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Natural IgM Anti-Leukocyte Autoantibodies Attenuate Excess Inflammation Mediated by Innate and Adaptive Immune Mechanisms Involving Th-17

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Little is known about the function of natural IgM autoantibodies, especially that of IgM anti-leukocyte autoantibodies (IgM-ALA). Natural IgM-ALA are present at birth and characteristically increase during inflammatory and infective conditions. Our prior clinical observations and those of other investigators showing fewer rejections in renal and cardiac allografts transplanted into recipients with high levels of IgM-ALA led us to investigate whether IgM-ALA regulate the inflammatory response. In this article, we show that IgM, in physiologic doses, inhibit proinflammatory cells from proliferating and producing IFN-γ and IL-17 in response to alloantigens (MLR), anti-CD3, and the glycolipid α-galactosyl ceramide. We showed in an IgM knockout murine model, with intact B cells and regulatory T cells, that there was more severe inflammation and loss of function in the absence of IgM after renal ischemia reperfusion injury and cardiac allograft rejection. Replenishing IgM in IgM knockout mice or increasing the levels of IgM-ALA in wild-type B6 mice significantly attenuated the inflammation in both of these inflammatory models that involve IFN-γ and IL-17. The protective effect on renal ischemia reperfusion injury was not observed using IgM preadsorbed with leukocytes to remove IgM-ALA. We provide data to show that the anti-inflammatory effect of IgM is mediated, in part, by inhibiting TLR-4-induced NF-κB translocation into the nucleus and inhibiting differentiation of activated T cells into Th-1 and Th-17 cells. These observations highlight the importance of IgM-ALA in regulating excess inflammation mediated by both innate and adaptive immune mechanisms and where the inflammatory response involves Th-17 cells that are not effectively regulated by regulatory T cells. The Journal of Immunology, 2012, 188: 1675–1685.

The physiologic relevance of natural IgM autoantibodies and the subset of IgM anti-leukocyte autoantibodies (IgM-ALA) remains to be elucidated. We have reviewed prior studies on natural IgM-ALA (1). Briefly, IgM-ALA were initially discovered because of their binding reactivity to lymphocytes. These IgM autoantibodies and the B-1 lymphocytes that produce them can be found in the umbilical cord blood, prior to exposure to foreign Ags; hence, such Abs are referred to as naturally occurring or natural IgM. Such autoantibodies that bind to leukocyte receptors (IgM-ALA) are present at low levels in normal individuals and increase during inflammatory disorders and various infections, including HIV-1. Previous studies in our laboratory and those of other investigators demonstrated that IgM-ALA are a heterogeneous group of several different Abs that are reactive to different receptors present on autologous and allogeneic leukocytes and other cells that express leukocyte receptors (1). IgM-ALA have been shown to bind to various undefined membrane receptors comprising glycoproteins, phospholipids, and glycolipids (1). Such naturally occurring IgM autoantibodies are encoded by minimally or nonmutated germline genes and, hence, are characteristically polyreactive with low binding affinity. Of particular importance, these IgM-ALA do not mediate cytolysis in the presence of complement at body temperature. Naturally occurring IgM differ from disease-producing autoantibodies, in that the latter are predominantly of the IgG isotype, bind with high affinity and specificity to the autoantigen, and mediate cytolysis at 37°C. Human kidney and heart transplants performed in the subset of patients having high levels of IgM-ALA were shown to have a lower incidence of acute rejections and of less severity, thus permitting better graft survival (1–6). This observation showing a strong correlation between high levels of IgM-ALA and protection from allograft rejection, together with the finding that IgM-ALA are noncytolytic to leukocytes at body temperature and increase in various inflammatory and infective states, led us to investigate whether IgM-ALA had a regulatory role in attenuating inflammation mediated by innate and adaptive immune mechanisms. We hypothesized that IgM-ALA could bind to different cell membrane receptors (i.e., receptors that initiate and activate the inflammatory process), as well as receptors that are important in enhancing chemokine production and facilitating chemotaxis. Several observations favored such a hypothesis. First, our studies with human B cell clones derived from umbilical cord clearly demonstrated that only 10% of IgM-secreting clones had IgM-ALA reactivity and that IgM-ALA from these clones had different receptor specificities (1). Second, we showed that IgM isolated from human serum immunoprecipitated CD3 and CD4, inhibited T cell activation/proliferation, and inhibited leukocyte production of certain cytokines (e.g., TNF-α). Additionally, we showed that

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Abbreviations used in this article: B6, C57/BL6; EGFP, enhanced GFP; α-gal, α-galactosyl; IgM-ALA, IgM anti-leukocyte autoantibody; IgMko, IgM knockout; IRI, ischemia reperfusion injury; Leu-Ads IgM, leukocyte-adsorbed IgM; μMT, B cell-deficient; Treg, regulatory T cell; WT, wild-type.

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human IgM immunoprecipitated chemokine receptors (e.g., CXCR4 and CCR5), inhibited the binding of chemokines (and HIV-1) to these receptors, and inhibited chemotaxis induced by chemokines (1, 7).

These in vitro findings with human leukocytes showing that IgM inhibit T cell function and leukocyte chemotaxis, as well as the clinical observations on human transplants, prompted us to study the inhibitory role of IgM in two murine models of acute inflammation: heart allograft rejection, in which inflammation is mediated by allorecipient-activated T cells, and kidney ischemia reperfusion injury (IRI), in which inflammation is mediated by NK and NKT cells that are activated by endogenous ligands released after ischemia. In these studies, we showed that C57BL/6 (B6) mice have IgM-ALA. In in vitro studies, we showed that purified murine serum IgM, but not IgM preabsorbed with splenic leukocytes to remove IgM-ALA, inhibited the production and activation of proinflammatory cytokines involved in innate and adaptive immune responses and inhibited TLR-4, which initiates the inflammatory process. Second, we showed that allograft rejection and kidney IRI is more severe in B6/S4-IgM knockout (IgMko) mice, although they possess competent regulatory T cells (Tregs) and administering physiologic doses of IgM to B6/S4-IgMko mice ameliorates this inflammatory response. More importantly, our studies showed that wild-type (WT)-B6 mice could also be protected from renal IRI and cardiac allograft rejection by pretreating with IgM to augment their IgM-ALA levels. Finally, we showed that IgM inhibited Th-17 cell differentiation, even when added 48 h after activation, and lack of such a mechanism in IgMko could explain the severity of the inflammatory process, especially involving Th-17 cells, although these IgMko mice have adequate levels of functional Tregs.

