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Natural IgM Is Required for Suppression of Inflammatory Arthritis by Apoptotic Cells

Clare A. Notley, Mark A. Brown, Graham P. Wright, and Michael R. Ehrenstein

The clearance of dying cells is vital for re-establishing tolerance during inflammation and has potent immunoregulatory consequences. Because natural IgM plays a key role in the removal of apoptotic cells, we investigated whether the immune modulatory properties of apoptotic cells depended on its presence. Using an Ab-independent, Ag-induced model of inflammatory arthritis, we tested whether natural IgM is essential for the arthritis-suppressing properties of apoptotic cells. Whereas administration of apoptotic cells reduced joint inflammation and damage in normal mice accompanied by suppression of the Th17 response, no protection was afforded in secreted IgM-deficient (S\(\mu\)\(^{-}\)) mice. The enhanced production of IL-10 by T cells from draining lymph nodes and splenic marginal zone B cells, driven by the infusion of apoptotic cells, was abrogated in the absence of natural IgM. Apoptotic cells were present shortly after administration in the splenic marginal zone, but their removal was substantially delayed in the absence of natural IgM. Incubation of apoptotic cells with natural IgM in vitro restored their arthritis-suppressing properties in S\(\mu\)\(^{-}\) mice. Moreover, these IgM-coated apoptotic cells were cleared rapidly after injection from the spleens of S\(\mu\)\(^{-}\) mice. Our results demonstrate that natural IgM is a critical factor in a chain of events triggered by the administration of apoptotic cells that promote IL-10–secreting B and T cells and restrain the development of inflammation. The Journal of Immunology, 2011, 186: 4967–4972.

Rapid removal of apoptotic cells (ACs) is considered central to the resolution of inflammation and in preventing autoimmune disease (1). In health, more than 1 \(\times\) 10\(^{9}\) ACs are cleared from the body each day (2), highlighting the importance of tightly regulated mechanisms to prevent the activation of proinflammatory responses. ACs express various autoantigens, and their persistence is thought to generate harmful autoimmunity through the activation of self-reactive cells (3, 4). Conversely, the removal of ACs can generate an immunoregulatory milieu and promote the resolution of inflammation, a process that relies on their recognition and engulfment by macrophages and dendritic cells (1, 5, 6). Indeed, infusion of ACs has been suggested as a possible therapy to induce tolerance (7). Release of IL-10, TGF-\(\beta\), or both by dendritic cells and macrophages after ingestion of ACs has been implicated in a number of model systems as the mechanism responsible for their anti-inflammatory properties (8, 9). More recently, the injection of ACs into a mouse model of inflammatory arthritis resulted in amelioration of disease via IL-10 producing regulatory B cells, although the precise mechanisms that led to their induction by ACs have not been elucidated (10). In particular, it has not been established whether the clearance of ACs is required for their arthritis-suppressing effects.

ACs display a number of unique determinants, such as phosphatidylserine, that can be recognized by distinct receptors expressed on phagocytic cells (1, 8). Natural, preimmune IgM can bind phosphatidylserine and phosphorylcholine, and it has been shown to facilitate phagocytosis of ACs by macrophages and dendritic cells either in vitro or in the peritoneum after local injection (11–13). It is unclear whether natural IgM is critical for the engulfment and removal of ACs from secondary lymphoid organs, such as the spleen. Secreted IgM-deficient (S\(\mu\)\(^{-}\)) mice are more prone to autoimmunity, which could be attributable to impaired removal of AC (14, 15). Indeed, the natural role of IgM in facilitating the clearance of ACs has provided an attractive explanation for its regulatory properties. In this study, we tested whether the arthritis-suppressing effects of ACs relied on the presence of natural, polyclonal IgM. We show that natural IgM is required for the efficient engulfment and clearance of injected ACs from the spleen in vivo. Moreover, natural IgM is essential for the immunomodulatory effects of ACs, including the induction of IL-10–secreting B cells and the suppression of inflammatory arthritis.

