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Exploiting Apoptosis for Therapeutic Tolerance Induction

Daniel R. Getts,1 Derrick P. McCarthy,1 and Stephen D. Miller

Immune tolerance remains the most promising yet elusive strategy for treating immune-mediated diseases. An experimental strategy showing promise in phase 1 clinical studies is the delivery of Ag cross-linked to apoptotic leukocytes using ethylene carbodiimide. This approach originated from demonstration of the profound tolerance-inducing ability of i.v. administered Ag-coupled splenocytes (Ag-SP) in mice, which has been demonstrated to treat T cell-mediated disorders including autoimmunity, allergy, and transplant rejection. Recent studies have defined the intricate interplay between the innate and adaptive immune systems in Ag-SP tolerance induction. Innate mechanisms include scavenger receptor-mediated uptake of Ag-SP by host APCs, Ag representation, and the required upregulation of PD-L1 expression and IL-10 production by splenic marginal zone macrophages leading to Ag-specific T cell regulation via the combined effects of cell-intrinsicnergy and regulatory T cell induction. In this paper, we discuss the history, advantages, current mechanistic understanding, and clinical potential of tolerance induction using apoptotic Ag-coupled apoptotic leukocytes. The Journal of Immunology, 2013, 191: 5341–5346.

A berrant or misdirected T cell responses constitute a major health concern in developed countries, contributing to the development of autoimmunity, allergy, and transplant rejection as well as immune responses against protein therapeutics. The spectrum of therapies currently available for treatment of immune disorders ranges from drugs that target pathways of immune activation and trafficking to mAb therapies that deplete subsets of lymphocytes. As a consequence of their nonspecificity, a number of these therapies have been associated with severe side effects such as tissue toxicity and increased susceptibility to infection and cancer. Therefore, Ag-specific tolerance, while elusive, remains the Holy Grail for treatment of these diseases. At present, peripheral T cell tolerance induction strategies, such as injection of soluble peptide, altered peptide ligands, or costimulatory molecule blockers (1–3), have been largely unsuccessful when tested in humans. One prospective treatment that was extensively developed in rodents (4–7) and has recently shown promise in an early phase 1 clinical trial (8) is the i.v. infusion of peptide Ags cross-linked to the surface of peripheral blood or splenic leukocytes (Ag-SP) using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (ECDI) to affect Ag coupling and induce cellular apoptosis. Ag-SP treatment has been shown to be highly effective both as a prophylactic therapy and as an acute and therapeutic treatment capable of regulating epitope spreading in rodent models of multiple sclerosis and type 1 diabetes (9, 10). Ag-SP tolerance is also effective in allergy (11) and allo- and xenograft rejection (6, 12–15) and therefore regulates responses mediated by naive and activated CD4+ Th1, Th17, and Th2 cells (9, 11) as well as CD8+ T cells (16).

Although the development of Ag-SP as a potential therapy dates back just over 30 years, it has roots in the Sulzberger–Chase tolerance phenomenon (17) that predates Billingham and Medawar’s report by several decades (18). In the 1920s, Sulzberger made a seminal observation while studying contact dermatitis in guinea pigs (19) when he demonstrated that hypersensitivity induced by the dermal application of neoarsphenamine could be prevented by i.v. treatment with the same agent if administered near the time of the sensitization. These observations were later confirmed by Chase who reported this unresponsiveness to be allergen-specific because oral treatment with dinitrochlorobenzene only prevented contact dermatitis if dinitrochlorobenzene was used as the sensitizing agent (20).

