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IgG1 Is Pathogenic in *Leishmania mexicana* Infection

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Abstract

There are over 2 million new cases of leishmaniasis annually, and no effective vaccine has been developed to prevent infection. In murine infection, *Leishmania mexicana*, which lives intracellularly in host macrophages, has developed pathways to hijack host IgG to induce a suppressive IL-10 response through FcγRs, the cell-surface receptors for IgG. To guide vaccine development away from detrimental Ab responses, which can accompany attempts to induce cell-mediated immunity, it is crucial to know which isotypes of IgG are pathogenic in this infection. We have found that IgG1 and IgG2a/c induce IL-10 from macrophages in vitro equally well but through different FcγR subtypes: IgG1 through FcγRIII, and IgG2a/c through FcγRI primarily, but also through FcγRIII. In sharp contrast, mice lacking IgG1 develop earlier and stronger IgG2a/c, IgG3, and IgM responses to *L. mexicana* infection and yet are more resistant to the infection. Thus, IgG1, but not IgG2a/c or IgG3, is pathogenic in vivo, in agreement with prior studies indicating that FcγRIII is required for chronic disease. This calls into question the assumption that macrophages, which should secrete IL-10 in response to both IgG1 and IgG2a/c immune complexes, are the most important source of IL-10 generated by IgG-FcγR engagement in *L. mexicana* infection. Further investigations are required to better determine the cell type responsible for this immunosuppressive FcγRIII-induced IL-10 pathway and whether IgG2a/c is protective.

Keywords

Antibodies; Fc receptors; Monocytes/Macrophages; Parasitic-Protozoan; Rodent; Transgenic/Knockout mice

Introduction

The protozoan parasite *Leishmania* causes 2 million new cases of leishmaniasis a year, afflicts 12 million people at any given time, and is a major health problem throughout the world (1). According to the World Health Organization, leishmaniasis is the second leading cause of death from parasitic infection and the 12th leading cause of death from infectious diseases worldwide (1,2). Furthermore, the incidence of leishmaniasis is increasing due to development into forested areas and because of population shifts due to wars and other conflicts. Unfortunately, drug therapies for leishmaniasis, such as pentavalent antimonial drugs and amphotericin B, are

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toxic, and drug resistance is on the rise for many species of *Leishmania* (3-5). To add to this problem, there are no highly effective vaccines for the infection, or arguably for any other protozoan parasite. In general, our most effective vaccines induce protective neutralizing Ab responses [e.g. to bacterial surface polysaccharides of pneumococcus (6) or to viral coat proteins of hepatitis B virus (7)]. We and other investigators showed that IgG bound to the surface of *Leishmania* amastigotes (the intracellular mammalian host-dwelling parasite stage) can induce the cytokine IL-10, which, in turn, can suppress the immune response to the parasite by downregulating inducible NO synthase (iNOS) and by inhibiting Th1 cell development and IFN-γ production (8,9). IFN-γ is essential to activate infected macrophages to kill *Leishmania* via the NO pathway, and mice lacking IFN-γ or iNOS have progressive infection rather than controlled chronic disease, when infected with *L. mexicana* (10). Hence, the IgG response, which is usually protective, is usurped by the parasite for its survival. Thus, understanding how *Leishmania* evades the immune response is central to the development of a usable vaccine. In particular, it is crucial to know if all Ab responses or only certain isotypes are pathogenic, to tailor these responses to the benefit of the host.

We previously showed that whereas IL-4 and IL-12, important drivers of Th2 and Th1 responses, respectively, are the main determinants of susceptibility for *L. major* infection, IL-10 plays the most important role in the more chronic *L. mexicana* infection (10). Mice lacking IL-10 are resistant to *L. mexicana* infection and generate a protective IFN-γ response, unlike wild-type (WT) C57BL/6 (B6) mice, which have chronic, non-healing lesions (9). Mice lacking all activating FcγR (FcRγ knockout [KO] mice), as well as mice lacking only FcγRIII, also heal with a protective Th1 response; in the latter case, it is clear that IL-10 is diminished in the lesion (9,11). In vitro, IgG bound to *Leishmania* amastigotes can generate an IL-10 response from LPS-stimulated macrophages (8,9). This IL-10 is FcγR- and IgG-dependent. We showed that IgG1 Abs to *L. mexicana* occur early in infection and are present and predominate at 10-12 wk, a time when IL-10 KO and FcγRIII KO mice begin to heal and when WT mice enter the chronic phase (11). Prior to the present studies, it was not known whether IgG2a/c responses, which are delayed when compared with IgG1, have a similar propensity for inducing the immunosuppressive IL-10 response. IgG3 responses also have not been the focus of much work in the field, because the Th1/Th2 association is less clear. Therefore, we examined the roles of different FcγR and IgG isotypes on bone marrow-derived macrophages (BMMΦs) and extended this work to the in vivo roles of IgG1 and IgG2a/c. We now demonstrate that although IgG2a/c and IgG1 are equally capable of inducing IL-10 from macrophages in vitro, IgG1 KO mice, which develop accentuated and early IgG2a/c and IgG3 responses, are more resistant to *L. mexicana* infection. This may require a rethinking of the model that IgG-*Leishmania*/FcγR-induced IL-10 must be coming from macrophages (8), requiring a further analysis of this question.

We also found that IgG1 KO mice have a stronger parasite-specific IgM response, as well as IgG2a/c and IgG3 responses early in infection. This demonstrates an interesting relationship of class switching to IgG2a/c and IgG3 in the absence of class switching to IgG1, which is not yet understood.