Materials and Methods

Mice

S\(\mu\)\(^{-}\) mice (14). B cell-deficient mice (\(\mu\)MT), or littermate C57BL/6 mice were bred and maintained in specific pathogen-free facilities under home office guidelines. Mice were used between 8 and 14 wk. All experiments were approved by the ethical review committee.

Preparation of ACs

Thymocytes were generated either by incubation with 1 \(\mu\)M dexamethasone (Sigma-Aldrich, U.K.) or 10 \(\mu\)M etoposide (Sigma-Aldrich) for 5 h at 37 C and then washed four times before in vivo transfer. The mode of preparation of ACs did not affect their capacity to suppress arthritis (unpublished observations). Cells were between 60 and 80% annexin V positive and propidium iodide negative. In some experiments, thymocytes were labeled with 1 \(\mu\)M PKH-26 (Sigma-Aldrich), and in others labeled or unlabeled ACs were incubated with 20 \(\mu\)g/ml of mouse IgM (Rockland) in PBS for 1 h and washed twice in PBS before i.v. injection.
Arthritis induction

Mice were immunized with methylated BSA (mBSA; Sigma-Aldrich) as described previously (16, 17). Seven days after intradermal immunization with mBSA and CFA, arthritis was induced by intra-articular injection of 200 μg mBSA or PBS into the joint cavity. Some mice were transferred i.v. with 3 × 10^7 ACs or 3 × 10^7 ACs precoated with IgM for 3 consecutive days after immunization. Clinical score was determined by the degree of limping, where 0 = normal walking, 1 = mild limping, 2 = severe limping, and 3 = unable to put weight on leg. Knee swelling was monitored by measuring knee diameter using dial gauge calipers (Kroepelin, Schluchem, Germany). For histopathologic examination, knees were removed, fixed in 4% formalin, decalcified in EDTA, and embedded in paraffin. Sections were stained with H&E and Safranin O. Histologic sections were scored in a blinded manner on a scale of 0–3 for degree of inflammation and joint destruction, where 0 = normal, 1 = mild inflammation/cartilage damage, 2 = moderate inflammation/cartilage destruction, and 3 = extensive inflammation/invasion of pannus and joint destruction. Total score was calculated by addition of the inflammation score and the destruction score.

In vitro assays

Inguinal draining lymph nodes were removed and stimulated with anti-CD3 mAb (0.1 ng/ml) for 72 h. Cells were incubated for the last 18 h of culture in 3 μg/ml LPS (Sigma-Aldrich). Supernatants from cultures were tested for IL-17A or IL-10 by ELISA (R&D Systems). Splenic B cells were negatively selected using CD43 acidified and subsequently tested for the presence of TGF-

Flow cytometry

Cells required for intracellular cytokine staining were cultured for 6 h in complete RPMI containing PMA (Sigma-Aldrich) and ionomycin (Sigma-Aldrich). Golgi-Stop (BD Biosciences) was added to the culture for the last 4 h. Cells were surface stained with anti–CD4-FITC or anti–CD4-PE, anti–B220-allophycocyanin, or CD19-PerCpCy7, anti–CD23-PE, anti–CD21-FITC or anti–CD23-biotin and anti–CD21-biotin, anti–CD24-biotin or anti–TGF-β-PE (R&D Systems), washed and incubated with streptavidin-allophycocyanin, streptavidin-PerCpCy7 or streptavidin-V450. Cells were then washed and stained with anti-mouse IFN-γ, anti-mouse IL-17, or anti-mouse IL-10 and analyzed by flow cytometry. Foxp3 staining was performed using the Foxp3 staining buffer kit following the manufacturer’s instructions (eBiosciences, Hatfield, U.K.). Abs used for confocal microscopy were CD19-biotin (BD Biosciences), CD68-PE-Cy3 (Serotec, Raleigh, NC), CD169-FITC (Serotec), and streptavidin-allophycocyanin (BD Biosciences). All Abs were purchased from BD Biosciences unless otherwise stated.