The Sulzberger–Chase phenomenon gained prominence when it was determined that these simple chemical compounds, or hapten, coupled with cellular constituents of the blood to induce hapten-specific tolerance when administered i.v. (21). This implied a crude role for cellular membranes in tolerance and the potential use of coupled cells for tolerance induction to foreign proteins with rudimentary coupling chemistry (22). Miller and Claman (23), examining T suppressor cells as a mechanism for tolerance induced by hapten-coupled cells, discovered the use of ECDI following a study by Doyle et al. (24) that used carbodiimide chemistry to couple Ag to RBCs for hemolytic plaque assays. By using water soluble ECDI to form a covalent bond between the primary amines on one protein and the free carboxyl groups on another protein, Ags could be covalently linked to cell membranes. Miller and Claman adapted this approach
for use as a flexible Ag-delivery platform that was capable of eliciting either immunity or tolerance, depending on the route of administration (4). Although it was not appreciated at that time, treating the cells with ECDI triggers the induction of apoptosis, and this secondary effect of Ag conjugation was subsequently found to be critical to the robustness of this platform. Their seminal observation followed by three decades of intense research has shown that Ag-SP–induced tolerance is the result of a complex immunological reaction involving innate and adaptive immune elements (Fig. 1).

How the innate immune response sets the scene for tolerance induction

Intravenous delivery to the marginal zone sinus. Ag-SP tolerance was originally thought to operate by direct presentation of Ag to T cells (5). However, the discovery that ECDI treatment induces rapid apoptosis after i.v. administration suggested operation via an indirect presentation pathway (7). Although the mechanism of ECDI-induced apoptosis has not been elucidated, it is clear that tolerance is indirectly induced by host APCs representing the coupled Ag because tolerance can be induced by Ag-SP if >90% of the cells are apoptotic prior to injection (S.D. Miller, unpublished observations) and by sonicated Ag-SP or Ag-RBCs (10). Fluorescently labeled Ag-SP localize to the splenic marginal zone sinus within minutes of infusion and are completely cleared within 18 h. Furthermore, Ag-SP tolerance is ineffective in splenectomized mice and cannot be induced via s.c. or i.p. administration (9). Thus, the available evidence suggests a model in which donor cell apoptosis, uptake of Ag via natural splenic clearance mechanisms, and the representation of Ags is the dominant pathway of Ag presentation to T cells in vivo (Fig. 1A) (7).

Marginal zone macrophage uptake of Ag-SP by scavenger receptors. The recognition of apoptotic debris within the spleen, a common pathway for the disposal of senescing RBCs and blood leukocytes, can be mediated through direct scavenger receptor recognition of dying cells and/or detection of serum proteins that opsonize apoptotic cells (25, 26). Scavenger receptors, such as the macrophage receptor with a collagenous structure (MARCO), CD68, the oxidized low-density lipoprotein receptor-1 (LOX-1), and the class B scavenger (SRB) receptors, have all been implicated in normal apoptotic cell clearance (25, 26). Using fluorescently labeled cells, we observed that Ag-SP colocalized with and upregulated the expression of SRBII (9), an isoform of SRBI (27). Furthermore, the scavenger receptor MARCO has also

