLR7, TLR8, and TLR9 are localize in the endosomes to sense intracellular pathogens. Following the dimerization of the receptor the TLRs engage the proteins MyD88 and MAL or TRIF and TRAM and stimulates downstream signaling pathways that lead to the activation of the MAPKs JNK, p38 and ERKs signalization and to the activation of transcription factors (NF-κB, IRFs, AP-1). TLRs signaling induces pro-inflammatory cytokines like type I IFNs. Adapted from [4].

The other role of the innate immune system is to recruit the adaptive immune system and induce inflammation by creating a local inflammatory environment. By secreting inflammatory cytokines, cells such as DCs or macrophages (called antigen-presenting cells or APC) can migrate and present parts of proteins that can be recognized (antigens) to the T lymphocytes of the adaptive immune system. Activation of the seric complement system can induce inflammation and induce killing of pathogens by opsonization (induction of phagocytosis) or by creation of pores in the membranes (membrane attack component)[5].

The innate immune cells are also able to learn from previously encountered pathogens by a process called innate immune memory[6]. This trained immunity is based on epigenetic modifications

and functional reprogramming. The first cells recognized to have these abilities were myeloid cells and natural killer (NK) cells[6]. Macrophages are also able to learn after a viral infection[7]. Indeed, in the alveolae, macrophages are programmed to overexpress major histocompatibility complex (MHC class II) (MHCII) molecules[7] and increase glycolytic metabolism upon re-stimulation. Effector T cells have been shown to play a role in this process, revealing a constant cross-talk between the adaptive and the innate components of the immune system.

Vaccination with bacille Calmette-Guérin (BCG) against tuberculosis induces T cell-independent protection against secondary infections through metabolic and epigenetic changes in the innate immune cells[8]. This concept raises the possibility of vaccinating populations with BCG to prevent virus epidemics such as COVID-19[9]. It is a promising approach to cancer vaccination and for the treatment of autoimmune diseases. However, more investigations are required to understand this newly discovered mechanism.

2. THE ADAPTIVE IMMUNE SYSTEM

An antigen is a molecular structure that can be bound by a soluble or membrane-bound antigen-specific antibody produced by B cells (BCR) or a T cell receptor (TCR) expressed by T cells. The mechanism of recognition of an antigen (from self or not) is the basis of the adaptive immune response. The ability to learn from the past (memory) and to have an antigen-specific response is an excellent advantage for organisms that have an adaptive immune system. The adaptive immune system is antigen-specific and provides long-term protection by developing long-lasting memory after the initial response, which leads to a better adapted and more specific response when a pathogen is re-encountered (Figure 2)[1].

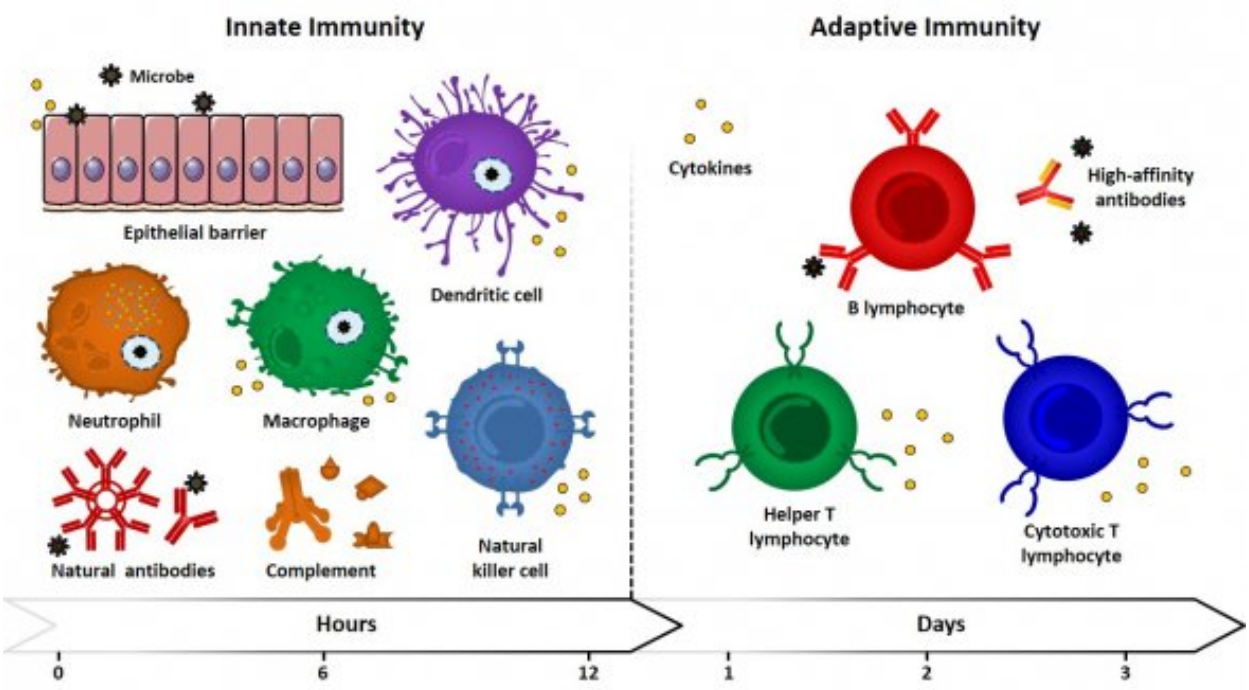


Figure 3 Cells of the immune system. The innate and adaptive immune systems have different characteristics of immune response. Adapted from [10].

2.1. B CELLS

B cells derive from hematopoietic stem cells in the bone marrow and process a long development and maturation. B lymphocytes can secrete antibodies in the bloodstream and in the lymph that recognize specific pathogens such as viruses and bacteria to protect the organism. Upon activation, naïve B lymphocytes can differentiate into long-lived memory cells, antibody-secreting short-lived plasma blasts, antibody-secreting long-lived plasma cells that can produce antibodies life-long in niches in the bone marrow[11]. The surface-expressed antigen receptors of B cells (called BCR or antibody) allows B cells to recognize, internalize, process proteins into peptides and present them to T cells. This presentation activates its endocytosis and later on its presentation to T cells via MHCII. Naïve B cells did not encounter an antigen and can circulate in the blood and the lymph. In the lymph nodes (LNs), they form B cell follicles, and there they screen for cell surface-bound or soluble antigens[11]. When B cells are activated, they can differentiate into different kind of cells: antibody-secreting cells like plasmablasts that are short-lived or into long-lived plasma cells or non-secreting antibody memory B cells. Those memory B cells survive in bone marrow niches or inflammatory foci, and need a cytokine environment and co-stimulations, (usually provided by $CD4^+$ T helper (Th) cells) and the innate system)[12]. The production of antibodies is not the only specificity of B cells they also present antigens to T cells and produce cytokines[13]. Antigen presentations and secretion of cytokines can shape the development of T cells by inducing proliferation and differentiation into effectors of T and B cells. Conversely, some B cells have a

tolerogenic role and can negatively regulate the immune system by secreting tolerogenic cytokines such as IL-10 or IL-35[14]. These cells are called regulatory B cells (Bregs) and will be discussed in another chapter.

Antibodies are localized on the membrane of B cells and can be used as cell surface receptors or can be secreted in body fluids. Most of the antibodies bind to all types of pathogens (like viruses and bacteria) and can inactivate toxins with their agglutination and neutralization properties[1]. Some antibodies can pass the placental barrier and give an immunity to the fetus. Indeed, antibodies are essential to fight pathogens. They recognize secondary, tertiary, and quaternary conformations of proteins, lipids, and carbohydrates. They are antigen-specific because of the variable region formed from the N-terminal part of their heavy and light chains. Antibodies can specifically bind to a pathogen to neutralize it and indirectly prepare them for destruction through opsonization. They can functionally neutralize the pathogen by different ways: they can neutralize viruses by blocking their ability to bind the receptor[15]. Even non-neutralizing antibodies can agglutinate and induce a precipitate to the pathogen. They opsonize the pathogen (even without precipitation) and attract innate immune cells (like macrophages) ready to phagocytize the opsonized pathogen. And they can induce complement to attack the membrane of microbes by direct lysis, opsonization and attraction of immune cells. However, it does not work for budding viruses that are able to protect themselves from complement attack by integration complement-inhibitory proteins in their membrane upon budding[16].

There are five classes of antibodies, called isotypes. The part that characterize their differences are the constant region of the heavy chain that differs between each class. There are well known differences in immunoglobin isotypes between mice and humans. Mice make several isotypes of immunoglobulin A (IgA), D (IgD), E (IgE) and M (IgM), and four subtypes of IgG: IgG_1, IgG_{2a}, IgG_{2b}, and IgG_3. In certain mouse strains (C57BL/6 and NOD) there is no expression of IgG_{2a}, these mice express instead IgG_{2c}[17]. Humans have some differences they express two subtypes of IgA (IgA_1 IgA_2), IgD, IgE, and IgM. There are also four subtypes of IgG called IgG_1, IgG_2, IgG_3, and IgG_4 that are named in order of their abundance in serum.
Even if the immunoglobins of humans and mice have many similarities they have important differences. In human and in mouse IgA is mostly found in mucus and it prevents opportunistic colonization by pathogens and prevent the evolution of the infection. It plays a crucial role in the mucous membranes. Several grams of IgA are secreted each day in the lumen of the intestine. IgAs protect the body against microbes but it also inhibit the inflammatory effect of other immunoglobins in the lumen[18]. IgM are expressed on the surface of all naïve B cells and can be also secreted in a pentameric form. This antibody has high avidity and is the first to be secreted in the early stages of an infection. After the recruitment of IgM an isoclass switching induce the future secretion of other isotypes (without changing the specificity of the antibody)[19]. IgEs are

involved in the fight against parasites and in allergies they can bind to allergens and induce the histamine release from mast cells and basophils. IgDs are never secreted, they are antigen receptors on naïve B cells. When B cells leave the bone marrow to go to the peripheral lymphoid tissues they start to express IgDs (they co-express IgM when they reach a mature state). The activation of IgDs induce antimicrobial, pro-inflammatory and B cell-stimulating programs[20].
IgGs are the antibodies responsible for most of the humoral immune response to pathogens. They are present in a high concentration in the blood and other body fluids. The different IgG subclasses have opposing properties. In humans IgG_1, IgG_2 and IgG_3 can activate the complement. In humans except for IgG_2 all the subtypes have a high affinity for the Fc receptor. Moreover, they have different half-life (IgG_3 has the lowest). In human the other two class IgG_3 and IgG_4 represent together around 10% of the IgGs. IgG_4 is known to be responsible of IgG_4 related diseases like fibrosis[21]. In human and mouse IgG_2 is associated with hypersensitivity reactions and in antibody-mediated autoimmune diseases.
An effective immune response against an antigen induce the secretion of a mix of the subclasses. A temporal model has been proposed[22].Early in the immune response IgG_3 and IgE are secreted. With its low affinity IgG_3 joins the IgM mediated defense against foreign antigen. After, IgG_1 and IgG_2 that have a higher affinity are secreted. If the threat is still present IgG4 are produced and help to dampen the inflammation (they don't induce FCR mediated processes)[22]. This model is helpful to determine the strength of the inflammation by the balance of these subclasses. However, the temporal model has some limitations, for example pathogens can disturb the balance of immunoglobins[23].
The germinal center is the site of the clonal expansion of B cells but also the place of affinity selection and where the B cells switch their isotypes[24]. B cells have the ability switch the isotype of their immunoglobulins, for example, from IgMs to IgGs. The constant region of the heavy chain of the antibody changes, but the variable regions (that binds to the epitope) remains the same. The antigen specificity is therefore unchanged but the new immunoglobin can interact with other effector molecules like Fc receptors and complement. Cytokines can induce class switching; for example, interferon IFN-γ can induce the class switching to IgG_2, and IL-4 can induce the switching of IgG_1 and IgE. In mice and in humans, TGF-β induces the switching to IgA. In human but not in mouse, IL-10 was shown to induce the switching to IgG_1 and IgG_3[25].
After the repeated exposures with the same antigen, activated memory B cells produce antibodies with increased affinity by a process called affinity maturation. Some programmed mutations on the variable regions of immunoglobulin genes occur. This process is called somatic hypermutation (SHM). This cellular mechanism allows immune cells to adapt to a new foreign element. This process is extremely regulated because it can lead to B-cell lymphomas[26]. A lot of mutations on the variable part (the one that binds to antigen) of immunoglobulin genes can alter and sometimes increase the affinity of antibodies. B cells that experienced SHM are selected by

their affinity to the antigen by follicular dendritic cells. Some of those mutations can also lead to B cells secreting self-reactive antibodies that need to be rapidly countered to avoid autoimmune diseases[27].

Follicular dendritic cells are stromal cells that reside in primary follicles and in germinal centers of secondary lymphoid organs. Follicular dendritic cells of the germinal centers present antigen to B cells. They form a large network allowing them to interact with B cells. Affinity maturation is the direct results of SHM and is selected for by competition for limited antigens presented to the B cell's surface Ig's by follicular dendritic cells, antigen processing in the B cells and presentation of the peptides together with MHCII to follicular T cells. The ensuing signals allow them to survive. If cells can grab enough of the antigens and present the processed peptides through MHCII they can survive due to the help by follicular T cells (TFH). The B cells that have the highest affinity have therefore the highest competitive advantage to capture sufficient antigen with their high-affinity surface Igs and hence present it to follicular T cells specific for these peptide/MHC complexes to receive a survival signal. B cells with a low affinity for the antigen cannot present sufficient peptides to TFH to receive help from TFH. This positive selection proceeds through several rounds of affinity selection and results in B cells secreting antibodies with an increased affinity[28,29].

2.2. T CELLS

Two main differences exist between T and B cell responses. First, T cells recognize a small fraction of the protein (peptide) presented by an APC (which is usually a DC or a B cell, sometimes a macrophage) by an MHC molecule. They require presentation by an APC to differentiate, whereas B cells can recognize the intact protein. Second, when they are activated, effector T cells need to go to the site of infection, whereas differentiated B cells can secrete antibodies from far away. T cells begin their development in the bone marrow but migrate to the thymus to mature (T indicates thymus). Naïve T cells (Tn) can be found in the blood, peripheral lymph nodes, Peyer's patches, and spleen. The activation of Tn generates a cascade of phosphorylation events, and many transcription factors activate the genes involved in differentiation and proliferation. An important cytokine in this process is IL-2, which induces the proliferation, survival and expansion of T cells by binding with its heterotrimeric receptor, called the IL-2 receptor. The cells also increase expression of different chemokine receptors and integrins that allow them migrate to the inflamed sites to fight the infection in place. Two classes of T cells can be distinguished by their expression of two surface molecules: CD4 (for helper T cells) and CD8 (for cytotoxic T cells).

CD4 T cells (helper T cells) can change the activity of other cells by the secretion of cytokines and by co-stimulation. They can increase suppress or regulate immune responses. They can increase the affinity to MHCII. When they are activated, they can help other cells by secreting

specific cytokines and displaying different surface molecules (Figure 4). They can differentiate into different types of T helper cells, controlled by lineage-specific transcription factors (Th1, Th2, Th17, and regulatory T cells (Tregs)). They differ mainly in the type of transcription factors they express and in the cocktail of cytokines they secrete[30]. Due to the epigenetic changes these subsets are mainly stable and hereditable but have some room for phenotype plasticity[31].
The activation of a naïve $CD4^+$ T cell with IFN-γ and IL-12 induces the STAT1 and STAT4 pathways, expression of the transcription factor Tbet, and production of IFN-γ. These activated cells are called Th1 cells. Th1 cells promote the activation of macrophages and increase the cytotoxic function of NK cells and CD8 T cells, and start the isotype switch toward IgG2a in B cells. The Th1 response is well suited to fight intracellular pathogens but it can also be responsible for autoimmune diseases[32].
The Th2 response promotes clearance of parasites (e.g., helminth infections). Th2 cells express the transcription factor GATA3 and secrete the cytokines IL-4, IL-5, and IL-13. Th2 cells are differentiated by IL-4, which induces activation of STAT6. Th2 cells increased the isotype switch toward IgE and IgG_1 in B cells. They are also responsible for allergic reactions[33].

The Th17 response was more recently described; Th17 cells got their name because they secrete the proinflammatory cytokine IL-17. They develop from a naïve $CD4^+$ T cell activated in the presence of transforming growth factor (TGF-β), IL-1, and IL-6[34]. After activation of STAT3, Th17 cells begin to express the transcription factor RORγT. By recruiting neutrophils, Th17 cells play a significant role in fighting extracellular pathogens. This response has been shown to be responsible for several autoimmune diseases, such as multiple sclerosis, rheumatoid arthritis, and inflammatory bowel disease[35].

In 2008, Stockinger et al. proposed the Th9 response. Th9 cells produce IL-9 and are mainly involved in defense against helminth infections and tumor suppression. Th9 cells derive from naïve T cells but they can also come from differentiated Th2 cells[36]. They are associated with pathological allergy responses and some autoimmune diseases[37].

Th22 cells are the source of IL-22 and are located at sites of infection and in a range of inflammatory diseases[38].

Follicular helper T (TFH) cells represent a group of CD4 T cells that have prior experience with an antigen. They are found in the B cell follicles of secondary lymphoid organs and express the chemokine receptor CXCR5. They maintain the germinal center through different mechanisms such as the secretion of IL-21. They have an essential role in the selection of high affinity B cells (selecting affinity maturated cells) and, therefore, select plasmocytes and immune memory. They negatively select B cells responsible for autoimmune diseases[39]. One additional role is the prevention of antibody-mediated autoimmune diseases such as lupus can promote by Breg and

prevent systemic lupus erythematosus[40]. A dysfunction of those cells can be central in the formation of this disease.

The CD4 subset of T cells generally does not follows a definitive lineage. A Th with no strong epigenetic modifications that fixed their fate can change its Th lineage. A CD4 T cells retains plasticity that enables them to adapt to different environments. The ability to adapt their functions in response to changing circumstances is a great advantage for protective immunity and immune control[41]. The environment can drive T cell reprogramming, through a cytosolic, metabolic and epigenetic signaling to change the established mechanisms that stabilize polarized T cell functions. T cell plasticity is observed in different pathologies such as autoimmune disorders, and may contribute to disease pathology[41]. For example patients with type 1 diabetes have a reduced stability of Foxp3 expression in Treg cells[42]. Moreover in the mouse models of inflammatory bowel disease the transition of Th17 cells to a mix of Th17-Th1 or to a fully differentiated Th17 cell phenotype is necessary to drive the disease[43]. The Th17 subtype has the highest plasticity; Th17 cells can dedifferentiate into all subtypes under different types of stimulation. For example, IL-12 and IL-23 induce differentiation from Th17 to Th1, whereas IL-4 induces primarily the switch from Th17 to Th2[44]. Transcription factors and cytokine can drive reprogramming, but they must have the epigenetic circuitry to allowed these changes in the cell. A fully differentiated epigenetically fixed Th cell has very little ability to change their lineage[41]. T cell plasticity is an important factor in autoimmune disease, and the capacity to control their re-programming is a promising axis of research for new therapies.

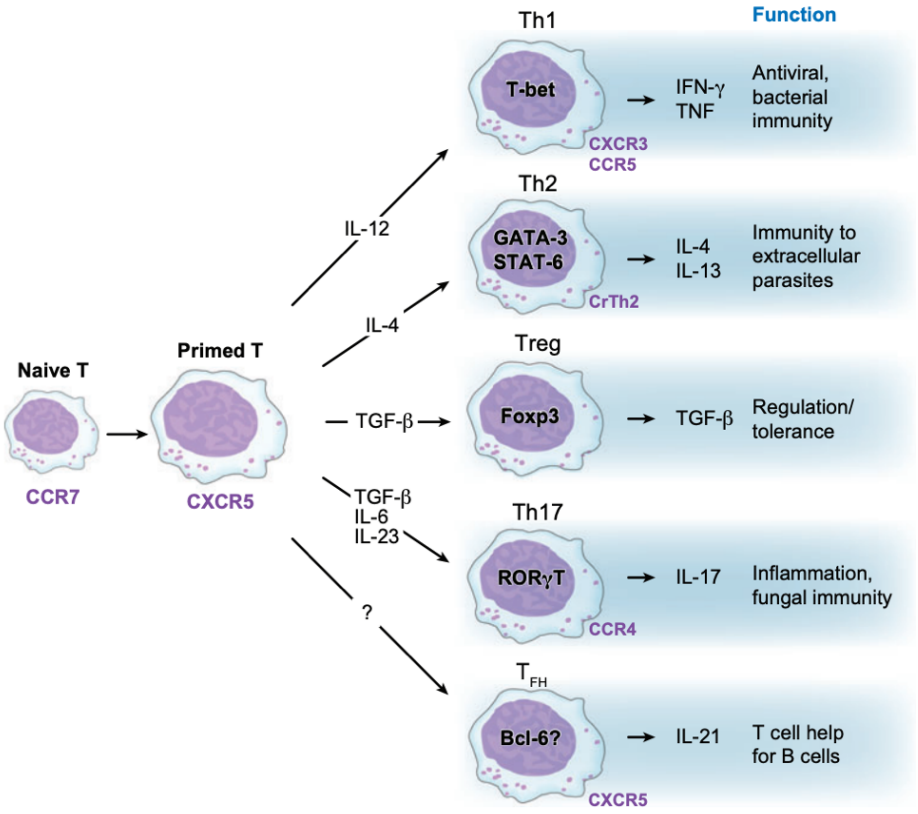


Figure 4 Different CD4 subsets and their major functions. Depending on the signals provided by the APC, different T effector phenotypes develop. Adapted from[39].

Cytotoxic T cells are T lymphocytes also called CD8 T cells express CD8 and can kill cancer and infected cells. CD8 T cells express a TCR protein that binds with CD8 to MHC class I (MHCI), which presents endogenous peptides[1]. Those peptides are generally from self but can also be tumor-associated, tumor-specific, or from intracellular pathogens. If the peptide is foreign and therefore has not induced immune tolerance, the T cells are activated and differentiation into memory and effector T cells is initiated. Then, the proliferation starts very quickly and the upregulation cytotoxic factors (such as perforin and Granzyme A) and the secretion of IFN-γ and TNF-α[45].

CD8 T cells are known to induce cell death by two pathways. Either through the binding of Fas to the Fas ligand (FAS-L) or, through other signaling, they can induce apoptosis. They can also kill directly the cell by releasing pore-forming and cytotoxic molecules such as perforins and entry of granzymes in the cytoplasm of the target cell inducing the apoptosis. Perforin perforates target cells and granzymes enter the cells via the pores and induces apoptosis through cleavage of different caspases. $CD8^+$ T cells can secrete cytokines such as IFN-γ, tumor necrosis factor (TNF-α), and IL-10. CD8 Tregs regulate immunity by, for example, secreting the tolerogenic cytokine IL-10 or by negative co-stimulation[46].

3. DENDRITIC CELLS: THE BRIDGE BETWEEN THE INNATE AND ADAPTIVE IMMUNE SYSTEMS

Dendritic cells (DCs) are essential cells of the immune system because of their ability to shape the adaptive immune system by direct contact, co-stimulation, and secretion of cytokines (other APCs also have this ability). This interaction can lead to the differentiation of pathogen-adapted effector and memory cells. DCs are located in the periphery, as well as in primary and secondary lymphoid organs in an immature state, filtering their environment for pathogens[47]. The functional activities of DCs mainly depend on their state of activation and state of differentiation. The classical view, that activated DCs are the main inducers of T effector cells, and that immature DCs are the inducers of tolerance is still debated[47]. Notably, because some observations have proven that some DCs (called tolerogenic DCs) can induce tolerance upon activation even after activation[48]. They can be activated by PRRs when they sense PAMPs; for example, when TLR9 is triggered by unmethylated CpG dinucleotides[49].

Activation of DCs changes their phenotype rapidly. They start to upregulate surface molecules able to present antigens and activate lymphocytes. They also induce the appropriate response by secretion of a specific cocktail of cytokines to fight the invading pathogen preferentially[2]. Together, DCs, macrophages, and B cells are professional presenting cells (APC). DCs derive, as do all blood leukocytes, from hematopoietic stem cell precursors. They originate from common myeloid progenitors in the bone marrow. The growth factor FLT3 ligand (FLT3L) is necessary to induce differentiation of DCs. Binding of FLT3L with its receptor (FLT3) activates a signaling cascade that commits a cell to the myeloid lineage. These common myeloid progenitors are the precursors of macrophages and DCs[2]. Differentiation to the DC subsets occurs in the periphery through multiple steps.

There are many different types of DCs; the two main subsets are plasmacytoid DCs (pDCs) and conventional DCs (cDCs). pDC is a rare cell type that specializes in the antiviral response. When pDCs are activated, they secrete large quantities of type 1 interferon[50,51]. They circulate in all the tissues but mainly reside in the periphery. They also have a substantial role in the maintenance of tolerance. Indeed, without activation, they induce tolerance by inducing deletion of CD8 and anergy (functional inactivation) of T cells[52].

The cDCs are different and considered as the classical DCs. They express a high level of intracellular MHCII when immature that gets to the surface immediately after activation and CD11c. This group of cDCs is highly heterogeneous[53]. Some of them are called cDC1 cells (Figure 5) depend on the transcription factors BATF3 and IRF8[54]. They have potent antitumor activity through their specialization to activate CD8 T cells. They can also, however, induce CD4 T cell differentiation. Upon activation, they can secrete large quantities of IL-12, which gives them the

ability to preferentially produce a Th1 response. In the spleen, they represent about 30% of DCs[2]. They are known to respond synergistically when they are activated simultaneously with TLRs, with signaling through MyD88 and TRIF, respectively[55]. cDC2s are characterized by the transcription factor IRF4. They express the markers CD11b and CD172a on their surface and represent the largest population of DCs in the spleen (Figure 5). They mostly specialize in activating the CD4 T cell response[56,57] but also have an essential antitumor immune function in their ability to prime CD4 T cells and to migrate in the tumor[56].

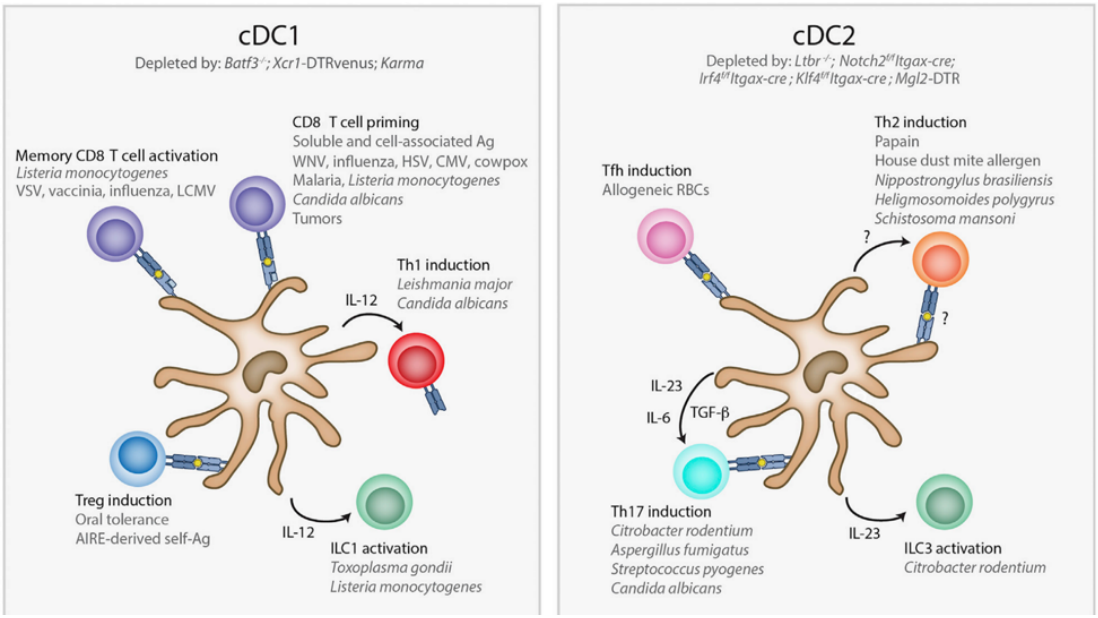


Figure 5 Different functions of conventional (c)DCs in the immune response. cDC1 cells direct Th1 immune responses to viruses and intracellular pathogens (left panel). They secrete IL-12 to induce the Th1 response. cDC1 cells can also induce Tregs against orally fed antigens and autoimmune regulator (AIRE)-dependent self-antigens expressed in the thymus. cDC2 cells are specialized to regulate Th2 (type 2) responses to parasites (right panel). cDC2s also control responses to extracellular bacteria and fungi (right panel) by activating Th17. cDC2s induce the T follicular helper (Tfh) cells that regulate the germinal center response and induce the Th17 response. Adapted from [58]

DCs generally remain in peripheral tissues and in primary and secondary lymphoid organs. When cDCs are activated by pathogens, they upregulate the CC-chemokine receptor 7 (CCR7), a chemotactic receptor expressed in many cells of the immune system but also in nonimmune cells. Together with its ligands (mainly CCL19 and also CCL21), CCR7 is involved in the homing of immune cells, inducing DCs to travel from the periphery through the lymphatic system to the draining lymphoid organs, where they present antigen to T cells. Many DCs reside in lymph nodes. They remain in the lymphoid organ to screen for pathogens entering via the lymph. cDCs undergo high turnover; the half-life of cDCs under nonpathological conditions varies between 3 and 7 days[59].

Some DCs are tolerogenic (tol DCs). Tol DCs are naturally present in the body; they prevent the development of autoimmune diseases and modulate inflammation to prevent immunopathology by different processes[60]. Immature DCs display tolerogenic features: low expression of MHC (class I and II) and low expression of co-stimulatory molecules (CD40, CD80, CD86). Immature DCs are those that have not yet been activated by PAMPs, and they reside mostly in peripheral organs and a bit in secondary lymphoid organs. Because of their localization and absence of sufficient pathogen-MHC complexes (co-stimulation molecules and inflammatory cytokines), T cells do not become activated. They play an essential role in inducing peripheral tolerance by presenting self-antigens with little co-stimulation (co-stimulatory molecules and inflammatory cytokines). When T cells become activated with low co-stimulation, negative co-stimulation, or tolerogenic cytokines, T cells die, become anergic or tolerogenic[61]. Only when DCs present high levels of co-stimulation, MHC-peptide, and secrete a high level of cytokines an efficient immune response ensues.

Tol DCs promote tolerance by subverting effector and memory T cells via distinct mechanisms, including T cell anergy, by inducing the expression of inhibitory receptors such as LAG-3, Tim-3, and TIGIT[62]. In another context, they can influence the apoptosis of memory T cells via expression of IDO[63]. Most of the time, tol DCs express high levels of programmed death-ligand 1 (PD-L1), which interacts with PD1 on T-cells. This interaction is a major promoter of cell death in effector T cells and inhibits apoptosis in Tregs[64]. One of the most interesting functions of tol DCs is the induction and expansion of several Treg and Breg populations[14,65,66]. One of the main mechanisms of tolerance is the secretion of tolerogenic cytokines such as IL-10, IL-27, and IL-35. Active TGF-β signaling can be required for the maintenance (and induction) of tol DCs[67] to exert regulatory functions. However, other cytokines such as IL-10[68], IL-27[69], or IL-35[70] can also induce tol DCs with strong and stable properties. Some compounds, such as vitamin D_3[71], can actively induce a tolerogenic phenotype on DCs. The development of therapies based on tol DCs to treat autoimmune diseases is currently the focus of much research and will be further discussed.

However, the mechanism that governs tol DC functions in the organism after their maturation under steady-state conditions remain unclear. Currently, tol DCs are considered to exert their function by inducing T cell apoptosis, anergy, and Tregs. They also have a stable phenotype even in inflammatory conditions. They generally do not respond to a stimulus[72] and secrete a larger quantity of tolerogenic cytokines such as IL-10 and IL-27[61]. Immature DCs, in contrast, induce more anergy by the lack of co-stimulation[47,73]. Their state is also less stable; they can become immunogenic under inflammatory conditions[61]. However, no studies have compared the tolerogenic potential of immature DCs and specialized tol DCs. It can be hypothesized, that tol DCs would induce more tolerance than immature DCs *in vivo*. Indeed, tol DCs secrete more tolerogenic cytokines and induce actively the tolerance. A better understanding of the mechanisms and

transcription factors involved is needed to understand the diversity of tol DCs and to determine which have the best tolerogenic potential.

4. THE ESTABLISHMENT OF IMMUNE MEMORY

4.1. SPECIALIZED MEMORY CELLS WITH DIFFERENT CHARACTERISTICS

After antigen exposure during primary infection or after vaccination, APCs activate naïve (Tn) and memory T cells (Tm). The recognition by a Tn clone of its cognate antigen results in the proliferation and differentiation into effector and memory cells. Tn populations can expand rapidly until they reach a limiting number. One cell can proliferate to reach 10^5 cells. Once the reaction is over, most of the effector cells die by apoptosis, and those that survive have differentiated into memory cells. These cells are present at a high frequency and help to increase the immune response when the body is re-exposed to the same pathogen or antigen. Memory cells have a lower activation threshold than naïve T cells; that is, they need fewer co-stimulation signals to differentiate and proliferate[74].

The concept of systemic memory was redefined by the division of circulating memory cells into subsets, independently from their CD4 or CD8 expression. Their ability to proliferate and migrate into different organs differentiates them further into various categories. Surface markers for these diverse populations are complex and many subpopulations have been described[74]. For this reason, we will simplify the marker distribution and discuss more details below. Central memory (Tcm) T cells, like naïve T cells, express CD62L (also called L-selectin) and the chemokine receptor CCR7, whereas effector cells (Te) are negative for both. Peripheral memory cells (Tpm) generally do not express CD62L or CCR7. They reside in peripheral organs and can proliferate in the recall response[75,76] (a summary of memory cells is given in Table 1). Another subset of memory cells called effector memory cells (Tem) is similar to Tpm because they are also in the periphery and they do not express CD62L[77,78]. However, Tpm and Tem can be differentiated by their level of CX_3CR1 or other markers that will be later described (Table 1).

If cells are Tcm or Tn, they can circulate and travel to the lymphoid organs because of the presence of CD62L and CCR7; these cell surface molecules are required for rolling and activating integrins allowing tight adhesion on high endothelial venules in secondary lymphoid organs such as the spleen, lymph nodes (LN), and Peyer's patches. Naïve T cells that have not yet encountered their antigen, need to enter the LN because a tiny percentage of T cells are specific for one microbial antigenic peptide. Tcm lymphocytes have encountered antigen and express CD62L and CCR7. Therefore, they also localize in secondary lymphoid organs (and are able to recirculate in the bloodstream). Effector cells (Te) cells do not express CD62L and they can move to peripheral

tissues, depending on their expression of integrins and chemokine receptors; they have a short half-life compared to other cell types and divide in a great extent. Tissue-resident memory T cells (Trm), on the other hand, are cells that stay only in the tissues and can self-renew; they do not express CD62L or CCR7. CD44 is another cell surface marker that helps to distinguish naïve from memory and effector cells. It is involved in cell–cell interaction and is upregulated when a lymphocyte is activated. CD25 is one of the three chains of the IL-2 receptor that is highly expressed on cells that are proliferating and Tregs. These four markers (CD62L, CD44, CCR7, CD25) are very useful for differentiating Tpm, Te, Tcm, Tem, and Tn cells[79].

Tcm cells are commonly found in the LN and peripheral circulation because of the surface markers CD62L and CCR7. Most Tcm produce large amounts of IL-2 and proliferate extensively upon activation. They can self-renew, mainly because of the high level of phosphorylation of crucial transcription factors. Tcm have a higher capacity to persist *in vivo* than effector memory cells[80]. They are more efficient in mediating protective immunity because of their increased proliferative potential. When a naïve T cell and a Tcm encounter an antigen through presentation by an APC, they do not respond in the same way. First, upon antigen encounter, there are many more Tcm than naïve T cells, which increases the chance of encountering their antigen via an APC (and to proliferate and differentiate into memory and effector T cells). This explains the increased amplitude and speed of the response to a challenge. Second, activated Tcm have increased expression of CXCR3 and several other chemokine receptors, allowing them to efficiently travel from the LN to the site of infection[81,82]. However, overexpression of CXCR3 is not necessary to leave the intrafollicular region of the LN. Some cells, including CD8 T and Th1 and Th2 cells, increase expression of chemokine receptor CXCR5, which enables them to migrate to B cell follicles once arrived in LNs[83]. Naïve T cells also increase the expression of chemokine receptors specific for CCL3 and CCL4 when they are activated, but not as efficiently and not as fast as Tcm.[84] When a naïve T cell is primed, it also reorganizes the intranodal DC network, showing the constant dialogue between DCs and T cells[84].

It is more difficult to strictly define $CD62L^-$ memory cells because these have different phenotypes and functions. Tpm and Tem preferentially reside in the periphery because of their lack of CD62L and CCR7. They are less proliferative but differentiate into effector T cells (Te) much more rapidly[75,85]. Because of their increased need for energy, activated Te increase aerobic glycolysis (in contrast to Tcm and naïve cells that are more anaerobic) by increasing the number and efficiency of their mitochondria to generate large quantities of Nad(P)H and ATP[86]. Those cells can express CXCR3 or CX_3CR1 to enable migration to the infection site[48].

CD4 and CD8 Tpm and Tem have the same self-renewal and proliferative properties as Tcm when they are activated. However, they do not have similar functional activity (IL-2 secretion, cytotoxicity, and secretion of cytokines). Interestingly, they might have the same migration pattern from

lymphoid tissues to the periphery[87]. In 2016, Tpm was identified by its intermediate level of expression of CX_3CR1, a chemokine receptor[87]. In mice infected by virus, Tpm cells were more likely than other memory cells to migrate to the peripheral tissue. Moreover, they seemed to have better proliferative capacity than Tem and seem to be able to be involved in the expansion of Tcm[87]. Circulating Tpm cells might be one of the principal sources of the tissue-resident memory T cells (Trm) in local sites[88]. It has been shown that the number of Trm in lung tissue is correlated with the number of circulating memory cells[88]. Those data suggest that circulating memory and effector cells, rather than naïve cells, are the main precursors of Trm. The difference (based on the level of CX_3CR1) between Tpm and Tem has been well described in CD8 T cells. However, CD4 T cells have different families (Th1, Th2, Th17) that have their own regulation[89]; their differentiation is more nuanced and will be discussed in the next chapter.

Indeed, Trm can reside permanently in peripheral tissues. They can self-renew and stay stably in tissues in the absence of antigens[78]. However, a recent publication reported $CD4^+$ Trm cells circulating between blood and the lymph by bloodstream[90]; thus, the principle of the residence might not be so strict, these cells might circulate more than it was initially though. When a Trm re-encounters an antigen, it alerts neighboring cells by releasing proinflammatory cytokines. The local tissue slowly differentiates into an “unwelcome for pathogens” state, and the cells start to activate other T cells polyclonally and NK cells by the secretion of inflammatory mediators. When these cells are activated, they produce cytokines, depending on their cell type, to promote local maturation of DCs and recruit innate immune cells from other tissues. It is the Trm cells that accelerate the local response against a re-infection[91]. They work in close association with local macrophages[92]. Trm cells express transcription regulators that are shared with both Tcm and Tem (Blimp1, Erg2, Runx3, and Nr4a1)[93]. Trm can also target self-antigens and lead to the development of autoimmune diseases like rheumatoid arthritis or psoriasis[94]. Recently, a role of Trm in multiple sclerosis has been identified. Patients with different stages of lesions had many CD8 Trm in the central nervous system that were highly reactive to specific antigens and sustain an inflammatory response[95].

To protect the organism against aberrant immune responses, a group of cells called memory regulatory T cells (Tregs) are present in the periphery and are antigen-specific[65]. These will be discussed later in the tolerance chapter.

Type of cell	Characteristic	Location	Properties
Tn (naïve T-cell)	$CD44^{low}$, $CD62L^{+}$ $CCR7^{+}$, $CD25^{low}$	Spleen, blood, lymph nodes	Multipotent, high proliferative capacity
Te (T effectors)	$CD44^{++}$, $CD62L^{-}$, $KLRG1^{(low\ or\ high)}$, $CD25^{high}$, CX_3CR1^{high}	Spleen, blood (some in lymph nodes), tissues (skin, lungs…)	High effector function, weak proliferative capacity
Tcm (T central memory)	$CD44^{+}$, $CD62L^{+}$, $CCR7^{+}$, $KLRG1^{-}$, $CD25^{low}$, CX_3CR1^{-}	Lymph nodes, spleen, blood, bone marrow	Self-renewal ability, intermediate effector function
Tem (T effector memory)	$CD44^{++}$, $CD62L^{-}$, $KLRG1^{(low\ or\ high)}$, $CD25^{low}$, CX_3CR1^{high}	Peripheral tissue, blood, spleen	Self-renewal ability, intermediate effector function
Tpm (T peripheral memory)	$CD44^{+}$, $CD62L^{-}$ (mostly), $KLRG1^{low}$, $CD25^{low}$, CX_3CR1^{inter}	Peripheral tissue, blood, spleen	Self-renewal ability, intermediate effector function; better motility
Trm (T resident memory)	$CD44^{++}$, $CD62L^{-}$ $KLRG1^{low}$ CX_3CR1^{-} $CD25^{low}$	Tissue (skin, gut, mucus, glands)	Self-renewal ability, intermediate effector function
Treg	$CD44^{+}$ $CD62L^{-}$ $CTLA4^{+}$ $Foxp3^{+}$ $CD25^{+}$ CX_3CR1^{-} $CD25^{high}$	Peripheral tissue	High immunosuppressive capacity

Table 1 Characteristics of memory cells.

