Tolerogenic dendritic cells and the priming of thymus-derived Tregs

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Abstract

Dendritic cell (DCs) play a crucial role in both the innate and adaptive immune system. The immunogenic role of DCs, which involves activation by pattern recognition receptor (PRR), migration to the lymph nodes and activation of conventional T-cells (Tconvs), is well understood. However, DCs can also be tolerogenic and hinder immune activation. In the intestine, the presence of tolerogenic DCs can be beneficial to prevent chronic inflammation. However, in the context of tumors, where immune activation is wanted, there is also often immune suppression caused by tolerogenic DCs. In the past years, there has been a growing interest in not only understanding immunogenic but also tolerogenic DCs. This review aims to provide an overview of the various characteristics that have been described for tolerogenic DCs. In addition, we elaborate on the priming of regulatory T-cells (Tregs) by tolerogenic DCs. Tregs can be thymus derived (tTregs) or be peripherally induced from Tconv cells (pTregs). The traditional research focus has always been on pTregs. However, current studies reveal that it are the tTregs that are predominantly found in tissues like tumors and the intestine and that tTregs play an important role in mediating tolerance. The mechanism by which these tTregs end up in the tissues is not known. In this review, we established a model using existing information to explain how tTregs are primed by tolerogenic DCs and migrate to tissues like the intestine or tumors and play an important role in mediating tolerance.

Plain Language summary

The immune system consists of different types of cells, and one important kind is called dendritic cells (DCs). These cells monitor the body for potential threats like bacteria of viruses. When DCs identify something that is potentially harmful, they become activated and move to lymph nodes. Here, they activate another type of immune cell called conventional T-cells (Tconvs). These activated Tconv cells eliminate the harmful factor or activate other type of immune cells. Interestingly, DCs do not only activate the immune system, they can also suppress it. This is useful in places like the intestine, where there is a constant exposure to bacteria that are not harmful. However, in the case of tumors, where an immune response is needed to eliminate the cancer cells, there is also often immune repression. So called tolerogenic DCs can cause immune suppression.

Unlike DCs that trigger an immune response, there is not a lot known about these immune-suppressing tolerogenic DCs. In this review, we provided an overview of various tolerogenic DC characteristics that have been described. Not all tolerogenic DCs have the same characteristics. It depends on the tissue and surroundings which proteins they express, what their metabolism is, and which immune suppressing substances they release. This is important to keep in mind when analyzing these cells. Also in the case for tumors, not every patient will have the same type of tolerogenic DCs in their tumors.

One of the ways how tolerogenic DCs can suppress immune response is by activating regulatory T-cells (Tregs). These type of T-cells do not trigger an immune response but instead hinder it. Tregs can either be regulatory from the start (tTregs) or they can be generated from Tconv cells (pTregs). While research has often focused on pTregs, evidence suggests that it are the naturally occurring tTregs that are crucial in suppressing immune responses in the intestine and tumors. It is however unclear how these tTregs, that are usually located in the lymph nodes, end up in these tissue. Using various information sources, we proposed a model of how these tTregs are activated by tolerogenic DCs and end up in intestine or tumor tissue. To start, tolerogenic DCs are induced in tissues after they have taken up apoptotic cells or substances released by the tissue. Once activated, these DCs move to the lymph nodes where they stimulate and activate tTregs. This stimulation is different compared to immunogenic Tconv cell stimulation. Additionally, tolerogenic DCs release substances that promote tTreg movement to the tissue. Upon arrival in the tissue, the tTregs help to maintain an immunosuppressive environment.

Understanding how tolerogenic DCs and Treg activation occurs brings us closer to comprehending suppressive environments, particularly in tumors. If we learn more about how these suppressive environments form, we can come up with ways to overcome them.

Introduction

Dendritic cells (DCs) are antigen presenting cells that are a key component of both the innate and adaptive immune system[1]. Immature DCs survey the body for various danger signals. Upon encountering a pathogen, DCs get activated through pattern recognition receptors (PRRs). Consequently, the DCs upregulate a combination of co-stimulatory and co-inhibitory molecules, as well as CCR7, permitting them to migrate to the draining lymph node (dLN). This immunogenic maturation state of DCs allows them to present pathogen-derived foreign antigens to naïve conventional T-cells (Tconv), promoting their differentiation into effector T-cells[2].While there is extensive knowledge on the role of DCs in initiating an immunogenic response, our understanding of DCs acting as tolerogenic cells is comparatively limited. In the steady-state, tolerogenic DCs are crucial in the induction and maintenance of central and peripheral tolerance. They do this by deleting self-reactive T cells during thymocyte development and by inducing clonal anergy and deletion, by production of anti-inflammatory cytokines and metabolites, and by priming and activation of regulatory T cells (Tregs).

The intestine is a prime example where tolerogenic DCs are induced in the steady state to maintain a suppressive environment essential to prevent chronic inflammation. In addition, the presence of tolerogenic DCs is often observed in tumors. These tolerogenic DCs contribute to an immunosuppressive tumor microenvironment (TME). It was long thought that this immunosuppressive TME resulted from a lack of tumor-specific T-cells. However, tumor-reactive T-cells have been identified in various cancer patients[3–5]. This is validated by the success of immune checkpoint inhibitors (ICIs) in treating cancer. Nevertheless, ICIs are not always effective[6]. This indicates that the frequently observed immunosuppressive TME cannot be solely attributed to the absence of tumor-specific T-cells. Tolerogenic DCs, generated within peripheral tissues like tumors, possess the capability to suppress an immunogenic response. These DCs can for example attract and promote the expansion of Tregs, who subsequently hinder tumor-specific Tconvs.

Immunogenic DCs have been extensively characterized, but the understanding of tolerogenic DCs remains limited while it is equally significant. In addition, Tregs can be generated in the thymus (tTregs) or at peripheral sites from CD4+ Tconv cells (pTregs). A lot of research has always focussed on pTregs, but there is growing evidence that suggests that it are the tTregs that play a pivotal role in mediation tolerance. In this review, we will provide an overview of tolerogenic DC characteristics in terms of expression of proteins, secreted molecules and changes in the metabolic state. In addition, we focus on Tregs and how DCs prime tTregs and pTregs. In the end, we propose a model elucidating the induction of tolerogenic DC state and the priming of tTregs within the context of both the intestine and tumors.

DC lineages

There are different subsets of DCs, namely conventional DCs (cDCs), monocyte-derived DCs (moDCs), plasmacytoid DCs (pDCs),) and Langerhans cells (LC). This review will focus on the cDC subsets, as they are particularly important in the activation of Tconvs and Tregs. The cDC lineage can be subdivided into cDC type 1 (cDC1s) and cDC type 2 (cDC2s), and can be distinguished by the expression of different transcription factors and cell surface proteins. In addition, they elicit different immune responses.

The cDC1 lineage relies on transcription factors IRF8 and BATF3 and are furthermore characterized by the expression of XCR1 and DNGR-1 (CLEC9A) on their cell surface[7–10]. In lymphoid tissue, cDC1s additionally express CD8α, while in non-lymphoid tissue, they express CD103. XCR1 serves as a chemokine receptor with ligands XCL1 and XCL2 predominantly produced by natural killer cells and activated T cells, recruiting cDC1c in the lymph node[11,12]. C-type lectin receptor DNGR-1 is important for the uptake of cell-associated antigen and subsequent antigen cross-presentation in major histocompatibility complex class I (MHC-I), a function restricted to the cDC1 subset[9]. cDC1s play a crucial role in promoting a Th1 response by secreting IL-12[13]. In addition, cDC1s can also cross-present antigens to also elicit a cytotoxic T-cell (CTL) response[14].

cDC2s on the other hand primarily induce Th2 and Th17 responses through the secretion of IL-23[10,15]. The cDC2s display a greater heterogeneity than cDC1s and express transcription factors IRF4 and Notch2, as well as signal regulatory protein α (SIRPα, also known as CD172a) and integrin CD11b on their cell surface[10,16,17]. SIRPα ligation by CD47 results in the inhibition of phagocytosis, mediated by the recruitment and activation of tyrosine phosphatases SHP1 and SHP2. One known target substrate of SHP-1 and SHP-2 is motor protein myosin IIA, which is involved in (F-actin-dependent) phagocytosis[18]. It is likely that SHP-1 and SHP-2 recruited to the SIRPα intracellular domain have more direct targets that might influence the phagocytic activity of cDC2s. Furthermore, it was discovered that the CD47-SIRPα interaction suppresses integrin activation, hence inhibiting phagocytosis[19].

Although cDC2s cannot acquire cell-associated antigens via phagocytosis, they can obtain soluble antigens via macropinocytosis for their presentation in MHC class II (MHC-II)[20]. In addition, antigen transfer between different cDC subsets has been reported[21–23]. These cDC subsets include resident cDC1s (CD8α+), migratory cDC1 (CD103+), resident cDC2s (CD11b+) and migratory cDC2s (CD11b+). *In vitro*, the migratory DCs were the most proficient donors and resident CD8α+ DCs the most effective recipients, potentially due to the superior phagocytic capacity of the cDC1s. However, *in vivo*, all types of cDCs were capable of receiving antigen[22,23]. Ruhland *et al*. discovered that this transfer of antigens occurs within formed synapses via vesicles[23]. In addition, Pirillo *et al*. recently found that this transfer of antigen is coencoded with the transfer of contextual information such as pathogen- or damage-associated molecular patterns (PAMPs or DAMPs)[22]. For example, in the case of an influenza A virus infection, DCs can cotransfer viral antigen with double-stranded RNA. This allows for mirroring of migratory cDC activation with the activation of resident cDCs in the LNs, at the site of infection or in the tumor.

