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Polyclonal and Specific Antibodies Mediate Protective Immunity against Enteric Helminth Infection

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SUMMARY

Anti-helminth immunity involves CD4$^+$ T cells, yet the precise effector mechanisms responsible for parasite killing or expulsion remain elusive. We now report an essential role for antibodies in mediating immunity against the enteric helminth *Heligmosomoides polygyrus* (*Hp*), a natural murine parasite that establishes chronic infection. Polyclonal IgG antibodies, present in naive mice and produced following *Hp* infection, functioned to limit egg production by adult parasites. Comparatively, affinity-matured parasite-specific IgG and IgA antibodies that developed only after multiple infections were required to prevent adult worm development. These data reveal complementary roles for polyclonal and affinity-matured parasite-specific antibodies in preventing enteric helminth infection by limiting parasite fecundity and providing immune protection against reinfection, respectively. We propose that parasite-induced polyclonal antibodies play a dual role, whereby the parasite is allowed to establish chronicity, while parasite load and spread are limited, likely reflecting the long coevolution of helminth parasites with their hosts.

INTRODUCTION

Enteric helminth infections represent a major cause of morbidity among populations living in developing countries with an estimated 400 million infected school children (WHO, 1997, 2005). Severe infections cause a variety of health problems including growth retardation, vitamin A deficiency, anemia, and poor cognitive function, and they contribute to potentially fatal malnutrition through malabsorption, protein-losing enteropathy, and intestinal obstruction (WHO, 1997, 2005). There is usually a clear correlation between parasite number and morbidity (Hayes et al., 2004; Hotez et al., 2005). Given that many enteric helminths reproduce outside the human host, parasite burdens increase mainly through reinfection—a process that could be halted by adequate vaccination. Protective immunity against enteric helminths is often dependent on Th2 responses (Finkelman et al., 1997) and associated with high levels of serum IgE and IgG1 together with an influx of eosinophils, basophils, and mast cells to the infected site. Yet, there is little evidence of an absolute requirement for any of these effector mechanisms in protective immunity, limiting rational vaccine design.

Heligmosomoides polygyrus is a natural enteric nematode parasite of murine rodents that enters the gastrointestinal tract at larval stage L3 then penetrates the epithelial cell barrier of the small intestine to mature within the submucosa to an L4 stage. Approximately 8–10 days after infection, the parasite exits the intestinal mucosa to populate the intestinal lumen and establish a chronic infection as a sexually mature adult producing viable eggs that are secreted through the feces (Anthony et al., 2006; Monroy and Enriquez, 1992). For many enteric helminths eventual expulsion of adult worms from the intestine is associated with changes in jejunal physiology, including IL-4- and IL-13-regulated changes to intestinal epithelial cell function (Madden et al., 2002, 2004; Shea-Donohue et al., 2001) and smooth muscle cells (Zhao et al., 2003).

In subsequent infections of immunocompetent mice with *H. polygyrus*, the parasite is quickly rejected and the development of sexually mature adult worms is prevented (Morimoto et al., 2004). Both primary and secondary infections with *H. polygyrus* are followed by a dramatic increase in the circulating levels of IgG1 and IgE and a robust eosinophilia (Anthony et al., 2006; Urban et al., 1991a). Protective immunity following secondary infection is directed against the larvae stages of the parasite and is dependent on CD4$^+$ T cells and IL-4 production (Anthony et al., 2006) but does not require eosinophils (Urban et al., 1991b). Surprisingly, mast cells are not found in the intestine following *H. polygyrus* infection, suggesting that this parasite has evolved mechanisms to evade mastocytosis (Behnke et al., 1993). Thus, the exact mechanisms of protective immunity against recurrent *H. polygyrus* infections remain unclear.

We set out to identify the primary effector arm of protective immunity against secondary infection with *H. polygyrus* and now

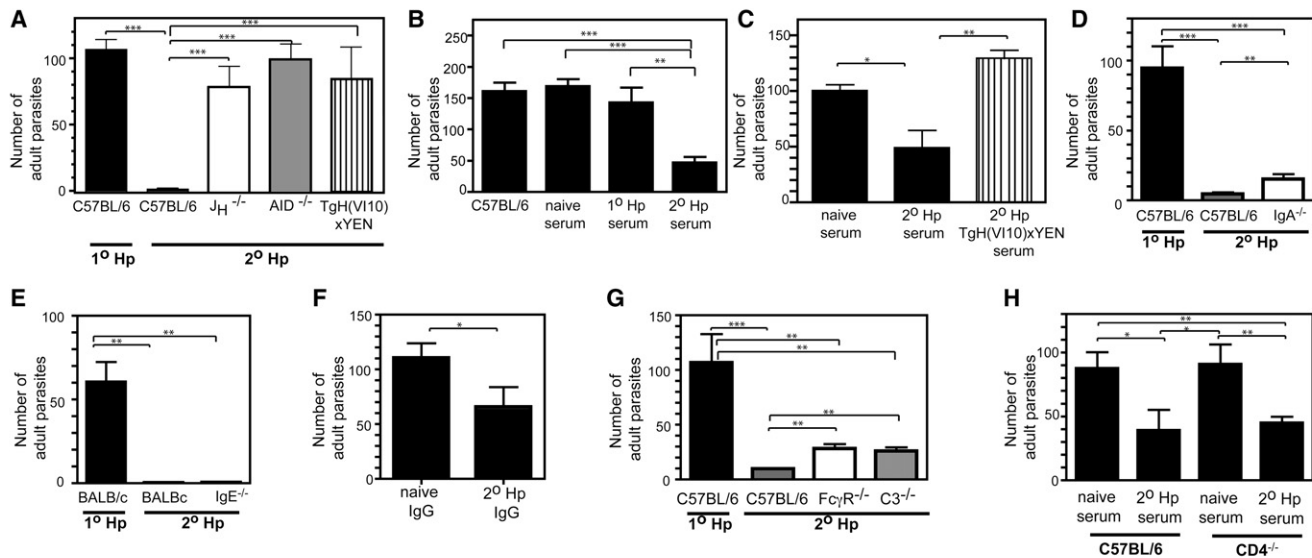

Figure 1. Antibodies Are Required for Protective Immunity against *H. polygyrus*

Mice were infected with 200 L3 *H. polygyrus* (Hp) on day 0 and the number of surviving adult parasites determined 14–20 days later. Adult worm numbers are given for (A) primary (1°) or secondary (2°) infected C57BL/6, $J_H^{-/-}$, $AID^{-/-}$, or TgH(VI10)xYEN mice.

(B) Naive C57BL/6 mice treated with intraperitoneal injections every 2nd day from the time of infection of 100 µl PBS or pooled serum from naive, 1°, or 2° Hp-infected C57BL/6 mice.

(C) Naive C57BL/6 mice treated with intraperitoneal injections every 2nd day from the time of infection of 100 µl PBS or pooled serum from 2° Hp-infected C57BL/6 or TgH(VI10)xYEN mice.

(D) 1° or 2° infected C57BL/6 and $IgA^{-/-}$ mice.

(E) 1° or 2° infected BALB/c and $IgE^{-/-}$ mice.

(F) Naive C57BL/6 mice treated with intraperitoneal injections every 2nd day from the time of infection of 100 µl PBS or IgG purified from the serum of naive or 2° Hp-infected C57BL/6 mice.

(G) 1° or 2° infected C57BL/6, $Fc\gamma R^{-/-}$, and $C3^{-/-}$ mice.