Materials and Methods

Mice

B6 WT and B6.129P2-Fcgr3^{tm1} (B6 FcγRIII KO) mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and B6.129P2-Fcerg1^{tm1} (B6 FcRγ KO) mice were purchased from Taconic Farms (Germantown, NY). B6 IgG1 KO mice (12) were a generous gift from Dr. R. T. Strait (University of Cincinnati, Cincinnati, OH) and were bred in-house. B6 FcγRI KO mice (13) were a generous gift of Dr. J. S.Verbeek (Leiden University Medical Center, Leiden, The Netherlands). Courses of infection consisted of groups of at least five mice per

experiment. Female mice were purchased at 4-6 wk and were age matched for all experiments. Animals were maintained in a specific pathogen-free environment; the animal colony was screened regularly, and tested negative, for the presence of murine pathogens. Studies were reviewed and approved by the Institutional Animal care and Use, Safety, and Research and Development Committees of the VA Medical Center of Philadelphia.

Leishmania parasites and Ags

L. mexicana (MNYC/BZ/62/M379) promastigotes were grown at 27°C in Grace's medium (pH 6.3; Life Technologies, Grand Island, NY) supplemented with 20% heat-inactivated FBS (Hyclone Laboratories, Logan, UT), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Stationary-phase promastigotes (day 7 of culture) were washed three times in PBS and 5×10^6 parasites (in 50 μl PBS or DMEM) were injected into the hind footpad of mice. Lesions were monitored using a metric dial caliper, and lesion size defined as footpad thickness in the infected foot minus thickness of the contralateral uninfected foot. Lesion-derived amastigotes were obtained by grinding footpad lesions of mice chronically infected with *L. mexicana*. Axenic amastigotes, grown free of mammalian cells, were prepared by placing *L. mexicana* stationary-phase promastigote cultures (day 7) at 33°C for 2 d with passage every 7-10 d at 1/100 into acidic Grace's medium (pH 5.5) supplemented as above. Freeze-thaw Ag (FTAg) was prepared from *L. mexicana* stationary-phase promastigotes that were washed four times in PBS, resuspended at 10^9/ml and frozen (−80°C) and thawed rapidly (37°C) for five cycles (14). Washed membranes were prepared from axenic amastigotes by hypotonic lysis, as described (11).

In vitro recall responses

Single-cell suspensions were prepared from draining lymph nodes (LNs) and 200-μl samples (8×10^5 cells) were cultured in duplicate in 96-well tissue-culture plates in DMEM supplemented with 10% heat-inactivated FBS (Hyclone Laboratories), 25 mM HEPES (pH 7.4), 50 μM 2-ME, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were stimulated with 10 μg/ml (~10^7 cell equivalents/ml) *L. mexicana* FTAg for 3 d at 37°C in a 5% CO_2 incubator, and supernatants were assayed by ELISA for IFN-γ and IL-4, as previously described (15), as well as for IL-10 using commercial antibodies as recommended by the manufacturer (BD Bioscience, San Jose, CA). Cells from uninfected mice had no detectable IL-10, IL-4, or IFN-γ production with Ag stimulation in these experiments. Macrophage supernatants were assayed by ELISA using Ab pairs for IL-10 per manufacturers recommendations (BD Bioscience).

Infection of BMMΦs

BMMΦs were prepared and infected as previously described (11). Axenic amastigotes (AAs) were opsonized for 30 min on ice with serum from infected mice or other Ab preparations and washed before infection of macrophages. Unopsonized parasites and those opsonized with uninfected B6 mouse serum showed no difference in IL-10 induction. LPS from *Escherichia coli* 0111:B4 (Sigma-Aldrich, St. Louis, MO) was added at 100 or 200 ng/ml. Purified anti-IL-10R (1B1.3a; a generous gift from DNAX), was added to cultures at 9 μg/ml. Chicken OVA (36 μg/ml) was incubated (rocking) with rabbit anti-OVA (3:10 dilution; Bethyl Laboratories, Montgomery, TX) for 30 min at room temperature to generate immune complexes, which were used at a final concentration of 1:10 as a positive control.

Measurement of Leishmania-*specific serum Ig*

Sera from infected mice were assayed for *Leishmania*-specific IgG1, IgG2a/c, IgG3, and IgM by ELISA using AA washed membranes for capture and biotin-conjugated anti-mouse IgG1, IgG2a/c, IgG3, and IgM (BD Biosciences), with detection by peroxidase-conjugated

streptavidin (Jackson ImmunoResearch laboratories, West Grove, PA). Ig quantitation shows mean and SEM for at least four mice per group. All OD values were calculated by first subtracting uninfected mouse serum OD values (background) before further calculations. Because ELISAs are quite nonlinear in response, relative quantities of anti-*Leishmania* Ig were calculated by plotting OD versus dilution factor for a standard serum and fitting with polynomial regression (third-to fifth-degree polynomial, depending on the data) using Kaleidagraph 3.64 for Mac (Synergy Software, Reading, PA). Relative Ig levels were calculated from the standard curve, determining the dilution of the standard that gave the same OD value as a defined dilution of the sample serum (1/40 or 1/80). Mean ODs for groups of mice were used to calculate relative Ig levels and the ratios (IgG1 KO/B6 WT).

