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*J Immunol* 2009; 183:1346-1359; Prepublished online 29 June 2009;
doi: 10.4049/jimmunol.0900948
http://www.jimmunol.org/content/183/2/1346

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**Supplementary Material**
http://www.jimmunol.org/content/suppl/2009/06/29/jimmunol.0900948.DC1

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Regulation of Dendritic Cells and Macrophages by an Anti-Apoptotic Cell Natural Antibody that Suppresses TLR Responses and Inhibits Inflammatory Arthritis

Yifang Chen,* Sahil Khanna,* Carl S. Goodyear,2* Yong Beom Park,3* Eyal Raz,* Steffen Thiel,† Caroline Grönewall,* Jaya Vas,* David L. Boyle,* Maripat Corr,* Dwight H. Kono,‡ and Gregg J. Silverman4*

Although natural Abs (NAbs) are present from birth, little is known about what drives their selection and whether they have housekeeping functions. The prototypic T15-NAb, first identified because of its protective role in infection, is representative of a special type of NAb response that specifically recognizes and forms complexes with apoptotic cells and which promotes cell-corpse engulfment by phagocytes. We now show that this T15-NAb IgM-mediated clearance process is dependent on the recruitment of C1q and mannose-binding lectin, which have known immune modulatory activities that also provide “eat me” signals for enhancing phagocytosis. Further investigation revealed that the addition of T15-NAb significantly suppressed in vitro LPS-induced TNF-α and IL-6 secretion by the macrophage-like cell line, RAW264.7, as well as TLR3-, TLR4-, TLR7-, and TLR9-induced maturation and secretion of a range of proinflammatory cytokines and chemokines by bone marrow-derived conventional dendritic cells. Significantly, high doses of this B-1 cell produced NAb also suppressed in vivo TLR-induced proinflammatory responses. Although inductions of apoptotic cells also suppressed such in vivo inflammatory responses and this effect was associated with the induction of high levels of IgM antiapoptotic cell Abs, apoptotic cell treatment was not effective at suppressing such TLR responses in B cell-deficient mice. Moreover, infusions of T15-NAb also efficiently inhibited both collagen-induced arthritis and anti-collagen II Ab-mediated arthritis. These studies identify and characterize a previously unknown regulatory circuit by which a NAb product of innate-like B cells aids homeostasis by control of fundamental inflammatory pathways. The Journal of Immunology, 2009, 183: 1346–1359.

To defend against infectious agents, yet also guard against autoimmune disease, complex activating and inhibitory pathways have evolved that interconnect the innate and adaptive immune systems and control their activation. The innate immune system senses for threats by recognizing microbe-associated molecular motifs using limited sets of cellular receptors, such as TLR, as well as soluble immune recognition opsonizing factors, such as complement, collagen-like ligands (i.e., collectins) and C-reactive protein. Some of these receptors also bind to stress-associated proteins and other self-ligands (reviewed in Ref. 1). Professional phagocytic cells, macrophages (Mφ)3 and dendritic cells (DC) thereby respond to environmental stimuli, microbial Ags, and cytokines, which by facilitating or forbidding differentiation changes control the capacity of Mφ and DC for overall inflammatory responses as well as the immunogenicity of foreign and self-Ags.

Although the innate immune system is important or even essential for modulating lymphocyte responses, innate immune responses themselves are also reciprocally influenced by specialized tiers of the adaptive immune system, such as NK, NKT, and γδ T cells, which can recruit DC into proinflammatory responses (2). We have wondered how B lymphocytes might also affect innate responses, especially by B-1 cells, the primordial tier of the B lymphocyte compartment that is the major source of the “innate” IgM NAb’s constitutively produced throughout life and which are also involved in responses to nonprotein Ags (3). This distinct set of self-replenishing mature B lymphocyte have been described as innate-like as they express a restricted and recurrent Ab repertoire that arises by a programmed sequence during immune development (3, 4). Indeed, certain B-1 cell clones appear to have regulatory roles through effects on innate immune cells even at remote sites in the body (5), although how this might occur is not known.

The prototypic T15 B-1 cell clonotype, defined by H-L paired canonical Ab gene rearrangements without hypermutation, was first characterized 40 years ago (6) with later repeated independent isolations (e.g., S107 (7), HPCM2 (8), EO6 (9), and others). T15

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Received for publication March 26, 2009. Accepted for publication May 12, 2009.

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1 This work was supported by Grants AI40305, AR47360, AR50659, and AI46637 from the National Institutes of Health, the Alliance for Lupus Research and Within Our Reach grant from Research Education Foundation (to G.J.S.), and an Arthritis Foundation fellowship (to S.K.) and Innovative Research Grant (G.J.S.).

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5 Abbreviations used in this paper: Mφ, macrophage; BM, bone marrow; CI, collagen type II; DC, dendritic cell; LDL, low-density lipoprotein; MBL, mannose-binding lectin; MDA, malondialdehyde; MHC II, MHC class II; NAb, natural Ab; ODN, oligonucleotide; PC, phosphorylcholine; poly(I:C), polyinosinic-polycytidylic acid.

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clonotypic B cells spontaneously arise and become highly represented within the first week of life, even in mice raised under germ-free conditions (10), which suggests that microbial ligands are not primary mediators of clonal selection. It has long been known that T15-NAbs bind to phosphorylcholine (PC) determinants and contribute to host defense to PC-containing pneumococci, and other microbes, and provide optimal protection from systemic infection (11). More recently, PC determinants were also identified on oxidatively modified low-density lipoprotein (LDL) generated during atherogenesis (9). Significantly, pneumococcal immunization, which induced active B cell responses that raised T15 Ab levels, greatly ameliorated the chronic inflammatory response in a murine model of hyperlipidemia and atherosclerosis (12). The mechanistic basis for these findings remains obscure, as the original hypothesis, that T15-NAB might enhance clearance of the proinflammatory oxidatively modified LDL, has subsequently been ruled out (13). Although more recent studies suggest the possibility that immunization may regulate B cells that serve as a source of inhibitory cytokines (14), we have suspected there are other Ab-mediated immune modulatory activities.

Other studies have previously shown that by immune recognition of the PC head group, T15-NAB can discriminate dead/dying cells from healthy cells (15–17). This is because the PC-head group, which is a ubiquitous component of cell membrane neutral phospholipids (e.g., phosphatidylycerol), is embedded within the lipid bilayer in healthy cells and therefore inaccessible to Abs unless exposed by membrane changes that occur during apoptosis (15–17). Importantly, we have shown that T15-NAB is structurally and functionally representative of the PC-specific anti-apoptotic cell Abs that are induced in vivo by apoptotic cell infections (18).

Herein, we show that the prototypic T15-NAB can play a general role in modulating innate immune responses by inhibiting the activation of phagocytes and thereby suppressing in vitro and in vivo inflammatory responses. We further show that these regulatory properties derive from the capacity to complex with apoptotic cells and recruit soluble innate immune recognition molecules, which together enhance uptake and clearance of apoptotic cells and inhibit TLR-induced phagocyte activation and maturation.

Materials and Methods

Antibodies

T15-IgM (from the E06 hybridoma) (9) and the IgM isotype control from the hybridoma, NC17-D8 (gift from L. Arnold, University of North Carolina, Chapel Hill, NC), both express J-chain transcripts. Hybridomas were grown under serum-free conditions in hollow fiber (10,000 MWCO) bioreactors in hybridoma serum-free media. For heat inactivation, Ig-deficient sera were incubated for 30 min at 56°C.

For stimulation studies, DC were further cultured for 24–48 h without/with agonists for TLR3, poly (I:C) at 3.3 μg/ml; TLR4, LPS at 0.1 μg/ml; TLR7, imiquimod (InvivoGen) at 1 μg/ml; or TLR9, phosphorothioate CpG oligo 1018 at 0.5 μg/ml for overnight culture.