(H) Naive C57BL/6 or $CD4^{-/-}$ mice treated with intraperitoneal injections every 2nd day from the time of infection of 100 µl PBS or pooled serum from naive or 2° Hp-infected C57BL/6 mice.

All data are shown as the combined mean ± SEM of individual mice (n = 3–17 mice per group) from one or multiple experiments and are representative of at least two independent experiments. For all data significant differences were determined by a one-tailed Student's t test and are depicted as *$p \leq 0.05$, **$p \leq 0.005$, or ***$p \leq 0.0005$.

demonstrate a crucial, nonredundant role for antibodies in this process. Surprisingly, the parasite-induced antibody response appeared to be dominated by polyclonal specificities. To further investigate the degree of polyclonal activation we employed TgH(VI10)xYEN mice, in which almost all mature B cells express a neutralizing immunoglobulin against vesicular stomatitis virus (VSV) (Hangartner et al., 2003). *H. polygyrus* infection of TgH(VI10)xYEN mice resulted in a marked production of VSV-specific IgE and IgG1, demonstrating that the polyclonal repertoire included nonrelevant specificities. Nevertheless, the polyclonal response present in normal wild-type C57BL/6 mice was found to act in favor of both the parasite and the host by allowing parasite persistence but limiting parasite spread by lowering female egg production following primary infection. In contrast, the later development of affinity-matured parasite-specific antibodies functioned to provide protection against reinfection.

RESULTS

Antibodies Are Essential for Protective Immunity against *Heligmosomoides polygyrus*

To investigate the role of B cells in protective immunity against secondary *H. polygyrus* infections we utilized $J_H^{-/-}$ mice that lack mature B cells (Chen et al., 1993b). These mice were completely unable to mount a protective response against secondary *H. polygyrus* infection as shown in Figure 1A. A role for B cells as antigen-presenting cells did not account for their protective effect as $J_H^{-/-}$ and control C57BL/6 mice exhibited comparable T cell responses following *H. polygyrus* infection (Figure S1 available online). Rather, protective immunity required isotype-switched or affinity-matured antibodies as $AID^{-/-}$ mice, which contain B cells and IgM-secreting plasma cells but have lost the ability to undergo isotype class switching or somatic hypermutation (Muramatsu et al., 2000; Revy et al., 2000), were also unable to mount a protective immune response following secondary infection with *H. polygyrus* (Figure 1A). Importantly, antibody-mediated protection following secondary *H. polygyrus* infection reflected parasite-mediated activation of memory cells as the same effect was observed when mice were subjected to anti-helminth treatment to clear remaining parasites 6 weeks prior to reinfection (data not shown). Moreover, protective immunity appeared to be absent rather than delayed in the absence of antibodies, as $J_H^{-/-}$ mice continued to harbor parasites at day 30 following secondary infection (data not shown).

Protective immunity against *H. polygyrus* appeared be directed against the tissue dwelling L4 stage, as equivalent

numbers of larvae were observed in the submucosa of primary and secondary infected C57BL/6 mice, while the development of luminal dwelling parasites was selectively prevented in secondary infected mice (Figure S2). A role for antibodies in killing tissue-dwelling L4 parasites was additionally supported by the finding that the cellular infiltrate surrounding these larvae was rich in IgG and IgE antibodies, some of which appeared to be bound to the surface of macrophages and granulocytes (Figure S3).

We next asked whether antibodies would be able to provide protection against secondary *H. polygyrus* infection when the B cell repertoire is fixed with an irrelevant specificity. For this purpose we infected TgH(VI10)xYEN mice, which were generated by crossing a B cell receptor transgenic strain expressing the VSV-specific light chain (YEN) to mice with a targeted VDJ insertion (knockin) encoding for the V_H-D-J_H heavy chain of the VSV-neutralizing antibody VI10 (Hangartner et al., 2003). As VI10 mice contain a targeted knocked-in VDJ insertion the immunoglobulin heavy chain locus can still undergo class switch recombination to all isotypes (Hangartner et al., 2003). TgH(VI10)xYEN were unable to mount protective immune responses against *H. polygyrus* challenge infection, indicating that the protective antibodies require parasite specificity (Figure 1A).

Protective specificities, present in C57BL/6 mice, were only found to arise after multiple infections as passive transfer of serum from secondary infected mice into naive wild-type C56BL/6 mice clearly reduced adult worm numbers, while serum from naive or primary infected mice had no effect on worm number (Figure 1B). As expected, serum from secondary infected TgH(VI10)xYEN mice was unable to limit adult worm development after passive transfer (Figure 1C). To determine which antibody isotype was responsible for mediating protective immunity we infected mice deficient in IgA or IgE antibodies. IgA deficiency resulted in a significant, but minor, increase in the development of adult worms (Figure 1D), while IgE deficiency had no impact on protective immunity (Figure 1E). These data indicated a major role for IgG antibodies, and passive transfer of purified IgG from secondary infected mice resulted in a significant reduction in adult worm numbers (Figure 1F).

We next investigated the role of Fc gamma-chain receptor (FcγR) ligation or complement activation and found that both pathways play very small, but reproducible, roles in mediating parasite rejection (Figure 1G). A requirement for CD4-dependent activation of accessory cells was determined by the passive transfer of serum from secondary infected mice into naive C57BL/6 or CD4-deficient recipients. A comparable reduction in the number of adult worms was found for C57BL/6 and $CD4^{-/-}$ mice receiving immune serum (Figure 1H), indicating that protective antibodies function, at least in part, through CD4-independent mechanisms. Taken together these data indicate that parasite-specific IgA and IgG antibodies can provide protective immunity through multiple mechanisms including complement activation, FcγR ligation, and possibly direct interference with essential parasitic enzymes required for tissue invasion, feeding, or development.

Parasite Infection Promotes the Production of Polyclonal IgE and IgG1

The finding that protective antibodies were only detected in mice having experienced multiple infections with *H. polygyrus* indicated that parasite-specific specificities arise slowly. Indeed, examination by ELISA of the specificity of the antibody response in C57BL/6 mice showed that although primary *H. polygyrus* infection resulted in a dramatic increase in total IgG1 and IgE antibodies (Figures 2A and 2B, left panels, closed symbols), L5 *H. polygyrus* excreted secreted protein (HES) specific antibodies were detectable only at late time points following primary infection (IgG1, Figure 2B, middle panel, closed symbols), or only following secondary infection (IgE Figure 2A, middle panel, closed symbols). The delayed appearance of HES-specific IgE compared to IgG1 corroborates the findings of Yoshida et al. (1990), who reported that the majority of parasite-induced IgE arises as a result of sequential class switch recombination events from C mu to C gamma 1 and then from C gamma 1 to C epsilon. HES-specific IgA could also be detected in the serum and intestinal wash but only following secondary infection (Figures 2C and 2D, middle panels, data for day 20 post-secondary infection shown, closed bars). Binding of IgE and IgA antibodies from secondary infected mice was only observed for HES antigens, while IgG1 antibodies were found to react against both HES antigens (as determined by ELISA, Figure 2) and L5 somatic and cuticle-associated antigens (as determined by immunofluorescent staining of sectioned worms, data not shown).

Histological analysis showed that at day 9 after primary infection IgE- and IgG-positive plasma cells were predominately present within extrafollicular regions of the draining mesenteric lymph nodes, while IgE and IgG staining within germinal centers was only detectable at later time points (Figure 3). No detectable increase in either extrafollicular or germinal center IgA staining was observed (data not shown). Immunoglobulin V region gene hypermutation within germinal centers promotes the development of high-affinity memory and plasma B cells (Berek et al., 1991; Jacob et al., 1991; Manser, 2004), while plasma cells in primary extrafollicular responses rarely exhibit evidence of V region mutation (Jacob and Kelsoe, 1992; MacLennan et al., 2003; Maizels and Bothwell, 1985; Sze et al., 2000). Thus, our data indicated that the majority of IgG1 and IgE antibodies arising after primary *H. polygyrus* infection represented polyclonal or low-affinity specificities as a result of the parasite driving a predominately extrafollicular response.