IgG sera and isotype purification

IgG1 serum was obtained from mice early in *L. mexicana* infection (6-7 wk) and was found to lack IgG2a/c and IgG3 by ELISA. IgG1 was purified from this serum using protein A agarose spin columns (Pro-Chem, MA) with elution at pH 5.5 as directed by the manufacturer. Protein A is specific for IgG and has differential binding to different mouse IgG isotypes, with IgG1 binding least tightly and IgG2a/c binding most tightly. This preparation also did not have detectible IgG2a/c or IgG3 present. IgG2a/c serum was obtained from a B6 IL-4 KO mouse immunized s.c. with *L. mexicana* washed membranes and IFAand then challenged with *L. mexicana*. This serum was found to have IgG2a/c and no detectable IgG1. For pure IgG2a/c, we used a mouse mAb selected for binding to washed membranes and the surface of *L. mexicana* amastigotes and purified by ammonium sulfate precipitation and protein G agarose (Invitrogen, NY). As expected, this Ab was devoid of IgG1 and IgG3. Similar results were obtained with purified IgG1 or IgG2a/c as with IgG1 and IgG2a/c sera, as described above.

IgG1 depletion of serum

IgG1 depletion was performed as per Su and Stevenson (16). Briefly, biotinylated goat anti-mouse IgG1 (0.5 mg/ml; BD Biosciences) was incubated with PBS-washed streptavidin agarose (Pierce/Thermo Fisher Scientific, IL) (2:1, Ab: 50% slurry) for 3 h at room temperature, with shaking on a rotary platform. This mixture was washed three times in PBS and incubated with serum at a 1:6 ratio (50% bead slurry: 1/40 dilution of serum) overnight on a rotating wheel at 4°C. The depleted serum was recovered by centrifugation. To control for any nonspecific effects of the beads, undepleted samples were mock depleted using streptavidin agarose beads without anti-mouse IgG1. IgG levels were calculated as described.

FcγRIV blockade

Anti-FcγRIV blocking mAb (9E9) (17) was kindly provided by Dr. J. V. Ravetch (Rockefeller University, New York, NY) and was used at 20 μg/ml in cell culture. Flow cytometry demonstrated that 9E9 saturated binding at <5 μg/ml, and no difference was seen for blockade in the range 5-50 μg/ml.

Parasite quantitation

Parasite quantitation was performed by limiting dilution, as described previously, for at least four mice per group (14). The limit of detection was 1.4 log = 25 parasites/lesion.

Statistics

A Student *t* test (two-tailed, unequal variance) was used to compare groups of samples, with $p < 0.05$ considered significant. For IgG1 depletion, a paired *t* test was used to compare mock and IgG1-depleted samples. For in vivo comparisons, values for groups of 5-10 individual mice were used; for in vitro macrophage assays, quadruplicate wells were used for each condition. All experiments were performed at least twice with similar results, and

representative data are shown. For Ig-quantitative calculations, statistical significance was determined by the raw OD values, and error was propagated using $\Delta f(x) = f(x+\Delta x) - f(x)$, where x is the OD value, $f(x)$ is the relative amount of Ig calculated from the standard curve, and Δx=SEM of x.

Results

IgG1 and IgG2a/c use different FcγR subtypes to induce IL-10 from macrophages

We stimulated BMMΦs with LPS and infected them with *L. mexicana* AAs that had been opsonized with anti-*L. mexicana* IgG1- or IgG2a/c-containing serum or uninfected serum or were unopsonized (see *Materials and Methods* for preparation of these sera). When we used macrophages from B6 WT mice, we observed the characteristically low IL-10 production from LPS stimulation alone (Fig. 1*A*). *L. mexicana* AAs lacking surface-bound IgG (unopsonized or opsonized with serum from uninfected mice) augmented this IL-10 slightly. However, opsonization of the parasites with either IgG1 (Fig. 1*A*) or IgG2a/c (Fig. 1*B*) induced large and comparable increases in IL-10. To confirm that the individual IgG isotype was responsible and that other serum components were not involved, we purified the IgG isotypes. Purified IgG1 and IgG2a/c yielded similar results to the IgG1 and IgG2a/c sera (Fig. 1).

FcγRIII KO macrophages had a severe, nearly complete defect in IL-10 augmentation in the presence of IgG1 on the parasites (Fig. 1*A*). This is consistent with a stronger binding of IgG1 to FcγRIII than to FcγRI or IV (18). When we used IgG2a/c, there was a very small reduction in IL-10 produced by FcγRIII KO cells compared to WT cells (Fig. 1*B*). It is known that IgG2a/c binds to FcγRI with high affinity (including uncomplexed, free IgG2a/c) and that the newly described FcγRIV also has a higher affinity for IgG2a/c than does FcγRIII (17). When we used FcγRI KO macrophages, there was a partial defect in IgG2a/c-stimulated IL-10 but not IgG1-stimulated IL-10. As a control, we show FcRγ KO macrophages, which lack expression of all of the activating FcγRs, (i.e. FcγRI, III, and IV) and have a complete defect in IL-10 production from both IgG1 and IgG2a/c. Because FcγRs are required for this IL-10, a role for other Ig subtypes, such as IgM, can be ruled out. In addition, in some experiments we purified IgG from serum using protein A columns and had similar results to unpurified serum (Fig. 1), also demonstrating that the active component is IgG rather than other Ig or non-Ig serum components. It should be noted that immune complexes (opsonized amastigotes) without LPS induced very low levels of IL-10 (data not shown) in agreement with the literature (8,19).

When taken together, this shows that macrophages can produce IL-10 equally well from IgG2a/c and IgG1 immune complexes, at least in vitro. These data do not distinguish between these isotypes in their abilities to stimulate immunosuppressive IL-10, which was shown to be crucial in lesions to induce chronic disease with *L. mexicana* infection. We conclude that IgG1 functions through FcγRIII, and IgG2a/c functions primarily through FcγRI and partially through FcγRIII or FcγRIV.

