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Maintenance of self-tolerance by apoptotic cell clearance

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1. ABSTRACT

Innate immune cells are genetically conferred the ability to recognize microorganisms as "non-self", and to induce appropriate immune responses to eliminate them. On the other hand, immune cells should recognize self cells in order to avoid attacking normal tissues. For this purpose, immune cells make use of self-cell corpses. When cells undergo apoptosis, cell corpses are rapidly phagocytosed by phagocytes, such as macrophages and dendritic cells. These phagocytes present self antigens derived from dead cell corpses to induce tolerance. Impairment of apoptotic cell clearance often results in autoimmune disorder. Intravenous injection of dead cell corpses can induce tolerance to cell-associated antigens, and this strategy has potential use in the treatment of various autoimmune and inflammatory disorders in human. Injected dead cell corpses are rapidly cleared by phagocytes located in the marginal zone (MZ) of spleen. Among those phagocytes, macrophages play a critical role in the rapid clearance of dead cell corpses, and the subsequent induction of tolerance to cell-associated antigens.

2. INTRODUCTION

The immune system has evolved to protect against invasion and proliferation of microorganisms from outside the body. For this purpose, it is necessary to discriminate "self" from "non-self." To recognize non-self "enemy", phagocytes, such as macrophages and dendritic cells (DCs) possess a wide variety of molecules that bind cellular components found on the surface of microorganisms (1, 2). These cells kill and phagocytose microorganisms for elimination. Engulfed microorganisms are digested in lysosomes of phagocytes, and peptides derived from the enemy are presented in an MHC-dependent manner to T cells for induction of acquired immunity.

On the other hand, the immune system should know what "self" is to avoid attacking self cells and organs. (3, 4). Self antigens are presented by antigen-presenting cells (APCs) in thymus, and T cells reactive to these self antigens are eliminated by apoptosis. Although the number of self antigens originally expressed in thymus is limited, a

wide variety of self antigens that are normally expressed in other tissues or cells are available in thymus due to the expression of AIRE in thymic epithelial cells (5). Therefore, almost all self-reactive T cells are theoretically eliminated in thymus, and never appear in peripheral tissues. However, a small population of self-reactive T cells escape selection in thymus, and appear in peripheral circulation and tissues. To avoid self-destruction caused by the attack of these self-reactive T cells, these cells have to be constantly eliminated in peripheral tissues. APCs, such as DCs localized in tissues and organs can collect self peptides by engulfing dead cell corpses, and make a presentation of these peptides. When self-reactive T cells escaping selection in thymus come across these APCs, deletion or anergy is induced in these cells.

This review describes the mechanisms and significance of apoptotic cell clearance by phagocytes and the subsequent induction of tolerance to cell-associated antigens.

3. MFG-E8-MEDIATED APOPTOTIC CELL CLEARANCE BY PHAGOCYTES

Phagocytes recognize dying cells for phagocytosis (6-9). The dead cell clearance is executed so rapidly by phagocytes that dead cell corpses are usually detected only within phagocytes by TUNEL staining in physiological conditions (10). The rapid clearance of dead cells prevents the release of potentially toxic or immunogenic intracellular materials from the cell corpses. Therefore, prompt elimination of dying cells is required for maintenance of tissue integrity, resolution of inflammation, and normal tissue repair. To engulf apoptotic cells, phagocytes recognize molecules on apoptotic cells through membrane-bound receptors or soluble proteins. The Milk fat globule-EGF-factor 8 (MFG-E8) was identified as one such soluble protein that is involved in apoptotic cell phagocytosis by phagocytes (11). MFG-E8 is produced and secreted by certain kinds of phagocytes including thioglycollate-elicited mouse peritoneal macrophages and bone marrow-derived DCs. MFG-E8 has two factor VIII-homologous domains in its C-terminus, which are responsible for binding to phosphatidylserine (PS) exposed on apoptotic cells. MFG-E8 also has EGF-like domains in its N-terminus, which can bind $\alpha_v\beta_3$ integrin expressed in phagocytes via its RGD motif. When recombinant MFG-E8 protein was added to 3T3 cells expressing $\alpha_v\beta_3$ integrin, the phagocytic activity of these cells was greatly enhanced, indicating that MFG-E8 acts as a bridge between apoptotic cells and phagocytes to promote dead cell clearance. MFG-E8 also induces integrin-dependent Rac-1 activation (12). Therefore, it can directly activate phagocytic machinery via integrins.