Materials and Methods

IgM and IgG purification from sera

IgM and IgG were purified by size-exclusion column chromatography (Sephacryl S-300 HR) from heat-inactivated (56°C for 1 h) WT-B6 murine sera (Innovative Research, Novi, MI), using previously described procedures and with modifications detailed below (1). IgM was not isolated by dialyzing sera in water or by ammonium chloride precipitation, because both of these techniques yielded IgM with impaired functional activity. Column-purified IgM was absorbed with protein G and repassaged through Sephacryl S-300 to remove other contaminating proteins. With this approach, >92% of the protein fraction contained IgM, as determined with protein electrophoresis, and there was <3% IgG and IgA contamination, as identified with ELISA. Purified IgM and IgG were concentrated to 1.3–1.5 mg/ml (higher concentrations lead to IgM aggregation and precipitation), dialyzed against RPMI 1640, and then丝路了用 a 0.45 μm filter, prior to use in cultures and for in vivo use. Purified IgM were stored at 4°C to prevent the precipitation that occurs when frozen. The effect of IgG on in vitro cultures was dose dependent, and its maximum effect was observed using a physiological dose of 15–15 μg/250×10^3 cells/0.5 ml culture.

Adsortion of IgM with splenic leukocytes

Aliquots of 1 mg IgM in 4.0 ml RPMI 1640 were absorbed at 37°C for 45 min with 1×10^11 splenic leukocytes that were preactivated for 24 h with soluble anti-CD3 and LPS. Preactivation of leukocytes increased receptor expression, whereas adsorption at 37°C prevented IgM-mediated cell cytolysis, which occurs at colder temperatures (e.g., room temperature). About 65% of IgM was recovered after the adsorption procedure. Adsorbed IgM were repassaged through Sephacryl S-300 HR column to remove cytokines and other contaminants resulting from the adsorption procedure. Absorbed IgM was redialyzed in RPMI 1640 prior to use.

Mice and surgical protocol for kidney IRI and heart transplantation

All experiments were performed in accordance with National Institutes of Health and Institutional Animal Care and Use Guidelines. The Animal Research Committee of the University of Virginia approved all procedures and protocols. We obtained BALB/c mice, C57BL/6 mice (WT-B6) expressing CD45.1 or CD45.2 on their leukocytes, and B6-hm12 mice [strain B6(C)-H2-Ab1hm12/KbEgJ] incompatible at MHC class II (Ia) with WT-B6 from The Jackson Laboratories (Bar Harbor, ME). B6/S4-IgMko mice were derived from a background of C57BL/6 and 129S4 mice (strain B6; 129S4-Igh-6mt1Cde/J) and obtained from The Jackson Laboratories (8). There was no detectable endogenous IgM in the sera of B6/S4-IgMko mice using an ELISA technique, nor could we detect the presence of IgM-ALA when B6/S4-IgMko sera were added to autologous or allogeneic leukocytes (data not shown). In experiments involving B6/S4-IgMko mice, we used mice with the same C57BL/6 and 129S4 background (referred to as WT/B6/S4) as WT controls, which were obtained from The Jackson Laboratories.

All experiments were performed on 6–8-wk-old male mice weighing 20 g.

Kidney IRI was performed under anesthesia with bilateral flank incisions, as we described previously (9). Both kidney pedicles were exposed and cross-clamped for either 26 min (mild ischemia) or 32 min (severe ischemia); thereafter, clamps were removed, and kidneys were reperfused for 24 h. Body temperature was maintained at 35–36°C (rectal temperature) during surgery with heating pads. Kidney pedicles were exposed, but not clamped, in sham-operated mice. Heart transplantation was performed according to previously described techniques (10). Briefly, the donor heart was harvested after heparinization and preserved in saline at 4°C. Following a midline laparotomy, the recipient inferior vena cava and aorta were cross-clamped proximal and distal to the anastomotic site. Donor aorta was anastomosed end to side to the recipient aorta, and donor pulmonary artery was anastomosed end to side to the abdominal inferior vena cava. Postoperatively, mice were administered analgesia and maintained at 35–37°C prior to returning to the vivarium.

IgM and IgG administration to mice

IgM predialyzed in RPMI 1640 were warmed to 37°C and administered to the tail vein of mice that were also kept prewarmed at 37°C to avoid IgM-mediated cytolysis in vivo at colder temperatures. In experiments requiring repeated doses, IgM were given every 48 h i.p. Mice received 150–200 μg IgM with each dose; at this dose, the serum IgM increased rapidly to 150 mg/ml. Lower doses of IgM did not inhibit the inflammatory response induced by IRI or rejection. Normal levels of IgM in serum of WT-B6 mice vary from 200 to 420 μg/ml.

Assessment of kidney function and histology

Plasma creatinine was determined using a colorimetric assay, according to the manufacturer’s protocol (Sigma) (11). For histology, kidneys were fixed in 0.2% sodium periodate–1.4% rat-lysine–4% paraformaldehyde in 0.13 M phosphate-buffered saline (pH 7.4), postfixed in 10% formalin, dehydrated in paraffin. Kidney sections (4 μm) were stained with H&E, and we quantitated tubular injury using a scoring sistem described previously (12).

Characterization of leukocytes eluted from kidney

Flow cytometry was used to analyze kidney leukocytes content 24 h post-reperfusion (9). Briefly, kidneys were weighed, minced, and digested with collagenase type 1A in EDTA. Cells were isolated by passage through a cotton column treated with 10% FCS. Cells were pretreated with anti-mouse CD16/32 (clone 2.4G2) to block for nonspecific Fc binding and with 7-aminoactinomycin D to distinguish between live/dead cells. Leukocytes were identified by labeling cells with CD45 (30-F11), and cell suspension was mixed with Caltag Counting Beads to normalize for differences in cell recovery among kidney samples. Surface labeling of leukocyte subsets was performed using Abs that we described previously (9). Appropriate fluorochrome-conjugated isotype-matched, irrelevant mAbs were used as negative controls. Seven-color flow cytometry was used to quantitate leukocyte subsets and their subsets. Flow cytometry data were analyzed with FlowJo software 9.1 (Tree Star, Ashland, OR).