Bone marrow (BM)-derived DC

BM cells from C57BL/6 femurs/tibias were washed and cultured in RPMI 1640 containing 10% FBS, glutamine, and 0.01 M HEPES, and then maintained for 30–45 days, by National Cell Culture Center. Supernatants were purified with a 300-kDa tangential flow ultrafiltration device, followed by a 104-kDa tangential flow ultrafiltration for further concentration, then dialyzed against PBS (pH 7.2), with documented low lysis in mouse (20, 25). Although more recent studies suggest the possibility that immunization may regulate B cells that serve as a source of inhibitory cytokines (14), we have suspected there are other Ab-mediated immune modulatory activities.

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Ab assays

Standard sandwich ELISA were performed with precoats of goat anti-IgM, PC-albumin for control Abs, and with detection with either biotinylated AB1–2 or FIJC anti-IgM, or anti-IgG, as described previously (20). Assays were adapted to buffer usage required to detect mannose-binding lectin (MBL) binding, as previously described (21), with limits of detection of ~5 ng/ml. In these studies, MBL binding by IgG could not be detected in sera either before or after thymocytes immunization. Array studies were performed as described previously (22).

Mice

Age and gender-matched adult C57BL/6, congenic B cell-deficient muMT, BALB/c, and DBA/1 mice were provided by The Jackson Laboratory or bred under specific pathogen-free conditions as supervised by University of California San Diego (UCSD) Animal Care Program. All animal protocols were approved by the UCSD Institutional Animal Care and Use Committee.

In vitro complement deposition

Apothecary thymocytes were incubated at 37°C with IgM at 20 μg/ml in TBS with 10 mM CaCl_2 and/or TBS with 20% Ig-deficient plasma for complement or TBS alone. After 40 min, cells were washed and studied for apoptosis (7-aminoactinomycin D (7-AAD) and annexin V), and with aliphohyocyanin-labeled goat anti-IgM, and anti-Clq (goat; Cedarslane Laboratories), or human recombinant MBL (6 μg/ml) and biotinylated mouse anti-human MBL (clone 131-1), or biotinylated rat anti-murine MBL A (clone 2B4) anti-murine MBL C (clone 14D12) (21), in the presence of Fc block (23).

In vivo apoptotic clearance assays

Using a standard in vivo approach (24), B cell-deficient mice received thioglycollate treatment and, 3 days later, received i.v. PBS or 1 mg of IgM. After 16 h, 5 × 10^8 SNARF-1-labeled apoptotic or fresh thymocytes were instilled, then peritoneal cells recovered after 10 min. For immunofluorescence microscopic studies, cytospins were prepared, and Mδ were stained with FITC-anti-F4/80, with >800 Mδ counted per mouse and the proportion determined of recovered Mδ that had ingested (and not just surface bound) one or more labeled thymocytes. Although longer time periods were also examined, 10 min of in vivo exposure yielded the greatest differences between groups, as described previously (25). Although dexamethasone-treated thymocytes yielded similar results, most studies used etoposide for apoptosis induction due to >95% Annexin V (i.e., apoptotic) thymocyte yields by flow cytometry. In other studies, to quantitate Mδ uptake, flow cytometric analyses were performed with 7-AAD and annexin V staining of apoptotic thymocytes that were tracked via CD3+CD4+CD8+ cell cultures

Cells were grown to 80% confluence with ~10^6 cells per well in 48-well plates in RPMI 1640 and 10% FBS, glutamine, and 0.01 M HEPES, then serum-starved overnight. To some, replicates were preincubated with T15 IgM or isotype control for 1 h, followed by addition of LPS (Escherichia coli 055:B5; List Biological Laboratories) at 0.1 μg/ml or polynosinic-polycytidylic acid (poly(I:C)) (Amersham Biosciences) at 3.3 μg/ml for overnight culture.

Bone marrow (BM)-derived DC

BM cells from C57BL/6 femurs/tibias were washed and cultured in RPMI 1640 containing 10% FBS 1% pen-strep-glutamine, GM-CSF (10 ng/ml), and IL-4 (400 pg/ml) and replenished on day 3 (26). On day 6, DC were selected in the presence of Fc block with magnetic anti-CD11c beads using LS magnetic columns (Miltenyi Biotec) to >94% CD11c+ purity. For phagocytosis assays, DC were cultured with GM-CSF but without IL-4 and harvested on day 5; CD11c+ cells were purified, then cultured at 0.5 × 10^6/100 μl in 96-well plates overnight in a 1:1 ratio with CFSE-labeled healthy or etoposide-induced apoptotic cells, as described previously (18). Some studies instead used STEMSPAN SF Expansion (StemCell Technologies) serum-free media. For heat inactivation, Ig-deficient sera were incubated for 30 min at 56°C.

For stimulation studies, DC were further cultured for 24–48 h without/with agonists for TLR3, poly (I:C) at 3.3 μg/ml; TLR4, LPS at 0.1 μg/ml; TLR7, imiquimod (InvivoGen) at 1 μg/ml; or TLR9, phosphorothioate CpG oligo 1018 at 0.5 μg/ml. Replicate cultures included serial concentrations of T15-IgM or IgM isotype control. Other cultures included blocking Ab to IL-10 or isotype control (R&D Systems) with Fc block, as per manufacturer’s directions. Cultures with T15-NAB blockade with AB1–2 anti-Id or isotype control also included Fc block. To assess DC maturation, cells were contained with PE-anti-mouse CD80 (clone 16-10A1) and for intracellular Alexa Fluor 647 anti-IL-12 p40 (clone C17-8), as per manufacturer’s protocol (eBioscience). qPCR was performed as described previously (20).

DC isolation

Splenic DC were isolated, as previously reported (26), then evaluated by flow cytometry for defined DC subsets and maturation/activation markers. Transcript analysis after 6 h of in vitro stimulation of CD11c-enriched BM-derived DC were performed by TaqMan (Applied Biosystems), using manufacturer’s directions.
In vivo challenge assays

On the basis of the pilot studies with outcomes assessed after weekly treatments, we selected a 2-wk treatment period, which is also the turnover period of most DC populations from stem cells (27). Hence, groups of adult C57BL/6 received three i.p. infusions (days 0, 7, and 14) of 1.5 mg of T15-IgM or isotype control. To assess the role of PC-binding specificity, some groups received 1.5 mg of T15-IgM incubated with 2 mg of PC-BSA for 30 min at room temperature before infusion. Other groups received i.v. 2.5 × 10⁷ freshly isolated (healthy), apoptotic, or necrotic (by repeated freeze-thawing) thymocytes in PBS, with bleeds obtained on day 16. To induce apoptosis, congenic murine thymocytes either received 600 rad using a 137Cs emission source of radiation or were treated with 10 μM etoposide, then incubated overnight in complete media at 37°C with 5% CO₂, then washed three times in media before use. Alternatively, mice received T15-NAb with 2 mg of PC-BSA (Biosearch Technologies) or BSA as a control. On day 17, at 18 h before sacrifice, mice received saline or challenge with 100 μg of poly(UUC), 30 μg of LPS, 100 μg of imiquimod, or 200 μg of PT Cpg ODN1018. As pilot studies did not demonstrate in vivo activation after imiquimod treatment, we instead used 300 μg of SM-360320 (28), because of 100-fold greater potency. Mice were bled at sacrifice, and suspensions of splenocytes and other lymphoid organs were evaluated by flow cytometry using standard Abs and methods (BD Pharmingen) (17, 20). Ab immunosassays and inhibitions were performed with PC-BSA, ABA-BSA (Biosearch Technologies), or BSA (Sigma-Aldrich) using IgG (sub)class and T15 clonotype-specific Abs, as described previously (20). Soluble factors in DC supernatants and sera were evaluated by Luminex assay (Invitrogen) or ELISA (BioSource International).