To further substantiate the largely polyclonal nature of the *H. polygyrus*-induced antibody responses we infected TgH(VI10)xYEN mice. Infection resulted in a dramatic increase in total serum IgE (Figure 2A, left panel, open symbols) and a smaller increase in total IgG1 (Figure 2B, left panel, open symbols) antibodies in these mice. Remarkably, the early production of IgE and IgG1 antibodies in TgH(VI10)xYEN mice receiving primary or secondary infections was largely represented by VSV-specific antibodies (Figure 2A and 2B, right panels, open symbols). The production of VSV-specific IgE after parasite infection was particularly striking with titers increasing by over 6 logs within the first 12 days after primary infection (Figure 2A, right panel, open symbols). No parasite-specific IgE, and severely reduced titers of parasite-specific IgG1, were detected in TgH(VI10)xYEN mice following *H. polygyrus* infection (Figures 2A and 2B, middle panels, open symbols). Interestingly, no significant increase in total or VSV-specific IgA was observed in parasite-infected TgH(VI10)xYEN mice, indicating that in contrast to IgE and IgG1, parasite infection does not drive the

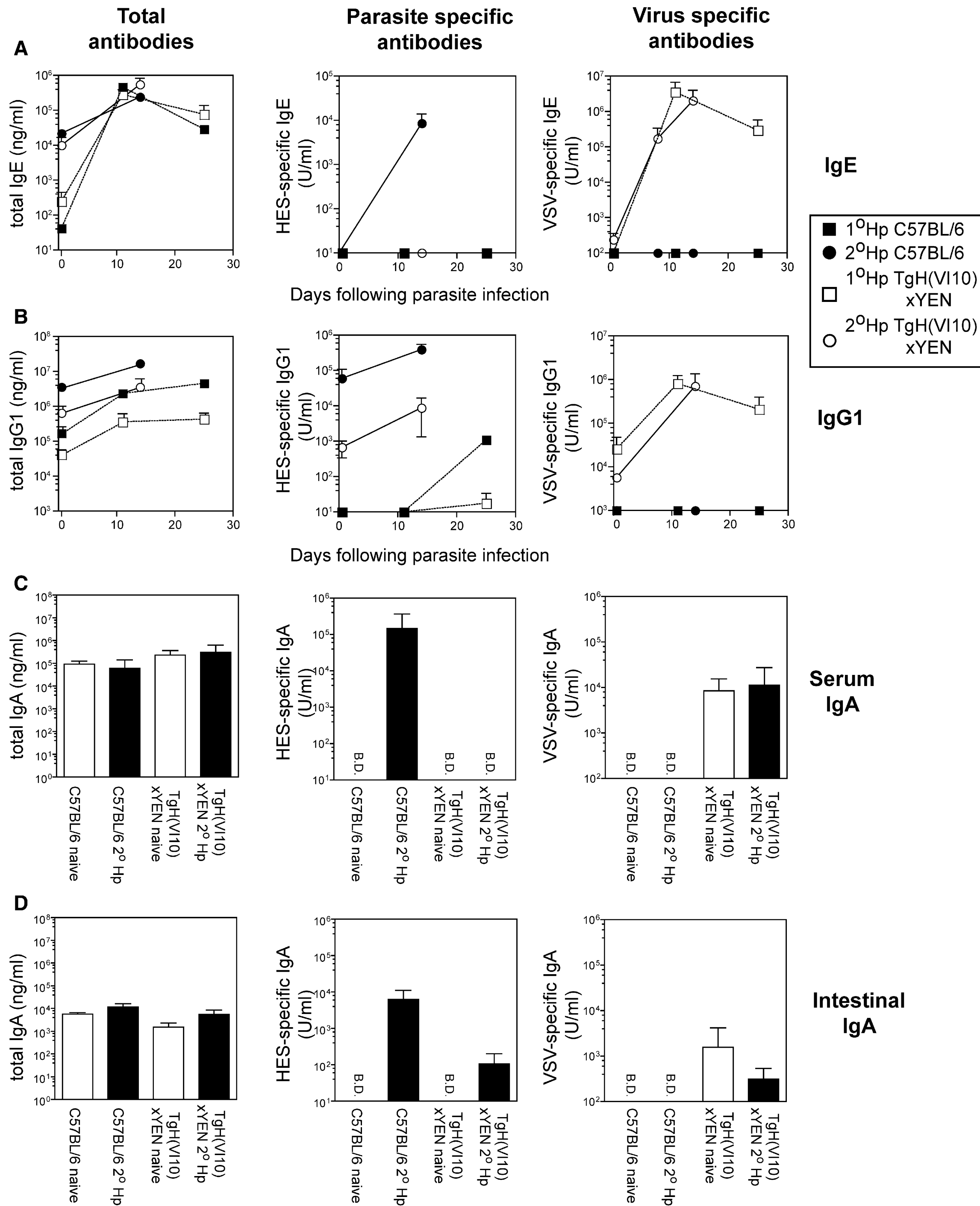

Figure 2. ***H. polygyrus*** **Infection Results in the Robust Production of Nonspecific IgE and IgG1 Antibodies**

(A and B) C57BL/6 (closed symbols) or TgH(VI10)xYEN (open symbols) mice were subjected to a primary (1°, squares) or secondary (2°, circles) infection with 200 L3 Hp and serum collected at the indicated time points following infection. Production of (A) IgE or (B) IgG1, including total, L5 HES (excretory/secretary products of adult L5 Hp) specific or VSV-specific antibodies was determined by ELISA. Data represent means ± SD of 3 mice per group or means of 2 mice per group for C57BL/6 mice given a 2° Hp infection. Data are from one experiment, with similar results obtained from three independent experiments.

(C and D) Production of IgA is shown for naive or day 20 2° Hp-infected C57BL/6 (closed bars) or TgH(VI10)xYEN (open bars) mice in the (C) serum and (D) intestinal wash. Data represent means ± SD from 3–4 mice per group. Data are from one experiment, with similar results obtained from two independent experiments. For all data, limits of detection for antigen-specific ELISA assays were 10 U/ml L5 HES-specific IgE, IgG1, and IgA, 100 U/ml VSV-specific IgE and IgA, and 1000 U/ml VSV-specific IgG1. B.D. indicates values below the detection limit.