Characteristics of tolerogenic DCs

For a long time, it was thought that tolerogenic DCs were simply immature DCs with a reduced expression of MHC and co-stimulatory molecules in the absence of maturation signals[24]. However, it has become evident that the distinction between immature and mature DCs does not align with the division between tolerogenic and immunogenic DCs. In fact, tolerogenic DCs undergo substantial transcriptomic changes comparable to those observed in immunogenic DCs.

Prior to describing the characteristics of tolerogenic DCs, it is essential to make an distinction between tolerogenic DCs and mature regulatory DCs (mregDCs). Recently, a conserved mature DC program activated within tumors has been described and is referred to as mregDCs[25]. This program has also been described in intratumoral LAMP3+ DCs[26] and more recently also in tumor-infiltrating DC3s[27], DC_S3[28] and helped cDC1s[29]. The transcriptome profiles of these various mature DC programs in tumors have been compared by Lei *et al*.[30]. It was found that these DC populations share common upregulated genes including genes associated with TLRs, MyD88 signaling, MHC and costimulatory proteins like CD80 and CD86, as well as *Cd40, Cd83, Lamp3, Relb, Ccr7, Ccl19* and *Cd274*. Although these genes were previously considered immunogenic, a collection of these genes may also be upregulated in tolerogenic DCs, as will be described in this review. Taken together, the mregDC program is characterized by the upregulation of genes involved in both immunogenicity and tolerogenicity. Therefore, mregDCs likely exist in a dynamic cellular state rather than being a separate subset, and they may exhibit flexibility in their functions. For example, in an immunosuppressive environment, they exhibit tolerogenic properties, but in the presence of an immune response, they may also act in an immunogenic manner. A recent study did show that mregDCs suppress CD8+ T-cell activation and promote pTreg differentiation[31]. Nevertheless, the precise role of mregDCs is not definitively understood, and further research is needed to elucidate their function. In literature, the term mregDCs is frequently used for referring to tolerogenic DCs. Nevertheless, as explained above, these two terms are not interchangeable.

In this review, we describe the characteristics of tolerogenic DCs and not mregDCs. We will give insight into proteins and immunomodulatory molecules that can play a role in regulating tolerogenicity of DCs, as well as the transcriptome alterations that occur during tolerogenic maturation. Additionally, we will discuss the metabolic state of DCs. It is important to note that not all tolerogenic DCs exhibit or are affected by every characteristic outlined in the section. The expression of specific proteins or changes in the metabolic state depend on the tolerogenic stimulus and tissue context. Our goal is to offer an overview of potential characteristics that may be observed in the context of tolerogenic DCs (Fig. 1).

Regulatory proteins

Various regulatory proteins are known to induce or maintain tolerogenic properties in DCs. These proteins may be located intracellularly or at the plasma membrane of DCs (Figure 1). In addition, regulatory proteins expressed by other immune cells can interact with or influence DCs. To start, two well-known proteins that play a role in the induction of tolerance in DCs via direct contact are cytotoxic T-lymphocyte associated protein 4 (**CTLA4)** and programmed cell death protein 1 (**PD1,** also known as CD274). Activation of T-cells generally requires the binding of the costimulatory receptor CD28 to its ligand CD80/86 in addition to the T-cell receptor (TCR)-MHC interaction. CTLA4 is expressed by Tregs and can interact with CD80 and CD86 on cDCs. This interaction leads to transendocytosis of these ligand into the Tregs

and thereby to downregulation of both proteins[32,33]. PD-1 is expressed on recently activated T-cells and can, upon binding to PD-L1 or PD-L2 on DCs, inhibit CD28 signalling[34,35]. Both CTLA-4 and PD-1 influence the CD28-CD80/86 costimulatory axis, thereby hampering T-cell activation and clonal expansion.

CD80 and **CD86** are often mentioned together and are generally considered interchangeable. However, there is evidence indicating that the reliance on CD80 or CD86 for costimulation varies when priming different T-cell subsets. It was demonstrated that CD80 and PD-L1 can form a heterodimer capable of binding exclusively to CD28 and not to PD-1 and CTLA-4, which favors Tconv activation[36]. CD86 cannot form this heterodimer, highlighting an distinction between CD80 and CD86. Furthermore, a study by Frijlink *et al*. revealed that CD86, and not CD80, enhances a Treg response and restrains a CTL response following radiotherapy of certain tumors[37]. Antibody-mediated blocking of CD86 resulted in the abrogation of the Treg response, which was not observed when CD80 was blocked. This difference could arise from the higher affinity of CTLA-4 for CD80 compared to CD86[38]. Because Tregs constitutively express CTLA-4, the CD80-CD28 interaction is impaired which results in CD86 being the selective CD28 ligand for Tregs[39]. These results suggest that CD86 plays a more pivotal role in initiating a tolerogenic cDC response indirectly by activating Tregs, while CD80 is more integral to promoting an immunogenic response by activating CTLs. Supporting this hypothesis, studies reveal that mice deficient in CD83, a protein that stabilizes CD86 surface expression, exhibited reduced levels of CD86[40,41]. DC-specific deletion of CD83 in experimental autoimmune encephalomyelitis (EAE) mice led to an increase in Tconv but a decrease in Treg numbers in peripheral lymphoid organs[41]. In addition, the uptake of apoptotic cells or lipid nanoparticles (LNPs), which induces tolerogenic DC maturation, led to a down-regulation of CD80. Conversely, the uptake of pIC-couples LNPs, which provoke an immunogenic DC response, increased CD80 expression by cDCs[42]. Furthermore, in response to influenza antigen uptake, cDCs upregulated both CD80 and CD86, while in a tumor setting, they only upregulated CD86[22]. Collectively, these observations underscore the role of CD80 in Tconv priming and CD86 in Treg priming.

Another protein expressed by DCs that engages in direct interactions with T-cells is B- and T-lymphocyte attenuator (**BTLA)**. The BTLA protein, predominantly found on tolerogenic cDC1s, can bind to the herpesvirus entry mediator (HVEM) receptor on T-cells. This interaction leads to an upregulation of CD5 on the T-cells. CD5 inhibits the PI3K/mTOR pathway that inhibits FoxP3 expression. Consequently, the upregulation of CD5 results in the induction of Foxp3 expression and the conversion of Tconv cells into pTreg cells[43,44].

There are also regulatory proteins expressed on DCs that do not directly engage with T-cells but nonetheless exert an influence on T-cell activation. Research has shown that **CD83** deletion in EAE mice causes Tconv expansion and Treg cell inhibition[41]. On these CD83-deficient cDCs, CD25 and OX40L showed increased expression, while the expression of CD86 and MHCII was decreased. OX40L serves as the ligand for OX40 and this interaction can inhibit Foxp3 expression and the induction of pTregs[45,46]. In addition, CD25 is crucial for IL-2 secretion, which can induce an immunogenic response. CD83-deficient cells indeed secreted increased amounts of IL-2. Given that the absence of CD83 supports an immunogenic response, the presence of CD83 is associated with tolerogenic DCs. This was additionally demonstrated by Kryczanowsky *et al*., who showed the emergence of two distinct peripheral blood mononuclear cells (PBMC)-derived DC populations following treatment with the suppressive cytokine IL-10, namely $CD83^{high}$ and $CD83^{low}$ DCs. pTregs induced by $CD83^{high}$ DCs exhibited significantly greater suppressive capacity[47].

A protein that also plays a role in tolerance is T cell immunoglobulin- and mucin-domain-containing 3 (**TIM-3)**. cDCs that lack TIM-3 fail to acquire a regulatory program[48]. Loss of TIM-3 activates the inflammasome and stimulates the STING pathway in these cDCs. The subsequent release of IL-1β and IL-18 promotes the differentiation of effector and memory CD8+ T-cells, which contributes to anti-tumor immunity. In addition, when the adaptor protein of TIM-3 that inhibits its function, **Bat3**, is absent, there is a hindered Tconv cell expansion[49]. The loss of Bat3 in cDCs promotes ER stress which results in the conversion of citrate from the tricarboxylic acid (TCA)-cycle in cytoplasmatic acetyl-CoA. This cytoplasmatic acetyl-CoA can be used for steroidogenesis. Consequently, Bat3-deficient DCs secrete more glucocorticoids, which inhibits the expansion of effector Tconv cells but promotes the differentiation of pTregs. In summary, the presence of TIM-3 and the absence of Bat3 are characteristic features associated with tolerogenic DCs.