In addition to activated peritoneal macrophages, tingible body macrophages (TBMs) also produce MFG-E8. These cells are located in the germinal center of spleen and lymph nodes, and show a unique marker (CD68 positive and F4/80 negative). TBMs are responsible for the removal of apoptotic B cells in germinal centers. In MFG-E8-deficient mice, a large number of apoptotic lymphocytes associate with TBMs in the germinal centers, but most of them are not engulfed by TBMs, indicating that MFG-E8 is involved in apoptotic cell clearance by TBMs (13). In addition, MFG-E8-deficient mice developed splenomegaly, and spontaneously produced autoantibodies, such as antinuclear antibodies, and anti-DNA antibodies in an age-dependent manner. In associated with autoantibody production, the mice developed proteinuria and glomerulonephritis.

The autoimmune phenotype is a common feature of various gene-targeting mice deficient in molecules involved in apoptotic cell clearance (14-16). These results indicate that apoptotic cell clearance plays a critical role in the maintenance of self-tolerance.

4. EFFECTS OF MFG-E8 MUTANT PROTEIN

An MFG-E8 derivative carrying a point mutation in the RGD motif (designated as D89E) was found to be a powerful tool for the analysis of the physiological and pathological roles of apoptotic cell clearance. D89E protein behaved as a dominant negative form inhibiting the phagocytosis of apoptotic cells by activated peritoneal macrophages *in vitro* and *in vivo*. Lately, we found that D89E inhibited the phagocytosis of apoptotic cells by various kinds of macrophages in a dose-dependent manner. Since another mutant, E1E2PT, lacking C1and C2 domains had no effects on phagocytic activity of these macrophages, the inhibitory effects of D89E were dependent on its binding activity to PS. These results indicated that a wide variety of macrophages recognize PS on apoptotic cells for phagocytosis, and that D89E can inhibit apoptotic cell engulfment by phagocytes by masking PS on dead cells. Then, we used D89E to evaluate the significance of apoptotic cell clearance *in vivo*. We intravenously injected the mutant protein into mice repeatedly, and found that the injection caused the production of autoantibodies, including antiphospholipid antibodies and antinuclear antibodies (17). The production of autoantibodies was enhanced by the coinjection of syngeneic apoptotic cells, indicating that the impairment of apoptotic cell phagocytosis led to autoantibody production.

Jinushi *et al.* recently applied MFG-E8 mutant protein to tumor vaccination (18). It is well known that vaccination with irradiated, GM-CSF-secreting tumor cells protected syngeneic mice from subsequent challenge with live wild-type tumor cells (19, 20). They reported that the presence of wild-type MFG-E8 protein abrogated the protective effects of the vaccination. On the other hand, in the presence of MFG-E8 mutant protein, the anti-tumor effects of the vaccination were potentiated. In these analyses, MFG-E8 mutant protein possibly inhibited the phagocytosis of vaccinated tumor cells by phagocytes, such as DCs migrated into the vaccination site by the action of GM-CSF, and this inhibition could induce immunity against tumor antigens instead of tolerance. Similar effects of annexin V on the induction of tumor immunity were reported (21). From these results, interference of apoptotic cell clearance induces immune responses to dead-cell-associated antigens; otherwise, tolerance is induced