Inflammatory arthritis models

For CIA studies, 8-wk-old DBA/1 male mice were immunized with avian CII/CFIA (Chondrex) at the tail base on day 0 and i.p. boosted on day 21 with CII/IFA. Anti-CII Ab levels were assessed, per manufacturer’s instructions (Chondrex). For histologic analyses, paws and knees of mice sacrificed on day 44 were decalcified, embedded, and sectioned. H&E-stained slides were scored for inflammatory infiltrates and joint erosions, and safranin O was stained for cartilage damage (29). Collagen Ab-induced arthritis was induced in BALB/c mice with 2 mg of CII-specific monoclonal IgG mixture injected i.v. on day 0, and 72 h later, each animal received 50 μg of LPS E. coli 011B4 i.p. (Arthogen-CIA kit; Chemicon International). Different groups received T15-IgM or control IgM at 2 mg, or buffer, given as a pretreatment and every 7 days thereafter. Clinical arthritis was scored visually from 0 to 4 per paw, with a maximum score of 16 (29).

Statistical analysis

Values are reported as mean ± SEM unless otherwise stated. Significance was assigned for p < 0.05 by two-tailed t test, with Welch correction, or ANOVA, as appropriate (Instat; GraphPad).

Results

NAb enhances local deposition of C1q and MΦ on apoptotic cells

To understand the immune-modulating properties of T15-NAb, we first characterized its Ab-effector capabilities and then assessed how these may affect the innate immune system. In earlier studies, purified monoclonal T15 clonotypic Abs were shown to recognize a subset of dying cells at both early (Annexin V⁻/7-AAD⁻) and late (Annexin V⁻/7-AAD⁺) stages of apoptosis in a PC-inhibitable fashion (17). As a physiologically relevant source of soluble opsonins, we used sera from B cell-deficient murine muMT mice that are therefore deficient in IgG (30). Whereas incubation with IgG-deficient sera results in low-level deposition of C1q on apoptotic cells (31), we found that the addition of T15-NAb of the IgM isotype increased the amount of C1q recruitment from IgG-deficient sera onto apoptotic cells (18). Notably, although neither T15-NAb nor C1q interacted with freshly isolated healthy thyocytes, T15-NAb was responsible for >3-fold relative increases in C1q deposition on cells at early stages of apoptosis, based on gating on 7-AAD⁻ cells (Fig. 1A).

MΦ is a multicellular collectin immune recognition protein that initiates the lectin pathway of complement activation, which plays a role in immune defenses but can also interact with certain self-glycoproteins (32). Although not well-known for contributions to Ab-effector functions, because of the reported roles of MΦ in apoptotic-cell recognition (23) and modulation of inflammatory responses (reviewed in Ref. 32), we also assessed the capacity of T15-NAb to recruit MΦ. Indeed, solid-phase immunosassays showed that both T15-NAb as well as the IgM-isotype control had dose-dependent binding to the labeled recombinant MΦ used to detect binding (Fig. 1B). However, only T15-NAb recognized the PC-albumin coated onto the wells and then also interacted with the labeled MΦ reagent. MΦ binding to T15-NAb was inhibited by mannose or N-acetylglucosamine, but not by N-acetylgalactosamine, and was also calcium-dependent (Fig. 1B), indicating that the carbohydrate recognition domain of MΦ is responsible for these IgM interactions presumably through Fcγ-associated N-glycans (33). In contrast to a single report that binding of a recombinant IgM-Ab to an experimental Ag disallows constant region interactions with MΦ (33), we found that T15-NAb, but not the isotype control, was capable of concurrent binding interactions with both PC and MΦ (Fig. 1B). This indicated that binding interactions with T15-NAb could potentially amplify recruitment of MΦ to immune complexes.

We therefore examined if T15-NAb could promote binding of human recombinant MΦ to apoptotic thyocytes. As previously reported (23), incubation with MΦ alone resulted in direct deposition of only low levels of this opsonin, predominantly on thyocytes at late stages of apoptosis and those undergoing secondary necrosis. By contrast, the addition of T15-NAb significantly enhanced MΦ deposition with the greatest increases on thyocytes at early stages of apoptosis (Fig. 1C and Supplemental Fig. 1). In further analyses, murine MBL-specific Abs (21) were used to directly detect mouse MΦ deposited on apoptotic cells from IgG-deficient sera. In this study, these specific detection Abs showed that T15-IgM similarly induced the recruitment of both MΦ A and C gene products from sera, either separately (data not shown) or together, and the specificity was again confirmed as these interactions were inhibited by mannose (Fig. 1D). Thus, a major function of T15-NAb is the recruitment of both C1q and MΦ to primarily early, but also late, apoptotic cells.

NAb enhances in vivo MΦ clearance of apoptotic cells

To assess whether the T15-NAb can affect the phagocytic clearance of apoptotic cells, we used a standard sterile peritonitis model (24) with B cell/Ig-deficient muMT mice, which received pretreatment infusions of either T15-IgM, control isotype IgM, or saline. Mice were then injected i.p. with labeled apoptotic thyocytes, and 10 min later, peritoneal MΦ were recovered and examined for phagocytized thyocytes (24). We found that in saline- or IgM isotype-treated mice, a mean of ~23% of recovered MΦ had engulfed a labeled apoptotic cell or bleb, whereas after T15-IgM treatment, the proportion of MΦ with ingested apoptotic thyocytes/fragments increased to ~36%. Hence, T15-IgM treatment resulted in a 50–60% increase in the level of apoptotic phagocytosis, compared with the isotype control (p < 0.0004; Fig. 1E). By contrast, we found that in T15-IgM-treated mice, after injection of labeled healthy thyocytes <3% of recovered peritoneal MΦ had engulfed a labeled thyocyte (data not shown). Notably, increases in the efficiency of apoptotic clearance in the same assay, akin to those mediated by T15-NAb, have also been documented when wild-type mice were compared with either C1q- or MΦ-deficient mice (24, 34). Indeed, we found that T15-NAb-coated apoptotic thyocytes formed chains and clusters, which were engulfed by peritoneal MΦ.

6 The online version of this article contains supplemental material.
Flow cytometric analysis of the recovered peritoneal cells demonstrated that T15-NAb enhanced the elimination of both early- and late-stage apoptotic cells (p < 0.004; supplemental Fig. 1). Thus, T15-NAb significantly enhanced the in vivo phagocytosis of apoptotic cells by peritoneal Mφ with an influence akin to the individual contributions of MBL and C1q for phagocytic clearance.

**NAb suppresses LPS-induced IL-6 secretion by RAW264.7 Mφ-like cell line**

As interactions with apoptotic cells are reported to blunt inflammatory responses (35) and we found that T15-NAb enhances interactions of phagocytes with apoptotic cells, we also assessed whether this NAb can affect proinflammatory responses of the
RAW264.7 Mφ-like cell line. In a representative study, T15 IgM displayed a significant dose-dependent inhibition of induced TNF-α secretion (p < 0.0001), with 55% inhibition by 1 µg/ml, 74% at 4 µg/ml, and 83% inhibition at 20 µg/ml, whereas the IgM isotype control had no effect (Fig. 2A). T15-NAb similarly inhibited TLR-induced IL-6 secretion (Fig. 2B). However, TLR-mediated stimulation also caused a significant level of cell death (data not shown), so we could not control for a requirement for apoptotic cells for T15-mediated inhibition. Nonetheless, these findings document that the T15-NAb can induce a dose-dependent inhibition of TLR4-induced Mφ secretion of the key proinflammatory factors, TNF-α and IL-6.

NAb associates with MBL and C1q to enhance DC phagocytosis of apoptotic cells

At early stages of differentiation, immature DC share many cell surface receptors as well as the phagocytic capacities of Mφ (36). We therefore used a standard culture system to generate CD11c+ immature DC (26) and studied the phagocytic capacity of BM-derived conventional DC (18, 25, 37, 38). After incubation with labeled thymocytes, the purified CD11c+ immature DC were discriminated in flow cytometry studies based on size (i.e., forward light scatter) and/or staining for CD11c (Fig. 3), and the proportion of immature DC subpopulation that ingested CFSE-tagged apoptotic cells was quantified based on the associated shift in fluorescence (Fig. 3A), using methods previously confirmed with side-by-side microscopic quantitation (18). Importantly, under serum-free conditions (i.e., devoid of Ig and opsonins), we have recently shown that DC displayed the same low frequency of phagocytosis of apoptotic thymocytes as with labeled healthy thymocytes (Ref. 18 and data not shown), which is consistent with the notion that efficient phagocytosis of apoptotic cells, compared with viable cells, is dependent on the availability of specific serum factors.

