Apoptotic cell clearance by macrophages and dendritic cells : immunoregulation in the context of innate immunity

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Summary and General Discussion

1. Summary of the thesis

1.1. Anti-inflammatory Mφ may be the default phagocytes that promptly and silently phagocytose apoptotic cells

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease with a yet unclear pathogenesis. To date, different cytokines, soluble mediators, genetic factors and as well as many immune cells such as T cells, B cells, and antigen presenting cells (APCs), are thought to be involved in the initiation and progression of this disease [1-6]. The major targets in SLE are nuclear components (DNA, histones, ribonucleoproteins) [1,2,7], which are mainly derived from dying cells (apoptotic and necrotic cells) that serve as a reservoir for such autoantigens [8,9]. It has been suggested that defective clearance of dying cells may lead to the breakdown of peripheral tolerance and initiation of autoimmune SLE [3,4,10,11]. In this thesis, we have investigated the role of the innate immune system in the processing of dying cells and its immunological consequences.

We first discussed the current understanding of different subsets of DCs and Mφ in the handling of dying cells (**chapter 2**). Both *in vivo* and *in vitro* data suggest that there are different subsets of professional phagocytes that are actively involved in the clearance of dying cells. Presumably, also neighboring non-professional phagocytes might actively participate in these processes. We hypothesized that under steady-state conditions, the fate of dying cells is ultimately determined by local conditions and the composition of the tissue. Therefore a further dissection of distinct functional properties of phagocyte subsets is of particular importance to understand how the clearance of apoptotic cells by phagocytes is regulated and how this may lead to induction or loss of peripheral tolerance.

To address the questions mentioned above, we generated 3 types of phagocytes, namely DCs, Mφ1 and Mφ2 in parallel from the same donor and compared their capacity for phagocytosis of different types of particles (**chapter 3**). We found that M-CSF-driven Mφ2 are potent phagocytes that have the unique capacity to preferentially bind and ingest early apoptotic cells. This macrophage subset has intrinsic anti-inflammatory properties, as characterized by high IL-10 production in the absence of pro-inflammatory cytokines, such as IL-6 and TNF-α. Importantly, whereas the IL-6 and TNF-α production by GM-CSF-driven Mφ1 is inhibited upon uptake of apoptotic cells, the anti-inflammatory status of Mφ2 is retained during phagocytosis. These findings are consistent with earlier data that ingestion of apoptotic cells by Mφ is a non-inflammatory process [12]. We have tried to identify the specific receptors on Mφ2 that are responsible for the unique recognition of early apoptotic cells. CD14 is one of the important molecules which recognize apoptotic cells [13] and it is highly expressed by Mφ2. However, we found that CD14 was only used by Mφ2 for the tethering of apoptotic cells, but not for ingestion. Interestingly, Mφ2 showed more potent macropinocytosis compared to DCs and

Mφ1, and uptake of apoptotic cells was inhibited by a macropinocytosis inhibitor. Our studies suggest that, under steady-state conditions, IL-10-producing Mφ2 are prominently involved in the clearance of early apoptotic cells.

In a normal adult, resident tissue Mφ are derived from circulating bone marrow–derived monocytes, and are largely heterogeneous [14,15]. GM-CSF and M-CSF are two primary growth factors for the differentiation of macrophages from monocytes [16]. Under steady-state conditions, M-CSF is the only primary Mφ growth factor that is detectable in peripheral blood [17]. In contrast, GM-CSF is a pro-inflammatory cytokine, mostly generated during inflammation and hardly detectable in the circulation [18]. Furthermore, knockout mice lacking M-CSF or GM-CSF confirm that M-CSF is a more crucial growth factor than GM-CSF in Mφ differentiation [19-21]. Thus under steady-state conditions, M-CSF could be the default cytokine in driving Mφ differentiation. A relevant question therefore is: do in vitro polarized Mφ1 and Mφ2 exist in vivo?