Aryl hydrocarbon receptor (**AhR**) is a transcription factor that upon activation by its ligands decreases NF-κB activation and decreases the production of proinflammatory cytokines such as IL-6 and IL-12, while concomitantly promoting the secretion of TGFβ, IL-10 and retinoic acid (RA)[50–53]. AhR mediates these effects by activating indoleomine-2,3-dioxygenerase (IDO) and suppressor of cytokine signaling 2 (SOCS2). **IDO** is an enzyme that converts tryptophan into the metabolite kynurenine. The depletion of tryptophan from the environment can lead to the starvation of T-cells. In addition, the tryptophan metabolites generated by IDO can suppress immunogenic responses while promoting the differentiation of pTregs[54–56]. This happens because kynurenine can bind to AhR in T-cells, which in turn promotes the conversion of Tconv into pTregs[57]. IDO is not solely triggered by AhR activation but can also be induced by other proteins, such as the β-catenin[58]. **SOCS2** on the other hand, as the name suggests, inhibits cytokine signaling. SOCS2 mediates the previously mentioned effects of AhR activation, such as diminishing the production of proinflammatory cytokines. This is achieved through the inhibition of the JAK/STAT pathway[52].

TAM receptors

TAM receptors play a crucial role in the engulfment of apoptotic cells. Given their regulatory influence on DCs, their expression is important in the context of tolerogenic DCs. There are three TAM receptors, namely Tyro3, Axl and MerTK[59]. All three of these receptors belong to the family of receptor tyrosine kinases and can form homodimers or heterodimers upon binding their ligands growth-arrest specific 6 (GAS6) and protein S (PROS1)[60–62]. Gas6 has the highest affinity for Axl but can also bind to Tyro3 and MerTK, while PROS1 exclusively binds to Tyro3 and MerTK. Many cells that express one or more of the TAM receptors also express one or both of the ligands. TAM receptors are involved in the regulation of two processes within DCs: (1) the innate inflammatory response triggered by pathogens and (2) the phagocytosis of apoptotic cells. In the first process, TAM receptors function as inhibitors to prevent chronic inflammation[63]. This inhibitory effect is mediated by STAT1, which is activated by the TAM receptors, especially Axl, in the presence of IFN-α. This activation can induce the expression of SOCS1/3[64]. Both of these proteins act to suppress toll-like receptors (TLRs) and cytokine signaling, thereby inhibiting an immunogenic response. Furthermore, the TAM receptors are needed for so-called homeostatic phagocytosis, which occurs in neonatal and adult organisms. This process involves the engulfment of apoptotic cells, resulting in an immunosuppressive effect. Even though apoptotic cells do not express Gas6 or PROS1, the cells do present phosphatidylserine. DCs secrete Gas6 and/or PROS1, which then bind to phosphatidylserine on the surface of apoptotic cells. The bound ligands subsequently engage with the TAM receptors and this interaction is further stabilized by phosphatidylserine[65,66]. Following TAM

receptor activation, the protein Vav is activated which subsequently activates the Rho-family GTPases Rac1, RhoA, and Cdc42[64]. Rho-family GTPases play a role in cytoskeletal rearrangements which is crucial for phagocytic capacity.

TAM receptors are expressed by tolerogenic DCs. While MerTK has been established as a significant player in the engulfment of apoptotic cells by macrophages, its expression in DCs is not evident and can vary depending on tissue[61]. Studies have shown that splenic DCs require Axl and Tyro3 for the engulfment of apoptotic cells[61]. On the other hand, in neonatal lungs, DCs express both Axl and MerTK but the engulfment was found to be dependent on MerTK[67]. However, the study exclusively utilized MerTK-/- mice, with no Axl-/- mice tested. Additional studies do support the notion that MerTK is required for inducing tolerance in DCs. In the pancreas, MerTK was found to be necessary to prevent NF-κB activation and suppress the production of proinflammatory cytokines after the engulfment of apoptotic cells[68,69]. In addition, MerTK stimulation by PROS1 increased IL-10 production by tumor-resident moDCs and splenic DCs[69,70]. It is possible that PROS1 has the same effect on cDCs. Nevertheless, it remains a challenge to draw conclusions about which TAM receptors are preferentially expressed by tolerogenic cDCs. All TAM receptors likely play a role, but the specific receptor preferred may depend on the tolerogenic stimulus and the tissue involved.

In the section "DC lineages", it was mentioned that SIRPα, after binding to CD47, inhibits phagocytosis by cDC2s. Given the presence of phagocytic TAM receptors on DCs, one might question if SIRPα has an inhibitory impact on these receptors. Fc-receptors signal, similar to TAM receptors, through tyrosine kinases. Studies have demonstrated that SIRPα can block Fc-receptor-mediated endocytosis through downstream activated SHP proteins[71]. As a consequence, the protective efficacy of antibodies blocking the SIRPα-CD47 axis was greatly enhanced when cells were additionally opsonized with IgG antibodies that could interact with the Fc-receptors[72,73]. Given that Fc-receptors signal through tyrosine kinases, and SIRPα can inhibit these receptors, one could speculate whether the SHPs activated by SIRPα also inhibit the TAM receptors. As mentioned earlier, phagocytosis by TAM receptors is mediated via the downstream proteins Vav and Rho-family GTPases. It has been observed that SHP-1 can negatively regulate Rac1. However, this finding was in a context unrelated to DCs[74]. Extensive research is required to substantiate this, but if the SHPs activated by SIRPα do inhibit Rac1 in DCs, it could potentially impact TAM-mediated endocytosis.

Secreted regulatory molecules

In addition to cell-resident proteins, various cytokines contribute to a tolerogenic response. Many of these immunomodulatory molecules are not only secreted by certain tolerogenic DCs but can also be released by other immune cells to induce a state of tolerance in DCs (Figure 1). The most widely recognized regulatory cytokine is IL-10[75]. It was discovered that **IL-10** can activate Tregs, which can subsequently suppress a Tconv response[76]. Other interleukins that are secreted by tolerogenic DCs are IL-27 and IL-35. **IL-27** inhibits the differentiation and expansion of effector Tconv cells while promoting the differentiation of Tconvs into IL-10 producing pTregs[77–79]. Besides its effect on T-cells, IL-27 can also directly influence DC function. Treatment of moDCs with IL-27 results in reduced T-cell stimulatory capabilities, attributed to IL-27-induced expression of PD-L1[80]. IL-27 could have a similar effect on cDCs. In addition, IL-27 induces upregulation of CD39 on cDCs. CD39 works together with CD37 to convert ATP, known for having immunogenic properties, into adenosine, which exerts immunosuppressive effects[81–84]. Furthermore, it has been observed that **IL-35** secreted by DCs can impair a Tconv response and induce a tolerogenic phenotype in DCs, characterized by

downregulation of MHCII and costimulatory receptor CD40 and increased production of IL-10[85]. IL-35 is additionally produced by Tregs, which further suppresses the proliferation of Tconvs[86].

Besides interleukins, other cytokines also contribute to the generation of a tolerogenic response. **TGF-β**, for instance, can facilitate the induction of Foxp3 in CD4+ T-cells and thereby mediate the differentiation of pTregs[87]. In addition, apart from the previously mentioned BTLA-HVEM interaction, the binding of TGF-β to the TGF-β receptor on T-cells is also essential for converting naive Tconvs into pTregs[88].

Lastly, metabolites possessing tolerogenic properties have been identified. One of these metabolites is the vitamin A metabolite retinoic acid (**RA**), which is present at high concentrations in the small intestine. RA promotes the expression of Raldh2 (also known as Aldh1a2) by cDCs, promoting further RA production by DCs. The RA secreted by cDCs contributes to an enhanced induction of pTregs[89,90]. It has additionally been revealed that RA serves as a critical regulator in modulating the TGF-β response. TGF-β can convert Tconvs into pTregs but in the presence of IL-6, TGF-β can also trigger an proinflammatory response. It was discovered that RA can inhibit IL-6-driven induction of a Th17 response, and thereby control the effect of TGF-β[91,92]. The production of another metabolite, **lactate**, is also identified as a characteristic of tolerogenic DCs. Tconvs become glycolytic upon activation but this glycolytic switch is inhibited in a lactate-rich environment, leading to suppression of Tconv activation and proliferation. Furthermore, lactate increases the differentiation of Tconvs into pTregs and expression of IL-10, RA and IDO by DCs[93].

Transcriptome analysis

As stated earlier, it has been observed that both tolerogenic and immunogenic DCs experience transcriptomic alterations during maturation. Cell renewal, including the process of apoptosis, is a constant process in multicellular organisms. DCs can engulf these apoptotic cells without eliciting an immune response. This makes DCs that perform this task suitable for studying the transcriptomic changes after tolerogenic maturation. We have summarized the genes identified to be upregulated during tolerogenic maturation by comparing findings from four studies that examined the transcriptome of tolerogenic DCs.