As levels of the opsonins, C1q and MBL, are reported to directly correlate with the efficiency of apoptotic cell elimination (39), we also used this system to look for potential interactions of T15-NAb NAb in serum-free media with the addition of these opsonins. In this study, we found that addition of recombinant MBL provided a significant dose-dependent increase in DC phagocytosis, with dramatic increases seen only when T15-NAb was present (Fig. 3). Strikingly, in similar serum-free cultures with a fixed amount of T15-NAb, MBL conveyed much greater dose-dependent increases in the efficiency of phagocytosis than we have found was associated with supplementation with purified C1q (Fig. 3, C and D) (18). Furthermore, in cultures with T15-NAb and the highest level of MBL, the further addition of C1q resulted in only a minor additional increases in DC phagocytosis. In fact, the level of phagocytosis seen in cultures with MBL at 20 µg/ml were comparable to those instead supplemented with Ig-deficient sera (Fig. 3, C and D). Hence, this NAb-dependent influence on innate immune function was limited by the availability of MBL and C1q, which were redundant in their capacity to enhance the influence of T15-NAb on DC phagocytosis. Unexpectedly, in this assay, MBL appeared to be more potent than C1q, because MBL alone conveyed nearly the full level of phagocytosis associated with Ig-deficient sera. We also compared levels of phagocytosis with T15-IgM (at 20 µg/ml) without the addition of sera, or with supplementation with 10% Ig-deficient sera, or after heat inactivation, which was thereby shown to reduce by 83% the contribution of serum factors to T15-mediated enhancement of iDC phagocytosis of apoptotic cells (data not shown).

To confirm the requirement of the Ag-binding specificity of the NAb, we performed studies with saturating amounts of MBL and a limiting concentration of T15 IgM. In this study, we found that with T15-NAb and MBL, the frequency of DC that engulfed apoptotic cells was still nearly twice as high as with serum-free conditions alone (Fig. 3, C and D). Importantly, preincubation with PC-BSA significantly reduced (≥50%) the T15-IgM-mediated increase in the phagocytic engulfment of apoptotic cells (p < 0.004), whereas incubation with an irrelevant control BSA conjugate instead increased phagocytosis by ~25% (p = 0.0021) (Fig. 3A). Hence, the capacity of T15-NAb to enhance apoptotic cell engulfment was also shown to be dependent on its PC-binding specificity.

NAb in association with MBL and C1q inhibits TLR-induced DC maturation

To assess the effects of NAb-apoptotic cell complexes on DC maturation, we studied how different culture conditions can affect co-expression of the membrane-associated costimulatory molecule, CD80, and intracellular IL-12 p40 expression, which can be up-regulated following TLR stimulation. Notably, control studies demonstrated that even without TLR agonists, these primary CD11c+ DC display a range in their phenotype (Fig. 4A), which in part reflects the persistent influence of GM-CSF and IL-4 (40). At first, we confirmed that either LPS or poly(I:C) induced the maturation of DC, based on evidence of decreased representation of less mature (i.e., CD80+Il-12p40−) DC, compared with culture without these stimuli (p < 0.0001) (Fig. 4A).

We next evaluated whether the addition of a large number of apoptotic cells alone can affect DC maturation. Notably, we uniformly found that in DC cultures stimulated with LPS or poly(I:C), the addition of equal numbers of apoptotic thymocytes significantly inhibited the TLR-induced DC maturation. This result was
were phagocytosed by viable immature DC (supplemental Fig. 2), increases in AC engulfment increases in the presence of T15-IgM (at 20 μg/ml) by control protein conjugate (ABA-BSA). PC significantly inhibited T15-NAb-mediated enhancement of AC phagocytosis, as shown in data compiled from replicate cultures. C. MBL is responsible for large dose-dependent increases in AC engulfment increases in the presence of T15-IgM (at 20 μg/ml), with significantly less effects in cultures without IgM or with isotype control (20 μg/ml). D. Replicate cultures demonstrate equivalent significant T15-IgM-mediated increases with addition of Ig-deficient sera, whereas C1q also conveys significant but smaller increases in T15-NAb-dependent AC phagocytosis, as reported recently (18).

We therefore tested the hypothesis that T15-NAb can suppress TLR-induced DC maturation, in the presence of suitable opsonins, and only the limited number of the dead cells/fragments that are continuously generated in culture. We uniformly found that addition of T15-NAb increased the proportion of less mature DC, compared with cultures with isotype control or without IgM (Fig. 4). This was also found in T15-NAb-containing cultures without supplemented opsonic factors, may reflect the potential carryover of serum factors on the apoptotic thymocytes, or the production by immature DC of small amounts of C1q and MBL (42, 43) and possibly other factors. Nonetheless, T15-NAb significantly suppressed DC maturation in cultures stimulated with either poly(I:C) or LPS (p < 0.05). There was also a trend toward less mature DC in cultures with T15-NAb but without TLR agonist (Fig. 4, B and C), presumably due to blunting of the residual influences of GM-CSF and IL-4.

There was a consistent hierarchy in the effects of C1q and MBL on T15-NAb suppression in these replicate LPS or poly(I:C)-stimulated serum-free cultures. The least inhibition of DC maturation was found in the absence of additional supplements, with greater inhibition with the addition of C1q, and even greater suppression with MBL. The greatest inhibition was seen when T15-NAb was added to TLR-stimulated cultures that had been supplemented with both C1q and MBL, because this resulted in significantly more immature DC than other T15-NAb-containing cultures, whether or not MBL or C1q were added (p < 0.015). Indeed, T15-NAb, in the presence of both C1q and MBL, effectively blocked DC maturation induced by LPS (Fig. 4B) or poly(I:C) (Fig. 4C) to the level found in cultures without TLR agonist and T15-NAb.

**NAb inhibits in vitro inflammatory responses of DC**

We next assessed whether T15-NAb, which binds apoptotic material in culture and enhances phagocytosis, can modulate other features of in vitro responses of DC in sera-containing media with a broad range of agonistic TLR ligands, including poly(I:C), LPS, imiquimod, and CpG DNA. Indeed, inhibition was again documented for surface maturation/activation markers, MHC class II (MHC II), CD40, CD86, and CD80 (Fig. 5A and our unpublished data) and for secretion of proinflammatory cytokines (TNF-α, IL-12, IL-6, IL-10, and IL-1ra) and chemokines (IL-8, CXCL10, and CXCL12).

**FIGURE 3.** T15-NAb enhances in vitro phagocytosis of apoptotic thymocytes by conventional DC. BM-derived purified DC were cultured with equal numbers of CFSE-labeled apoptotic cells in serum-free media for 1 h. Cultures were supplemented with IgM, purified human C1q, recombinant human MBL, or Ig-deficient murine sera as indicated. For analysis, cell fragments were removed based on forward scatter, which enabled the discrimination of the proportion of DC that have engulfed apoptotic cells (AC). A. Cultures included limiting concentrations of T15 or isotype control IgM (5 μg/ml), with saturating amounts of MBL (40 μg/ml), as indicated. High levels of AC phagocytosis requires coculture with both T15-NAb and MBL, whereas the level of phagocytosis is inhibited by preincubation with PC-BSA (50 μg/ml) but not by control protein conjugate (ABA-BSA). B. PC significantly inhibited T15-NAb-mediated enhancement of AC phagocytosis, as shown in data compiled from replicate cultures. C. MBL is responsible for large dose-dependent increases in AC engulfment increases in the presence of T15-IgM (at 20 μg/ml), with significantly less effects in cultures without IgM or with isotype control (20 μg/ml). D. Replicate cultures demonstrate equivalent significant T15-IgM-mediated increases with addition of Ig-deficient sera, whereas C1q also conveys significant but smaller increases in T15-NAb-dependent AC phagocytosis, as reported recently (18).
IL-6, and IL-12p70), CC chemokines (KC, MCP-1, and MIP-1α), and CXC chemokine (IP-10) (Fig. 5B and our unpublished data). By real-time PCR analysis, T15-NAb also inhibited LPS induction of TNF-α, IL-1β, IL-6, and IL-12 transcripts (Fig. 5C and our unpublished data). By contrast, at even high concentrations, the B-1 cell-derived IgM isotype control, which showed only minor binding to late-stage apoptotic cells, resulted in little or no inhibition. Further studies showed that T15-NAb-mediated inhibition of IL-6 production was >80% reduced by a T15-specific idiotypic Ab that blocks the T15 PC binding site (19). Hence, our findings support the hypothesis that the specific interactions of T15-NAb with dead and dying cells can inhibit DC maturation and suppress activation-associated expression of cytokine and chemokine factors.