In **Chapter 4**, a partial answer to the question raised above was obtained. We isolated human peritoneal Mφ (pMφ) freshly from patients on peritoneal dialysis and found that pMφ have phenotypical characteristics, including CD163 surface expression and lack of CD16, as Mφ2 generated *in vitro* upon stimulation of monocytes with M-CSF. Furthermore, we show that like Mφ2, pMφ have the capacity for endocytosis and macropinocytosis, and preferentially bind and ingest early apoptotic cells. These cells produce large amounts of IL-10 upon stimulation with LPS. Moreover, upon LPS stimulation both pMφ and Mφ2 exhibit a down-regulation of CD86, resulting in a reduced capacity of these cells to stimulate the proliferation of allogeneic T cells and an inhibition of Th1 cytokine release by these T cells. Thus, our data provide evidence for the first time that a countpart of *in vitro*-polarized Mφ2 exist in vivo, and that human pMφ resemble anti-inflammatory Mφ2. We propose that pMφ potentially maintain an anti-inflammatory condition in the peritoneal cavity.

Based on the data from **chapter 3** and **chapter 4**, we were intrigued by the opposing functions of polarized Mφ1 and Mφ2. Therefore, we questioned whether Mφ1 and Mφ2 are stable in terms of phenotype and function. In **chapter 5**, we described that Mφ1 exposed to M-CSF for an additional 6-day culture showed an increase in expression of CD163 and CD14, an increased capacity for phagocytosis of early apoptotic cells, and decreased capacity to stimulate T cell proliferation and inhibition of production of pro-inflammatory cytokines. *Vice versa*, Mφ2 exposed to GM-CSF converted these cells into Mφ1-like cells as envisioned by phenotype, cytokine profiles and capacity for phagocytosis and T cell stimulation. The data described above indicate that Mφ1 and Mφ2 can undergo reversible functional changes, namely into cells with anti-inflammatory or pro-inflammatory properties depending on the exposure to GM-CSF or M-CSF, respectively. Our

data support the importance of dynamic changes in cytokine environment in driving Mφ plasticity and provide moral perspectives for local therapeutic targeting.

1.2. Properdin is an important regulator in the processing of dying cells

The innate immune system has a key role in host defense against invading pathogens. Recent studies indicate that molecules of the innate immune system, including complement components and pentraxins, can also target their effect to self tissue such as dying cells. **Chapter 6** reviewed the pivotal role of the innate immune system in the clearance of apoptotic cells. Among the opsonins of apoptotic cells, complement factors, including C1q, and complement-activating members of the pentraxin family play an important role. We proposed that some soluble factors, such as C1q, prevent autoimmunity and maintain self-tolerance by supporting the efficient clearance of apoptotic material, as well as by actively modulating phagocyte function.

Consequently, we identified that another complement component, properdin, binds predominantly to late apoptotic and necrotic cells, but not to early apoptotic cells (**chapter 7**). Although previous studies indicated that properdin binds to pre-existing clusters of C3b on surfaces of cells, our current data suggest that binding of properdin to late apoptotic and necrotic cells can occur independently of C3b. Furthermore, we showed that properdin serves as a focus point for local amplification of alternative pathway complement activation. Importantly, properdin exhibits a strong interaction with DNA that is released and exposed on these cells. It is of interest to note that properdin does not compete with C1q and MBL for binding to late apoptotic or necrotic cells, although C1q and MBL interact with DNA as well [22,23], suggesting that next to DNA, properdin may bind to other as yet unknown ligands. We found no direct effect of purified properdin in the enhancement of phagocytosis of late apoptotic or necrotic cells. However, we did find that opsonisation of necrotic cells by properdin limits the pro-inflammatory potential of necrotic cells by reducing TNF-α release by DCs and Mφ, whereas IL-10 production is retained. Our data indicate that recognition of dying cells by properdin is essential to drive alternative pathway complement activation and that properdin actively promotes an anti-inflammatory response. The studies above provide additional knowledge on complement-mediated phagocytosis of dying cells, involving all three pathways of complement activation [24-26].

It will be a major challenge in the near future to integrate the information obtained in the past years and to distinguish the relative contribution of different components of the innate immune system to the clearance of apoptotic cells. However, also other parts of the immune system might have a direct or an indirect role. Opsonization of apoptotic cells with autoantibodies could potentially facilitate uptake, either via Fc receptor (FcR)-mediated process or via activation of the

complement system. We recently observed that SLE sera contain variable amounts of antibodies (Abs) directed against early apoptotic, late apoptotic /necrotic cells. The contribution of autoantibodies to phagocytosis is still under investigation. Importantly, as already stated in our hypothesis, since expression of FcR is different among phagocytes (**Xu, unpublished**), the effector mechanisms will also strongly depend on the type of phagocyte which encounters the apoptotic cells.

