Long-Term Antibody Memory Induced by Synthetic Peptide Vaccination Is Protective against *Streptococcus pyogenes* Infection and Is Independent of Memory T Cell Help

Manisha Pandey, Michelle N. Wykes, Jon Hartas, Michael F. Good and Michael R. Batzloff

*J Immunol* published online 11 February 2013

http://www.jimmunol.org/content/early/2013/02/10/jimmunol.1202333
Long-Term Antibody Memory Induced by Synthetic Peptide Vaccination Is Protective against *Streptococcus pyogenes* Infection and Is Independent of Memory T Cell Help

Manisha Pandey,* Michelle N. Wykes, † Jon Hartas,* Michael F. Good,* and Michael R. Batzloff*

*Streptococcus pyogenes* (group A *Streptococcus* [GAS]) is a leading human pathogen associated with a diverse array of mucosal and systemic infections. Vaccination with J8, a conserved region synthetic peptide derived from the M-protein of GAS and containing only 12 aa from GAS, when conjugated to diphtheria toxoid, has been shown to protect mice against a lethal GAS challenge. Protection has been previously shown to be Ab-mediated. J8 does not contain a dominant GAS-specific T cell epitope. The current study examined long-term Ab memory and dissected the role of B and T cells. Our results demonstrated that vaccination generates specific memory B cells (MBC) and long-lasting Ab responses. The MBC response can be activated following boost with Ag or limiting numbers of whole bacteria. We further show that these memory responses protect against systemic infection with GAS. T cell help is required for activation of MBC but can be provided by naive T cells responding directly to GAS at the time of infection. Thus, individuals whose T cells do not recognize the short synthetic peptide in the vaccine will be able to generate a protective and rapid memory Ab response at the time of infection. These studies significantly strengthen previous findings, which showed that protection by the J8-diphtheria toxoid vaccine is Ab-mediated and suggest that in vaccine design for other organisms the source of T cell help for Ab responses need not be limited to sequences from the organism itself. *The Journal of Immunology*, 2013, 190: 000–000.

Infection with *Streptococcus pyogenes* (group A *Streptococcus* [GAS]) causes many clinical manifestations including pharyngitis, impetigo, scarlet fever, invasive infections such as toxic shock syndrome, and nectrotizing fasciitis as well as the postinfectious sequelae of rheumatic fever and rheumatic heart disease (RHD). The latter are a major problem in developing countries and indigenous populations worldwide, particularly in indigenous Australians, who have the highest reported disease incidence rate (1). There is strong evidence that RHD is autoimmune in etiology (2). Current control strategies to prevent streptococcal infection that would prevent RHD and other associated diseases are proving ineffective, and it is believed that development of a vaccine represents the best primary prevention solution. However, because RHD is autoimmune in etiology, it is important for safety concerns to use the minimal amount of GAS sequence required in the vaccine.

A number of potential GAS vaccine candidates have been identified and are at various phases of development as reviewed elsewhere (3); however, the M-protein is a major candidate, and Ab responses specific for it can protect against *Streptococcus pyogenes* (4). J8 is a minimal epitope derived in part from the conserved region of the M-protein (12 aa) and contained within a sequence of 16 aa from the yeast DNA-binding protein GCN4 (designed to maintain the α-helical coiling of the 12-mer insert (5). J8 conjugated to diphtheria toxoid (DT) is a leading vaccine candidate designed to protect against all strains. Studies investigating the mechanism of protection by J8-DT demonstrated that immunization or transfection of J8-DT–specific antisera/Ab protected mice against lethal GAS challenge (6). CD4+ T cells were also shown to be important for protection because depletion of this subset prior to challenge resulted in reduced protection. The data suggested that CD4+ T cells functioned as Th cells for the vaccine-induced B cell response. Neither the duration of protection nor the factors controlling any memory/recall response were known. This was a significant issue because the vaccine contained minimal streptococcal sequence and specifically was designed not to contain any immunodominant T cell epitopes derived from the M-protein. T cell help following vaccination came from stimulation by the DT conjugate partner, not GAS sequences.