![FIGURE 1. Proposed mechanisms of Ag-SP tolerance. (A) Innate immune responses required for Ag-SP tolerance induction. The splenic marginal zone is the primary interface between the splenic nonlymphoid compartment and the lymphoid. It is composed of B cells and macrophages important for capturing exogenous Ags and debris, which may be processed for subsequent presentation to T cells in T cell zones. For efficient tolerance, Ag-SP must be delivered (1) via i.v. administration. Once within the marginal sinus, the Ag-SP rapidly degrade via apoptotic pathways (2), with debris and cells recognized and rapidly taken up via scavenger receptors on MZMs. DCS may take up Ag directly from the marginal zone sinus or via membrane transfer (3). The uptake of Ag-SP triggers the production and secretion of soluble mediators including IL-10 and TGF-β (4), which have multifarious functions including the regulation of costimulatory molecules, such as PD-L1, on APCs (5). (B) Mechanisms of Ag-SP–induced T cell regulation. Ag-SP tolerance is the result of a number of independent but overlapping regulatory mechanisms. The upregulation of negative costimulatory molecules on APC, including CTLA-4 and PD-L1, can trigger effector cell anergy and apoptosis (6). In the context of naive T cells, TCR stimulation (signal 1), without positive costimulation results in T cell anergy (7). In addition, IL-10/TGF-β secreted in response to Ag-SP infusion supports the differentiation of naive T cells into Tr1 and/or induced (iTREGs) (8). Although the precise temporal contribution of each regulatory mechanism requires further examination, it seems that tolerance is the result of early anergy, with iTREGs playing a major role in long-term tolerance maintenance.](http://www.jimmunol.org/)
been shown to be important for the induction of tolerance using Ag coupled to polystyrene nanoparticles. Understanding differential scavenger receptor utilization is critical because the activation of receptors, such as MARCO, can have a profound impact on cellular function(s) including the induction of apoptosis, cytokine production, and even T cell activation and differentiation (28–30). Furthermore, differential roles for scavenger receptors in promoting T cell function recently have been observed using Ag–Ab complexes targeting specific scavenger receptors (31). Ag delivered to human dendritic cells (DCs) via LOX-1 or DC-SIGN increased the expansion of IFN-γ–expressing CD4+ T cells, but the same Ag delivered via the DC-asialoglucoprotein receptor favored the expansion of IL-10–secreting, CD4+ T cells, presumably of a regulatory phenotype. Taken together, the data showing a differential impact on T cell function depending on scavenger receptor activation and the observation that MARCO is redundant for Ag-SP tolerance, but not Ag-NP tolerance, highlight the need for further study into the precise scavenger receptors and the downstream signaling cascades involved within marginal zone macrophages (MZMs) that result in tolerance induction. **TGF-β and IL-10 production.** The infusion of apoptotic Ag-SP by MZM corresponds with the expression of immunoregulatory cytokines in the spleen (Fig. 1A) (6, 9). We have observed that IL-10 production, after Ag-SP infusion occurs rapidly, is sustained for a considerable length of time and that blockade of IL-10 signaling or genetic deletion of IL-10 prevents the induction of Ag-SP tolerance (9). Furthermore, in vitro studies, the use of IL-10–deficient donors, localization of Ag-SP, and the kinetics of IL-10 protein secretion support macrophages as the major source of this cytokine (9). Interestingly, studies using 2-mercaptoethanol–induced apoptotic thymocytes as a tolerogenic strategy have shown B cell–produced IL-10 to be important for immune regulation (32). However, in the case of Ag-SP, genetic B cell deficiency or anti-CD20 depletion does not impact the ability to induce T cell tolerance (9). Nonetheless, a functional role for IL-10 has been partially defined and may involve the differential regulation of APC-expressed costimulatory molecules, especially PD-L1 on MZMs (see below).

Similar to IL-10, inhibition of TGF-β secreted in response to Ag-SP also inhibits Ag-SP tolerance induction (6, 9). TGF-β secretion by macrophages and other APC populations has been commonly described. Generally speaking, TGF-β plays an important role in the framework for Ag-SP tolerance because it is important for the development of induced regulatory T cells (Tregs) (Fig. 1B), which are critical for long-term maintenance of Ag-specific nonresponsiveness, as described below. **Costimulation and Ag specificity.** APCs express numerous costimulatory proteins that act in conjunction with TCR stimulation to regulate T cell activity. An in-depth discussion on costimulation is beyond the scope of this review—for a review on costimulation refer to Ref. 33. The focus in this paper will be to summarize observations regarding PD-L1. Similar to the B7/CTLA-4 pathway of negative costimulation, the PD-1/PD-L1 pathway is also a negative regulator of T cell activation that functions to suppress T cell proliferation and cytokine secretion, including IL-2 and IFN-γ (34). The PD-1 receptor is expressed on T cells following TCR stimulation while its ligand, PD-L1, is expressed on multiple cell types including APC subsets. In the context of Ag-SP, we have shown that PD-L1 is integral to Ag-SP tolerance induction (Fig. 1) (6, 9). Ag-SP induced IL-10 acts in an autocrine fashion to upregulate PD-L1 on MZMs (9). This suggests that MZMs may have a direct role in regulating autoreactive T cells through the provision of PD-L1/PD-1–negative costimulation. Furthermore, PD-L1 expression also may reduce T cell interactions with DCs through its ability to modulate T cell motility (35). This raises important questions regarding the role of DCs in Ag-SP tolerance induction. Little colocalization of Ag-SP has been observed in vivo with CD11c+ cells (9). Furthermore, Ag-SP infusion induces few phenotypic changes in DCs, including PD-L1 expression.