As discussed, these T cells differ in their migration patterns. Depending on their expression of CD62L and CCR7, naïve T cells and Tcm can circulate between blood and the T cell zones of secondary lymphoid organs and back to the blood via lymph. In contrast, Tem can enter peripheral tissues and reach the bloodstream again via lymph passing via the sinuses into LNs (without entering the T zone) and re-entering the bloodstream via the lymphatics. Trm are in the tissues and circulate very rarely in the blood[96].

4.2. CD4 MEMORY T CELLS CAN CHOOSE THE FLAVOR OF THE IMMUNE RESPONSE

We have seen that T helper cells are essential to choose the "flavor" of the response to an infection. A naïve CD4 can differentiate into Th1, Th2, Th17, or Treg cells in a memory or an effector state. $CD4^+$ T cells have the same ability as $CD8^+$ T cells to retain memory of previous antigenic encounters. In humans and mice, CD4+ Te and Tcm differ in their capacity to proliferate and produce cytokines *in vitro*[97]. Both cells have high sensitivity for TCR-mediated proliferation. However, the cells have been shown to produce more effective Th1- and Th2-specific cytokines, and activated Tcm secreted more IL-2. The very high diversity in CD4 T cells complicate their study, and two or three cell surface markers are not sufficient to identify different memory populations.

Memory CD4 T cells can come from naïve cells or from $CD4^+$ T cells that remember the profile (Th1, Th2) from their previous effector states. The qualitative composition of the $CD4^+$ T-cell memory cells is then determined during the primary response. A naive T cell primed by a DC in the LN is influenced during and after clonal expansion to become Th1, Th2, Th17, or induced Treg (iTreg) by the early cytokines secreted by the innate immune response and the antigen-presenting DC. The cell fate of effectors is determined by the type of cytokines present in the environment and by the type of interaction with APCs. After the effector phase, most effector cells die by apoptosis. A theory suggests that the survivors give rise to most of the Tpm population, whereas Tcm derives mostly from the lineage of naïve cells[89]. According to this hypothesis, the "flavor" is remembered by the Tpm cells that encountered the pathogen, and the strength of the proliferation (IL-2) is remembered by the CD4 cells in the lymph nodes.

4.3. THE TWO LINEAGE MODELS BETWEEN EFFECTORS AND MEMORY CELLS

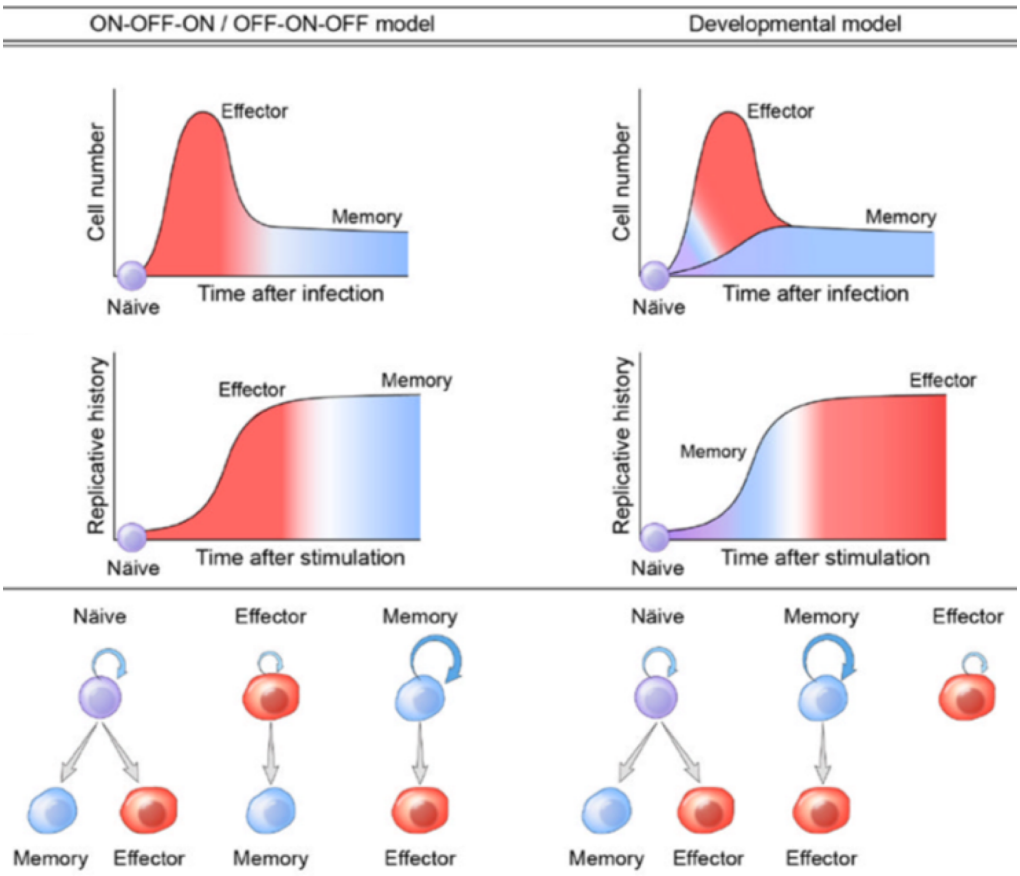


Figure 6 Two dominant competing models of T cell differentiation. The conventional model ("on-off-on" model) in which all memory cells are derived from effector cells (left panel) and the developmental model where memory cells arise directly out of naïve precursors without going through an effector stage (right panel). Adapted from [98]

The state of memory is characterized by a readiness to respond quickly to a re-challenge. After re-infection, the differentiated memory cell subsets (

Figure 6) are sharply increased in numbers and react more rapidly with fewer co-stimulation requirements. Just after the infection, the specific T cells become effector cells and, after the clearance of the pathogens, some cells become memory cells for a long time. But on a single-cell level, predicting and explaining the fate of a T cell is very complex.

Several models for differentiation have been proposed. In the dominant model for $CD8^+$ T cell differentiation, the memory population is differentiated from the effector population by their fitness or avidity for the pathogen[80]. This model implies that all memory cells originally derive from an effector cell and that the most efficient ones can kill pathogens that are capable of giving memory cells. It also implies that the phenotypical changes of effectors are not definitive.

Another model, the developmental model, originated from the hypothesis that an effector cell is in a terminally differentiated state and can no longer differentiate into a memory T cell. An effector T cell can only perform effector functions or die after initial strong proliferation. Only naïve cells can change into effectors and memory cells upon activation. This model relies on the concept of the pluripotency of stem cells that can maintain themselves, and a change into an "effector" state implies the loss of pluripotency. In this model, memory T cells have experience with the antigen but not with the inflammatory environment of the infection site. To become an effector, a T cell has to pass through drastic epigenetic modifications such as DNA methylation and histone modifications that regulate the chromatin. Those modifications can activate and silence many different genes. Effector $CD8^+$ T cells can epigenetically silence genes responsible for longevity and plasticity through methylation and chromatin modifications via the polycomb repressive complex 2-mediated chromatin repression[99]. Memory precursors do not show those changes. Many reversible chromatin modifications can be made to become more stable[100].

Initially, the classical model was intensely debated because differentiation into effector T cells showed many transcriptional changes that are not reversible[101], including the length of telomeres and radical transformation of telomerase activity[102,103]. The idea that a fully differentiated effector cell would have a pluripotent potential is difficult to imagine. Second, the inflammatory environment and activities of perforins and granzymes can be toxic for the cell, and it is difficult to believe that a "fresh" memory cell could differentiate from an effector T cell. The developmental model is also consistent with different experimental data. First, memory T cells can differentiate into both memory and effector T cell subsets, but fully differentiated effector cells are not able to differentiate into memory T cells *in vitro*.[76] The repeated stimulation of specific T cells does not imply an increase in memory cells *in vivo*[104]. However, the classical model was validated by a large amount of experimental data on memory and effector T cells.

As explained before, memory and effector cells are divided into different subsets based on expression of CX_3CR1. It was shown that effectors with intermediate and low expression of CX_3CR1 were able to increase the expression of CD62L and CCR7 and start showing a Tcm phenotype[87]. Effectors do not seem to have a fixed fate; they also seem to have—on the single cell level—the ability to convert into memory cells under certain conditions[105,106]. Other experiments on CD8 T cells have shown that cells can be classified using their KLRG1 expression levels. KLRG1 is an immune checkpoint expressed predominantly on late-differentiated effector T cells[107] (Figure 7). When effector cells are not fully differentiated, they do not yet express KLRG1, but they can still become a memory cell with highly protective potential memory. A transcriptional repressor, Bach2, can stop the terminal differentiation into Te and promote plasticity between the effector state and the memory state[108,109].

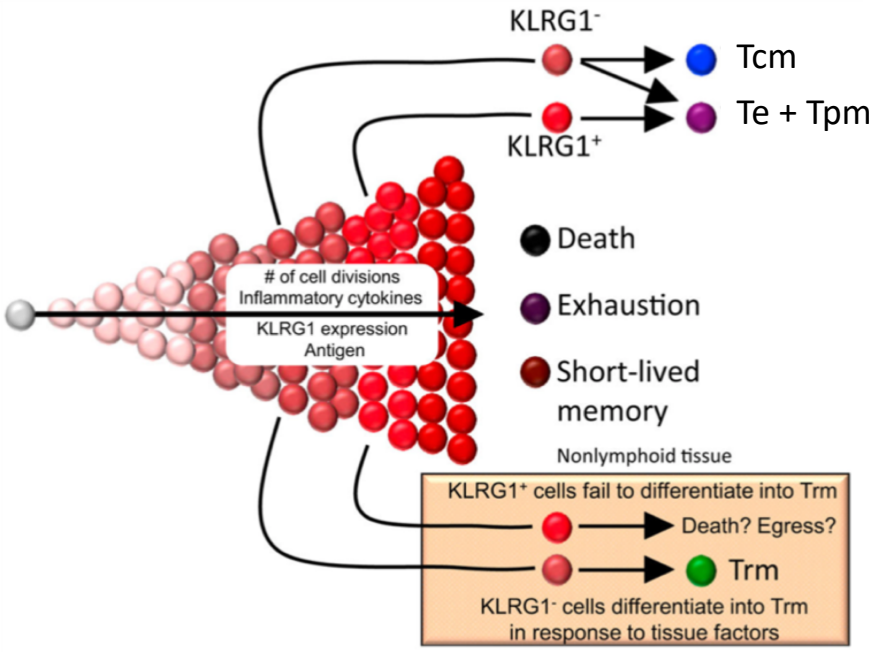


Figure 7 Memory T cell differentiation based on KLRG1. Tcm and Trm cells derive partially from KLRG1⁻ precursors in mice and are less terminally differentiated. KLRG1⁺ T cells are more differentiated and have greater proliferation and effector potential. Numerous divisions and effector functions result in apoptosis or exhaustion. Adapted from [96].

The ON-OFF-ON model and the developmental model are not reconcilable. Recent studies indicate that even if T cell fate was an extremely regulated process, there is still room for plasticity when an effector cell is not fully differentiated and the chromatin is not stably modified. This then raises the question of how immune memory could be manipulated for a vaccination or to treat autoimmune disease.

To change immune memory, it is therefore essential to differentiate long-term memory and short-term memory. Each pool differs extensively by the types of receptors present on the surface[86,90,] and by other characteristics. The long-term memory pool is less sensitive to changes because they present fewer chemokine receptors and are therefore less susceptible to change its state into a Treg or an exhaustive cell[111].

5. IMMUNE TOLERANCE

5.1. CENTRAL TOLERANCE

Before their release into the periphery, T lymphocytes need to differentiate in the thymus and B cells need to differentiate in the bone marrow. During maturation of T lymphocytes, double-positive precursor cells (i.e., that express both CD4 and CD8 molecules) migrate from the bone marrow to the cortex of the thymus, where they rearrange their T cell receptors. In the cortex, cells are positively selected by cortical epithelial cells to be able to recognize self-MHC-peptide complexes. In the medulla, they are selected to do not act destructively towards self-antigens by medullary epithelial cells and DCs. Most of the thymocytes will die in this process. This process is critical because it ensures that T cells have an affinity for MHC molecules. The cells that interact well with MHCII will become CD4+ and those that interact better with MHCI will mature into CD8+ cells. Medullary epithelial cells express a wide variety of self-antigens because of the expression of different transcription factors: AIRE, Fezf2, and, probably, a third unknown transcription factor. The thymic medullary epithelia positively selects $CD25^+$ regulatory T cells that will prevent autoimmune reactions towards self-antigens not expressed by DCs in the thymus[112].

Cells that react too strongly with self-antigen peptide could be responsible for autoimmunity. To avoid autoimmunity, lymphocytes must be eliminated by negative selection in the thymic medulla. Cells that react too strongly to self-peptides presented by DCs are deleted. The cells that leave the thymus are self-restricted, self-tolerant, and positive for CD8 or CD4[113].

However, there is one exception: some cells that react to self-antigen during the negative selection do not die and become regulatory T cells. The cells that respond with medullary thymic epithelial cells presenting self-peptides (mTEC) are forced to become natural Tregs (Figure 8). Once differentiated, they react to self-antigens but in a tolerogenic way, to maintain tolerance. Those cells start to express the transcription factors Foxp3 and helios[114]. They are called thymus-derived Tregs or natural Tregs and are very important for the prevention of autoimmune diseases[112,115].

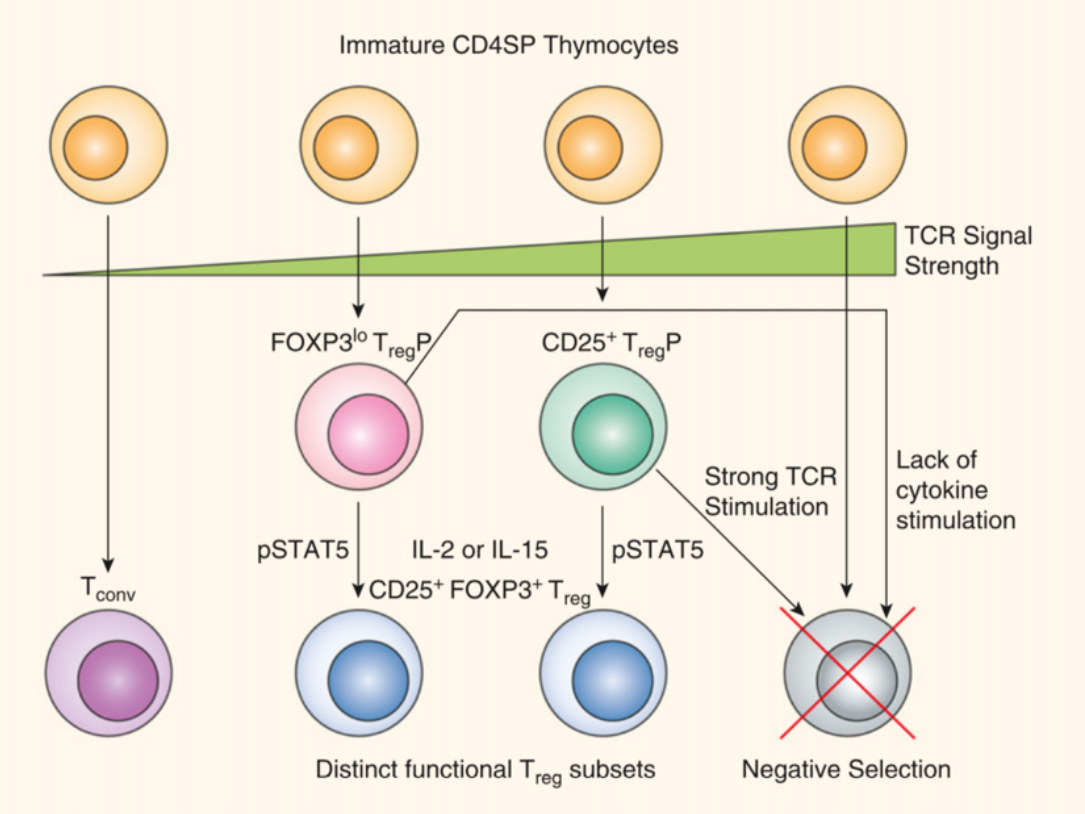


Figure 8 Model of thymic regulatory T cell (Treg) development. CD4 single-positive thymocytes interact with different affinities for self-antigens presented by thymic APC subsets. Adapted from [112].

5.2. TOLERANCE IN THE PERIPHERY

Central tolerance is not sufficient to ensure a global balance of the immune system. Many other mechanisms maintain peripheral tolerance. One such mechanism is called “anergy.” When an antigen is presented by an APC to a lymphocyte with insufficient co-stimulatory signals or with checkpoint inhibitors such as PD1, Tim3, or LAG-3, they become hyporesponsive[62,116].

Another state, called exhaustion, occurs when a cell is chronically stimulated by the same antigen. This term has been used in the setting of chronic viral infection but has also been described in response to tumors[117]. An exhausted T cell is almost unable to proliferate, begins to increase expression of inhibitory receptors, including PD-1, CTLA-4, Tim-3, and LAG-3, and secrete very few cytokines. This mechanism is essential in setting a maximum level of inflammation and preventing lethal inflammation[117].

The difference between anergy and exhaustion is difficult to define. Their phenotype is sometimes similar. Anergic cells are hypo-responsive because they were incompletely activated and do not produce IL-2, they are long lived and have an immunosuppressive potential[118]. On the

other hand, a T cell become exhausted in the case of chronic antigen stimulation (viral or tumour antigens). Those cells express and cannot produce effector cytokines and have an arrested cell cycle. Through the expression of inhibitory receptors (such as PD1 or Tim-3) they also exert inhibitory function and this state is reversible[119].

Some other specialized cells called Tregs actively work to maintain peripheral tolerance. The best-known are the $CD4^+$ $Foxp3^+$ Tregs that can specifically and systematically suppress the immune response. There is substantial diversity of regulatory cells and not all express Foxp3[120]. Some have a temporary regulatory phenotype only and some are not CD4 T cells (e.g., Bregs or $CD8^+$ T regulatory cells) with essential functions that will be discussed in another chapter.

5.3. FOXP3: THE MAIN TRANSCRIPTION FACTOR

The discovery of specialized cells able to regulate the immune response emerged as a crucial step in our understanding of the immune system[121]. Generally, the term "Treg" refers to $CD4^+$ T cells that express the transcription factor Foxp3 and CD25. A vast diversity of regulatory T cells has been described, and no single molecule can differentiate them *in vitro* or *in vivo*. T cells with a regulatory potential were first observed in 1970[121] but their definition was not established and their role in autoimmune disease not clarified until 1985[122]. The discovery of Foxp3 has increased our understanding of Tregs[123]. Mice deficient for Foxp3 cannot survive because they spontaneously develop severe autoimmunity[124]. Foxp3 collaborates with several other transcription factors to form a multimeric complex activating several genes that make the transcriptional and effector signature of a Treg[125]. Foxp3 consolidates the pre-existing features of Treg precursors, not as the inducer of the Treg lineage[126]. The targets of this transcription factor represent a vast landscape that include increased expression of *Cd25, Ctla4, Tgfb,* and *Ebi3* and the downregulation of inflammatory genes such as *Ifng* or *Il17* but also metabolic genes[127]. Tregs are very plastic cells: they can lose their phenotype and reacquire it[115,128,129].

In nonpathological conditions, thymus derived $Foxp3^+$ Tregs represent 10% of the total $CD4^+$ T cells. $Foxp3^+$ Tregs derive from 2 distinct origins: the thymus (tTreg) and the periphery for inducible Tregs (iTreg)[130]. This last group of cells are derived from naïve CD4 T cells and can be induced, for example, through secretion of active TGF-β without IL-6. Indeed, the addition of TGF-β without IL-6 to activated naïve CD4 T cell *in vitro* induces expression of Foxp3[131]. In contrast, addition of TGF-β with IL-6 induces the development of Th17. *In vivo* iTregs develop outside the thymus under different of conditions. Antigen presentation is needed under immunogenic or noninflammatory conditions to develop iTreg[126]. They are also generated in the homeostasis in the gut and in cancer. The stability of iTregs depends on their origins[126]. Depending on their different origins and their functions, tTregs and iTregs have very different TCR repertoires. As previously mentioned, tTreg

exclusively expresses specific TCR for self-antigens (e.g., myelin, which is targeted by the immune system in multiple sclerosis). The TCR of the iTregs also have specificity against self-antigen but are more dedicated toward environmental antigens (e.g., gluten, food, commensal bacteria); iTreg cells are essential for expanding TCR diversity within regulatory responses[132].

5.4. CYTOKINES INDUCING TOLERANCE

Cytokines are the soluble messengers of the immune system. They are, for the most part, not considered hormones because most do not act at distance. There are exceptions, however, such as TGF-β, IL-1, TNF-α. They have, for the most part, autocrine and paracrine effects. The others can be secreted form far away from the target cell and have an endocrine effect (for example TGF-β). Cytokines are generally small glycoproteins secreted mainly but not only by immune cells in response to different stimuli. They can act on both the adaptive and the innate immune system. They can also act on nonimmune cells. There is a large variety of tolerogenic cytokines, and we decided to focused on TGF-β, IL-10, and IL-35; in another section, we will discuss in detail IL-27.

TGF-β is one of the most immunosuppressive cytokines produced by iTreg and tTreg; many cells of the body produce it and it is present in an inactive form in the bloodstream at high concentration. Mice deficient in this cytokine have severe autoimmune symptoms similar to Foxp3-deficient mice (scurfy mouse phenotype). Several weeks after their birth mice die by a multiple inflammatory cell responses that lead to organ failure[133]. However, TGF-β seems to be dispensable for the development of tTreg in young mice[134]. It appears to play a significant role with IL-10 at the mucosal interface. Associated with IL-6, it induces the differentiation of the Th17 response and, alone, TGF-β precipitates the expression of Foxp3 in activated CD4 T cells[135]. TGF-β is secreted by many cell types, not only immune cells, in large quantities in a latent inactive form[67]. The protein circulates in a complex with two other polypeptides: latency-associated peptide (LAP) and latent TGF-β-binding protein (LTBP). TGF-β needs to be cleaved or reduced to become active[135]. It can be cleaved by proteases such as plasmin or thrombospondin-1 by direct interaction with the latent TGF-β complex and it can become activated by low pH *in vitro*. The formation of active TGF-β generally occurs on the surface of immune cells. This process is one of the fundamental mechanisms for the regulation of immune homeostasis in the gut. There are three isoforms of TGF-β that share the same structure but have different functions. The most important is TGF-β1, which induces production of IgA in mucosal sites (e.g., in the intestine)[136]. Active TGF-β signals through serine/threonine kinase type I and II TGFβ-receptors, which initiate several intracellular signaling transduction pathways leading to the transcription of target genes[137].

IL-10 is a tolerogenic cytokine discovered in 1991. It was initially reported to inhibit the secretion of other immunogenic cytokines (IL-6, IL-1, TNF-α) and antigen presentation to CD4+ cells[138]. Later,

it was discovered that this anti-inflammatory cytokine is produced by many different immune cells including APCs, Th1, Th2, Th17, type 1 regulatory cells (Tr1), Tregs, and $CD8^+$ T cells[139]. Mice deficient in IL-10 have autoimmune diseases, but their symptoms are not as severe as those in mice deficient in TGF-β or Foxp3[140]. They mainly develop inflammatory bowel disease in specific-pathogen-free conditions, showing the importance of IL-10 for mucosal tolerance[141]. Indeed, without IL-10, the TLR recognition of commensal bacterial patterns results in an immune response leading to tissue damage and, in turn, to fatal colitis. Reciprocally, overexpression of IL-10 is associated with chronic infections[142,143]. IL-10 acts on the innate and adaptive components of the immune system. It signals mainly through Jak1 and STAT3. It inhibits DC maturation, antigen presentation, co-stimulation, and secretion of inflammatory cytokines by APCs. IL-10 blocks the secretion of IL-2 and, therefore, the proliferation of T cells. It can also prevent the Th1 response by blocking secretion of IFN-γ and can block secretion of other immunogenic cytokines such as IL-17[144]. IL-10 can alter the Th response. *In vitro* and *in vivo,* the priming of CD4+ T cells in the presence of IL-10 has been shown to develop $Foxp3^-$ Tregs able to secrete IL-10 called Tr1[145]. Like most tolerogenic cytokines, IL-10 is expressed in tumor cells to stop antitumor activity[146]. The protective role of IL-10 in autoimmunity has been widely discussed[143,146,147]. It can, for example, protect mice from inflammatory bowel disease (IBD)[139]. Indeed, mice that are deficient in IL-10 develop spontaneously severe pathologies in the gut[139]. It has a crucial preventive role in the experimental allergic encephalomyelitis (EAE) model, preventing evolution of the disease[148]. In the same study Bettelli and colleagues observed that the secretion of IL-10 by Tregs was observed in the EAE model to prevent infiltration and decrease the aggressivity of pathogenic autoreactive T cells in the central nervous system.

IL-10 is a powerful immune mediator but it also has an inflammatory side. IL-10 signaling induces the proliferation and maturation of B cells and enhances production of antibodies[149]. It can also increase the cytotoxic function of CD8 T cells in a tumor[150]. Recently, IL-10 overproduction has been implicated in lupus[151].

IL-35 is a heterodimeric cytokine with very strong tolerogenic power. It shares the subunit p35 with the inflammatory cytokine IL-12. The second subunit of IL-35 is the Epstein-Barr virus-induced gene 3 (EBI3).[152] These two proteins are not covalently attached, which raises the question of its stability. Together with IL-27, IL-12, and IL-23, IL-35 is part of the enigmatic IL-12 family that will be discussed in the IL-27 chapter[153]. The receptors of IL-35 are unusual for an IL-12 family member. This cytokine binds to the homodimer of gp130, the homodimer of IL-12Rb2, and the heterodimers IL-12Rb2–gp130, and IL-12Rb2–IL-27Ra[154]. After binding to one of these receptors, signal transduction occurs through JAK1, JAK2, TYK2, and STAT1, STAT3, and STAT5. STAT1 and STAT4 lead the expression of tolerogenic genes[155]. IL-35 can induce expression of IL-35 in activated T cells, leading to a tolerogenic feedback loop.[154] For more than ten years, IL-35 has

been studied in a variety of different models. It has been shown that IL-35 can inhibit the proliferation of T cells[70,156,157], and can change the differentiation of Th17 into Tregs. IL-35 can induce *in vitro* iTR35 cells that do not express Foxp3 and can secrete large quantities of IL-35 and IL-10.[152] This cytokine is also able to change B cells into Bregs able to secrete IL-35 and IL-10[14]. Because of those properties, IL-35 has been tested in many different autoimmune disease models. Ebi3 and p35 knockout (KO) mice develop much stronger EAE[158]. Mice deficient for p28 developed the same symptoms as controls, showing the importance of the heterodimer p35-EBI3 to prevent autoimmune diseases. The delivery of IL-35 inhibited and decreased the severity of clinical symptoms in EAE and collagen-induced arthritis (CIA)[70,158–161]. Very early in the history of this cytokine, in 2007, it was demonstrated that iTR35 (CD4 T cells stimulated by exogenous IL-35 *in vitro*) had an immunosuppressive effect in inflammatory bowel disease (IBD) model mice[157]. In the type I diabetes mouse model (non-obese diabetic mouse), administration of IL-35 did not increase the number of Foxp3$^+$ cells However, it decreased the numbers of Th1 and Th17 cells and reduced the infiltration of mononuclear cells in the islets, therefore reducing evolution of the diabetes[162]. In humans, the concentration of IL-35 in serum is negatively correlated with type I diabetes[163]. Some tumors express IL-35, benefitting from its extremely tolerogenic property, which leads to protection from immune attack[152]. Several publications have shown that tumors grow faster in the presence of IL-35 because it limits anti-tumor immunity[70,164]. Vignali and collaborators recently demonstrated the synergetic effect of IL-35 with IL-10 on T cell exhaustion[165]. This synergy was observed in tumor microenvironments in which Tregs producing IL-10 and Tregs producing IL-35 cooperated to induce intra-tumoral exhaustion with induction of co-inhibitory receptors (LAG-3, Tim-3, PD1) and promotion of transcription factor BLIMP1[165]. The same synergy was observed in our laboratory (manuscript in preparation) in mouse models of autoimmune disease, in which the combination of DCs secreting IL-10 and DCs secreting IL-35 were shown to cooperate to induce tolerance through the inhibitory receptor LAG-3.

5.5. MECHANISMS OF TREGS

The secretion of cytokines is not the only suppressive mechanism of Tregs. At least four other mechanisms to suppress the immune response were described: metabolic disruption, cytolysis, inhibition of the innate system, and regulation of humoral activity[166] (Figure 9).

Metabolic disruption. Foxp3$^+$ Tregs show high expression of CD25. This empowers cells to consume local IL-2, preventing Tem from self-renewal and proliferation[44,145]. In 2015, it was reported that autoreactive T cells for self-antigen form clusters with Tregs that inhibit their activation with the consumption of IL-2 but also with physical interactions[169]. IL-2 is necessary for the maintenance and proliferation of T cells. Its feedback system, based on the secretion of IL-2, is

essential for the homeostasis of those clusters. The consumption of IL-2 is not the only metabolic disruption that Tregs mediate. The surface-expressed enzyme CD39 can hydrolyze ATP and ADP into AMP on the cell surface. Tregs highly express this enzyme on their membrane and it has high suppressive effects on T cells[170,171]. Very recently, CD36 was found to be a central metabolic modulator in intra-tumoral Tregs. CD36 can tune mitochondrial fitness via the peroxisome proliferator-activated receptor-β and induce Treg cells to adapt to a lactic acid-enriched tumor microenvironment[172].

Suppression by cytolysis. Cytolysis is a major effector function of NK cells and activated CD8 cells. However, several CD4+ T cells also have cytotoxic activity. Tregs induce cytolysis via granzyme A and perforin-dependent cytolysis by cell contact to stop the immune response. The transfer of tTreg of a granzyme A-deficient mouse induced immune tolerance efficiently in secondary lymphoid organs but did not prevent local inflammation in a gastrointestinal autoimmune disease mouse model[173]. Granzyme B was also reported as a critical component of the Treg activity[174]. The cytolytic activity of Tregs is mainly directed against B cells[175]. Tregs can kill innate immune cells in tumors through the activity of granzyme B[176]. However, the exact targets of the cytotoxic activity of Tregs remain unclear.

Tregs can inhibit cells of **the innate immune system.** Lymphocytes are not the only target of Tregs; Tregs can also change DCs, macrophages, and other cells with a different mechanism. Recently it was found that Tregs can bind specifically to DCs and reduce MHCII expression.[177] Microscopy studies have revealed that a lack of Tregs increased the duration of contacts between T cells and antigen-loaded DCs *in vivo*[178]. By direct contact through CTLA-4, it was reported that Tregs downmodulate DCs[179]. Tregs can induce DCs to express indoleamine 2,3-dioxygenase (IDO), an enzyme that catalyzes the degradation of tryptophan and helps to control inflammation and immune response by inducing tryptophan starvation[63,180,181]. Tolerogenic molecules associated with exhaustion give reliable tolerogenic power to Tregs[62]. The PD1/PD-L1 interaction, for example, is often discussed from the T cell point of view, but it also induces tolerogenic features to DCs. For example, the PD1/PD-L1 interaction can induce a tolerogenic phenotype in DCs through expression of IDO[182], and LAG-3 (a CD4 homolog) can bind to MHCII to suppress maturation and immunogenicity of DCs[183].

Tregs are essential for the regulation of humoral immunity. Foxp3-deficient mice have an abnormal accumulation of plasma cells in their spleen[184]. Tregs can suppress the emergence of autoimmune long-lived plasma cells and, therefore, avoid the persistence of humoral autoimmunity. They play this role in different ways. They can induce apoptosis of B cells (with PD1). Some hypotheses state that Tregs can inhibit the differentiation of plasmablasts (through direct interaction with CTLA4 or PD1). They can also downregulate the production of antibodies, specifically IgG[185].

Negative co-stimulation. Co-stimulations in general are strong regulators of T cell activity. Costimulatory pathways are important for T cell differentiation[186]. Negative co-stimulation constrains the possible cell states that T cells can acquire. For example, it has been shown that PD1 limits the effector functions of CD8 T cell and CTLA-4 limits the differentiation of CD4 T cells into a Th subtype[187].

Tolerogenic cytokines. Tregs do not work only by cell contact; they can also secrete cytokines. IL-35, TGF-β, IL-10, and IL-27 are the primary soluble mediators of Tregs. Cytokines give Tregs the ability to change locally activated cells.

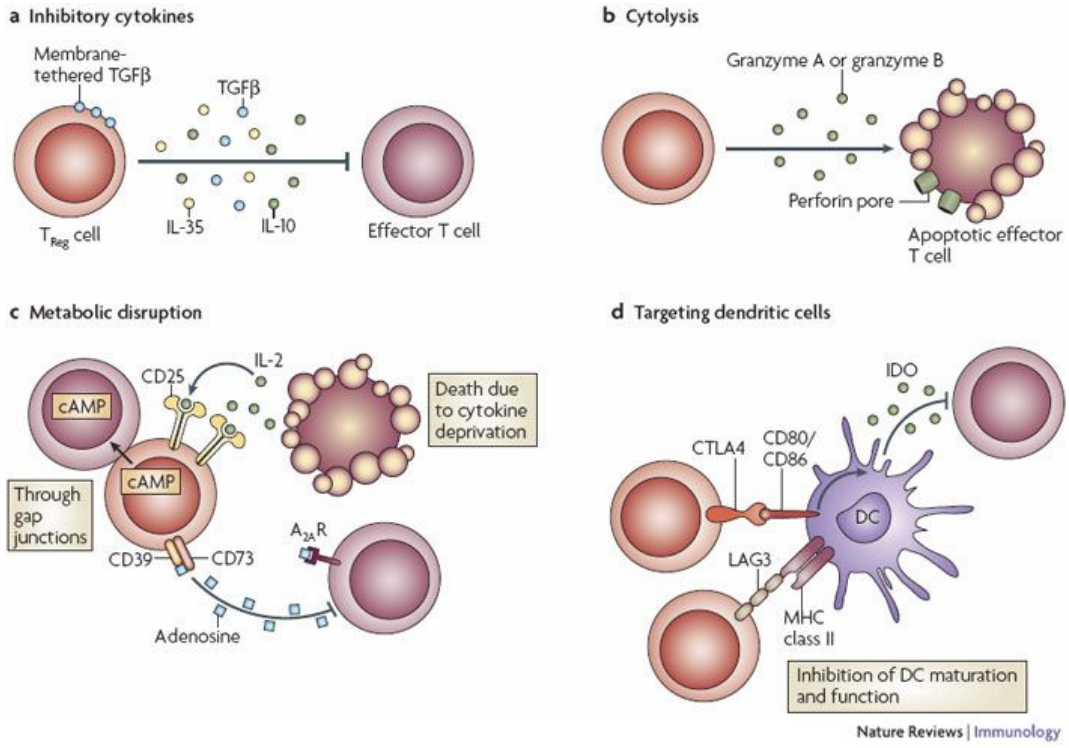


Figure 9 The 4 tolerogenic features of Tregs. (a) Secretion of tolerogenic or inhibitory cytokines such as IL-35, IL-10, and TGF-β; (b) cytolysis of effector cells through granzyme A or granzyme B; (c) metabolic disruption through high expression of CD25, which removes IL-2 and deprives effector T cells; (d) targeting DCs through expression of LAG-3, CTLA-4, and other negative co-regulators. Adapted from [166].

5.6. OTHER TREGS

As mentioned, Foxp3 Tregs are not the only regulatory cells in the immune system; several Foxp3$^-$ Treg populations are critically important in maintaining immune homeostasis.

iTR35. As previously mentioned, IL-35 is able to induce a subset of Foxp3$^-$ T cells with tolerogenic properties called iTR35[152]. The treatment of naïve human or mouse activated T cells with IL-35 induces a regulatory population that able to secrete IL-35 but also IL-10 and TGF-β. Thus, iTR35 cells constitute a crucial mediator of infectious tolerance and are still studied. This population of cells is a promising target in the research for treatments[156].

Tr1 are CD4$^+$ cells that secrete a large amount of IL-10. They can be induced very rapidly to decrease inflammation[188]. They downregulate the immune response toward self and non-self antigens[189]. Some confusion surrounds this population because IL-10 is secreted by a variety of cells with both suppressive and nonsuppressive activity. The following criteria for Tr1 cells were proposed: (1) Tr1 produce mainly IL-10, even if they can also secrete other cytokines; (2) Tr1 cells have suppressive activity and they do not express Foxp3; (3) the negative co-stimulatory molecules LAG-3, Tim-3, and PD1 are often expressed on the surface of Tr1 cells[120].

A cell can differentiate into a Tr1 cell in several ways (Figure 10**).** For example, under an activated condition, Tr1 can be induced by IL-27, IL-10, and TGF-β from most of the CD4+ subtypes (Th1, Th2, Th17, or naïve CD4 T cells). Blimp-1 is one of the transcription factors that boosts secretion of IL-10 by Tr1. CD4$^+$ T cells deficient for Blimp-1 produce less IL-10, whereas if Blimp-1 is overexpressed, IL-10 expression is increased[190]. c-Maf is one of the transcription factors involved in the production of IL-10[140].

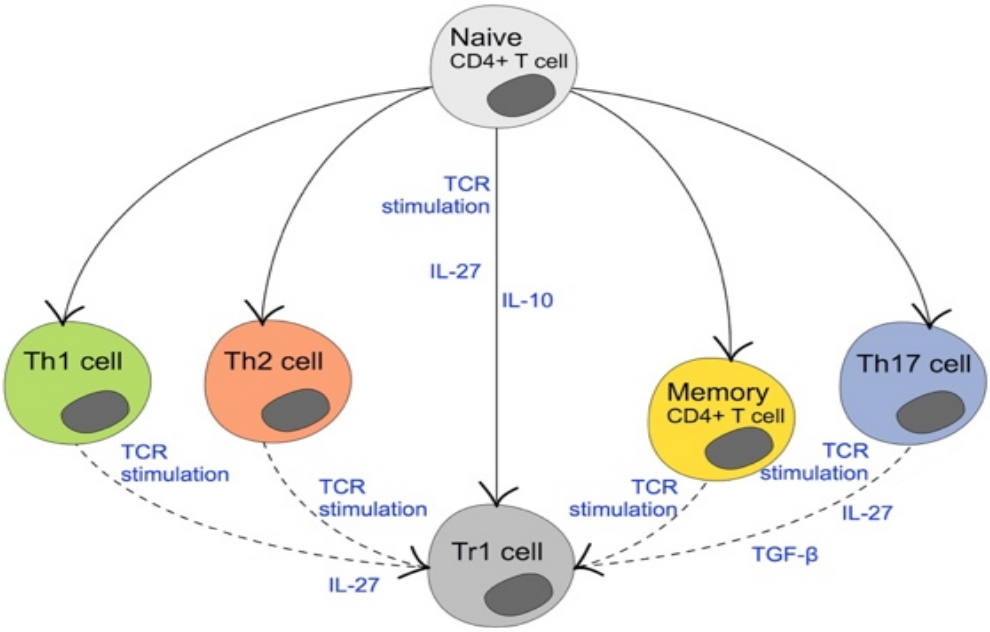


Figure 10 How to become a Tr1 cell. Adapted from [120]

Tr1 cells prevent the development of EAE and other autoimmune diseases in mouse models[191–193]. Tr1 cells have been used in several clinical trials, including one involving Crohn's disease[194]. That study evaluated the efficacy of antigen-specific Tr1 cells to treat patients. T cells from the blood were polyclonally expanded in the presence of IL-2 and IL-4, and then clones were selected for Tr1 by IL-10 secretion (ELISA) and low level of IFN-γ in reaction to the antigen. The cells were then administrated intravenously a few days later. Six of 8 patients had a statistically significant reduction in symptoms after several months[194].

CD8 Tregs. $CD8^+$ T effector lymphocytes have cytotoxic effects and secrete cytokines upon activation but some perform regulatory roles in the immune response. Those cells are called $CD8^+$ Tregs and they have immune-suppressive functions different from those of $CD4^+$ Tregs.[46] Their importance in autoimmune disease has been increasingly recognized in recent years[195]. The major challenge in working with these cells, as for Tr1 cells, is the lack of clear marker definition of the subsets. The role of Foxp3 in this population is controversial. Some groups say Foxp3 is less expressed in the $CD8^+$ T cell population than in the CD4+ T cell population in mice[196]. It was observed in mice that had a green fluorescent protein (GFP) reporter for Foxp3 expression (and with anti-Foxp3 antibodies), $CD4^+$ T cells were the predominant cell type expressing Foxp3, although a small population of $CD8^+$ T cells also expressed Foxp3[127,196]. Other studies have found that it is the transcription factor Helios that identifies $CD8^+$ Tregs[197]. The mechanism of action of those cells is still unclear. However, some classical tolerogenic cytokines can be secreted in $CD8^+$ Tregs such as IL-10 and TGF-β[198]. IL-10-mediated suppression for natural $CD8^+$ Tregs in mice has been described[199]. The authors of that study suggested that secretion of IL-10 by $CD8^+$ Tregs is a crucial mechanism for tolerance in the allograft survival model (more than the secretion of IL-10 by CD4 T cells). Like any other CD8 T cell, CD8 Tregs secrete IFN-γ. This inflammatory protein is another "double-edged sword" in that it also has tolerogenic properties in the long term.[200] IFN-γ seems to be a mechanism of suppression for these cells. Surprisingly, the CD8 Tregs of mice deficient for IFN-γ do not show any suppressive activity[201,202]. Like other Tregs, CD8 Tregs express negative co-receptors such as TIGIT, TIM-3, Lag-3, and PD1, thus are a robust tool to induce tolerance[165,203–205].