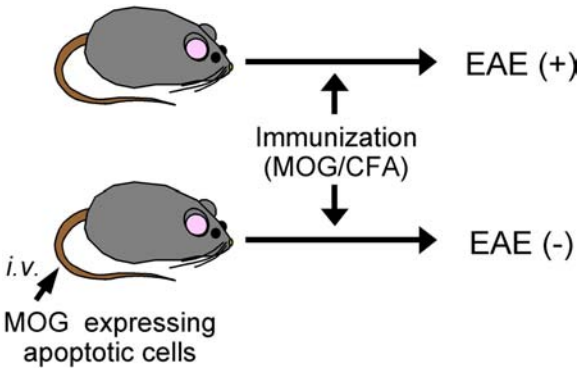

Figure 1. Intravenous injection of MOG-expressing cells suppresses the development of EAE.

5. INTRAVENOUS INJECTION OF DEAD CELLS INDUCES TOLERANCE TO CELL-ASSOCIATED ANTIGENS

The analysis of gene-targeting mice and of the inhibitor of dead cell clearance described above has revealed that dead cell clearance plays a critical role in the maintenance of self-tolerance. Peripheral tolerance to self antigens is thought to be maintained by APCs, including DCs localized in peripheral tissues (22, 23). Tissue-resident DCs constantly phagocytose apoptotic cells generated during normal tissue turnover, and migrate to draining lymph nodes where they present the antigens taken up from apoptotic cells. This presentation of self antigens leads to deletion or anergy of self-reactive T cells, thereby maintaining T cell tolerance to self antigens.

In addition to the peripheral tolerance induced by residual DCs in lymph nodes, circulating hematopoietic dying cells are cleared, and tolerance to self antigens expressed in these cells is induced in spleen. When apoptotic cells loaded with an exogenous protein, ovalbumin, were injected intravenously into mice, immune tolerance to the protein was induced (24). Analysis using ovalbumin-specific TCR transgenic mice revealed that $CD8^+$ T cells reactive to cell-associated antigens are deleted by intravenous injection of dying cells. Several groups successfully controlled immune responses by intravenous injection of dying cells. Sun *et al.* reported that intravenous transfusion of apoptotic splenocytes from the donor strain prevented rejection of heart allografts (25). Xia *et al.* reported that transfusion of apoptotic β-cells induced immune tolerance and subsequent suppression of diabetes in MOD mice (26).

We recently found that the induction of tolerance to cell-associated antigens could be applied to prevention of autoimmune diseases in a mouse model. Immunization of myelin oligodendrocyte glycoprotein (MOG) peptide with complete Freund's adjuvant (CFA) induced experimental autoimmune encephalomyelitis (EAE), a mouse model of human multiple sclerosis. We established transformants expressing MOG fragments, and treated these cells with Fas ligand to induce apoptosis. Injection of apoptotic cells expressing the MOG fragment suppressed the development of EAE (27) (Figure 1). Intravenous injection of apoptotic cells expressing a MOG fragment reduced MOG-specific T cell responses, while T-cell responses to an irrelevant antigen were not affected, indicating that tolerance to cell-associated antigens was specifically induced by apoptotic cell injection. For the tolerance induction to cell-associated antigens, the characteristics of early phase of apoptotic cells were required. The suppressive effects of apoptotic W3/MOG-L cells were largely, but not completely, eliminated when the mice were injected with D89E, while the suppressive effects were not affected by co-injection with E1E2PT, another MFG-E8 mutant protein that does not bind to PS. This result suggests that PS-dependent engulfment of apoptotic cells is required, at least in part, for tolerance induction to cell-associated antigens.

6. CLEARANCE OF CIRCULATING DYING CELLS IN SPLEEN

Intravenously- injected apoptotic cells are initially accumulated in splenic marginal zone (MZ), and then rapidly cleared from this site. This observation indicates that some phagocytes located in the spleen phagocytose the injected dead cell corpses and that these cells presents antigens derived from the dead cell corpses for tolerance induction.