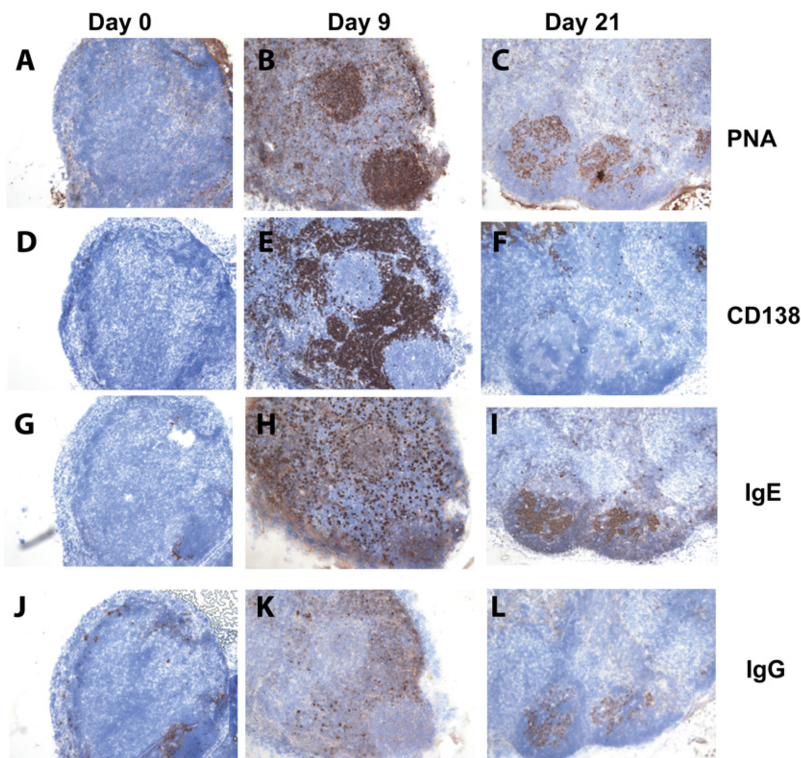

Figure 3. Early Production of IgE and IgG Antibodies following *H. polygyrus* Infection Occurs Predominately at Extrafollicular Sites

C57BL/6 mice were infected with 200 L3 Hp and the draining mesenteric lymph node removed for analysis at day 0, 9, or 21 post-infection. Immunohistochemical staining of serial sections shows (A–C) PNA-positive germinal centers, (D–F) CD138-positive plasma cells, (G–I) IgE-positive cells, and (J–L) IgG-positive cells. Note that anti-IgE staining reveals both IgE-producing plasma cells (dark stain) and germinal center IgE-positive or CD23^{+}B220^{+} positive naive B cells (light stain). Photographs were taken under 5× magnification and represent individual mice. All sections are representative of four mice from two independent experiments.

production of polyclonal IgA (Figures 2C and 2D). This observation fits with that of Sangster et al., who reported that influenza-specific IgG2a, but not IgA, responses included a large nonspecific component (Sangster et al., 2003).

To confirm that the production of VSV-specific IgE and IgG1 reflected parasite-induced isotype class switching, as opposed to the expansion of an existing memory population, we analyzed the draining mesenteric lymph nodes of *H. polygyrus*-infected TgH(VI10)xYEN mice for the presence of ε and γ1 germline transcripts (GLT). Both εGLT and γ1GLT could be detected in mesenteric lymph nodes from infected TgH(VI10)xYEN mice (Figure 4A). Furthermore, the transfer of purified naive B cells from TgH(VI10)xYEN mice into recipient $J_H^{-/-}$ mice followed by *H. polygyrus* infection resulted in the production of εGLT and γ1GLT (Figure 4A) and similar levels of VSV-specific IgE and IgG1 as observed for TgH(VI10)xYEN mice (Figures 4B and 4C).

To exclude the possibility that serum from TgH(VI10)xYEN mice exhibited crossreactivity to *H. polygyrus* antigens, we infected these mice with the unrelated helminth parasite, *Nippostrongylus brasiliensis*. As seen for *H. polygyrus, N. brasiliensis* infection of TgH(VI10)xYEN mice induced a dramatic increase in VSV-specific IgE antibodies (Figure S4). We next examined whether the VSV-specific antibodies had been subjected to somatic hypermutation, which might indicate affinity selection by parasite antigens. IgE and IgG1 transcripts were amplified from the draining mesenteric lymph nodes of *H. polygyrus*-infected $J_H^{-/-}$ mice previously injected with naive TgH(VI10)xYEN B cells. None of the analyzed clones revealed evidence for somatic hypermutation since all sequences corresponded to the knocked-in VI10 sequence (Tables S1 and S2). These data indicated that *H. polygyrus* infection drives the activation and heavy chain class switching of B cells expressing an irrelevant specificity, without necessarily exerting any selective pressure to increase antibody affinity via somatic hypermutation.

As a final confirmation of the polyclonal nature of *H. polygyrus*-induced antibody response we performed an extensive analysis of IgE transcripts isolated from primary or multiply infected mice. IgE was chosen as the large majority of transcripts represent *H. polygyrus*-induced specificities. Transcripts were first analyzed by H-CDR3 length spectrotyping. This method detects differences in CDR3 fragment lengths reflecting the use of various V, D, and J genes, as well as insertions or deletions of nucleotides during the V(D)J rearrangement process (Stoel et al., 2005). Figures 5A and 5B show the pattern of H-CDR3 lengths among IgE transcripts isolated from mice at day 10 following primary or multiple infections with *H. polygyrus*. The data show no difference in diversity following the primary infection, or following a fourth infectious challenge, indicating that in both cases polyclonal antibodies dominate the parasite-induced response. Thus, any parasite-specific response is likely to represent only a small fraction of the total IgE production.

We next performed extensive sequencing of the IgE transcripts and, in keeping with the spectrotyping data, found a high degree of diversity. This was evidenced by the majority (94%) of IgE clones isolated being unique and exhibiting different usage of V, D, and J elements (Figure 5C). There was also no evidence for somatic hypermutation since accumulation of mutations within the CDRs was essentially absent, although for each mouse analyzed we did observe a potential bias in the usage of certain germline V_H sequences. This may indicate preferential selection of these V_H sequences by the parasite, or a murine strain-specific preferential usage of V_H sequences. In contrast, multiple infection rounds did lead to the isolation of IgE transcripts exhibiting evidence of clonal selection and affinity maturation. In this case transcripts with the same VDJ recombination event were amplified from more than one clone for each mouse analyzed (Figure 5C). These clones exhibited evidence of affinity maturation in the form of sequential acquisition of mutations, particularly within CDR2 region (data not shown). Such observations are in keeping with the appearance of HES-specific antibodies detectable by ELISA following multiple infections and