Bregs are immunosuppressive cells that can produce large quantities of IL-10, IL-35, and TGF-β[206] (Figure 11). Bregs can suppress unwanted immune pathology by inhibiting proinflammatory lymphocytes. Multiple Breg cell subsets can be induced in response to inflammation at different stages in development. The importance of Bregs in autoimmune diseases was highlighted by the observation that B cell-deficient mice were unable to recover from EAE[207]. The production of IL-10 by Bregs was later shown in different autoimmune disease models[208,209]. In 2014, the power of Bregs was highlighted by their ability to stop the differentiation of Th1 and Th17 and the immunogenic properties of DCs[210,211]. In the EAE model, plasmablasts in the draining LN are

powerful IL-10 producers that limit autoimmune inflammation. If B cells lack expression of EBI3 or p35, EAE clinical scores are increased. The adoptive transfer of activated Bregs induced *ex vivo* with IL-35 inhibited strongly Th17 and Th1 responses and induced Treg expansion.

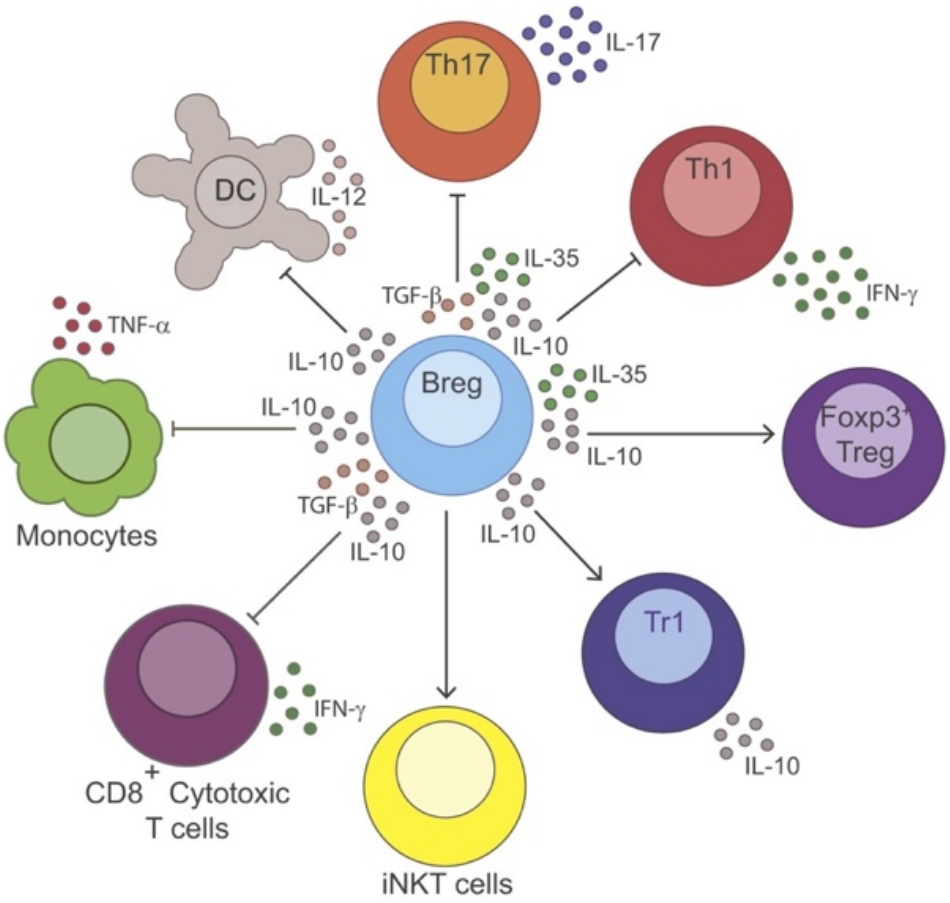


Figure 11 Mechanisms of action of regulatory B cells (Bregs). Adapted from[206]

6. IL-27: A KEY CYTOKINE THAT REGULATES IMMUNITY

6.1. IL-27: A MEMBER OF THE IL-12 FAMILY

In 1989, when IL-12 was purified for the first time, several surprising findings were noted. The structure was unusual—most cytokines were known to be monomeric, whereas IL-12 was heterodimeric, with subunit p40 covalently linked with subunit p35[212]. For several years, it was the only heterodimeric cytokine known. IL-12 was considered immunogenic and produced almost exclusively by the innate immune system (macrophages, neutrophils, and DCs) to induce the IFN-γ producers (Th1 cells). Later, three other heterodimeric proteins from the IL-12 family were characterized: IL-35, IL-27, and IL-23[212] (Figure 12). The subunit p40 was shown to associate covalently with p19 to form the cytokine IL-23. IL-23 was proposed to induce the expansion of Th17.

It was demonstrated that IL-23, but not IL-12, could induce expansion of Th17 and increase the severity of autoimmune diseases[213,214]. In induced CIA, p19 KO mice failed to develop bone and joint lesions because of their lack of Th17 cells[213].

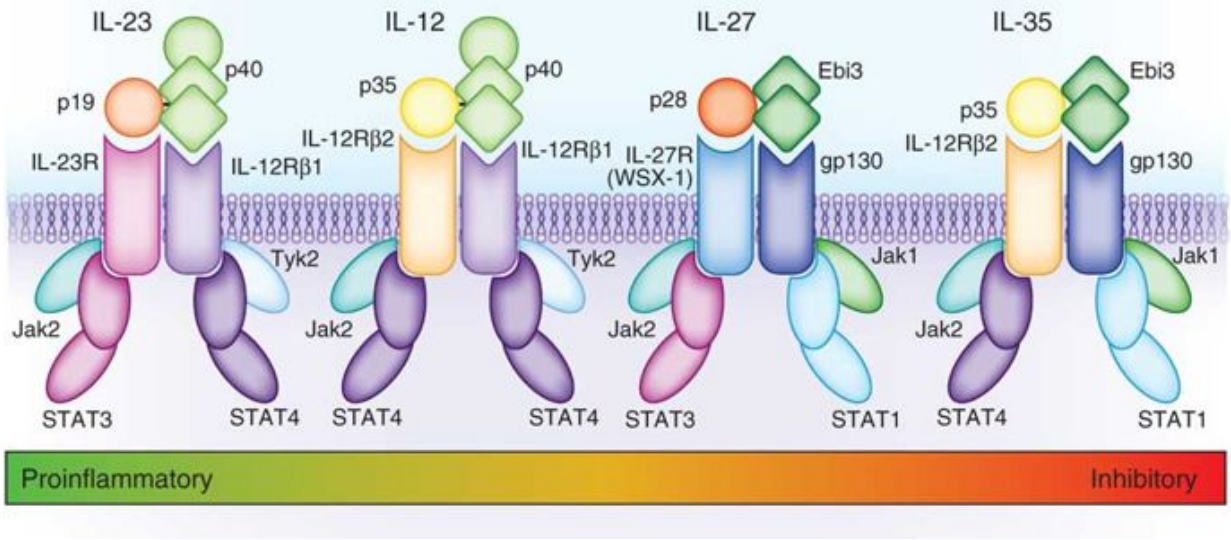


Figure 12 The IL-12 family: a family of heterodimeric cytokines, including IL-12, IL-23, IL-27, and IL-35, that shares the same structure, they are heterodimers sharing one chain, and they have different functions. Adapted from [153].

In 2002, two other cytokines called, IL-27 and IL-35, were identified as part of the family. They also are heterodimeric proteins and they share a subunit, EBI3[215]. p28 was first recognized as a part of IL-27 based on research for proteins that could associate with EBI3[215]. EBI3 is structurally related to IL-12p40, and it can also bind p35 to form IL-35. The structure of these two cytokines is similar: EBI3 is also not covalently bound with its partners, p35 or p28[153,216]. This raises the question of their stability. It was suggested that IL-35 is poorly secreted in transfected cells compared with IL-12, suggesting that its formation is unfavorable or the secreted protein in unstable[156]. A similar property was observed for IL-27[217]. To be secreted and functionally active, the four-helical subunit p28 requires association with EBI3. Computational analysis and site-directed mutagenesis have proven that p28 and EBI3 are linked by an aromatic–aromatic (tryptophan-phenylalanine) interaction that is surrounded by salt bridges[217]. The binding of EBI3 and p28 seems to be necessary to allow secretion and the addition of a synthetic disulfide linker between the two subunits did not affect its function[218].

IL-27 is produced by many cell types: DCs, monocytes, NK cells, endothelial cells, and macrophages. Its secretion is generally induced upon a microbial stimulus via a TLR receptor [212].

The genes of EBI3 and p28 are not close neighbors, and they are regulated independently[219]. The triggers of TLRs activate p28 and EBI3 separately. However, even if the mechanism of activation of *Ebi3* and *p28* genes is increasingly understood, the mechanism that induces p28 and EBI3 subunits to dimerize and to be secreted remains unclear[220].

The receptor of IL-27 (IL-27R) is composed of two chains: the alpha chain IL-27R (WSX-1) is linked to gp130 (Figure 13). The two chains have significant amino acid similarities[221]. gp130 is a receptor utilized by many different cytokines (e.g., IL-35, IL-6, IL-11) and it is expressed on many kinds of cells[222]. WSX-1 is more expressed on immune cells (lymphocytes, macrophages, DCs)[223]. IL-27 induces the dimerization of these two membrane proteins and activates a signal transduction cascade via the JAK/STAT and the MAPK signaling pathways. On naïve CD4 cells, IL-27 induces the activation of JAK1, JAK2, and TYK2 and induces the phosphorylation of STAT1 and STAT3[224,225]. The MAPKs were seen to induce, through the transcription factor AP1, the transduction of tolerogenic cytokines. IL-6 and IL-27 have the same gp130 chain on their receptor, and they both activate STAT1 and STAT3. However, their mechanisms of activation are different. IL-6 is more inflammatory by promoting the development of Th17 cells, whereas IL-27 is both inflammatory and tolerogenic[35,225–227].

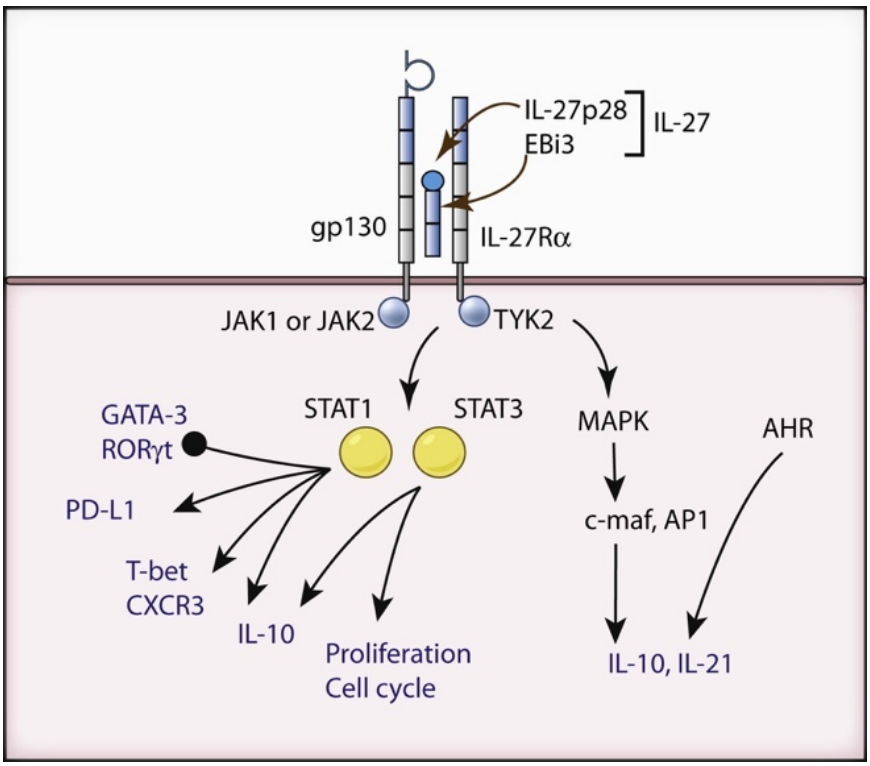


Figure 13 IL-27 induces the dimerization of gp130 and IL-27Ra (WSX1), which engages JAK1, JAK2, and TYK2 to induce the MAPK pathway and the phosphorylation of STAT1 and STAT3. STAT1 is involved in the increase of PD-L1 expression and inhibition of GATA-3, whereas STAT3 and MAPK increase secretion of IL-10. Adapted from [228].

6.2. IL-27 CAN SHAPE THE IMMUNE SYSTEM: GRAY AREA

Initially, IL-27 was described to be proinflammatory because of its similarities to IL-12 and IL-6 and its ability to enhance IFN-γ production in some cell types[214,220,229]. Discovery of the IL-27 receptor and STAT1 and T-bet promotion by IL-27 strongly suggested that IL-27 promoted the Th1 response[230]. Later, however, IL-27 receptor-deficient mice were challenged with different pathogens, and IL-27 was eventually classified as a tolerogenic cytokine. In a study from 2002, WSX-1-deficient mice were infected with the intracellular parasite *Toxoplasma gondii*. The WSX-1-deficient mice efficiently established protective T cell responses with increases in inflammatory cytokines (IL-12, IFN-γ) but failed to downregulate T cell responses induced by infection and developed a lethal CD4+ T cell-mediated immune pathology associated with excessive production of IFN-γ[221]. Further studies with these mice deficient for the IL-27 receptor showed that IL-27 limits IL-2 production during Th1 differentiation[231]. WSX-1 deficiency is also implicated in Th17 responses, as mice infected with *Toxoplasma gondii* developed severe neuroinflammation that was associated with the secretion of IL-17 by CD4+ T cells[232]. In 2006, it was shown that IL-27 increased production of IL-10 in $CD8^+$ and $CD4^+$ T cells via STAT3[225]. Still, in the same model of chronic infection with *Toxoplasma gondii*, it was shown that IL-27 promoted expression of T-bet and CXCR3 in Treg cells at mucosal sites[233]. The transfer of Tregs decreased the pathology of the infection observed in WSX-1-deficient mice dependent on their ability to produce IL-10. During *Leishmania* infection, $CD4^+$ T cells from WSX-1 deficient mice produced less IL-10 and more IL-4, but no differences were seen in numbers of parasites. However, massive augmentation in the lesions caused by the Th17 response was observed, demonstrating the role of IL-27 in downregulating an inappropriate Th17 response during infection[234].

One analysis showed that IL-27 upregulated expression of programmed death-ligand PD-L1 on activated naïve $CD4^+$ T cells[235]. Those cells, when they were co-activated with other T cells, inhibited differentiation of Th17 cells. The overexpression of co-inhibitory receptors might be the critical mechanism of inhibition via IL-27 in different mouse models such as EAE. Several inhibitory receptors, such as PD1, CTLA4, Tim-3, and LAG-3, exert their inhibitory functions by overlapping and discrete functions[204]. IL-27 induces different transcriptional regulators that activate a module of co-inhibitory receptors like PD1. Recently it has been shown that Tim-3, a critical regulator of the T cell exhaustion that develops during chronic viral infections, is upregulated with IL-27[205,236]. In 2016, a study showed that Tregs stimulated with IL-27 substantially improved suppressive function and induced expression of LAG-3[237]. This mechanism seems to be critical in IBD. Both Tim-3 and LAG-3 co-inhibitory receptors are expressed in CD4 and CD8 T cells and are part of a co-inhibitory gene program that is shared by nonresponsive T cells driven by the immunoregulatory cytokine IL-27 (Figure 14). Recently, a study found that *in vivo* overexpression of IL-27 (with TCR stimulation) in mice led to increased T cell expression of PD-L1, LAG-3, TIGIT, and Tim-3 in CD4 and CD8 T

cells[238]. The same study found that the TCR stimulation *in vitro*, combined with addition of IL-27, resulted in synergistic induction of LAG-3, CTLA-4, and TIGIT. Another group showed that IL-27 induced the expression of the ectonucleotidase CD39 on Foxp3$^+$ T cells[171]. The inhibition of CD39 reduced IL-27–induced suppressive activities of Tregs[171].

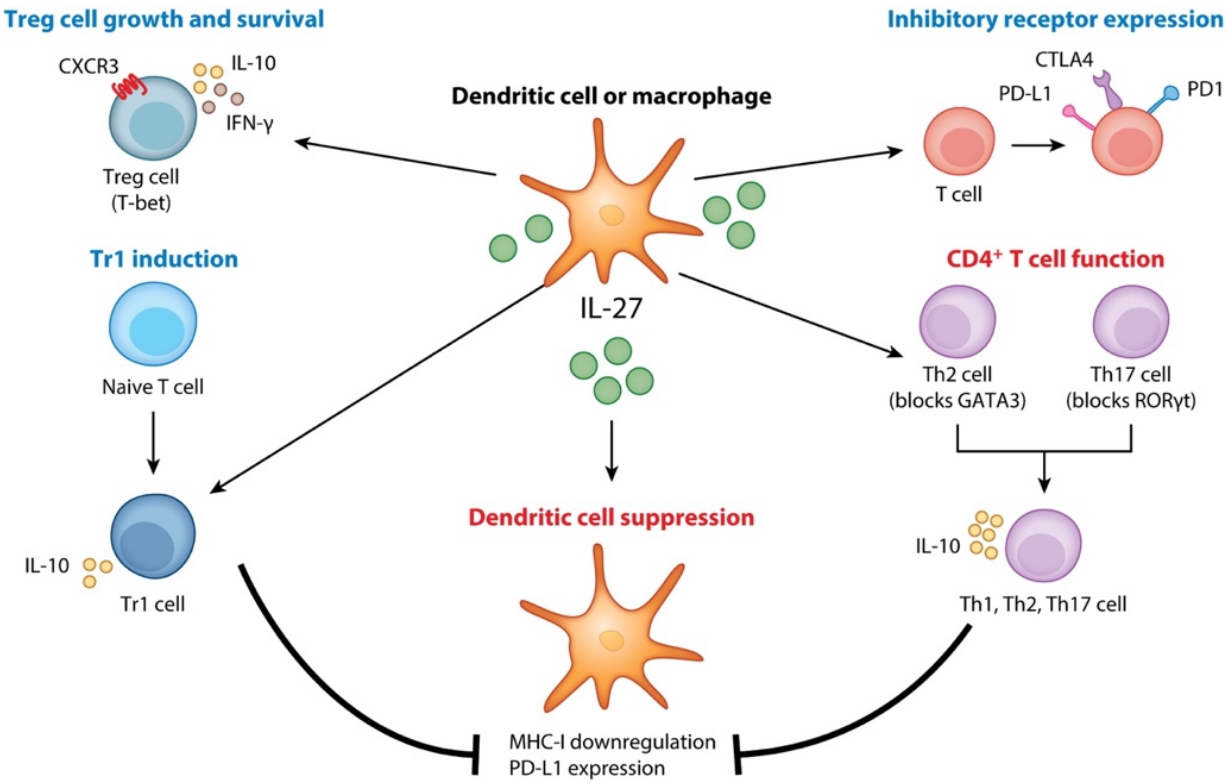


Figure 14 IL-27 can regulate many activities. IL-27 induces expression of CXCR3 in Foxp3$^+$ Tregs and induces Tr1 cells that secrete IL-10. IL-27 inhibits the development of Th2 and Th17 cell subsets, which induce secretion of IL-10. IL-27 can inhibit the function of DCs by stopping their ability to present antigen and to promote their expression of PD-L1. Adapted from [220]

However, IL-27 remains in a gray area between tolerance and immunity. As previously mentioned, IL-27 can limit inflammation in some contexts but promote it in other settings. IL-27 positively affects survival of activated CD4 T cells by inducing downregulation of FasL and the antiapoptotic protein cFLIP[239]. By inhibiting cell death, IL-27 can indirectly induce antigen-specific T cell expansion. It has been observed that IL-27 can work synergistically with IL-12 to trigger IFN-γ production of naïve CD4$^+$ T cells[215]. IL-27 was shown to promote the proliferative and cytotoxic functions of CD8 through the increase of the T-box transcription factor[240]. The addition of IL-27 to activated CD8 T cells significantly increased their cytotoxic activity by increasing their proliferation, IFN-γ secretion, and granzyme B production[241,242]. IL-27 was involved in the mobility of CD8 T cells by upregulating the expression of ICAM-1 and sphingosine-1-phosphate and increasing their attachment to

integrins[191,243,244]. However, IL-27 does inhibit expression of CCR5, which impedes the ability of cells to migrate to infection sites[245]. The role of IL-27 in the effector functions of CD8 was proven by *in vivo* studies using cancer cell lines that "synthetically" produce IL-27[224,246,247]. The CD8 response was enhanced and tumor regression was significantly stronger. This represents a significant difference from IL-35, which presents mostly tolerogenic functions toward CD4 and CD8 T cells[164,165]. However, this remains unclear because, as mentioned, mice that do not express the IL-27 receptor have more active CD8 T cells than wild-type mice when they are challenged with a pathogen. It seems that IL-27 is necessary for an optimal CD8 response (especially for the secretion of IL-10)[248]. Very recently, intra-tumoral injection of IL-27 was tested in mice, and stimulation of antitumor immunity was shown by infiltration of CD8 and NK cells. Into the tumor and an increase of CXCR3[249]. IL-27 is also able to inhibit cancer cell proliferation and migration directly[214,250,251]. IL-27 can enhance the expression of MHCI of NK cells[252]. However, even if the literature indicates that IL-27 directly inhibits proliferation of tumor cells and can induce antitumor immunity, IL-27 is currently considered a marker of poor prognosis in cancer[220,251,253]. For example, an elevated level of IL-27 in patients with breast and lung cancers is correlated with progression of the tumor[254,255]. Despite the tolerogenic features of IL-27, it could be used as a treatment against solid tumors to induce infiltration of immune cells, but it would have to work in synergy with other drugs that could counter its tolerogenic effects[249].

6.3. IL-27: A CYTOKINE ABLE TO CHANGE THE INNATE IMMUNE SYSTEM

IL-27 is secreted by a diversity of cell types, but its main producers remain cells of the innate immune system. The cytokine itself has significant autocrine and paracrine effects on those cells. IL-27 has a positive effect on the survival of eosinophils. Most of the cells from the innate immune system have a high expression the two chains of the IL-27R and its activation reduces apoptosis, modulates expression of adhesion, and induces release of IL-6 and chemokines[256]. IL-27 has been reported to modulate the activity of neutrophils but it is still unclear if this represents a direct action of IL-27[257]. Macrophages and DCs, which are the main producers of IL-27, are very sensitive to IL-27. The DCs of mice that deficient for WSX-1 (IL-27R) activate better the CD4 T cells to differentiate them in Th1 cells[258]. IL-27 was shown to reduce the ability of DCs to present antigens, express co-stimulatory molecules, and secrete immunogenic cytokines in mouse and human DCs[69,258–261]. IL-27 also increased expression of CD39 on DCs[69]. Macrophages were seen to respond in a tolerogenic fashion to IL-27, producing much less type I IFN and more IL-10 upon LPS activation[256,262]. IL-27 appears to be a good mediator of the antiviral response for macrophages[263].

IL-27 was shown to inhibit directly innate lymphoid cells from group 2 (ILC2). It seems that the deficiency of IL-27 is directly linked to an increase of ILC2 in a model of inflammatory lung disease.

Moreover, the addition of IL-27 decreases the antiparasitic response to a helminth infection[264]. IL-27 is able to antagonize to directly suppress tissue-resident ILC2 but not Trm Th2 cells in lung inflammation[265]. These contradictory results mean that the exact role of IL-27 in the innate immune system remains unclear.

6.4. THE TOLEROGENIC ROLE OF IL-27 TO TREAT IMMUNE DISORDERS

The fact that IL-27 can inhibit Th17 responses and increase production of IL-10[266–268] gave hope for the use of this cytokine against inflammation-associated diseases. In mice deficient for WSX-1, the increased severity of EAE was associated with direct development of Th17 activity[232]. Moreover, subcutaneous injection of IL-27 was shown to inhibit symptoms of EAE during passive and active induction of EAE[268]. The direct and indirect immunoregulatory effects of IL-27 in CNS immunity showed many promising results in EAE[69,268–270]. However, IL-27 was not able to regulate fully differentiated Th17 cells[232,268,271]. Indeed, IL-27 gene therapy was shown to be very effective in preventing EAE (i.e., before the first symptoms) but did not have any therapeutic effect once the disease was established (i.e., just after the first symptoms appeared)[272]. It was suggested that the ability of IL-27 (and other cytokines) to dedifferentiate Th17 cells depends mainly on the ratio of phosphorylated (p)STAT3 to pSTAT1[273]. In 2019, a study showed that the systematic delivery of IL-27 (with an osmotic pump) efficiently prevented the development of symptoms of EAE[274]. This study showed that IL-27 signaling in Tregs is necessary and LAG-3 dependent, but surprisingly independent of IL-10. In humans, type I IFN is said to be an effective treatment for patients with multiple sclerosis, even if its efficiency is debated[260]. The ability of type I IFN to block symptoms in EAE was shown to be dependent on IL-27 secretion by innate immune cells[275]. For example, IFN-β increases IL-27 production by innate immune cells, which seems to be critical for its immunoregulatory role in CNS autoimmune disease[275,276]. In the case of neuroinflammation, the secretion of IL-27 and its receptor was reported in the microglia[277]. The authors of that study showed that this could play a role in immune tolerance in the brain. This has also been demonstrated in human practices. For example, the level of IL-27 was found to be significantly lower in plasma and serum of autoimmune disease patients than in healthy donors[278,279]. Furthermore, the level of IL-27 is often negatively correlated with the level of IL-17 in plasma[278].

In another Th17-driven autoimmune disease, Sjögren's syndrome (characterized by decreased salivary and lacrimal gland secretions, resulting in severe dry mouth and dry eyes), intravenous gene therapy with IL-27 was tested in a mouse model. The mice treated had a reduced level of IL-17 and a less severe clinical score several weeks post-injection[280]. However, mice deficient in IL-27 or its receptor are resistant to Sjögren's disease, which suggests that IL-27 is essential for the development of this disease.

Unexpectedly, IL-27 seems to have a role in neuropathic pain; it is produced after peripheral nerve injury that counteracts neuropathic pain development through induction of the antinociceptive cytokine IL-10[281]. The immunomodulatory role of IL-27 was also observed in the ovalbumin (OVA)-induced asthma model. The intranasal administration of IL-27 twice a day during seven days of sensitization upregulated the Th1 and Treg subgroups and downregulated the Th2 response in the lung microenvironment and therefore decreased airway inflammation[282].

The role of IL-27 has been studied in many other autoimmune diseases. In antibody-mediated diseases, such as rheumatoid arthritis, IL-27 has been shown to have a therapeutic effect in models of CIA[283–285].

Many mouse models of autoimmunity show that IL-27 can prevent and treat disease, but it is more challenging to apply this cytokine as a treatment to a human disease. IL-27 can limit the protective immune response and might be more dangerous than the autoimmune disease itself. This is why, despite the numerous studies previously cited, 25 years of promising research, and several research patents deposited on the role of IL-27 in multiple sclerosis (patent numbers: US8178308B2, US20130189262A1, CA2824805A1, US8691227B2), no treatment based on IL-27 has developed to clinical trial.

6.5. A POSSIBLE SYNERGY BETWEEN IL-27 AND VITAMIN D3

Vitamin D is a group of fat-soluble steroids responsible for many biological activities. The two main compounds of this group are vitamin D_3 (cholecalciferol and calcitriol) and vitamin D_2 (also called ergocalciferol). Vitamin D levels are dependent on two sources: dietary and exposure of the skin to sunlight. Exposure to the sun converts 7-dehydrocholesterol (7-DHC) to the previtamin precholecalciferol, which is then converted into activated 7-dehydrocholesterol. The vitamin D from the two sources then has to undergo two metabolic modifications in the liver and kidney. It is finally converted to the active form, 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], also called calcitriol[286–289]. Its half-life is short but it has many targets. Calcitriol represents a critical physiological factor in autoimmune diseases (Figure 15).

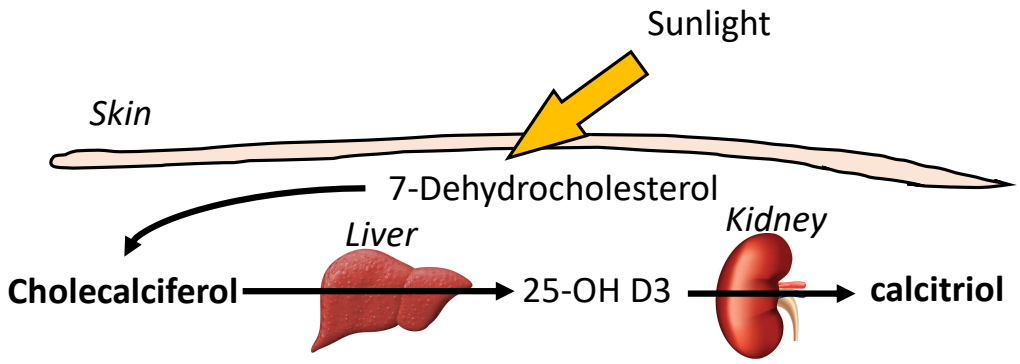


Figure 15 The metabolism of vitamin D_3 in the organism.

It has been shown, for example, that the combination of IL-27 and TGF-β increases the production of IL-10 by Tr1 cells[192]. Vitamin D_3 (VitD3) is a well-known immunomodulator that has been tested in many autoimmune mouse models by its ability to suppress the Th17 response[286,290]. IL-27 and the biologically active form of VitD3, calcitriol, have the same effect on the polarization of Th17 but one signals through an extracellular receptor and the other through the vitamin D receptor (VDR), a nuclear receptor that also acts as a transcription factor. *In vitro*, when CD4 cells were polarized to Th17 with IL-6 and TGF-β, it was observed that the combination of IL-27 and calcitriol cooperated to dedifferentiate the cells into Tr1 and produce IL-10[290]. Indeed, treatment with a combination of IL-27 and calcitriol generated a significantly higher amount of IL-10 compared with that induced by IL-27 alone.

Moreover, in a recent study, venous samples from patients with multiple sclerosis before and after supplementation of vitamin D_3. After supplementation, the concentration of IL-27 in the plasma was significantly higher. The mechanism underlying the tolerogenic properties of vitamin D_3 could be related to its ability to induce at least partially the secretion of IL-27[291].

The cellular activity of calcitriol in immune tolerance has been studied for a long time. Its role in the induction of tolerance of DCs has been proven: DCs that are treated with calcitriol for several days showed a decrease in CD40, CD80, CD86 and MHCII, low secretion of IL-12, and enhanced IL-10 production[292]. Those cells were shown to induce tolerance *in vitro* and *in vivo.* Also, a single injection of VitD3 in the mouse has been shown to increase $Foxp3^+$ and $Helios^+$ T cell population in the spleen and LN and enhance evolution of EAE[293].

7. AUTOIMMUNE DISEASES

7.1. DIFFERENT CAUSES OF AUTOIMMUNE DISEASES

The ability to discriminate self from non-self is the central pillar of the immune system, rendering the ability to attack a pathogen while avoiding self-destruction. However, the distinction between a foreign antigen and a self-antigen is not always obvious. In some exceptional conditions, the immune system can make mistakes and attack the wrong targets. There is a very long list of autoimmune diseases that correlate with the diversity of self-antigens. They range from very low physiological effects to extreme clinical consequences resulting from organ failure caused by the immune system. In western countries, autoimmune diseases have a high prevalence, affecting between 5 and 10% of the population. They are categorized by the type of organ they affect. Some have already been discussed here, such as MS, IBD, rheumatoid arthritis (RA), and type 1 diabetes. Autoimmune diseases are clinically heterogeneous between individuals and have different causes, including genetic or environmental factors[294]. There are very few genes in which a single mutation causes an autoimmune disease: *CTLA4*, *FOXP3*, *IL35,* and *AIRE*. In these diseases, the immune system is not well regulated and it starts to attack one or several organs. If there is a dysfunction in central tolerance (the mechanism to exclude cells that react against self-antigen), autoreactive B or T cells escape tolerance selection and can be the cause of an autoimmune disease[295]. The APECED syndrome, for example, is a disease caused by mutations of the gene encoding AIRE. This gene is expressed by epithelial cells and tDC in the thymus that help to suppress autoreactive T cells[296]. This rare autosomal disease is characterized by the autoimmune destruction of organs.

Most autoimmune diseases are due to complex combinations of risk factors that alone would not be sufficient. More than 100 loci have been found to be associated with RA, MS, diabetes, lupus, or IBD; these are called predisposition factors. Mutations in MHC class II have the strongest associations[297]. However, many risks associated with loci occur in poorly characterized and poorly understood coding and noncoding regions of the genome.

In lupus B cells are the central players in this disease by the secretion au autoantibodies. This disease has a high genetic susceptibility. Autoreactive B cells are negatively selected during the maturation in the bone marrow. A dysregulation of this mechanism due to a mutation leads to a high genetic susceptibility for the development of this disease[298].

The role of the microbiota flora in autoimmunity is still only partly understood. Several complex interactions occur between the mucosal immune system and the commensal flora. It was shown that if this ecosystem is disrupted, it could be the cause of autoimmune disease like IBD but also multiple sclerosis and others (type I diabetes). The exact mechanisms are unknown, and research is slowly progressing in this area[299].

Some other mechanisms are linked with autoimmune diseases, such as the association of vitamin D and MS. In the case of MS, many epidemiological studies have shown the importance of genetic factors in susceptibility to the disease[300,301]. A genome-wide association study from 2011 identified regions of the genome associated with MS; among several regions, 2 were related to the catalyzation of calcitriol[300]. Another study on data from 7 million US military personnel suggested that high circulating levels of vitamin D were associated with a lower risk for MS[302]. A survey of MS patients has shown an inverse linear correlation between the concentration of VitD3 in the blood and the probability of having a relapse of the disease in a dose-dependent way[303]. VitD3 seems to be an excellent immune modulator but it has not shown any efficacy as a treatment in any autoimmune disease.

7.2. TOLEROGENIC DC-BASED THERAPIES

Peripheral DCs maintain peripheral tolerance through the induction of anergic regulatory cells with their low expression of co-stimulatory signals or secretion of tolerogenic cytokines such as IL-10. Immature DCs can induce unresponsive lymphocytes through, for example, the induction of expression of negative co-stimulator molecules such as CTLA4, PD-L1, and LAG-3[304]. The ablation of DCs causes directly spontaneous autoimmune diseases[305]. Other experiments on ablations of DCs have shown that their lineage is critical. For example, CD11c seems to be essential, and mice that lack this protein have more severe EAE[60]. Other experiments *in vitro* have shown that DCs can acquire tolerogenic properties[292]. This acquired tolerance is a way to control immune homeostasis against commensal antigens under inflammatory conditions[48] (Table 2).

DCs can shape the adaptive immune response and, because of this powerful property, they are an interesting target for the development of new cellular therapies. The molecular identification of cancer-specific antigens has allowed the development of antigen-specific immunotherapy[306]. Much research has been performed on the use of DCs for cancer vaccines, but they have shown many limitations for long-term treatments, even for a well-understood cancer such as melanoma[307]. With a better understanding of DCs and by combining DCs with antibodies specific for immune checkpoint inhibitors (anti-PD1 and anti-PD-L1), we may be able to develop new therapies for these diseases[308].

For human autoimmune diseases, the approach is more complicated for different reasons. First, tol DCs can change the immune memory but not the underlying factors (genetics and environmental) that lead to autoimmune diseases. For example, if the environment is fertile to autoimmune disease, the memory will be changed to attack self-antigens. Second, it is difficult to know precisely the self-antigens that are attacked by the immune system. In the case of the dysfunctional immune system, there is a vast diversity between individuals. And there are many

epitopes of the same autoimmune target organ that might be implicated in the disease. The third reason is that from the point of view of the clinical research industry, it is more difficult to propose innovative cell therapies that potentially have many secondary effects for autoimmune diseases that are nonlethal and already have “efficient” therapies. Even though immune suppressors have critical unwanted effects and they do not definitively cure the disease, they do decrease symptoms efficiently. Recently, several strategies to modulate antigen-specific T cells have been evaluated in clinical trials for patients with MS. The main advantage of using an antigen-specific therapy is that they lack the general immunosuppression that is usually correlated with susceptibility to infections, cancers, and metabolic disorders. Several phase I clinical trials are currently assessing the safety and efficacy of administration of an ascending dose of tolerogenic DCs[309,310]. Most of the antigen-specific therapeutic approaches were well tolerated by MS patients except a phase II clinical trial based on altered peptide ligand that had to be stopped because patients started to have exacerbations of MS symptoms[311]. Several methods can be used to induce antigen peptide tolerization: DNA, RNA, or peptide vaccines or administration of blood mononuclear cells chemically coupled with myelin peptides[66,312].

Several obstacles hinder successful implementation of DC vaccination. The appropriate DC subtype needs to be used and, once chosen, the appropriate protocol needs to be established to differentiate them in a large quantity. The route, dose, and time of administration need to be established: where should it be injected? Should it be injected during the crisis or when there are no symptoms? The question of how to produce a large quantity of tol DCs is a challenge in finding an efficient therapeutic strategy for mice model experiments. Many researchers have shown that tol DCs can be differentiated *in vitro* from mature or immature DCs with many different immunosuppressive agents such as calcitriol, estriol, and tolerogenic cytokines[61,292,313]. After being cultured (between 4 and 8 days) with different agents, all generated tol DCs present the same characteristics, down regulation of MHCI, MHCII, and co-stimulatory molecules. When matured they generally produce tolerogenic cytokines (e.g., IL-10 or IL-27), they generally poorly activate T cells, and they express negative co-stimulation molecules (checkpoint inhibitors)[314]. The administration of those DCs in autoimmune disease mouse models generally presents the same results (depending on the DCs and administration): improvement in mean clinical score, an increase in Tregs ($Foxp3^+$), an increase of IL-10 level in lymph nodes, and increases in regulatory surface markers such as PD1, CTLA4, and LAG-3. In table 2, We summarize different studies that have used tol DCs in autoimmune disease models. The selected publications are differentiated between those that attempted to cure symptoms and those that aimed to prevent symptoms (i.e., were injected before the arrival of symptoms).

Model	Prevention / treatment	Number of DCs (and route of administration)	Generation of tol DCs	Reference
EAE	Prevention	5×10^5 (in the footpad)	Cholecalciferol	315
EAE	Treatment	8×10^6 (*i.v.*)	Vitamin D_3	71
EAE	Treatment	1×10^6 (*i.v.*) many times	Vitamin D_3	316
EAE	Treatment	1×10^6 or 10×10^6 (*i.v.*)	pDCs	317
EAE	Prevention	2×10^6 (*i.v.*)	IL-35 (transduction)	70
EAE	Prevention	2×10^6 (*i.v.*)	Estriol	313
EAE	Prevention/ treatment	No injection	DCs targeting MOG expression	318
Diabetes (NOD mice)	Treatment	2×10^6 (*i.v.*)	NF-kB–specific oligodeoxyribonucleotide (ODN)	319
Diabetes (NOD mice)	Prevention/ treatment	Injection of a drug that targets DCs *in vivo*	TLR7 ligand to 6 units of polyethylene glycol (PEG)	320
CIA	Prevention	2×10^6 (*i.v.*)	IL-10 and TGF-β	321
DTH	"Prevention" / treatment	5×10^5 (*i.v.*) (2 times)	IL-10	322

Table 2 A few examples of beneficial application of tolerogenic DCs in different animal models of autoimmune diseases.

8. MUTU DC LINE

Research on DCs has always been challenging. Even though they are valuable players in immune response and immune tolerance, their study has been limited for the following reasons: (1) DCs are challenging to isolate, and after isolation, they quickly become activated and die by apoptosis; (2) DCs in the same subgroup are very heterogeneous depending on their age, state of activation, origin, and subtype.

Another problem is that DC isolation is time consuming and expensive, and the number of cells collected is limited. The other technique to generate large quantities of DCs is to derive them from bone marrow or blood precursors. Stem cells or monocytes can differentiate into DCs by using granulocyte macrophage colony stimulating factor (GM-CSF) or FLT3, with or without IL-4. However, the problems with this approach are diverse and include limitation of the amount of bone marrow available, time and reproducibility of differentiation, and the heterogeneity of DC subpopulations.

The need for a stable, homogeneous cell line is obvious (Table 3). The CD11c integrin promoter is important during myeloid differentiation. CD11c is a type I transmembrane protein found on DCs but also on monocytes, macrophages, neutrophils, and some B cells. Using our minimal promotor, we could achieve DC-specific expression of the oncogene in transgenic mice. The generation of the SV40 large T oncogene under the control of the minimal promoter of CD11c allowed DC-specific transformation *in vivo*. The expression of GFP under the same promoter allowed us to identify the cells expressing the transgene. Mice with the transgene spontaneously develop tumors and die in a dose-dependent fashion between 1 month and more than 1 year after birth.

For our DC lines, we chose mice that died at 4 months or 12 months. Sick mice developed large tumors of GFP-positive cells in the spleen and liver. Culture of the splenocytes of sick transgenic mice led to a cultivable DC line after several months of adaptation.

These cells were characterized by flow cytometry and showed the same characteristics as freshly isolated DCs. They are able to present antigen to CD4 and CD8 T cells. Upon activation, they secrete a large quantity of cytokines and upregulate MHCII, CD40, CD80, and other co-stimulatory signals. They can be efficiently transduced with lentivirus to express or delete other genes. By crossing with KO mice or by using lentiviral CRISPR-Cas9 strategies, KO lines can be generated. These cells are called Mutu DCs and they have a comparable phenotype to *in vivo* $CD8^+$ cDCs and function similarly, with or without activation. They are stable for over 40 passages[70,323].

Many laboratories currently use Mutu DCs, indicating that they are an excellent tool for research in DC immunology. Recently, our laboratory has developed a new Mutu DC line generated from a $Batf3^{-/-}$ Mushi1 murine line that shares characteristics with cDC2s[324].