2. General discussion and future directions

2.1. Is SLE a DC-driven disease?

Most experiments described in this thesis have been performed with Mφ and Mφ subsets. However, several non-published experiments have been performed in parallel using monocyte-derived DCs. In this part of the discussion some of these results will be presented and these findings will be discussed in the context of recent developments on the involvement of DCs, or specific DC subsets, in the pathogenesis of SLE.

Many factors are involved in the initiation and progression of SLE, such as cytokines, soluble mediators, genetic factors as well as many immune cells such as T cells, B cells, and cytokines [1-6]. Among the latter, one important cytokine is type I interferon (IFN-α) [5,6], as in agreement with an earlier report that IFN-inducible genes are up-regulated in peripheral blood mononuclear cells (PBMCs) from patients with SLE [27]. Importantly, it was suggested that IFN-α mediates unabated differentiation of DCs, which drive the autoimmune response [28]. DCs acquire antigens from apoptotic cells and (cross-)present these antigens to class I- or class II-restricted T cells [29-31]. It was suggested that late apoptotic cells and necrotic cells, but not early apoptotic cells induce maturation of DCs, and present antigens in the absence of exogenous danger signals [31,32]. However, others suggest that such maturation is due to possible contamination /infection [33]. Therefore it remains controversial whether late apoptotic and necrotic cells one the one hand activate DCs, and that early apoptotic cells on the other hand are immunologically “null”.

To address this controversy, we have tried to study how dying cells at different stages of cell death influence DC biology. We found that DCs loaded with early apoptotic, late apoptotic, or necrotic cells all inhibited pro-inflammatory cytokine release (**Xu, unpublished**). In contrast to the findings of others, we observed that neither late apoptotic nor necrotic cells induce DC maturation. Furthermore, We were unable to demonstrate that DCs loaded with early apoptotic, late apoptotic, or necrotic cells (cross-)present antigen to T cells. This finding is in agreement with a recent study showing that DCs that had captured apoptotic cells only in the presence, but not in the absence of TLR4 triggering by LPS, could present antigens to CD4 T cells [34]. Thus extra danger signals provided by TLR ligands

could make a substantial difference in the initiation of autoimmunity by apoptotic cell-loaded DCs.

Plasmacytoid DCs (pDCs) are a specialized subset of DCs that produce IFN-α upon activation by virus. Although *in vitro* pDCs hardly ingest apoptotic cells [35], they do internalize immune complexes (ICs). It has been shown that apoptotic or necrotic cells combined with IgG from patients with SLE induce the production of IFN-α by purified pDCs [36]. Furthermore, ICs derived from patients with SLE, upon intracellular delivery via CD32, were able to activate pDCs through toll like receptor 9 (TLR9) [37]. This observation was extended in another study showing that small nuclear RNAs within ribonucleoprotein particles activate pDCs through TLR7 [38], suggesting a link between pDCs and autoimmmunity to autoantigens containing DNA or RNA. These studies have provided novel insights in the possible mechanisms of loss of peripheral tolerance to autoantigens in SLE.

Nevertheless, it is too early to conclude that SLE is indeed a DC-driven disease. Firstly, whether late apoptotic or necrotic cells activate DCs is still a matter of debate. Secondly, the pivotal contribution of soluble factors in controlling danger singles requires further investigation. Existing data suggest that factors such as properdin (**Chapter 7**) and C4 binding protein (C4BP) [39] are able to bind DNA exposed or released by necrotic cells, and potentially limit their pro-inflammatory potential. Furthermore, we recently found that complement component C1q is able to modulate DCs into a cell type with tolerogenic properties (**Castellano *et al*., Eur. J. Immunol. in press**). These findings indicate that the host itself is able to minimize the effect of danger signals and control inflammation by the use of inherent innate immune factors.

2.2. Tolerogenic Mφ

Plasticity of pro-inflammatory Mφ1 and anti-inflammatory Mφ2 is a matter of interest for further exploration. We showed in this thesis that both Mφ1 and Mφ2 are not at the end stage of cell differentiation and are adaptable to changes in the microenvironment concerning their immunological functions (**chapter 5**). For example, exposure of pro-inflammatory Mφ1 to M-CSF resulted in down-regulation of their pro-inflammatory cytokine profile such as IL-6 and TNF-α production and up-regulation of the production of the anti-inflammatory cytokines such as IL-10. Similar functional changes were also reflected in their capacity to stimulate T cells.