The exquisite Ag specificity of Ag-SP tolerance induction is arguably its most attractive feature. Initially, Ag-SP were shown to be capable of directly influencing activated T cells (5); however, subsequent experiments have shown this feature is predominately observed in vitro. In vivo, Ag-SP processing plays an important role as cell donors deficient in MHC class I and MHC class II are both highly efficient at inducing tolerance (7). However, the precise APC subset(s) responsible for driving Ag-SP tolerance Ag specificity remains unknown. DCs are known to acquire Ags from the marginal zone sinuses (36) as well as directly from the plasma membrane of macrophages in the spleen and subsequently traffic to T cell zones (37); however, this does not appear to be critical for Ag-SP–induced tolerance. This difference may be a result of Ag-SP not triggering danger signals. Furthermore, DC behavior in the spleen mainly has been described in the context highly immunogenic Ag carrying inherent TLR signals. **T cell inhibition results from coordinate mechanisms.** Multiple regulatory mechanisms have been postulated to be involved in Ag-SP–induced peripheral tolerance. These include immune deviation, deletion, anergy, and suppression. Although each of these mechanisms may contribute to Ag-SP–induced unresponsiveness, current evidence supports a biphasic tolerance induction process, with anergy and Tregs playing key roles (Fig. 1B). **Anergy induction.** Observations from the 1970s suggested that tolerance induced by coupled-cell treatment was the result of a rapid and long-lasting clonal inhibition, and the short-term activation of suppressor T cells (4). With respect to the former, Jenkins and colleagues ascribed the state of clonal inhibition caused by Ag-SP to the induction of T cell anergy, demonstrating for the first time that the two-signal model of B cell activation (38) also applied to T cells (5). In these experiments, the authors observed that when AE7 T cells specific for pigeon cytochrome c fragment 84–104 were incubated with pigeon cytochrome c fragment 84–104-SP in vitro, they failed to proliferate, upregulate IL-2R expression, or respond to secondary stimulation with cognate peptide/MHC molecules. This anergic state of Ag-SP–experienced T cells was found to be dependent on a defect in IL-2 production, because anergy was reversed when exogenous IL-2 was added to the re-stimulation cultures (5). Thus, the authors postulated that the anergic state was induced following ligation of the TCR (signal 1) in the absence of a critical, APC-derived costimulatory molecule (signal 2). Subsequent studies implicated signaling through the B7/CD28 costimulatory pathway as a critical factor in determining the fate of Ag-SP–experienced T cells, because in the absence of CD28 stimulation, T cells fail to sustain IL-2 production and become anergic (39, 40). As further proof of principle, direct stimulation of CD28 with an agonist mAb has been reported to prevent the induction of T cell anergy by Ags cross-linked to the surface of peripheral blood in a human coculture system (41).

Although the two-signal hypothesis of T cell activation is an elegant paradigm of T cell biology, it presents a limited view of the...
events leading to T cell–mediated immunity or tolerance. Within the past two decades, it has become apparent that multiple signals converge upon a T cell to influence its fate during Ag recognition. CTLA-4 is an inhibitory receptor that competes with CD28 for B7-1/2 ligation (42–44) and is a critical regulator of T cell activation. Its role in the maintenance of peripheral tolerance is exemplified by the observation that CTLA-4 deficiency results in spontaneous autoimmunity, exacerbation of inflammation, and epitope spreading (45, 46). To this extent, CTLA-4 has been demonstrated to function at the induction and maintenance of peripheral tolerance mediated by Ag-SP treatment. Although CTLA-4 blockade prevented the induction of Ag-SP–mediated tolerance in a BDC2.5 transfer model of type 1 diabetes (47), the blockade of CTLA-4 at the time of experimental autoimmune encephalomyelitis induction also abrogated tolerance in mice that had been previously treated with Ag-SP (48).