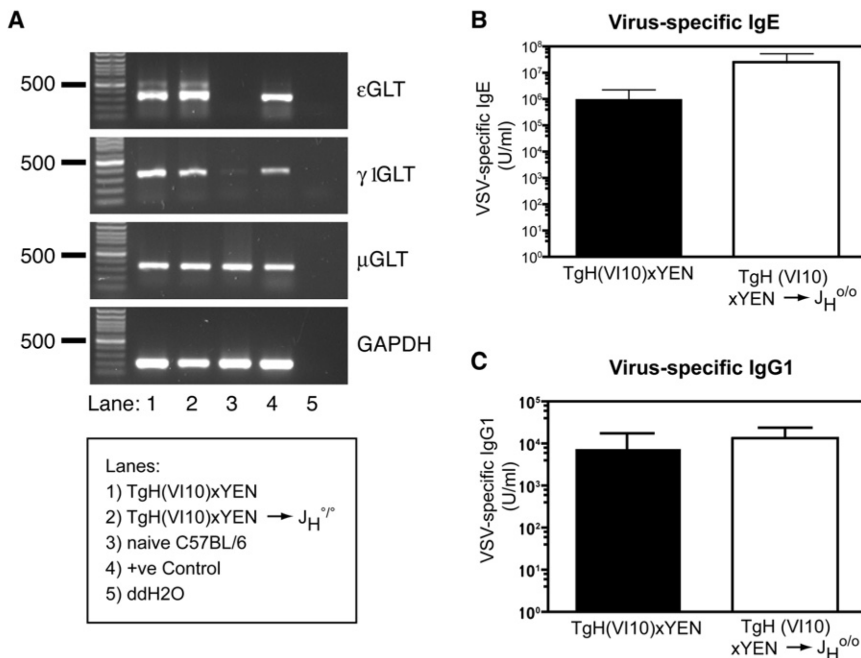

Figure 4. *H. polygyrus*-Induced Production of Nonspecific IgE and IgG1 Antibodies Requires Ig Isotype Switching of Naive B Cells
Naive B cells (5×10^6) purified from TgH(VI10)xYEN were injected into $J_H^{-/-}$ hosts on day 0. On the same day recipient mice were infected with 200 L3 Hp. Naive TgH(VI10)xYEN mice were also infected as a positive control. (A) Total RNA was isolated from mesenteric lymph nodes at day 9 post-infection and reverse transcribed into cDNA, and germline transcripts specific for IgE (εGLT), IgG1 (γ1GLT), and IgM (μGLT) were amplified by PCR. The expected sizes of the εGLT, γ1GLT, and μGLT PCR products are 300, 325, and 325 bp, respectively. GAPDH transcripts (220 bp) are shown in the lower panel. Serum titers of VSV-specific (B) IgE or (C) IgG1 were also determined by ELISA at day 9 following infection. Data represent means ± SD from 3–4 mice per group from one experiment. Similar results were obtained from two independent experiments. For all data, significant differences were determined by a one-tailed Student's t test and are depicted as $^*p \leq 0.05$, $^{**}p \leq 0.005$, or $^{***}p \leq 0.0005$.

suggest that protective parasite-specific antibodies arise as a result of clonal selection and affinity maturation within germinal centers.

Parasite-Mediated Induction of Both Polyclonal and Specific Antibodies Is Dependent on MHC Class II-Dependent Cognate T Cell Help

We recently identified a novel pathway for the induction of natural polyclonal IgE that was IL-4 and CD4 T cell dependent but did not require cognate MHC class II interactions or the presence of organized lymphoid structures (McCoy et al., 2006). Interestingly, the IgE antibodies in this study did not exhibit evidence of affinity maturation. Thus we next determined whether MHC class II-dependent cognate interactions were required for parasite-induced polyclonal IgE and IgG1 production. To address this question we generated mixed bone marrow chimeras in which only B cells lacked MHC class II expression. C57BL/6 mice were lethally irradiated and then reconstituted with equal numbers of bone marrow cells from $J_H^{-/-}$ and MHC Class $II^{-/-}$ mice ($J_H^{-/-}$:$MHCII^{-/-}$ → C57BL/6). The inability of B cells to present MHC class II-restricted peptides in $J_H^{-/-}$:$MHCII^{-/-}$ → C57BL/6 chimeric mice prevented the production of both polyclonal and parasite-specific IgE and IgG1 (Table 1). Thus, despite efficient induction of nonspecific IgE and IgG1 antibodies early following *H. polygyrus* infection, B cells undergoing class switch recombination to IgE and IgG1 must first receive cognate help from $CD4^+$ T cells. Of note, although these mice failed to exhibit early polyclonal IgE production or parasite-specific IgE production (Table 1), increased titers of natural IgE did eventually arise. However this occurred at very late time points (day 90) and was not related to parasite infection (data not shown).

Polyclonal Antibodies Reduce Parasite Fecundity

The dominant production of nonspecific polyclonal antibodies following *H. polygyrus* infection was surprising given the essential role of isotype-switched antibodies in mediating protective immunity against this parasite. We therefore examined the role of polyclonal antibodies, present even within naive mice, by comparing the outcome of primary *H. polygyrus* infection in C57BL/6 and $J_H^{-/-}$ mice. Although the presence of the polyclonal antibody repertoire did not alter the number of *H. polygyrus* larvae that developed into luminal dwelling adult worms (Figure 6A), such antibodies clearly impacted on parasite fecundity by attenuating parasite egg production (Figure 6B). We also observed that parasite fecundity could be attenuated by polyclonal serum antibodies following the reconstitution of naive $J_H^{-/-}$ mice with naive serum, which exhibited decreased egg output as compared to $J_H^{-/-}$ mice receiving PBS (Figure 6C). Definitive proof that polyclonal antibodies were mediating this effect was obtained by transferring purified IgG from naive mice into $J_H^{-/-}$ recipients, resulting in a reduction of the egg output to a level comparable to that observed in wild-type C57BL/6 mice (Figure 6D).

Collectively our data indicated that antibodies arising after multiple infections of *H. polygyrus* functioned to limit the development of adult worms, while polyclonal antibodies—present in naive mice and increasing rapidly following primary *H. polygyrus* infection—acted to limit parasite fecundity. We reasoned that the comparative impact of IgG antibodies present in naive mice, or those having experienced multiple parasite infections, likely reflected differences in the abilities of these antibodies to recognize worm antigens. We therefore performed western blot analysis of IgG binding to HES antigens using serum from naive, primary infected, or secondary infected mice. Although no binding of naive serum IgG to parasitic proteins could be determined by ELISA assay (Figure 2B, middle panel) or immunofluorescence (data not shown), western blot analysis revealed a weak binding of polyclonal IgG to HES antigens of approximately 50 kDa in size (Figure 6E). Serum from primary infected or secondary infected mice resulted in an increasing degree of IgG binding to HES antigens present in the 50 kDa band and in the