Next to growth factors, mediators from the innate immune system might impact these polarized cells to mount or tolerize an immune response. For example, during an infection pathogens trigger Toll-like receptors (TLRs) on myeloid cells and activate these cells. It is of interest to investigate how Mφ1 and Mφ2 respond to TLR triggering. Our preliminary data showed that TLR2 engagement e.g. by

peptidoglican (PGN) is able to boost both IL-10 and IL-6 production by Mφ1 (Fig 1) (**Xu, unpublished**). In contrast, anti-inflammatory Mφ2 failed to produce IL-6 by any means of TLR triggering as indicated. Our data imply a role for TLR agonists in modulation of immunological function of myeloid subsets (under investigation).

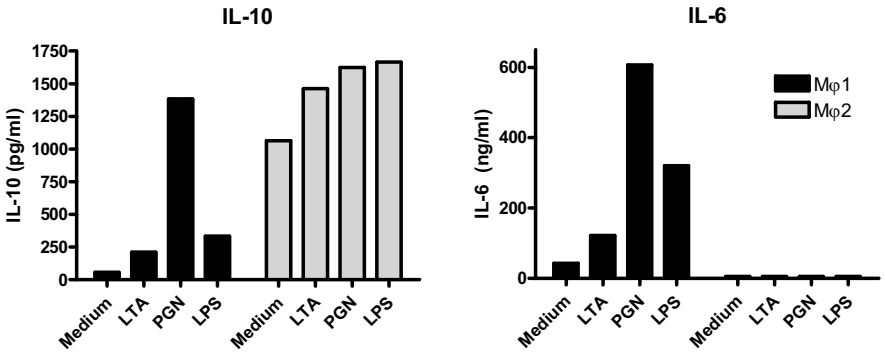

Figure 1. TLR activation by DCs, Mφ1 and Mφ2. Mφ1 and Mφ2 (25,000 cells) were stimulated with TLR agonists Lipoteichoic Acid (LTA) or peptidoglican (PGN) (1 ug/ml), or with the TLR4 agonist LPS (200 ng/ml) for 24 hours in 48-well plates. Supernatants were harvested and measured for IL-10 and IL-6 production by ELISA.

Another way to activate Mφ is via T cell cytokines such as IFN-γ (Th1 cytokine) or IL-4 (Th2 cytokine) [14]. IFN-γ has long been considered the most powerful cytokine produced by Th1 cells to exert antiviral and bactericidal activities against pathogens [40]. However, recent data provide evidence that both DCs [41] and Mφ [42] are able to secrete IFN-γ themselves. Therefore these cells are susceptible to autocrine activation by IFN-γ. Furthermore, IFN-γ actively induces indoleamine 2,3-dioxygenase (IDO) expression on DCs and Mφ. IDO is an tryptophan-degrading enzyme involved in immune suppression and tolerance induction [43,44]. In pilot experiments, we showed that IFN-γ induces IDO expression on both Mφ1 and Mφ2 at RNA level, as measured by RT-PCR (Figure 2A) and real-time PCR (Figure 2B) (**Xu, unpublished**). Furthermore, we found that IFN-γ-stimulated Mφ1 and Mφ2 are impaired in the induction of allogeneic T cell proliferation (data not shown). We are currently actively investigating the mechanisms that are involved in the inhibition of T cell proliferation by IFN-γ.

Together, the plasticity of Mφ subsets opened the door for further characterization of these cells, and we have obtained insight in modulating their functions towards cells with tolerogenic properties. It sheds some light on the potential application of these tolerogenic Mφ in preventing transplant rejection.