To assess for potential pathways responsible for these T15-NAb-mediated inhibitory activities, we first examined expression of IL-10 and TGF-β1, which are both implicated in the inhibitory properties of regulatory DC responses. Neither, however, were induced, at either the transcript or protein level by T15 exposure, and in fact, T15-NAb inhibited the LPS-mediated induction of IL-10 (Fig. 5C and our unpublished data). The suppressive effects of T15-NAb were also unimpaired by IL-10-neutralizing Abs or in DC from IL-10-deficient mice (our unpublished data).

**T15-NAb inhibits in vivo inflammatory responses**

To determine whether T15-NAb can also inhibit in vivo inflammatory responses, we investigated the effects of infusions of purified T15-IgM on in vivo innate immune proinflammatory responses. Indeed, after 2 wk of T15-NAb exposure, which corresponds to the approximate turnover period for DC populations (27), the T15-NAb group had 17–21% less splenic CD11c<sup>hi</sup> DC (p < 0.02, n = 7–8/group) and significantly lower levels of surface-expressed MHC II (p < 0.02), which is consistent with evidence that T15-NAb can inhibit in vitro DC maturation. Importantly, responses to the TLR agonists poly(I:C) (TLR3), LPS (TLR4), and CpG nucleotides (TLR9) were also inhibited by T15-NAb pretreatment, with impaired induction of activation and maturation markers, CD86 and MHC II, on splenic Mφ and CD11c<sup>hi</sup> DC (Fig. 6, A and B). Furthermore, T15-NAb also significantly inhibited responses to the potent TLR7 agonist, SM-360320 (28) (Fig. 6A), as well as poly(I:C) induction of other costimulatory molecules such as CD40, CD80, and B7-DC (our unpublished data). T15-NAb treatment also blunted poly(I:C)-induced blood levels of proinflammatory cytokines (IL-6, IL-12, IL-17, and TNF-α) and chemokines (MIP1α, MCP-1, KC, and IP-10) (Fig. 6C). In addition, NAb treatment significantly reduced the production of IL-6 and IL-12 by peritoneal Mφ (our unpublished data). Confirming the role of the PC-binding specificity, preincubation of apoptotic cells with an excess of PC-conjugate before T15-NAb infusion antagonized T15-IgM on in vivo innate immune proinflammatory responses. Indeed, after 2 wk of T15-NAb exposure, which corresponds to the approximate turnover period for DC populations (27), the T15-NAb group had 17–21% less splenic CD11c<sup>hi</sup> DC (p < 0.02, n = 7–8/group) and significantly lower levels of surface-expressed MHC II (p < 0.02), which is consistent with evidence that T15-NAb can inhibit in vitro DC maturation. Importantly, responses to the TLR agonists poly(I:C) (TLR3), LPS (TLR4), and CpG nucleotides (TLR9) were also inhibited by T15-NAb pretreatment, with impaired induction of activation and maturation markers, CD86 and MHC II, on splenic Mφ and CD11c<sup>hi</sup> DC (Fig. 6, A and B). Furthermore, T15-NAb also significantly inhibited responses to the potent TLR7 agonist, SM-360320 (28) (Fig. 6A), as well as poly(I:C) induction of other costimulatory molecules such as CD40, CD80, and B7-DC (our unpublished data). T15-NAb treatment also blunted poly(I:C)-induced blood levels of proinflammatory cytokines (IL-6, IL-12, IL-17, and TNF-α) and chemokines (MIP1α, MCP-1, KC, and IP-10) (Fig. 6C). In addition, NAb treatment significantly reduced the production of IL-6 and IL-12 by peritoneal Mφ (our unpublished data). Confirming the role of the PC-binding specificity, preincubation of apoptotic cells with an excess of PC-conjugate before T15-NAb infusion antagonized >78% of the in vivo inhibitory effects. Hence, elevated levels of T15-NAb drastically reduced the in vivo responsiveness of the innate immune system to a range of proinflammatory stimuli.

**Apototic cells induce anti-inflammatory NAbs with the properties of T15**

We reasoned that apoptotic cells might be the main antigenic target for in vivo T15-related NAb responses. To examine the in vivo relationship between T15-NAb, apoptotic cells, and inflammation, we infused large numbers (25 × 10<sup>6</sup>) of apoptotic thymocytes into naive mice that had low but detectable natural T15 levels (supplemental Fig. 3, A with data deposited in the GEO repository under accession number GSE14969; www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc = GSE14969). Notably, this treatment also blunted Mφ and DC activation responses (Fig. 6, B and C). Moreover, cotreatments of apoptotic cells plus T15-NAb trended toward greater suppression of in vivo poly(I:C)-induced activation responses, compared with either apoptotic cells alone, or apoptotic cells plus isotype control (Fig. 6, B and C). Inhibition was also
FIGURE 5. T15-NAb treatment blunts in vitro DC responses to TLR agonists. A, CD11c⁺-selected myeloid DC were cultured in replicate with agonists for TLR3 (poly(I:C)), TLR4 (LPS), TLR7 (imiquimod), or TLR9 (PT CpG ODN1018), without or with T15-IgM or isotype control at indicated concentrations. A, Histograms of MHC II and CD40 on DC after culture without or with stimulant (indicated above panel) are depicted. Mean fluorescence intensity is listed without and with IgM, at indicated concentrations. B, Supernatants from these overnight cultures of conventional BM-derived DC were assessed for levels of proinflammatory cytokines and chemokines, which were determined from standard curves (mean ± SEM). Results are shown without (none) or with stimulants (poly(I:C), pIC; imiquimod, imiq) without or with T15-IgM, isotype IgM control, at 10 μg/ml. The isotype control was associated with minor inhibition of MCP-1. C, Transcript levels were determined by real-time PCR for murine BM-derived CD11c⁺ DC, under indicated cultured conditions over time (minutes). DC were preincubated with T15-NAb or isotype control before time “0” sampling, then LPS was added. Amplification for TGF-β is β1 isoform specific. Results are representative of three or more independent experiments.
observed for peripheral blood proinflammatory cytokines and chemokines (Fig. 6C). Hence, in vivo apoptotic-cell treatment unexpectedly produced a similar inhibitory effect as demonstrated for T15-NAb.

Although apoptotic cells were anti-inflammatory, primary necrotic thymocytes, which can exacerbate autoimmune disease (44), were not. In fact, infusion of necrotic cells alone significantly increased the expression of activation markers after poly(I:C) agonist challenge, dark solid line; isotype control followed by TLR agonist challenge from thick gray dashed line; bottom, T15-NAb followed by TLR agonist, black dashed line. Compared with naive mice, challenge with TLR agonist induces high expression levels of MHC II and CD86 but was inhibited by T15-NAb. B, T15-IgM or apoptotic cell treatments inhibited poly(I:C)-induced activation of splenic Mφ (F4/80+ and myeloid DC (CD11c+)) from C57BL/6 mice. Groups received buffer alone (PBS), necrotic (Necr.) thymocytes, apoptotic (Apop.) thymocytes, isotype control (Iso IgM), T15, or the indicated combination. Results are shown for individual mice at 18 h after challenge. Horizontal bars depict mean values for each group. There were no significant differences in the representation of splenic F4/80+ Mφ and CD11c+ DC between different treatment groups. C, T15-IgM or apoptotic cell treatments inhibits poly(I:C) induction of serum levels of proinflammatory cytokines and chemokines in C57BL/6 mice. Values of p from two-tailed t test are shown. Results were pooled from three or more independent experiments.