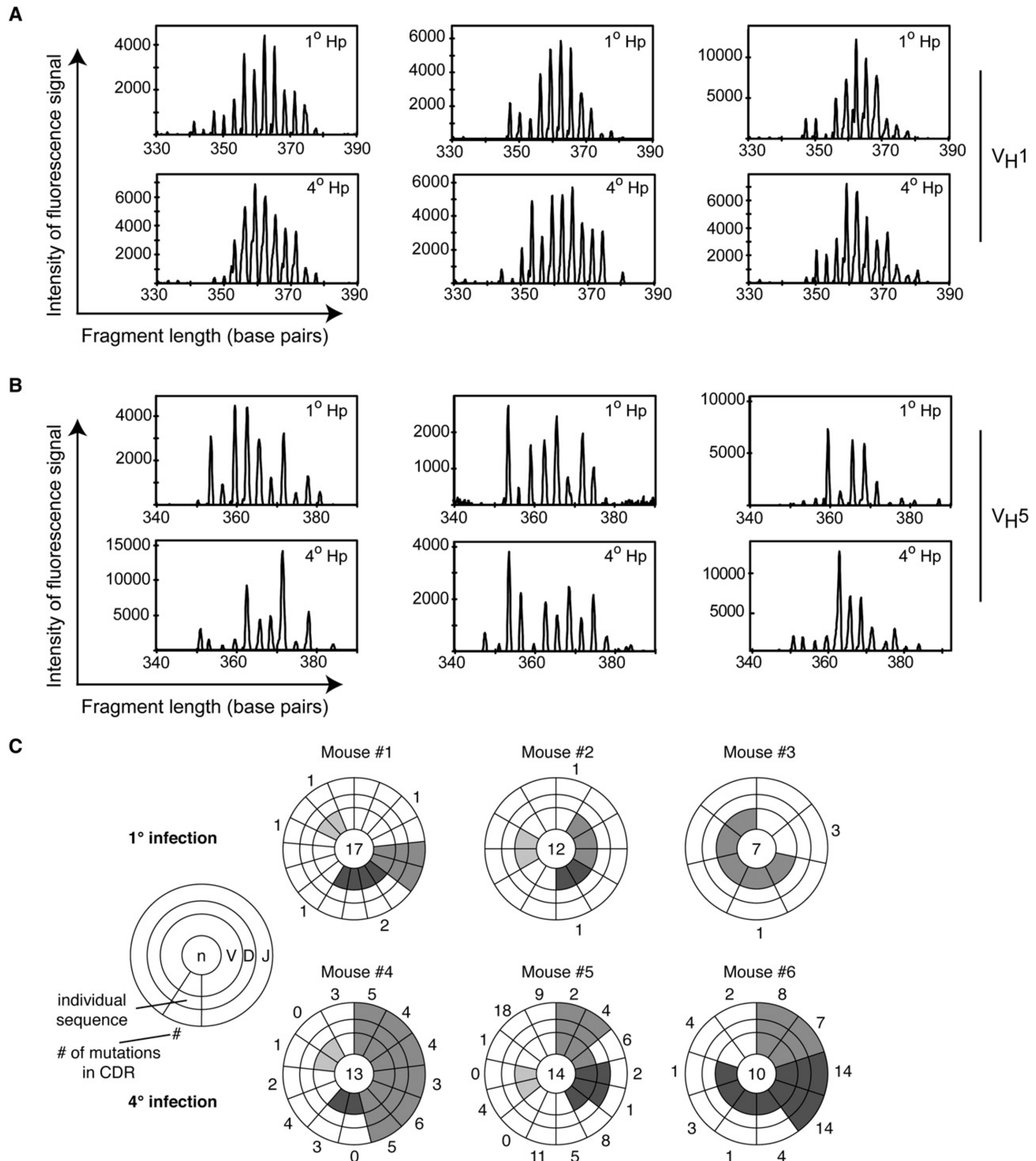

Figure 5. Analysis of IgE Genes Shows a High Degree of Diversity

RNA was extracted from the draining mesenteric lymph nodes of C57BL/6 mice at day 9 following primary (1°) or four (4°) rounds of infection with *H polygyrus*. IgE V_H genes from the (A) J558 V_H1 or (B) PC 7183 V_H5 families were amplified and the length of the CDR3 region determined by spectrotyping. Plots represent the analysis of RNA isolated from individual mice.

(C) IgE transcripts were amplified by RT-PCR subcloned into pGEM-T and sequenced. The closest germline V_H clone (indicated by the VBASE2 ID) was assigned using the DNAPlot program (http://www.dnaplot.de). Each slice of the pie chart indicates one isolated V_H-D_H-J_H clone. The left pie chart indicates which segments of the pie represent the V_H, D_H, and J_H elements (inner: V_H; middle: D_H; outer: J_H). All white pie slices and segments represent unique sequences whereas shaded slices indicate identical sequences, such that shading of all three elements of more than one sequence indicates clonal expansion of a single specificity, whereas shading of only the V_H elements in individual clones indicates usage of identical V_H elements but combined with different D_H and J_H elements. The number of sequences analyzed per mouse is indicated in the center of each pie chart and ranges from n = 7 to n = 17.

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Table 1. Both Specific and Nonspecific Antibody Production following *H. polygyrus* Infection Require MHC Class II-Dependent Cognate T Cell Help

	Total Antibody[a]		Parasite Specific Antibody[b]	
	Naïve	Parasite-Infected	Naïve	Parasite-Infected
IgE				
JHT:B6→B6	$2.6 \times 10^1 \pm 3.6 \times 10^1$	$1.1 \times 10^4 \pm 6.0 \times 10^3$	<1	$8.7 \times 10^2 \pm 8.0 \times 10^2$
JHT: MHC II$^{0/0}$→B6	<1	<1	<1	<1
IgG1				
JHT:B6→B6	$2.7 \times 10^5 \pm 3.0 \times 10^5$	$5.9 \times 10^7 \pm 5.5 \times 10^7$	<1	$6.4 \times 10^3 \pm 4.0 \times 10^3$
JHT: MHC II$^{0/0}$→B6	$1.0 \times 10^4 \pm 9.5 \times 10^3$	$1.8 \times 10^4 \pm 1.5 \times 10^4$	<1	<1

The indicated bone marrow chimeras were infected with Hp and serum collected 40 days later for analysis of serum antibodies; naïve mice are shown for comparison.

[a] All values indicate mean ± SD (n = 3–6) of serum IgE (ng/ml) or IgG1 (ng/ml) or

[b] mean ± SD (n = 3–6) of HES-specific IgE or IgG1 (U/ml).

further detection of antigens of approximately 70 kDa (primary and secondary challenge serum) and >110 kDa in size (secondary challenge serum only) (Figure 6E). Thus, affinity-matured parasite-specific antibodies, present in mice infected multiple times with *H. polygyrus,* were directed against a larger variety of parasitic antigens, and present in greater quantities, than those observed for naive or primary infected mice.

These data indicated that polyclonal IgG functioned to limit parasite fecundity by low-affinity interactions with parasitic antigens. Natural antibodies, or low-affinity polyclonal responses to unrelated pathogens, have previously been demonstrated to provide protection against bacterial infections through a mechanism requiring complement activation (Benedict and Kearney, 1999; Moxon and Anderson, 1979). We therefore investigated

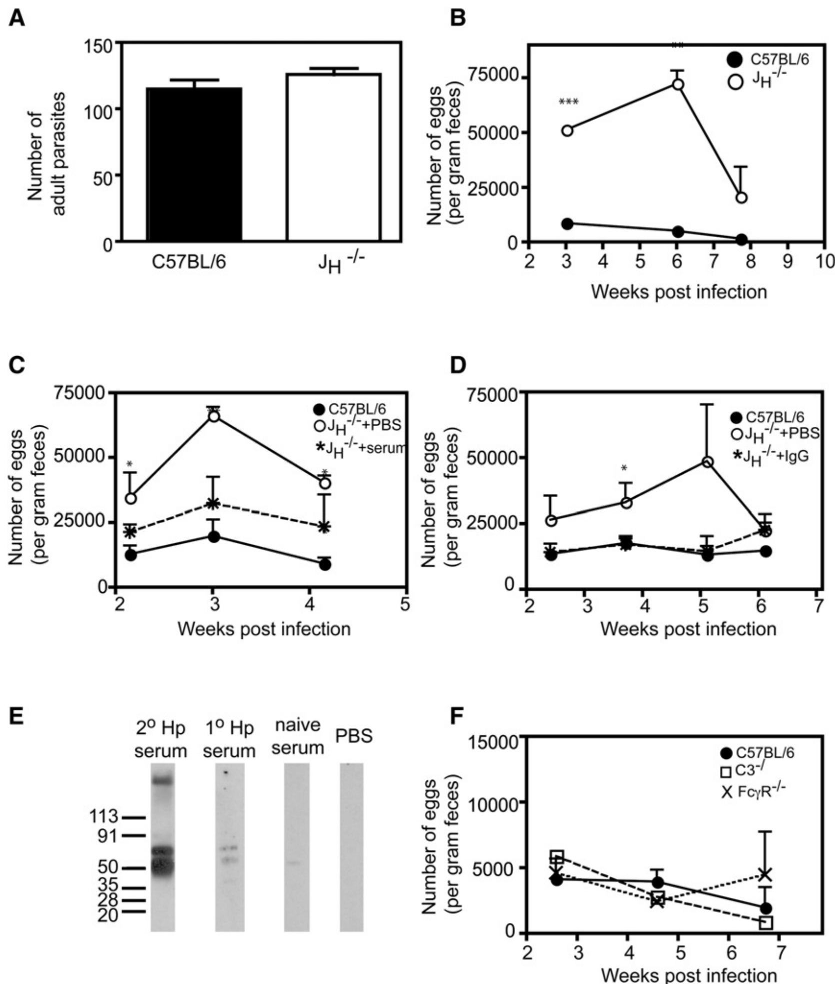

Figure 6. Polyclonal Antibodies Reduce the Fecundity of Female *H. polygyrus* Worms

(A and B) Naive C57BL/6 or $J_H^{-/-}$ mice were infected with 200 L3 Hp and (A) adult parasite development (day 14 post-infection) or (B) egg production determined. Naive C57BL/6 or $J_H^{-/-}$ mice were infected with 200 L3 Hp and $J_H^{-/-}$ mice treated with intraperitoneal injections every 2nd day from the time of infection with (C) 200 μl PBS or naive C57BL/6 serum until day 10 or (D) 400 μl PBS or purified naive IgG (5 mg/ml) throughout the experimental protocol. Egg production was determined at the indicated time points.