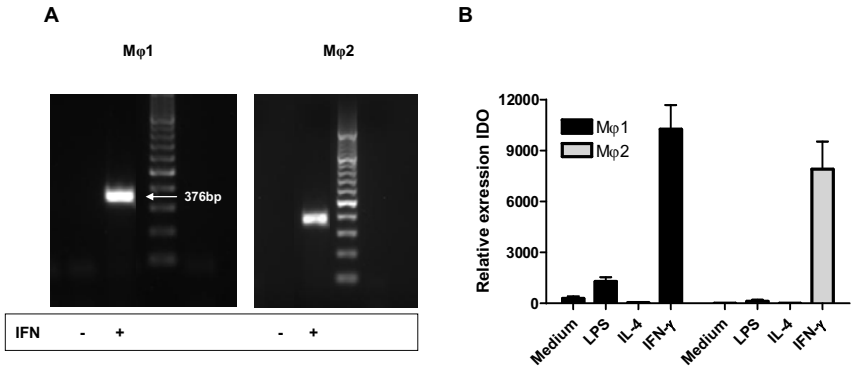

Figure 2. Induction of IDO by IFN-γ on Mφ1 and Mφ2. Mφ1 and Mφ2 were stimulated with IFN-γ for 24 h and RNA was isolated from these cells. IDO expression was measured by RT-PCR (A) or real-time PCR (B).

2.3. Apoptotic cells in the regulation of immune tolerance via regulating Treg and TH17 cells

As discussed throughout the current thesis, apoptotic cells play an essential role in the regulation of immune system in terms of self-tolerance and autoimmunity. How such a balance is regulated is not fully understood. We showed here (**Chapter 3**) that a subset of phagocytes with anti-inflammatory properties (namely Mφ2) have the capacity to preferentially phagocytose early apoptotic cells, resulting in a sustained high production of IL-10. These type of phagocytes exist also *in vivo*, namely in the peritoneal cavity and share characteristics with *in vitro*-polarized Mφ2 (**Chapter 4**). We therefore speculate that *in vivo* clearance of early apoptotic cells is largely confined to a specialized subset of phagocytes with anti-inflammatory properties, leading to the maintenance of self tolerance. If overloaded early apoptotic cells are not cleared promptly, these cells may progress into late apoptosis or secondary necrosis, which consecutively activate newly recruited pro-inflammatory phagocytes. Thus an immunogenic self-antigen presentation might occur which facilitates the development of autoimmune disease.

Failure of self-tolerance often leads to the development of autoimmune disease, whereas maintenance of self-tolerance is critically engaged with the regulatory CD4 T cells (Treg) [45]. Treg cells produce immune suppressive cytokines such as IL-10 and /or TGF-β. One of major Treg cells is defined as $CD4^+CD25^+FOXP3^+$ T cells, and can be differentiated by TGF-β [46]. A breakthrough has been made recently by Bettelli *et al.* who showed that IL-6 completely inhibits the generation of $Foxp3^+$Treg cells induced by TGF-β. Instead, IL-6 and TGF-β together induce the

differentiation of newly described pathogenic Th17 cells from naïve T cells [47]. Th17 cells are the cells producing IL-17 and have a crucial role in the induction of autoimmune tissue injury [48]. Thus there is a reciprocal developmental pathway for the generation of pathogenic TH17 cells and protective Treg cells in the immune system depending on the cytokine balance between IL-6 and TGF-β, probably some other unknown cytokines. As many cells produce IL-6 and TGF-β, the main source of these cytokines remains to be investigated [49], and in addition it is of importance to study how the cytokine balance might be ticked.

As discussed earlier, IL-10 and TGF-β are the main cytokines released by phagocytes which have ingested (early) apoptotic cells. Therefore, we hypothesize that a local production of cytokines during clearance of apoptotic cells *in vivo* by anti-inflammatory phagocytes (such as Mφ2 like cells) might server as a primary source of IL-10 and TGF-β to initiate the differentiation of Treg cells for the maintenance of peripheral tolerance. It has been proposed that apoptotic cells induce transplant tolerance via the generation of Treg cells [50,51]. The very first study showed infusion of apoptotic spleen cells induces TGF-β-dependent CD4+CD25+ T cell expansion in a bone marrow transplantation setting [52]. It was further demonstrated in another study that administration of donor apoptotic cells results in indefinite graft survival mediated by generation of Treg [53]. Therefore, use of the inhibitory effects of apoptotic cells on the anti-donor response provides a new approach to treat transplant rejection.

On the other hand, delayed clearance of apoptotic cells (or overload with late apoptotic cells /necrotic cells) might be a trigger to shift the cytokine balance in terms of IL-6, TGF-β and probably other cytokines, and thereby leads to a skewing of differentiation of TH17 cells over Treg cells (see Figure 3). It is worthwhile to explore how local apoptotic cells and phagocytes regulate T cell subset differentiation. It should be noted that most of data mentioned above have been obtained in the murine system. Although it could be anticipated that a similar balance of Th cells exists in the human system, the exact regulation by the cytokine environment is not fully established.