The first study done by Cummings *et al*., DCs that engulfed apoptotic intestinal epithelial cells (IECs) were characterized[94]. A specific CD103+ cDC subset displayed significant transcriptomic alterations after engulfing apoptotic IECs. Second, a study by Bosteels *et al*. identified the transcriptomic changes of homeostatic late mature CCR7+ cDCs after phagocytosis of apoptotic cells in the spleen[42]. Third, neonates experience a high degree of cell renewal. cDCs in neonatal mouse lungs were examined by Silva-Sanchez *et al*. between birth and 2 weeks of age. It was observed that a specific $CD103^{int}$ cDC subset underwent a process of homeostatic (tolerogenic) maturation, underscored by their lack of efficient stimulation of IFN-γ production by CD8+ T cells[67]. These CCR7+ $CD103^{int}$ cDCs appeared in response to an apoptotic wave in the developing lungs. Therefore, this $CD103^{int}$ subset probably arose after the engulfment of pulmonary apoptotic cells. Lastly, Ardouin *et al*. characterized the transcriptome of homeostatically matured thymic and peripheral XCR1+CCR7+ cDCs. Thymic DCs were identified as tolerogenic due to their capability to present thymic epithelial cell (TEC) peptides[95]. All four of these studies describe or focus on cDC1s. As previously mentioned, cDC2s express SIRPα, limiting their ability to engulf apoptotic cells. The difference in phagocytic ability is therefore probably the reason that the cDCs described in these studies are cDC1s.

The transcriptome profiles of the four tolerogenic DC subsets from the aforementioned studies were compared. Each transcriptome profile comprised both up- and downregulated genes. The downregulated genes were primarily associated with immunogenic functions such as pathogen sensing, antigen presentation and innate immune response. There were only minimal to no similarities in the downregulated genes across the analyzed DCs. However, there were multiple genes that showed upregulation in the tolerogenic DCs from two or more studies. These upregulated genes are listed in Table 1.

A total of 18 genes were upregulated in two or more studies. Among these, four genes (*Cd274, Cd83, Ido, Socs2*) encode for proteins and molecules discussed earlier. In addition, the gene *Aldh1a2* encodes for the aldehyde dehydrogenase ALDH1A2 (also known as RALDH2), which is an enzyme that catalyzes the synthesis of RA[96]. The function and effect of RA were covered in the section "Immunomodulatory molecules". The remaining 11 genes encode for proteins and molecules that have not been previously discussed.

The expression of *Tnf* encoding TNF was increased in tolerogenic DCs. This cytokine is important in the induction of pTregs[97]. TNF exists as a soluble protein and as membrane-bound (mTNF). It was discovered that mTNF expressed by tolerogenic moDCs plays a crucial role in inducing pTregs[97,98]. In addition to TNF itself, genes encoding TNF receptor superfamily proteins (*Tnfrsf4*, *Tnfrsf11a, Cd40*) are also upregulated. These receptors are known to play a role in inducing an immunogenic response. Nevertheless, some tolerogenic properties of these receptors have been described. TNFRSF11a, also known as RANK, shares close homology with CD40. When this receptor binds to its ligand RANKL, it can promote survival of cDCs and activate CD4+ Tconvs[99]. However, in the skin and intestine, RANK present on DCs can control the number of peripheral Tregs[100]. In addition, blocking the RANK-RANKL interaction has demonstrated therapeutic benefits in treating cancer[100,101]. Another upregulated TNF receptor superfamily member, TNFRSF4, also known as CD134 (or OX40), is typically expressed on T-cells, while its ligand OX40L is typically expressed by DCs. The OX40-OX40L interaction is important for the expansion and survival of Tconvs. As of now, no tolerogenic properties have been attributed to OX40 expressed by DCs. Lastly, CD40 is upregulated. While CD40 can trigger immune activation upon binding to CD40L on T-cells, it has been discovered that CD40 signaling in bone marrow derived DCs (BMDCs) enhances the expression of IL-10[102].

Genes encoding various chemokines (*Ccl5, Ccl17, Ccl22)* were also upregulated. All of these chemokines can facilitate the accumulation of Tregs in tumors, consequently promoting tumor growth[103–105]. As expected, the chemokine receptor CCR7 (*Ccr7*) is upregulated as well. This protein is essential for the migration of DCs to lymph nodes, where CCR7 ligands CCL19 and CCL21 are produced[106].

In addition to cytokines and chemokines, there is an elevated expression of the gene encoding IL-12b (*Il12b*) in tolerogenic DCs. IL-12b (also known as IL12p40) can combine to form either IL-23 (p10/p40) or IL-12 (p35/p40). Both IL-12 and IL-23 are proinflammatory cytokines that induce a Th1 response and Th17 response, respectively[107,108]. However, it has been demonstrated that IL-12b, as a monomer or dimer, can inhibit Tconv responses by serving as an IL-12 antagonist[109]. Furthermore, the gene for the IL-15 receptor (*Il15ra*) is upregulated. This alpha chain of the receptor can cross-present IL-15 to T-cells to promote their survival[110]. Currently, no tolerogenic properties have been described for IL15Ra.

CD63 (*cd63*, also known as LAMP-3 or DC-LAMP) is also upregulated upon DC maturation. This tetraspanin is enriched on intraluminal vesicles within late endosomes/lysosomes in DCs. CD63 has been identified as a marker of mregDCs. The infiltration of CD63+ mregDCs has shown a positive correlation with a favorable prognosis in various types of cancer[111]. Furthermore, the genes *Scube3* and *Itgb8* show increased expression. The protein SCUBE3 is a secreted glycoprotein that binds to the TGF-β receptor[112]. This binding activates the TGF-β signaling pathway, which in turn can enhance an immunosuppressive environment The effect of TGF-β is discussed in the section "secreted regulatory molecules". *Itgb8* encodes for integrin β8, a pivotal player in the activation of TGF-β through the αvβ8 integrin, a process essential for pTreg generation[113]. Lastly, RelB is a member of the NF-κB family and its expression is usually correlated with proinflammatory T-cell responses. RelB inactivation in cDCs or RelB-deficiency in mice leads to the accumulation of Tregs in peripheral tissues[114,115]. Even though these results support a role for RelB in immunogenic responses, the gene coding for RelB (*Relb)* is upregulated in all tolerogenic DCs described in the four studies, so it probably has a function in tolerogenic responses.

NF-κB signaling encompasses the RelA-dependent canonical and the RelB-dependent non-canonical pathway[116]. Various genes involved in the non-canonical NF-κB pathway are upregulated in tolerogenic DCs, suggesting a potential role for this pathway in inducing or maintaining tolerance. To begin, there is increased expression of the non-canonical NF-κB pathway protein RelB in all four tolerogenic DCs described in the transcriptome analysis. Secondly, genes encoding for members of the TNFR superfamily (*Tnfrsf4*, *Tnfrsf11a* and *Cd40)* are upregulated. While the canonical NF-κB pathway can be induced by a wide variety of immune receptors, the non-canonical NF-κB pathway is specifically activated by TNFR superfamily members. It was found that CD40, RANK and OX40 can all activate the non-canonical pathway[117–119]. In addition, it was demonstrated that CD40L stimulation in moDCs, in combination with targeted inhibition of the canonical NF-κB pathway, resulted in the generation of a regulatory DC phenotype[120]. Lastly, various proteins that can intervene with the NF-κB pathways are upregulated in tolerogenic DCs. The SOCS2 protein can inhibit the canonical NF-κB pathway. It was found that delivering nanoparticles containing an AhR ligand to splenic DCs leads to the induction of SOCS2, which subsequently inhibits NF-κB signaling and the production of proinflammatory cytokines[52]. Specifically, SOCS2 targets RelA, a critical component of the canonical pathway. Furthermore, the upregulation of IDO can be linked to the non-canonical NF-κB pathway, as IDO can produce kynurenine, which has the potential to activate AhR. AhR can activate SOCS2 which results in inhibition of the canonical NF-κB pathway. Additionally, AhR and RelB can form a transcription factor heterodimer that can increase the expression of IL-8[121]. In tumors, IL-8 promotes angiogenesis and disrupts moDC migration[122]. Altogether, there is a possibility that the non-canonical NF-κB pathway is important in tolerogenic cDCs.

However, what contradicts this hypothesis is that silencing of RelB in BMDCs results in the generation of tolerogenic DCs[123,124]. This is interesting because, in our transcriptome comparison, RelB was found to be upregulated in all tolerogenic DCs analyzed (Table 1). A possible explanation is that RelB is required for the maturation of DCs. It is plausible that the observed generation of tolerogenic DCs upon silencing RelB may be attributed to their immaturity rather than a specific tolerogenic phenotype. However, more research is needed to elucidate the role and function of RelB and the non-canonical NF-κB pathway in tolerogenic DCs.