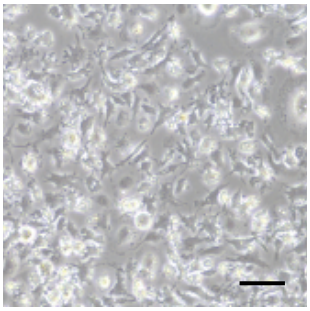
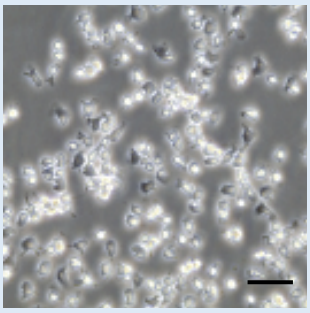
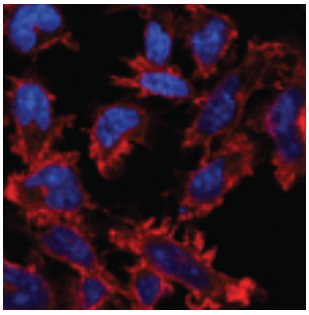
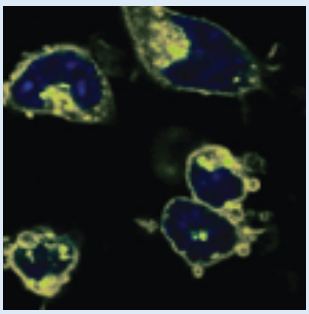
Light microscopy images of representative Mutu DC lines at early passages.	
Light microscopy images of representative Mutu DC lines at later passages.	
Immunofluorescence microscopy of representative Mutu DCs showing CD11c in red. **Nuclei were stained with DAPI.**	
Immunofluorescence microscopy of representative Mutu DCs showing MHCII in yellow. **Nuclei were stained with DAPI.**	

Table 3 Morphology of Mutu DC line cultures. Scale bar = 20 µm. Adapted from [323]

9. CELL ENCAPSULATION TECHNOLOGY TO SECRETE CYTOKINES

Osmotic pumps implanted in mice to provide continuous delivery of proteins have been used in research for a long time[325,326]. In research, osmotic pumps are primarily used to study the effect of a high concentration of a protein in the serum[268]. However, the use of an osmotic pump to constantly deliver recombinant proteins has some limitations. This approach is generally expensive and the cost of purified recombinant proteins is high. Limited quantities of proteins can be stored inside the pump and, depending on the model, the pump may need to be changed every week. In addition, osmotic pumps cannot be used with an unstable protein that would quickly be degraded. Except with very modern osmotic pumps (e.g., the iPRECIO Programmable Pumps from Alzet[327]), the release of proteins cannot be induced or stopped on demand; instead, the device needs to be surgically removed to adjust the delivery of proteins.

The team of Prof. Patrick Aebischer and Dr. Bernard Schneider at EPFL developed a new kind of implant able to encapsulate cells in an environment that is not accessible to the immune system[328]. It has many advantages although it does share the problems of unstable proteins and cost of proteins with osmotic pumps. The cost of other components (gel, cells, and implant) is relatively low. The implant can protect the encapsulated cells from the immune system of the host; therefore, the same optimized producer cell line can be used for all patients. The cells can grow in a biodegradable gel until they reach confluency and form a tissue able to secrete the protein of interest. The selected myoblast cell line C2C12 is physically resistant, and it can survive at high confluency in the implant for at least one year after implantation, continuously secreting large quantities of the desired protein. C2C12 cells were chosen because they stop growing and continue to survive when they reach confluency in the implant or culture plate, and they are resistant to hypoxia.

Encapsulated cells have been shown to survive for more than a year and secrete high quantities of proteins when implanted subcutaneously in the back of the mouse. Another advantage also that the cells allow continuous delivery of freshly produced proteins. The team of Prof. Aebischer and Dr. Schneider has demonstrated that this implant can deliver a high amount of recombinant anti-amyloid-β antibodies that can efficiently prevent the formation of amyloid plaques in a mouse model of Alzheimer disease[329]. Maxivax, a Geneva-based clinical-stage biotechnology company, is currently testing this technology in humans for cancer vaccination[330]. The device with encapsulated cells is implanted in the arm of the patient to secrete locally a small quantity of GM-CSF. At the same time, irradiated cells from tumors are injected next to the implant to recruit DCs and induce an immunogenic reaction against the tumor. Several clinical trials have shown that this technology is safe and effective in humans and does not have unwanted secondary effects[331].

The use of encapsulated cells instead of an osmotic pump provides another advantage; they can be tuned to be inducible and to activate the gene of interest in the function of the medical need. Synthetic biology aims to redesign organisms for useful purposes by engineering them to acquire new abilities. Organisms can, for example, be engineered to sense a protein and respond to this stimulus by secreting a protein of choice. Logic gates made via recombinases, G-protein-coupled receptors (GPCRs), or CRISPR (clustered regularly interspaced short palindromic repeats) represent important tools by which to tune the expression of genes[332]. Cells inside an implant could be activated with an inducible promoter to secrete a tolerogenic cytokine only when it is needed. For example, in a study on psoriasis, genetically engineered cells encapsulated in alginate were able to sense TNF and IL-22 (the proinflammatory cytokines responsible for the psoriasis mouse model) and to secrete a corresponding therapeutic level of the immunomodulatory cytokines IL-4 and IL-10[333]. That study showed that encapsulated cells can do more than just provide cytokines; they can decide to release, or not, a therapeutic protein according to precise sensing of the concentration of the disease marker in the bloodstream. This could be a great advantage because the cytokines would be secreted before the onset of the symptoms and prevent their onset. However, in the case of autoimmune diseases, it is more challenging because inflammatory cytokines are not exclusively disease markers. They are useful to protect the organism, and the induction of tolerance is very dangerous. A proof of concept of this idea was made with an “AND” gate to sense TNF AND IL-22 to secrete tolerogenic cytokines only for a pathogenic immune response[333]. Indeed, psoriasis induced by imiquimod is characterized by high secretion of those two proteins. An implant made of genetically engineered HEK cells that sense TNF AND IL-22 and secrete, upon activation, the anti-inflammatory cytokines IL-4 and IL-10 has been designed. Mice implanted with the “microencapsulated cytokine converter” did not show symptoms of the induced psoriasis[333]. More studies are needed to design a cellular sensor capable of detecting the “cytokine signature” of an autoimmune disease.

AIMS OF THE PROJECT

This thesis is mainly focused on the tolerogenic potential of IL-27 in autoimmune disease mouse models.

The first aim of our project was to investigate whether expression of IL-27 could modulate DC maturation and give a tolerogenic phenotype to lymphocytes. For that, we used our $CD8\alpha^+$ cDC1 cell line (Mutu DCs) that was transduced to constitutively secrete an IL-27 construct. We analyzed whether the transduced cells had a tolerogenic phenotype *in vitro* and whether they could change the immune-specific memory *in vitro*.

The second aim was to test whether our IL-27-secreting DCs could change the established immune memory *in vivo.* The DTH response model was ideal because it allowed us to quantify the degree to which immune memory was altered by monitoring the degree of footpad swelling. We also wanted to determine whether those DCs could induce the Tr1 response and reduce the Th1 response.

The third aim was to test whether IL-27 DCs had therapeutic potential in the EAE model and whether they could positively alter development of the disease. We wanted to understand whether IL-27 DCs could help to restore the tolerance of the adaptive immune system and aid in recovery from the disease.

The fourth aim was to understand the mechanisms of IL-27 DCs on T cells. We wanted to determine if the tolerogenic potential of IL-27 DCs is due to IL-27 or if it is due to the induction of negative co-stimulators such as: Tim-3 and PD1.

We also wanted to determine if IL-27 DCs would act synergistically with the active form of vitamin D_3 *in vitro* and *in vivo* to induce tolerance. To this end, we evaluated IL-27 DCs and vitamin D_3 in the DTH and EAE models to see whether they worked together to further enhance the established immune memory.

Finally, we aimed to propose a new way of changing the adaptive immune system through systemic secretion of tolerogenic cytokines. The encapsulated cell technology developed by Prof. Aebischer allowed us to test a subcutaneous cellular implant containing genetically engineered cells able to secrete high quantities of IL-27. We compared systemic secretion of IL-27 with IL-27 DCs in the DTH model. These experiments helped us to understand whether the cytokine had to be secreted by an antigen-presenting DC producing locally high concentrations of the cytokine or whether similar effects could be achieved when IL-27 was secreted systemically by an implant.

1. GENERATION OF THE IL-27 DC LINE

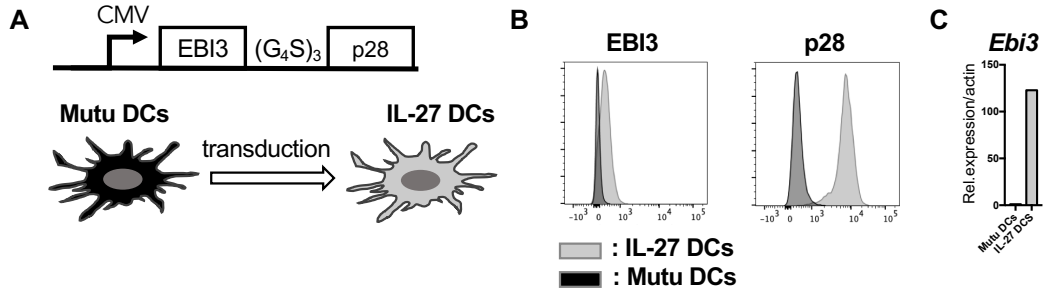


Figure 16 IL-27 transduced dendritic cells (DCs) are able to secrete IL-27. (A) Mutu DCs were transduced to produce IL-27 constitutively with a gene coding for a fusion protein of EBI3 and p28 linked by a $(G_4S)_3$ linker. (B) EBI3 and p28 intracellular proteins were detected by flow cytometry after 5 hours of culture with brefeldin A. (C) The IL-27 subunit gene *Ebi3* was detected in the IL-27-transduced DC line by quantitative reverse transcription PCR using primers specific for *Ebi3*.

Our group has previously generated tolerogenic $CD8\alpha^+$ MuTu DCs able to secrete IL-10, latent or active TGF-β (Koga et al. – manuscripts in preparation), or IL-35[70]. The referred tolerogenic Mutu DC lines secrete large quantities of the respective cytokine. Specifically, the IL-35 construct in the IL-35-secreting Mutu DC line (referred to as IL-35-DCs) was engineered by linking the two IL-35 subunits EBI3 and p35 with the flexible (Glycine4Serine)3 [$(G_4S)_3$] linker used here also for IL-27 DCs. This type of linker allows the secretion of both subunits under the same promoter, avoiding dimerization with other subunits and therefore generating other cytokines, such as IL-12 (IL-12p35 + p70), IL-27 (EBI3 + p28), or IL-35 (EBI3 + IL-12p35). Surprisingly, EBI3 alone has inflammatory activity by activation of STAT3 and induction of proliferation through IL-6 trans-signaling[334]. The linker to associate EBI3 covalently with another subunit might prevent the inflammatory activity of the EBI3 subunit alone. The IL-35 construct was shown to be stable over passages and was biologically active[70].

Taking advantage of the same strategy, we generated an IL-27-secreting Mutu DC line (hereafter, IL-27 DCs). IL-27 is a cytokine structurally similar to IL-35, in which the EBI3 subunit is associated not with p35 but with p28. To generate this stable DC line that secretes IL-27 constitutively, Mutu DCs were transduced with a single-chain construct linking the two subunits of IL-27 (EBI3 and p28) with a linker $(G_4S)_3$ (Figure 16**A**). The polypeptide linker is reported to be biologically neutral[335] and previous work done in our laboratory has shown that the linker does not change the biological activity of its relative, IL-35 DCs[70]. In another publication, the same construct was used and the construct shown to be biologically active in DCs[336]. The linker allowed expression of both cytokine subunits at a 1:1 ratio, forcing the dimerization of the two linked chains. The human

cytomegalovirus promoter (CMV) is often used to constitutively produce a high quantity of protein. This same promoter has been shown to efficiently produce cytokines in our DC line (in most cells except lymphocytes)[70,337].

The DCs were transduced using the highest lentiviral concentration that did not reduce their viability. Infected cells were selected with puromycin at 0.5 µg/mL for 5 days. It has been shown previously that lentiviral transduction does not alter gene expression of Mutu DCs[70,323]. The expression of EBI3 and p28 proteins was measured by flow cytometry after 5 hours of culture with brefeldin A to inhibit cytokine secretion (Figure 16**B**). Unfortunately, the secreted IL-27 was not detectable by a commercial ELISA kit, probably because the linker can inhibit binding of the cytokine to one of the antibodies. The same phenomenon was observed previously by Haller with IL-35[70]. Expression of the transgene was maintained for at least 20 passages. There is an advantage in not detecting IL-27 and IL-35 by the available ELISA kits, in that we can distinguish endogenously produced cytokine from recombinant cytokine. Transgene transcription was assayed by measuring *Ebi3* by reverse transcription real-time qPCR (Figure 16**C**).

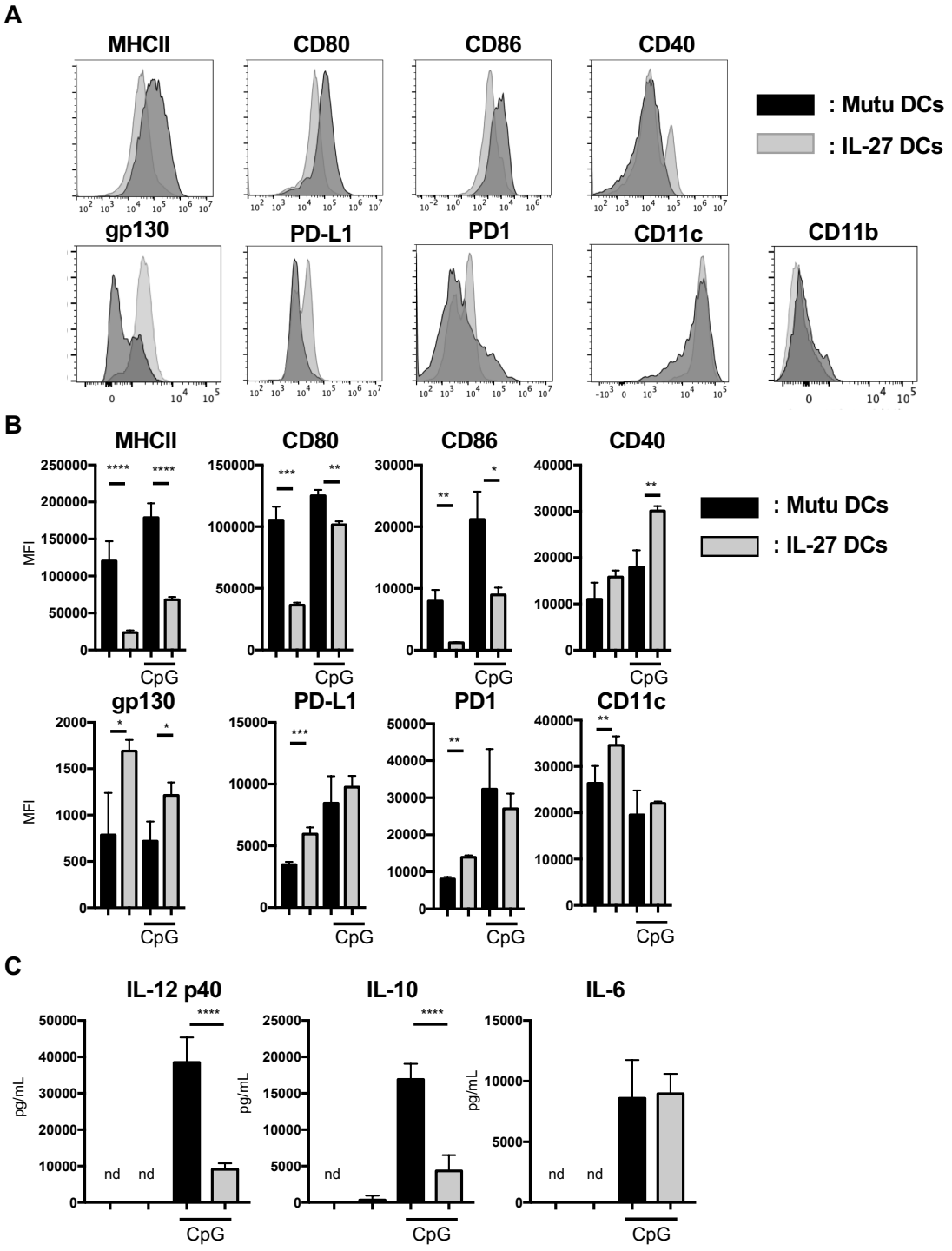


Figure 17 IL-27-transduced DCs have partially tolerogenic characteristics and show a lower response upon CpG stimulation. (A) IL-27 and untransduced DCs were cultured under the same conditions and analyzed by flow cytometry for the expression of different cellular markers. (B) Mean fluorescent intensity (MFI) of the DC markers under resting conditions or stimulated with CpG (1 µg/mL). (C) Secretion of cytokines was measured by ELISA. Cells were seeded at 5 × 10^5 cells/well, cultured for 3 days, and then activated with CpG for 16 hours. Results are shown as the mean of biological triplicates ± SD. Data represent

2 to 4 independent experiments. Statistical analysis was performed applying Student's *t*-test: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

As discussed in the introduction, IL-27 is a cytokine with both immunogenic and tolerogenic properties. With IL-35 DCs, it was shown that the cytokine has a powerful autocrine and paracrine effect on the DCs themselves. To determine whether the constitutive expression of IL-27 also had an autocrine/paracrine effect in DCs, we tested a panel of surface markers used to distinguish the immunogenicity of DCs. Flow cytometry showed that compared with the MuTu DCs, IL-27 DCs had lower expression of MHCII, CD80, and CD86 (Figure 17**A**) and similar expression of CD40. When IL-27 DCs were activated by TLR9 agonist CpG (1 µM) overnight, they also increased surface expression of MHCII and the co-stimulatory molecules CD80 and CD86, but to a lesser extent than MuTu DCs (Figure 17**B**). CD40 expression, however, was higher on IL-27 DCs after TLR9 stimulation. CD11c expression was comparable to that of Mutu DCs, and both were negative for CD11b. In the resting state, IL-27 DCs showed higher expression of the immune checkpoint inhibitors PD1 and PD-L1. It has also been proposed that like IL-35, IL-27 could bind to the homo-dimeric receptor made with two gp130 molecules[153]. IL-27-DCs were shown to have higher expression of gp130 compared with Mutu DCs in both resting and activated conditions (Figure 15B). We also assessed the ability of DCs to secrete cytokines. Under resting conditions, no secretion of IL-12 (p40) or IL-6 was detected, but upon stimulation with CpG, IL-27 DCs secreted less IL-12 p40 and IL-10 and similar quantities of IL-6 compared with untransduced DCs (Figure 17**C**).

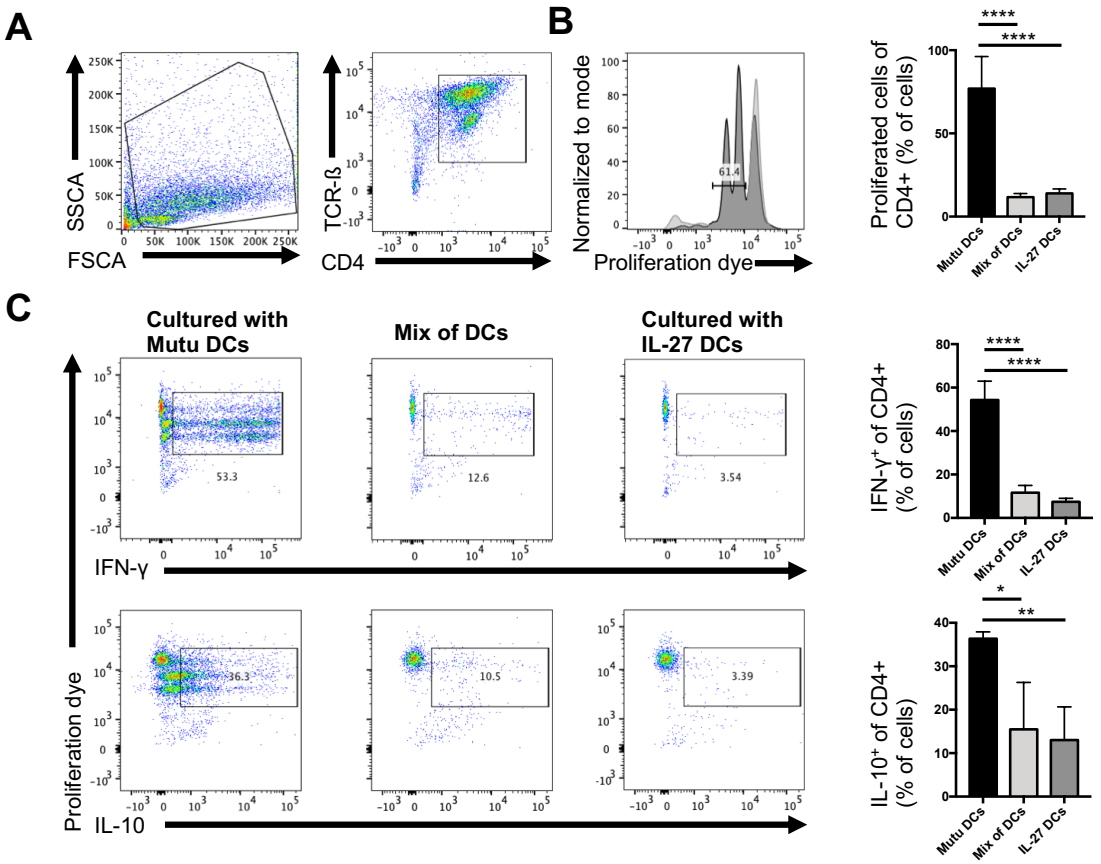


Figure 18 IL-27 DCs induce reduced proliferation of OVA-specific CD4 T cells. Mutu DCs, IL-27 DCs, or a 1:1 mix of the two cell lines were pulsed for 5 h with OTII peptide and CpG before the addition of OT-II-specific CD4 T cells. The T cells were analyzed by flow cytometry 72 h later. **(A)** Gating strategy. **(B)** Proliferation of CD4 T cells cultured with IL-27 DCs, wild type DCs (Mutu DCs), or a 1:1 mix of DCs (Mutu DCs and IL-27 DCs). **(C)** Cytokine production by CD4 T cells. Results are shown as the mean of biological triplicates ± SD. Data represent 4 independent experiments. Statistical analysis was performed applying one-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Mutu DCs are known to induce antigen-specific proliferation of $CD4^+$ T cells. IL-27 was described to inhibit the proliferation of CD4 T cells. An OTII proliferation assay was made with IL-27 DCs to see whether DCs exposed continuously to IL-27 would show altered antigen presentation to CD4 T cells. MuTu and IL-27 DCs were pulsed for 5 h with OVA peptide (OT-II peptide, 100 nM) in the presence of CpG (1 µM). They were cultured for 3 d with OVA-specific $CD4^+$ T cells magnetically purified from OT-II mice stained with a proliferation dye to track cell divisions. The cells were gated for living cells and $CD4^+$ TCR-β^+ double-positive cells (Figure 18**A**). Mutu DCs were able to induce two peaks of division after 3 days, but IL-27 DCs induced only marginal proliferation (Figure 18**B**). As IL-27 DCs have a lower expression of MHCII and co-stimulatory molecules and they produce

less IL-12 (Figure 17), reduced antigen presentation and co-stimulation could explain the lowered T cell activation properties. To address whether this reduced proliferation was due to IL-27 or to alterations in DCs growing in the presence of IL-27, we made a 1:1 mixture of IL-27 DCs and untransduced DCs. Even in the presence of untransduced DCs, T cells did not proliferate more, highlighting the role of IL-27 in this effect. Furthermore, IL-27 DCs were able to inhibit the differentiation into IFN-γ^+ or IL-10^+ T cells (Figure 18**C**).

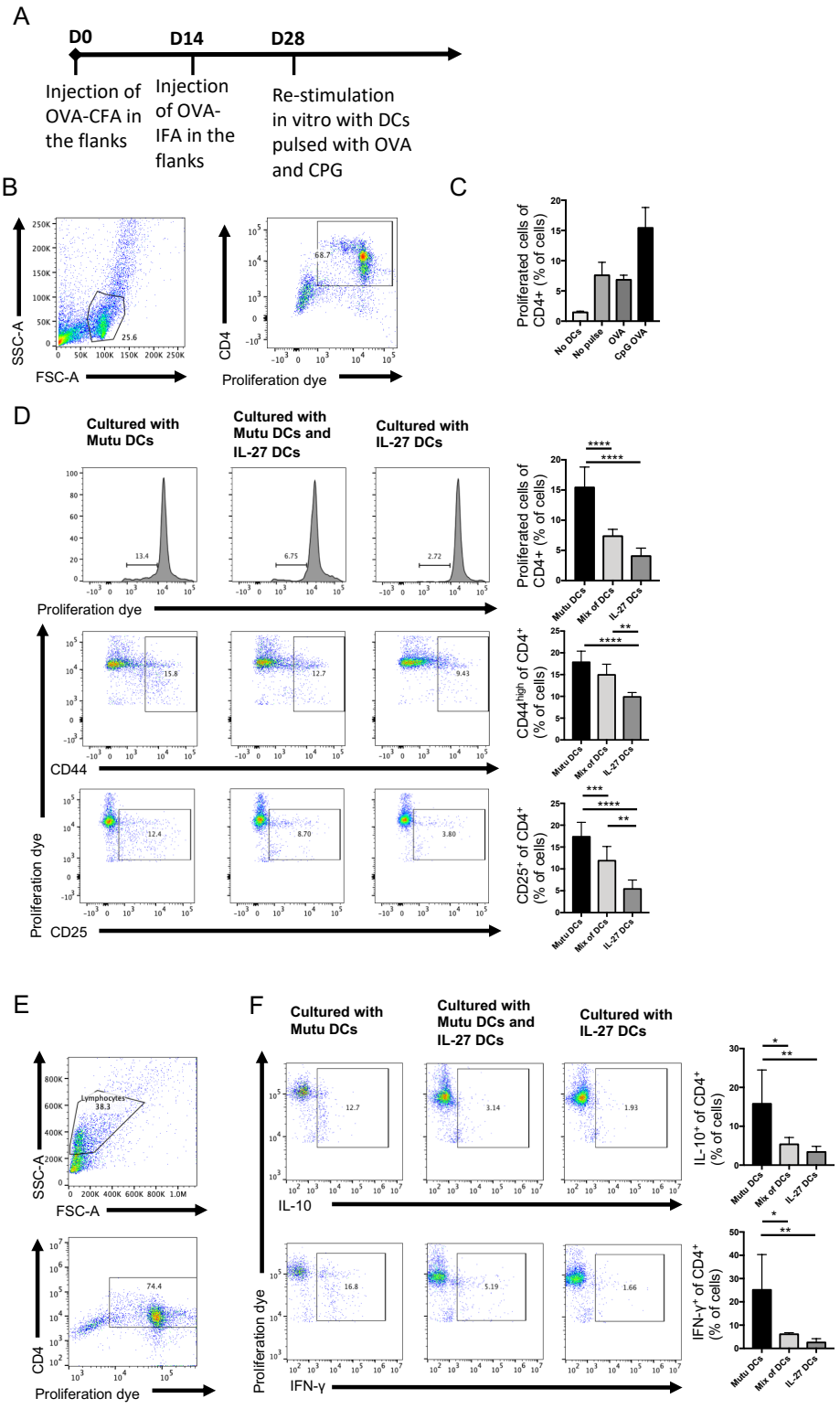


Figure 19 IL-27 DCs change the antigen-specific proliferation of memory CD4⁺ T cells *in vitro*. (A) Scheme of the experiment: C57BL/6 mice were immunized with ovalbumin (OVA)

grade V (1 mg/mL) in complete Freund's adjuvant (CFA) and boosted 2 weeks later with OVA (same concentration) in incomplete Freund's adjuvant (IFA). CD4 T cells were collected from the spleen and lymph nodes after 28 days, when stable memory was developed, and restimulated *ex vivo* with OVA-pulsed IL-27 and/or MuTu DCs for 3 days. **(B)** Gating strategy. **(C)** Analysis of CD4 subpopulations for proliferation. **(D)** Analysis of CD4 subpopulations for IFN-γ^+, IL-10^+, $CD25^+$, and $CD44^{high}$ of the T cells by flow cytometry after 3 days. Results are shown as the mean of biological triplicates ± SD. Data represent one out of three experiments. Statistical analysis was performed with one-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

To determine whether IL-27 DCs can modify restimulation of memory T cells, we induced stable immune memory against a specific antigen (OVA) *in vivo*. C57BL/6 mice were immunized with OVA grade V (1 mg/mL) in complete Freund's adjuvant (CFA) at day 0 (Figure 19**A**). Two weeks later, the immune system was boosted with the same concentration of OVA in incomplete Freund's Adjuvant (IFA). After 28 days, $CD4^+$ T cells from the spleen and inguinal lymph nodes (ILN) were magnetically purified and stained with the Tag-it Violet proliferation dye (5 µM; Biolegend). Mutu DCs and IL-27 DCs were pulsed with OVA (OVA grade VI, 100 µg/mL) with or without CpG (1 µM) for 4 hours, and then co-cultured with the magnetically purified $CD4^+$ T cells (2×10^5 DCs were mixed with 10^6 T cells, making a 1:5 DC:T cell ratio). After 3 days, the cells were analyzed by flow cytometry and gated on $CD4^+$ cells. Double-negative cells (proliferation $dye^- CD4^-$) could be the DCs (Figure 19**B**). Mutu DCs were able to induce proliferation of 15% of the cells when they were pulsed with OVA and CpG (Figure 19**C**). Without CpG, fewer cells proliferated independently of the addition of OVA (background proliferation; data not shown). IL-27 DCs induced very low specific proliferation on $CD4^+$ T cells. To show the tolerogenic role of IL-27, a mixture of IL-27 DCs and Mutu DCs (1:1) called "mix of DCs" was added. In the presence of IL-27 DCs, T cells proliferated very little, even in the presence of Mutu DCs. The activation markers CD44 and CD25 were also analyzed on the T cells. Fewer cells cultivated with IL-27 DCs had high expression of CD44 compared with those cultivated with Mutu DCs or the mix of the two DCs. After co-culture with IL-27 DCs, the percentage of $CD25^+$ T cells was reduced (a decrease of 3-fold) compared to those cultivated with Mutu DCs, and an intermediate percentage of cells cultivated with the mixture of DCs expressed (Figure 19**D**). The cells were also stained for intracellular cytokines. The cells were gated for $CD4^+$ T cells (Figure 19**E**). T cells cultivated with IL-27 DCs with or without Mutu DCs secreted less IL-10 and less IFN-γ. For IFN-γ, the difference was even more pronounced: 25% of the cells were IFN-γ^+ if they were pulsed with

Mutu DCs, but only 5% were IFN-γ$^+$ when IL-27 DCs were present.

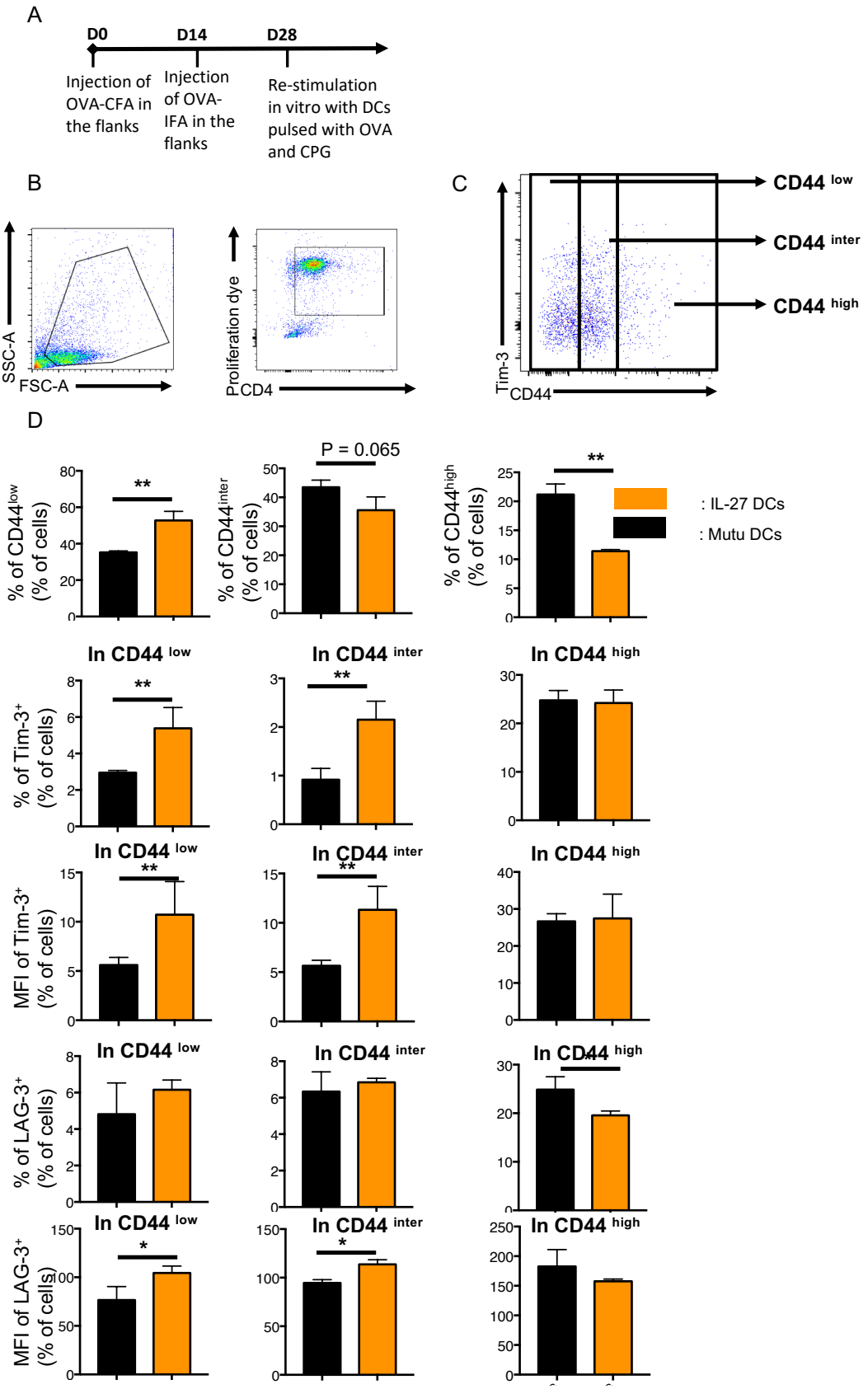


Figure 20 IL-27 DCs can induce Tim-3 in CD44^{low} and CD44^{inter} in CD4 T cells. (**A)** Scheme of the experiment: C57BL/6 mice were immunized with ovalbumin (OVA) grade V (1 mg/mL) in complete Freund's adjuvant (CFA) and boosted 2 weeks later with OVA (same concentration) in incomplete Freund's adjuvant (IFA). CD4 T cells were collected after 28 days, when stable memory was developed, and restimulated *in vitro* with OVA-pulsed and CpG-stimulated IL-27 and/or MuTu DCs for 3 d. **(B)** Gating strategy cells were divided in 3 groups: CD44^{high}, CD44^{inter}, and CD44^{low}. **(C)** Mean fluorescence intensity (MFI) and percentage of Tim-3 and LAG-3 in each population of CD44. Results are shown as the mean of biological triplicates ± SD of one experiment. Statistical analysis was performed with one-way ANOVA: *$p < 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Different co-inhibitory receptors have important roles in regulating the T cell response and seems to be an interesting target in different autoimmune diseases. CTLA-4, PD1, LAG-3, Tim-3, and TIGIT belong to the same class of receptors but have different functions and are regulated differently[62]. IL-27 is a key inducer of LAG-3 and Tim-3[236,274]. On Th1, the addition of IL-27 was shown to promote expression of Tim-3 and IL-10[236]. To establish whether IL-27 was able to induce the expression of LAG-3 and Tim-3 in memory cells, an experiment with the same set-up as figure 17 was done, and expression of LAG-3 and Tim-3 was checked in different populations of CD4 T cells (Figure 20**A**). Cells were gated for CD4^{+} cells and then separated in 3 groups (CD44^{high}, CD44^{low}, and CD44^{inter}) according to their expression of CD44 (Figure 20**B**). Our results showed a a reduced number of CD44^{high} and a higher percentage of CD44^{low} in cells primed with IL-27 DCs (Figure 20**C**. The mean fluorescence intensity (MFI) of LAG-3 and Tim-3 on CD4 T cells was also analyzed (Figure 20**D**). After 3 days of culture with IL-27 DCs, cells in the CD44^{inter} and CD44^{low} groups had a higher percentage of Tim-3^{+} cells and a higher MFI of Tim-3. Expression of LAG-3 was also observed. The MFI of LAG-3 was higher in CD44^{inter} and CD44^{low} but not in CD44^{high}. The percentage of LAG-3^{+} cells was higher only in CD44^{high}, not in the other two subpopulations. This could reflect the mechanism of IL-27 DCs, where they inhibit the activation of T cells through the increase of the expression of those two receptors. However, it is not possible to distinguish whether this overexpression was a direct effect of IL-27 or whether it was caused by direct priming of IL-27 DCs (no mix of Mutu DCs + IL-27 DCs was used in this experiment).

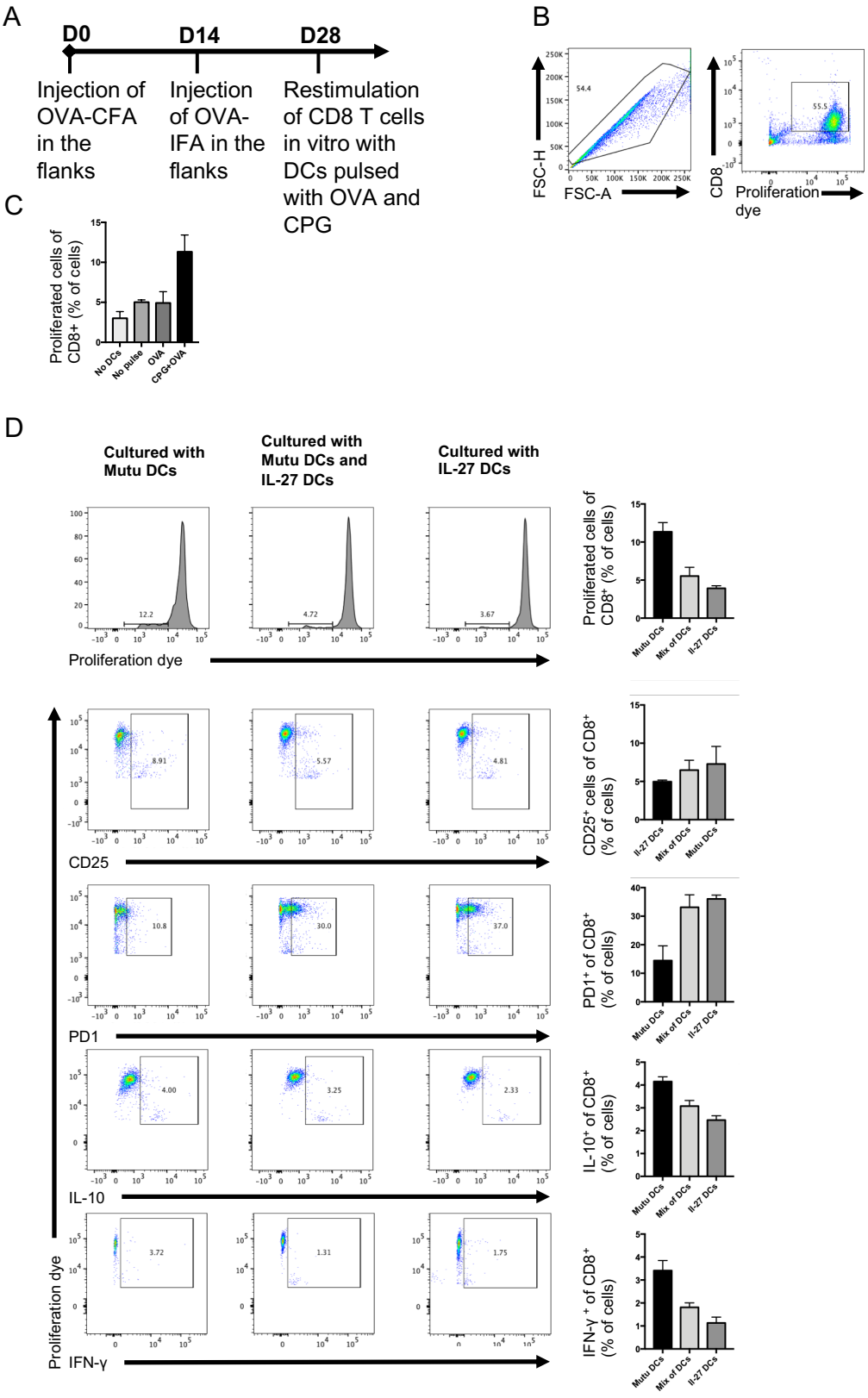


Figure 21 IL-27 DCs change the antigen-specific proliferation of memory CD8⁺ T cells *in vitro*. (A) Scheme of the experiment: C57BL/6 mice were immunized with ovalbumin (OVA) grade V (1 mg/mL) in complete Freund's adjuvant (CFA) and were boosted after 14 days with OVA grade V in incomplete Freund's adjuvant. CD8 T cells were collected after 30 days and

restimulated *in vitro* with IL-27 and/or Mutu DCs previously pulsed for 4 h with OVA (100 µg/mL) in the presence of CpG (1 µg/mL) stimulation for 3 days. **(B)** Gating strategy. **(C)** Analysis of proliferation in CD8 T cells after restimulation. **(D)** IFN-γ, IL-10, CD25, and PD1 expression of T cells was analyzed by flow cytometry after 3 days. Results are shown as the mean of biological duplicates ± SD. The data are from 1 of 2 independent experiments.