FcγRIV is not important in macrophage induction of IL-10

The recently described FcγRIV is an activating FcγR that contains the common FcRγ-signaling component and has affinity for IgG2a/c and IgG2b but little binding by IgG1 (17,20). FcγRIV is important in B cell depletion during anti-CD20 therapy of lymphomas and autoimmune diseases (20) and in autoimmune thrombocytopenia (17). We wished to determine whether some of the IgG2a/c stimulation that was not induced by FcγRIII might be occurring through FcγRIV, in addition to or instead of FcγRI. We stimulated WT and FcγRIII KO macrophages with LPS and IgG2a/c-*L. mexicana* complexes in the presence or absence of FcγRIV blocking Abs (mAb 9E9) (Fig. 1*C*). Binding of anti-FcγRIV was found to be saturating at <5 μg/ml, as demonstrated by flow cytometry; in addition, no effect was observed even when 50 μg/ml anti-

FcγRIV was used (data not shown). We found that blocking FcγRIV did not have any major role in IgG2a/c-induced IL-10, suggesting that FcγRI and, to a lesser extent, FcγRIII were likely responsible.

IgG1 KO mice are more resistant to L. mexicana *infection than WT mice*

It is known that FcγRIII KO mice heal *L. mexicana* infection (11), indicating that this receptor is required for the establishment of chronic disease. We demonstrated above that IgG1 acts almost exclusively through FcγRIII. Furthermore, anti-*L. mexicana* IgG1 levels are detectible by 8 wk of infection, before IgG2a/c is seen (11). However, IgG1 and IgG2a/c are equally capable of inducing IL-10 from B6 WT macrophages in vitro. Therefore, we asked whether IgG1 is required for chronic disease or whether IgG2a/c is sufficient. We found that B6 mice lacking IgG1 are more resistant than WT mice to *L. mexicana* infection (Fig. 2*A*). Parasite burdens were not different at 12 wk, but IgG1 KO mice had 4000-fold ($3.6\log_{10}$) fewer parasites than WT mice by 27 wk of infection (Fig. 2*B*). IFN-γ production by draining LN cells was significantly higher (4.5-fold) in IgG1 KO mice than in WT mice at 12 wk of infection, before the decrease in parasite burdens (Fig. 2*C*). IL-4 and IL-10 levels were not consistently different between WT and KO mice (data not shown). These findings demonstrate a stronger Th1 response in IgG1 KO mice, indicating that IgG1 has a role in suppressing the IFN-γ response.

IgM levels continue to increase throughout chronic disease and are greater in IgG1 KO mice than in B6 WT mice early in infection

IgG1 KO mice have a deletion in the Igγ1 H chain, which prevents class switching to IgG1. We wished to determine whether this caused a build up of IgM-producing cells with a concomitant increase in serum IgM levels specific for *Leishmania*. We found more IgM in the serum of IgG1 KO mice than in B6 WT mice at 10 wk of infection (4.4-fold more) (Fig. 3*A*). The IgM levels were greater at 28 wk of infection than at 10 wk in IgG1 KO and B6 WT mice, reaching similar levels at this latter time point in both mouse strains.

IgG1 KO mice have earlier and stronger IgG2a/c responses to L. mexicana *infection than WT mice*

The Ab response to *L. mexicana* infection of B6 WT mice shows an early peak in IgG1, followed by a later increase in IgG2a/c (11). We bled IgG1 KO and B6 WT mice throughout the course of *L. mexicana* infection to examine the kinetics of the IgG responses. As expected, IgG1 KO mice had no detectable IgG1, and B6 WT mice had detectable levels ~6 wk of infection, with relative amounts that peaked ~12 wk of infection (Fig. 3*B*). B6 WT mice developed IgG2a/c later than IgG1 (Fig. 3*C*). Unexpectedly, IgG1 KO mice had significantly more IgG2a/c than did B6 WT mice at nearly all time points, with a range of 2.2-5.3-fold more (Fig. 3*C*). Thus in the absence of class switching to IgG1, B cells switched to IgG2a/c earlier in infection.

Different IgG isotypes may compete with each other for binding to the Ag-capture material in an ELISA. We wanted to be certain that the increase in IgG2a/c in early infection of IgG1 KO mice seen in ELISAs was not an artifact due to reduced competition for Ag. To assess this, we depleted IgG1 from serum samples of *L. mexicana*-infected B6 WT mice and compared these to mock depleted samples. Depletion was 97.3-99.8% effective at reducing IgG1 in B6 WT serum samples, and a 2.6-fold increase was seen in IgG2a/c detected by ELISA in a quantitative assay, although these were not statistically significant changes (Fig. 3*D*). This was not sufficient to explain the 24-fold increase in IgG2a/c detected in IgG1 KO versus B6 WT mice; thus, the large increase in IgG2a/c in IgG1 KO mice is not artifactual.