(E) Binding of IgG in pooled serum from naive, primary (1°), or secondary (2°) Hp-infected C57BL/6 mice was assessed on identical western blot strips of L5 HES proteins.

(F) Egg production was determined for C57BL/6, $Fc\gamma R^{-/-}$, or $C3^{-/-}$ mice infected with 200 L3 Hp on day 0. For all experiments showing egg counts, values were obtained from 2–3 individual cages per group containing 1–4 mice per cage. Data are shown as the combined mean ± SEM of individual cages from one or multiple experiments and are representative of at least two independent experiments. Significant differences between the indicated groups and control C57BL/6 mice were determined by a one-tailed Student's t test and are depicted as *$p \leq 0.05$, **$p \leq 0.005$, or ***$p \leq 0.0005$.

parasite egg production following *H. polygyrus* infection of mice lacking the complement component C3 ($C3^{-/-}$) or the Fc gamma-chain receptor ($Fc\gamma R^{-/-}$). Neither pathway was observed to impact significantly on parasite egg production (Figure 6F). This indicated that polyclonal IgG likely functions to limit parasite fecundity by binding to, and interfering with, the function of secreted parasite antigens required for essential functions such as feeding or reproduction.

DISCUSSION

Because many enteric helminths do not replicate within the mammalian host, morbidity only occurs when the host suffers repeat or very large doses of parasitic larvae (Hotez et al., 2005). Limiting the infective dose and the possibility of reinfection would therefore prevent host morbidity and the associated consequences. Protective immunity functions to limit reinfection, as highlighted by the finding that children usually exhibit the most severe worm burdens, while the lowest parasite burdens are observed in those adults exhibiting indicators of antiparasite immune responses including high serum IgE (Hayes et al., 2004). Yet despite epidemiological evidence for an association between immune responsiveness and decreased total parasite loads, to date very little is known about the exact effector mechanisms responsible for protection.

We have shown that antibodies form an integral arm of the effector response against the murine enteric helminth *H. polygyrus*. Parasite infection induced a robust IgG and IgE response, but these antibodies were dominated by nonspecific specificities, with parasite-specific antibodies arising only late after primary infection or following multiple infections. We suspected that the ability of this parasite to promote nonspecific antibody production may account for the delayed production of parasite-specific antibodies thereby allowing the long-term survival of adult worms. Remarkably however, polyclonal antibody production was found to be beneficial to the host by reducing parasite fecundity and thereby limiting parasitic spread through the population, and possible reinfection of the same individual. This effect could be replicated by the transfer of polyclonal IgG into mice devoid of antibodies but did not require FcγR ligation or complement activation. These data indicated that antibody-mediated reductions in parasite fecundity were most likely mediated through low-affinity interactions of the antibodies with parasite antigens required for the development of healthy egg-producing female worms. Thus we hypothesize that parasite-induced polyclonal antibody production plays a dual role, whereby the parasite is allowed to establish chronicity, while parasite load and spread are limited by the host. Such a relationship likely reflects the long coevolution of helminth parasites and their mammalian hosts.

We further demonstrated that *H. polygyrus*-induced polyclonal IgG1 and IgE production required T helper cell MHC class II cognate interactions. These observations fit with previous findings that LCMV-induced nonspecific IgG production is dependent on cognate help from virus-specific T cells (Hunziker et al., 2003) and indicated that naive B cells, regardless of BCR specificity, are able to acquire parasitic antigens. How this process occurs is not fully defined but is likely to involve low-affinity BCR binding and/or uptake of complement coated antigens (Villiers et al., 1996), resulting in antigen presentation and extrafollicular plasma cell differentation.

Following worm explusion and reinfection, full protective immunity was observed and was entirely dependent on the production of isotype-switched antibodies. In this case parasitic larvae were prevented from developing into adult worms and protection was associated with an increased titer of parasite-specific antibodies that arose following multiple infections. The mechanism by which parasite-specific antibodies functioned to limit adult worm development could largely be attributed to the IgG istoype, although IgA antibodies contributed to a small degree. A dominant role for IgG antibodies in protective immunity is supported by earlier studies where only IgG1-enriched serum fractions were observed to impact on parasite survival following passive transfer into naive mice (Pritchard et al., 1983). IgE was not found to play an important role in protective immunity against *H. polygyrus*, perhaps reflecting the unique ability of this parasite to suppress intestinal mastocytosis (Behnke et al., 1993).

Antibody-mediated protective immunity appeared to involve multiple mechanisms including FcγR ligation and complement activation. As mentioned, eosinophils are not essential for protective immunity against *H. polygyrus.* However, neutrophils have been reported to play a protective role during *N. brasiliensis* infection (Pesce et al., 2008). In addition, a recent study by Anthony et al. showed large numbers of Th2 cell-dependent alternatively activated macrophages within intestinal granulomas of *H. polygyrus*-infected mice and further demonstrated an essential role for these cells in protective immunity (Anthony et al., 2006). These findings raise the intriguing possibility that IgG binding of Fc receptors on alternatively activated macrophages present within the granulomas surrounding L4 *H. polygyrus* may function to promote their protective function. In the same study Anthony et al. demonstrated that although mice given a secondary *H. polygyrus* infection and treated with clodronate or ariginase 1 inhibitors (to block the function of alternatively activated macrophages) failed to reject adult worms, a reduction in worm fecundity was noted. These data agree with our own observations that antibody-mediated reductions in worm fecundity likely function through direct interactions with parasitic antigens. Of note, neither FcγR ligation nor complement activation could fully account for the ability of parasite-specific antibodies to provide protection against secondary infection, and passive transfer of immune serum to wild-type C57BL/6 or CD4-deficient mice resulted in comparable reductions in parasite numbers. These data indicate that IgG may also impact on adult worm development by directly interfering with the function of essential parasitic antigens.

We have recently reported that transfer of immune serum from mice infected multiple times with *H. polygyrus*, but not from mice infected only once, can provide protective immunity to neonates (Harris et al., 2006). In this case protection was dependent on the transfer of specific IgG into the neonatal intestinal lumen via the FcRn receptor, which likely prevented larvae from invading the intestinal wall. These data support our current observations that antibodies capable of limiting adult worm development only arise after multiple infections and indicate that specific antibodies function largely to provide immune memory and to protect vulnerable neonates.