Immunofluorescence staining of heart and kidney sections and endothelial cells

Kidney and cardiac tissue were fixed and frozen, as we described in detail for kidney tissue (13). Briefly, these tissues were fixed in 1% paraffinmaldehyde, 1.4% rat-lysine, 0.2% sodium periodate in 0.1 modified PBS (pH 7.4) overnight; incubated in 30% sucrose for 48 h at 4°C, and embedded and frozen in OCT (Ted Pella, Redding, CA). Frozen sections (5 μm) of kidney or heart were permeabilized with 0.3% Triton X-100, and nonspecific binding was blocked with 10% horse serum and anti-mouse CD16/32. Tissue sections were stained with the following conjugated Abs: rat anti-neutrophil (clone 7/4; Cedara Laboratories, Burlington, NC), rat anti-CD45 (clone 30-F11; Serotec, Raleigh, NC), rat anti-CD3 (clone 30-H12; Caltag, Burlington, NC), rat anti-CD19 (clone 6D5; Caltag), rat anti-CD8 (clone 53-6.7; Caltag), and rat anti-CD4 (clone 145-2C11; Caltag). Immunofluorescence was analyzed in double-blind manner using an Olympus BX60 microscope (Olympus, Center Valley, PA).
anti-Foxp3 (clone FJK-16s; eBioscience, San Diego, CA), rabbit anti–IL-17A (polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA), rat anti-CD31 (clone MEC 13.3; BioLegend, San Diego, CA), rat anti-F4/80 (clone BM8; Invitrogen, Carlsbad, CA), rat anti–TLR-4/MD-2 (clone MTS 510; eBioscience), rabbit anti-murine JE (MCP-1, polyclonal; PeproTech, Rocky Hill, NJ), and rabbit anti-murine CXCL1 (polyclonal, PeproTech). Nuclei were visualized using DAPI. Specimens were mounted with ProLong Gold Antifade Reagent ( Molecular Probes, Eugene, OR) and examined using a Zeiss Axiovert 200 microscope with Apo Teme (Zeiss).

LPS-activated adherent endothelial cells on glass cover slips were fixed in 4% formaldehyde and were permeabilized and blocked using the same reagents as described for kidney tissue. Intracellular staining was performed with conjugated rabbit anti–NF-κBp65 (clone C22B4; Cell Signaling, Technology, Danvers, MA), phallolidin-conjugated actin (Sigma), and Abs to detect chemokines.

**Real-time RT-PCR**

Kidney sections were immediately transferred into RNA Later (Ambion, Austin, TX). RNA and cDNA were prepared from these reagents, as we described previously (13). Subsequently, real-time RT-PCR was performed using a MyiQTM Single Color Real-Time PCR detection system (Bio-Rad, Hercules, CA). Primers were obtained from Integrated DNA Technologies (Coralville, IA); primer sequences used to detect IFN-γ, TNF-α, and chemokines were described previously (13). Sample values in triplicate were calculated with normalization to GAPDH.

**Alloantigen activation of splenocytes (MLR)**

We performed a one-way MLR, in which 2.5 × 10⁵ WT-B6 responder splenocytes were cocultured with 7.5 × 10⁵ radiated (3000 rad) BALB/c splenocytes, which are fully MHC incompatible with WT-B6. MLR cocultures were set up in a total of 0.5 ml RPMI 1640 culture medium, which was supplemented with 10% FCS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μM 2-μM for murine in vitro cultures. To evaluate the proliferative response, we used WT-B6 responder splenocytes that were labeled with CFSE and added to cocultures. A well-described approach was used to label cells with CFSE (14). Cells were cocultured in 5% CO₂ at 37°C for 5–6 d prior to quantitating proliferation. Splenocytes were cocultured with 7.5 × 10⁵ cells in 0.5 ml RPMI 1640 culture media, which was supplemented with 10% FCS, 10 mM HEPES buffer, 1 mM sodium pyruvate, 2 mM l-glutamine, and 50 μM 2-μM for murine in vitro cultures. To evaluate the proliferative response, we used WT-B6 responder splenocytes that were labeled with CFSE and added to cocultures. A well-described approach was used to label cells with CFSE (14). Cells were cocultured in 5% CO₂ at 37°C for 5–6 d prior to quantitating proliferation and intracellular cytokine production. TGF-β, IL-6, IL-21, and IL-23 (2 ng each) were added to MLR cocultures to maximize Th-17 differentiation, whereas IL-4 (2 ng) was added to maximize Th-2 differentiation. Intracytoplasmic staining of cytokines was performed on cells that were incubated with ionomycin/phorbol dibutyrate (500 μM) in the presence of brefeldin A for the last 4 h of the coculture incubation to inhibit cytokine secretion. Cells were then washed twice in RPMI 1640, stained for 30 min with LIVE/DEAD Near-IR stain (according to the Invitrogen kit protocol), fixed with 4% paraformaldehyde, washed twice, permeabilized using Becton Dickson (Franklin Lakes, NJ) permeabilization kit, and stained using the following fluorochrome-labeled Abs obtained from eBioscience: rat anti-mouse IFN-γ (clone XMG1 2), rat anti–IL-17A (clone 17B-7), rat anti–IL-4 (clone 11B11), rat anti-Foxp3 (clone FJK-16s), and mouse anti–CD45.2 (clone 104). A five-color flow cytometer (BD FACSCalibur; BD Biosciences) was used to acquire data, which were analyzed with FlowJo software 9.1 (Tree Star).

**Anti-CD3 activation of splenocytes and T cells**

Freshly isolated splenocytes or isolated T cells or T cell subsets (2.5 × 10⁵ in 0.5 ml RPMI 1640 culture media) were activated with 12 μl soluble anti-CD3/CD28 beads (Invitrogen-Dynal bead, T cell activators), and cells were cultured for 4–5 d in 5% CO₂ at 37°C.

In certain experiments, splenocytes (2.5 × 10⁵ in 0.5 ml RPMI 1640 culture media, which were activated with 12 μl CD3 (1.0 μg/ml), and soluble anti–CD3 (1.0 μg/ml), and cells were cultured for 3–4 d. We used the same cytokines as described for MLR to maximize Th-2 and Th-17 differentiation. Intracytoplasmic staining of cells and staining of leukocyte subsets were performed as described in the MLR assay.

**α-Galactosyl ceramide activation of splenocytes**

α-Galactosyl (α-gal) ceramide (100 ng) was added to 400,000 splenic leukocytes in 0.5 ml RPMI 1640 culture media. Cells were cultured for 48 h at 37°C in 5% CO₂. IFN-γ in supernatant was quantitated by an ELISA kit.

**LPS activation of cultured endothelial cells**

Murine glomerular endothelial cells, a kind gift from Dr. Michael Madaio (Medical College of Georgia, Augusta, GA), were cultured on glass cover slips (2.5 × 10⁵ cells/slip) in a tissue culture well containing 1 ml DMEM/HamF12 (ratio 3:1) culture media, supplemented with 10% FCS and 2 mM l-glutamine (15). IgM (50 μg) were added 1 h prior to cell activation with LPS (serotype 0.55:B5, 1 μg/ml media, Sigma). After 1 h, adherent cells on cover slips were fixed, permeabilized, and stained with anti–NF-κB (1:50 dilution; Cell Signaling). Intracellular chemokine production was evaluated after cells were cultured for 6–12 h.