Immunohistochemistry

PKH-26 labeled ACs (3 × 10^7) were injected i.v. into WT or S– mice. Spleens were harvested and frozen at indicated time points and frozen in OCT medium; 7-μm sections were cut in a cryostat microtome. Sections were left to air dry, fixed with methanol for 5 min, washed in PBS-Tween 20 and blocked for 1 h in 5% serum and 4% BSA in PBS. Sections were incubated with anti-CD19, anti-CD169, or anti-CD68 (Serotec) Abs, washed, mounted with VECTASHIELD (Vector Labs, Burlingame, CA) containing DAPI. Images were collected using a confocal scanning microscope (Leica SPE). All confocal images are taken at ×40 magnification with oil immersion. The number of ACs in one field was calculated by counting the number of ACs in one specified area of each image. Three images were taken for each section, and the mean number was calculated.
Statistical analysis
When appropriate, a one-way ANOVA or Students t test was performed. A p value < 0.05 was considered statistically significant.

Results
Natural IgM is not required for the induction of Ag-induced arthritis
To distinguish the role of natural IgM in the amelioration of inflammatory arthritis by ACs from its role in the disease process itself, it was important to use a model of inflammatory arthritis that was independent of Ab. Collagen-induced arthritis (CIA) relies on the presence of B cells and Abs, and thus would not be an appropriate model (18). We therefore tested whether Ag-induced arthritis (AIA) was affected by the absence of either B cells or natural IgM using μMT and Sμ− mice, respectively. The incidence, development, and severity of arthritis were not significantly affected in the absence of B cells (Supplemental Fig. 1A) or secreted IgM (Supplemental Fig. 1B), based on clinical score and knee swelling. Disease incidence was 100% in all groups. Consistent with these observations, we found no increase in the production of IL-12, IFN-γ, or IL-17 by splenocytes in naïve Sμ− mice (Supplemental Fig. 1C).

ACs can suppress AIA and Th17 production in WT but not in Sμ− mice
Previous data have shown that transfer of ACs at the time of immunization can suppress the development of CIA (10). Following a similar protocol, WT mice treated with ACs for 3 consecutive days from the time of immunization with mBSA and adjuvant showed suppression of clinical disease, assessed by limping, at 4 and 7 d after knee injection (Fig. 1A). Joint involvement, as quantified by inflammatory cell infiltrate and degradation of cartilage and bone, was significantly reduced in WT mice treated with ACs (Fig. 1B, 1C). In contrast, ACs were unable to suppress the development of AIA in Sμ− mice (Fig. 1). Substantial cartilage loss was evident in Sμ− mice regardless of whether ACs had been administered (Fig. 1B).

Because AIA is a T cell-dependent model of arthritis, we investigated the ability of ACs to suppress cytokine production by Th1 and Th17 subsets in the draining lymph nodes. Transfer of ACs reduced the total number and the percentage of Th17 cells in WT mice (Fig. 2A). However, the number and percentage of Th17 cells was unaffected by ACs in Sμ− mice (Fig. 2A). There was a trend (p = 0.16) toward a lower absolute number of Th1 cells following ACs in WT mice, whereas the reverse was seen in Sμ− mice treated with ACs (Fig. 2B). ELISA of supernatants from cultured draining lymph node cells confirmed that transfer of ACs suppressed IL-17 production in WT mice, but not in Sμ− mice (Fig. 2C). Furthermore, anti-CD3–driven proliferation of lymph node cells was substantially diminished by AC transfer in WT arthritic mice, whereas there was no change in proliferation of lymph node cells from Sμ− mice (Fig. 2C).