A protective role for antibodies during helminth infection has been hypothesized for a long time (Ogilvie, 1964), with positive correlations being noted between antibodies specific for antigens from the enteric helminths *Necator americanus* and *Trichinella spiralis* and reduced worm burdens (Bethony et al., 2005; Gu et al., 2008). Partial immunity can also be observed against *T. spiralis* following the passive transfer of immune serum (Love et al., 1976), and mice deficient in IgE exhibit defective parasite rejection (Gurish et al., 2004). We have previously isolated an antigen from the enteric helminth *N. brasiliensis* that induced specific IgE, and antigen crosslinking of mast cell-bound IgE could induce degranulation, suggesting a potential for IgE-mediated protective effects (Pochanke et al., 2007). Collectively, these studies indicate that vaccination strategies designed to promote antibody production against targeted parasite antigens may serve as a successful means of providing immunity against enteric helminths—with the additional potential that such immunity may function to protect breastfeeding infants. In support of this hypothesis Loukas et al. recently demonstrated that vaccination of adult laboratory dogs with Ancylostoma caninum aspartic protease 1, APR-1 from human hookworm, induced specific antibody production and enhanced protection against L3 challenge (Loukas et al., 2005).

In summary, we have shown that enteric helminth infection results in the robust production of polyclonal antibodies, including nonrelevant specificities, alongside a delayed and comparatively weak production of parasite-specific antibodies. Importantly, both types of antibody were demonstrated to play key but distinct functional roles with polyclonal antibodies acting to limit parasite fecundity, while specific antibodies provided immune protection.

EXPERIMENTAL PROCEDURES

Mice and Generation of Bone Marrow Chimeras

C57BL/6 and $J_H^{-/-}$ (Chen et al., 1993a) mice were bred at the Labortierkunde of the University of Zürich. MHC Class $II^{-/-}$ (I-A^b) (Kontgen et al., 1993), TgH(VI10)xYEN, $AID^{-/-}$ (Muramatsu et al., 2000), $IgA^{-/-}$ (Harriman et al., 1999), $IgE^{-/-}$ (Oettgen et al., 1994), $Fc\gamma R^{-/-}$ (Takai et al., 1994), and $C3^{-/-}$ (Wessels et al., 1995) mice were bred and maintained at the University of Zürich and Bio-Support, Zürich. All animal experiments were performed according to institutional guidelines and Swiss law. For generation of bone marrow (BM) chimeras, C57BL/6 mice were lethally irradiated (9.5 Gy) using a ^{60}Cobalt source then injected intravenously with 5–10 × 10^6 BM cells from MHC $II^{-/-}$ and $J_H^{-/-}$ or C57BL/6 mice.

Parasites and Antibody Treatment

Mice were infected orally with 200 L3 *H. polygyrus.* For challenge studies mice were treated with 250 mg of the anti-helminth pyrantelum (Cobantril, Pfizer, Zürich, Switzerland) 40–50 days following primary infection, then subjected to a second infection of 200 L3 2 weeks later. Numbers of adult parasites were determined by examining whole intestine plus luminal contents under a dissecting microscope. For experiments using pooled serum, serum was collected from naive mice, at days 9–13 post-primary (1°), or at days 13–20 post-secondary (2°) *H. polygyrus* infection. Murine IgG from pooled naive or infected serum was purified by affinity chromatography (Protein G Sepharose 4 Fast Flow, Amersham Pharmacia Biosciences, GE Healthcare, Munich, Germany). Egg production was quantified by collection of moist feces, flotation using saturated NaCl, and counting using a McMaster Worm Egg Counting Chamber (Weber Scientific International, Ltd, Hamilton, NJ, USA).

Immunohistochemistry and ELISA

Immunohistochemistry was performed as previously described (Macpherson et al., 2001). Briefly, tissues were snap frozen, sectioned, fixed in acetone, then rehydrated with PBS. Stainings were performed using monoclonal antibodies against IgE (clone 3–11), CD138 (clone 281-2) and IgG1 (clone A85-3) or biotinylated PNA. Antibody binding was revealed as described (Macpherson et al., 2001). ELISA assays were performed as previously described (Macpherson et al., 2001). Antigen-specific IgG1 and IgE were measured in a similar manner after coating with 1–5 μg/ml HES (excretory/secretary products collected from adult L5 *H. polygyrus* cultured for a period of 2 days in RPMI plus antibiotics and 1% glucose and concentrated using a 10,000 MWCO cellulose membrane, Centriprep, Millipore, MA) or VSV (PEG precipitated). Bound antibody was detected with secondary peroxidase–conjugated anti-IgG1 (Zymed, CA, USA) or biotin-conjugated anti-IgE (clone RIE-4) followed by HRP-conjugated streptavidin (Zymed) and developed as described (Macpherson et al., 2001). To calculate concentrations an internal standard consisting of pooled serum from C57BL/6 or TgH(VI10)xYEN mice infected two times with *H. polygyrus* was used.

RT-PCR, Sequencing, and Spectrotyping

B cells were purified to >95% by MACS (Miltenyi Biotech, Bergisch Gladbach, Germany) using anti-B220 microbeads, RNA isolated, and reverse transcribed into cDNA and mature IgE transcripts, IgE or IgM germline transcripts, or GAPDH transcripts detected by RT-PCR performed as previously described (McCoy et al., 2006). For IgG germline transcripts the primer sequences and PCR conditions were as follows: upper 5′-CAGCCTGGTGTCAACTAG-3′ and lower 5′-GCAAGGGATCCAGAGTTCCAG-3′, 94°5′[94°1′, 55.0°1′, 72°1′]×35, 72°7′. Sequence analysis of mature IgE transcripts was performed as described (McCoy et al., 2006) and the closest germline V_H clone assigned using the DNAPlot program (http://www.dnaplot.de). For spectrotyping, a PCR for IgE was performed for the J558 and PC7183 VH gene families. Amounts of cDNA were used that encoded for equal amounts of IgE as measured by quantitative PCR. The primers and PCR conditions for the J558-IgE PCR are as follows: upper 5′- GCCTGACATCTGAGGACTCT-3′ and lower 5′ FAM-TCGTTGAATGATGGAGGATGTGTCACGT-3′, 94°2′[94°1′, 60.0°1′, 72°1′]×35, 72°10′. The primers and PCR conditions for the PC7183-IgE PCR are as follows: upper 5′- ACAGTCTGAGGTCTGAGGACAC-3′ and lower 5′FAM- TCGTTGAATGATGGAGGATGTGTCACGT-3′, 94°2′[94°1′58.0°1′, 72°1′]×35, 72°10′. Products were analyzed by gel electrophoresis on a Megabase-1000 system (Amersham Biosciences).

Transfer of B Cells and Flow Cytometry

Naive B cells were isolated by MACS using anti-IgM microbeads (>95% purity) and 5 × 10^6 cells injected intravenously into naive $J_H^{-/-}$ mice. Transferred cells were monitored in blood using anti-B220 (Becton Dickinson Biosciences, CA, USA), VI10 mAb (clone 36.51), and anti-IgM (Becton Dickinson Biosciences) and analyzed by flow cytometry (FACSCalibur; Becton Dickinson).

Western Blot

HES proteins were loaded in a single broad well of an SDS polyacrylamide gel and transferred to nitrocellulose transfer membranes (Protran; Schleicher & Schuell) using a semi-dry transfer unit (Hoefer SemiPhor, GE Healthcare, Munich, Germany). Protein bands were visualized by reversible Ponceau Red staining and the membrane sectioned into strips then blocked with 5% milk/PBS. Strips were incubated for 1 hr with the indicated serum samples and bound immunoglobulin detected by incubation with HRP-conjugated rabbit anti-mouse IgG (Zymed) preincubated on additional membrane strips to remove nonspecific binding. Bands were visualized using enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce, IL, USA).

SUPPLEMENTAL DATA

Supplemental Data include four figures and two tables and can be found with this article online at http://www.cellhostandmicrobe.com/supplemental/S1931-3128(08)00294-1.

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