**Isolation of Tregs and EGFP-labeled Foxp3⁺ cells**

CD4⁺CD25⁺ Tregs were isolated from splenocytes using the Miltenyi Biotech isolation kit, which uses magnetic beads (cat. #130-091-041). Isolated cells were >93% CD4⁺CD25⁺, and 70–75% of these cells were Foxp3⁺. Magnetic bead-isolated CD25⁺ Tregs from splenocytes of Foxp3⁻EGFP mice were sorted using a flow cytometer to obtain purified (99% pure) Foxp3⁺EGFP cells.

**Statistics**

GraphPad Instat 3 (Graph Pad, La Jolla, CA), Sigma Plot 11.0 (Systat Software), and Canvas X (ACD Systems of America, Chicago, IL) were used to analyze and present the data. Data were analyzed, after transformation if needed to generate a normal distribution, by the two-tailed t test or one- or two-way ANOVA, with post hoc analysis as appropriate; p < 0.05 was used to indicate significance.

**Results**

**Murine serum IgM binds to non-FcγR receptors on the cell membrane**

We wanted to confirm whether WT-B6 and WT-B6/S4 mice have IgM-ALA present in their sera. IgM were purified from serum using size-exclusion chromatography and interacted with splenic-derived pronase-treated T cells for 45 min at 37°C prior to staining at 4°C for binding of IgM. Splenic cells (2 × 10⁵ cells/ml) were pronase digested (250 μg/ml at 37°C for 30 min) to remove FcγR receptors (16). Data in Fig. 1A and 1B clearly show that purified polyclonal IgM from WT-B6 sera bind to T cells in a dose-dependent manner. IgM binding to macrophages was much more pronounced compared with T and B cells (data not shown). Adsorbing polyclonal IgM with splenic leukocytes significantly decreased the binding of adsorbed IgM to T cells (Fig. 1C). A murine monoclonal IgM (isotype control) failed to bind to leukocytes. Additionally, we could not detect IgM in the sera of B6/S4-IgMko mice using an ELISA technique, nor could we detect the presence of IgM-ALA when B6/S4-IgMko sera were added to autologous or allogeneic leukocytes (data not shown).

IgM purified from murine serum is a polyclonal preparation. Hence, based on our studies with human monoclonal IgM, it became important to determine whether polyclonal murine serum IgM, like human IgM, would bind and immunoprecipitate several different receptors expressed on the cell membrane (1). In these studies, we used surface biotinylation of leukocyte membrane proteins, followed by immunoprecipitation and Western blotting.

Initially, we compared the binding of purified serum IgM with mouse cell lines WEHI7.1 (T cell), Sp2/0 (B cell), and J774 (macrophage). Based on flow cytometry analysis, maximum binding of IgM was observed with the macrophage cell line J774. Fig. 1D shows that murine IgM, purified from serum, immunoprecipitates biotin-labeled J774 cell membrane protein having different molecular weights. Two isotype-control IgM Abs did not immunoprecipitate these membrane proteins.

**Murine IgM inhibits production of proinflammatory cytokines induced by innate and adaptive immune mechanisms in vitro**

Endogenous glycolipids, released by ischemia-injured cells, initiate inflammation associated with IRI. Endothelial and dendritic cells present these Ags to NK and NKT cells, which, when activated, augment cytokine production (IFN-γ and IL-17) of infiltrating leukocytes, especially neutrophils (9, 13). To test the in vitro effect of IgM on this innate immune response, we activated splenic leukocytes in the presence of the glycolipid α-gal cer-
IgM can inhibit postactivation intracellular processes (Fig. 2). Th-1 and Th-17 subsets produce proinflammatory cytokines (primarily NK cells, macrophages, and effector T cells) to mediate the alloraft rejection. To test the in vitro effect of IgM on this adaptive immune response, T cells were activated with either LPS and anti-CD3 Ab or with alloantigens using a one-way mixed MLR in which CFSE-labeled WT-B6 responder splenocytes were cocultured with fully MHC-incompatible BALB/c-irradiated splenocytes. As depicted in Fig. 2B, purified IgM significantly inhibited IFN-γ production of the T cell subset activated by alloantigens in the MLR. Additionally, IgM slowed down the proliferative response of the activated T cells. These inhibitory effects of IgM on Th-1 differentiation were not observed using leukocyte-adsorbed IgM, which is deficient in IgM-ALA (Fig. 1C).

Allograft rejection is mediated by alloantigen-activated T cells, which proliferate and differentiate into cytokine-producing T cell subsets. Th-1 and Th-17 subsets produce proinflammatory cytokines (IFN-γ and IL-17), which activate the infiltrating leukocytes (primarily NK cells, macrophages, and effector T cells) to mediate the alloraft rejection. To test the in vitro effect of IgM on this adaptive immune response, T cells were activated with either LPS and anti-CD3 Ab or with alloantigens using a one-way mixed MLR in which CFSE-labeled WT-B6 responder splenocytes were cocultured with fully MHC-incompatible BALB/c-irradiated splenocytes. As depicted in Fig. 2B, purified IgM significantly inhibited IFN-γ production of the T cell subset activated by alloantigens in the MLR. Additionally, IgM slowed down the proliferative response of the activated T cells. These inhibitory effects of IgM on Th-1 differentiation were not observed using leukocyte-adsorbed IgM, which is deficient in IgM-ALA (Fig. 1C). Interestingly, IgM did not inhibit the compensatory increase in IL-4 production that occurs in the setting of decreased IFN-γ production induced by IgM (Fig. 2C). Therefore, these data indicated that the inhibitory effect of polyclonal IgM is mediated by the subset of IgM that binds to leukocyte receptors (i.e., IgM-ALA) and that murine IgM, like human IgM, inhibit T cell proliferation and production of certain proinflammatory cytokines (1).