The persistence of long-term Ab titers for any vaccine is dependent on memory B cells (MBC) and long-lived plasma cells (LLPC). MBC differentiate rapidly (4 to 5 d) into Ab-secreting cells (ASC), which produce high-affinity IgG Ab, whereas a new primary immune response would take 10–14 d (7, 8). In contrast, LLPC survive in the bone marrow (BM) in the absence of Ag for several years and continuously secrete Abs (9–11), although titers diminish significantly over time (12). For many organisms, a boost of Ab responses via an MBC response may be critical for ongoing protection (13, 14).

Whether or not B cells require T cell help for a primary response depends on the type of Ag (15). The protein Ags possess the ability to recruit cognate CD4+ T cell help through the TCR recognition
of peptide–MHC class II complexes on the surface of APCs. On the contrary, the polysaccharides use multivalent membrane-Ig–dependent B cell signaling (15). However, there is controversy as to whether MBC specific for protein Ags require a memory T cell response for optimal help (16, 17). Because the J8-DT vaccine was designed to contain a minimal B cell epitope (defined by J8) but not a dominant T cell epitope from GAS (to reduce the likelihood of any untoward autoimmune response), this issue is critical for success (18–20). Although T cell help following vaccination came from DT, there was great concern as to whether natural infection with GAS would boost the J8-specific Ab response. Any T cell help for boosting would need to come from naive T cells responding to GAS at the time of challenge.

The current study was therefore designed to assess whether immunization with J8-DT/aluminum hydroxide (alum) would result in development of a long-lived protective immune response that could be boosted by exposure to limited numbers of GAS organisms, as might occur during natural exposure. We further dissected the role of T cells in the memory responses and assessed whether and how these cells could protect against GAS infection. Our results demonstrate that the stimulation of a memory B cell response requires T cells, but these need not be memory T cells. The data suggest that vaccine design for other organisms could successfully use foreign T cell epitopes chosen solely on their ability to induce a strong vaccine response.

Materials and Methods

Mice

Four- to 6-week-old BALB/c or SCID mice were obtained from the Animal Resource Centre (Perth, Western Australia). All protocols were approved by the Institute’s ethics committee (Queensland Institute of Medical Research Animal Ethics Committee and Griffith University Animal Ethics Committee) in accordance with the National Health and Medical Research Council of Australia guidelines.

Peptide synthesis

Peptides used in this study were synthesized in-house or synthesized commercially by Auspep. Peptide J8 was conjugated to DT as described elsewhere (21). All peptides were stored lyophilized or in solution at −20°C.

Immunization, sample collection, and challenge

Cohorts of 20–30 BALB/c mice were s.c. immunized at the tail base with 30 μg J8-DT or DT. The Ags prepared in PBS were adsorbed onto alum (Alhydrogel; Brenntag Biosector) at room temperature for 1 h with slow mixing before administering into mice. To control for the effect of adjuvant, parallel cohorts of mice were given alum without Ag. To confirm the J8-DT vaccine was effective, the percentage of proliferating CD4+ T cells in the spleens of mice immunized with J8-DT and DT were counted, resuspended in 200 μl sterile PBS, and transferred intravenously into SCID mice. Each mouse received one spleen equivalent.

To assess the role of different lymphocyte populations, postimmunization with J8-DT/DT or PBS the BALB/c mice were rested for 14–16 wk. The B or T cell populations from spleens of these mice were used (prepared using the method described earlier) and used for adoptive transfer studies. For the mice receiving B or T cells, each SCID mouse received one mouse equivalent of splenic B or T cells.

In vivo activation of memory response

To measure memory Ab responses in vivo, immunized mice with memory cells or recipient SCID mice with transduced memory cells were boosted i.v. with 10 μg J8-DT/DT or 1000 CFU GAS M1 strain. The Ag-specific ASC were measured in the serum of mice 5 or 7 d postboost, with Ag or infection, by ELISA. To determine protective efficacy of long-lived and memory immune responses, the immunized-boosted-boosted (1-R-B) BALB/c and recipient SCID mice postboost were challenged with GAS M1 strain.

ELISPOT assay

Ag-specific ASC in the BM and spleens of donor and recipient mice postimmunization, postboost, and postchallenge were quantified by ELISPOT. Multiscreen-HA plates (Millipore) were coated with 5 μg/ml J8 or DT in carbonate coating buffer (pH 9.6). Isolated spleen or BM cells were directly tested for ASC using a published method (22, 23).