6.1. Macrophages in spleen

Several types of macrophages are found in spleen. Among them, red pulp macrophages have the largest population in spleen. Red pulp macrophages are $CD11b^+$ and $F4/80^+$, and are scattered in the red pulp of spleen. They are responsible for clearance of aging erythrocytes, and consequently recycling of iron (28). In addition to red pulp macrophages and TBMs (described in the previous section), MZ of spleen contains two types of macrophages. Marginal metallophilic macrophages (MMMs) were originally detected by immunohistochemistry using MOMA-1 (29) and SER-4 (30) antibodies. SER-4 antibody was found to recognize sialoadhesin (CD169), a sialic acid-binding receptor (31-34). Sialoadhesin is an adhesion molecule of 185 kDa, and can bind sialic acid expressed on erythrocytes. However, the function of sialoadhesin *in vivo* has not been revealed.

The other population of macrophages located in MZ is called marginal zone macrophages (MZMs). MZM are found at the outer rim of MZ, which is close to the red pulp. MZMs are detected by immunohistochemistry using ER-TR9 antibody (35). It was reported that the antibody recognized SIGN-R1 (36), a C-type lectin that has strong binding activity for the capsular polysaccharide of Streptococcus pneumoniae (37, 38). When fluorescence-labeled S. pneumoniae were injected into wild-type mice, the labeled bacteria were exclusively localized in MZ of spleen. On the other hand, in SIGN-R1-deficient mice, the injected bacteria were no longer restricted to MZ; instead, they were disseminated throughout the red pulp. Consistent with this observation, SIGN-R1-deficient mice showed increased susceptibility to S. pneumoniae infection, indicating that SIGN-R1- expressing MZMs play a critical role in the clearance of the bacteria. (39). MZMs are reported to have phagocytic activity with the expression of

several pattern-recognition receptors, suggesting that these cells are responsible for the recognition and clearance of several invading microorganisms.

6.2. Dendritic cells in spleen

In spleen, two major conventional DC subpopulations, $CD8\alpha^+$ $CD11b^-$ DCs and $CD8\alpha^-CD11b^+$ DCs exist. $CD8\alpha^-$ DCs can be further divided into two populations, $CD4^+$ and $CD4^-$. $CD8\alpha^+$ and $CD8\alpha^-$ DCs are located in different regions of the spleen. $CD8\alpha^+$ DCs are located in MZ and T-cell-rich areas of periarteriolar lymphatic sheaths, while $CD8\alpha^-$ DCs are mainly found in MZ of spleen (40). The functional differences between these subpopulation have been reported. One major difference is their ability to phagocytose dead cells. When dead cells are intravenously injected into mice, $CD8\alpha^+$ DCs preferentially engulf circulating dying cells in physiological conditions (27, 41). Consistent with this observation, $CD8\alpha^+$ DCs have a stronger activity of presentation of antigens derived from injected dead cells in normal mice. Functional differences were demonstrated by using monoclonal antibodies against surface molecules specifically expressed in each DC subpopulation (42-45). DEC-205 is specifically expressed in $CD8\alpha^+$ DCs, while DCIR2 is exclusively expressed in $CD8\alpha^-$ DCs. The antigens of interest can be delivered to either $CD8\alpha^+$ or $CD8\alpha^-$ DCs by conjugation with anti-DEC-205 or anti-DCIR2 antibody, respectively. This analysis revealed that different subsets of DCs induce distinct immune responses, but the delivery of antigens to both DC subsets without any maturation signals leads to T cell tolerance**.** On the other hand, Corbett *et al.* reported that targeting of antigens to $CD8\alpha^-$ DCs induced humoral immunity even in the absence of adjuvants (46).

6.3. Role of macrophages in marginal zone in tolerance induction to cell-associated antigens

Recently, a method for conditional cell ablation by transgene of the human diphtheria toxin receptor (DTR), named TRECK (Toxin REceptor-mediated Cell Knockout), has been used in a number of research fields (47-54). Human, but not mouse, heparin-binding EGF-like growth factor (HB-EGF) exhibits strong binding activity to diphtheria toxin (DT). Thus, mouse cells are more resistant to DT than human cells. When the human HB-EGF gene is transduced into mice under the control of a cell-specific promoter, the target cells are transiently depleted by DT administration *in vivo*.