Natural IgM is required for the induction of B and T cell IL-10 production by AC
ACs can induce the production of IL-10 by different cell types in the spleen (8–10). We examined whether ACs induced IL-10 production by B or T cells in the spleens and draining lymph nodes of WT and Sμ− mice. AC administration led to an increase in the number and percentage of IL-10–producing splenic B cells (Fig. 3A). Further analysis revealed an increase in the number and percentage of marginal zone (MZ) B cells producing IL-10, which was responsible for the increase in B cell IL-10 production seen in the WT arthritic mice treated with AC (Fig. 3B). There was no significant increase in the number or percentage of splenic follicular, T2, or CD5+ B cells producing IL-10 (Fig. 3B). B cells from Sμ− mice were not intrinsically defective in their ability to
produce IL-10 as indicated by their capacity to respond to stimulation in vitro with anti-CD40 and LPS (Fig. 3C). An increase in IL-10 production by T cells isolated from lymph nodes (Fig. 4A) was also observed in WT arthritic mice treated with AC, but not from mice lacking secreted IgM that had received ACs. IL-10–producing T cells present in the lymph nodes of WT mice treated with ACs did not express Foxp3 (Fig. 4B), suggesting that ACs induce a Tr1 type of regulatory T cell. We found no increase in the number or percentage of IL-10–secreting T cells in the spleens of WT or Spμ– mice after injection with ACs (Fig. 4C). Although ACs have been shown to induce TGF-β production and regulatory T cells (8, 19), no change was seen in TGF-β serum levels (Supplemental Fig. 2A) or expression in the spleen (Supplemental Fig. 2B). In addition, there was no difference in the percentage of regulatory T cells in the spleens (Supplemental Fig. 2C) or lymph nodes (Supplemental Fig. 2D) of either WT or Spμ– mice that received AC. There was no increase in IgM or IgG anti-phosphorylcholine (PC) Abs in this model of arthritis (Supplemental Fig. 2A) or expression in the spleen (Supplemental Fig. 3), suggesting that preimmune natural Abs are critical for protection in this model.

Reduced engulfment and clearance of AC in Spμ– mice

We hypothesized that the lack of suppressive effects of ACs in Spμ– mice was due to their defective clearance from the spleen. To address this question in vivo, PKH-26–labeled ACs were injected i.v. and spleens were harvested at different time points. At 1 h after transfer, similar numbers of ACs were detectable in the spleen in both WT and Spμ– mice (Fig. 5A). At 6 and 12 h after transfer, AC numbers were similar to those measured 1 h after transfer in Spμ– mice. In contrast, AC numbers in WT mice were significantly reduced at 6 and 12 h after transfer, indicating that ACs were being efficiently cleared from the spleen only when secreted IgM was present (Fig. 5A). Confocal microscopy of spleen sections 6 h after transfer revealed that in WT mice few ACs could be identified in the spleen (Fig. 5B, 5C), whereas ACs were readily found in mice lacking secreted IgM, particularly in the MZ (Fig. 5B, 5C). Apoptotic cells were localized with macrophages around B cell (CD19) zones in the spleens of Spμ– mice (Fig. 5B, 5C).

Suppression of arthritis by AC in Spμ– mice is restored by polyclonal IgM

We next investigated whether in vitro incubation of ACs with IgM prior to their injection could restore their suppressive properties. Spμ– mice were either untreated or injected with ACs with or without preincubation with polyclonal IgM at the time of immunization and for an additional 2 consecutive days. Whereas ACs failed to suppress arthritis in Spμ– mice, in vitro incubation of ACs with IgM resulted in a significant reduction in clinical score 4 and 7 d after disease onset (Fig. 6A). Suppression of disease was associated with a reduction in Th17 cells in the draining lymph nodes (Fig. 6B). In mice treated with IgM-coated ACs, a significant increase in IL-10 production by MZ B cells (Fig. 6C) and CD4+ T cells from draining lymph nodes (Fig. 6D) was observed compared with mice that had received ACs alone. These data confirm that natural IgM is critical for the arthritis suppression properties of ACs.