ELISA

At the indicated time points, blood samples (10 μl) were collected from individual mice by tail snip. J8- and DT-specific IgG titers were determined as described elsewhere (24). Nunc Immunoplates (Flow Laboratories) were coated with 100 μl J8 or DT at 5 μg/ml concentration. The end-point titers were determined as the highest dilution of serum for which the OD was 3 SD above the mean OD of control wells containing serum from naive mice. The IgG subclass composition of sera was analyzed by ELISA using subclass-specific HRP-conjugated secondary Abs (Zymed; Invitrogen).

In vitro cell proliferation studies

To measure T cell responses to Ags, an in vitro proliferation assay that uses 5-ethyl-2′-deoxyuridine (EUDU; a thymidine analog) incorporation into the DNA of proliferating cells was used (25). Briefly, T cell–enriched lymphocyte fractions from J8–DT–immunized mice were seeded into round-bottom, 96-well plates at 2.5 × 10⁶ cells/well in complete IMDM media. The T cells were then cultured in the presence or absence of various Ags including J8i, J8, DT, and J8–DT (Table 1). T cells stimulated with 2 μg/well Con A were used as positive controls. Following incubation at 37°C/5% CO₂ on day 3 (72 h poststimulation), 20 μM EUDU was added to each well for 18–24 h. On day 4 (96 h poststimulation), the cells were harvested, washed, and labeled with various cell-surface markers for T cells. Detection of EUDU incorporated into the proliferating CD4⁺ T cells was accomplished by using the Click-IT EUDU Alexa Fluor 647 kit for Flow Cytometry (Molecular Probes) following the manufacturer’s instructions. The cell population positive for CD3-AP-488 and CD4-PerCP-Cy5.5 was analyzed for the percentage of proliferating CD4⁺ T cells.
Flow cytometry labeling

Abs used to check the purity of isolated B and T cells include CD19-PE, CD3-FTTC, CD4-allophycocyanin, CD8-PerCP, and their corresponding isotype controls. CD3-488 and CD4-PerCP-5.5 were used in an assay. All of the Abs were sourced from Becton-Dickinson Biosciences. Flow cytometry was conducted on an FACS Canto II (Becton-Dickinson Biosciences) and data analysis achieved on FCS Express (De Novo Software).

Results

Primary immune responses to J8-DT

To assess the efficacy of J8-DT immunization in inducing long-lived immune responses, multiple cohorts of BALB/c mice were immunized with J8-DT, DT, or PBS (all in alum) following a protocol that is established in our laboratory. Within 35 d of immunization, J8-DT/alum–immunized mice developed significantly higher (p < 0.001) titers of J8-specific IgG1 compared with the negligible titers in cohorts of mice immunized with DT/alum or PBS/alum (Fig. 1A, 1B). As expected, the incorporation of alum with the vaccine induced Th2 responses resulting in a predominance of serum J8-specific IgG1 Abs over IgG2b and -1b. Similarly, very high (∼10^6) DT-specific IgG1 titers were achieved in cohorts immunized with J8-DT or DT (Fig. 1C).

Enumeration of Ag-specific ASC in spleen and BM, on day 37 postimmunization, found that following immunization, the majority were resident in the spleen, and only a small fraction had migrated to the BM (Fig. 1A, 1D, 1E). The ASC in the spleen represent primary B cells, LLPC, and short-lived plasma cells, whereas the majority of the ASC in the BM would be LLPC. Mice immunized with J8-DT had similar number of J8- (Table I) and DT-specific ASC in spleen and BM, whereas DT-immunized mice showed significantly higher (p < 0.001) numbers of DT-specific ASC both in spleen as well as in the BM compared with J8-specific ASC (Fig. 1D, 1E).

Long-term immune responses in mice immunized with J8-DT

The above cohorts of immunized mice were then rested for ∼14–16 wk (∼110 d) to allow all primary immune cells and short-lived plasma cells to subside resulting in only MBC and LLPC surviving (Fig. 1A). These LLPC maintain a basal level of Abs in the serum. A comparison of J8-specific serum IgG1 titers in J8-DT–immunized mice between days 35 and 110 postimmunization found an ∼16-fold reduction in IgG1 titers (Figs. 1B, 2A). However, these titers were still significantly higher (p < 0.001) compared with the levels in DT- or PBS-immunized cohorts (Fig. 2A). DT-specific IgG1 titers in J8-DT– or DT-immunized cohorts were also maintained in the serum post 110 d of immunization (Fig. 2B). IgG1 still remained the predominant isotype postrest (Fig. 2A, 2B).