Table 1: transcriptome comparison of upregulated genes after tolerogenic maturation

Gene	Function	Study context	Type of DC	Study
Ccl22	Encodes Ccl22, facilitates Treg accumulation in the tumor via CCR4[105].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
Ccl17	Encodes Ccl17, facilitates Treg accumulation in the tumor via CCR4[104].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
Ccl5	Encodes CCL5, which is a chemoattractant for immature DCs. It was also found to facilitate the accumulation of Tregs tumors[103].	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Engulfment apoptotic cells neonatal lungs	$CD103^{int}$ DCs	Silva-Sanchez *et al.*, 2023
Cd274	Encodes CD274 (PD-L1), which is a negative regulator of T-cell activation, but can be in a costimulatory dimer with CD80[34–36].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Engulfment apoptotic cells neonatal lungs	$CD103^{int}$ DCs	Silva-Sanchez *et al.*, 2023
Cd63	Encodes CD63. The infiltration of CD63+ DCs has shown a positive correlation with a favorable prognosis in various types of cancer[111].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
Cd40	Encodes CD40 which causes immune activation after binding to CD40L. However, CD40 signaling in IL-10 producing DCs can also enhances the expression of IL-10[102].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
		Engulfment apoptotic cells neonatal lungs	$CD103^{int}$ cells	Silva-Sanchez *et al.*, 2023
Cd83	Encodes CD83. CD83 deficiency causes Tconv expansion and Treg inhibition[41].	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Engulfment apoptotic cells neonatal lungs	$CD103^{int}$ DCs	Silva-Sanchez *et al.*, 2023
Ccr7	Encodes the chemokine receptor CCR7, which is necessary to redirect DCs to LNs[106].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
		Engulfment apoptotic cells neonatal lungs	$CD103^{int}$ DCs	Silva-Sanchez *et al.*, 2023
Aldh1a2	Encodes ALDH1A2, an enzyme that catalyzes the synthesis of retinoic acid (RA). [96]	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023

Relb	Encodes the transcription factor RelB, which is a protein of the non-cannonical NF-kB pathway	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
		Engulfment apoptotic cells neonatal lungs	CD103^{int} DCs	Silva-Sanchez *et al.*, 2023
Itgb8	Encodes integrin β8 (ITGB8), which is essential for activating TFG-β (via integrin αyβ8) and thereby pTreg generation[113].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
Socs2	Encodes SOCS2, which inhibits NF-κB and proinflammatory cytokine production[52].	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
Ido	Encodes IDO, an enzyme that converts tryptophan into the metabolite kynurenine. IDO can suppress immunogenic responses while promoting the differentiation of pTregs[54–56].	Sampling apoptotic IEC	CD103+ DCs	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
Scube3	Encodes SCUBE3, a TGF-β receptor ligand[112]. The activation of the TGF-β receptor can enhance an immunosuppressive environment.	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
Tnfrsf4	Encodes CD134 (TNFRSF4, OX40 receptor). OX40 is mostly expressed on T-cells and usually the ligand is expressed on DCs. On T-cells, OX40 promotes Tconv expansion and survival and suppresses Tregs after binding to OX40-L of APCs. As of now, no tolerogenic properties have been attributed to OX40 expressed by DCs.	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Homeostatic maturation	Late mature CCR7+ DCs	Bosteels *et al.,* 2023
		Engulfment apoptotic cells neonatal lungs	CD103^{int} DCs	Silva-Sanchez *et al.*, 2023
Tnfrsf11a	Encodes TNFRSF11A (also known as RANK), which is a NF-kB activator. RANK present on DCs can control the number peripheral Tregs and blocking the RANK-RANKL interaction has demonstrated therapeutic benefits in treating cancer[100,101].	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Engulfment apoptotic cells neonatal lungs	CD103^{int} DCs	Silva-Sanchez *et al.*, 2023
Il15ra	Encodes IL-15RA, the receptor for IL-15. No tolerogenic properties have been described for IL-15RA.	Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Engulfment apoptotic cells neonatal lungs	CD103^{int} DCs	Silva-Sanchez *et al.*, 2023
Il12b	Encodes beta subunit of IL-12, (also known as IL-12p40). This protein can inhibit Tconv responses by serving as a IL-12 antagonist[109].	Sampling apoptotic IEC	CD103+ DC	Cummings *et al*., 2016
		Homeostatic maturation	XCR1+ CCR7+ DCs	Ardouin *et al.,* 2016
		Engulfment apoptotic cells neonatal lungs	CD103^{int} DCs	Silva-Sanchez *et al.*, 2023
Tnf	Encodes TNF-α. This cytokine can induce pTregs[97].	Sampling apoptotic IEC	CD103+ DC	Cummings *et al*., 2016
		Engulfment apoptotic cells neonatal lungs	CD103^{int} DCs	Silva-Sanchez *et al.*, 2023

Metabolic state

Besides the upregulation of proteins and secreted molecules, the metabolic state of DCs is also very important in inducing immunogenic or tolerogenic characteristics. It is important to note that the metabolic state of tolerogenic and immunogenic DCs is predominantly described in BMDCs and moDCs, with less focus on cDCs[125]. Nevertheless, discussing the metabolic programming of DCs is important to discuss in the context of tolerogenic DCs. Upon activation by PRRs, DCs undergo metabolic changes. They switch from a catabolic metabolism, characterized by fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS), to an anabolic metabolism, characterized by increased glycolysis and fatty acid synthesis (FAS)[126]. The switch to glycolysis leads to increased lactate expression, providing energy for the cell. In addition, pyruvate is produced which can be used in the TCA cycle for citrate production that can subsequently be used for FAS[127]. Immunogenic activation of DCs results in significant production of proteins, including transcription factors and signaling molecules, which leads to ER stress. Expansion of the ER and Golgi by additional fatty acid synthesis relieves this stress.

The pathway through which FAS is increased involves TANK-binding kinase 1 (TBK1) and inhibitor of NF-κB kinase subunit-ε (IKKε). These proteins activate AKT, which subsequently activates hexokinase 2 (HK2). HK2 enhances pyruvate production, leading to an elevated supply of citrate available for FAS. Additionally, HK2 activates the pentose phosphate pathway, resulting in an increased availability of NADPH, which is crucial for FAS. Lastly, ER stress induces the unfolded protein response (UPR) which activates the XBP1. This transcription factor facilitates the expression of enzymes necessary for FAS. In summary, PRR-activated DCs have increased citrate, NADPH and XBP1 production which enhances their glycolytic activity and fatty acid synthesis[128].

The mammalian target of rapamycin (mTOR) is also essential in orchestrating the metabolic switch towards glycolysis. Activation of mTOR is initiated by the PI3K pathway. Activated mTOR triggers the expression of MYC, a transcription factor responsible for inducing the expression of genes necessary for the glycolysis pathway[129]. In addition, mTOR can stimulate hypoxia-inducible factor 1α (HIF1α), which in turn induces the expression of genes required for glycolysis. Lastly, the PI3K-mTOR pathway can activate iNOS. This leads to the suppression of OXPHOS due to the production of nitric oxide (NO)[127,130]. Interestingly, early activation of FAS is not dependent on the mTOR-HIF1α pathway but on the previously described TBK1/IKKε-AKT-HK2 pathway [127,131]. The mTOR-HIF1α-iNOS was found to be important for the prolonged switch to glycolysis and inhibition of OXPHOS by DCs post-activation[126,132].

It was discovered that the balance between anabolic and catabolic pathways is important in the switch between an immunogenic or tolerogenic DC response. As described above, the anabolic pathway of FAS after PRR activation is a characteristic of immunogenic DCs. A protein that can antagonize this anabolic pathway, and promote catabolic pathways, is AMP kinase (AMPK). AMPK can activate PPARγ co-activator 1α (PGC1α), which in turn supports mitochondrial biogenesis, leading to an increase in OXPHOS[133]. It was found that when the expression of this catabolic protein PGC1α was increased, while the anabolic protein HIF1α was suppressed, PMBC-derived DCs exhibited a tolerogenic phenotype and produced IL-10[134]. Furthermore, AMPK can enhance FAO[135]. An increased level of FAO can result in the induction of IDO, an enzyme that suppresses proinflammatory cytokine production and promotes the differentiation of pTregs[58]. Taken together, these findings underscore that immunogenic DCs predominantly rely on glycolysis for energy while engaging in FAS, whereas tolerogenic DCs primarily utilize FAO and OXPHOS for their metabolic needs.

DCs can alter their metabolism in response to metabolites. Certain metabolites have the capacity to induce a tolerogenic phenotype in DCs. For instance, butyrate, which is produced by bacteria, can stimulate the expression of IL-10 and AhR in splenic DCs through HCAR2 (GPR109A)[136]. In addition, exogenous metabolites such as lactate and adenosine are capable of inducing tolerogenic DCs that produce IL-10[137–139]. It is important to notice that not every metabolite induces the same degree of metabolic programming. This change in metabolism can vary depending on the tolerogenic stimulus or the tissue in which the DC is located[140].

Another metabolic regulator that is correlated with tolerogenic DCs is sterol response element binding protein 2 (SREBP2). SREBPs are transcription factors that play a key role in regulating lipid biosynthesis. SREBP2 specifically controls cholesterol biosynthesis[141]. It was discovered that the engulfment of apoptotic cells by cDC1s activated the liver X receptor (LXR) pathway, leading to the activation of cholesterol efflux and anti-inflammatory genes. The increased cholesterol efflux, in turn, activates SREBP2. Subsequently, SREBP2 triggers the mevalonate (MVA) pathway, ultimately resulting in cholesterol biosynthesis[42]. The excess cholesterol produced gives rise to so-called lipid bodies, promoting FAO. In addition, the MVA pathway hinders antigen cross-presentation by cDCs. Lastly, besides activating cholesterol synthesis, SREBP2 increases the expression of the RA-synthesizing enzyme RALDH2[142]. Multiple studies investigating tolerogenic DCs consistently observed the upregulation of cholesterol biosynthesis and SREBP2. Tolerogenic matured cDCs capable of displaying TEC peptide[95] and mregDCs were both found to be enriched in SREBP2[31]. Altogether, the expression of SREBP2 by DCs induces tolerance by increased FAO, reduced antigen presentation and increased RA production. These findings suggest a potential role for SREBPs in the induction and maintenance of tolerogenic characteristics in DCs.