The same protocol as in the previous experiment was used to establish a large number of memory T cells specific for OVA. The CD8 T cells from spleen and LN were isolated on day 30, stained with Tag-it Violet proliferation dye (Biolegend), and cultured with DCs (Figure 21**A**). After 3 days, the cells were analyzed by flow cytometry and gated for $CD4^+$ cells (Figure 21**B**). Mutu DCs were able to induce proliferation of 11% of cells when they were pulsed with OVA and CpG (Figure 21**C**). Without CpG, fewer cells proliferated, independently of the addition of OVA (background proliferation). IL-27 DCs were able to induce very little specific proliferation on $CD8^+$ T cells (Figure 21**D**). As in the previous experiment, the tolerogenic role of IL-27 was verified to determine whether this reduced proliferation was due to IL-27 or to alterations in DCs growing in IL-27. We made a 1:1 mixture of IL-27 DCs and Mutu DCs. In the presence of IL-27 DCs, CD8 T cells proliferated very little, even in the presence of Mutu DCs. The activation marker CD25 was analyzed on the T cells. The percentage of $CD25^+$ cells did not change between cells activated with IL-27 DCs and those activated with Mutu DCs. Inversely, cells that were activated with IL-27 DCs showed increased expression of the PD1 receptor (3-fold increase in expression compared with cells activated with Mutu DCs). The tolerogenic potential of IL-27 DCs, even mixed with Mutu DCs, on proliferation and expression of PD1 was dominant (Figure 21**D**). CD8 cells were also stained for intracellular cytokines. Cells cultivated with IL-27 DCs with or without Mutu DCs expressed less IL-10 and IFN-γ. For IFN-γ, the difference was even more pronounced (Figure 21**D**).

5. IL-27 DCS CAN DECREASE ANTIGEN-SPECIFIC INFLAMMATION *IN VIVO*

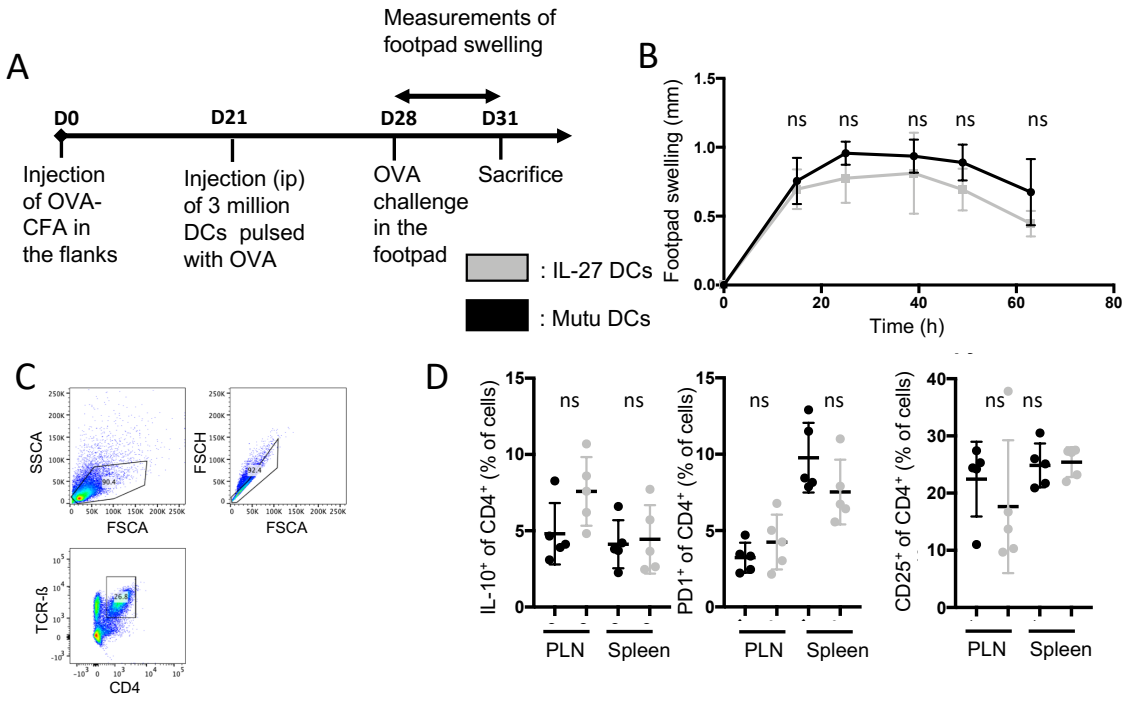


Figure 22 IL-27 DCs induced no significant changes in footpad swelling when mice were immunized against OVA. (A) Scheme of the experiment: C57BL/6 mice were immunized with ovalbumin (OVA) grade V (1 mg/mL) in complete Freund's adjuvant (CFA) on d 0. Then, 21 d after immunization, mice were injected with DCs (IL-27 DCs or Mutu DCs). **(B)** Footpad swelling was measured 6 times during the 3 days following injection. The data shown are the calculated size differences of the OVA-challenged and PBS-challenged footpads. Splenocytes and popliteal lymph nodes were isolated 3 d after challenge and analyzed by flow cytometry. **(C)** Gating strategy. **(D)** $CD4^+$ $TCR\text{-}\beta^+$ cells were analyzed for expression of $IL\text{-}10^+$, $PD1^+$, and $CD25^+$. The experiment was done once with 5 mice per group. Statistical analysis was performed with one-way ANOVA: $*p < 0.05$.

To see whether IL-27 DCs were able to change immune memory *in vivo*, a delayed-type hypersensitivity reaction (DTH) was performed. DTH is a good model to quantitate the immune memory against a specific antigen. Mice were immunized as in the previous experiment with OVA in CFA at day 0 (Figure 22**A**). Two weeks later, when T cell memory was established, mice were injected (*i.p.*) with 3×10^6 IL-27 DCs or Mutu DCs that had been previously pulsed with OVA (100 µg/mL), not activated with CpG. The right footpad was challenged with OVA and the left one was injected with PBS. Footpad swelling was calculated as the difference in size of the two footpads. In the 3 days after the challenge, no significant differences could be observed between the 2 groups of mice (Figure 22**B**), although mice injected with IL-27 DCs tended to have thinner footpads (4 of 5 mice in the IL-27 DCs group had thinner footpads than 5 of 5 control mice). The population of $CD4^+$ cells in the popliteal LN (PLN) and spleen were then analyzed by flow cytometry (Figure 22**C**). No significant differences were observed for expression of IL-10, PD-1, or CD25 (Figure

22**D**). The population of IL-10^{+} cells in the PLN tended to be superior to that in the IL-27 DCs mice. The number of CD25^{+} cells in the PLN tended to be inferior to that in mice injected with IL-27 DCs. One injection of IL-27 DCs did not seem sufficient to significantly change the established T cell memory.

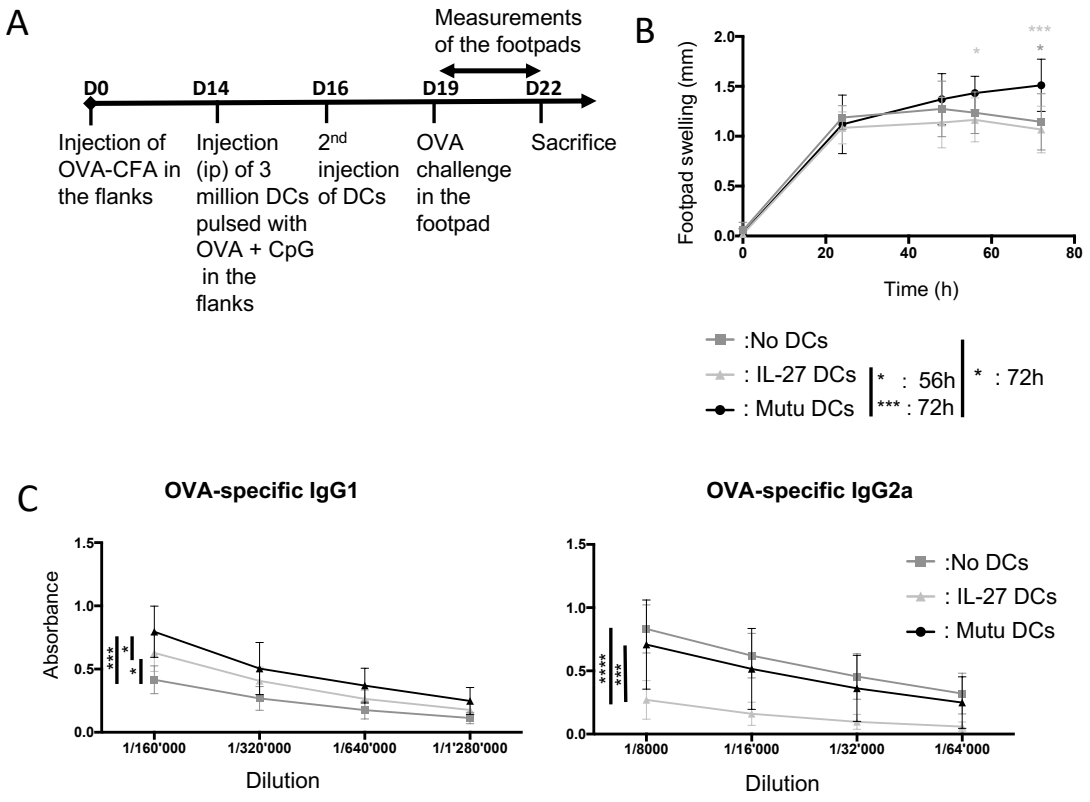
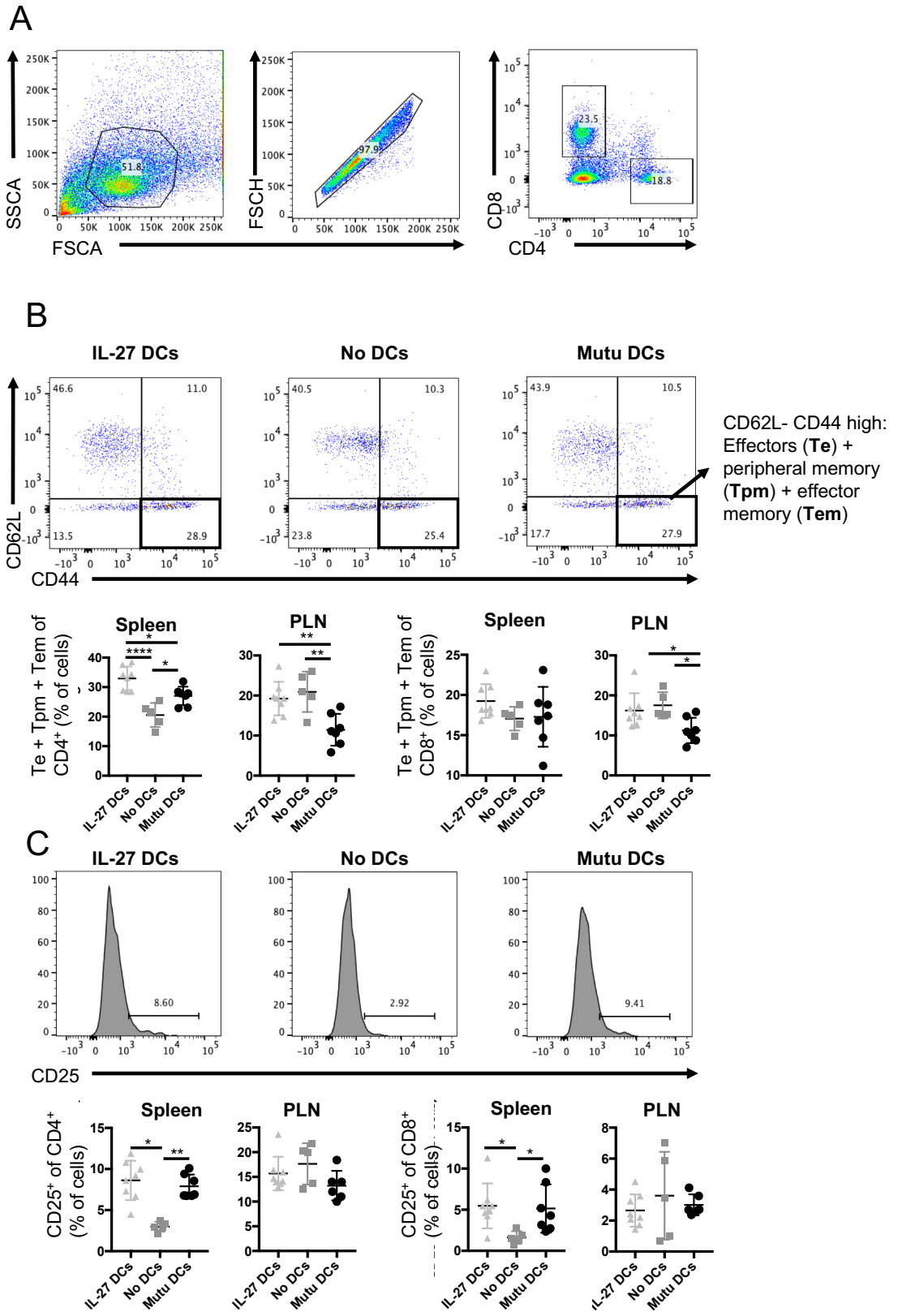


Figure 23 IL-27 DCs reduce antigen-specific inflammation in DTH. (A) Scheme of the experiment: mice were immunized with ovalbumin (OVA) in complete Freund's adjuvant (CFA) on day 0, and mice were separated into three groups. The IL-27 DC group (n=8) and Mutu DCs (n=7) received two injections (*i.p.*) of 3 × 10^6 DCs (IL-27 DCs or Mutu DCs) on day 14 and day 16, previously pulsed for 4 h with OVA and CpG. The third group (no DCs) had only control injections of PBS. On day 23, all mice were challenged with OVA in the right footpad and injected with PBS in the left footpad. **(B)** Footpad swelling was measured 5 times and calculated as the difference in size of the right footpad (challenged with OVA) and the left footpad (injected with PBS). **(C)** After 72 h, mice were sacrificed and the concentration of OVA-specific IgG_{2a} and IgG_1 in the serum was measured by ELISA (with different dilutions). Results are shown as the mean ± SD. The experiment was done once with at least 5 mice per group. Statistical analysis was performed with two-way ANOVA: *$p < 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

We then decided to modify the protocol. We injected IL-27 DCs twice, on days 14 and 16, instead of only once. In this setup, DCs were pulsed with OVA and activated with CpG (Figure 23**A**).To determine whether injection of DCs influenced the immune response, one control group of mice did not receive DCs, instead being administered only injections of PBS (control) on days 14 and

16. Mice were challenged with OVA in the right footpad 3 days after the second DC transfer. After the OVA challenge, footpad swelling was measured as previously described. Footpad swelling of mice injected with IL-27 DCs was significantly less than that of mice injected with Mutu DCs (Figure 23**B**). The difference was even more pronounced 3 days after the challenge. Mice that were not injected with any DCs had lesser inflammation 3 days post-challenge compared with mice injected with Mutu DCs. The footpad swelling of mice injected with IL-27 DCs was comparable to that of mice that did not have any OVA-pulsed DCs transferred, which raised the question of whether the decreased footpad swelling was due to the tolerogenic effect of IL-27 or to the absence of co-stimulation of the DCs. This was answered by analysis of OVA-specific antibodies in the sera of the mice. We observed that the IL-27 DC-injected group had higher levels of IgG_1 than the "no DC" group, and a lower level of IgG_{2a}. (Figure 23**C**). Mice injected with Mutu DCs had a significantly higher concentration of OVA-specific IgG_1 than mice in the other two groups. Mice that were not injected with DCs showed a lower concentration of OVA-specific IgG_1 compared with mice injected with DCs. The concentration of OVA-specific IgG_{2a} was significantly lower for mice injected with IL-27 DCs than for mice that were not injected or were injected with Mutu DCs.


A
SSCA
FSCA
FSCH
CD8
CD4
51.8
97.9
23.5
18.8
B
IL-27 DCs
No DCs
Mutu DCs
CD62L
CD44
46.6
11.0
13.5
28.9
40.5
10.3
23.8
25.4
43.9
10.5
17.7
27.9
CD62L- CD44 high: Effectors (Te) + peripheral memory (Tpm) + effector memory (Tem)
Te + Tpm + Tem of CD4+ (% of cells)
Te + Tpm + Tem of CD8+ (% of cells)
Spleen
PLN
C
CD25
8.60
2.92
9.41
CD25+ of CD4+ (% of cells)
CD25+ of CD8+ (% of cells)

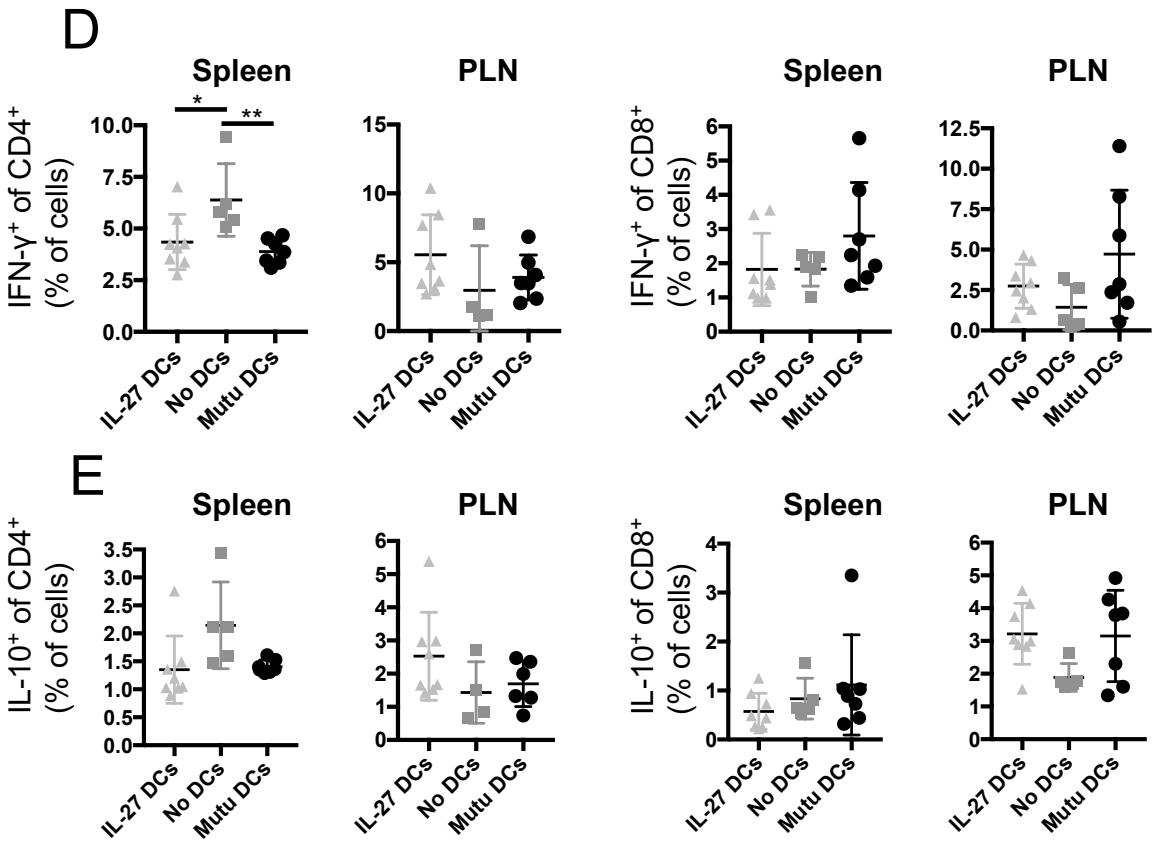


Figure 24 Injection of DCs changed cell populations in the spleen and popliteal lymph node (PLN) in DTH. (A) Gating strategy to analyze CD4 and CD8 populations. **(B)** Effector + memory populations ($CD44^+$ and $CD62L^-$) in the spleen and PLN. **(C)** $CD25^+$ populations in the CD4 and CD8 populations in the spleen and PLN. **(D)** IFN-γ^+ in the $CD4^+$ and $CD8^+$ populations. **(E)** IL-10^+ cells in the $CD4^+$ and $CD8^+$ populations. Results are shown as the mean ± SD. Statistical analysis was performed with one-way ANOVA: *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

The spleen and PLNs of footpads injected with OVA were isolated and analyzed by flow cytometry. The cells were gated for $CD4^+$ and $CD8^+$ populations (Figure 24**A**). In the $CD4^+$ population, mice that were not injected with DCs had a significantly lower percentage of "Te + Tpm + Tem" T cells ($CD44^+$ $CD62L^-$) cells in the spleen (Figure 24**B**). In the PLNs of the footpad that was challenged with OVA, the group injected with Mutu DCs unexpectedly had a significantly lower number of effectors than the other two groups. This difference was also observed in the $CD8^+$ population in PLNs and in spleen. This is probably due to the fact that the cells migrated more in the footpad. The mice that did not receive any injections had a significantly smaller $CD25^+$ cell population in the spleen compared with the two other groups (Figure 24**C**). The $CD4^+$ IFN-γ^+ cell population was smaller for mice injected with Mutu DCs compared with mice that received no DCs (Figure 24**D**).

$CD4^+$ $IL\text{-}10^+$ cells in PLNs showed no difference between groups, although it tended to be higher for mice injected with IL-27 DCs (Figure 24**E**).

The control with no DCs helped reinforce that the boost of DCs has a powerful effect on the immune system. After the challenge, mice that did not receive any injection had less footpad swelling than mice injected with Mutu DCs, which was undoubtedly because of the lack of restimulation with DCs. This was also observed in the percentage of $CD25^+$ cells and the number of effector cells in the spleen. The double injection of 3×10^6 activated and OVA-pulsed DCs is an important boost and changed the immune system completely. It is, however, complicated to compare mice that were not injected and those that were injected with IL-27 DCs. One has low inflammation because of the lack of boost and the other because of the tolerogenic potential of DCs

After the injection (*i.p.*) of DCs, it is difficult to know where they establish in the periphery of the lymphatic system. However, in preliminary data (not shown), activated IL-27 DCs injected (*i.v.*) were observed 1 d later in ILN and the spleen. This tracking experiment needs to be repeated, but it suggests that IL-27 DCs act directly in secondary lymphoid organs to prime memory cells.

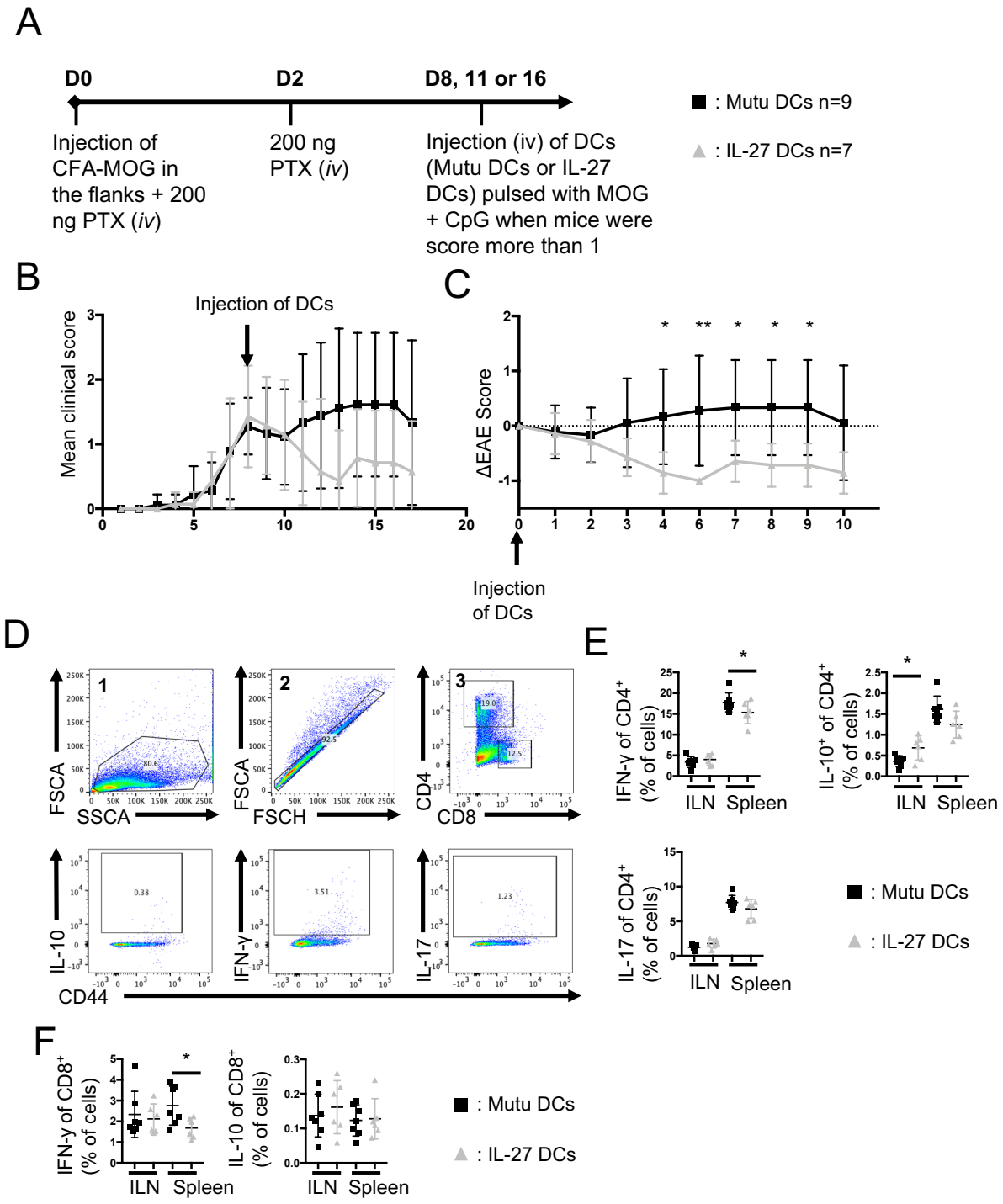


Figure 25 Il-27 DCs have a therapeutic effect on experimental autoimmune encephalitis (EAE). (A) Scheme of the experiment: C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG; 1 mg/mL) in complete Freund's adjuvant (5 mg/mL *M. tuberculosis*) in the flanks and were injected (*i.v.*) with pertussis toxin (200 ng) at days 0 and 2. Mice were observed daily using a clinical EAE scoring system ranging from 0 (no symptoms) to 4 (complete paralysis). When the clinical score of EAE reached 1 (limp tail), treatment was initiated by injection of 3 × 10^6 DCs (IL-27 DCs or MuTu DCs) pulsed for 3 h with MOG and

CpG (one injection each on d 8, 11, or 16). **(B)** Evolution of the mean clinical score of the EAE before and after mice received an injection of DCs. **(C)** Difference in EAE score between the day of injection and the following days. Spleen and inguinal lymph nodes were isolated and analyzed by flow cytometry. **(D)** Gating strategy. **(E)** Analysis of $CD4^+$ T cells in the spleen and ILN for the cytokines IFN-γ, IL-10, and IL-17. **(F)** Analysis of $CD8^+$ T cells for the cytokines IFN-γ, IL-10, and IL-17. Results are shown as the mean ± SD. The experiment was done once with at least 7 mice per group. Statistical analysis was performed with two-way ANOVA for the EAE scores in (B) and (C), and unpaired Student *t*-test was performed for flow cytometry analysis: $*p < 0.05$, $**p < 0.01$.

To test the potential of IL-27 DCs in treating established memory on an autoimmune disease model, an EAE was performed (Figure 25**A**). Mice were immunized against the myelin oligodendrocyte glycoprotein (MOG_{35-55}) peptide in CFA (5 mg/mL *Mycobacterium tuberculosis*). On the same day and 2 days later, the mice received an injection (*i.v.*) of 200 ng of pertussis toxin (PTX). Mice were observed daily using a clinical EAE scoring system ranging from 0 (no symptoms) to 4 (complete paralysis). The scoring table is described in Material and Methods. After 1 week, the mice started to show symptoms of EAE. After 8 days, all mice that started to show symptoms [with an EAE score >1 (limp tail)] were injected (*i.v.*) with DCs previously pulsed with MOG and CpG in paired groups. At days 11 and 16, other mice that started to show symptoms were also injected in paired groups. Mice were paired by day of injection and EAE score; 7 mice were injected with IL-27 DCs and 9 mice were injected with Mutu DCs pulsed with MOG (10 µg/mL) and stimulated with CpG. The mean clinical score was calculated as the mean EAE score of all mice from the group. After injection, mice that received the IL-27 DCs had the tendency to show ameliorated symptoms compared with the control group (Figure 25**B**). The ΔEAE score was calculated as the difference in EAE score of the day of observation and the EAE score on the day of injection. This calculation allowed us to see whether the DCs had a therapeutic effect in the EAE model and it considers the variation in EAE score that can occur on the day of treatment. Mice injected with IL-27 DCs had a significant decrease in ΔEAE score 3 days after injection compared with mice injected with Mutu DCs. Mice that were injected with Mutu DCs did not show a change in their symptoms or showed an increased. Mice injected with IL-27 DCs showed a decrease of 1 in EAE score. At the end of the experiment, mice were sacrificed and cells of the spleen and ILN were isolated and analyzed by flow cytometry (Figure 25**C**). In the CD4 T cell population of the spleen, significantly more IFN-γ^+ cells were detected in mice injected with Mutu DCs than in those injected with IL-27 DCs (Figure 25**D**). The mice that were treated with IL-27 DCs had more IL-10^+ cells in the ILN in the $CD4^+$. No differences were seen in the IL-17^+ population (Figure 25**E**). Injection of IL-27 DCs decreased the IFN-γ^+ cell population of the $CD8^+$ cells from the spleen but not in the ILN (Figure 25**F**).

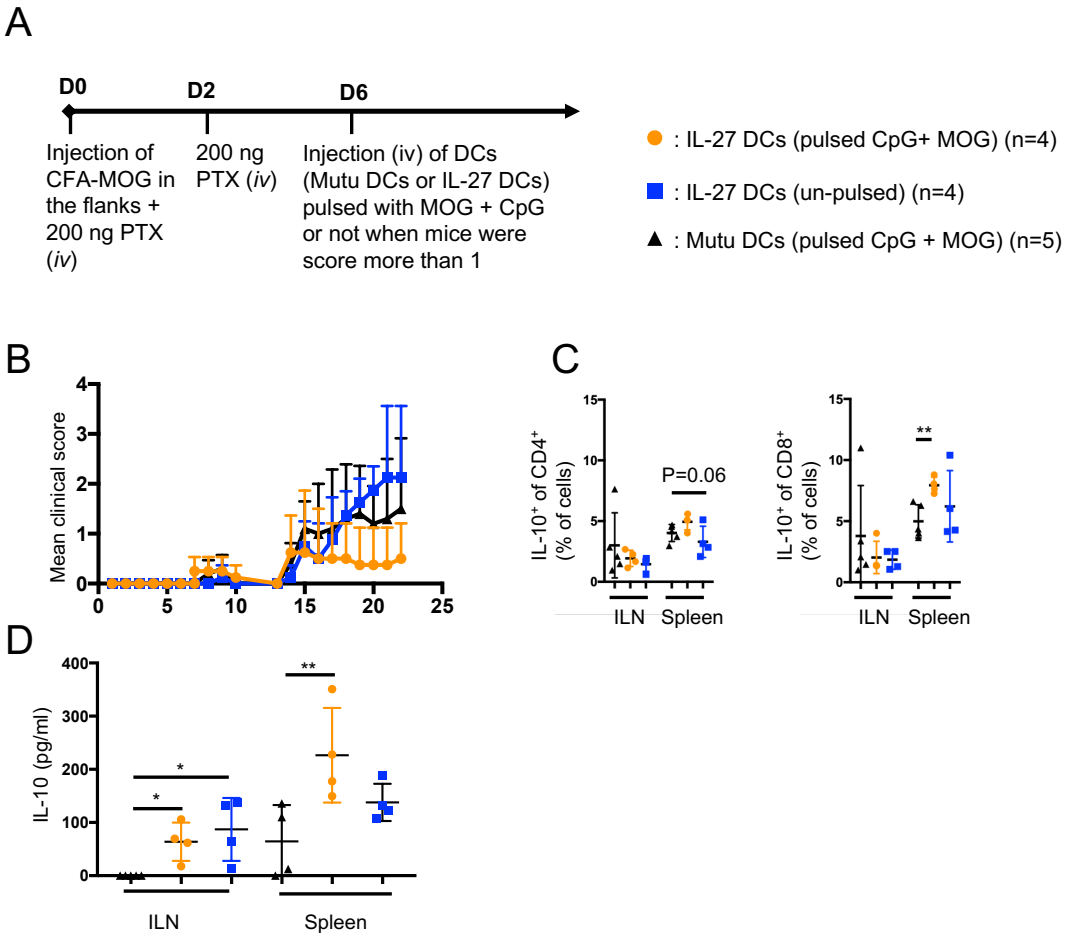


Figure 26 IL-27 DCs injected before symptoms appear were able to induce a MOG-specific IL-10 response. (A) Scheme of the experiment: C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG; 1 mg/mL) in complete Freund's adjuvant (5 mg/mL *M. tuberculosis*) and were injected (*i.v.*) with pertussis toxin (200 ng) at days 0 and 2. Mice were observed daily using a clinical EAE scoring system ranging from 0 (no symptoms) to 4 (complete paralysis). Six days after immunization, mice were treated with an injection of 3 × 10^6 DCs (IL-27 DCs or MuTu DCs) pulsed for 4 h with MOG (10 µg/mL) and CpG 1 µM; one group was injected with unpulsed and unstimulated IL-27 DCs. **(B)** Evolution of the mean clinical EAE score. **(C)** Analysis of $CD4^+$ cells in the spleen and inguinal lymph nodes (ILN) for IFN-γ, IL-10, and IL-17. **(F)** Analysis of expression of IL-10 in $CD8^+$ and $CD4^+$ cells by flow cytometry. **(D)** Cells from the ILN and spleen were restimulated with MOG (10 µg/mL) and the concentration of IL-10 secretion was measured by ELISA 24 h later. Results are shown as the mean ± SD. Statistical analysis was performed with two-way ANOVA for EAE scores and by one-way ANOVA for the flow cytometry analysis and ELISA: $*p < 0.05$, $**p < 0.01$.

The process underlying EAE prevention can be explained by different mechanisms. Tolerogenic DCs can enter LNs before the onset of the first symptoms and prevent or decrease the symptoms of EAE by reducing the production of brain-infiltrating pathogenic T cells. To see whether IL-27 DCs could prevent pathological symptoms, mice were immunized against MOG_{35-55} (1 mg/mL) and

CFA (5 mg/mL) in the flanks. PTX (200 ng) was injected (*i.v.*) on the same day and 2 days later (Figure 26**A**). Before the onset of symptoms, mice were injected (*i.v.*) with 3×10^6 DCs previously pulsed with MOG (10 µg/mL) and activated with CpG. To understand whether IL-27 DCs need to be pulsed and activated to achieve their tolerogenic potential, a group of mice injected with IL-27 DCs not pulsed was included. Twelve days after the first immunization, symptoms started to appear, and mice that were injected with pulsed IL-27 DCs tended to have a lower mean clinical score than the two other groups (Figure 26**B**). In contrast, injection of IL-27 DC not pulsed with antigen did not change the clinical symptoms. The mean clinical score of this group was even higher than that of the control group 19 days after injection. However, no significant differences were observed among all groups, indicating that larger groups need to be used for EAE experiments. Cells from the ILN and spleen were analyzed by flow cytometry for IL-10 expression in CD4 and CD8 cells (Figure 26**C**). In the spleen, $CD4^+$ and $CD8^+$ T cells of mice that were injected with IL-27 DCs expressed more IL-10 than mice injected with Mutu DCs. Cells from the ILN and spleen were restimulated with MOG (10 µg/mL) for 24 h (Figure 26**D**) and IL-10 secretion was then analyzed by ELISA. Cells from ILNs of mice injected with IL-27 DCs (pulsed or not) showed increased secretion of IL-10. In the control group (injected with pulsed Mutu DCs), secretion of IL-10 could not be detected in the ILN. The same result was observed in the spleen: cells from mice injected with IL-27 DCs (pulsed or not) secreted more IL-10 upon restimulation. However, only the group injected with pulsed IL-27 DCs showed a significant difference compared with the control group.

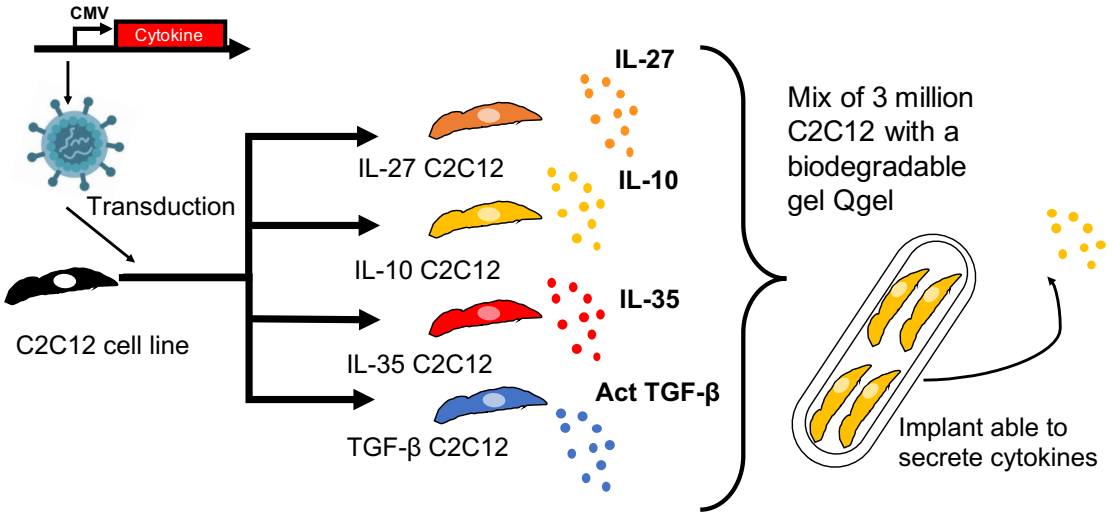


Figure 27 Scheme of the design of the tolerogenic implant.

The systemic secretion of cytokines is a powerful tool to influence the immune response. This measure is widely used for studying the role of cytokines in mouse disease models. Many study use an osmotic pump that delivers a constant release of recombinant cytokines[268], but some prefer the use of bioengineered cells encapsulated in immune isolated implants. This method has several advantages: it allows the constant release of freshly made functional proteins, it is cheaper, and unstable proteins can stay functional for a long time *in vivo* (Figure 27). The gene of interest generally encodes a protein with therapeutic potential such as an antibody, a hormone, or a cytokine. In this study, we also compared the systemic secretion of cytokines with the treatment of DCs presenting antigens and secreting tolerogenic cytokines. The technology developed in the laboratory of Prof. Patrick Aebisher[329] allowed us to encapsulate cells able to secrete a high amount of cytokines in the bloodstream over a long period. The implant helps to protect the cells of the implant from the immune system of the host. The controlled delivery of cytokines in an immune-isolated implant containing genetically engineered cells is also an inexpensive and safe approach for chronic treatment with cytokines. Production can be interrupted when the implant is removed or if the gene of interest is controlled with an inducer promoter. We chose to use C2C12 myoblasts, which have demonstrated prolonged high physical and chemical resistance under the conditions in the implant and a high capacity to produce proteins. We created 4 different stable cell lines able to secrete 4 tolerogenic molecules: IL-27, IL-35, IL-10, and active TGF-β (Figure 27). The cells were transduced with the same vector as used for the DCs. The mice were able to live a normal life with the implant on the back after surgery (Figure 28**A**). The cells survive well inside an implant that is well vascularized by the host. (Figure 28**B**)

A

B

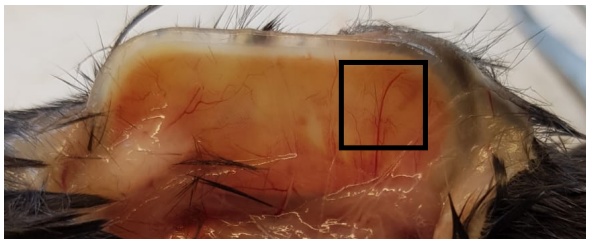


Figure 28 The implant is integrated and well vascularized subcutaneously in the mouse. **(A)** Three days after surgery, the mouse can walk and move normally (the back of the mouse was shaved for surgery). **(B)** Implant 1 month after surgery showing visible vascularization.