Next, ELISPOT assays were used to quantify J8- and DT-specific ASC (LLPC) in the BM and spleens of J8-DT–, DT–, or PBS–immunized-rested (I-R) mice. It was apparent that at 110 d postimmunization, only few ASC were residing in the spleen (Fig. 2C), whereas the majority of LLPC had migrated to the BM (Fig. 2D). The spleens from mice immunized with J8-DT or DT had typically around or <100 LLPC/spleen specific for DT or J8, respectively (Fig. 2C). J8-DT–immunized mice had similar numbers of J8- and DT-specific LLPC in the BM (∼2000 ASC/10^7 BM cells), whereas DT-immunized mice had a significantly higher (p < 0.01) number of DT-specific LLPC (∼2000 LLPC/10^7 BM cells) compared with J8-specific LLPC (∼80 LLPC/10^7 BM cells) (Fig. 2D).

J8-DT–immunized mice have a memory response to J8-DT

To determine if J8-DT immunization generated memory B cells that were specific for the vaccine, mice were immunized with the vaccine and rested for 14–16 wk. To recall memory responses, half (n = 10/group) of the mice from each cohort of I-R mice were boosted (I-R-B) with a small dose (10 μg) of Ag (J8-DT or DT), whereas the remaining mice (n = 10/group) were left nonboosted (I-R). Following a boost with 10 μg of Ag, we observed an 8-fold rise in J8-specific Ab titers by day 7 compared with titers on day 4 (p < 0.01) (Fig. 3A). To confirm that this rise in J8-specific IgG titers was due to activation of memory B cells and not a new primary immune response, which typically takes 9–14 d to generate, we also included control naive groups that were immunized with J8-DT/alum via an s.c. route or J8-DT alone via an i.v. route. These control cohorts did not show any detectable J8-specific IgG until day 14 postimmunization, thereby confirming that the rise in Ab levels in I-R-B mice is not a primary immune response. Further, the Ab levels in the I-R cohort (without boosting) did not show any change, further supporting that it was the activation of memory B cells that resulted in the rapid rise in Ab levels. There was no noticeable rise in DT-specific IgG titers following boosting (Fig. 3B).

J8-specific MBC are functional against a GAS infection

To measure recall of memory responses by GAS, splenocytes from parallel cohorts of mice (immunized with J8-DT, DT, or PBS and rested for 14–16 wk) were transferred to naive SCID mice and either boosted with the vaccine (10 μg of J8-DT) or given a low-dose infection (1000 CFU of GAS) (Fig. 4A). The J8-DT–immunized mice demonstrated a significant increase (p < 0.001) in J8-specific IgG following boost with Ag or infection by day 5 postboost compared with their nonboosted counterparts. By day 7, there was a further 10–14-fold increase (p < 0.001) in J8-specific IgG titers following Ag and infection boost, respectively, which reflected activation of memory B cells (Fig. 4B). Further, transfused MBC did not differentiate to ASC in the absence of Ag boost or infection. The absence of significant Ab titers in nonboosted control groups excluded the possibility that short-lived or LLPC were responsible for the increase (Fig. 4B). These results thus confirmed that Abs were derived from activated MBC and not from plasma cells.

We then quantified the numbers of J8-specific memory B cells in the spleen and BM of SCID mice, on day 7 poststimulation, using an ELISPOT assay (Fig. 4C, 4D). The SCID mice transfused with J8-DT splenocytes whether boosted with Ag (>3000 MBC/spleen) or infection (>4000 MBC/spleen) had significantly higher (p < 0.01) numbers of J8-specific MBC in their spleens compared with basal numbers in DT (<100) or PBS (<20) control mice (Fig. 4C). As expected, the number of ASC that had migrated to the BM, at that stage, was negligible (Fig. 4D). This confirmed that the rise in serum IgG levels was due to activation of MBC that resulted in their differentiation into Ab secreting plasmablasts and that the LLPC were not a source of these Abs.