IgG1 KO mice have earlier and stronger IgG3 responses to L. mexicana *infection than WT mice*

Although IgG1 is a Th2-associated isotype, and IgG2a/c is a Th1-associated isotype, IgG3 is not as clear-cut in terms of expression in Th1 and Th2 responses. We determined the time course of the anti-*Leishmania* IgG3 response in B6 WT and IgG1 KO mice. We found that in *L. mexicana*-infected B6 WT mice, like IgG2a/c, IgG3 responses are not present early but appear much later in infection (Fig. 4*A*). At 9 wk of infection, B6 WT mice had very low levels of parasite-specific IgG3. In contrast, IgG1 KO mice had a robust IgG3 response that was 84-fold higher than that of B6 WT mice. In fact, the IgG1 KO mice had IgG3 levels at 9 wk that were comparable to B6 WT mice at 28 wk of infection. IgG3 levels in IgG1 KO mice plateaued ~8 wks of infection (Fig. 4*B*), whereas in B6 WT mice the levels of IgG3 were much lower for >8 wk and plateaued ~16 wk of infection. In vitro depletion of IgG1 increased IgG3 levels in B6 WT sera 1.3-fold, similar to what was observed above for IgG2a/c. This is a small effect compared with the 106-fold increase in IgG3 in IgG1 KO sera versus B6 WT sera at the same time point (Fig. 4*C*) and even compared with the 12.7-fold difference seen in Fig. 4*B*. Thus, the IgG3 response was much stronger and earlier in IgG1 KO mice than in B6 WT mice.

Discussion

We previously found that FcγRIII is required for chronic disease with *L. mexicana* infection in B6 mice, and *L. mexicana*-specific IgG1 appears early in infection and predominates at the branch point between healing and chronic disease (10-15 wk). We have now shown that IgG2a/c and IgG1 are equally capable of inducing IL-10 from macrophages in vitro. IgG1-parasite immune complexes act almost exclusively through FcγRIII. However, in this infection, IgG2a/c immune complexes, act through FcγRI and, to some extent, through FcγRIII, but likely not through FcγRIV.

It was shown that IL-10 from regulatory T cells is critical to the outcome of *L. major* infection of BALB/c mice (21). However, we showed that regulatory T cells ($CD25^+$ $CD4^+$ $CD3^+$ cells), which are the main $CD4^+$ T cell subset making IL-10 in the draining LNs in *L. mexicana* infection, can be depleted without changing the immune response, lesion development, or parasite load (11). We also showed that IL-10 in lesions is diminished in healing FcγRIII KO mice as compared with B6 WT mice, whereas IL-10 is not different in draining LNs (either by ELISA or flow cytometry) (11). This argues that a crucial cell type for IL-10 production through FcγRIII engagement is found in lesions rather than in draining LNs. *L. mexicana* lesions have large numbers of macrophages and very small numbers of lymphocytes (10), and macrophages express high levels of FcγRs, whereas conventional T cells do not express these receptors (reviewed in Ref. 22). When these data are combined with the fact that macrophages readily secrete IL-10 in response to IgG-*Leishmania* immune complexes, it was reasonable to assume that macrophages were the important source of IL-10 in *L. mexicana* infection (8).

In sharp contrast to the in vitro macrophage data, IgG1 KO mice are more resistant to *L. mexicana* infection; thus, IgG1 is the critical isotype in vivo for the chronic disease phenotype. In itself, this does not demonstrate a special role for IgG1 over IgG2a/c; IgG1 may be the only IgG isotype present at the critical time when a Th1 response needs to develop to lead to parasite control and healing. However, we also showed that IgG1 KO mice surprisingly develop a much stronger and earlier IgG2a/c response. This argues against a simple kinetic model for the importance of IgG1 over IgG2a/c. It suggests, instead, that the cell types responsible for IL-10 production in lesions must show a preference for IgG1, possibly due to exclusive FcγRIII expression or FcγRIII function dominating FcγRI and IV function. Furthermore this suggests the possibility that macrophages, which are so abundant in *L. mexicana* lesions, may not be the main important players in IL-10 production. Experiments are underway to test this more

directly and may suggest a role for other cell types in this chronic disease picture caused by *L. mexicana* and potentially many other pathogens.

Immune complexes through FcγR have at least three major roles: uptake of parasites into cells potentially facilitating infection, suppression of IL-12, and induction of IL-10. In prior studies, we addressed the role of FcγR for parasite uptake in vivo. β2-microglobulin KO mice have undetectable Ab levels (because FcRn contains β2-microglobulin and protects IgG from catabolism) and yet have higher parasite loads early in infection compared with WT mice, before they heal and control parasites (9). This is not due to a lack of CD8 T cells, because CD8 KO mice have no phenotype. Also in FcγRIII KO mice we showed that early parasite loads are the same as those of WT mice before healing occurs (11). Thus, a lack of IgG or FcγRIII in vivo does not impair parasite uptake (because other receptors can take their place), conversion to amastigotes, or parasite survival, but it does lead to less IL-10, greater IFN-γ production, and ultimately, to parasite control and disease resolution.

We have also addressed the FcγR effects on IL-12. The major pathway of IL-12 suppression is dependent on IL-10 induction by FcγR activation, with a minor IL-10-independent pathway also occuring through FcγR (11). When IL-10R was blocked in vitro, IL-12 levels were 10-fold higher in macrophage experiments in which immune complexes were present. When IL-10 is absent, the suppression of IL-12 through FcγR (which is intact) is not sufficient to cause chronic disease. In vivo, IL-10 KO mice are resistant to *L. mexicana* infection, showing that IL-10 is absolutely required for chronic disease; thus, the IL-10-dependent pathway predominates.

This leaves the induction of IL-10 as the most important known role of FcγR engagement in *L. mexicana* infection. IL-10 then has a mutiplicity of effects from suppressing T cell activation, suppressing IL-12, and directly suppressing iNOS expression.