We next wanted to determine whether polyclonal IgM inhibited T cell differentiation toward Th-17 cells, especially in the presence of cytokines that favor maximum Th-17 differentiation. Fig. 2D clearly shows that physiological doses of IgM inhibited activated T cells from differentiating into Th-17 cells. Because Foxp3+ CD4+ Tregs can also differentiate into Th-17 cells (especially in the presence of IL-6/21), it became important to determine whether IgM can prevent Tregs from differentiating into Th-17 cells under these cytokine conditions. Accordingly, CD 45.1 WT-B6 responder splenocytes were cocultured with CD 45.2 WT-B6 purified Tregs and then activated with LPS and soluble anti-CD3 in the presence of cytokines favoring Th-17 differentiation. As depicted in Fig. 2D, CD45.2 Tregs failed to inhibit CD45.1 splenocytes from differentiating into Th-17 cells. Instead, relatively more Th-17 differentiation occurred in CD45.2 Tregs than in CD45.1 splenocytes. Addition of IgM inhibited both CD45.1 splenocytes and CD45.2 Tregs from differentiating into Th-17 cells and decreased the downregulation of Foxp3 that occurred in CD45.2 Tregs under Th-17 cytokine conditions (Fig. 2D). To more conclusively determine the effect of IgM on differentiation of Foxp3+ Tregs into Th-17 cells, we obtained 99% purified Foxp3+EGFP cells by cell sorting and activated these cells with insoluble anti-CD3/CD28 beads under Th-17 conditions. Data in Fig. 2E depict that IgM also inhibited Th-17 differentiation of CD4+Foxp3+ Tregs. Isotype IgM failed to inhibit Th-17 differentiation of both non-Tregs and Tregs (data not shown). These inhibitory effects of polyclonal IgM on T cells were not a consequence of isolating and purifying IgM, because similar inhibitory effects were observed with WT-B6 sera containing similar quantities of purified IgM (Fig. 2G).

Because IgM were added at the initiation of cultures in all of these in vitro experiments, it became important to determine whether IgM could also inhibit intracellular processes induced after cell activation. Accordingly, IgM was added 48 h after T cells were activated by alloantigens in an MLR. Data in Fig. 2F clearly show that IgM, when added at 48 h, inhibited both cell proliferation and Th-17 differentiation of the T cell subset activated by alloantigens in the MLR. Reduction of cell proliferation, in the presence of IgM, was also associated with less cell death (Fig. 2F). The lack of apoptosis on T cells with physiological doses of IgM and the antiproliferative effect of IgM on T cells were also observed with human IgM (1, 17). IFN-γ production was similarly inhibited by IgM when added at 48 h (data not shown). Therefore, these data indicated that IgM can mediate its inhibitory effect by binding to cell membrane receptors designed to inhibit postactivation intracellular processes. However, these data do not negate the possibility of IgM-mediated inhibition of early events that occur during T cell activation.
IgM-ALA protects B6 mice from renal IRI

Because polyclonal IgM inhibited the in vitro activation of NK and NKT cells by α-gal ceramide (Fig. 2A), we proceeded to determine whether IgM inhibited the in vivo inflammatory response induced by kidney IRI. We used two approaches to test the protective role of IgM in the suppression of this innate inflammatory response. First, we performed renal IRI in B6/S4-IgMko mice. In these studies, rectal temperatures of mice were maintained at 35–36˚C with a heating pad during renal ischemia. The IgM-expressing B cells in these IgMko mice cannot secrete IgM, but they can differentiate into plasma cells and secrete normal levels of other Igs. As can be seen from Fig. 3A, 26 min of ischemia, which was insufficient to cause renal IRI in WT-B6/S4 mice, led to severe IRI (as assessed by plasma creatinine and by histology) in B6/S4-IgMko mice. Importantly, i.v. administration of a physiologic dose of purified IgM (250 μg), given 24 h prior to ischemia, protected B6/S4-IgMko mice from developing IRI (Fig. 3A), thus indicating that it is the IgM deficiency that predisposes these mice to IRI after minimal ischemia.

In the second approach, we determined whether increasing IgM levels would protect WT-B6 mice from developing IRI with more severe (32 min) renal ischemia. As can be seen from Fig. 3B and 3C, i.v. administration of 150 μg IgM, 24 h before ischemic injury, protected WT-B6 mice from severe IRI, as determined by plasma creatinine and tubular necrosis score (Fig. 3B) and by histology (Fig. 3C). This protective effect was mediated by the IgM-ALA subset of polyclonal IgM, because leukocyte-adsorbed IgM had no protective effect on IRI. Most of the renal injury seen after IRI is associated with infiltration of neutrophils and increased production of IFN-γ, as seen in mice pretreated with albumin and leukocyte-adsorbed IgM (Leu-Ads IgM) (Fig. 4). Natural IgG, unlike natural IgM-ALA, binds to leukocytes via its Fc domain (i.e., to FcγR on the cell membrane). Hence, we used IgG as another control to determine whether other natural
Abs can inhibit inflammatory processes even if they bind to leukocytes via their Fc domain. Data from these experiments indicated that IgG or FcγR do not inhibit the innate inflammatory processes that occur in renal IRI (Figs. 3, 4). Data in Fig. 4 clearly demonstrates that the protective effect mediated by IgM is associated with minimal neutrophil infiltration into the ischemic renal parenchyma (Fig. 4A, B), as well as with decreased production of CXCL1, which attracts neutrophils into the interstitial space. IgM also prevented the IRI-induced increase in IFN-γ and IL-17, which have an important role in promoting renal IRI (13). For example, IFN-γ production, as determined by mRNA levels in renal parenchyma, was significantly suppressed, and levels were lower than in sham-treated mice (Fig. 4C). Additionally, IgM suppressed intracytoplasmic INF-γ and IL-17 of infiltrating leukocytes (Fig. 4D). Furthermore, the mRNA levels of TNF-α and CXCL1 in IgM-pretreated mice did not increase after renal IRI, and levels were similar to that of sham-treated mice (Fig. 4C).

IgM protects B6 mice from cardiac allograft rejection

Because the in vitro studies demonstrated that IgM inhibited alloantigen-activated T cell proliferation and differentiation into Th-1 and Th-17, we performed studies aimed at determining whether IgM could also inhibit allograft rejection, which is an in vivo model of inflammation mediated by alloantigen-activated T cells. Two approaches were used. First, cardiac transplants were performed in B6/S4-IgMko mice using B6-bm12 donor hearts, which are mostly incompatible at the MHC class II locus (1a). In this transplant model, there is a chronic from of cellular rejection and a vasculopathy that is initiated by a T cell-mediated inflammatory process not involving anti-MHC Abs (18, 19). As a result, a rejection-induced decrease in cardiac function is detected between days 17 and 28, when, with finger palpation, one can clearly detect a diminution in cardiac contractility. This palpation technique was found to be reliable for detecting significant rejection that impairs cardiac function and was initially described in detail by Corry et al. (10). However, cardiac allograft ceases having a heart beat at 2–3 mo in this model (18). In Fig. 5A, one can observe that rejection (defined by a decrease in cardiac contractility) occurs significantly earlier (i.e., between days 10 and 18), when B6-bm12 donor hearts are transplanted into B6/S4-IgMko recipients. Furthermore, Fig. 5A clearly shows that, by day 10, the rejection-induced inflammatory process in cardiac allografts is severe when transplanted into B6/S4-IgMko recipients but is minimal in WT-B6/S4 recipients. Additionally, cardiac allografts in B6/S4-IgMko recipients cease having a heart beat in 2–3 wk, which is significantly earlier compared with their WT-B6/S4 counterparts, in which cessation of heart beat occurs after >2 mo.

