Cell Research (2006)16: 154-161
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REVIEW

Regulation of cytokine production during phagocytosis of apoptotic cells

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Loss of self-tolerance and expansion of auto-reactive lymphocytes are the basis for autoimmunity. Apoptosis and the rapid clearance of apoptotic cells by phagocytes usually occur as coordinated processes that ensure regulated cellularity and stress response with non-pathological outcomes. Defects in clearance of apoptotic cells would contribute to the generation of self-reactive lymphocytes, which drive autoimmune disorders such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE). The IL-12 family of cytokines (IL-12, IL-23, and IL-27) and IL-10 are produced by phagocytic macrophages and play critical roles in the regulation of antigen-presenting cells (APCs) and effector lymphocytes during an immune response to pathogens. Inappropriate expression of these cytokines and their dysregulated activities have been strongly implicated in the pathogenesis of several autoimmune diseases. The production of pro- and anti-inflammatory cytokines by phagocytic APCs is delicately regulated during the ingestion of apoptotic cells as part of an intrinsic mechanism to prevent inflammatory autoimmune reactions. How apoptotic cell-derived signals regulate cytokine production is poorly understood. A recent study by our group demonstrated that phagocytosis of apoptotic cells by activated macrophages results in strong inhibition of IL-12 p35 gene expression by activating a novel transcription repressor, which we named GC-binding protein (GC-BP), through tyrosine dephosphorylation. We are also beginning to understand the molecular mechanisms underlying apoptotic cell-triggered production of IL-10 by phagocytes. These studies will help to elucidate some novel immune regulatory mechanisms and explore the regulation of immune responses to autoantigens with potentials to discover new therapeutic targets for the treatment of autoimmune disorders.

Cell Research (2006) **16**:154-161. doi:10.1038/sj.cr.7310021; published online 13 February 2006

Keywords: cytokines, apoptotic cells, phagocytosis, autoimmunity

Clearance of apoptotic cells by professional phagocytes

Multicellular organisms have evolved genetic and epigenetic mechanisms of programmed cell death (apoptosis) to eliminate cells that are no longer needed or damaged. Physiological apoptosis has an essential role in development, differentiation and tissue homeostasis [1]. The elimination of apoptotic cells and cell bodies by phagocytes represents an evolutionarily conserved means to prevent exposure of surrounding tissue to potentially cytotoxic, immunogenic, or inflammatory cellular contents [2, 3]. When apoptosis occurs at moderate rates such as during normal adult tissue turnover, neighboring cells such as fibroblasts can act as phagocytes in their ingestion and clearance. When apoptosis occurs on large scales such as during embryonic morphorgenesis, ionizing radiation, and acute infections, macrophages are the major professional phagocytes that play important roles in the clearance of apoptotic cells. Macrophages are attracted to sites of high rate of apoptosis such as the thymus and the follicles of secondary lymphoid tissues in the immune system.

Phagocytic receptors and apoptotic cell ligands

Apoptotic cells exhibit numerous changes including

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alteration of membrane lipid molecules and carbohydrates. There are four major phospholipids in the plasma membrane of many mammalian cells, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and sphingomyelin. PS is normally limited to the inner layer of the lipid bilayer [4] but is found at higher levels on the outer leaflet of cell membranes that are undergoing apoptosis due to a poorly understood inhibition of an aminophospholipid translocase [5] and the activation of a lipid scramblase [6]. The observation that apoptotic cells that do not express PS are poorly phagocytosed suggests that PS provides an important signal in the recognition and/or clearance of apoptotic cells [7]. The process of removing dead cells is carried out by a wide variety of cell types and involves multiple receptors [8], such as scavenger receptors, oxidized low-density lipoprotein receptors, CD14, CD68, CD36, and vitronectin receptor, and apoptotic cell ligands. The exposed PS on apoptotic cells is recognized by several phagocyte receptors including a presumptive phosphatidylserine (PS) receptor (PSR) [9]. Ligation of this presumptive PSR has been proposed to be the primary mechanism through which these responses are initiated [10], although experimental demonstration of such a receptor has been quite controversial [11].