This work highlights the importance of different isotypes of IgG in *L. mexicana* infection, as has been seen in other disease processes, such as autoimmune hemolytic anemia and thrombocytopenia (17) and with anti-CD20 depletion of B cells (20). The distinct effects of IgG isotypes are likely due to different affinities for various FcγR subtypes (20), which have now been determined in humans as well (23). Our studies also give guidance for vaccine development for leishmaniasis in that IgG1 production [or the equivalent Th2 isotype in humans, likely IgG4 (24,25)] should be avoided, whereas the human equivalents of IgG2a/c may be protective. Thus, the IgG responses to *Leishmania* vaccines may be critical, in addition to the established need for an IFN-γ-driven cell-mediated immune response. Although the Th1-associated IgG2a/c likely will be fostered by vaccines that induce IFN-γ responses, this cannot be taken for granted as FcγRIII KO mice had stronger early IFN-γ responses and did not alter the IgG isotype character of the Ab response (11).

We also uncovered an aspect of IgG class switching. In *L. mexicana* infection, class switching to IgG2a/c and IgG3 occurs at an earlier time in mice that lack IgG1 than in B6 WT mice, and IgG1 KO mice produce considerably more IgG2a/c and IgG3 than WT controls. Thus, IgM-producing cells switch to IgG2a/c or IgG3 when IgG1 H chain genes are unavailable. This contradicts prior data that indicate specific pathways for IgG class switching for each isotype (26). It might be hypothesized that a stronger early IFN-γ response in the IgG1 KO mice could stimulate the IgG2a/c class switching, because IFN-γ is known to favor this process (27). However, IgG3 should be suppressed by IFN-γ (27) rather than stimulated, as our data indicate (Fig. 4). Also against this explanation is the finding that FcγRIII KO mice, like IgG1 KO mice, have a stronger early IFN-γ response than do B6 WT mice when infected with *L. mexicana*, but the IgG isotype kinetics are identical in FcγRIII KO and B6 WT mice (11). Further analysis of the isotype-switching mechanism in IgG1 KO mice may prove interesting. IgG3 KO mice

immunized with protein-conjugated pneumococcal polysaccharide had greater and earlier total Ig, IgM, and IgG1 responses to the polysaccharide than WT mice (28). Surprisingly, however, in vivo infection of mice with pneumococcus did not yield increased levels of IgG1 or IgM in IgG3 KO mice over WT responses (29). This indicates that the types of immune responses induced by pathogens in vivo are more complex than seen in immunization model systems.

It has been reported that IgG3 does not interact with any of the FcγR in vitro (13,17) or in vivo (22) and is frequently T cell independent. Most effector functions of IgG3 in mice are mediated by complement and not FcγR (22,30). Given the clear dependence of chronic disease in *L. mexicana* infection on FcγRIII, it seems that IgG3 would not have a role. In addition, IgG3 is essentially absent early in infection (Fig. 4), when IgG is crucial to induce IL-10 through FcγRIII to engender chronic disease. However, IgG3 may still have a role in IgG1-deficient mice in competing with IgG2a/c for targets or late in infection in WT mice.

Our examination of IgM in *L. mexicana* infection showed some surprises. We found that early parasite-specific IgM production is greater in IgG1 KO than in WT mice. Thus, there may be a population of IgM-producing B cells that stall in class switching when γ1 H chain genes are lacking. Interestingly, IgM responses continued to rise during chronic infection with *L. mexicana* in both IgG1 KO and WT mice, with high levels persisting at 28 wk of infection, well into the chronic phase (Fig. 3*A*). Because IgM has a half-life of 28 h in mice (31), this suggests continued IgM production and likely continued stimulation of naïve B cells to become IgM producers. This is unusual, even for chronic infections, in which IgM declines as IgG and other Ig types increase (32). There is some evidence for persistent IgM to *Plasmodium* infection in mice, despite parasite clearance (33), but few other examples were found in the literature. Although it generally wanes in titer, human serum IgM can persist in West Nile virus infection for > 17 mo (34,35), and it can persist in the cerebrospinal fluid for >6 mo without clear evidence of continuing disease (36). Also, persistent IgM to phase II Ag at 1 y is a bad prognostic sign in Q fever (*Coxiella burnetii*) endocarditis in humans (37). Because we found a specific role for FcγRIII, which does not bind IgM, in chronic disease, a direct pathogenic role for IgM, as recently seen in *L. infantum* infection of BALB/c mice (38), would not be expected. However, we cannot rule out a partially protective role for IgM as is seen in lupus nephritis, in which anti-dsDNA IgM may protect from the harmful effects of IgG (39). The immunologic role of this IgM in *L. mexicana* infection remains unclear.

Our current studies showed that IgG1 KO mice, which develop stronger and earlier IgG2a/c responses, are more resistant than WT mice. One explanation is that IgG2a/c is protective and that IgG1 is merely irrelevant. However, the in vitro data indicate that IgG2a/c, through FcγRI, and to a lesser extent FcγRIII, can efficiently induce IL-10. We have already shown that IL-10 is critical for chronic disease and, thus, is pathogenic. Clearly, IgG2a/c and IgM, in the absence of IgG1, as seen in IgG1 KO mice, do not induce sufficient IL-10 to suppress a protective Th1 response or directly block iNOS sufficiently. This leaves us with the alternative hypothesis that IgG1 induces IL-10 and chronic disease through FcγRIII. Dr. Ravetch's group (17,18) showed that FcγRIII has highest affinity for IgG1 and that IgG1 acts almost exclusively through FcγRIII, whereas IgG2a/c binds efficiently to FcγRI and FcγRIV. The high affinity of IgG1 for FcγRIII and the clear dependence of chronic disease on FcγRIII argue that IgG1 is, in fact, pathogenic by inducing immunosuppressive IL-10 through FcγRIII. The early appearance of IgG1 and late appearance of IgG2a/c in *L. mexicana*-infected B6 mice is consistent with this role for IgG1. This suggests that the relevant IL-10-producing cell expresses FcγRIII but not FcγRI or FcγRIV, unlike macrophages. It remains to be seen if IgG2a/c, IgG3, and IgM are actually protective, either by competing with IgG1 for targets or by signaling through receptors other than FcγRIII. They may just be neutral and unable to induce the immunosuppressive IL-10 pathway. However, late in B6 mouse infection, when IgG2a/c, IgG3, and IgM increase in titer, *L. mexicana* lesions and parasite loads plateau. This may be

evidence that IgG2a/c, and potentially IgG3 and IgM, compete with IgG1 and reduce the FcγRIII-IL-10 pathway. We hope to address this point directly in the future.