D, Representative flow cytometric analyses after gating on early apoptotic thymocytes (i.e., Annexin V+ 7-AAD+) of IgM and MBL from sera of naive adult C57BL/6 or mice that received three weekly apoptotic cell (AC) treatments, with level of binding shown to be dose-dependent based on the percentage of sera. PC-BSA incubation greatly reduced the level of MBL deposition. Representative of three independent studies.
was also T15-NAb, MBL recruitment by postimmune anti-PC responses produced high levels of MBL-binding anti-PC Abs (384 anti-PC immunoassay and found that apoptotic cell treatments inhibited poly(I:C)-induced activation of splenic Mφ and myeloid DC in B cell-deficient (muMT) mice. Results are for mean fluorescence index from flow cytometry studies, as shown in Fig. 6. A and B. Results are for individual mice are the pooled from two independent experiments.

Extending recent observations (18), we also tested whether infusions of apoptotic cells in these studies induced relevant T15-like Ab responses similar to pneumococcal immunization (12). Indeed, 10 days after a single infusion of such cells without adjuvant, 8-fold increases in circulating IgM anti-PC levels were detected. Although responses varied in individual mice, three weekly apoptotic-cell infusions generally raised circulating levels of T15-clonospecific (19) and IgM anti-PC Abs >40-fold higher than in naive mice (p < 0.01) (supplemental Fig. 3, A and B) (18). In contrast, infusions of healthy or necrotic cells yielded only minor changes, which is consistent with findings that apoptotic cells induced ~20-fold increased numbers of PC-specific splenic IgM-secreting cells (18). In fact, apoptotic-cell infusions induced IgM Abs to PC-containing determinants to levels equivalent or higher than those that followed T15-NAb infusions (supplemental Fig. 3C), which required substantial doses due to the short half-life of IgM (45). Apoptotic cells also induced IgG anti-PC responses, but these levels were much lower (5.5 ± 2.5 μg/ml) and overwhelmingly of the IgG3 subclass, indicating a mainly T cell-independent response (supplemental Fig. 3D and our unpublished data). Hence, despite evidence that apoptotic cells may suppress some innate immune functions, i.e., treatment nonetheless induced robust B cell responses, even without the use of adjuvant.

To assess the relevance of these active immune responses to the above described T15-NAb studies, we have recently shown that incubation of sera from apoptotic cell-treated mice resulted in IgM binding to apoptotic cells and enhanced Ab-dependent C1q recruitment to apoptotic cells, both of which were inhibited by PC preincubation (18). We now used the solid-phase MBL recruitment anti-PC immunoassay and found that apoptotic cell treatments induced high levels of MBL-binding anti-PC Abs (384 ± 89 μg/ml, n = 4), whereas negligible levels were found before treatment (<2 μg/ml, p < 0.0001). Akin to the properties of the monoclonal T15-NAb, MBL recruitment by postimmune anti-PC responses was also >90% inhibited by preincubation with mannose or N-acetylglucosamine, or with EDTA in the media that demonstrated calcium dependence, whereas MBL binding was not inhibited by N-acetylgalactose (data not shown). Compared with naive sera, incubation in apoptotic-cell postimmune sera greatly increased levels of IgM binding to apoptotic cells, with similar increases in the recruitment of C1q and MBL. Notably, the greatest IgM-associated enhancement was seen on early apoptotic cells (i.e., 7-AAD) (18) (Fig. 6D). Preincubation of T15-NAb with PC-BSA also greatly reduced the deposition of IgM and also MBL (Fig. 6D) and C1q (18) on apoptotic cells. Furthermore, similar to purified T15-NAb (Fig. 3A), sera obtained after apoptotic-cell treatment, which had markedly increased levels of IgM Abs to apoptotic cells, also suppressed in vitro TLR-mediated activation of cultured DC (our unpublished data).

Suppression of inflammation by apoptotic cells requires B cells or T15-NAb

B cell-deficient muMT mice were next used to assess the requirement for IgM in the in vivo inhibitory properties of apoptotic cells. Strikingly, infusions of apoptotic cells alone had little or no effect on poly(I:C)-induced cellular activation or cytokine/chemokine responses in B cell-deficient mice (Fig. 7 and our unpublished data). In contrast, T15-NAb, but not isotype control, treatment of B cell-deficient mice induced the same blunting of TLR-induced cell activation and cytokine/chemokine production as demonstrated in C57BL/6 mice. Overall, these findings indicate that the suppressive effect of apoptotic cell infusions in vivo is dependent on the induction of Abs with certain specificities, which include anti-PC reactivity.

NAb protects from inflammatory arthritis

As inflammatory pathways involving Mφ, DC, and TLR have been implicated in the pathogenesis of autoimmune arthritis (46), we studied collagen-induced arthritis (CIA) in DBA/1 mice (47) to test the hypothesis that high levels of T15-NAb might suppress the development of inflammatory disease (Fig. 8 and supplemental Fig. 4). Significantly, pretreatment with the anti-PC NAb markedly reduced clinical disease activity, synovial leukocytic infiltrates, and bone and joint damage (Fig. 8, A–C). Notably, there were no differences in total IgG, or in IgG1 and IgG2a subclass anti-CII levels induced by collagen immunization in

**FIGURE 7.** B cell-deficient mice are not protected by apoptotic cell treatments. T15-IgM but not apoptotic cell treatments inhibits poly(I:C)-induced activation of splenic Mφ and myeloid DC in B cell-deficient (muMT) mice. Results are for mean fluorescence index from flow cytometry studies,
the different treatment groups (our unpublished data), suggesting that T15-NAb was primarily inhibiting the end organ inflammatory response. In other studies, infusions of apoptotic cells into DBA/1 mice yielded increased IgM anti-PC levels and protection from clinical arthritis, while infusions of primary necrotic cells did not (Fig. 8A).

To further define the adaptive immune systems role in this process, we studied the effects of T15-NAb on passive transfer arthritis induced by anti-CII IgG, in which lymphocytes do not play a central role (48). In this study, we again found that T15-NAb treatment significantly diminished joint swelling (Fig. 8D). Together, these findings indicate that the regulatory properties of T15-NAb in these models of arthritis act through the blunting of proinflammatory effector mechanisms mediated by the recruitment of IgG-autoantibody immune complexes.

Discussion

In health, inflammatory responses are critical for combating infection and tissue injury, but of equal importance are control mechanisms that remove dying cells and prevent overexuberant responses detrimental to the host. In this study, we document several previously unrecognized and functionally important features of the prototypic anti-PC NAb, T15. First, by virtue of its capacities for PC-specific binding to apoptotic cells, in addition to C1q (18), T15-NAb also facilitates the deposition of MBL onto apoptotic cells, particularly early apoptotic cells that are generally less susceptible to direct opsonin recruitment. Second, T15-NAb, by recruiting the deposition of both C1q and MBL, enhances the phagocytosis of early and late apoptotic cells. Third, T15-NAb, by forming complexes with MBL and C1q on apoptotic cells, can effectively suppress TLR-induced maturation of conventional DCs. Fourth, T15-NAb inhibits Mφ and DC secretion of proinflammatory cytokines and chemokines in response to agonists for a broad range of TLR, and it is also capable of inhibition of in vivo phagocyte activation and suppression of potentially harmful inflammatory responses. Fifth, infusion of high doses of T15-NAb, or large numbers of apoptotic cells that induce IgM anti-PC Abs, can inhibit the development of autoimmune inflammatory arthritis. Taken together, these findings identify a hitherto unsuspected set of regulatory functions of this type of NAb.