A 4-step process has been proposed to account for the recognition and ingestion of apoptotic cells by phagocytes. Ligation of the "PSR" on phagocytes delivers a "tickle" signal, which stimulates the internalization of apoptotic cells, including bystander cells, that are "tethered" to the phagocytes through other recognition receptors. Simultaneously, the immune response is modulated through secretion of immunosuppressive cytokines [12].

Phagocytosis of apoptotic cells and regulation of immune responses

Resolution of inflammation depends not only on the removal of apoptotic cells but also on active suppression of inflammatory mediator production. Aberrations in either mechanism are associated with chronic inflammatory conditions and autoimmune disorders [13-15]. Uptake of apoptotic cells by phagocytes is thought to suppress autoimmune responses through the release of anti-inflammatory cytokines IL-10, TGF-β, platelet activating factor (PAF), and prostaglandin E_2 (PGE_2), and inhibition of pro-inflammatory cytokines TNF-α, GM-CSF, IL-12, IL-1β, and IL-18 [16-18].

In human systemic lupus erythematosis (SLE), impaired phagocytosis of apoptotic materials by macrophages has been reported [19, 20], providing an explanation for increased levels of early apoptotic cells, DNA, and nucleosomes observed in the circulation of SLE patients [21-24]. The impaired clearance of apoptotic cells resulting in an accumulation of late apoptotic and secondary necrotic cells including oligosomes might lead to an activation of autoreactive T and B cells [16].

IL-12 family of cytokines in autoimmunity

IL-12 is an important cytokine in both the innate and adaptive phases of host immune defenses against intracellular pathogens. IL-12 is a heterodimer produced primarily by macrophages and DCs. It is a key factor in the induction of T cell-dependent and independent activation of macrophages, NK cells, generation of T helper type 1 (Th1) cells and CTL, induction of opsonizing, complement-fixing antibodies, and resistance to intracellular infections [25]. The genes encoding the two heterologous chains of IL-12, p40 and p35 are located on different human and mouse chromosomes. Together, p40 and p35 form the biologically active IL-12 (also called p70). The p40 chain is also shared with another IL-12-related cytokine, IL-23, which is composed of p40 and an IL-12-independent subunit p19 [26]. The highly coordinated expression of p40 and p35 genes to form IL-12 p70 in the same cell type at the same time is essential for the initiation of an effective immune response. IL-12 is also an important player in T-cell-mediated autoimmunity [27, 28]. Specifically, IL-12 administration exacerbates autoimmune phenomena by inducing the differentiation of Th1 autoreactive cells [29, 30] whereas the lack of IL-12/IL-23 p40 in genetically deficient mice or mice treated with anti-IL-12 antibody abrogated diseases in experimental models of autoimmunity such as insulin-dependent diabetes mellitus (IDDM) in NOD mice [31, 32], experimental allergic encephalomyelitis (EAE) [33, 34], experimental autoimmune uveitis (EAU) [35, 36], and collagen-induced arthritis (CIA) [37]. Aberrant levels of IL-12 are produced by macrophages isolated from young mice prone to lupus (MRL and NZB/W) [38]. The diabetes-associated quantitative trait locus, Idd4, was found to be responsible for the IL-12 p40 overexpression in nonobese diabetic (NOD) mice.[39] Administration of IL-12 to aging mice renders them vulnerable to the induction of experimental SLE induced by the monoclonal anti-DNA autoantibody bearing the 16/6Id.[40] In human patients with SLE, elevated levels of IL-12, IL-18 is observed [41-43], and the higher serum IL-12 levels are correlated with fever in subjects but not with renal diseases [44]. Moreover, PBMCs from patients with active SLE were found to be more sensitive to IL-12 by inducing phosphorylation of STAT3 and STAT4 [45]. IL-12 produced by DCs in a $CD4^+$ T cell-dependent manner is able to break tolerance and activates $CD8^+$ T cell effector functions in graft rejection [46]. Administration

of IL-12 and IL-18 in vivo can break oral tolerance to ovalbumin with abrogated suppression of specific IgG2a production, delayed-type hypersensitivity responses and IFN-γ production by antigen-specific T cells [47].