J8-specific MBC protect against a GAS infection

To measure protection against GAS by MBC per se, cohorts of mice were immunized with J8-DT, DT, or PBS to generate MBC and rested for 14–16 wk prior to challenge. SCID mice were then

Table I. Peptides used in the study to investigate in vitro and in vivo responses

<table>
<thead>
<tr>
<th>Name of Peptide</th>
<th>Peptide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>J8</td>
<td>QAEDKVKQSSRARKQVERKALKQLEDKVKQ*</td>
</tr>
<tr>
<td>J8i</td>
<td>SRARKQVERKAL</td>
</tr>
</tbody>
</table>

*Sequence of chimeric peptide J8 derived from the M-protein of GAS. M-protein–derived sequence is underlined, and GCN4-derived sequence is italicized.
transfused with splenocytes from these immunized mice and
either boosted with Ag or 1000 CFU of GAS. A parallel cohort
was also left as a nonboosted control. On day 8 posttransfusion,
all mice were challenged with a GAS infection (5 × 10^4 CFU/
mouse), and bacteremia was monitored from day 1 (Fig. 4A).
All SCID mice that received J8-DT splenocytes (whether boosted
with vaccine or low-dose infection) and challenged with GAS
infection controlled disease by days 8–12 (Fig. 5A–C). In
contrast, all control SCID mice given splenocytes from DT-
(Fig. 5D–F) or PBS-immunized mice (Fig. 5G, 5H) developed high
bacteremia and had to be sacrificed after ∼20 d. SCID mice
given J8-DT splenocytes cleared the infection more rapidly if
given an Ag boost or infection prior to challenge, correlating with
the activation of MBC prior to challenge. These mice developed
peak bacteremia by day 3, and infection cleared by day 8. Re-
cipient cohorts that did not receive any prior boosting before GAS
challenge had negligible J8-specific IgG titers and a slightly
more prolonged peak bacteremia with a very rapid clearance by day
12 (Fig. 5A). Finally, SCID mice recipients of DT splenocytes had a
progressive increase in bacteremia starting from day 1 postchallenge
irrespective of the level of DT-specific IgG, highlighting the re-
sduction in bacterial counts seen in J8-DT–immunized cohort was
due to J8-specific Abs (Fig. 5D–F). SCID mice given PBS spleno-
cytes whether nonboosted or boosted with Ag or infection were
unable to control the infection (Fig. 5G, 5H).

At 20 d postchallenge, the numbers of J8-specific ASC were
enumerated in these mice. SCID mice given J8-DT splenocytes
had ∼4000 J8-specific ASC per spleen with (Ag or infection)
or without boosts and challenge with GAS compared with <100
ASC/spleen in mice given splenocytes from DT- or PBS-im-
munized mice (Fig. 6A). These numbers were comparable to the
numbers of MBC per spleen seen in SCID mice given J8-DT
splenocytes and vaccine and/or infection to quantify MBC (Fig.
4C). These studies show that GAS infection does not compromise

FIGURE 1. Ag-specific IgG isotypes and ASC in the spleens and BM of BALB/c mice immunized with J8-DT or DT. (A) A schematic of experimental
protocol employed to investigate the longevity of Ab responses induced by vaccination with J8-DT. Cohorts of BALB/c mice (n = 30/group) were im-
munized s.c. with J8-DT/alum or DT/alum on days 0, 21, and 28. One week after the last boost (day 35), J8-specific (B) and DT-specific (C) IgG subclass
compositions were detected in the serum samples. Data representative of three or more independent experiments, and results are shown as means ± SEs
of the means for 15–20 mice in each group. To enumerate the Ag-specific ASC, spleens and BM were analyzed on day 35 postimmunization. The numbers of
J8- and DT-specific ASC that were resident in spleen (D) or had migrated to the BM (E) were quantified by ELISPOT using five mice per group. Data are
representative of three or more independent experiments, and results are shown as means ± SEs of the means for at least five mice in each group. Sig-
nificance determined by two-way ANOVA throughout the figure. * * * p < 0.001.
the MBC responses as noted for other infections (26). Further, although ASC numbers in recipient mice were negligible in the absence of vaccine and/or infection (Fig. 4C), GAS challenge alone activates MBC to secrete Ab (Fig. 6A). Finally, small numbers of ASC had also migrated to the BM (Fig. 6B).