We showed that IgG1 and IgG2a/c are equally capable of inducing IL-10 from macrophages in vitro; however, in vivo, mice that lack IgG1 have earlier and greatly enhanced IgG2a/c and IgG3 responses and control *L. mexicana* infection better than do WT mice. Because macrophages are known to express high levels of multiple FcγRs, it is unlikely that they are the important source of FcγR-induced IL-10 responsible for chronic disease in this infection. We are currently addressing this issue directly and hope to find this other cellular source of IL-10 to help explain the mechanism of this important immunosuppressive pathway. In addition, the increased parasite-specific IgM, IgG2a/c, and IgG3 found in mice unable to class-switch to IgG1 indicates an unexpected interrelationship between class switching to different isotypes that deserves further examination. Furthermore, we now have important information indicating which isotypes are pathogenic to help guide vaccine development away from IgG1 production (or the equivalent isotypes in humans).

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Abbreviations used in this paper

AA	axenic amastigotes
BMMΦ	bone marrow-derived macrophage
B6	C57BL/6
FTAg	freeze-thaw Ag
iNOS	inducible nitric oxide synthase
KO	knockout
LN	lymph node
WT	wild-type

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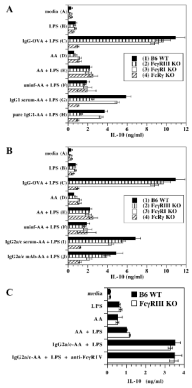

FIGURE 1. IgG1 and IgG2a/c can induce IL-10 from macrophages through FcγRIII, and FcγRI and III respectively, but FcγRIV is not involved

BMMΦs were prepared from B6 WT, FcγRIII KO, FcγRI KO, and FcRγ KO mice (strains are numbered 1-4) and incubated with anti-IL-10R and with the following additions (conditions are labeled A-J): media; LPS; chicken OVA-rabbit anti-OVA complexes (IgG-OVA); unopsonized AAs; or AAs opsonized with uninfected serum (uninf-AA) or anti-*L. mexicana* IgG1 serum (IgG1 serum-AA), IgG2a/c serum (IgG2a/c serum-AA), purified IgG1 (pure IgG1-AA), or IgG2a/c mAb 3E4-G1-G4 (IgG2a/c mAb-AA) for 20 h, and IL-10 was measured in the supernatants by ELISA. IgG-OVA + LPS was used as a positive control. Opsonized parasites generate similar amounts of IL-10 to unopsonized parasites when LPS is not present. IgG-OVA also does not induce significant amounts of IL-10 when LPS is absent. *A*, IgG1-induce IL-10 with controls. $p < 0.05$ for 1B versus 1E, 1F; 1F versus 1G, 1H; 2F versus 2G; 4G versus 1G; 4H versus 1H; 2G versus 1G; 3G versus 2G; 4G versus 2G; 2H versus 1H; and 3H versus 2H. *B*, IgG2a/c-induced IL-10 with controls. $p < 0.05$ for 1B versus 1E, 1F; 1F versus 1I, 1J; 3F versus 3I; 4I versus 1I; 4J versus 1J; 3I versus 1I; 4I versus 1I; 3J versus 1J; and 4J versus 1J. *C*, BMMΦs were prepared from B6 WT and FcγRIII KO mice and incubated with anti-IL-10R and media alone, LPS, unopsonized AAs, AAs with LPS (AA + LPS), or AAs opsonized with IgG2a/c serum with LPS (IgG2a/c-AA + LPS) for 20 h, and IL-10 was measured in the supernatants by ELISA. Anti-FcγRIV-blocking Ab (9E9; 20 μg/ml) was added to the cultures indicated. No statistically significant differences were seen between B6 and FcγRIII KO cells or for IgG2a/c-AA + LPS in the presence or absence of anti-FcγRIV-blocking Ab.

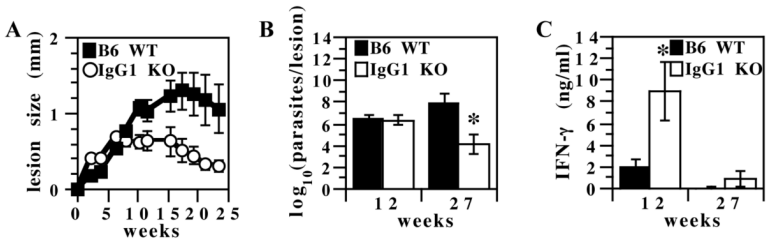

FIGURE 2. IgG1 KO mice are more resistant to *L. mexicana* infection with an earlier IFN-γ response than WT mice

A, IgG1 KO and B6 WT mice were infected in the right hind footpad with 5×10^6 stationary-phase *L. mexicana* promastigotes, and lesion size was monitored. Lesion sizes were different at 15 wk and thereafter ($p < 0.05$). *B*, At the times indicated post-infection, lesion parasite burdens from IgG1 KO and B6 WT mice were determined by limiting dilution. *C*, At the times indicated post-infection, draining LN cells were stimulated with FTAg for 3 d, and supernatants were assayed for IFN-γ. *$p < 0.05$.