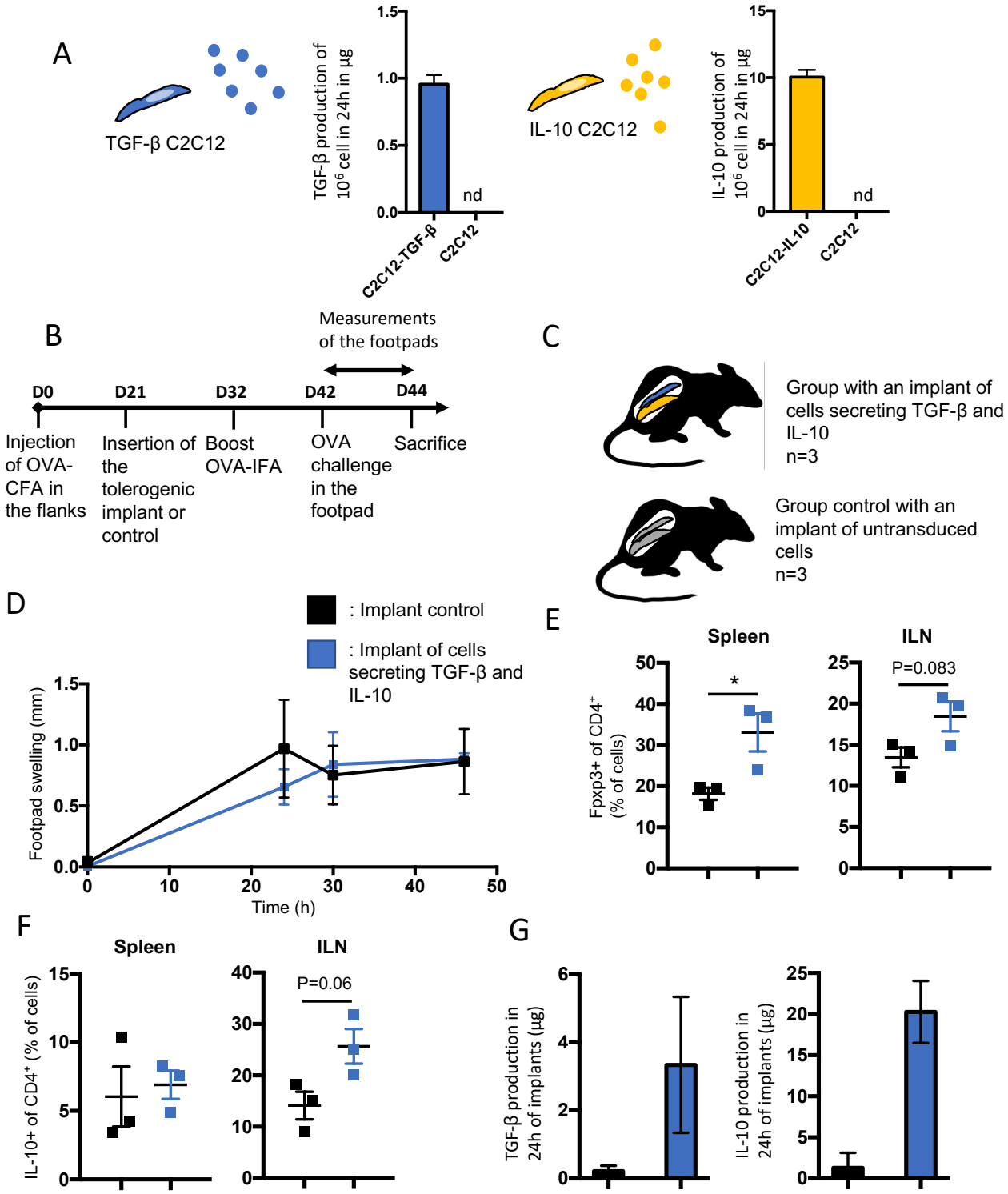


Figure 29 A subcutaneous implant of cells able to secrete IL-10 and TGF-β in a mouse model of DTH. (A) Two types of C2C12 cell were transduced to secrete high amounts of IL-10 or active TGF-β. **(B)** Scheme of the experiment: mice were immunized against ovalbumin (OVA) with an emulsion of OVA (1 mg/mL) in complete Freund's adjuvant (1 mg/mL *M. tuberculosis*) in the flank, and the implant was inserted subcutaneously on d 21. Implants were made with 5 × 10^6 C2C12 cells encapsulated in a porous membrane that was inaccessible to the immune system. Five million cells (2.5 × 10^6 TGF-β C2C12 and 2.5 × 10^6 IL-10 C2C12 or 5 × 10^6 untransduced C2C12 for control mice) were embedded in the matrix with

biodegradable gel (from Qgel). A boost with OVA (1 mg/mL) in incomplete Freund's adjuvant was injected in the flank at day 32. After 42 days, mice received an injection of OVA (right footpad) or PBS (left footpad). **(C)** The size of each footpad was measured twice a day (average of 3 independent measurements for each footpad). Footpad swelling was calculated as the difference in size of the PBS-injected footpad and the OVA-injected footpad. **(D)** Mice were sacrificed after 44 days. Inguinal draining lymph nodes (OVA-challenged side) and spleen were collected, and CD4 T cells were analyzed by flow cytometry for $Foxp3^+$ cells. **(F)** $CD4^+$ cells were stained intracellularly for IL-10. **(G)** The implant was removed from the mouse after sacrifice and left for 24 h in medium at 37°C. The medium was then analyzed by ELISA to estimate production of IL-10 and TGF-β of the implants. The experiment was done once with 3 mice per group. Results are shown as the mean ± SD. Statistical analysis was performed with Student's *t*-test: $*p < 0.05$, $**p < 0.01$.

To establish the efficiency of the implant with cytokines, we chose two cytokines that have well-known effects when added together: TGF-β and IL-10. Their synergy is known to induce strong $Foxp3^+$ Treg cell-dependent responses and to decrease inflammation[131]. The two transformed C2C12 cell lines (C2C12-IL10 and C2C12-active TGF-β) were able to secrete a high quantity of their respective cytokines *in vitro*, as shown by ELISA (Figure 29**A**). To determine whether the cells were efficiently producing in the implant and could have an *in vivo* effect, we performed a DTH experiment (Figure 29**B**). The mice were immunized against OVA (1 mg/mL) in CFA (1 mg/mL *M. tuberculosis*); then, on day 21, when stable T cell memory was established, an implant was subcutaneously inserted in the back of each mouse. Mice were divided in two groups: (Figure 29**C**) one with an implant with 5×10^6 untransduced C2C12 (control group) and a group with a mix of 2.5×10^6 of C2C12-active TGF-β and 2.5×10^6 C2C12-IL-10. The mice were boosted with IFA and OVA on day 32 and then challenged in the footpad on day 42. Evolution of footpad swelling was measured twice a day and no significant differences were observed between the two groups of mice during the 48 h following the challenge (Figure 29**D**). Cells from the spleen and ILN were analyzed by flow cytometry. The $Foxp3^+$ Treg population was significantly higher in the CD4 T cells in the spleen of mice with the implant secreting both active TGF-β and IL-10 and the same trend was observed in the ILN (Figure 29**E**). The $IL\text{-}10^+$ population of the $CD4^+$ was higher (but not significantly) in the ILN for mice with the tolerogenic implant (Figure 29**F**). The implants were removed when the mice were sacrificed and incubated in medium for 24 h at 37°C. The medium was then collected and the concentration of IL-10 and TGF-β quantified by ELISA (Figure 29**G**). After 21 days inside a mouse, the implants were still secreting high amounts of IL-10 and TGF-β. The control implants with C2C12 produced small amounts but this was probably because of the tissue full of immune cells that stays attached on the implant.

8. THE TOLEROGENIC EFFECTS OF IL-27 DCS AND IL-27 IMPLANTS ARE COMPARABLE

C2C12 cells were bioengineered to secrete constitutively IL-27 and later be encapsulated in an implant. The cells were transduced with EBI3 and p28 bound with a linker to form IL-27 C2C12 (as previously described) (Figure 30**A**). The expression of EBI3 and p28 was verified by flow cytometry (Figure 30**B**). *Ebi3* gene expression was also assessed by quantitative PCR (Figure 30**C**), but we could not detect IL-27 protein production for the same reasons previously mentioned (our ELISA kit does not detect the recombinant IL-27 with the linker).

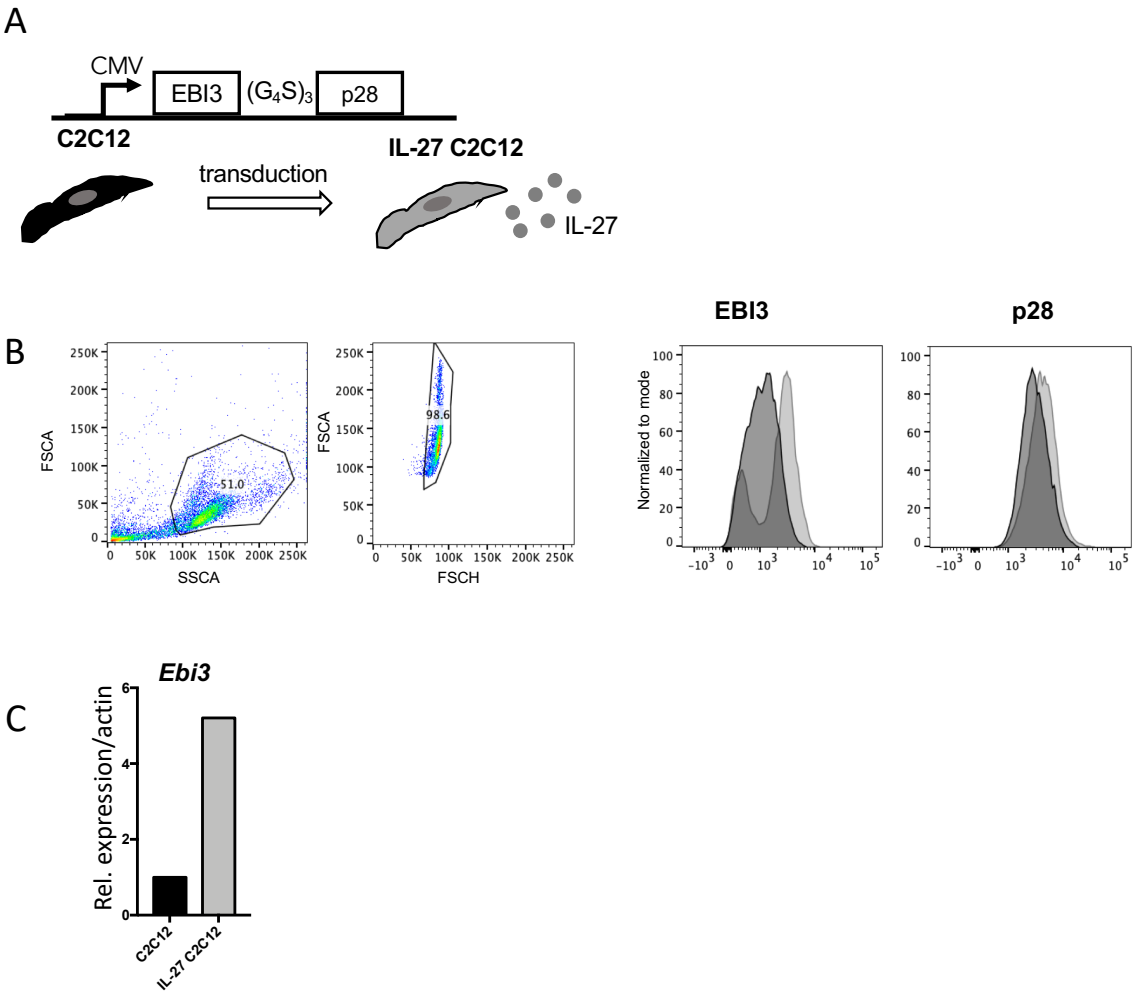
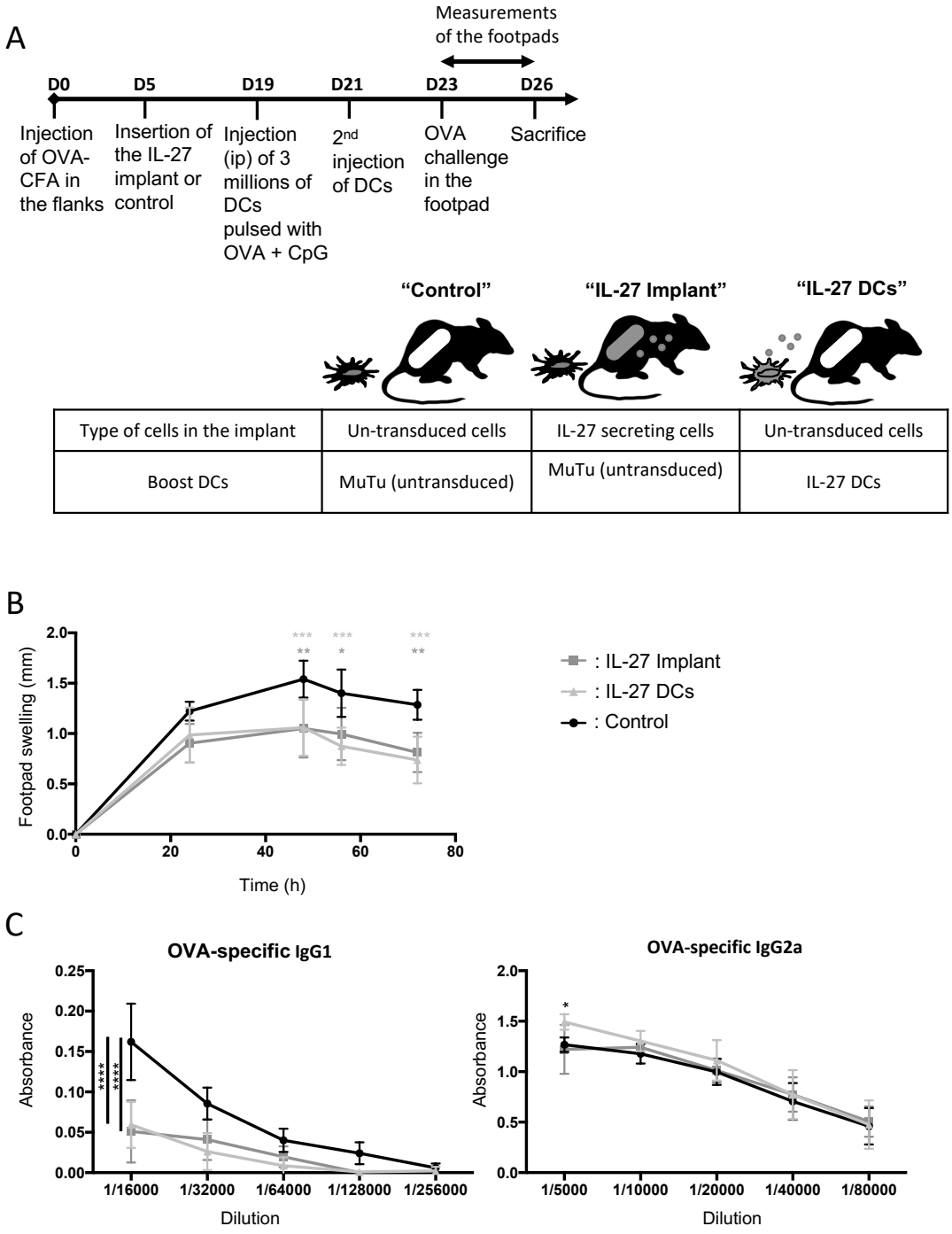


Figure 30 Generation of C2C12 cells producing IL-27. (A) Scheme of the design of the IL-27 construct and the production of C2C12-IL-27 cells. **(B)** Cells were positive for EBI3 and p28 by flow cytometry. **(C)** C2C12-IL-27 cells expressed the *ebi3* transcript, as shown by quantitative real-time PCR.



Type of cells in the implant	Un-transduced cells	IL-27 secreting cells	Un-transduced cells
Boost DCs	MuTu (untransduced)	MuTu (untransduced)	IL-27 DCs

Figure 31 IL-27 DCs and IL-27-producing implants during vaccination modulate the inflammatory response during DTH. (A) Scheme of the experiment: after immunization against ovalbumin (OVA), mice were separated into three groups: one with an implant that produced IL-27 constantly (n=5), one that was injected with IL-27 DCs (n=8), and a control group (n=4). All mice were implanted subcutaneously with an implant of cells that constantly produced IL-27 or with an implant with untransduced cells. At days 19 and 21, mice received an injection of 3 × 10^6 DCs (IL-27 DCs or Mutu DCs) previously pulsed with OVA and CpG. On day 23, mice received a challenge with OVA in the footpad. **(B)** Footpad swelling was measured in size of the right footpad (challenged with OVA) compared with the left footpad (injected with PBS). **(C)** After 72 h, mice were sacrificed and the concentration of OVA-specific IgG_{2a} and IgG_1 in the serum was measured by ELISA (with different dilutions). The experiment

was done once with at least 4 mice per group. Results are shown as the mean ± SD. Statistical analysis was performed with two-way ANOVA: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

We also compared the systemic secretion of IL-27 and the effect of tolerogenic DCs on the established immune memory in the DTH model (Figure 31**A**). The encapsulation process of the cells in the implant was the same as previously described[328]; the cells survived well in the implant and were able to secrete IL-27 for prolonged times. Mice were divided in three groups: one group (IL-27 implant) was implanted in the back with a subcutaneous implant containing cells able to secrete IL-27. The other two groups (IL-27 DCs and Control) had the same implant inserted but with untransduced cells. Implants were subcutaneously inserted in mice 5 days after immunization with OVA (1 mg/mL) in CFA (1 mg/mL). At day 19, mice were injected with DCs pulsed with OVA and CpG (4 h). Mice in all three groups (control, IL-27 DCs, and IL-27 implant) were injected twice (day 19 and day 21) with 3×10^6 Mutu DCs or IL-27 DCs. On day 23, mice were challenged with OVA in one footpad and PBS in the other footpad. Footpad swelling differences were assessed daily for 72 h. Two days after the challenge, mice in groups IL-27 DCs and IL-27 implant showed a clear decrease in footpad swelling compared with the control group (Figure 31**B**). No significant differences in the footpad swelling were noted between the IL-27 DCs group and the IL-27 implant group. OVA-specific IgG_1 and IgG_{2a} were quantified in sera of mice 3 days after the challenge. The IL-27 DCs and IL-27 implant groups had almost the same concentration of OVA-specific IgG_{2a}, which was lower (3 fold) than that of the control group (Figure 31**C**). The concentration of OVA specific IgG_1 was almost the same between the three groups, although mice in the IL-27 DCs group had a slightly higher concentration. These results suggested that IL-27 DCs and the IL-27 secreting implant could both decrease antigen-specific inflammation to the same degree.

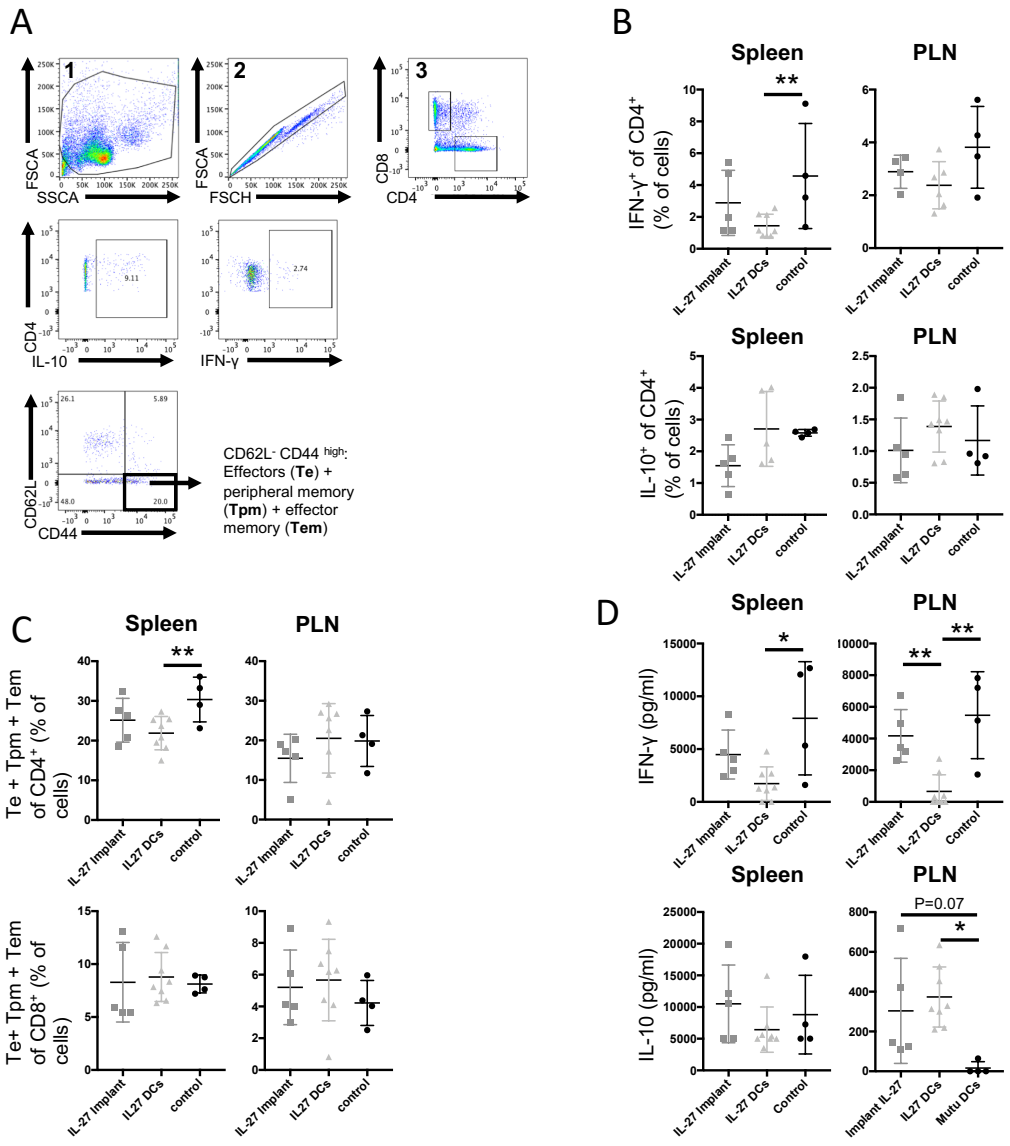


Figure 32 IL-27 DCs were able to induce OVA-specific IL-10-secreting cells. Spleen and popliteal lymph nodes (PLN) from mice of figure 31 were isolated and analyzed by flow cytometry. **(A)** Gating strategy for CD4 and CD8 T cells. **(B)** IFN-γ and IL-10 production by CD4 T cells. **(C)** Effector T (Te) + effector memory (Tem) + peripheral memory cells (Tpm) ($CD44^+$ $CD62L^-$) subsets in the CD4 and CD8 populations. **(D)** Cells from the spleen and PLN were isolated and restimulated with 100 µg of ovalbumin (OVA) *ex vivo* and their cytokine secretion was quantified 24 h after OVA restimulation. Supernatant was collected and analyzed by ELISA for IL-10 and IFN-γ. Statistical analysis was performed with one-way ANOVA: $*p < 0.05$, $**p < 0.01$.

The mice were sacrificed 3 days after the challenge and cells from the spleen and the PLN were isolated and analyzed by flow-cytometry for IFN-γ production in CD4 and CD8 T cells (Figure 32**A**). The control group showed a higher IFN-γ^+ population in the CD4 cells in the spleen compared with mice that were injected with IL-27 DCs. We observed a slight tendency for an increase in IFN-γ^+ cells in the control group in the PLN (Figure 32**B**). No differences in the IL-10^+ population were

observed in CD4 cells of the spleen or PLN. In the CD4 T cells of the spleen in the control group, the $CD44^{high}$ $CD62L^{-}$ population was higher than that of the other groups. This difference was not observed in CD8 T cells (Figure 32**C**). Cells from the PLN and spleen, plated at the same concentration (5×10^6 cells per well), were re-stimulated for 1 d with OVA (100 µg/mL) in medium to measure the antigen-specific secretion of cytokines. Splenocytes of the control group were shown to secrete more IFN-γ than splenocytes of the IL-27 DCs group (Figure 32**D**). Secretion of IFN-γ by cells in the IL-27 implant group was not significantly different from that of the two other groups. No significant differences in IL-10 secretion from splenocytes were observed between the groups after restimulation. However, in draining LN cells of the OVA-injected site (PLN), a significant difference was observed in the concentration of secreted IFN-γ. Cells from mice in the control group and from mice with the IL-27 implant had a significantly higher secretion of IFN-γ than mice injected with IL-27 DCs. The PLN cells of mice with the IL-27 implant and those injected with IL-27 DCs were shown to produce more IL-10 than PLN cells of mice in the control group.

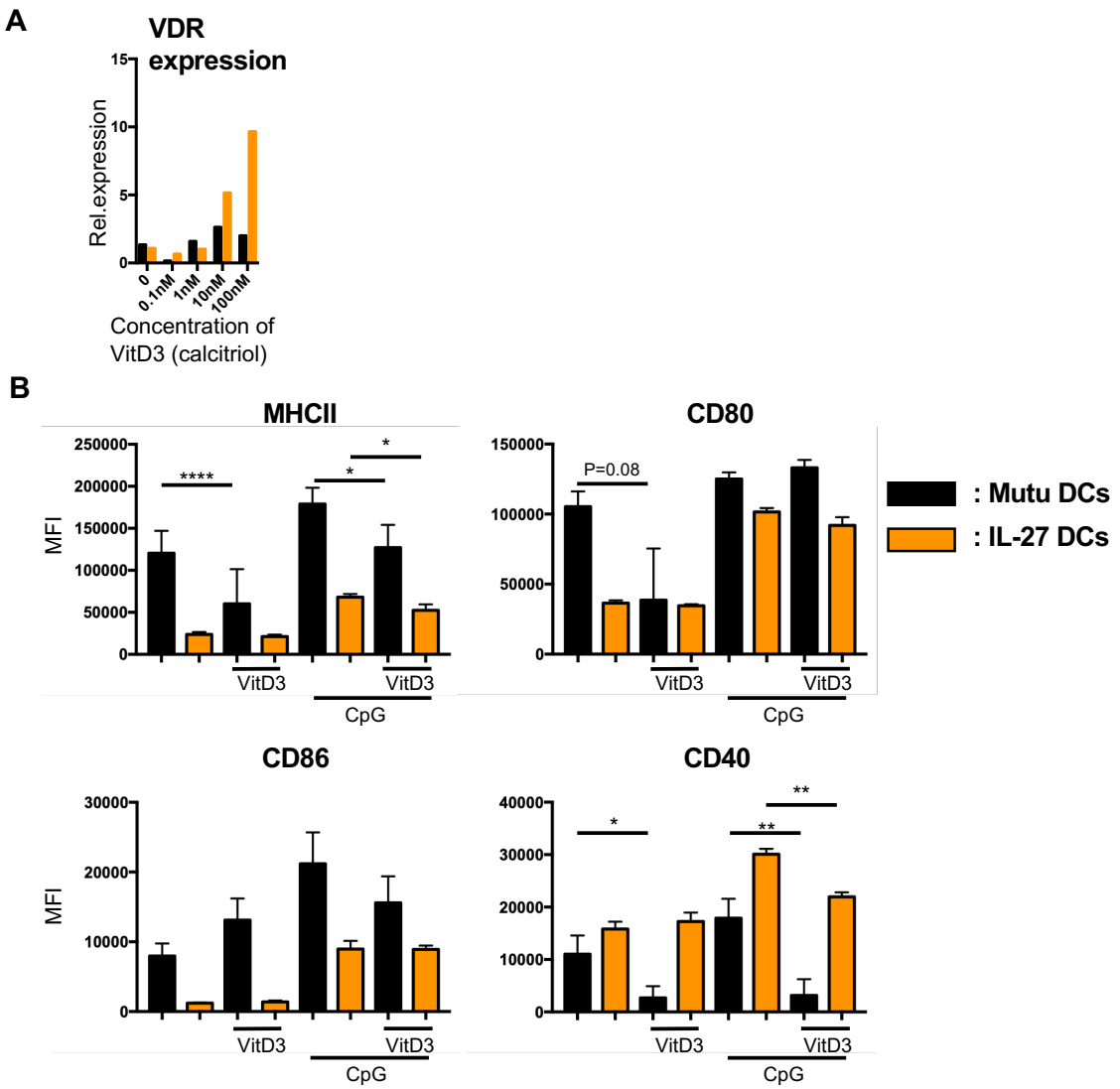


Figure 33 The active form of vitamin D3 (calcitriol) has a tolerogenic potential on IL-27 DCs and Mutu DCs. (A) mRNA expression of the vitamin D receptor (*VDR*) in IL-27 DCs and in untransduced DCs after 48 h of culture with different concentration of calcitriol. **(B)** IL-27 and untransduced DCs were cultured under the same conditions with or without vitD3 for 72 h and analyzed by flow cytometry for expression of different cellular markers under resting conditions or stimulated with CpG (1 µg/mL) for 14 h. Results indicated as the mean of biological triplicates ± SD. Data represent 2 to 3 independent experiments. Statistical analysis was performed applying Student's *t*-test: **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

The tolerogenic effects of VitD3 on Mutu DCs and on IL-27 DCs was tested with calcitriol, the active form of VitD3. Cells were cultivated with different concentrations of calcitriol and the RNA transcript of the vitamin D receptor (VDR) was tested by quantitative PCR. The RNA transcript of the VDR is upregulated in dendritic cells when they are cultured with VitD3. IL-27 DCs showed the same

expression profile of VDR but with 10 nM of calcitriol, they showed double the expression compared with control DCs. With 100 nM of VitD3 for 48 h, the relative expression of VDR was extremely high; however, high cell toxicity was observed by microscopy. We chose a concentration of 10 nM to study the effects of VitD3 *in vitro* (Figure 33**A**). In the literature, a concentration between 10 and 50 nM has been used to test the tolerogenic effects of calcitriol in human and mouse.[71,290,338,339]. Culture for 48 hours with 10 nM calcitriol was shown to reduce the expression of MHCII and CD40 on Mutu DCs (Figure 33**B**). Upon stimulation for 15 hours with CpG (1 µg/mL), Mutu DCs treated with VitD3 responded differently, with lesser expression of MHCII and CD40. The expression of CD40 of Mutu DCs treated with VitD3 did not change with stimulation by CpG. IL-27 DCs were also responsive to treatment with VitD3 and showed slightly downregulated expression of MHCII after stimulation. The expression of CD40 also decreased after stimulation by CpG between IL-27 DCs treated for 2 days with VitD3 and IL-27 DCs that were not treated. However, treatment with VitD3 for 2 days did not change expression of CD80 and CD86 of IL-27 DCs.

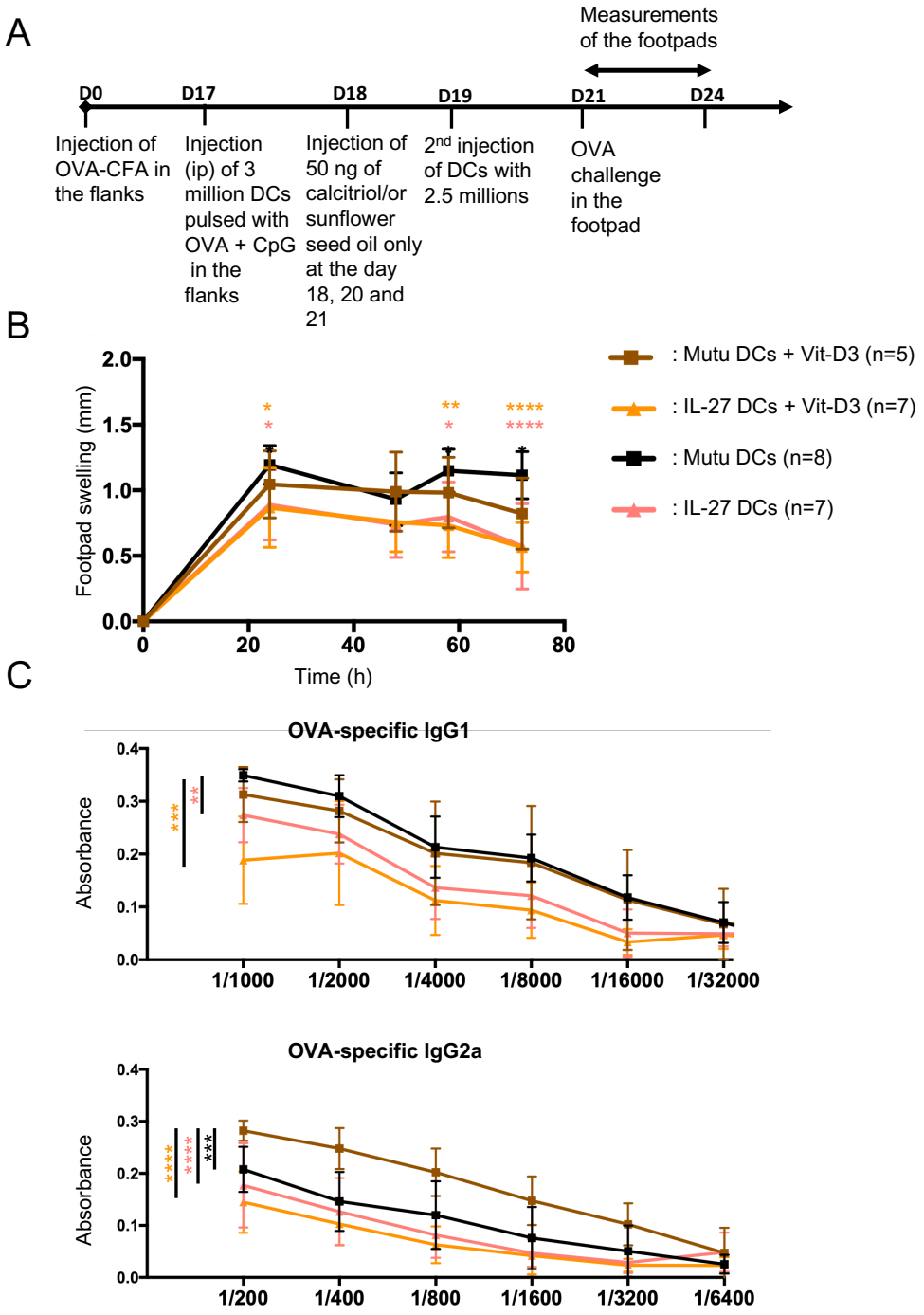
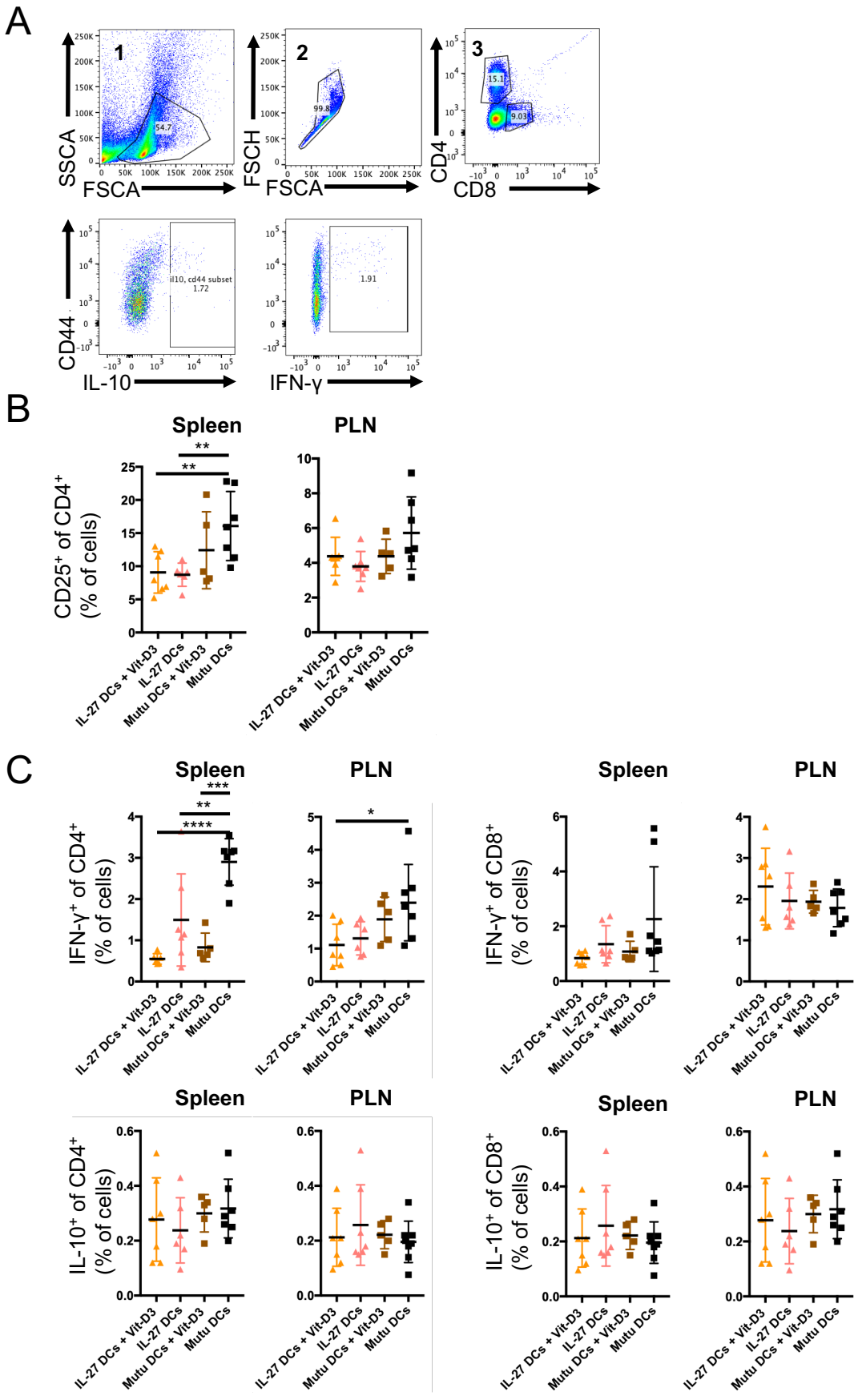


Figure 34 IL-27 DCs could change the antigen-specific inflammation in combination or not with VitD3. (A) Scheme of the experiment: mice were immunized with ovalbumin (OVA) (1 mg/mL) in complete Freund's adjuvant (1 mg/mL *M. tuberculosis*) on days 0 and separated into 4 groups: IL-27 DCs + VitD3 (n=7), IL-27 DCs (n=7), Mutu DCs (n=8), and Mutu DCs + VitD3 (n=5). Mice received two injections (*i.p.*) of 3 million DCs (IL-27 DCs or Mutu DCs) on days 18 and d 19, previously pulsed for 4 h with OVA and CpG. Mice received 3 injections of VitD3 in sunflower seed oil or sunflower seed oil only on days 18, 20, and 21. On d 21, all mice were challenged with OVA in the right footpad and had an injection of PBS in the left

footpad. **(B)**. Footpad swelling was measured 4 times and calculated as the difference in size of the right footpad (challenged with OVA) compared with the left footpad (injected with PBS). **(C)** After 72 h, mice were sacrificed and the concentration of OVA-specific IgG_{2a} and IgG_1 in the serum was measured by ELISA (with different dilutions). The experiment was done once with at least 5 mice per group. Results are shown as the mean ± SD. Statistical analysis was performed with two-way ANOVA: $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

To determine whether injection of IL-27 DCs and VitD3 treatment were able to change established memory *in vivo*, a DTH experiment was performed using 4 groups of mice. Mice were immunized in the same way as the previous experiments, with OVA (1 mg/mL) in CFA (1 mg/mL *M. tuberculosis*). After 17 days, mice received an injection of 3×10^6 DCs (*i.p.*) previously pulsed with OVA and stimulated with CpG, and received the same injection 2 days later (IL-27 DCs or Mutu DCs). At day 18, mice in the IL-27 DCs + VitD3 and Mutu DCs + VitD3 groups received an (*i.p.*) injection of 50 ng of VitD3 (calcitriol) diluted in sunflower seed oil and mice in the two other groups (IL-27 DCs and Mutu DCs) received a control injection of sunflower seed oil only. The same injections were repeated at day 21. Mice were challenged 21 days after the first immunization against OVA (Figure 34**A**). Footpad swelling showed that the two groups of mice that received the injection of IL-27 DCs had significantly lower swelling than the group injected with Mutu DCs after 24 h, 54 h, and 72 h (Figure 34**B**). No differences were significant after 48 h but the difference in footpad swelling was more pronounced after 72 h. Footpad swelling was similar in the two groups of mice injected with IL-27 DCs. The group injected with VitD3 (Mutu DCs + VitD3) had an intermediary level of footpad swelling but they did not differ significantly with any other group. After 3 days, mice were sacrificed and the concentration of OVA-specific IgGs were compared by ELISA. Mice injected with IL27 DCs showed a lower level of OVA-specific IgG_1 than mice injected with Mutu DCs (Figure 34**C**). No significant differences were observed between mice that received Mutu DCs and VitD3 and the control group (Mutu DCs). Surprisingly, no differences in OVA-specific IgG_{2a} were observed between mice that were injected with IL-27 DCs (with or without VitD3) and mice injected with Mutu DCs (without VitD3). Mice injected with Mutu DCs and VitD3 had a significantly higher level of OVA-specific IgG_{2a} than mice in the 3 other groups.


A
1
SSCA
FSCA
54.7
2
FSCH
FSCA
99.8
3
CD4
CD8
15.1
9.03
CD44
IL-10
il10, cd44 subset
1.72
IFN-γ
1.91
B
Spleen
PLN
CD25+ of CD4+ (% of cells)
**
**
IL-27 DCs + Vit-D3
IL-27 DCs
Mutu DCs + Vit-D3
Mutu DCs
C
Spleen
PLN
IFN-γ+ of CD4+ (% of cells)
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IFN-γ+ of CD8+ (% of cells)
IL-10+ of CD4+ (% of cells)
IL-10+ of CD8+ (% of cells)

Figure 35 IL-27 DCs and VitD3 could regulate the immune response in the DTH. Spleen and popliteal lymph nodes (PLN) from the mice of figure 32 were isolated and analyzed by flow cytometry. **(A)** Gating strategy. **(B)** $CD25^+$ in CD4 T cells in the spleen and PLN. **(C)** IFN-γ and IL-10 expression in CD4 and CD8 T cells of the spleen and PLN. Results are shown as the mean ± SD. Statistical analysis was performed with one-way ANOVA*$p < 0.05$, **$p \leq 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Cells from the spleen and PLN were isolated and analyzed by flow cytometry and gated for CD4 and CD8 T cells (**Figure 35A**). Mice injected with IL-27 DCs (with or without VitD3) were shown to have CD4 T cells with a lower percentage of $CD25^+$ in the spleen. This difference was observed but not significant in the PLN (**Figure 35B**). Mice injected with Mutu DCs and VitD3 had intermediate expression of CD25 on CD4 T cells compared with the other groups; no significant differences were observed among groups. The expression of intracellular cytokines was compared in CD4 and CD8 T cells of the spleen and PLN (**Figure 35C**). Mice injected with Mutu DCs and without VitD3 had a higher percentage of IFN-γ^+ CD4 T cells than mice in the other three groups. The only significant difference on the percentage of IFN-γ^+ observed was between mice injected with Mutu DCs and mice that received the injection of IL-27 DCs and VitD3. In CD8 cells, no differences in IFN-γ were observed between spleen and PLN. In cells from the spleen and PLN, no significant differences were observed in the percentage of IL-10^+ cells in CD4 and CD8 T cells.