To reveal the role of macrophages in MZ in the induction of immune tolerance to injected cell-associated antigens, we generated transgenic mice in which macrophages in MZ could be transiently deleted by DT injection (27). In CD169-DTR mice, DTR cDNA was introduced into CD169 gene locus. DT administration to the mice resulted in transient deletion of MZMs and MMMs, while red pulp macrophages and TBMs were not affected. We found that tolerance induction to cell-associated antigens failed in the MZ macrophage-depleted mice, indicating that macrophages in MZ are indispensable for tolerance induction to cell-associated antigens.

In the mice, two interesting findings were observed in association with the failure of tolerance induction. First, the clearance of injected apoptotic cells was changed in the absence of macrophages in MZ. Circulating dead cells or non-self materials, such as injected latex beads and bacteria, were first trapped and accumulated in MZ of spleen (55), and then rapidly cleared. Even in the absence of macrophages in MZ, injected apoptotic cells were still accumulated in MZ, indicating that other types of cells localized in MZ are responsible for trapping circulating dying cells in spleen. However, there was a dramatic delay in cell corpse clearance in the absence of macrophages in MZ, indicating that these macrophages play a critical role in clearance by phagocytosis. It is possible that this delayed clearance of injected dying cells critically affects tolerance induction to cell-associated antigens. Several cytosolic materials, such as uric acid (56), and HMGB1 (57-59) released from late phase of apoptotic cells or necrotic cells are reported to stimulate immune responses, interfering with the tolerance induction to cell-associated antigens. These materials may be released from dying cells that are not cleared rapidly in MZ.

The second finding in MZ macrophage –depleted mice is the aberrant phagocytosis of injected apoptotic cells by $CD8\alpha^-$ $CD11b^+$ DCs in spleen (Figure 2). As described above, $CD8\alpha^+$ DCs preferentially engulf circulating dying cells in wild-type mice . $CD8\alpha^+$ DCs engulfing apoptotic cells can present cell-associated antigens and stimulate proliferation of T cells *in vitro*. On the other hand, the deletion of macrophages in MZ caused aberrant phagocytosis of injected dying cells by $CD8\alpha^-$ DCs. These results indicate that $CD8\alpha^-$ DCs potentially possess the ability to phagocytose apoptotic cells *in vivo*. The precise mechanisms by which $CD8\alpha^-$ DCs are silent for apoptotic cell clearance in physiological conditions are unknown. Although macrophages in MZ may compete with $CD8\alpha^-$ DCs for apoptotic cell phagocytosis, the physiological relevance of this competition and the preferential clearance of apoptotic cells by $CD8\alpha^+$ DCs remain unclear. To resolve these, it is important to elucidate the functional differences between $CD8\alpha^+$ and $CD8\alpha^-$ DCs for immune responses.

7. CLINICAL RELEVANCE/SIGNIFICANCE

As described above, intravenous injection of apoptotic cells can induce tolerance to cell-associated antigens. This strategy may be applied to novel therapies for various allergies as well as inflammatory or autoimmune diseases in human. For this purpose, the use of "cell corpses" may not be appropriate for practical reasons, and the so-called "artificial cell corpses" should be developed. For the induction of tolerance, artificial cell corpses should be cleared (probably by $CD8\alpha^+$ DCs) in the same way as dying cells. However, it remains unknown what characteristics of the dead cells are minimum requirements for phagocytosis by DCs and consequent induction of tolerance to target antigens. Although it has been confirmed that the exposure of PS is required for