In the second approach, we wanted to determine whether IgM, when administered to WT-B6 mice, inhibited the severe and rapid
rejection that occurs in the setting of fully MHC-incompatible donor hearts (i.e., from BALB/c donors). In this model, rejection in WT-B6 recipients is detectable by day 5 with finger palpation, and the heart ceases having a heart beat by days 7–9 (18). In these studies, 175 μg IgM was administered 24 h after ascertaining that cardiac surgery was successful, and the dose of IgM was repeated on days 3 and 5. Mice were euthanized on day 6. Fig. 5B clearly shows that IgM inhibited the severe inflammation in the cardiac allograft induced by rejection on day 6, as detected by H&E staining and immunofluorescence staining for neutrophils (7/4, green), macrophages (F4/80 [white]), and CXCL1 (red) in outer medulla (original magnification \( \times 400 \)). C, Renal parenchyma mRNA for IFN-γ, TNF-α, and CXCL1 were quantitated relative to GAPDH. D, CD45+ leukocytes eluted from kidneys were examined by flow cytometry for intracytoplasmic IFN-γ and IL-17. Data are mean ± SEM. \( ^*p = 0.016, ^{**}p < 0.01 \), two-way ANOVA.

**FIGURE 4.** Protective effect of IgM after renal ischemia is associated with lack of leukocyte infiltration into renal parenchyma and with suppression of ischemia-induced cytokine/chemokine production. Data were obtained from all WT-B6 kidneys in Fig. 3B. A, Leukocytes eluted from kidneys were quantitated and subjected to flow cytometry to identify neutrophils, dendritic cells, and macrophages. B, Immunofluorescence staining of neutrophils (7/4, green), macrophages (F4/80 [white]), and CXCL1 (red) in outer medulla (original magnification \( \times 400 \)). C, Renal parenchyma mRNA for IFN-γ, TNF-α, and CXCL1 were quantitated relative to GAPDH. D, CD45+ leukocytes eluted from kidneys were examined by flow cytometry for intracytoplasmic IFN-γ and IL-17. Data are mean ± SEM. \( ^*p = 0.016, ^{**}p < 0.01 \), two-way ANOVA.

Tregs in IgMko mice are competent but do not effectively suppress Th-17

Because Tregs are the predominant suppressors of inflammatory processes mediated by adaptive immune responses, it became necessary to determine whether the enhanced inflammatory response in B6/S4-IgMko mice results from inadequate Treg levels or function. Such a possibility seemed unlikely, because B6/S4-IgMko mice had similar levels of CD4+CD25+ splenic Tregs compared with their WT counterparts (Fig. 6A), and B6/S4-IgMko Tregs possessed more suppressive activity compared with their WT counterparts, as evidenced by the suppressive ability of isolated CD4+CD25+ Tregs to inhibit Th-1 differentiation of splenic leukocytes activated with LPS and anti-CD3 (Fig. 6B). In Fig. 6B, one also observes more Th-17+ cell differentiation in the setting of adding more Tregs, especially IgMko Tregs. A potential explanation is that IL-17+ cells could arise from the added Tregs that differentiate into Th-17 cells (Fig. 2D), especially in the setting of decreased IFN production induced by Tregs. IFN is known to regulate Th-17 differentiation (20). These in vitro findings may explain our observations in vivo; more severe rejection-induced inflammation was noted with Th-17 cells in IgMko recipients (Figs. 5A, 6C), despite similar levels of Tregs in cardiac allografts from both IgMko and WT-B6/S4 recipients.

**IgM inhibits chemokine production by preventing TLR-4-induced NF-κB translocation into the nucleus**

Because the protective effect of IgM, in both renal IRI and cardiac allograft rejection, is associated with minimal leukocyte interstitial infiltration, it became important to determine whether IgM inhibited early innate events that lead to cell recruitment to injured sites. The initial ischemic injury in both the kidney and donor heart releases endogenous ligands, which bind to TLRs, in particular TLR-2 and TLR-4 (21, 22). Activation and up-regulation of TLRs present on leukocytes and endothelial and epithelial cells induce the expression of adhesion molecules (e.g., ICAM-1), chemokines, and chemokine receptors that regulate cell migration to the sites of inflammation. Two approaches were used to determine whether IgM inhibited activation of TLRs and chemokine production. In the in vivo approach, WT-B6 mice, subjected to severe renal IRI (32 min), were sacrificed 3 h after ischemia reperfusion. At 3 h, the ischemic reperfused kidney had increased TLR-4 expression in both renal tubular cells and endothelial cells, as well as increased production of MCP-1 in renal tubular cells and CXCL1 in endothelial cells, but with minimal or no leukocyte infiltration (Fig. 7B). However, pretreatment of mice with IgM, 24 h before ischemia, partially inhibited up-regulation of TLR-4 expression on both endothelial cells and renal tubular cells, but it significantly reduced production of MCP-1 and CXCL1. Furthermore, pretreatment with IgM prevented the ischemia-induced increase in mRNA levels of TLR-4 (Fig. 7A).

In the second approach, the effect of IgM was tested on cultured renal glomerular endothelial cells, in which TLR-4 was activated with the bacterial LPS ligand to induce chemokine production. As can be seen from Fig. 8A, IgM (but not leukocyte-adsorbed IgM deficient in IgM-ALA) significantly inhibited TLR-4 activation-induced translocation of the transcription factor NF-κB into the nucleus. As a result, NF-κB–induced CXCL1 and MCP-1 chemokine production was also inhibited by IgM (Fig. 8B, 8C). Data in Fig. 7 indicated that IgM inhibit TLR-4–induced NF-κB translocation by preventing up-regulation of TLR-4 in response to LPS. By flow cytometry, we could not show that IgM inhibited the binding of FITC-labeled LPS to murine glomerular endothelial cells or to the murine macrophage cell line J774 (data not shown).
In this study, we provided evidence to show that polyclonal IgM inhibited inflammatory processes that occur after renal ischemia reperfusion and cardiac allograft rejection, indicating that IgM inhibits inflammatory processes mediated by both innate and adaptive immune mechanisms. Mice lacking only secreted IgM (i.e., IgMko) had a more severe inflammatory response to both renal IRI and cardiac allografts; replenishing IgM in these mice made them less sensitive to renal injury. Importantly, increasing IgM in WT-B6 mice made them more resistant to renal IRI and to cardiac allograft rejection. Our studies also indicated that the anti-inflammatory effect is mediated, in part, by a subset of polyclonal IgM that binds to leukocyte receptors (IgM-ALA).