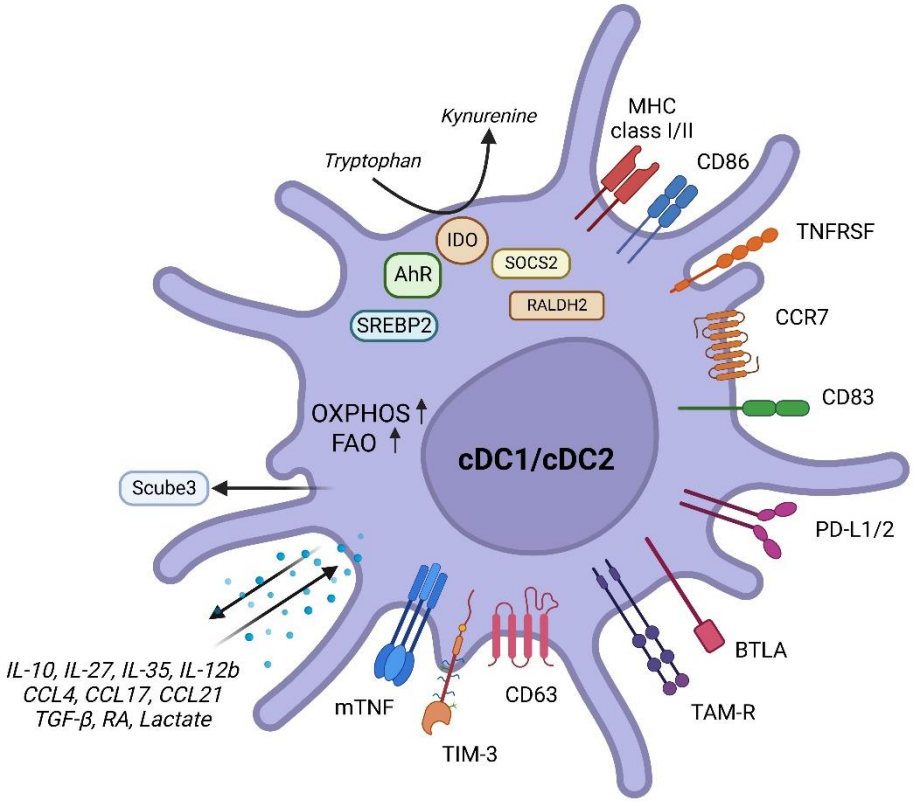

Figure 1. Illustration depicting the possible characteristics of tolerogenic dendritic cells in terms of proteins and secreted molecules. OXPHOS: oxidative phosphorylation, FAO: fatty acid oxidation, mTNF: membrane-bound TNF, TAM-R: TAM-receptors, RA: retinoic acid.

Activation of T-cells by tolerogenic DCs

Up to this point, we have discussed several characteristics that can be exhibited by tolerogenic DCs. Another important aspect is how these tolerogenic DCs are induced. This process of induction can take place in various tissues. In this section, we will delve deeper into and propose a model of the induction of tolerogenic DCs and the subsequent priming of Tregs in the intestine and tumors, which are two tissues recognized for their association with tolerogenic priming.

Inducing tolerogenic DCs

In the intestine, there is an abundant supply of the tolerance-inducing metabolites RA and lactate. Following exposure to RA, it was found that moDCs activate AMPK signaling, leading to enhanced RALDH activity and CD103 expression[140]. Consequently, the majority of tolerogenic DCs in the intestine exhibit the CD103+ phenotype and secrete RA due to increased RALDH activity. Important to note is that RA diminished moDC differentiation, which makes this finding also interesting in the context of cDCs. Besides RA, lactate also plays a role in mediating tolerance. In the intestine, lactate uptake by DCs has several effects: (1) it suppresses the HIF-1α pathway and thereby glycolysis and (2) it binds to GPR81, activating β-arrestin, which in turn activates AMPK and induces the expression of RA, IL-10, and IDO[93]. These combined effects contribute to the development of tolerogenic DCs. Besides intestinal metabolites, DCs in the intestine engulf apoptotic cells as a result of continuous turnover of epithelial cells. Especially cDC1s play a key role in the uptake of apoptotic intestinal cells, as SIRPα expressed on cDC2 limits their phagocytic capacity. The engulfment by cDC1s results in the upregulation of genes encoding various proteins and secreted molecules, as observed in the transcriptome analysis[94]. One general DC maturation gene that is upregulated by the engulfment is *Ccr7*. The upregulation of CCR7 causes the migration of the tolerogenic DCs to dLNs, where they can prime Tregs. In CCR7-deficient mice, tolerogenic DCs in the intestine fail to migrate to the dLNs, failing to induce tolerance[143]. In summary, both the uptake of metabolites and the engulfment of apoptotic cells contribute to the development of tolerogenic characteristics in DCs.

Another site where the priming of tolerogenic DCs occurs is within tumors. Tumors release diverse molecules and metabolites that can impact the metabolic state of intratumoral DCs and the activation of T-cells. There are various mechanisms through which a tumor can achieve this: (1) Nutrient starvation is a hallmark of tumors. In a nutrient-deprived environment, AMPK can be activated, which results in a tolerogenic DC phenotype[144]. (2) DCs have the capability to uptake lipids from the TME, leading to the formation of lipid droplets within the cell. Although lipid droplets are associated with immunogenicity[145], it was found that lipid droplets found in CD103+ DCs located in the TME contain oxidized polyunsaturated fatty acids. These specific fatty acids can capture MHC complexes in the endosomes and lysosomes, which limits cross-presentation, contributing to tolerogenic DC properties[146]. (3) Tumor cells secrete high levels of lactate due to the Warburg effect[147]. As discussed earlier, lactate is correlated with an immunosuppressive environment. (4) The nucleoside adenosine is present in the TME[148,149]. In response to adenosine, DCs present in the tumor lower the production of proinflammatory cytokines while increasing the production IL-10[150,151]. (5) Tumor cells can increase the expression of SOCS3 by BMDCs[152]. As discussed for the TAM receptors, that induce SOCS3 as well, this protein can inhibit the JAK/STAT pathway. In addition, SOCS3 can suppress pyruvate kinase type M2 (M2-PK), which is a crucial enzyme in glycolysis[153,154]. As a consequence, tumor cells can inhibit glycolysis and FAS in DCs, resulting in a tolerogenic DC phenotype. (6) Within tumors, hypoxia occurs and triggers the induction of HIF1α in moDCs.

This hypoxia-induced HIF1α leads to AMPK activation and the stabilization of PD-L1, ultimately resulting in the development of tolerogenic DCs[155]. (7) Finally, Wnt5a can stimulate β-catenin expression in DCs. This leads to the expression of PPARγ, subsequently enhancing FAO and suppressing IL-6 and IL-12. Additionally, β-catenin induces IDO expression, contributing to the differentiation of Tconvs into pTregs[58].

All the aforementioned effects shut down glycolysis and increase FAO and OXPHOS in DCs. A glycolytic metabolism was found to be essential for CCR7-mediated trafficking of BMDCs and splenic DCs[156]. Consequently, a tolerogenic stimuli that will shut down the glycolytic metabolism will inhibit DC migration. As a result, the DCs that have a changed metabolism are likely to remain in the TME. Tumor cells can also release factors, such as oxysterols or IL-6, which inhibit the expression of CCR7 and thereby hinder DC migration[157,158]. In addition, there are mregDCs identified that do express CCR7 but nevertheless reside within the tumor[159]. It remains unclear whether these mregDCs are retained in the tumor due to altered migration behavior or due to interaction with tumor proteins or secreted molecules. Some tumor cells do have the capacity to secrete CCR7 ligand CCL19 or CCL21, which might potentially contribute to retaining DCs within the TME[160,161]. Despite their reduced migratory capacity, these tumor-resident DCs retain tolerogenic properties, marked by the upregulation of immunosuppressive proteins and secreted molecules. This, in turn, influences the activity of Tconvs and Tregs, contributing to the maintenance of an immunosuppressive TME.

Besides influencing the metabolism and migration of DCs, tumors can exert other effects on DCs within the TME. An example of such an effect is the expression of prostaglandin PGE2 by tumors. It was observed that the receptors of PGE2, EP2 and EP4, were highly expressed by cDCs in the TME[162]. PGE2-EP2/EP4 signaling in cDCs increased the production of CCL17 and CCL22, which are cytokines that recruit CCR4+ Tregs to the tumors. In addition, PGE2-activated Tregs are present in the TME. It is not known if these recruited and activated Tregs are tTregs or pTregs. Interestingly, while PGE2 recruits and activates Tregs, *de novo* Treg conversion from CD4+ T-cells *in vitro* was inhibited by PGE2. Furthermore, it was also discovered that PGE2 binding to EP2/EP4 inhibited CXCL9 and IL-12 production by cDC1s[163]. The cDC1s efficiently sampled and processed tumor antigens, underwent activation and transported tumor antigens to the dLNs. However, the presence of CXCL9 and IL-12 in the TME was found to be crucial for maintaining effector activity of dLN-primed CD8+ T-cells. Consequently, because PGE2 hinders the production of CXCL9 and IL-12 by DCs, the activation of CD8+ T cells is limited. These findings indicate that DCs within the TME can undergo priming and migration to dLNs, while activated Tconvs are suppressed in and Tregs are recruited to the TME.