IL-27 is the latest addition to the IL-12 family. IL-27 is a heterodimeric protein consisted of Epstein–Barr virus (EBV)-induced gene 3 (EBI3), a p40-related protein, and p28, a newly discovered IL-12 p35-related polypeptide. IL-27 appears to be produced early by activated antigen-presenting cells. It is able to induce clonal proliferation of naïve but not memory $CD4^+$ T cells and synergizes with IL-12 in IFN-γ production by naïve $CD4^+$ T cells [48]. Recently, an orphan receptor was described with 26% homology and 37% similarity to the IL-12Rβ2 subunit and to gp130, respectively, designated TCCR [49] or WSX-1 [50]. This receptor was identified as one of the receptor subunits for IL-27 and is necessary but not sufficient for IL-27 function [48]. It has been suggested that IL-27 and IL-12 function sequentially in initiating and maintaining Th1 responses, respectively [48, 50]. Recombinant IL-27 expressed from tumor cells has been shown to elicit potent tumor-specific immune responses in vivo and result in complete regression of orthotopic primary and metastatic murine neuroblastoma tumors [51], and Colon 26 murine colon carcinoma [52].

Li *et al*, reported that IL-27 p28 and EBI3 subunits and WSX-1 mRNAs were markedly upregulated in inflammatory cells in the CNS during EAE [53]. Furthermore, neutralizing the *in vivo* function of IL-27 by antibodies against IL-27 p28 rapidly suppressed an ongoing long-lasting disease in C57BL/6 mice [54]. These studies strongly suggest the involvement of IL-27 in autoimmunity.

Mechanisms of apoptotic cell-induced inhibition of IL-12 production

Phagocytosis of apoptotic cells usually results in an anti-inflammatory state with an inhibition of proinflammatory cytokines such as IL-12. How apoptotic cell-derived signals regulate IL-12 gene expression was not understood. We demonstrated recently [55] that cell-cell contact with apoptotic cells is sufficient to induce profound inhibition of IL-12 production by activated macrophages. PS could mimic the inhibitory effect. The inhibition does not involve autocrine or paracrine actions of IL-10 and TGF-β. Moreover, we reported the identification, purification and cloning of a novel zinc finger-like nuclear factor, named GC-binding protein (GC-BP), that is induced following phagocytosis of apoptotic cells by macrophages or by treatment with PS. GC-BP selectively inhibits IL-12 p35 gene transcription by binding to its promoter in vitro and in vivo, thus decreasing IL-12 production. Blocking GC-BP by RNA interference restores IL-12 p35 transcription and IL-12 p70 synthesis. Upon contact with apoptotic cells, GC-BP, which is present in both the cytoplasm and nucleus, undergoes dephosphorylation possibly at tyrosine 15. The tyrosine-dephosphorylated GC-BP binds the IL-12 p35 gene promoter between +13 and +19, and blocks its transcription, thereby inhibiting IL-12 production [55]. The reduced capacity of macrophages to produce IL-12 is associated with an impaired ability to promote IFN-γ production by activated T cells [56].

GC-binding protein (GC-BP)