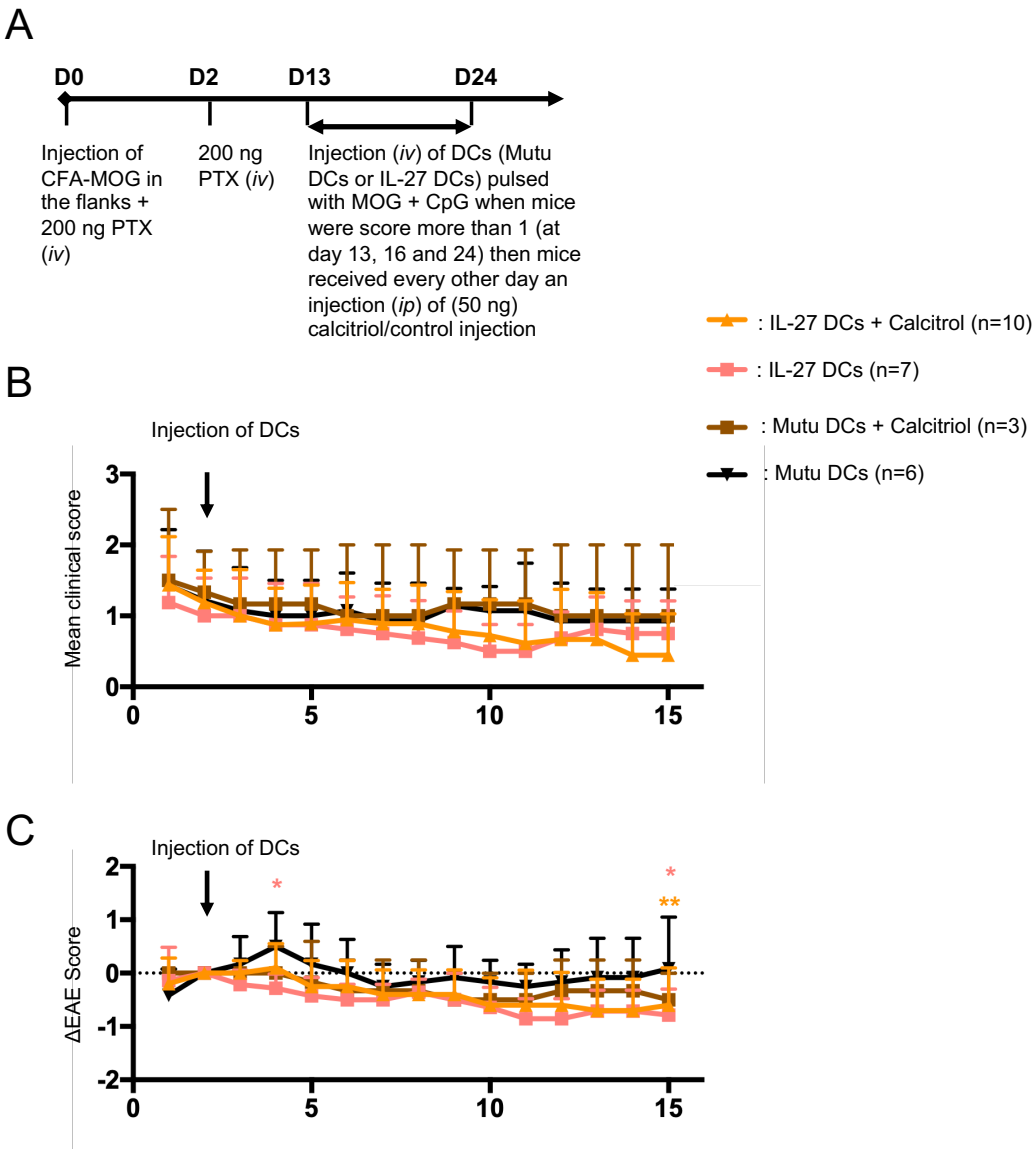

Figure 36 Effect of IL-27 DCs and VitD3 on experimental autoimmune encephalitis (EAE) **(A)** Scheme of the experiment: C57BL/6 mice were immunized with myelin oligodendrocyte glycoprotein (MOG; 1 mg/mL) in complete Freund's adjuvant (5 mg/mL *M. tuberculosis*) in the flank and were injected (*i.v.*) with pertussis toxin (200 ng) on days 0 and 2. Mice were observed daily using a clinical EAE scoring system ranging from 0 (no symptoms) to 4 (complete paralysis). When the clinical score of EAE was >1 (limp tail), treatment was initiated. Mice were separated into 4 groups, IL-27 DCs + VitD3, IL-27 DCs, Mutu DCs, and Mutu DCs + VitD3, and received one injection (*i.v.*) of 3 × 10^6 DCs (IL-27 DCs or Mutu DCs), previously pulsed for 4 h with MOG_{35-55} and CpG. Mice received an injection of 50 ng of VitD3 in sunflower seed oil or sunflower seed oil only every second day after injection of the DCs until the end of the experiment. **(B)** Evolution of the mean clinical EAE score before and after mice received an injection of DCs. **(C)** Difference in EAE score between the day of injection and the following days. Results are shown as the mean ± SD. The experiment was done once with at least 3 mice per group. Statistical analysis was performed with two-way ANOVA: *$p < 0.05$, **$p < 0.01$.

To understand whether IL-27 DCs and VitD3 are able to synergize to induce tolerance to treat EAE symptoms, mice were immunized against MOG_{35-55} (1 mg/mL) in CFA (5 mg/mL *M. tuberculosis*) and injected with 200 µg of PTX (*i.v.*); 2 days later, mice were injected again with 200 µg of PTX (*i.v.*) (**Figure 36A**). The first symptoms appeared on different days after the first immunization; some mice began to get sick after 10 days and some others began to have visible symptoms after 3 weeks. To early treated we did 3 rounds of injections at days 13, 16, and 24, where mice received an injection of 3×10^6 of DCs (Mutu DCs or IL-27 DCs) previously pulsed with MOG and activated with CpG. At this stage, mice were separated in four groups: IL-27 DCs + VitD3, Mutu DCs + VitD3, IL-27 DCs, and Mutu DCs. Mice in the VitD3 groups received an injection (*i.p.*) of the active form of VitD3 (calcitriol) diluted in sunflower seed oil. The injections were repeated every 2 days until the end of the experiment. Mice that were not injected with VitD3 received a control injection of the same quantity of sunflower seed oil. To be treated, mice had to present obvious symptoms (with an EAE score >1). Mice were scored daily beginning 1 day before the first injection (**Figure 36B**). Mice injected with IL-27 DCs (with or without VitD3) had a mean clinical score lower than two other groups of mice injected with Mutu DCs, no significant differences were observed. The ΔEAE score was calculated as the difference in EAE score on the day of observation and that on the day of injection. Mice injected with IL-27 DCs had a significant decrease in ΔEAE score 2 days after the injection compared with mice injected with Mutu DCs, but this difference was not significant on subsequent days. Only after 13 days did this difference reach significance again. Indeed, mice injected with Mutu DCs had an increase in ΔEAE score shortly after injection, followed by a decrease (**Figure 36C**). Mice injected with IL-27 DCs and VitD3 showed a slight decrease in ΔEAE after injection and had significantly lower ΔEAE scores than mice injected with Mutu DCs 13 days after injection. No significant difference in ΔEAE scores were observed between mice injected with Mutu DCs and VitD3 and the other three groups.

DISCUSSION

1. MUTU DC LINES: POWERFUL TOOLS TO STUDY ANTIGEN PRESENTATION

The abilities to learn from the past (memory) and to adapt the immune response toward pathogens or tumor cells are unique advantages for multicellular organisms. The adaptive immune system defends us against a broad spectrum of infectious agents; however, disequilibrium in the system can cause an immune reaction against self-antigens and lead to life-threatening immune pathologies and autoimmunity. Tolerance to self-antigens and control of overshooting immune responses are, therefore, necessary to prevent potentially harmful immune responses. DCs play a critical role in the maintenance of immune homeostasis. In the resting state, they have low surface expression of MHC class II and co-stimulatory molecules. Upon activation, they transfer MHC class II molecules to the cell surface, upregulate gene expression, and begin to secrete cytokines to induce a specific immune response. DCs have superior activity in the presentation of antigens and are therefore called professional APCs. The role of DCs is central in the control of immunity and in the control of an immune response. They can improve qualitatively and quantitatively the outcome of the immune response, making them an exciting target for cell therapy against cancer and autoimmune diseases.

However, the scarcity of DCs represents a difficult challenge in their study. First, they are very heterogeneous and, even in the same subtype, they can have different states of maturation. Second, depending on the subtype, DCs have a very short half-life; once differentiated, they survive *in vivo* and *in vitro* for only a few days. Finally, the classical derivation of DCs from bone marrow with GM-CSF is time consuming and expensive, and the outcome is generally a partially activated, heterogeneous population of cells, which makes them difficult to handle. The laboratory of Prof. Acha-Orbea established the MuTu CD8α$^+$ DC line in 2010, and it has many advantages in research on DCs[323,324,340]. The cell line is homogeneous and easy to culture, and it represents an infinite source of cells with the same phenotype and function as natural DCs. These cells can also be efficiently transduced with lentiviruses to secrete or express a protein or to generate stable CRISPR knockouts[323].

Mutu DCs represent a powerful tool, by which the modalities of DC vaccination can be investigated. Research in DCs to investigate potential treatment and prevention options against cancer and autoimmune disease is limited by the difficulty of obtaining sufficient homogeneous and nonactivated DCs in both, humans and mouse. Mutu DCs offer a great advantage in studying how bioengineered modifications could enhance the strength and outcome of therapy. They are also well characterized (e.g., migration properties, and subtypes).

2. DC VACCINATION IN CANCER AND AUTOIMMUNE DISEASES

The concept of reprogramming the immune system with the most efficient APCs to fight tumors is old [307]. Nevertheless, personalized vaccination with DCs is not yet efficient enough to be integrated as a standard treatment of tumors. The safety profile of this technique is proven but clinical studies show that improvements are needed[341]. There are several possible approaches to engineering DCs and improving their efficiency in cancer vaccine models. The main one involves the optimization of DC maturation, subset selection, and knowledge about the migration and survival behavior of transferred cells *in vivo*. In clinical practice, activation is generally based on TLR stimulation, cytokines, or CD40 ligands.

Enhancing antigen presentation would also augment the cellular response toward tumor cells. Because activated DCs are already very efficient in priming T cells, pulsing them with oxidized tumor lysates could increase immunogenicity. The pulsing of tumor antigen with tumor-derived mRNA via electroporation would also increase their immune specificity[341]. Some studies have shown that inhibition of the indoleamine 2,3-dioxygenase (IDO) enzyme of DCs was able to stop the induction of T cell anergy[342]. The inhibition of STAT3 signaling through IL-6/JAK/STAT3 signaling blockers has also been studied in DCs, with siltuximab, tocilizumab, or ruxolitinib preventing the tolerogenic function of DCs[343]. The indirect inhibition of STAT3 with those drugs was able to change maturation and enhance the immunogenic function of DCs by inhibiting the transcriptional regulator ID2[344]. The clinical efficiency of this inhibition is still being evaluated in clinical trials[345].

Another idea is to improve the transport of tumor antigens from the tumor to the lymph nodes. For example, intranodal injection of tumor antigen-pulsed DCs has shown increased efficacy in inducing the antitumoral immune response[346]. Most strategies based on improving DC trafficking to secondary lymphoid organs focus on the expression of CCR7 in different ways; for example, by improving DC maturation or by lentiviral transduction[347,348].

It is also possible to enhance the tolerogenic properties of DCs, an approach great potential to decrease the specific immune memory against self-antigen[60]. Some recent clinical trials have shown promising results with DC cell therapy against autoimmune diseases[349]. However, even if tolerogenic DCs have a well-accepted safety profile, their efficiency needs to be improved before this can become a standard therapeutic protocol[66,350,351].

Many studies have been conducted to determine how to use DCs to induce tolerance and how to generate them in large quantities[351]. For DC therapy for autoimmune diseases to be efficient in clinical practice, the main challenge is in maintaining the tolerogenic activity. The first attempt to use DCs in humans (in 2001) showed that immature DCs pulsed with antigens were able to

suppress antigen-specific CD8 T cells[352]. Despite an excellent safety profile, the effects were time-limited to several months[352]. Indeed, when DCs are treated *ex vivo* to become tolerogenic or to have an immature state, they generally lose their properties under inflammatory conditions[351]. As stated in the introduction, the tolerogenic state for DCs or T cells can be temporary. A method to generate a large quantity of stable tol DCs is needed to avoid inducing an undesired effect on the immune system. Human DCs are generally differentiated with GM-CSF and IL-4 from monocytes[306]. Another problem in clinical practice is the volume of blood needed to generate DCs (300 mL); removing this amount of blood from a very sick patient would be dangerous. Many protocols have been developed to produce human tol DCs. The addition of a factor to differentiate the cells in a tolerogenic state differs between studies. Some of the strategies proposed include the addition of self-antigens to DC culture, the use of VitD3 or NF-κB inhibitors, the inhibition of CD40 or CD80, or the overexpression of co-inhibitory receptors[351]. As an example, Moderna Inc. (Cambridge, MA, USA) recently patented mRNA-6981, a method based on mRNA transduction to enhance the expression of PD-L1 specifically on myeloid cells, improving their specific tolerogenicity[353]. This technology is currently being tested in type 1 autoimmune hepatitis in patients. In general, the primary goal for tol DCs therapies would be to know precisely what type of tol DC is suited for a specific autoimmune disease.

Much progress has been made since 2001, but improvements in patients suffering from autoimmune diseases have been limited. Currently, several tol DC therapies are in phase I clinical trials, including treatments for type 1 diabetes, multiple sclerosis, Crohn's disease, and skin graft transplantations[66,354,355]. The therapeutic potential of tol DCs for Lupus also seems to be promising[350]. However, knowledge is lacking in many aspects of tol DC therapies. Except in antibody-mediated diseases like rheumatoid arthritis[356], antigens responsible for autoimmune diseases are not always precisely known. They also differ between conditions and between patients with the same disease. Moreover, It has even been observed that immune damage of an organ can greatly increase autoimmune target antigens[357,358].

For the last decade, our laboratory has focused on finding the most efficient way to enhance the tolerogenic properties of DCs. Mutu DCs represent an infinite resource to create tolerogenic cell lines, and Dr. Anais Duval has generated several tolerogenic DC lines with different properties. Overexpression of tolerogenic molecules such as IL-10, latent TGF-β, active TGF-β, arginase 1, IDO, PD-L1, and CTLA4 completely changes the behavior of the cells, with interesting results *in vitro* and *in vivo*.[359] The combination of active TGF-β DCs and IL-10-producing DCs strongly induces the development of $Foxp3^+$ Tregs cells, whereas overexpression of CTLA4 induces an anergic antigen-specific T cell response. This work was continued by Dr. Sergio Haller, who engineered Mutu DCs to produce a larger quantity of IL-35[70,360]. This cell line had a pronounced tolerogenic phenotype, and injection of these DCs in mice promoted tumor growth. The reduced

severity of autoimmune encephalitis demonstrated the impressive potential of these cells to change fully differentiated effector and memory cells specific for self-antigens[70].

In the present study, we chose to engineer Mutu DCs constitutively secreting IL-27. This cytokine has a central role in different steps of immune regulation[361]. In humans, the lack of this cytokine is associated with autoimmune diseases[278,362]. In mice, deficiency of IL-27R leads to an increase in the severity of pathologies in autoimmune disease models and better resistance to infections[232]. This cytokine has been shown to inhibit Th1 and Th17 responses and to induce regulatory CD4 Tr1 cells through different mechanisms[361]. IL-27 was also chosen because it is naturally secreted by activated DCs and regulates their immunogenicity[361]. Its overexpression might induce natural and unknown features to Mutu DCs to give them therapeutic potential.

It is possible that other DC subtypes, such as cDC2 and pDC, have tolerogenic properties when they continuously secrete IL-27, but they would undoubtedly have different properties. However, we chose cDC1 because this cell line is well characterized and has excellent properties to induce T cells. Naturally, these cDC1s have a unique role in shaping the immune system after migration to secondary lymphoid organs[363]. We hypothesized that this cell type could be engineered to have a strong tolerogenic potential on memory T cells.

3. THE FLAT-SHEET IMPLANT

We have tested the efficiency of the implant developed in the laboratory of Prof. Aebischer in different models: in the CIA model with IL-10 and/or IL-35 (manuscript in preparation) and in the DTH model with IL-10 and/or IL-35, TGF-β, and IL-27; however, we have not tested it in the EAE mouse model for several reasons. First, in the EAE model, systemic secretion of IL-27 using an osmotic pump has already been shown to prevent symptoms efficiently[268,274]. In those studies, the implant was encapsulated in the back of the mouse several days after vaccination with CFA and MOG but before appearance of EAE symptoms. It would be interesting to install the implant during the acute phase of the disease to show its possible therapeutic potential, as done in the experiment with IL-27 DCs in the EAE model. However, symptoms in the EAE mouse model are extreme. Sometimes mice die or have to be sacrificed for ethical reasons. The surgery to install the implant is hard to support and a paralyzed mouse would probably die or suffer beyond ethical limits. However, several modifications could be implemented to conduct this experiment. It is possible to use a smaller implant subcutaneously in the mouse (in the form of a tube that is easier to insert) without the need for anesthesia[329]. However, because of the smaller size, the implant would contain fewer cells and might not yield sufficient cytokines. It is possible to make an implant with a gene switch with recombinases or optogenetics[364]. The implant would be installed in the mouse before the first appearance of symptoms, and secretion of cytokine would be activated only when

symptoms appear. It might also be possible to use cells capable of sensing IL-17 or IFN-γ levels and secreting a tolerogenic cytokine in a dose-responsive manner. This technology could be used to prevent relapse in a patient. However, it is perhaps simpler to design a therapy based on a device that would be implanted for only a short period that would secrete a tolerogenic cytokine for a short time, when the patient has a relapse.

Therapies based on implants that secrete tolerogenic cytokines are probably simpler to execute in clinical practice than tolerogenic DC therapy even tough sufficient systemic levels would be difficult to obtain. For example, we could envision encapsulated cells being installed next to a site of local inflammation to secrete cytokines for a limited period and the implant could be removed once symptoms are resolved. This kind of therapy is less invasive than gene or cell therapy because the device can be removed at any time. It also reduces one of the main areas of concern with immunomodulatory treatments—the oncogenicity. The cytokines would be secreted for a short time only during antigen-specific autoimmune T cell stimulation (induced by vaccination), probably not enough to overwhelm the immune system with opportunistic infections or cancers. It is certainly easier to develop an implant able to secrete tolerogenic cytokines for local inflammation. Indeed, the design of a small implant able to secrete a large quantity of cytokines systemically in humans is more difficult due to the size of the implant and the producing capacity of the cells[365]. Research to optimize production of recombinant proteins in mammalian cells would probably make this technology feasible for systemic production in humans. It is also possible to imagine multiple implants inserted in different parts of the body.

We decided to compare implant therapy and tolerogenic DCs in the DTH response to evaluate the best treatment to change immune memory into a regulatory phenotype. To our knowledge, this is the first time that systemic secretion of cytokines and tol DCs have been compared in a mouse model. This comparison will be further discussed.

4. THE IL-27-SECRETING DENDRITIC CELL LINE HAS A TOLEROGENIC PHENOTYPE

Unfortunately, due to interference of the linker (between the two subunits EBI3 and p28) in the ELISA of IL-27 we were not able to quantify the secreting capacity od IL-27 DCs. However, IL-10 DCs that were transduced with a similar vector with *Il10* under the control of the same promoter (CMV) were able to produce high levels of the protein. When 2.5×10^5 cells/mL were cultured for 24 h in resting conditions, we were able to detect up to 4 ng/mL of the cytokine in the supernatant, indicating that IL-27 DCs would be able to produce high amounts of the respective cytokine as well.

We first tested whether the secreted IL-27 had paracrine and autocrine effects. IL-27 DCs had a lower level of MHCII and other co-stimulatory signals. However, the level of CD40 was increased

in IL-27 DCs when they were stimulated with CpG, showing that IL-27 did not induce an entirely immature phenotype in DCs. Expression of the gp130 subunit of the IL-27 receptor was increased in IL-27 DCs, indicating possible regulation of the cytokine on its receptor. The effect of IL-27 has already been associated with elevated expression of the two subunits of the IL-27 receptor (WSX-1 and gp130)[366]. Many other proteins can associate with the gp130 subunit, such as cardiotropin 1, leukemia inhibitory factor, ciliary neurotrophic factor, IL-6, IL-35, IL-27, and IL-11[367]. We hypothesize that overexpression of IL-27 might have induced the expression of its receptor and therefore enhanced its sensitivity to this cytokine.

Engagement of the IL-27 receptor has been shown to trigger signaling phosphorylation of STAT1/STAT3 in B and T cells[220]. IL-27 has also been shown to induce phosphorylation of STAT1 and STAT3 on cDCs, and this signaling is a way to induce their tolerogenic properties[171]. We can assume, therefore, that the systemic production of IL-27 induces STAT1 and STAT3 signaling on the DCs and on T cells.

The presence of PD-1 on DCs is known but not well explained; it has been reported that PD-1-deficient DCs are more immunogenic, showing that both ligand and receptors have similar effects on DCs[368]. It is also interesting to note that PD-1-deficient mice were shown to be highly resistant to infection by a effect on the innate system even in the absence of T cells and their DCs were able to produce more immunogenic proteins[369]. PD1 and PD-L1 both have a tolerogenic function on DCs. The fact that IL-27 DCs overexpress the ligand and the receptor might indicate their tolerogenic features. The complex PD1-PDL-1 is known to induce cell death and to suppress T cell inflammatory activity. When IL-27 DCs present antigen they would have probably have a higher chance to inhibit T cell activity. More research needs to be done to see if negative costimulations are involved in the tolerogenic features of IL-27 DCs.

Taken together, these observations confirm a direct tolerogenic effect of IL-27 on DCs that was previously shown[69,259]. The DCs are still able to upregulate MHCII and co-stimulatory molecules and to secrete inflammatory cytokine IL-12 (in a lower quantity); secretion of IL-6 did not change upon activation. Our previous work with IL-35-secreting DCs showed that producing cells were not able to upregulate MHCII, CD40, and CD80 upon stimulation of TLR3 and TLR9 ligands. In contrast to IL-27 DCs, IL-35 DCs secreted more IL-10 than Mutu DCs[70]. We conclude that IL-27 DCs are not hyporesponsive and have an intermediate tolerogenic phenotype (high level of CD40). The fact that they are still able to upregulate antigen-presenting molecules is useful information and can enhance their tolerogenic behavior by improving their capacity to present antigens to lymphocytes *in vivo* and *in vitro*.

The mobility of DCs and T cells can be changed in different ways. For example, DCs via an induced calcium signal selectively attenuate transmigration on a gradient of the chemokine CCL21,

necessary for migration to the LN in the capillaries[370]. It would be interesting to see whether cells stimulated with IL-27 DCs have a different migration pattern on chemokine gradients[371].

5. IL-27 DCS ALTER IMMUNE MEMORY T CELLS

To determine whether tolerogenic DCs have immune-suppressive properties and can inhibit differentiation of memory T cells into effectors, we performed an *in vitro* antigen-specific proliferation assay. CD4 T cells primed with IL-27 DCs did not proliferate and were unable to produce IFN-γ or IL-10. Surprisingly, no Tr1 was observed in this experiment. T cells polyclonally activated with anti-CD3 and anti-CD28 in the presence of IL-27 are reported to become Tr1 and to produce a high quantity of IL-10[192,372]. We did not observe T cell proliferation in the presence of IL-27 DCs after co-culture, which indicated the high inhibitory effect of the cell line but hindered our investigation into what types of cells the co-cultures would generate.

As previously mentioned, immune memory cells cause different pathologies when their specificity is directed toward self-antigens or upon reactivation. We wanted to use IL-27 DCs to change the memory cells individually and chose CFA as the adjuvant; CFA is a solution of antigen emulsified in non-metabolizable oil with inactivated and dried *M. tuberculosis* bacteria and it is one of the strongest possible adjuvants for animal use. CFA has ligands for many receptors, including TLR2, TLR4, and TLR9, and it induces a mixed but Th1-dominant response. An intense inflammatory reaction at the site of the injection is visible few days after the injection. We chose this adjuvant because it is classically known to induce a Th1- and Th17-shifted immune response. We wanted to test IL-27 DCs on the Th1 and Th17 memory that generally causes many autoimmune diseases. This adjuvant is s strong that, associated with self-antigen, it is able to break self-tolerance[373].

The CD8 T cells cultivated with IL-27 DCs showed decreased proliferation. Interestingly, the percentage of PD1 positive cells was more important with cells pulsed with IL-27 DCs indicating that the cells were probably in an exhausted state. We suspected therefore checkpoint inhibitors such as Tim-3 and LAG-3 to be responsible of this mechanism.

6. TIM-3 AND LAG-3: POSSIBLE MECHANISMS

Inhibitory receptors regulate T cell responses. Currently, many studies are focused on the more recently discovered inhibitory receptors, including PD1, LAG-3, Tim-3, and TIGIT. These receptors exert unique functions and are linked directly or indirectly with IL-27[236–238].

LAG-3 is upregulated on activated T cells; it has structural similarities to CD4. This receptor can bind MHCII with more affinity than CD4, and it has been shown to negatively regulate T cell proliferation. Indeed, mice deficient in LAG-3 have uncontrolled T cells responses they show that T cells cannot be suppressed anymore by TRegs[374].

Tim-3 was shown to be selectively expressed on Th1 and CD8 T cells and on innate immune cells (specifically APCs)[62]. This receptor was initially identified as a negative regulator of the Th1 response, and its inactivation through antibodies exacerbated symptoms of the active induction of EAE[375]. Mice deficient in Tim-3 have more severe EAE[376]. In the same study, Tim-3 KO mice were shown to have an increased Th1 response, indicating the tolerogenic potential of this receptor. Tim-3 has two ligands: galectin-9 and the carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1). The interaction of Tim-3 and galectin-9 is an essential element of T cell regulation. The administration of soluble galectin-9 can decrease the severity of symptoms in the EAE mouse model[377], and knockdown of galectin-9 has the opposite effect[377]. Tim-3 is also a marker of the Tr1 cell population[120]. Interestingly Tim-3$^+$ and LAG-3$^+$ Tregs have increased regulatory function, and both can be upregulated with cytokines[378,379].

IL-27 is a crucial inducer of these two receptors. On Th1 cells, addition of IL-27 was shown to induce NFIL3, which promotes expression of Tim-3 and IL-10[236]. On Tregs, the stimulation of IL-27 induces expression of LAG-3 and is critical in regulating the inflammatory response in the intestine[237]. The systemic delivery of IL-27 was unable to prevent the appearance of EAE symptoms in mice deficient in LAG-3[274].

With our *in vitro* data, we have shown that IL-27 DCs can induce expression of LAG-3 and Tim-3 on CD4 T cells. Interestingly, the mean fluorescence intensity of Tim-3 and LAG-3 in the CD44^{inter} and CD44^{high} populations was almost doubled but not in the CD44^{high} population.

Induction of Tim-3 could be the mechanism by which IL-27 DCs inhibit the proliferation and differentiation of CD4 T cells. It confirms the observation that IL-27 works through an NFIL3 axis to induce Tim-3 and T cell dysfunction[236]. This discovery was made in two *in vivo* models. IL-27 was observed to induce NFIL3, which in turn induced overexpression of Tim-3 that was associated with a decrease in gut pathology (inflammation score and body weight)[236]. In a tumor growth model, IL-27R-deficient (WSX-1 KO) mice were resistant to tumor growth, which was associated with (almost complete) loss of expression of Tim-3 in the CD8 T cells infiltrating the tumor[236]. Nevertheless, decreased proliferation induced by IL27-DCs requires further study to conclude whether this effect is partially Tim-3 dependent.

IL-27 also has direct-action LAG-3 dependent on Tregs. As previously mentioned, an osmotic pump providing a constant supply of IL-27 protects mice from EAE but fails to protect mice that lack expression of LAG-3 on iTregs[274]. This study confirms another study in an IBD mouse model[237]. IL-27R-deficient Tregs had no tolerogenic power, whereas IL-27-stimulated Tregs showed high expression of LAG-3 and could inhibit the expansion of T cells *in vitro* and *in vivo*[237]. However, in our assay, we did not specifically target Tregs. DCs primed a mix of Tpm, Tem, Tcm, and Tn CD4 T cells from the spleen. We could not, therefore, conclude whether the tolerogenic potential of IL-27 DCs passed through the same mechanism.

In our study, we could not conclude that the tolerogenic potential of IL-27 DCs on the DTH response and in the EAE model was due directly or indirectly to increased expression of Tim-3. To determine whether IL-27 DCs induce tolerance through Tim-3 effects, the same experiments (DTH and EAE) should be repeated on Tim-3 KO mice. However, our *in vitro* data strongly suggest that the tolerogenic potential of IL-27 DCs works through overexpression of Tim-3. This mechanism would, therefore, create significant T cell dysfunction and induce strong tolerance. Tim-3 is also a marker of the Tr1 cell population[120], showing that IL-27 DCs probably influence this cell type.

7. IL-27 DCS CHANGE IMMUNE MEMORY *IN VIVO*

The tolerogenic power of IL-27 DCs has a strong effect on the immune system *in vitro*. To test this effect *in vivo,* we chose a simple way to quantify the tolerogenic capacity of IL-27 DCs. Although DTH is not an autoimmune disease model (because the antigen used is not from the self), it mimics closely what happens in autoimmunity. For our experiments, it was an attractive model because it generated immune memory that could be modulated. With the DTH model, we were able to test our Mutu DCs *in vivo,* and it was easy to quantify whether immune memory was changed after challenge. The DTH response is mediated mainly by the activation of memory CD4 and CD8 T cells and the release of mediators. The activation and recruitment of cells into the site of inflammation are necessary for the development of its response. DTH is mainly mediated by the Th1 response and to a lesser extent by Th2. Macrophages, neutrophils, and T cells accumulate at the site of the DTH and become activated by the secreted cytokines. There are many different ways to create local inflammation (injection in the skin, absorption in the skin, or absorption in the gut); we chosen to dose the antigen in the footpad, which is the traditional manner for DTH and the easiest to measure. This inflammatory response starts 12 h after the OVA challenge and gradually decreases after 72 h[380].

Different ways exist to boost the tolerogenic effect of DCs. First, the number of cells injected can be increased or the number of injections can be increased. According to previous experiments done in our laboratory, mice injected with tumoral Mutu DCs eliminate the tumor cells after 5 days by a cytotoxic T cell response to the highly immunogenic large T transgene[323,340]. According to those experiments, no more than 5 days, better 2-3 days, should separate the first and the last injection. Second, the cells can be activated through TLR activation. As we saw, IL-27 DCs and Mutu DCs can upregulate MHCII and co-stimulatory signals after TLR stimulation with CpG. With tol DCs, the increase in antigen presentation might also increase the tolerogenic power by increasing the probability of encountering memory T cells. Indeed, the cells are more mobile activated *in vivo*. We have seen that *in vitro,* even activated with CpG, the cells inhibited the antigen-specific proliferation of CD4 and CD8 T cells. Finally, the injection location can be changed to *i.v.* instead *of i.p.* When DCs are injected in the peritoneum, an unknown percentage of cells is

able to reach the bloodstream, and *i.v.* injection might be better. However, previous experiments in our laboratory have shown that Mutu DCs had a strong effect when injected *i.p.* in the DTH, CIA, and tumor models[70,323,381].

The immunoglobulin isotypes IgG_{2a} and IgG_1 are associated with different types of response: IgG_{2a} is commonly induced by a Th1 response, whereas IgG_1 is mostly associated with a Th2 response. It is difficult to know whether IL-27 DCs have a direct or indirect effect on humoral immunity. The decreased concentration of IgG_{2a} is probably due to inhibition of the Th1 response by IL-27 DCs. Indeed, our *in vitro* experiments showed that IL-27 DCs were able to inhibit production of IFN-γ and proliferation of CD4 T cells.

We can also hypothesize that IL-27 DCs have a direct tolerogenic effect on B cells. Indeed, naïve and memory B cells were shown to express the two subunits of the IL-27 receptor[382], and IL-27 was able to modulate their differentiation[382]. Tol DCs were shown to be able to directly decrease the proliferation and survival of B cells[383]. The decrease in OVA-specific IgG_{2a} might be due to both indirect effects with a reduced Th1 response and a direct inhibitory effect of IL-27 DCs on B cells.

8. COMPARISON OF IL-27 DCS AND IL-27-SECRETING IMPLANT

We observed that both, IL-27 DCs and the IL-27 implant, resulted in lesser footpad swelling and lower concentration of OVA-specific IgG_{2a}. After restimulation with OVA in PLN cells from mice injected with IL-27, DCs were shown to increase secretion of IL-10. This allowed us to hypothesize that IL-27 DCs induced the OVA-specific secretion of IL-10 and inhibited secretion of IFN-γ in the draining LN, reducing footpad swelling. However, further experiments with specific separation of CD4 T cells would be needed to confirm whether the IL-10 secretion is due to Tr1 and not CD8 T cells or cells from the innate immune system. Admitting the hypothesis that IL-27 DCs expand the Tr1 population, it is difficult to conclude whether IL-27 DCs induced proliferation of these Tregs or whether they induced their recruitment to the draining LN of the challenged footpad.

IL-27 implants were also able to increase OVA-specific secretion of IL-10 in PLN, but this was not associated with a reduction in OVA-specific secretion of IFN-γ. In the spleen, the OVA-specific secretion of IFN-γ was intermediate for mice that received the IL-27 implant, and no significant differences were found between the two groups. This indicated that IL-27 DCs might be more powerful and appear to reduce the local antigen-specific Th1 response associated with induction of the antigen-specific secretion (perhaps Tr1) in the draining LN of the inflammation. However, the IL-27 implant was also able to decrease footpad swelling. The systemic secretion of IL-27 likely induced different mechanisms, with strong tolerogenic capacities on local inflammation and on humoral immunity in a non-antigen-specific way.

To our knowledge, this is the first time that systemic secretion of tolerogenic cytokines and tolerogenic DCs have been compared *in vivo*. IL-27 has an essential immune-modulatory role in the DTH model. Mice deficient in the IL-27 receptor were shown to have increased swelling in the footpad after challenge[270]. Exogenous IL-27 provided by an osmotic pump was shown to potently suppress the encephalitogenic ability of cells from the lymph node and spleen in an EAE mouse model. The mice with the osomotic pump constantly delivering IL-27 had a decreased infiltration and secretion of IL-17 and IFN-γ[268]. However, delivery of exogenous IL-27 did not reduce the IFN-γ MOG-specific secretion (after restimulation of the cells of the spleen)[268]. In another study using the DTH model, mice injected with recombinant IL-27 had less footpad swelling than controls, and restimulation of the LN with the antigen did not result in a difference in secretion of IL-10 and IFN-γ[384]. However, according to the two previous studies, IL-27 decreases the antigen-specific Th17 response[268,384]. On the basis of our data, we can conclude that continuous delivery of IL-27 has a tolerogenic effect independent of the antigen-specific Th1 response. It was not, therefore, possible to observe a difference after a "recall assay."

The constant release of IL-27 in a mouse would probably induce dysfunction in all T cells upon activation, independently of their antigen specificity. In a study, in which osmotic pumps able to deliver 50 ng of IL-27 per day were installed *s.c.* in EAE experiments, mice showed reduced inflammation and diminished Treg accumulation in the central nervous system (CNS). Surprisingly, this study showed that the direct effect of IL-27 was independent of IL-10 secretion but rather was caused by the increase in LAG-3 expression in the CNS during EAE[274]. As previously mentioned, IL-27 induces T cell dysfunction through induction of Tim-3[236]. We can hypothesize that the constant release of IL-27 would create the same dysfunction systemically in all activated T cells of the mouse. The low degree of footpad swelling would be caused by lower immunogenicity as a result of T cell dysfunction. Nevertheless, more experiments are needed to prove whether our results are directly linked to this phenomenon.

These results show that, directly or indirectly, the systemic delivery of IL-27 and antigen-specific IL-27 DCs can change immune memory, making these approaches an attractive therapeutic strategy to fight autoimmune diseases. We can make the hypothesis that the IL-27-secreting implant seems to reduce antigen-specific inflammation without establishing antigen-specific tolerance and might not be the best way to build long-term tolerance. On the other hand, IL-27 DCs seem to induce a specific tolerance that might be a great tool to modify, in the long term, the immune memory responsible for an autoimmune disease.

9. THE THERAPEUTIC POTENTIAL OF IL-27 DCS IN A MULTIPLE SCLEROSIS MOUSE MODEL

The symptoms of EAE are mainly caused by infiltration in the CNS of Th1 and Th17 cells specific for myelin that induce mature myeloid cells to destroy the parenchyma of the CNS[385]. Indeed, inflammatory myeloid cells were shown to have an essential role in inducing the disease a few days after immunization[386]. They can prime T cells at the beginning of the development of the disease. For example, KO mice for CD80 and CD86 are not susceptible to EAE and do not show symptoms of EAE[387]. This implies that the priming of myeloid cells is necessary to activate the lymphocytes in the LN, and it shows the importance of antigen presentation in this model. The activated cells will then migrate and pass the brain–blood barrier and infiltrate the CNS at different locations, causing the symptoms of EAE. Tolerogenic DCs (induced in several ways) have been shown to prevent appearance of symptoms[71,315,316]. Some researchers have demonstrated the ability of tol DCs to treat symptoms immediately after the first sign of the disease[316,388]. pDCs also help in recovery of EAE, enhancing the therapeutic potential of MOG-pulsed DCs[317]. In the same study, clinical improvements were associated with migration of MOG-pulsed pDCs to the site of inflammation in the CNS. Their tolerogenic properties resulted from their capacity to secrete a lot of type I interferons. The high expression of IDO in pDCs is necessary to generate Tregs capable of stopping the activity of inflammatory T cells[389]. This might be one of the mechanisms underlying their suppressive function in EAE.

In another study, our laboratory demonstrated the importance of Mutu DCs transduced to secrete tolerogenic cytokine (IL-35) in the context of EAE. Several experiments with DCs secreting IL-35, IL-10, or active TGF-β were done by Dr. Sergio Haller and Dr. Anais Duval[359,360]. The transfer of DCs secreting the active form of TGF-β efficiently prevented (before the appearance of symptoms) the development of EAE[359]. No differences were observed in the Th1 or Th17 CD4 T cells, whereas the number of Foxp3 Tregs was significantly increased. TGF-β did not suppress the effector function of the cells but induced the expansion of Foxp3 T cells that inhibited the infiltration of T cells in the CNS. The role of DCs in the infiltration of Tregs was highlighted in a study showing that Tregs injected into EAE mice tended to accumulate in the spleen and LN and were not able to migrate to the CNS[390].

IL-10 DCs showed a completely different action on the development of EAE; they did not change its development. Indeed, injection of recombinant IL-10 or transduced cells protected mice from EAE symptoms[139,391]. Cytokines secreted in our Mutu DC model have different effects from injected cytokines.

IL-35 DCs, in contrast, were able to sharply limit progression of symptoms in an adoptive transfer of T cells reactive against myelin. This study highlighted the fact that tolerogenic DCs can inhibit,

directly or indirectly, fully differentiated effector T cells *in vivo*. IL-35 is a well-known tolerogenic molecule with a proven tolerogenic potential on EAE.[156,158] The restimulation *in vitro* with MOG-pulsed IL-35 DC inhibited the proliferative response of donor mice-derived Th17 cells.[70]

We have observed that IL-27 DCs might have the potential to modulate the symptoms of EAE in a preventive way. Even though the experiment was preliminary and needs to be repeated, we observed that IL-27 DCs were able to increase the percentage of IL-10-secreting cells in the CD4 and CD8 population. As mentioned previously, CD8 Tregs can have an essential role in the induction of tolerance[392]. These cells act by secreting IL-10, which might indicate that IL-27 DCs induce directly or indirectly the secretion of this cytokine in CD8 T cells. The MOG-specific secretion of IL-10 in ILN for mice that were injected with unpulsed IL-27 DCs was also high. This difference might be due to the presence of IL-27 for a short period. Other cytokines, such as IFN-γ, IL-17, and IL-2, also need to be checked to see whether IL-27 DCs are able to change the profile of the "cytokine cocktail" secreted after the MOG restimulation. This experiment could not be repeated because the pertussis toxin (PTX) was changed after some time and induction of the disease was shallow (20 to 30%) with the new PTX. This "batch-dependent" phenomenon is known and yet still misunderstood[385]. However, our preliminary data seem to confirm results from our previous study with IL-35 DCs[70]. It appears that IL-27 DCs are also able to limit the progression of the disease directly or indirectly by inducing tolerance.