The J8-DT–induced MBC response is T cell dependent

To define the role of J8-DT-specific memory B and T cells, independently, in protection against GAS, these cell populations were purified from splenocytes of mice immunized with J8-DT, DT or PBS that had then been rested for 14–16 wk. Assessment of in vitro proliferative capacities of the enriched T cells was carried out using a flow cytometry–based EDU uptake assay in which EDU added to the cell cultures is incorporated by cells replicating DNA. The enriched T cells from J8-DT–immunized and rested mice were stimulated in vitro with Ags including J8i (the minimal 12-mer B cell epitope from within J8, Table I), J8, J8-DT, or DT and their proliferation studied. The cells were labeled to detect EDU uptake into CD3+CD4+ T cells by flow cytometry (Fig. 7A–D). The CD4+ T cells from J8-DT–I-R mice responded to J8-DT (p < 0.038) and DT (p < 0.0001), but not to J8 (p > 0.05) or J8i (p > 0.05) compared with unstimulated T cells (Fig. 7E). A recall proliferative T cell response was observed to rM1 (data not shown), which may be attributed to cross reactivity between DT and the M-protein of GAS. This observation suggested that J8-DT–specific memory T cells are functional and can be recalled upon stimulation with J8-DT or DT.

Further, to investigate the role of purified B and T cells in protection against GAS, in vivo protection assays were conducted. For in vivo studies, naive SCID mice received purified B or T cell from J8-DT, DT, or PBS I-R mice. In addition, one cohort that received total splenocytes from J8-DT/DT/PBS I-R mice was also included as a control (Fig. 7F). Following a sublethal M1 GAS challenge, we observed 83% survival (p < 0.01) in J8-DT splenocyte-recipient mice compared with 16% in J8-DT B cell or T cell recipients by day 18, suggesting the requirement of both B and T cells to mount a protective immune response (Fig. 7F).

Naive T cells can help J8-DT memory B cells to mount a protective immune response

We next analyzed the T cell dependency of memory B cell responses. Adoptive transfer experiments were conducted in which naive SCID mice were trans fused with various combinations of J8-DT memory as well as naive B or T cells as depicted in Fig. 8. Twenty-four hours following adoptive transfer, the mice were infected i.v. with 50,000 CFU of M1 GAS. As readouts of protection, bacterial bioburden was followed in blood samples collected at various time points. Our data demonstrated that the cohort recipient of J8-DT memory B and naive T cells was protected significantly better (p < 0.01) compared with the cohort receiving naive B/memory T cells (Fig. 9A). These mice also fared significantly better than mice receiving MBC alone or memory T cell alone (Fig. 9C). The cohort recipients of memory splenocytes were able to clear bacterial burden very efficiently,

The J8-DT–induced MBC response is T cell dependent

To define the role of J8-DT-specific memory B and T cells, independently, in protection against GAS, these cell populations were purified from splenocytes of mice immunized with J8-DT, DT or PBS that had then been rested for 14–16 wk. Assessment of in vitro proliferative capacities of the enriched T cells was carried out using a flow cytometry–based EDU uptake assay in which EDU added to the cell cultures is incorporated by cells replicating DNA. The enriched T cells from J8-DT–immunized and rested mice were stimulated in vitro with Ags including J8i (the minimal 12-mer B cell epitope from within J8, Table I), J8, J8-DT, or DT and their proliferation studied. The cells were labeled to detect EDU uptake into CD3+CD4+ T cells by flow cytometry (Fig. 7A–D). The CD4+ T cells from J8-DT–I-R mice responded to J8-DT (p < 0.038) and DT (p < 0.0001), but not to J8 (p > 0.05) or J8i (p > 0.05) compared with unstimulated T cells (Fig. 7E). A recall proliferative T cell response was observed to rM1 (data not shown), which may be attributed to cross reactivity between DT and the M-protein of GAS. This observation suggested that J8-DT–specific memory T cells are functional and can be recalled upon stimulation with J8-DT or DT.

Further, to investigate the role of purified B and T cells in protection against GAS, in vivo protection assays were conducted. For in vivo studies, naive SCID mice received purified B or T cell from J8-DT, DT, or PBS I-R mice. In addition, one cohort that received total splenocytes from J8-DT/DT/PBS I-R mice was also included as a control (Fig. 7F). Following a sublethal M1 GAS challenge, we observed 83% survival (p < 0.01) in J8-DT splenocyte-recipient mice compared with 16% in J8-DT B cell or T cell recipients by day 18, suggesting the requirement of both B and T cells to mount a protective immune response (Fig. 7F).