The GC-BP gene is uniquely present in the mouse genome, located on chromosome 7, with a postulated 3-exon structure spanning ~ 10 kb. The 615-amino acid residues of GC-BP with a calculated molecular weight of 68.3 kDa predict strongly that it is a zinc finger-containing protein and transcriptional repressor. The mouse cDNA was originally isolated from a tissue biopsy of the MMTV-LTR/INT3 mammary tumor. PSORT II analysis indicates with a very high probability and reliability (>95%) that it is a nuclear protein with 16 putative C2H2 type zinc finger motifs that could interact with DNA. However, in reality it is present in approximately equal amounts in the cytoplasm and in the nucleus, and the importance of these putative zinc fingers is uncertain since the N-terminal 27 amino acids with only one zinc finger retains ~ 50% of GC-BP's transcriptional capacity [55]. There is a human ortholog of the predicted protein sequence of mouse GC-BP: hypothetical protein FLJ13479 (NCBI database), which is 93% homologous to its mouse counterpart. Mouse GC-BP shares 33% homology with mouse zinc finger protein 51(Zfp-51), with 19 contiguous zinc fingers and being ubiquitously expressed [57]. The rat homologue of GC-BP (40% at amino acid level) is zinc finger protein 37 (Zfp-37), a novel peroxisome proliferator responsive cDNA isolated originally from rat hepatocytes [58]. In this context, it is curious to note that there is a good putative binding site, AGGTCT, in the 5' UTR of GC-BP for peroxisome proliferator-activated receptor (PPAR)/retinoid X receptor (RXR) heterodimers. PPARs as well as another family of nuclear hormone receptors, liver X receptors (LXRs), are activated by trigleride-rich lipoproteins, oxidized low-density lipoprotein (LDL), and apoptotic cells in macrophage lipid metabolism [59]. Relevantly, it has been shown that PPARγ agonists can inhibit experimental autoimmune encephalomyelitis (EAE), a Th1 cell-mediated inflammatory demyelinating disease model of multiple sclerosis (MS), by blocking IL-12 production, IL-12 signaling and Th1 differentiation [60]. Thus, GC-BP may have additional unidentified targets involved in broader areas of biology than the original data revealed.

Model of GC-BP-mediated inhibition of IL-12 production during phagocytosis

Apoptotic cells, upon contact with professional phagocytes induce a profound inhibition of IL-12 production, in part, by selectively targeting the transcription of the p35 gene. The chief findings of our recently published study [55, 61] and our working hypothesis are summarized as follows:

1) A target cell undergoing apoptosis displays characteristic surface changes including externalization of PS, which serves as a marker recognized by phagocytes such as macrophages.

2) The interaction between the apoptotic cell and the macrophage, particularly between PS and PSR, initiates a signaling event that acts on the GC-BP.

3) Dephosphorylation of GC-BP on tyrosine 15 occurs.

4) The dephosphorylated GC-BP binds to the GC-element in the IL-12 p35 promoter.

5) The binding of GC-BP prevents IL-12 p35 gene transcription, thus IL-12 protein synthesis.

6) In the meantime, the interaction between the apoptotic cell and the macrophage including the PS-PSR interaction results in the production of anti-inflammatory cytokines such as TGF-β? and IL-10.

7) Together, the suppression of pro-inflammatory cytokines such as IL-12 and anti-inflammatory cytokines prevent T cell activation and induce tolerance.

Role of IL-10 in homeostatic regulation of inflammation and immune response

IL-10 is a pleiotropic cytokine produced by both T and B cells and macrophages and possesses both anti-inflammatory and immunosuppressive properties [62]. The ability of IL-10 to inhibit cytokine production by both T cells and NK cells was found to be indirect, via inhibition of accessory cell (monocyte/macrophage) function [63-66]. These initial studies were soon followed by extensive research showing that IL-10 is an inhibitor of a broad spectrum of monocyte/macrophage functions, including cytokine synthesis, nitric oxide production, and expression of MHC class II and costimulatory molecules such as CD80/CD86 [67-74]. Investigations in numerous inflammatory disease models including chronic enterocolitis, cutaneous inflammatory condition, endotoxic shock and Shwartzman reaction, and autoimmune encephalomyelitis in IL-10-deficient mice have yielded strong evidence that IL-10 plays a central role in vivo in restricting inflammatory responses [75-79]. However, endogenous IL-10 production and systemic administration can also exacerbate macrophage- and T-cell dysfunction, decrease T-cell apoptosis, blunt antimicrobial activity, and increase mortality in other less acute bacterial models of sepsis or after thermal injury [80]. In addition, IL-10 also processes immunostimulatory effects that have not attracted sufficient attention. IL-10 is a potent growth factor for B lymphocytes. It promotes B cell proliferation, antibody production, and class II expression [81]. IL-10 enhances, paradoxically, the development of cytotoxic T lymphocytes (CTL) [82-84]. It induces NK cytotoxicity against NK-resistant tumor cells in vitro and increases IL-2-induced NK cell proliferation [85]. It acts as a co-factor for colony formation by mast cell progenitors [86] and thymocytes [87]. The B cell-stimulating property of IL-10 is thought to be the basis of several antibody-mediated autoimmune disorders [88].