Coating of ACs with natural IgM enhances their clearance in Spμ– mice

To determine whether the suppression of arthritis in Spμ– mice that received IgM-coated ACs was associated with efficient clearance of AC from the spleen, PKH-26 stained ACs with or without IgM were transferred into Spμ– mice. By 6 h after transfer, IgM-coated ACs were rapidly cleared from the spleens of Spμ– mice, similar to levels observed in WT mice (Fig. 6E). Therefore, the coating of ACs by natural IgM facilitates their efficient removal from the spleen after i.v. injection.

Discussion

These results demonstrate that natural IgM is essential for the suppression of inflammatory arthritis by transfer of ACs. In particular, natural IgM appears to govern a chain of events triggered by the clearance of ACs, restraining the development of inflammatory arthritis and promoting the generation of IL-10–secreting B and T cells. Previous data have shown that regulatory B cells mediate the protective effect of ACs in the context of inflammatory arthritis via IL-10 production (10). Our results reveal that the increased IL-10 production driven by ACs depends on the presence of natural IgM, suggesting that AC engulfment is necessary for their immunoregulatory properties in the context of inflammatory arthritis. All the immunologic and pathologic consequences of injecting ACs into mice with inflammatory arthritis, including suppression of IL-17 and T cell proliferation, were lost in the absence of natural IgM.

To our knowledge, this report is the first to demonstrate that natural IgM accelerates apoptotic cell clearance in the spleen and that precoating ACs with natural IgM facilitates their removal. The increased number of injected ACs in the spleen in the absence of secreted IgM indicates that IgM promotes their clearance from this organ and that their presence alone is not sufficient to exert an
Immunomodulatory effect. Although natural IgM is dispensable for ACs to enter the spleen, loss of this molecule leads to differences in their persistence and localization. Few ACs were found in the spleens of WT mice a few hours after injection, whereas in the absence of natural IgM, ACs were far more abundant in the splenic MZ. Previous studies have confirmed that macrophages concentrated in the MZ of the spleen are efficient scavengers, engulfing and clearing trapped ACs within 5 h of them reaching the spleen (2, 20). One report has suggested that MZ macrophages are required to regulate efficient clearance of ACs and the selective engulfment of ACs by CD8α+ dendritic cells to maintain immune suppression (6). A number of receptors for ACs have been identified on phagocytic cells (1, 8), but appear to have a limited ability to compensate for the loss of natural IgM in vivo; this reinforces the central position that IgM has in AC clearance and the immunoregulatory circuits that ensue. Incubation of ACs with polyclonal natural IgM enhanced their removal from the spleen and restored their ability to suppress inflammatory arthritis in mice lacking endogenous secreted IgM. Furthermore, all the immunologic consequences of ACs were again observed after a short preincubation of ACs with natural IgM, reinforcing the importance of natural IgM in governing the arthritis-suppressing properties of AC.

The suppression of CIA by ACs has been shown by two groups (10, 21). Our findings reveal that AC injection can also inhibit arthritis induced by mBSA, in which joint inflammation is characterized by bone erosions and a substantial inflammatory infiltrate. This model of arthritis was associated with a significant IL-17 response, which was inhibited by injecting ACs in WT mice. Production of IL-17, rather than IFN-γ that was not significantly suppressed by ACs, is considered critical in the pathogenesis of AIA (22, 23). The infusion of ACs to inhibit the differentiation of Th17 cells illustrates the potency of this approach in suppressing inflammation. It is likely that IL-10 mediates the suppression of Th17 cell development, because IL-10 production has been shown to be essential for the suppressive effects of ACs (10), and IL-10 can potently inhibit IL-17–mediated responses (24). It is also possible that IL-10–secreting B cells may account for the increased number of IL-10–producing T cells found in the lymph nodes of WT mice treated with ACs. These IL-10–secreting T cells could differentiate in the spleen after AC injection, but migrate to the lymph nodes when mice are challenged by intraarticular injection with mBSA. We found no increase in IL-10–secreting T cells in the spleen after AC injection, which could be explained by their migration to the draining lymph node. Splenic T cells were unable to suppress CIA in contrast to splenic B cells (10). B cells from IgM-deficient mice were capable of responding in vitro to stimuli known to induce IL-10 production, indicating that there was no intrinsic defect in their IL-10 response. Spontaneous IL-10 production was increased in Sμ− mice compared with WT controls, but this difference was lost in arthritic mice, raising the possibility that the inflammatory milieu inhibits IL-10 production in Sμ− mice. Nevertheless, the administration of apoptotic cells is sufficiently potent to induce a sustained increase in IL-10 production in the presence of natural IgM.