Naive T cells can help J8-DT memory B cells to mount a protective immune response

We next analyzed the T cell dependency of memory B cell responses. Adoptive transfer experiments were conducted in which naive SCID mice were trans fused with various combinations of J8-DT memory as well as naive B or T cells as depicted in Fig. 8. Twenty-four hours following adoptive transfer, the mice were infected i.v. with 50,000 CFU of M1 GAS. As readouts of protection, bacterial bioburden was followed in blood samples collected at various time points. Our data demonstrated that the cohort recipient of J8-DT memory B and naive T cells was protected significantly better (p < 0.01) compared with the cohort receiving naive B/memory T cells (Fig. 9A). These mice also fared significantly better than mice receiving MBC alone or memory T cell alone (Fig. 9C). The cohort recipients of memory splenocytes were able to clear bacterial burden very efficiently,
Similar to the mice receiving MBC/memory T cells (Fig. 9B, 9D). Furthermore, these cohorts demonstrated an early decline in bacterial burden that led to faster clearance (Fig. 9B, 9D) compared with the recipients of J8-DT MBC and naive T cells (Fig. 9A). As expected, the cohort recipient of naive B/naive T cells or naive splenocytes did not show any reduction in bioburden until day 12 (Fig. 9B, 9D). These data further demonstrated the need for both B and T cells in J8-DT–mediated protection.

Discussion

Although Abs have been implicated in protection mediated by J8-DT/alum (6, 21), in this study, we show that exposure to GAS can stimulate memory B cells to respond rapidly and that this response can protect mice from the extant infection. For several vaccines, there is good evidence that immunity can persist after immunization when Ag-specific Abs are no longer detected (27–30). It was not known whether this applied to GAS, although previous work showing that the degree of protection was proportional to the serum J8-specific Ab titer (6, 21, 31) suggested that Ab would need to be present in high titer at the time of challenge for protection. We were particularly keen to know what titer of Ab might correlate with protection. By adoptively transferring memory cells into naive immunodeficient mice, we have been able to show that the titer at the time of challenge is in fact not critical; rather it is the presence of an MBC population per se that is important. Memory T cells alone offered no protection. It is known that MBC continuously recirculate though the body, thus allowing them to encounter and react to Ags at tissue sites (32). Following appropriate stimulation, MBC undergo rapid proliferation, culminating in differentiation into plasma cells and in the secretion of high-affinity IgG. The quick rise in Ab levels that we observed after 5–7 d of stimulation confirms that they are coming from MBC, as a new immune response would take 9–14 d to develop. It seems that this quick response is critical. LLPC are also important for ongoing protection. It is known that LLPC can survive and continue to secrete Abs for extended periods of time (>$1$ y) (10). The majority of these nondividing cells are present in the BM, and their main function is to continuously secrete large amounts of specific Ab. Thus, we observed that high levels of J8-specific IgG were present in the serum of mice immunized 110 d previously. At 110 d postimmunization, the majority of the ASC are detected in the BM, confirming that the source of the Abs detected in serum were LLPC. However, titers measured by ELISA had declined significantly over that time. For example, on day 110 postimmunization, a 16-fold drop in J8-specific IgG titers was noticed. Thus, the vaccine-specific primary immune response had subsided, and the major fraction of Abs detected in the serum thereafter were coming from LLPC at that time.