IL-10 gene expression in microbe- and cytokine-activated macrophages

A key feature of macrophages is their ability to produce both proinflammatory cytokines such as IL-1, IL-6, IL-8, TNF-α, and IL-12, and anti-inflammatory cytokines such as IL-10 and TGF-β in response to microbial stimuli. The balance of pro- and anti-inflammatory cytokine production is of critical importance to the outcome of an immune response. Understanding this delicate balance is essential to appreciate the complexity of macrophage biology. A large number of studies have been devoted to the dissection of the molecular mechanisms involved in the regulation of proinflammatory cytokine gene expression, which unveiled several important transcription factor families that mediate inflammatory response of macrophages such as NF-κB, NF-IL6, C/EBP, and interferon regulatory factors [89-96]. In contrast, much less is known about the regulation of anti-inflammatory cytokines.

IL-10 gene expression in macrophages is usually triggered by the same typical inflammatory stimuli such as lipopolysaccharides (LPS) that induce the release of proinflammatory cytokines. However, the kinetics of its induction differs from those of the proinflammatory mediators [63, 68, 97]. Recent molecular analyses of the murine IL-10 promoter show that IL-10 transcription in macrophage cell types can be regulated by constitutive and ubiquitous transcription factors such as Sp1 and Sp3, suggesting that IL-10 may be produced at low levels constitutively to maintain certain level of control over "baseline" inflammation [98, 99]. Another study provided evidence that post-transcriptional regulation of IL-10 gene expression through sequences in the 3'-untranslated region of the IL-10 mRNA contributes to its overall production as well [100, 101]. A critical role for Stat3 but not other Stat proteins in LPS-induced IL-10 transcription in a human

B cell line was reported by Benkhart and colleagues who demonstrated a direct interaction of Stat3 with the human IL-10 promoter at -120 [102]. Since Stat3 is also the mediator of IL-10 signaling via the IL-10 receptor [103], this finding provides a mechanistic explanation for the noted autoregulation of IL-10.

IL-10 gene expression during phagocytosis of apoptotic cells

Our group has carried out studies to elucidate the molecular mechanism whereby apoptotic cells induce the production of IL-10 by phagocytic macrophages. Our preliminary data (unpublished) suggest that:

1) Apoptotic cells induce IL-10 gene transcription and protein production in macrophages.

2) Phagocytosis of apoptotic cells is not required for IL-10 production by macrophages. Cell-cell contact is sufficient. It's partially CD36-dependent.

3) The major apoptotic cell-response element (positive regulator) in the human IL-10 promoter is mapped to -106/-98, to which binding with specific nuclear protein(s) is induced by apoptotic cells.

4) A negative element is mapped to -171/-129 where a novel nuclear binding activity has been identified, and this activity is inhibited by contact with apoptotic cells.

5) The p38 mitogen-activated protein kinase (MAPK) is critically involved in apoptotic cell-medicated IL-10 transcription and post-transcriptional regulation.

6) CD36 is essential in the induction of p38 MAPK activation by apoptotic cells.

Conclusion

Immunoregulatory cytokines IL-10 and IL-12 play important roles in the etiology and pathology of many autoimmune diseases. Elucidation of the apoptotic cell-mediated signaling mechanisms involved in the control of IL-10 and IL-12 production during cell turnovers under normal and pathological conditions may help us counter the cytokine dysregulation and control inappropriate host immune reactions in disorders such as autoimmunity, infectious diseases, graft-versus-host disease, and cancer.

Acknowledgements

This work was supported by a NIH grant (AI45899) and a grant from the Mary Kirkland Foundation for Lupus Research, both to Xiao Jing Ma.

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