In summary, there are various tolerogenic stimuli in the intestine and tumors that cause the induction of tolerogenic DCs. Some of the primed DCs remain in the tissue, directly contributing to the maintenance of a tolerogenic environment, while others migrate to dLNs for the priming of Tregs, indirectly promoting a tolerogenic environment.

Priming of Tregs

Upon migration to dLNs, DCs can prime Tconvs and Tregs. The Tconv population consists of CD4+ helper and CD8+ cytotoxic T cells. Both cell types are important in shaping the immune response. CD4+ helper T cells can fulfill various functions. They can activate cellular responses and macrophages (Th1-response), enhance neutrophil responses (Th17-response) and activate antibody responses (Th2-response)[164]. In contrast, Tregs control and suppress immunogenic responses. Tregs exert an inhibitory effect on Tconvs through various mechanisms: (1) Tregs express CD25, depleting the environment of IL-2 essential for the expansion and differentiation of Tconvs. (2) Tregs can secrete immunomodulatory cytokines like TGF-β, IL-10 and IL-35. (3) Tregs continuously express CTLA4, leading to the downregulation of the CD80/86 needed for T-cell priming. (4) Lastly, Tregs express CD39 and CD73. As mentioned before, CD39 converts ATP to AMP and CD73 subsequently converts AMP to the immunosuppressive nucleoside adenosine[83,84].

As mentioned in the introduction, Tregs can be generated in the thymus (tTregs) or at peripheral sites (pTregs). tTregs are generated in the thymus through stimulation with self-antigen presented by TECs[165]. Because tTregs are largely specific for self-antigens, their activation is primarily induced during immune response against the body's own tissues, as observed in situations such as tissue damage[166]. Following their activation, the tTregs migrate to lymphoid and peripheral tissues, where they maintain peripheral tolerance[167]. tTregs are the most abundant Treg population in secondary lymphoid organs. Conversely, pTregs differentiate from antigen-activated CD4+ T-cells in the periphery[168,169]. These type of Tregs predominantly recognize non-self antigens such as allergens, bacteria or pathogens[169]. Both tTregs and pTregs are characterized by expression of the transcription factor Foxp3. tTregs can be distinguished from pTregs by their additional expression of the transcription factor Helios[170]. However, there is some debate on the exclusivity of Helios as a marker for tTregs, as some studies suggest its expression by other cell types[171]. Besides Helios, tTregs have a Treg-specific demethylated region (TSDR) in the *Foxp3* gene locus. In pTregs, this region is highly methylated[172]. Demethylation of the TSDR is associated with stable FoxP3 expression, making tTregs a more stable Treg population than pTregs[173].

A lot of research has traditionally concentrated on the induction of pTregs. However, mounting evidence suggests that it is, in fact, the tTregs present in tolerogenic tissues that play a pivotal role in mediating tolerance. It was discovered that the majority of Tregs present in the intestine are tTregs[174]. Similarly, in the context of tumors, evidence points to the prevalence of tTregs. Tregs found in tumors express Helios, which is a tTreg marker [175,176]. In addition, there is a limited overlap in TCRs between CD4+ T cells and Tregs within tumors, suggesting the presence of tTregs in tumors rather than a conversion of Tconvs into pTregs[177–179]. Due to the predominant focus on pTregs in research, there is still limited understanding of how tTregs, primarily located in LNs, are primed. The mechanisms and stimuli driving the migration of tTregs to tissues also remains a question.

The priming of Tregs, like Tconvs, requires TCR stimulation. In addition to the MHC-TCR interaction, T-cells also require co-stimulation. A recent study demonstrated that the costimulatory molecule TNFR2 plays a role in expansion of tTregs[180]. Tregs highly express TNFR2 compared to Tconvs[181]. TNF-α serves as a ligand for TNFR2. As mentioned earlier, mTNF is upregulated by DCs upon tolerogenic maturation. mTNF has been identified as a crucial factor in inducing Tregs by tolerogenic moDCs[97,98,181]. It is conceivable that mTNF also plays a crucial role in Treg induction by cDCs. If that is the case, the interaction between TNFR2 and mTNF may be critical for the priming of tTregs by cDCs. On the other hand, Tconv priming was found to rely more on CD28 costimulation[180].

In addition to TCR and co-stimulatory stimulation, molecules secreted by tolerogenic DCs also play a role in the priming of tTregs. As previously mentioned, TGF-β is essential for inducing FOXP3 expression in Tregs. While TGF-β is extensively studied in the context of pTreg conversion from Tconvs, it has been established that TGF-β is also essential for tTreg development[182]. It could be that TGF-β not only induces *de novo* expression of FOXP3 but also increases the expression of pre-existing FOXP3 in tTregs. This increase in FOXP3 expression could have diverse effects, potentially influencing the migration or protein expression of tTregs. However, there is limited to no research on this aspect. Besides TGF-β, other molecules also influence the priming of tTregs. The CD103+ tolerogenic cDCs induced in the intestine secrete RA, a critical regulator in modulating the TGF-β response to favor Treg priming and inhibit Tconv priming[91,92]. In addition, the enzyme IDO is a characteristic of some tolerogenic DCs. While the expression of IDO is typically associated with the induction of pTregs[58], it has also been shown to induce the expansion of tTregs[183]. The study that demonstrates this IDO effect primarily focuses on pDCs, but it is conceivable that a similar mechanism may be occurring for cDCs.

Following activation in dLNs, tTregs can migrate to nonlymphoid tissues[37,184]. The migration of immune cells is typically regulated by chemokines. During their development, tTregs experience a switch in trafficking receptors. In the thymus, they initially express low levels of CCR7 but upon maturation, they express high levels of CCR7. This elevated CCR7 expression facilitates the trafficking of tTregs to secondary lymphoid organs, where CCR7 ligands CCL19 and CCL21 are expressed. Upon arrival in these lymphoid organs, tTregs regain expression of CXCR4, resulting in the presence of $CXCR4^{high}CCR7^{high}$ tTregs[184]. After antigen-specific priming by DCs in the LNs, the tTregs can undergo a switch in the expression of trafficking receptors, leading to the upregulation of specific receptors such as CCR4, CCR8 or CCR9. This switch enables the migration of tTregs to non-lymphoid tissues[184].

As mentioned before, the increase in FOXP3 expression during tTreg priming can have various effects. It was found that an increased expression of FOXP3 can enhance the expression of chemokine receptor CCR4[185]. Tumor cells and tolerogenic DCs present in the TME may secrete CCR4 ligands CCL17 and CCL21, thereby recruiting tTregs into the tumor[186]. In the intestine, cDCs also increase the production of CCL17 and CCL21 after engulfment of apoptotic IECs which can attracts tTregs[94]. Additionally, RA secreted by tolerogenic DCs upregulates CCR9. The ligand for CCR9, CCL25, is consistently expressed in the intestine[187]. This results in the migration of tTregs to the intestine. Upon reaching the intestine or tumor, tTregs contribute to the maintenance of a tolerogenic environment. They achieve this by secreting immunomodulatory molecules that inhibit Tconvs, by inhibiting immunogenic DCs or by assisting the induction of tolerogenic DCs.

tTregs in action: a proposed model

Combing all the information on the induction of tolerogenic cDCs and priming of tTregs, we propose a model illustrated in Figure 2. In the intestine (Fig. 2, right), the uptake of apoptotic epithelial cells and the presence of RA and lactate collectively induce CD103+ CCR7+ tolerogenic cDCs. These cDCs migrate to dLNs. Predominantly, these migrating cDCs are cDC1s, as cDC2s are unable to phagocytose apoptotic cells due to the presence of SIRPα. Some tolerogenic cDCs, including cDC2s, remain in the intestine and help to maintain a tolerogenic environment in a way similar to non-migrating cDC1s. In the LNs, thymus-derived tTregs are present. These tTregs are activated by tolerogenic cDCs through the TCR/MHC-II/self-peptide interaction and TNFR2/mTNF costimulation. Additionally, the tolerogenic cDCs secrete RA and TGF-β. The combination of these metabolites inhibits Tconvs and increases the FOXP3 expression in the tTregs. Furthermore, RA induces the expression of CCR9 by tTregs. The intestine produces the CCR9 ligand CCL25, promoting the migration of tTregs to the intestine. Within the intestine, tTregs play a crucial role in maintaining immune homeostasis and preventing chronic inflammation.