It is possible that AC administration can induce distinct regulatory cascades, because we did not observe induction of TGF-β or regulatory T cells as documented in other reports (8, 19). The mechanisms that govern whether IL-10 or TGF-β are produced after injection of ACs have not been elucidated, but may depend on either the provenance of the AC or its encounter with either macrophages or dendritic cells (25). Alternatively, the inflammatory milieu that occurs with Ag-induced arthritis may favor an IL-10 response after exposure to ACs. The favoring of IL-10 over TGF-β could also occur at the level of the macrophage subset that encounters the AC and its state of activation and location (26).

To determine whether natural IgM was required to mediate the effects of ACs to suppress inflammatory arthritis, it was important to use a model in which Abs do not play a significant role in the
development of arthritis. Consistent with previous data (16), arthritis induced by mBSA is strongly dependent on CD4+ T cells, but was not affected by the absence of B cells and therefore Abs, in contrast to other models of inflammatory arthritis such as CIA. In addition to confirming this observation, we found that natural IgM did not have a significant effect on the development of arthritis. Therefore, our data indicate that natural polyclonal IgM is not anti-inflammatory of itself in the context of arthritis, but mediates the disease-suppressing effects of apoptotic cells. Moreover, we found no increase in the splenic production of IL-17, IFN-γ or IL-17 in nonarthritic mice. An IgM monoclonal anti-PC (T15-IgM) administered i.v. has been shown to suppress CIA regardless of the presence of apoptotic cells and without inducing IL-10 production (21). These apparent differences could be explained by the polyclonal nature of natural IgM studied here compared with the single specificity of the T-15-IgM against PC. Of relevance, an earlier report demonstrated that mice with B cells that functioned through IgD rather than IgM developed significantly less CIA and a reduction in the IgG2a anti-collagen response. The authors concluded that this was due to alterations in the surface BCR (IgD) rather than an absence of secreted IgM (27). Therefore, whereas natural, polyclonal IgM is required for the prophylactic effect of ACs, it has little effect on the arthritis itself. The absence of secreted IgM has been associated with worsening of lupus (14, 15), raising the possibility that natural IgM influences autoimmunity driven by abnormal processing of ACs, and therefore not inflammatory arthritis. We were unable to detect any significant IgM or IgG anti-phosphorylcholine response in WT mice during the relatively short time course of this inflammatory model. This finding is in contrast to the effects of AC in nonarthritic mice (21), although in our study the ACs were administered only around the time of the first immunization and not repeated 1 wk later to induce a more marked immune response. It is possible that the strong immune response induced by CFA masked the development of anti-PC Abs or skewed the immune response toward other targets. Whatever the explanation, our data support the notion that natural IgM rather than an induced specificity is responsible for the clearance of the ACs and their immunosuppressive effects. It is possible that an array of specificities may be important for apoptosis cell clearance before substantial quantities of anti-PC Abs are generated. Because ACs were given only at the outset of the experiment, there would be insufficient time for an Ab response given the rapidity of apoptotic cell clearance, measured in hours rather than days or weeks. In summary, the results presented in this study demonstrate that natural IgM is necessary for efficient clearance of ACs from the spleen after their injection and that other receptors for AC cannot compensate for its absence. Moreover, the arthritis-suppressing properties of ACs require the presence of natural IgM, suggesting that the engulfment of ACs coated with IgM has potent disease-suppressing properties. Further examination of the mechanisms underpinning these effects will facilitate the adaptation of this process for the therapy of inflammation.

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Disclosures

The authors have no financial conflicts of interest.

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