3. Concluding remarks

Throughout this thesis, we have tried to obtain insight in how apoptotic cell clearance is regulated by cells or mediators from the innate immune system. We provide evidence that anti-inflammatory Mφ2 fulfil the demand to efficiently clear early apoptotic cells in a silent manner. This probably provides an explanation on how apoptotic cell clearance contributes to the maintenance of self-tolerance under steady-state conditions. However, it remains largely unclear how the handling of dying cells translates into a break in peripheral tolerance and induction of autoimmunity.

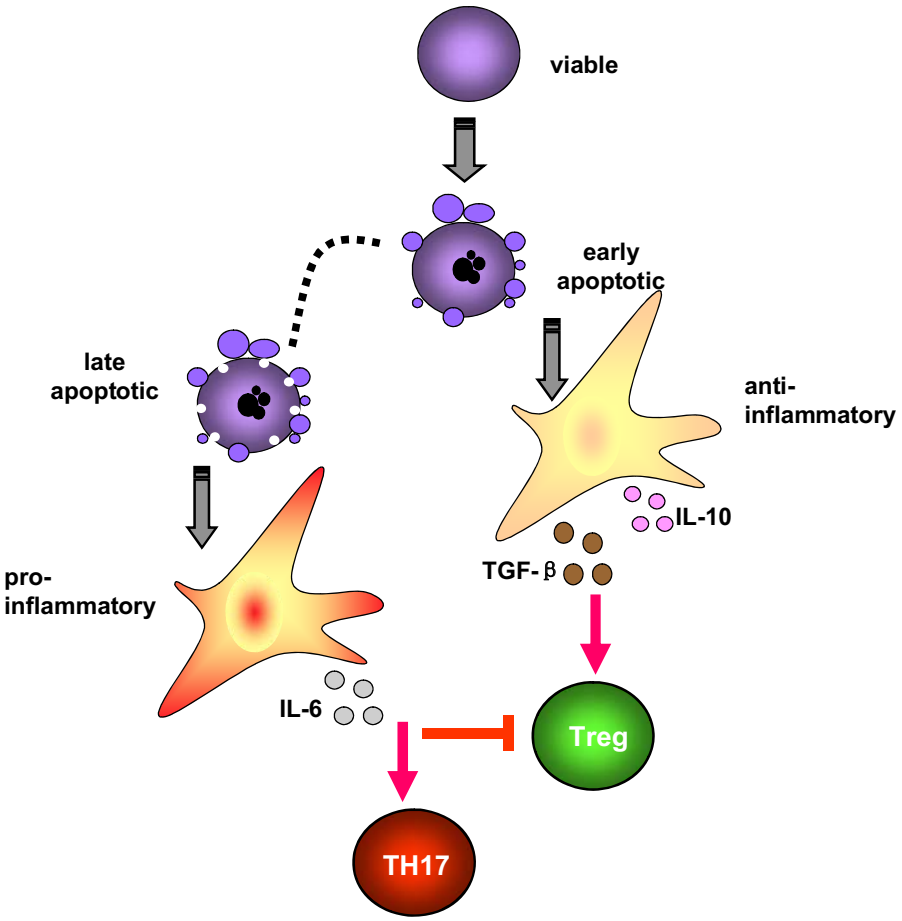

Figure 3. Hypothesis on apoptotic cells in the regulation of immune tolerance via regulating Treg and TH17 cells. We speculate that early apoptotic cells are cleared promptly and efficiently by resident anti-inflammatory phagocytes, leading the release of anti-inflammatory cytokines such as IL-10 and TGF-β. These cytokine serves the primary triggers to the differentiation of Treg cells. When early apoptotic cells are not cleared promptly, they may progress into late apoptosis or necrosis, leading to recruitment of newly elicited pro-inflammatory phagocytes to participate in the clearance. Consequently, pro-inflammatory cytokines such as IL-6 are release, and a cytokine balance is disturbed. As a result, Treg cells are inhibited by IL-6, and pathogenic TH17 cells are promoted by the combination of IL-6 and TGF-β.

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