In the tumor (Fig. 2, left), various tumor-derived metabolites and secreted molecules can induce tolerogenic cDCs. Some of these molecules prompt a shift in the metabolic state of the cDCs, transitioning from FAS and glycolysis to FAO and OXPHOS. This metabolic change results in the induction of tolerogenic properties in these cDCs. Glycolysis is essential for migration to the dLNs, so these metabolically altered cDCs do not migrate but instead remain in the TME, contributing to a tolerogenic environment. Other molecules do not alter the metabolic state of cDCs but do make them tolerogenic. These tolerogenic cDCs can migrate to the LNs in a CCR7-dependent manner and induce the activation and expansion of tTregs. The activation of tTregs is again dependent on the TCR/MHC-II/self-peptide interaction and the costimulatory interaction of TNFR2 and mTNF. In addition, TGF-β can enhance the expression of FOXP3, which in turn can increase the expression of CCR4. Tumor cells and TME-resident tolerogenic cDCs can produce CCL17 and CCL22, which causes migration of the CCR4+ tTregs to the tumor. Within the tumor, tTregs can inhibit Tconvs or suppress immunogenic cDCs[188]. Furthermore, it has been demonstrated that certain tumor-specific Tconvs, such as CD8+ T-cells, are primed via the CD28-CD80/86 co-stimulatory axis. However, these activated Tconvs are inhibited in the TME by tTregs and tolerogenic DCs[163]. The presence of tolerogenic DCs, the priming of tTregs, and the inhibition of primed Tconvs collectively contribute to the establishment of a tolerogenic environment in certain tumors.

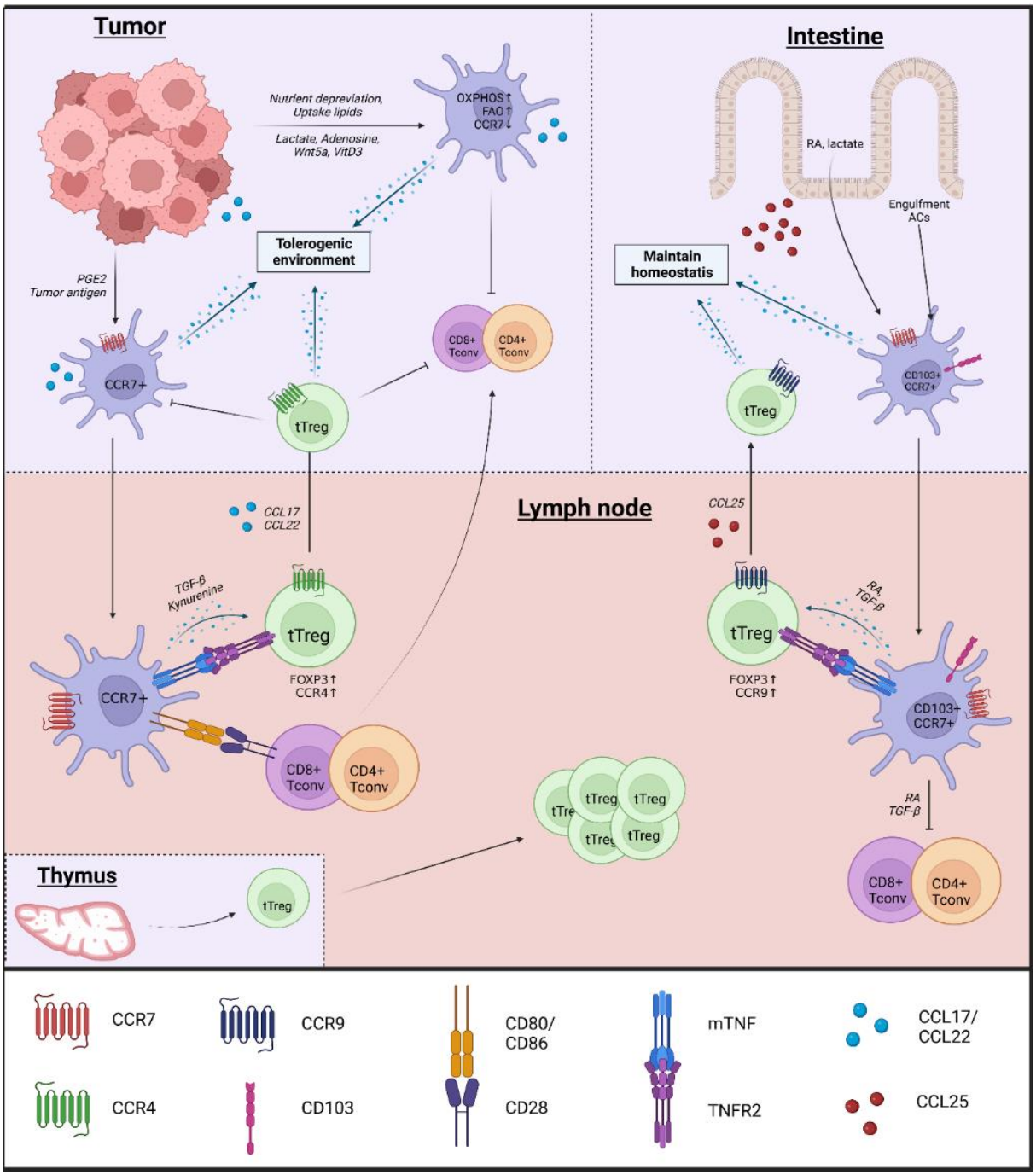

Figure 2. A proposed model of the induction of tolerogenic dendritic cells (DCs) and the priming of tTregs in the intestine (right) and tumors (left). DCs are primed in the periphery and subsequently migrate to the lymph node or remain in the tissue. In the lymph node, tolerogenic DCs prime tTregs and/or inhibit Tconvs. Chemokines attract the tTregs back to the tissue, where they assist in maintaining or inducing a tolerogenic environment. tTreg: thymus-derived Treg, pTreg: peripherally induced Treg, Tconv: conventional T-cell, OXPHOS: oxidative phosphorylation, FAO: fatty acid oxidation, mTNF: membrane-bound TNF, RA: retinoic acid, AC: apoptotic cell.

Future perspectives

There is a gap in our understanding when it comes to tolerogenic DCs and the priming of Tregs. The predominant focus has consistently been on immunogenic responses, overlooking the crucial aspect of understanding tolerogenic responses, especially in the context of anti-tumor immunity. In this review, we summarized the characteristics of tolerogenic DCs. As mentioned, there is not one tolerogenic state for DCs. Every tolerogenic stimulus elicits a distinct type of response. Various proteins or immunomodulatory molecules can be upregulated. In addition, DCs can shift their metabolic state towards a more tolerogenic profile. Within a specific tissue, various types of tolerogenic DCs may coexist. This poses challenges for diagnosis and intervention with the tolerogenic state of DCs. An ideal scenario would involve personalized tolerogenic DC screening for patients. Extracting tumor tissue from the patient and screening both DCs and the immunomodulatory molecules present could reveal the nature of the tolerogenic DCs. This information can then guide the administration of tailored medications that specifically target and neutralize the identified tolerogenic molecules or proteins.

In addition to detailing the characteristics of tolerogenic DCs, we elaborated on Tregs and proposed a model elucidating the priming and tissue migration of tTregs. In this model, pTregs are not taken along but that does not mean that pTregs are not primed. We decided to focus on tTregs as they are underrepresented in existing studies and evidence suggested their predominant presence in tolerogenic tissues. It is also important to keep in mind that this model serves as a conceptual framework, and extensive research on Tregs and their role in tolerance is still necessary. Numerous questions still persist: What are the different roles of tTregs and pTregs in tolerogenic tissues? We proposed a model of how tTregs are primed, but why are tTregs preferentially primed instead of pTregs? How are other chemokine receptors upregulated that target tTregs to various tissues? What are the effect of FOXP3 upregulation in tTregs?

In the clinic, ICIs targeting CTLA-4 and PD-1 are widely used to treat the tolerogenic nature of some tumors. However, these ICIs are not always effective[6]. It was discovered that both CTLA-4 and PD-1 blockade have been associated with an amplified Treg response[37]. Consequently, in tumors with elevated Treg levels, the application of PD-1 and CTLA-4 blocking antibodies may only enhance Treg expansion. This occurs because PD-1 and CTLA-4 blocking antibodies enable CD28 costimulation. In a TME that predominantly supports Treg priming over Tconv priming, Tregs can benefit from this. The introduction of an additional CD86 blocking antibody did prove to be effective in reducing tumor burden, whereas blocking CD80 did not abrogate the Treg response. Although CD80 and CD86 are commonly presumed to be similar, this study demonstrates their distinct roles. Overall, this highlights the necessity to broaden our existing understanding of the tolerogenic DCs and the associated costimulatory proteins.

Utilizing the proposed model, it is possible to formulate potential inhibitors for the priming of tTregs. Multiple studies have demonstrated positive outcomes by targeting TNFR2 either alone or in combination with ICI targeting PD-1[189–191]. By inhibiting TNFR2, the activation of tTregs in the dLNs is prevented, impeding their migration. Another possibility for blocking migration involves targeting the chemokine receptor CCR4[192,193]. Further research is imperative to assess the impact of these blockers on the priming process of tTregs

Altogether, there is a rising interest in exploring the tolerogenic aspect of the immune response. Understanding the tolerogenic nature of DCs and their role in sustaining a tolerant milieu is pivotal for improving therapeutic strategies for cancer patients, where tolerance must be abrogated, and for chronic infections, where tolerance needs to be induced. Conducting additional studies specifically focused on tTregs and the difference between tTregs and pTregs has the potential to yield valuable insights.

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