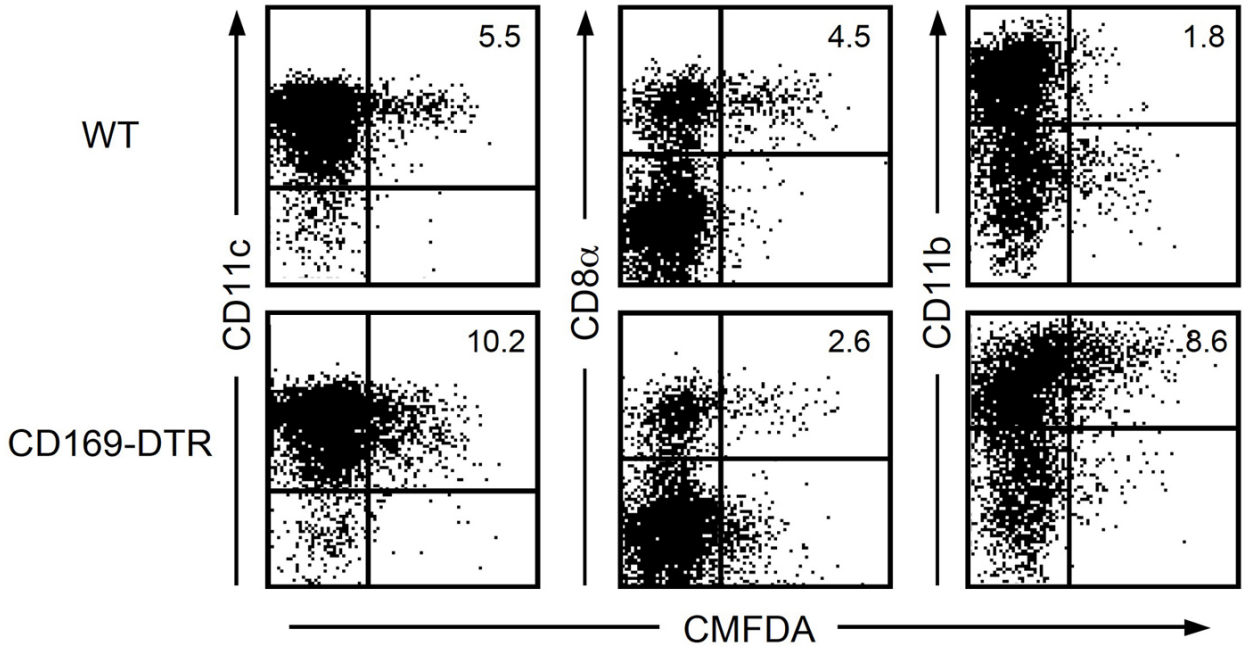

Figure 2. Aberrant phagocytosis of apoptotic cells by $CD8\alpha^-$ $CD11b^+$ DCs in marginal zone macrophage-depleted mice. Fluorescent (CMFDA)-labeled apoptotic cells were intravenously injected into wild type (WT) and marginal zone macrophage-depleted mice (CD169-DTR). The spleens were obtained 1 hour after injection. Splenic DCs were enriched by cell sorting with anti-CD11c microbeads, and were stained with anti-CD11c, CD8α, or CD11b. CMFDA positive cells were considered to phagocytose the injected apoptotic cells. Note that the number of CMFDA-positive $CD11b^+$ DCs was greatly increased in CD169-DTR mice.

phagocytosis of apoptotic cells and tolerance induction, it is very important to examine whether PS exposure on artificial cell corpses is sufficient for tolerance induction.

Another clinical application of apoptotic cell clearance is the induction of tumor immunity. As described above, inhibition of clearance of irradiated tumor cells enhanced the effects of tumor vaccination. In addition, it is reported that the efficiency of tumor vaccination largely depended on the nature of cell death in vaccinated tumor cells (60, 61). These findings strongly suggest that clearance of dying tumor cells plays a critical role in the induction of immunity or tolerance to tumor cells. Therefore, the control of tumor cell clearance can be a novel strategy for cancer therapy.

8. ACKNOWLEDGEMENT

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