In our setup, we decided to inject DCs only when the mice had an EAE score ≥ 1 (limp tail) to demonstrate their therapeutic potential. As was previously demonstrated, IL-27 was not able to change the phenotype of fully differentiated Th17[271,285]. We therefore chose to inject the mice as soon as possible after the first symptoms appeared. IL-27 DCs dampened the severity of EAE symptoms compared with the first injection; however, mice were not able to fully recover. The induction of tolerance does not repair existing damage in the spinal cord; it only prevents exacerbation of the disease. We can compare these results with a previously cited study done with pDCs[317]. In that study, mice received a single injection (when the first symptoms appeared at day 11 after immunization) of 10, 5, or 1 × 10^6 cells of bone marrow-derived pDCs loaded with the peptide MOG. Recovery was associated with a decrease in Th1 and Th17 and an increase of PD1 on CD4 T cells[317]. Interestingly, in the same study, cDCs were not shown to alter the mean clinical score. The setup chosen for our study was different; we injected a different type of cells with an entirely different phenotype, and the cells were pulsed with MOG before injection (not loaded with MOG when they were injected). The previous study found pDCs in the CNS a few days after injection[317], showing that the effect of those DCs probably took place at the site of inflammation. We have not tracked whether IL-27 DCs were present in the CNS or LN after injection in the EAE model. The migration pattern of pDCs and cDCs is very different, and we would not expect IL-27 DCs to migrate the same way as pDCs. We conclude that IL-27 DCs can exert their tolerogenic

properties in LN and spleen and that this effect is probably responsible for the decline in disease severity.

Another study showed the tolerogenic potential of bone marrow derives DCs (BMDC) treated with VitD3 also after the first appearance of the symptoms (as a therapeutic)[388]. They suggested that DCs could be loaded with MOG-encoding mRNA instead of being pulsed with the peptide and facilitate the pulse of DCs[388]. BMDCs treated with VitD3 seemed to have a strong tolerogenic potential when they were pulsed with MOG (peptide or RNA electroporated). In that study, mice received three injections of 1×10^6 cells every 4 d. It is difficult to compare this method with injection of IL-27 DCs. First, BMDCs represent a heterogeneous mix of DCs, and it is not possible to know what type of DC is responsible for the induction of tolerance. Second, the tolerogenic properties of VitD3-treated DCs (BMDC) are different and not fully understood[71,315]. They have the potential to restore antigen-specific tolerance in various autoimmune disease mouse models (e.g., diabetes, multiple sclerosis)[71,286,393]. It is possible to hypothesize that stable secretion of IL-27 in DCs makes a more stable phenotype and increases the time of action of tol DCs. However, a comparative study of those two techniques is necessary to support this statement.

Together these studies and our data validate the relevance of DC therapies for multiple sclerosis. Our data indicate that a combination of IL-27 and cDC1 pulsed with MOG_{35-55} might have potential as a therapy for EAE. It seems also that the amelioration of the symptoms is associated with an increase in IL-10 expression (possibly Tr1) and a reduction in Th1 response.

10. TREATMENT WITH VITAMIN D3 AND IL-27 DCS

VitD3 can induce immune-modulatory function in DCs[339]. By activation of the vitamin D_3 receptor (VDR), DCs acquire a regulatory phenotype and develop therapeutic potential in EAE[388]. Moreover, a single injection of the active form of VitD3 changes the evolution of clinical severity therapeutically[293]. VitD3 working synergistically with IFN-γ was observed to induce a tolerogenic phenotype on DCs. Indeed, the mechanism of those two molecules is interdependent and their combination results in a more robust tolerogenic phenotype: higher secretion of IL-10 and higher expression of PD-L1 associated with a decrease of MHCII and co-stimulatory molecules[394]. Another study found that IL-27 and VitD3 can increase the percentage of IL-10 positive cells in activated CD4 T cells[290]. This might be a key element to switch the tolerogenic properties of IL-27. Indeed, cytokines and VitD3 act on very different pathways to induce tolerance, and they could increase the production of IL-10 in Tr1 cells.

Our data indicated that IL-27 DCs are sensitive to vitD3, whereby it decreases expression of MHCII and increases the RNA expression of *VDR*. Untransduced Mutu DCs were the most responsive. The lowest level of MHCII was observed in IL-27 DCs treated with VitD3. Our findings agree with the literature showing that vitamin D_3 decreases expression of co-stimulatory molecules and MHCII

cDC1s[339,393,395]. The constitutive expression of IL-27 and VitD3 seems to decrease the expression of MHCII, and the effect appears to be additive.

These findings suggested to us that injection of VitD3 could increase the tolerogenic properties of IL-27 DCs in the DTH model. Our data led us to conclude that both IL-27 DCs and VitD3 have a tolerogenic effect on the Th1 response, but these effects seem cumulative rather than synergistic in this context. This could also be due to an insufficient dose of VitD3. However, the dose injected was similar to doses injected in previous studies in mouse models[290,293,338].

Interestingly, in the EAE model, mice injected with IL-27 DCs showed the same amelioration of symptoms independently of injection of VitD3. The group injected with Mutu DCs and VitD3 showed a small decrease in severity of EAE symptoms. Still, this difference was not significant compared with that observed in any other group.

Our data showed that VitD3 has a tolerogenic potential in the evolution of EAE but its effect is not as strong as a single injection of IL-27 DCs pulsed with MOG and CpG. The moderate effect of the infusion of VitD3 could be due to an insufficient dose. In a previously cited study, mice were injected with a single injection of 200 ng of VitD3 (before appearance of the first symptoms), and VitD3 was added at the same time in the water[290]. In another study where a single injection of VitD3 had a therapeutic effect on the evolution of the severity of EAE, mice were fed a synthetic diet that contained all essential nutrients except VitD3[293]. This would explain why in our DTH and EAE experiments, VitD3 had a very moderate effect. Mice already had sufficient nutritional intake of VitD3, and the injection had minimal effects between groups. Following this hypothesis, if a lack of VitD3 increases the severity of EAE, its addition to mice that already have a sufficient intake of VitD3 would not have a therapeutic effect. This would explain results of a clinical study done in US military personnel, which showed that people with high circulating levels of VitD3 have a lower risk of multiple sclerosis[302]. This it also consistent with a genome-wide association study that showed an association of multiple sclerosis with mutations in the enzymes responsible for catalysis of calcitriol[300]. Today, treatments based on dietary supplementation of VitD3 have unproven benefits for patients with multiple sclerosis and other autoimmune diseases. Those observations, coupled with our data, showed that even if VitD3 can modulate the immune system, its supplementation is not sufficient for a therapeutic effect.

We did not observe, in our experiments, synergy between IL-27 DCs and VitD3. We found a small cumulative tolerogenic effect of VitD3 on the IL-27 DCs that decreased expression of MHCII. We found another possible cumulative effect *in vivo* that is difficult to compare with the strong tolerogenic potential of IL-27 DCs in the DTH model. Perhaps the injection of VitD3 in the mouse was not strong enough to see the outcome previously observed[293]. It is also possible that the tolerogenic potential of IL-27 DCs is so strong that injection of an immunomodulator does not change the established T cell memory.

CONCLUSION

The results we generated show that IL-27 can regulate the inflammatory response efficiently. We have not only demonstrated that through the over-expression of IL-27 it was possible to generate tol DCs with strong tolerogenic properties but we have also shown that an implant secreting a high quantity of IL-27 is able to limit the inflammatory response. It underlines the importance of this cytokine in the regulation of the immune response and it shows that IL-27 is a promising target in the research for new therapies against autoimmune diseases.

One of the main difficulties in tol DC therapy is how to induce the tolerogenic properties of the DCs isolated from the patient. Our results show that the induction of secretion of IL-27 by lentivirus gives the ability to decrease inflammation and acts as a therapy in the EAE model. This can be a interesting axis of research in order to reprogram the immune memory responsible of an autoimmune disease.

Our results show that IL-27 does not only regulate inflammatory responses of T cells but it can affect dendritic cells. We could furthermore show that IL-27can increase the expression of the negative costimulatory receptor Tim-3 and probably induce the anergy of T cells.

On the other hand, our data show that encapsulated cell technology producing a constant secretion of IL-27 efficiently decreases antigen specific inflammation. The mechanisms are different: IL-27 DCs -secreting implants do not allow the production of quantities that reach similar local levels at the site of T cell stimulation compared to IL-27 DCs. Also, the negative co-stimulation (with PD1 for example) is certainly higher with IL-27 DCs. These results led us to consider that tol DCs might be better able to induce stable tolerance with the potential to cure autoimmune diseases.

However encapsulated cell technology has a lot of advantages, it is easier to apply in humans, and their application in a local and easy-to-access autoimmune disease (such as lupus or polyarthritis rheumatoid) holds promise. The implant contrary to the DC can be easily taken out, the secondary effects would be therefore more controlled. However, in our setup the implant cannot be huge enough to secrete sufficient cytokines in human organisms. Further studies are needed to study if the systemic secretion of tolerogenic cytokines can treat autoimmune diseases in clinics.

OUTLOOK

IL-27 DCs were able to decrease footpad swelling in the DTH model, and a single injection of pulsed DCs changed the evolution of the clinical score in the EAE model. Nevertheless, it is still unclear how long tolerance persists over time. We hypothesize that IL-27 DCs induce Tr1 and inhibitory receptors such as Tim-3, but negative co-stimulation blocking experiments are needed to understand this mechanism. Our investigations with the DTH model showed that IL-27 DCs could decrease the humoral-specific immunity, indicating that IL-27 DCs could be effective in antibody-mediated autoimmune diseases such as the CIA model or in allergy.

IL-27 DCs could also synergize with other drugs. We observed that the injection of VitD3 did not seem sufficient to have a visible effect on DTH or EAE. A vast library of compounds could synergize with IL-27 DCs. For example, the systemic secretion of other tolerogenic cytokines such as IL-10 or TGF-β with encapsulated cell technology could exert a cumulative effect with IL-27 DCs. Indeed, the IL-27 secreting implant and the IL-27 DCs reach different cells, and we think that they would in various autoimmune diseases.

It is difficult to determine if IL-27 DCs induce more Tregs or anergic T cells. More researches need to be done to understand how IL-27 DCs are able to induce the expression of Tim-3 and if they are able to induce other negative co-stimulations.

The systemic secretion of IL-27 with encapsulated cell technology seems to be a promising therapy. Implants could be used to test IL-27 in combination with other cytokines in different mouse models. For example, IL-10 could synergize with IL-27. They reach different receptors, and have independent mechanisms; those two cytokines would probably synergize together to dampen inflammations. Our study raises new questions about implants: does systemic secretion act only on cells of the immune system (innate or adaptive)? In which time window is the implant effective in changing established T cell memory in the long term?

Consequently, the engineering of DCs secreting tolerogenic cytokines or encapsulated cell technology could lead to new strategies for the treatment of autoimmune diseases, allergies, and skin graft transplantation.

MATERIALS AND METHODS

1. GENERAL METHODS

1.1. CELL CULTURE

All cells were handled in a laminar flow bench under sterile conditions.

All dendritic cell lines (Mutu DCs), T cells and splenocytes were cultured in IMDM + GLUTAMAX completed with 10% FCS, 50 µM β-mercaptoethanol, 10 mM HEPES, 50 U/ml penicillin and 10 µg/ml streptomycin, 0.075 % sodium bicarbonate at 37°C in incubators with 5% CO_2 supply. Upon splitting, Mutu DCs were never divided below 10^5/ml. They were detached from the cell culture plates with cell dissociation buffer (10 mM HEPES and 5 mM EDTA in PBS) for 5 or 10 min at 37°C and centrifuged for 10 min at 350g. Before distribution into new cell culture plates or bottles, cells were resuspended in fresh medium.

All other cells used in this study: HEK 293 T cells and C2C12 were cultured in DMEM + Glutamax with 10% FCS and 1% Penicillin /streptomycin solution at 37°C and 5% CO_2. Before reaching maximal confluency or before the change of the color of the cultured medium, cells were detached centrifuged and split in new flasks. HEK 293T were mechanically detached. C2C12 were detached with the addition of a solution of 0.25% trypsin for 20 min temperature or could be detached with the same dissociation buffer as Mutu DCs (10 mM HEPES and 5mM EDTA in PBS).

1.2. FLOW CYTOMETRY

Flow cytometry staining were performed on single cell suspensions from PLN, ILN spleen and cell lines like C2C12 or Mutu DCs. For intracellular staining cells were activated for 4h with phorbol myristate acetate (PMA) at a concentration of 10 ng/ml and ionomycin 500 ng/ml and then, to quantify intracellularly secreted molecules, brefeldin A (10 µg/mL) was added for 2h at 37ºC. Cells were then washed 2 times with FACS buffer (2 mM EDTA, 2% FCS in PBS). Then cells were stained for surface molecules during 40 minutes on ice (4°C). Samples were then washed twice with FACS buffer. If the cells needed to be stained for intracellular staining, they were permeabilized with Fox3 Fixation/Permeabilization solution (ebioscience) during 1 hour. The fixation was followed by two washes with permeabilization buffer and intracellular staining (with antibodies diluted in permeabilization buffer) at room temperature for 40 minutes in the dark RT. Flow cytometry data was bacquired on BD LSR-II, BD LSR Fortessa or BD FACSCanto 1 cytometers (BD Biosciences) and analyzed with FlowJo (version 10.6.1).

1.3. RNA EXTRACTION AND QPCR

To isolate the RNA, 500 µL Trizol were added on the cells. The lysate was then isolated with 200µl chloroform. The lysate was then centrifuged for 20 min at $1.5x10^4$ g. The pellet was washed 3 times with 500µl 75% ethanol. Ethanol was then removed after centrifugation and pellet resuspended in 30 µL of RNA secure. RNA was incubated at 60°C for 15 minutes and stored at -80°C. Random nanomer primers were added (2 µL of a 200 ng/µL) to the 10µL of RNA and the solution was then denatured for 5 minutes at 70°C. To align RNA and primers they were incubated together at 4°C for 10 minutes. Mixture was pre-incubated for 10 minutes at room temperature 4 µL of 5x RT buffer, 1 µL of 10 µM dNTP, 100 mM DTT, 1 µL of 0.5 µg/µL BSA, 2 µl and 0.5 µl RNAse inhibitor (RNAseIN). The Superscript reverse transcriptase was added (0.5 µL) and the reaction incubated for 50 minutes at 42°C. Then the enzyme was heat inactivated at 70°C for 5 minutes. The reaction was then purified using a Qiaquick PCR purification kit. All the purified cDNA samples were all set at the same concentration. Then 2 µl cDNA was mixed with 0.25 µL of each primer (10 µM) and 2,5µl SYBR Fast Green qPCR Master mix.

Following the standard protocol of our lab was used for all of the analyzed genes: Initial denaturation at 95°C for 5 minutes. 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 normalized to β-actin.

1.4. CELL COUNTING

Cells were counted with cell counter (CASYcounter, Innovatis).

1.5. ELISA

Cytokines

To compare expression levels of cytokines secreted by DCs or T cells, standard enzyme linked immunosorbent assays were performed. In order to compare the secretion between different cell types/mice/condition/treatment cells were plated at the exact same concentration in wells of the same size and cultivated in fresh medium. Cell supernatant was taken at one or at several time points and immediately frozen at -20°C until further processing. ELISA assay was then performed with serial dilutions of the supernatants using the respective Kits, following the manufacturer's instructions. The wash buffer used was made with 0.05% Tween-20 in PBS. TMB Substrate (Thermo Scientific) was added and incubated in the plates for 30 min RT in the dark Data read out was performed with a microplate reader 560 (BioRad) at 450nm within 10 minutes after stopping

of the color reaction. Absorbance sample values were calculated after subtraction of wells incubated with assay diluent.

IgG1 IgG2a ELISA

In order to compare the concentration level of antigen specific immunoglobins. To determine OVA-specific antibodies (but it can be also used for other antigens), plates were coated overnight at 4 °C with OVA in (20 µg/mL) in PBS. Plates were washed three times with wash buffer), and blocked with assay diluent (PBS with10% heat-inactivated fetal bovine serum) for 1 h. M**ous**e sera : a 2 h incubation at RT plates were washed five times, and anti-IgG1 (clone 2H12B4, Chondrex) or anti-IgG2a (clone 1F10C2, Chondrex) conjugated with peroxidase were used as secondary antibodies. Plates were incubated for 1h and washed seven times. TMB Substrate (Thermo Scientific) was added and plates were left for 30 min RT in the dark. Colorimetric reaction was stopped by the addition of 2 N H_2SO_4. Absorbance was acquired at 450 nM in the microplate reader (Ledetect 96, LabExim). The absorbance was calculated with the subtraction of the "blank" (wells incubated with assay diluent). Data are shown in optical density (OD) units.

2. *IN VITRO* METHODS

2.1. GENERATION OF TRANSGENIC CELL LINES

Cloning strategy

The cloning construct EBI3-linker-P28 (see plasmid section) was ordered to Genscript. Codon optimization was made for mammalian cells. The construct was delivered in a vector called (pUC57). Restrictions sites specific for Not1 and Nhe1 were used to cut the sequence of the construct (1396 bp). The gene of interest was inserted into the Not1/Nhe1 cloning site of the lentiviral expression vector pCDH-CMV-MCS-EF1α-RFP+Puro Cloning and Expression Lentivector System Biosciences. Lentiviral expression vectors were cut with the enzymes Nhe1 and Not1. Expression vectors were dephosphorylated using Antarctic phosphatase in a buffer (NEB) to avoid plasmid self-ligation. The reaction was incubated for 1h at 37°C. Ligations were performed with 1µL T4 ligase (NEB) and 2µl of the according buffer. Insert and Vector DNA were added at different molar ratio, and dH2O and the mix were set to a volume of 20 µL. The reaction was incubated for 1 hour at 16°C. Competent *Escherichia coli* were thawed on ice. 10µl of the ligation mix was added to 50µl bacteria and incubated for 30min on ice. The transfection was assessed by heat-shocking for 45 secondes at 42°C. The cells were placed back on ice for 30 minutes, and 500 µL LB medium was subsequently added to each transfection. Bacterial suspension was then plated on agar plates containing the ampicillin n and put overnight into a 37°C incubator.

The next day, single colonies were transferred into reaction tubes containing LB medium with ampicillin and were grown overnight at 37°C. Plasmid extraction was done with miniprep or maxiprep extraction kit (Qiagen) according to the manufacturer's protocol. Test cuts were done to verify if the insert was inside the plasmid (with the enzymes Nhe1/Not1), and the plasmid was sequenced with the appropriate primers to see if point mutation(s) appeared.

Transduction of Mutu DCs and C2C12 cells

The 3rd generation lentiviral transduction system consists of 4 different vectors needed to be transfected in the same time. However, the 3rd generation transfer plasmid can be packaged by either a 2nd generation or 3rd generation packaging system. The expression vectors pCDH-CMV-MCS-EF1α-RFP+Puro containing the IL-27 construct. The envelope vector pVSV-G and the packaging psPAX provide the genes required for the assembly of replication.

With 30µg psPAX, 20µg pVSV-G in 3 mL of serum-free, of serum-free DMEM. Then, 130µg of Polyethylenimine (PEI) was added to the mix (1.5 µg PEI/ 1µg DNA). After gentle mixing, the transfection mix was incubated at room temperature for 30min. The mix was then diluted with 15mL of completed DMEM. A confluent T150 flask of HEK 293 T (More than 6x106 cells) was incubated with the mix in DMEM for 24h. After the incubation time, the supernatant was removed and replaced with 20 mL of viral-collection medium (Opti-ME + 10% heat-inactivated FCS). The efficiency of the transfection was assessed by the presence of RFP by fluorescent microscopy. After 24h, the supernatant was collected and replaced with 20mL of fresh viral-collection medium. The harvested viral-particle-containing supernatant was filtered through a syringe filter unit and stored at 4°C. (or -80°C for a prolonged time).

In each well of a 6-well plate 2x105 MuTu DCs were plated and incubated overnight. On the following day, the media was removed, and 1mL of the 293T derived supernatant containing the viral particles was added to the culture. A serial dilution was done to assess the toxicity of the medium. The medium was replaced with completed IMDM after 24 hours. Five days later, cells were selected for puromycin resistance with 0.5 µg/mL of puromycin in completed IMDM for five days (the medium was changed every two days).

The efficiency of the transduction was checked by microscopy or fluorescent microscopy ten days later to observe the presence of the RFP marker. The presence of the gene construct (EBI3 and P28) can be checked by flow cytometry.

C2C12 cells were transduced the same way but with small differences:

105 C2C12 cells per well of a 6-well plate were plated an incubated overnight. On the following day, the media was removed, and 1mL of the 293T derived supernatant containing the viral particles was added to the culture. A serial dilution was done to assess the toxicity of the medium. The medium was replaced with completed DMEM after 24 hours. Five days later, cells were selected for puromycin resistance with 7 µg/mL of puromycin in complete DMEM for five days (the

medium was changed every two days). The efficiency of the transduction was checked by microscopy or fluorescent microscopy ten days later to observe the presence of the RFP marker. The presence of the gene construct (EBI3 and P28) can be checked by flow cytometry.

2.2. ANTIGEN SPECIFIC OT-II PROLIFERATION ASSAY

The antigen-specific T cell presentation was made the following way: Mutu DCs transduced, or un-transduced were seeded in a 96-well round-bottom one day before at a density of 2x104 DCs per well. For the control group "mix of DCs" the same quantity of IL-27 DCs and Mutu DCs were added in one well (2x104 IL-27 DCs and 2x104 Mutu DCs). The next day DCs were pulsed for four h with the OVA-peptides and stimulated, or not, with CpG (1 µM) at the same time. CD4+ T cells were purified from TCR-transgenic mice (OT-II CD45.1) and magnetically isolated using the EasySepTM Mouse CD4+ T Cell Isolation Kit (#19852, STEMCELL™ Technologies). The T cell isolation kit was used following the manufacturer's protocol. The CD4+ T cells were then stained with Tag-it Violet (Biolegend) or efluortm 450 proliferation dyes (Invitrogen). The staining with the proliferation dye was done according to the manufactured protocol. Finally, 10^5 of stained T cells were added in each well of the 96 well-plate and co-cultured with DCs for three days.

2.3. *EX VIVO* ANALYSIS OF MEMORY CELL

C57BL6 mice (between 7 to 14 weeks old) were immunized against OVA (1mg/mL – Grade IV, Sigma Aldrich) in CFA (1mg/mL *M.Tuberculosis* (InvivoGen)). The mice were then boosted either with OVA (1mg/mL – Grade IV, Sigma Aldrich) in Incomplete Freund's Adjuvant (IFA, Invitrogen). For each immunization, mice were injected with 50 µl of the emulsion in each flank.
Similarly, as the OT-II experiment, mice were sacrificed, and spleens, inguinal and were harvested. After the second immunization, DCs (IL-27 DCs, Mutu DCs or the mix of the two cell lines) respectively were all pulsed for 4h with OVA (100 µg/mL) and stimulated with CpG (1µM). CD4+ or CD8+ T cells were isolated using EasySep. CD4+ Isolation and MACS CD8+ Isolation respectively and stained with Tag-it Violet Proliferation dye. After the stimulation of DCs (4h with OVA at 100 µg/mL, $1x10^5$ prepared T cells were co-cultured with DCs in 96-well culture plates for three days. T cells were collected and analyzed by flow cytometry.

2.4. ORGAN COLLECTION

Popliteal lymph nodes (PLN) and Inguinal lymph nodes (ILN) and spleens were mashed through 40 µm cell strainers, cells. Cells from the spleen were treated with ACK lysis buffer to lyse erythrocytes (NH_4Cl 0.155 M, $KHCO_3$ 0.01 M, EDTA 0.1 mM).

Blood was obtained by cardiac puncture. Samples were let one hour at RT and centrifuged for 10 min at 5000 x g in a refrigerated centrifuge to have to sera saved a -80°C.

3. *IN VIVO* EXPERIEMENTS

3.1. MOUSE HANDLING

All mice experiments were performed in accordance with Swiss federal legislation and had been approved by the Cantonal Veterinary Office (license VD.3324 and VD.3322 for EAE experiments).

C57BL/6 mice were purchased from Harlan Laboratories. OT-II CD45.1 mice are bred in the mouse facility of Epalinges. Maximal 5 adult animals can live together in individual ventilated cages. Males were kept together only with littermates.

All the experiment with DTH and EAE were performed with female animal with an age between 9 weeks and 14 weeks.

3.2. INDUCTION OF THE DTH

Mice were immunized *s.c.* with 50 µl/flank of an emulsion of OVA (1mg/mL – Grade IV, Sigma Aldrich) in CFA (1mg/mL *M. Tuberculosis* InvivoGen). Two weeks later mice were boosted with IL-27 DCs or Mutu DCs pulsed for 4h with OVA (100 µg/ml) and activated or not with CpG (1µM). After the stimulation, DCs were harvested and injected intraperitoneally (*i.p*) in mice ($3x10^6$ DCs/mouse). Some mice received IL-27 DCs or MuTu DCs and some others received only PBS. Two days later (day 16), mice received a second *i.p.* injection of the same DCs ($3x10^6$ DCs/mouse) pulsed with the same protocol. Three days later (day 19 after immunization), mice were challenged *s.c.* in right footpad with a solution of OVA prepared as follows: OVA (Grade IV, Sigma Aldrich) diluted in PBS to form a 20 mg/ml solution and heated at 80°C for 1 hour (to form a gel) and kept at 4°C overnight. The gel was injected (25 µL) in the footpad with a syringe

As a control, the same volume of PBS was injected in the left footpad. Finally, footpad thickness was measured at different time point after the challenge (24h, 48h, 56h and 72h) using a dial vernier caliper. Seventy-two hour after the challenge, blood, spleen, inguinal and popliteal lymph nodes were collected from each mouse. A single cell solution was prepared for each organ of each mouse. A red blood cell lysis was used for the spleens and all the single cell solution were put at the same concentration. Finally, 200 µL of each were incubate at 37°C overnight in 96-well culture plates ($1-3x10^6$/well). Then, cells were analyzed by flow cytometry.

3.3. SUBCUTANEOUS IMPLANT

The murine myoblast C2C12 cell line was kindly given by the lab of Prof. Patrick Aebischer. They were cultured with DMEM (GIBCO) supplemented with 10% FBS, penicillin (100 U/mL) and streptomycin (100 µg/mL) (BoConcept). To generate the C2C12 cell lines expressing IL35, we used the same lentivirus as Mutu DCs. They were transduced and selected with puromycin. The encapsulation of the cells in an immune-isolated implant was previously described. Briefly, cells were detached with Trypsin-EDTA solution or cell dissociation buffer, and cell suspensions were mixed with PEG biodegradable gel premix (two vials of NS13 provided by Qgel) immediately before the loading of 3-5x10^6 cells (in 250 µL) into the cell encapsulation devices called “flat sheet” device”. The device is a flat sheet implant 27 mm long, 12 mm wide and 1.2 mm thick and was assembled by the Cell Encapsulation Technology Team at Nestlé Health Science. The internal volume is approximately 40 µl. It is constituted of a semi-permeable membrane made of either polypropylene membrane with 45 µm pore (PP45) or a polytetrafluoroethylene (PTFE) membrane and a polyester mesh. Devices were placed on a rocking platform until hydrogel crosslinking was complete, when they were sealed with polymerizing medical-grade glue (Loctite, Henkel). Devices were maintained in DMEM over-night under cell culture conditions and washed with PBS before subcutaneous (s.c) implantation in the back of mice. Surgeries were performed under ketamine (100 mg/kg)/xylazine (10 mg/kg) anesthesia and mice recovered in their home cages.

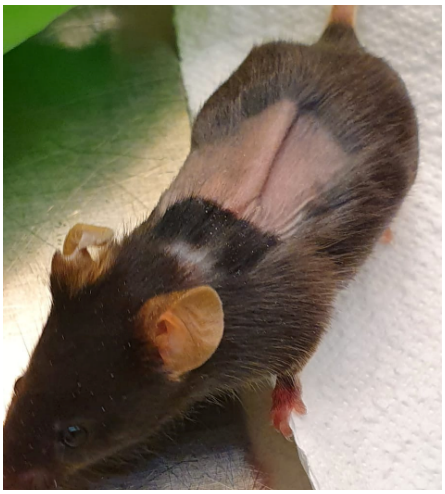

Figure 37 Mouse with a subcutaneous implant one week after the surgery. The back of the mouse was shaved for the surgery.

3.4. ACTIVE INDUCTION OF EAE

Active EAE was obtained by administering subcutaneous injections of 200µg MOG35-55 peptide in CFA (5 mg/ml *M. tuberculosis, (*Sigma) at day 0 in mouse flanks. In addition, mice were injected with 200 ng pertussis toxin (Sigma). Mice were then monitored daily for limb and tail

paresis/paralysis until full development of the disease. Different incidences and severity of the diseases were seen with the different badge of pertussis toxin used.

Passive EAE were tried but not successfully.

Mice were controlled daily and clinical EAE symptoms were scored as previously described[385]. When possible, the double blinded scoring was done.

0 - no obvious changes in motor functions

0.5 - decreased tail tonus but still able to move. Or small hind limb weakness

1 - Limp tail a or hind limb weakness but not both

2 - Limp tail and hind limb weakness

3 - Partial hind limb paralysis (mice sick for more than 2 days at a level 3 are sacrificed for humane reasons)

4 - Complete hind limb paralysis

5 - Moribund state

For the use of DC as treatment. When mice started to have clear symptoms, mice were paired according to their disease symptoms and put in the treatment or control group in randomized way in order to have the same proportion of very sick and mildly sick mice in all the groups.

4. STATISTICAL ANALYSIS

Statistical analysis was performed applying unpaired, One-way Anova or Student's *t* – test using Graphpad Prism. Differences were considered significant when * $p<0.05$, ** $p\leq0.005$, *** $p\leq0.001$.

5. MATERIALS

5.1. REAGENTS

Product	Supplier	Reference number
2-Mercaptoethanol 50 mM	Gibco	31350-010
Brefeldin A	BioLegend	420601

Cell proliferation dye eFluor 470	eBioscience	65084285
Tag-it VioletTM Proliferation and Cell Tracking dye (cat. 425101, BioLegend	BioLegend	(cat. 425101, BioLegend
CFA/IFA	SIGMA	F5881/F5506
Chloroform	SIGMA	C2432
Collagenase D	Roche	11088866001
Magnetic cell sorting for CD4 cells	EasySep Mouse CD4+ T Cell Isolation Kit	Stemcell (19852)
Biodegradable gel for implants	QGel	NS013
CpG	Trilink Technologies	N/A
Ethanol	Fischer	10048291
Fc blocking antibody 24G2	Made in the lab	N/A
FCS	Pan Biotech	Lot: P140112
HEPES	Gibco	31980-022
IMDM	Gibco	31980-022
Ionomycin	Calbiochem	407950
Isopropanol	Fluka Analytical	59310
MOG35-55	SIGMA	163913-87-9
OVA	SIGMA (grade III, IV, V)	A5503
PEI	SIGMA	408727
Pen/strep	Life Technologies	N/A
RNA secure	Thermofischer	AM7005

Sunflower seed oil	SIGMA	S5007-250ML
VitD3 (calcitriol)	SIGMA	D1530-10UG
TRI Reagent	SIGMA	T9424

5.2. RT PCR PRIMERS

p35RTfw: AAATGAAGCTCTGCATCCTGC

p35RTrev: TCACCCTGTTGATGGTCACG

ebi3RTfw: ATG TCC AAG CTG CTC TTCCT

ebi3RTrev: AGA GGA GTC CAG GAG CAG TC

vdrRTfw: CACCTGGCTGATCTTGTCAGT

vdrRTrv: CTGGTCATCAGAGGTGAGGTC

5.3. FACS ANTIBODIES

CD4 (clone RM4-5, FITC or PerCP-Cy5.5, BioLegend),

CD40 (clone 1C10, APC)

CD44 (clone IM7, APC, PE-Cy7, or Pacific Blue)

CD8 (clone 53-6.7, Pe-Cy7, BD Biosciences),

CD62L (clone MEL-14, PE, BioLegend),

CD11b (clone M1/70, APC)

CD11c (clone N418, PeCy7)

PD-L1 (clone 1-111A, PE),

CD86 (clone GL-1, AlexaFluor700),

MHCII (clone M5, PerCp)

CD44 (clone IM7, APC, eBioscience or Pacific Blue, BioLegend),

CD25 (clone PC61, PerCP-Cy5.5 or PE, eBioscience),

MHCII (clone M5/114.15.2, PERCP)

CD3 (clone 145-2C11, BioLegend),

FOXP3 (clone FJK-16s, PE-Cy5, Invitrogen),

FOXP3 PE (clone FJK-16s, eBioscience)

EBI3 (clone 1C18341A, APC, R&D System),

GP130 (clone 4H1B35, PE, Biolegend)

IL-10 (clone 554467, PE, BD Pharmingen),

IL-10 FITC (clone JES5-16E3, FITC)

IFN- γ (clone XMG1.2, PE/Dazzle, BioLegend),

IL17a (clone TC11-18H10.1, APC or BUV395)

IL-6 (clone MP5-20F3, PE, BioLegend),

IL-2 (clone 3ES6-5H4, eF450, Invitrogen),

5.4. PLASMID SEQUENCE

IL-27 EBI3-Linker-P28 plasmid sequence:

ATGAGTAAACTCCTGTTCCTCAGCCTCGCACTGTGGGCCAGTAGAAGCCCAGGGTATACTG
AGACTGCACTCGTGGCCCTGAGCCAGCCCAGGGTCCAGTGTCACGCCTCAAGATACCCCGT
CGCCGTGGATTGTAGCTGGACTCCCCTGCAGGCACCAAACTCAACCAGGAGCACTAGTTTC
ATCGCAACTTACAGGCTCGGAGTGGCTACTCAGCAGCAGAGCCAGCCATGTCTGCAGAGGA
GCCCTCAGGCAAGCCGGTGTACAATCCCAGATGTGCACCTGTTCAGCACAGTGCCCTATAT
GCTCAATGTGACAGCAGTGCACCCAGGGGGTGCAAGCAGCTCACTCCTCGCATTCGTCGCT
GAACGAATCATCAAGCCAGATCCACCCGAGGGTGTCCGGCTGCGGACTGCCGGTCAGCGG
CTCCAGGTCCTCTGGCATCCACCTGCAAGCTGGCCATTCCCCGACATTTTCAGCCTGAAATA
TCGGCTGAGATACAGGAGACGAGGGGCAAGCCATTTCAGGCAGGTCGGTCCAATTGAAGCT
ACTACCTTCACACTGAGGAATAGCAAACCACACGCAAAGTACTGCATTCAGGTCAGCGCACA
GGACCTGACTGACTACGGTAAACCATCCGATTGGAGCCTGCCCGGTCAGGTGGAAAGCGC
CCCACATAAGCCTGGTGGAGGCGGAAGTGGTGGGGGTGGAAGCGGCGGAGGGGGTTCAT
TTCCTACAGACCCACTGTCCCTGCAGGAGCTGCGGCGGGAGTTTACAGTGAGCCTGTATCT
CGCTAGAAAGCTCCTCTCCGAAGTGCAGGGTTACGTGCATAGCTTCGCCGAGTCACGGCTC
CCAGGGGTCAACCTCGATCTGCTGCCACTGGGGTATCACCTCCCTAATGTCAGCCTGACAT
TCCAGGCTTGGCACCATCTCAGCGACAGTGAACGGCTGTGCTTCCTGGCAACTACACTGAG
GCCCTTCCCAGCCATGCTCGGGGGACTCGGGACCCAGGGTACATGGACTAGCAGTGAGCG
GGAACAGCTCTGGGCCATGCGGCTCGATCTGCGGGACCTGCATCGGCACCTCAGATTCCA

GGTGCTCGCAGCAGGTTTCAAGTGCAGCAAGGAGGAGGAGGACAAGGAAGAGGAAGAGGA
GGAGGAAGAAGAAGAGAAGAAACTCCCCCTGGGTGCCCTGGGCGGTCCTAATCAGGTCTC
ATCACAGGTGTCATGGCCACAGCTCCTGTATACATACCAGCTCCTCCACAGCCTGGAGCTC
GTGCTGAGCAGAGCAGTCAGGGACCTCCTCCTGCTGAGCCTCCCAAGGAGGCCTGGAAGC
GCATGGGATAGCTAA

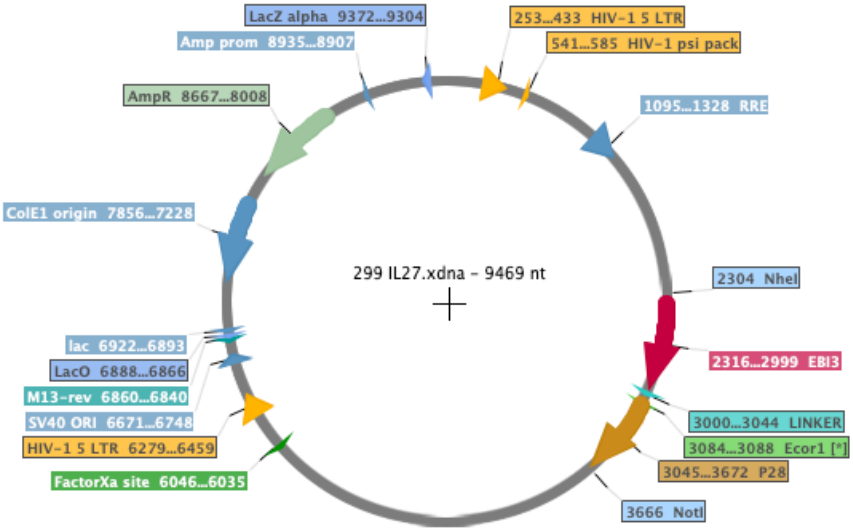

Figure 38 IL-27 EBI3-Linker-P28 plasmid.

ABREVIATIONS

AD	Autoimmune Diseases
APC	Antigen Presenting Cell
BCR	B-cell Receptor
C2C12	Immortalized Mouse Myoblast Cell Line
CCR7	C-C motif Chemokine Receptor 7
CD	Cluster Differentiation
cDC	Conventional Dendritic Cell
CFA	Complete Freud's Adjuvant
CIA	Collagen-Induced Arthritis
CpG	CpG oligodeoxynucleotides
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DC	Dendritic Cell
DTH	Delayed Type Hypersensitivity
EBI3	Epstein-Barr virus induced gene 3
ELISA	Enzyme-Linked Immunosorbent Assay
Foxp3	Forkhead Box P3
Gp130	Glycoprotein 130
i.p.	Intraperitoneal
IDB	Inflammatory Bowel Disease
IDO	Indolamin-2,3-Dioxygenase
IFA	Incomplet Freund's Adjuvant

IFN	Interferon
Ig	Immunoglobulin
IL-	Interleukin
IL-10 DCs	Dendritic Cell able to secrete IL-10
IL-27 DCs	Dendritic Cell able to secrete IL-27
IL-35 DCs	Dendritic Cell able to secrete IL-35
ILN	Inguinal Lymph Node
iTregs	Induced regulatory T cells
LAG-3	Lymphocyte Activation Gene-3
LAG-3	Lymphocyte Activation Gene-3
LN	Lymph nodes
LPS	Lipopolysaccharide
MACS	Magnetic-Activated Cell Sorting
MHC	Major Histocompatibility Complex
MHCI	Major Histocompatibility Complex class I
MHCII	Major Histocompatibility Complex class II
mTEC	Medullary Thymic Epithelial Cells Presenting Self-Peptides
Mutu DCs	Murine Tumor DCs
MyD88	myeloid differentiation primary response gene 88
nd	Non-Detectable
NF-κB	Nuclear Factor-kB

NK	Natural Killer
ns	Non-Significant
nTregs	Natural Regulatory T Cells
OVA	Ovalbumin
PAMP	Pathogen-associated-molecular pattern
PBS	Phosphate-buffered saline
PCR	Polymerase Chain Reaction
PD-1	Programmed cell death 1
PD-L1	Programmed cell death ligand 1
pDC	Plasmacytoid Dendritic Cell
pLN	Popliteal Lymph node
PRR	Pattern recognition receptor
s.c.	Subcutaneous
SV40LgT	Simian virus 40 large T oncogene
Tcm	Central memory T cells
TCR	T cell receptor
Te	Effectors
TGF	Transforming growth factor
Th	Helper T cells (CD4)
Tim-3	T-cell immunoglobulin and mucin domain-3
Tn	Naïve T cells

tol DC	Tolerogenic DCs
Tpm	Perypheral memory T cells
Treg	Regualtory T cells
Trm	Tissue-resident memory T cells
tTregs	Thymus-derived Tregs
VitD3	Active form of Vitamin D3 (calcitriol)

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SCIENTIFIC PUBLICATIONS 2016-2020

1. ARTICLE I

Synthetic IL-27 producing dendritic cells as a tool to change the specific immune memory in autoimmune disease models. (manuscript in preparation)

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2. ARTICLE II

IL10- and IL35-secreting MuTuDC lines act in cooperation to inhibit memory T cell activation through LAG-3 expression. (manuscript in preparation)

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ATTENDED CONFERENCES 2016-2020

1. POSTER I

Adrien Engel, Marianna M. Koga, Patrick Aebisher, Bernard Schneider, Hans Acha-Orbea. **A tolerogenic implant against autoimmune diseases.**

Poster presented at: 6th joint Novartis-EPFL-UNIL meeting; Feb 22, 2019, Basel, Switzerland.

2. POSTER II

Adrien Engel, Marianna M. Koga, Diana Vitorino-Francisco, Patrick Aebisher, Bernard Schneider, Hans Acha-Orbea. **Tolerogenic dendritic cells and subcutaneous implant as tools to change pre-established T- cell memory.**

Poster presented at: Next Gen Immunology. Weizmann Institute of Science; Feb 2-5, 2020, Rehovot, Israel.

3. ORAL PRESENTATION I

Adrien Engel, Marianna M. Koga, Patrick Aebisher, Bernard Schneider, Hans Acha-Orbea. **IL-27 producing dendritic cells to change established immune memory.**

Oral presentation at: XXXI Wolfsberg meeting of Swiss PhD students in immunology. March 13-15, 2019, Thun, Switzerland

4. ORAL PRESENTATION II

Adrien Engel, Marianna M. Koga, Diana Vitorino-Francisco, Patrick Aebisher, Bernard Schneider, Hans Acha-Orbea. **Tolerogenic dendritic cells as a tool to change pre-established T- cell memory.**

Oral presentation at: Department of Biochemistry Day (DB Day), UNIL. Sep 24, 2019, Lausanne, Switzerland.

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