Similarly, PD-1 signaling has been significantly implicated in tolerance mediated by Ag-SP treatment. Tolerance induction and maintenance in the BDC2.5 transfer model of diabetes was susceptible to PD-1/PDL-1 blockade (47), and we also have demonstrated that treatment with allogeneic Ag-SP failed to prevent islet allograft rejection when administered to PDL-1−/− graft recipients (6). Although the roles of CTLA-4 and PD-1 may at first appear redundant, it is possible that although CTLA-4 limits the priming and activation of naive T cells (49), PD-1 may function to inhibit the reactivation and function of T cells at the effector stage through the modulation of TCR signals (50, 51).

The presentation of Ag to cognate TCRs results in formation of the immunological synapse (IS), an area of structural rearrangement and molecular clustering around the TCR at the T cell–APC interface (52). The IS plays a pivotal role in the activation of T cells during antigenic stimulation: although synapse formation is dictated by the quality of the Ag being presented (53), its organization also affects the strength of TCR signaling (54, 55). Multiple adhesion and costimulatory molecules are present at the IS that augment Ag processing, including CD4/CD8, LFA-1, CD2, and CD28 (56), which has been reported to directly facilitate TCR signal transduction at the IS (57, 58). Interestingly, both CTLA-4 and PD-1 are recruited to the IS where they have been reported to abrogate TCR stop signals and limit TCR signal transduction (51, 59). CTLA-4 in particular is recruited to TCR-CD28 microclusters where it competes with CD28 to prevent the recruitment of key signaling molecules (60), and a similar role for PD-1 also has been described previously (61). Although the initial characterization of anergy in vitro reported no defect in TCR stimulation during the encounter with Ag-SP (5), anergy induction may result in a loss of the ability to receive sufficient stimulation through the TCR during subsequent interaction with cognate peptide/MHC ligands in addition to blocking positive costimulation. In this context, it is interesting to speculate that anergy maintenance may in function constitute a de facto form of immunological ignorance.

The CD40/CD154 pathway is a potent activator of the innate immune system and exerts significant influence on T cell activation. The positive costimulatory ligand CD154 is expressed by T cells during TCR stimulation, whereas its receptor, CD40, is functionally expressed by APCs and accumulates at the IS during T cell activation. We have observed that CD154 is weakly expressed by OT-II T cells during Ag encounter in vitro if they were previously tolerized to OVA323–339−SP in vivo (14), confirming a prior study on anti–CD3-induced anergy (62). Anergy induction could be reversed in vivo if Ag-SP was delivered in the presence of an agonist anti-CD40 Ab (14). Although CD40 stimulation upregulates the expression of B7-1 and B7-2, it also enhances expression of ICAM-1 and peptide/MHC molecules (63). By possibly stabilizing the IS and increasing the avidity of TCR-peptide/MHC interactions, CD40 stimulation may overcome the effects exerted by negative costimulation to enhance TCR signaling. With recent work highlighting the importance of TCR signal strength for efficient effector T cell activation (64), the potential for molecules such as costimulatory molecules to promote or inhibit synapse formation has important implications in clonal anergy and peripheral tolerance induction (65).

**Tolerance maintenance and TREG induction.** The coordinated response resulting in prolonged Ag-SP tolerance appears to require an early anergic phase combined with an active regulatory phase (9). Anergy induction and active suppression/regulation are often thought of as exclusive mechanisms of immunological tolerance, because it is unclear how (or rather why) the intrinsic regulation of a response by anergy might require and support extrinsic regulation of the same response by TREGs. This is especially perplexing in the context of diminished IL-2 signals that are essential for TREG biology in the periphery (66). Moreover, short-term Ag-SP tolerance can be induced in the absence of natural TREGs. Tolerance was found to be successfully induced by Ag-SP when activated BDC2.5 T cells depleted of TREGs were transferred into TCR−/− hosts that were replete with wild-type NOD CD4+CD25− T cells (47). In another study, NOD,CD28−/− mice (which are deficient in TREGs) developed autoimmune pancreatitis that could be ameliorated if mice were tolerized to the appropriate autoantigen (67). However, Ag-SP treatment has been shown to increase the frequency and number of TREGs (68), and tolerance could be partially or completely abrogated in mice that were depleted of TREGs before the time of tolerance induction (6, 28). Furthermore, the passive transfer of tolerance from an Ag-SP–treated animal to a naive mouse is critically dependent on transferring a population of cells that includes CD25+ purportedly TREGs (4). Although these reports warrant further examination of the precise role of TREGs in Ag-SP–mediated tolerance, the question remains as to how anergy and suppression are related?