The anti-inflammatory protection afforded by IgM was associated with a significant lack of infiltrating leukocytes in the affected organs, suggesting that IgM mediates the protective effect by inhibiting processes involved in leukocyte activation and migration. Our in vitro and in vivo data indicated that IgM-ALA inhibits activation of TLR-4 receptors on APCs and endothelial cells. IgM inhibited LPS-induced translocation of NF-κB into the endothelial cell nucleus, indicating that IgM inhibits processes involved in TLR-4 activation. The transcription factor NF-κB plays a central role in the generation of an inflammatory response because it regulates production of proinflammatory cytokines, such as IL-1β, TNF-α, and IFN-γ; chemokine production, such as CXCL1, MCP-1, and RANTES; and expression of adhesion molecules (e.g., LFA-1 and ICAM-1). Leukocyte migration through capillaries is dependent on chemokines and up-regulation of adhesion molecules on endothelial cells, whereas activation of effectors that mediate the innate immune response (e.g., NK cells) or the adap-
tive immune response (e.g., Th cells) is dependent on cytokine production and Ag presentation by activated APCs and endothelial cells. Data from this study indicated that IgM inhibits the inflammatory process, through inhibiting up-regulation of TLR-4 (Fig. 7) and preventing TLR-4–induced activation of APCs and endothelial cells (Fig. 8). In support of such a premise are other studies clearly showing that absence of TLR-4 or inhibition of TLR-4 activation in mice protects them from renal IRI, as well as from allograft rejections (21–23). IgM-ALA could also inhibit the inflammatory process by other mechanisms. One potential mechanism is that IgM could directly inhibit cell differentiation toward effectors that participate in the adaptive immune response, and such a mechanism would explain the protective effect of IgM on cardiac rejection (Fig. 5), although IgM was administered 24 h posttransplant (i.e., after APC and T cell activation occurred). Our in vitro data (Fig. 2) supported this possibility, because IgM inhibited proliferation and differentiation of cells into Th-1 and Th-17 effectors, even when IgM was added 48 h after T cells were activated in an MLR. Another possibility is that IgM could inhibit leukocyte chemotaxis by decreasing chemokine production (Figs. 4, 6, 7) or by inhibiting binding of chemokines to their receptors.

We previously demonstrated that IgM bind to chemokine receptors and inhibit chemokine-induced chemotaxis (1).

Natural IgM can inhibit inflammatory processes, including Th-17–mediated inflammation, via several mechanisms. In our in vitro and in vivo inflammatory models, we showed that IgM-ALA can directly inhibit activation, proliferation, differentiation, and migration of inflammatory cells. Other investigators showed that natural IgM can inhibit altered self-Ag–induced inflammation by another mechanism that involves clearing of self-Ags (e.g., dsDNA, apoptotic cells, and oxidized lipids) (reviewed in Refs. 24–26). In these murine models of inflammation, which include systemic lupus erythematosus, arthritis, and atherogenesis, polyreactive monoclonal IgM with specificity for the self-Ag was used to show that natural IgM can ameliorate the inflammatory process. Additionally, in a murine model of complement-mediated glomerular inflammation, natural IgM was shown to ameliorate the inflammatory process by scavenging C3 and C4 (27). It is important to note that many of these anti-inflammatory mechanisms of natural IgM can operate simultaneously in an inflammatory disorder. For example, high levels of IgM-ALA have been detected in systemic lupus erythematosus (28). Additionally, in our

**FIGURE 7.** IgM protects against ischemia-induced leukocyte infiltration in renal IRI by preventing upregulation of TLR-4 and suppressing chemokine production. WT-B6 mice were pretreated with IgM or bovine albumin, as in Fig. 3B. Kidneys were subjected to severe ischemia (32 min) and were examined after 3 h of reperfusion. A, mRNA levels of TLR-4 in kidney tissue after 3 h reperfusion (n = 4 mice/group). **p < 0.01, two-way ANOVA. B. Representative examples from the 3-h reperfusion kidneys. Original magnification ×400. CXCL1 (red) colocalizes with CD31 (white), a marker of endothelial cells (top panels). TLR-4 (red) is expressed by renal tubular cells and endothelial cells (CD31+). Colocalization of TLR-4 (red) and CD31 (green) in endothelial cells creates a yellow fluorescence (bottom panels). MCP-1 production by tubular cells (middle panels). Albumin-pretreated control mice have significantly more CXCL1, TLR-4, and MCP-1 compared with IgM-pretreated mice. Data are representative examples from one of three mice.

**FIGURE 8.** IgM inhibits TLR-4–induced NF-κB translocation into the nucleus of cultured endothelial cells (A) with a resulting decrease in MCP-1 (B) and CXCL1 (C) production. Cultured glomerular endothelial cells were activated with LPS in the presence or absence of 50 µg IgM, which was added 1 h prior to adding LPS. One hour after LPS activation, cells were fixed to determine whether NF-κB had translocated into the nucleus. In A, intracytoplasmic NF-κB stained brownish-red, and intranuclear NF-κB stained purple. Other endothelial cells were fixed at 12 h to determine the presence of intracytoplasmic MCP-1 by immunofluorescence staining (B, red) or to quantitate mRNA levels of CXCL1 (C). A and B. Original magnification ×400. Data are representative examples from one of three separate experiments. **p < 0.01, two-way ANOVA.
in vivo murine models of renal IRI and rejection, we cannot exclude the anti-complement contribution of natural IgM.

Based on our findings, one would expect RAG-1−/− mice, which lack both IgM and Tregs, and B cell-deficient (μMT) mice, which lack IgM and other Iggs, to be more sensitive to renal ischemia compared with their WT counterparts; however, this was not observed (13, 29, 30). The absence of NKT cell effectors in RAG-1−/− mice could explain their lack of increased sensitivity to renal IRI (9). However in μMT mice, the concomitant lack of B cells or their secreted products could have altered the immune reactivity of T cell effector cells that mediate the inflammatory process. There is evidence to support such an explanation in other inflammatory models involving μMT mice, in which effector T cell function was found to be significantly reduced (31–33). Such findings may explain why Burne-Taney et al. (30) failed to observe renal IRI in their μMT mice, despite the presence in the renal parenchyma of infiltrating leukocytes and T cells, which were quantitatively similar to those in WT controls with renal IRI. The IgMko mice that we used differ from μMT mice in that they have intact B cells expressing IgM on their cell membrane but lack the ability to secrete IgM but not other Iggs. Additionally, function of T cells and other effector cells in IgMko mice is not reduced, as evidenced by the heightened inflammatory response in these mice after IRI or an allograft transplant.