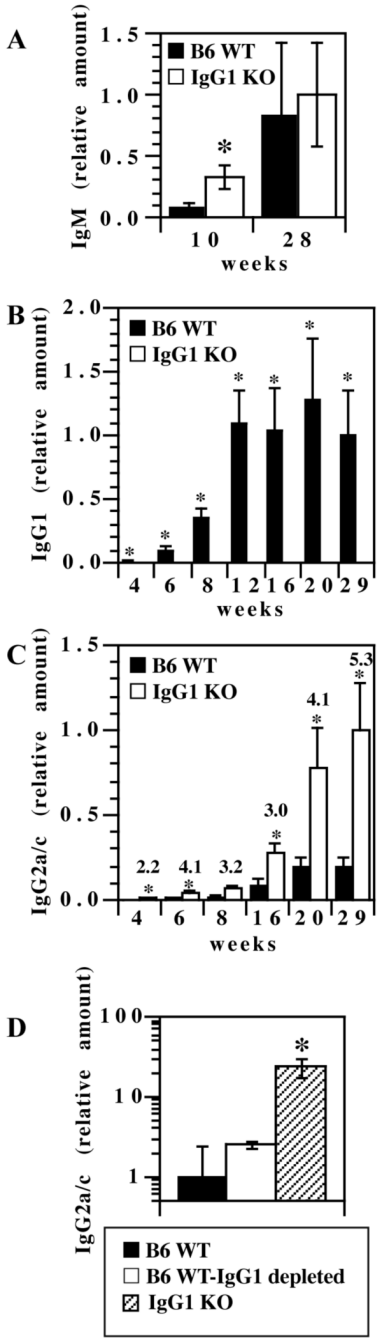

FIGURE 3. IgG1, IgG2a/c, and IgM levels in IgG1 KO and B6 mice infected with *L. mexicana*

IgG1 KO and B6 WT mice were infected as in Fig. 2, and serum samples collected at the times shown were evaluated for *L. mexicana*-specific IgM (*A*), IgG1 (*B*), and IgG2a/c (*C*) by ELISA. In C, the ratio of relative abundances (IgG1 KO/B6 WT) is shown for each time point. *$p <$ 0.05 (*A*-*C*). *D*, Sera from IgG1 KO and B6 WT mice infected with *L. mexicana* for 12 wk were depleted of IgG1 and then ELISAs were performed for IgG2a/c. *$p < 0.05$ for IgG1 KO versus other groups; $p > 0.05$ for B6 WT versus B6 WT-IgG1 depleted (paired *t* test). Undepleted samples received streptavidin beads without biotinylated anti-IgG1.

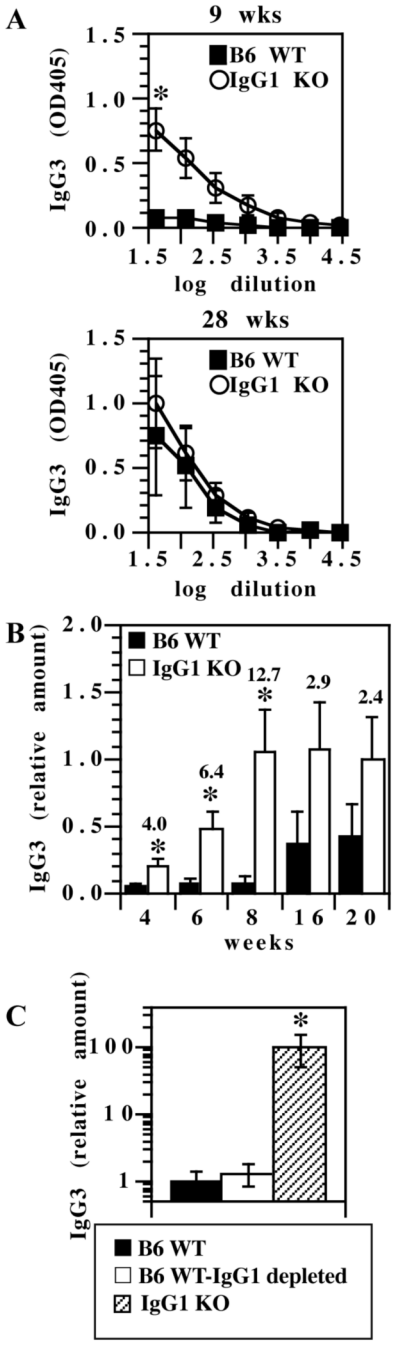

FIGURE 4. IgG1 KO mice have stronger and earlier IgG3 responses

A, IgG1 KO and B6 WT mice were infected, as in Fig. 2 and serum samples (collected at the times shown) were evaluated for *L. mexicana*-specific IgG3 by ELISA. *$p < 0.05$ at all dilutions. *B*, The kinetics of *L. mexicana*-specific IgG3 levels were determined. The ratio of relative abundances (IgG1 KO/B6 WT) is shown for each time point. *$p < 0.02$. *C*, Sera from IgG1 KO and B6 WT mice infected with *L. mexicana* for 12 wk were depleted of IgG1 and then ELISAs were performed for IgG3. *$p < 0.05$ for IgG1 KO versus other groups; $p > 0.05$ for B6 WT versus B6 WT-IgG1 depleted (paired *t* test). Undepleted samples received streptavidin beads without biotinylated anti-IgG1.