Our findings therefore characterize the potential functional roles of T15-NAb, which is representative of a dominant Ab response induced by apoptotic cells. Multiplex autoantigen-microarray assays have shown that apoptotic-cell treatment of C57BL/6 mice induces a dominant IgM response directed toward PC-containing Ags, including PC-albumin, pneumococcal vaccine, and capsular polysaccharide alone, or the self-Ag, oxidized LDL, with little or no reactivity to a large panel of other autoantigens (18) (supplemental Fig. 3). In C57BL/6 mice, T15-related Abs represent up to half of all induced Abs that recognize apoptotic cells (18). In fact, apoptotic cells induced relatively low levels of anti-DNA IgM (18)
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physiologic repertoire, our findings may explain why mice without circulating IgM spontaneously develop IgG-autoantibodies and lupus-like disease (53, 54), as this may result in impaired clearance of apoptotic breakdown products, as well as the loss of an NAb regulatory influence that otherwise can suppress overexuberant responses of innate phagocytes.

Although the potential immunosuppressive roles of NAbs have been generally overlooked, there is nonetheless overwhelming evidence that C1q and MBL have immune-mediating properties (32).

On the basis of evidence that humans with a homozygotic deficiency in C1q have a high penetrance of lupus-like systemic autoimmunity (55), studies of murine models confirmed this also occurs on certain genetic backgrounds and is associated with an accumulation of apoptotic bodies in the kidney and glomerulonephritis in 25% of animals (56). These data are consistent with the hypothesis that C1q deficiency, resulting in inefficient clearance of apoptotic cells, leads to the release of intracellular components, exposure of the immune system to self-Ags, and subsequent autoimmunity. Like C1q deficiency, MBL-deficient mice also display defects in apoptotic clearance and are prone to inflammatory conditions (34), although it is controversial whether MBL deficiency also influences autoimmune predisposition or disease severity (57).

Although MBL is well-known for its contributions to the clearance of microbial pathogens and apoptotic cells, its potential role in IgM-Ab effector functions has been little explored. Although these opsonins can be directly deposited at low levels onto apoptotic cells, this is primarily on late-stage apoptotic and secondary necrotic cells (23).

Our data show that MBL (and C1q) are recruited by T15-NAb to early-stage apoptotic cells, and this is associated with both enhanced phagocytosis and reduced inflammation. Significantly in vivo apoptotic-cell immunization was shown to induce IgM-anti-PC responses that also recruited MBL and C1q binding, confirming the physiologic relevance of their essential yet redundant roles in NAb-effector functions. The effector function for MBL recruitment has also been associated with B-1 cell-derived IgM Abs with a different self-specificity (58).

Apoptotic-cell phagocytosis by immature DC enables constant sampling and presentation of self-Ags in a manner believed es-

rived IgM Abs with a different self-specificity (58).

The anti-inflammatory influences of T15-NAb are no doubt aided by the capacity to flag cells and fragments at earlier stages of apoptosis for clearance.

The association of MBL with an apoptotic cell alone has been reported to be inadequate for activation of the complement cascade (23), and host cells themselves express specific complement inhibitors that also provide protection from inappropriate complement activation (61). However, other recent reports (62, 63) have shown that polyclonal serum IgM can induce the deposition of C3 products, which enable recognition by Mac-1/CR3 on Mφ also implicated in the clearance of apoptotic cells. We therefore repeated our in vitro challenge studies with TLR agonists in serum-free cultures of C3-deficient DC but found no reduced capacity of T15-NAb to enhance phagocytic clearance and inhibit inflammatory responses (18) (data not shown). Our pilot studies also indicate that the in vivo anti-inflammatory activities of T15-NAb are unimpaired in C3-deficient mice (our unpublished data). Taken together, we believe these findings rigorously rule out an absolute requirement for downstream propagation of complement pathway activation in the immune modulatory properties of T15-NAb.

Our studies highlight the otherwise overlooked capacity for Ag-specific IgM Abs to affect the fundamental process of apoptotic clearance and regulation of TLR responses. As discussed above, we postulate that T15-NAb, which recognizes apoptosis-specific determinants, by increasing the amount of C1q and MBL bound on apoptotic cells facilitates their phagocytosis and also enhances apoptotic cell-mediated inhibition of Mφ and DC activation. This process potentially involves receptors, such as CD36, CD93 αβγ, calreticulin, and the LDL receptor (LRP-1 and CD91) that have implicated in MBL- and C1q-enhanced apoptotic clearance (39, 64, 65). However the topic remains controversial, especially in light of the recent report, which also used etopside-induced ap-

optotic thymocytes, to demonstrate that LRP-1 was not essential for the enhancement of phagocytosis by murine Mφ (38). In addition, T15-NAb also appears to enhance immunomodulatory cellular interactions of complexed apoptotic cells and fragments, for which there are also a number of candidate receptors and at times conflicting literature (reviewed in Ref. 66). Yet, even though T15-

NAb enhanced the phagocytosis of cell corpses and also blunted TLR-induced DC maturation, the underlying pathways are not necessarily entirely identical, because these effects can be mediated by distinct residues in the intracellular domain of an apoptotic-cell receptor (67). Our preliminary studies indicate that the suppressive effects of T15-NAb on DC involve inhibition of inflammatory sig.

naling pathways (our unpublished observations). However, al-

though previous studies implicated IL-10 and TGF-β in the anti-infl.

flammatory effects of apoptotic cells (reviewed in Ref. 66), we were unable to find evidence to support this for T15-NAb, but this may in part reflect that such responses may primarily occur at later time points. Albeit, it remains possible that IL-10 and TGF-β might contribute to the in vivo effects of T15-NAb.

We first appreciated the prohomeostatic properties of T15-NAb in the setting of the chronic inflammatory disease, atherosclerosis. Indeed, earlier in vitro studies initially suggested that T15-IgM might act by blocking apoptotic-cell binding to elicited Mφ (15).

By contrast, we now document that these NAbs-mediated properties are linked to the contributions of the opsonins, MBL and C1q, that were likely absent or in low amounts in the earlier study (15).

Indeed, our current studies, which used more physiologically relevant assays, instead clearly showed that T15-NAb enhanced in vivo phagocytic clearance by Mφ and directly inhibited inflammatory responses. These mechanisms now appear to be a more likely explanation for previous evidence that immunization with a PC-vaccine-suppressed atherosclerosis (12).
The current studies of autoimmune arthritis models also showed that treatment with T15-NAB significantly reduced inflammatory joint injury despite grossly unimpaired collagen-specific lymphocyte responses. Our studies were limited to prevention of the initiation phase of disease. However, we believe these findings may still be clinically relevant, despite the fact that C1q and MBL deficiency do not specifically predispose to inflammatory arthritis, and such conditions are not known to result from defects in apoptotic clearance. Yet in vitro and in vivo studies in C57BL/6 did demonstrate a potent capacity to inhibit phagocyte production of TNF-α, IL-6, IL-17, IL-12, and key chemokines that recruit other cell types into inflammatory responses. Hence, we postulate that raising levels of such NAbs may provide benefits in conditions as diverse as rheumatoid arthritis, and perhaps also atherosclerosis (12), that are associated with innate immune cell recruitment and uncontrolled activation at sites of pathologic inflammation. Overall, these findings suggest that administration of T15-NAB, or induction of T15-NAB by apoptotic cells or microbial products, might be of general use for the suppression of deleterious inflammatory conditions.

Earlier studies of T15 Abs suggested that the driving force for the recurrent expression of these germline-encoded NAbs was linked to protection against microbial pathogens that bear PC molecular patterns, which is reminiscent of functions of receptors of innate immune cells. Our studies may now provide clues to unsuspected primary functional roles of the earliest B lymphocytes, the strong evolutionary pressure to maintain the anti-PC specificity, and why we are born with Abs in our bloodstreams. Indeed, NAbs with the binding properties of murine T15, to PC and other phospholipid-derived determinants, are also highly conserved in humans from birth (5, 68), with documented increases in healthy aging Shared Resource of the UCSD Moores Cancer Center. We appreciate helpful discussions with L. A. Herzenberg, M. Botto, and J. Savill. 2007. Apoptotic cells protect mice from autoimmune inflammation by the induction of regulatory B cells. 2007. Proc. Natl. Acad. Sci. USA 104: 14080–14085.