Data from our studies differ from those of Thurman’s laboratory, in which B1 cells producing natural IgM were depleted by lysing i.p. B1 cells with distilled water (34). In their studies, reduction of peritoneal B1 cells did not worsen the ischemia-induced acute renal injury. It is possible that their technique did not deplete the subset of B1 cells that produce IgM-ALA. In their studies, mice were not evaluated for IgM-ALA.

Data from our studies also differ from studies involving IRI of bowel, skeletal muscle, or cardiac muscle in rodents (35–37). In these ischemic rodent models, IgM was found to be pathogenic and worsened the inflammatory process, leading to more reperfusion injury. A likely explanation is that the mechanism of IRI for these organs is not the same as that in the kidney, especially because these investigators convincingly showed that ischemia induced a complement-mediated inflammatory response that occurs after a specific clone of natural IgM Ab binds to a neoantigen, which is exposed by ischemic injury. Furthermore, they showed that this neoantigen is common tobowel, skeletal muscle, and cardiac muscle. IgM binding and C4/C3 (classical pathway) are readily detectable in these tissues (35, 38). RAG-1−/− mice, which lack B cells and IgM, are protected from IRI of bowel and cardiac muscle. In murine renal IRI, IgM is not pathogenic and one does not detect IgM binding to the tubular basement membrane or to other tubular structures. Additionally, RAG-1−/− mice are not protected from renal IRI (13, 29). Furthermore, peritubular C3 deposition in murine renal IRI does not involve natural IgM, but it results from ischemia-induced tubular injury that activates complement via the alternative complement pathway independently of Ab (39, 40).

Further studies are clearly necessary to better define the anti-inflammatory role of IgM-ALA in the context of Tregs, especially with suppression of excess inflammation mediated by innate and adaptive immune mechanisms (reviewed in Refs. 41, 42). Prior observations indicated that the lack of IgM in B6/S4-IgMko mice does not predispose them to T cell-mediated autoimmune diseases, as seen with Treg deficiency in both humans and mice, implying that Tregs in IgMko do not need IgM to control the type of T cells that are autoaggressive (8, 43). However, our studies indicated that regulation of excess inflammation involving Th-17 effectors (e.g., in allograft rejection) requires both Tregs and high levels of IgM-ALA. In both humans and rodents, the rejection-induced inflammatory response involves Th-17 effectors, primarily because IL-6 and IL-21 in the inflammatory environment enhance Th-17 differentiation; hence, in this environment, polyclonal natural Tregs and TGF-β cannot effectively suppress Th-17 differentiation (reviewed in Refs. 44–46). Furthermore, natural Tregs can convert into Th-17 cells and become proinflammatory with the amount of IL-6 present in the inflammatory environment (19, 47, 48). This latter observation has led to some concern regarding the use of cultured Tregs for therapy. Data in Fig. 2D and 2E suggested that natural IgM can directly inhibit Th-17 differentiation and prevent Foxp3+ Tregs from becoming proinflammatory under Th-17-inflammatory conditions.

Our studies in mice (Fig. 5) and observations in human renal transplants (reviewed in Ref. 1) indicated that high IgM-ALA levels can, in the presence of Tregs, attenuate severe inflammatory processes (e.g., in rejection) by inhibiting Th-1 and Th-17 proliferation and differentiation. Allograft rejection of several organs in humans also involves Th-17 cells, making our observations with IgM more even relevant (49, 50). High IgM-ALA levels can be naturally induced, as we observed in a subset of end-stage renal dialysis patients, or achieved by administering IgM, as we did to protect WT-B6 mice with competent Tregs from developing an aggressive rejection with fully incompatible BALB/c cardiac allografts where rejection in this model also involves IL-17 (51).

These studies, prompted by observations in human renal and cardiac transplants, highlight the importance of naturally occurring IgM-ALA in providing another mechanism to regulate excess inflammation involving IL-17–producing cells and mediated by both innate and adaptive immune mechanisms and could explain why these Abs increase in various infective and other inflammatory conditions and are designed to be noncytolytic to leukocytes at 37°C, despite the presence of complement. Such Abs could be used pre-emptively, either alone or together with Tregs, to prevent renal IRI and to treat severe inflammatory disorders involving Th-17 cells.

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Disclosures
The authors have no financial conflicts of interest.

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Corrections


Under the first heading (IgM and IgG purification from sera) in the Materials and Methods, the third sentence should read “Column-purified IgM was not absorbed with protein G but repassed through Sephacryl S-300 to remove contaminating proteins.”

We did not absorb IgM with protein G, as after the absorption procedure residual protein G in the IgM preparation enhanced lymphocyte activation and proliferation.

The corrected full method is as follows:

Sera from WT-C57BL/6 (referred to as WT-B6) were heat treated (56˚C for 1 h) to inactivate complement. CaCl₂ (30 µl 1 M solution) and dextran sulfate (40 ml 10% solution) were added to each milliliter sera, which was then incubated at room temperature for 30 min to precipitate out fibrin and lipids from the serum. Serum was then diluted 1:2 with PBS and centrifuged at 12,000 × g to remove the precipitate. Approximately 15 ml of this pretreated, diluted serum was loaded on a column (2.5 cm diameter × 100 cm length) containing Sephacryl S-3000 HR (GE Health Care Biosciences, Upsalla, Sweden) suspended in PBS (Gibco, Grand Island, NY) to separate serum proteins by size. All effluent from the first protein peak was saved for IgM whereas effluent from the peak portion of the second larger protein peak was saved for IgG. Effluents were filtered to remove large particles and then concentrated on a centrifugal concentrator (100-kDa pore size; Amicon Ultra, EMD Millipore, Cork, Ireland) to achieve IgM and IgG levels of 1.0–1.2 mg/ml as quantitated by ELISA assays. Approximately 10 mg of concentrated IgM and IgG was then repassaged through the same column to further purify these preparations, after which effluents were reconcentrated (1 mg/ml), dialyzed against RPMI 1640, and then millipored using a 0.45 µm filter. There was <5% contaminating IgG in the IgM preparation. With this procedure, we obtained 6–8 mg IgM from 30 ml mouse serum. Purified IgM or IgG was aliquotted and stored at 4˚C and not frozen. IgM forms aggregates when frozen. The effect of IgM on in vitro cultures was dose dependent and maximal functional effects were observed using a physiological dose of 5–15 µg/250 × 10⁶ cells/0.5 ml culture.

IgM was not isolated by dialyzing in water or by ammonium chloride precipitation as both these techniques yielded IgM with impaired functional activity. Additionally, we did not use protein G to remove contaminating IgG from IgM as residual protein G in the IgM preparation activated T cells to proliferate.

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