We have observed that during the initial encounter with Ag-SP, some Ag-specific T cells can undergo up to 7 rounds of cell division, whereas others remain undivided as measured by CFSE dilution (28, 69). Indeed, TCR stimulation alone is sufficient to promote limited cycles of IL-2-dependent proliferation (70), but CD28 costimulation is necessary to stabilize IL-2 production and to sustain the proliferative state (71). We speculate that the immunosuppressive milieu that is present following administration of Ag-SP (9) may support the induction or expansion of TREGs responding to low levels of paracrine IL-2 transiently produced by Ag-specific conventional T cells. These TREGs in turn may further deprive Ag-SP–experienced conventional T cells of IL-2 signals during activation, thereby facilitating the establishment of anergy. This may explain why IL-2 supplementation to T cells during Ag-SP encounter failed to inhibit tolerance in vitro, although these studies were performed prior to the discovery of TREGs (72). Conversely, TREGs may also limit the activation of naive T cells by sequestering B7 molecules from APCs through the constitutive expression of CTLA-4 (73) or by suppressing APC maturation through other means. This hypothesis is consistent
with our transfer-of-tolerance experiments and our observations that long-term tolerance maintenance is dependent on TREGS (9). Mice treated with anti-CD25 at the time of PLP139–151-SP administration and then immunized 60 days later showed no protection from PLP139–151 induced experimental autoimmune encephalomyelitis disease, whereas tolerized mice that were treated with the control Ab or tolerized mice that were immunized 28–35 days post-tolerance were still significantly protected (9). It is reasonable to speculate that at >60 days posttolerance, the lymphoid organs of tolerized mice were seeded with fresh thymic emigrants of cognate specificity that were unlikely to have been Ag-SP experienced and therefore likely to have retained the potential to induce EAE upon immunization. That mice tolerized 60 days previously and treated with isotype control Ab were significantly protected from disease when compared with the anti–CD25-treated group suggests that TREGS may have limited the activation or effector function of these recent thymic emigrants. Although this interpretation would imply that TREGS induced or expanded during the induction of tolerance to Ag-SP go on to develop immunological memory, a recent report has established such a precedent (74).

Conclusions

Ag-SP tolerance induction is a highly efficient, versatile, and safe process that harnesses natural systems of apoptotic cellular debris clearance and homeostasis to induce T cell nonresponsiveness. Findings to date highlight the importance of responses in the splenic marginal zone, particularly MZMs, to Ag-SP. Furthermore, it is clear that successful long-term tolerance appears to hinge on a biphasic T cell regulation program, with energy critical for short-term tolerance, whereas TREGS expanded during early Ag-SP exposure are required for long-term tolerance. These observations have important clinical ramifications, because they suggest that current attempts to simply induce anergy or providing TREGS alone to patients suffering from autoimmunity or transplant rejection may be insufficient individually to promote long-term immune tolerance. Furthermore, these findings may spur examination of whether current immune suppressive agents, such as calcineurin and niTOR inhibitors, may inadvertently inhibit the initiation of these pathways and subvert clinical attempts to induce durable tolerance. In summary, Ag-SP have provided a sound scientific platform for determination of cellular and molecular mechanisms critical for the induction of peripheral tolerance and have been proven safe and effective in early clinical studies for treatment of human autoimmune disease (8). How the extensive manipulation and processing of autologous PBLs required for preparation of Ag-coupled tolerogen may affect the widespread clinical applicability of this therapy is not yet known, nor is the potential for transmission of blood-borne pathogens in the context of alloantigen-specific tolerance. However, based on investigation of the mechanisms underlying Ag-SP tolerance, Ag-NPs (referred to as tolerizing immune modifying nanoparticles), have been shown to serve as surrogates for apoptotic Ag-SP, inducing efficient tolerance by targeting to MZMs (28) and are currently in clinical development.

Disclosures

The authors have no financial conflicts of interest.

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