References
Supplemental Figures

Supplemental 1: T15-NAb enhances the clearance of both early and late apoptotic thymocytes. Treatment of B-cell deficient mice with T15-IgM but not isotype control enhances in vivo clearance of apoptotic thymocytes. Mice received 1 mg IgM (T15-NAb or isotype control) or saline intravenous 16 hr before i.p. instillation of fresh thymocytes or apoptotic thymocytes. At 10 min after thymocyte instillation, peritoneal cells were recovered with ice cold HBSS/EDTA, and flow cytometric analyses using staining for CD3 (for thymocytes/T cells), and to identify dying cells with 7 AAD and Annexin V (top panel). To evaluate clearance of instilled dying cells, after gating on CD3+ cells that primarily identified transferred thymocytes, the clearance of thymocytes at early stages (7AAD- AnnexinV+) and late stages of death (7AAD+AnnexinV+) were evaluated. Values represent the proportion of mononuclear peritoneal CD3+ cells at early (Annexin V+ 7AAD-) and late (Annexin V+ 7AAD+) stages of death from a representative mouse in each group. T15-NAb greatly enhanced clearance of early apoptotic cells compared to saline/negative control treatment (P=0.002), or isotype control (P=0.004). There were no differences in the numbers of overall recovered peritoneal cells. Four or more mice in each group were evaluated.

Supplemental 2: Cultures of purified BM-derived CD11c+ immature DC contain T15-NAb coated apoptotic cells, which are identified by characteristic morphologic changes of nuclear condensation, but not viable cells. The phagocytic capabilities of these cultured DC enables engulfment of cells that spontaneously die in culture, which was confirmed by Annexin V and TUNEL staining. Apoptotic cell-T15-NAb complexes, identified by APC fluorescence, are engulfed by viable DC via pseudopodia lined with CD11c. Under these same conditions, apoptotic cells were not stained by the IgM-isotype control (not shown). Differential interference contrast (DIC) microscopy.
**Supplemental 3: Apoptotic cells but not necrotic cells induce high levels of T15 anti-PC antibodies.** (A) T15-clonotypic anti-PC antibodies are induced by apoptotic cells. For assays, wells were coated with PC-albumin and developed with biotin-tagged anti-T15 clonotypic antibody (AB1-2). Values for individual mice were derived from T15-IgM standard curves, with horizontal bars representing mean values. (B) Apoptotic-cell infusions induce high levels of IgM anti-PC antibodies. For assays, wells were coated with PC-albumin and developed with tagged anti-IgM antibody. Serum values for individual mice were derived from T15-IgM standard curves, with horizontal bars representing mean values. (C) IgM-binding with an autoantigen microarray with replicate arrays reacted with monoclonal Abs (concentrations shown) or post-treatment sera at 1:200, unless otherwise indicated. Sera were obtained from naïve age-matched mice, or at d16 from mice that received three infusions of T15-IgM (T15 A and T15 B), saline (PBS A & PBS B), apoptotic (Apop) thymocytes, primary necrotic (Nec) thymocytes or freshly isolated (Healthy) thymocytes, on d0, 7 and 14. Naïve 6-month-old lupus-prone MRL/lpr mice, which have spontaneous high levels of IgM anti-PC antibodies, are included for comparisons. Results are representative of two independent experiments, with a limited version previously reported (1). (D) IgG anti-PC antibodies are induced by apoptotic cells. Values from ELISA as described above, using an IgG anti-PC Moab standard. Sera were obtained d16 from mice that received treatment of apoptotic (Apop), necrotic (Nec) or freshly isolated (Healthy) thymocytes of d0, 7 and 14.

**Supplemental 4: T15-NAb protects from inflammatory arthritis.** DBA/1 mice were immunized with CII and boosted with CII on d20. T15-NAb at 2mg/dose or control treatments were administered weekly. Images of representative paws from CIA mice in
T15-NAb-treatment and IgM-isotype control-treatment groups. Other data from these studies are presented in Figure 8.

Supplemental 1: T15-NAb enhances the clearance of both early and late apoptotic thymocytes. Treatment of B-cell deficient mice with T15-IgM but not isotype control enhances in vivo clearance of apoptotic thymocytes. Mice received 1 mg IgM (T15-NAb or isotype control) or saline intravenously 16 hr before i.p. instillation of fresh thymocytes or apoptotic thymocytes. At 10 min after thymocyte instillation, peritoneal cells were recovered with ice cold HBSS/EDTA, and flow cytometric analyses using staining for CD3 (for thymocytes/T cells), and to identify dying cells with 7AAD and Annexin V (top panel). To evaluate clearance of instilled dying cells, after gating on CD3+ cells that primarily identified transferred thymocytes, the clearance of thymocytes at early stages (7AAD- AnnexinV+) and late stages of death (7AAD+AnnexinV+) were evaluated. Values represent the proportion of mononuclear peritoneal CD3+ cells at early (Annexin V+ 7AAD-) and late (Annexin V+ 7AAD+) stages of death from a representative mouse in each group. T15-NAb greatly enhanced clearance of early apoptotic cells compared to saline/negative control treatment (P=0.002), or isotype control (P=0.004). There were no differences in the numbers of overall recovered peritoneal cells. Four or more mice in each group were evaluated.
Supplemental 2: Cultures of purified BM-derived CD11c+ immature DC contain T15-NAb coated apoptotic cells, which are identified by characteristic morphologic changes of nuclear condensation, but not viable cells. The phagocytic capabilities of these cultured DC enables engulfment of cells that spontaneously die in culture, which was confirmed by Annexin V and TUNEL staining. Apoptotic cell-T15-NAb complexes, identified by APC fluorescence, are engulfed by viable DC via pseudopodia lined with CD11c. Under these same conditions, apoptotic cells were not stained by the IgM-Isotype control (not shown). Differential interference contrast (DIC) microscopy.
Supplemental 3: Apoptotic cells but not necrotic cells induce high levels of T15 anti-PC antibodies. (A) T15-clonotypic anti-PC antibodies are induced by apoptotic cells. For assays, wells were coated with PC-albumin and developed with biotin-tagged anti-T15 clonotypic antibody (AB1-2). Values for individual mice were derived from T15-IgM standard curves, with horizontal bars representing mean values. (B) Apoptotic-cell infusions induce high levels of IgM anti-PC antibodies. For assays, wells were coated with PC-albumin and developed with tagged anti-IgM antibody. Serum values for individual mice were derived from T15-IgM standard curves, with horizontal bars representing mean values. (C) IgM-binding with an autoantigen microarray with replicate arrays reacted with monoclonal Abs (concentrations shown) or post-treatment sera at 1:200, unless otherwise indicated. Sera were obtained from naïve age-matched mice, or at d16 from mice that received three infusions of T15-IgM (T15 A and T15 B), saline (PBS A & PBS B), apoptotic (Apop) thymocytes, primary necrotic (Nec) thymocytes or freshly isolated (Healthy) thymocytes, on d0, 7 and 14. Naïve 6-month-old lupus-prone MRL/lpr mice, which have spontaneous high levels of IgM anti-PC antibodies, are included for comparisons. Results are representative of two independent experiments, with a limited version previously reported. (D) IgG anti-PC antibodies are induced by apoptotic cells. Values from ELISA as described above, using an IgG anti-PC Moab standard. Sera were obtained d16 from mice that received treatment of apoptotic (Apop), necrotic (Nec) or freshly isolated (Healthy) thymocytes of d0, 7 and 14.
**Supplemental 4: T15-NAb protects from inflammatory arthritis.** DBA/1 mice were immunized with CII and boosted with CII on d20. T15-NAb at 2mg/dose or control treatments were administered weekly. Images of representative paws from CIA mice in T15-NAb-treatment and IgM-isotype control-treatment groups. Other data from these studies are presented in Figure 8.