Having shown that both vaccine Ag and GAS could boost a MBC response, we then analyzed the role of T cells in this activation. Some earlier studies suggested that MBC require CD4+ T cells for their survival (17, 33) and activation (34, 35). Thus, using a vesicular stomatitis virus infection model, Ochsenbein et al. (17). have demonstrated that differentiation of MBC into short lived ASC always depends on primed specific T cell help. Similarly, in another study, a polyclonal stimulus by bystander T cell help was shown to be critical for proliferation and differentiation of human MBC (33). However, there are also data showing that boosting of a B cell response does not require memory T cell help (16, 36, 37). Our data serve to dissect this issue. We have demonstrated that MBC with the help of T cells generate an Ab response that protects mice from a GAS challenge. Furthermore, our data also demonstrate that the T cell help for J8-DT–specific immunity can come from naive T cells. Thus, naive T cells were capable of stimulating J8-DT MBC, resulting in a protective immune response postexposure to either immunogen or the pathogen. This highlights the usefulness of the J8-DT vaccine that contains a pathogen, but to DT. It has recently been shown that memory T cells that express B cell follicle–homing molecule CXCR5 are capable of providing accelerated help to B cells (38). We observed that naive as well as memory T cells, when transferred with MBC, were both capable of reducing GAS bioburden. However, there was a difference in the kinetics of bacterial clearance between the two groups with the naive T cell recipient cohort, demonstrating a delayed decline in bacterial burden compared with the cohort receiving memory T cells. This outcome
FIGURE 5. Ag-specific memory IgG levels and GAS bioburden in SCID mice following a challenge with M1 GAS. SCID mice (n = 12/group) were transfused with J8-DT splenocytes (A–C), DT splenocytes (D–F), or PBS splenocytes (G, H) from I-R BALB/c mice. They were then boosted with either Ag or infection on day 1 posttransfusion. A parallel cohort did not receive any boosting (AT-Ch) prior to challenge. Seven days postboost (day 0 for challenge, on the graph), mice were challenged with a low dose (5 × 10⁴ CFU/mouse) of GAS infection. The J8- and DT-specific serum IgG titers were monitored for up to day 18 postchallenge and are shown on right y-axis. The CFU counts were also monitored at indicated time points and are shown as solid lines on left y-axis. Data are representative of three or more independent experiments, and results are shown as means ± SEs of the means for 10–12 mice in each group.
is likely to be due to the delayed proliferation of naive CD4+ T cells resulting in delayed T cell help to MBC.

Apoptosis of MBC following infection with Plasmodium (26) and Trypanosoma brucei (39) had been implicated in the abolition of long-term protection in mice. Our studies demonstrated that vaccination with J8-DT resulted in an MBC response that was capable of responding to a low inoculum of GAS. Of interest was the observation that this memory response was also protective without any prior boosting. The demonstration that the MBC response can reduce bacteremia following a high-dose challenge was further reassuring.

In vitro proliferation assays showed that J8 did not contain a T cell epitope from GAS because there was no recall proliferative response to either J8i (a minimal B cell epitope from within J8) or to J8. J8-DT was designed to contain the absolute minimum component of GAS (12 aa from the M-protein), and previous studies in different mouse strains (21, 24) and in humans (40) have also shown that it does not contain a dominant GAS-specific T cell epitope. In the in vivo protection studies, T cells alone did not offer any protection, further suggesting their role is primarily in the regulation of vaccine-induced B cell immunity, as cognate CD4+ Th cells. There was thus concern that GAS infection would not boost a vaccine-induced memory response. In showing in this study that memory T cells are not required for boosting an Ab response, we have allayed many concerns that this vaccine would not induce enduring protection. Furthermore, based on these data,

FIGURE 6. Persistence of ASC in the recipient SCID mice post-GAS challenge. The SCID mice recipients of J8-DT/DT or PBS splenocytes were boosted with Ag or infection. One cohort was left without any boost. On day 7 postboost, the mice (n = 12/group) were challenged with a low-dose GAS infection. The number of J8-specific ASC in the spleen (A) and BM (B) of SCID mice (n = 7/group) on day 20 postinfection are shown. Data are representative of three or more independent experiments, and results are shown as means ± SEs of the means for seven mice in each group.

FIGURE 7. In vitro proliferation of memory T cells and in vivo protection assay with MBC, T cells, and splenocytes. To measure activation of CD4+ T cells in response to various Ags, EDU assay was carried out on T cell–enriched fraction from J8-DT I-R mice. Following antigenic stimulation for 72 h, the cells were incubated with optimized concentration of EDU. The percentages of CD3+CD4+ T cells proliferating in the unstimulated cell population (A) or stimulated with J8 (B), DT (C), and J8-DT (D) are shown. Con A was used as a positive control. (E) The numbers of EDU-positive cells per well were determined after 16–24 h of incubation with EDU and are shown on y-axis. To assess the protective efficacy of lymphocyte subsets, SCID mice recipient of purified B or T cells or total splenocytes (n = 10/group) from J8-DT/DT- or PBS-immunized mice were challenged with M1 GAS strain. (F) Protection induced in recipient SCID mice following GAS challenge is shown. Log-rank analysis was carried out to compare the survival curves in (F). (A–F) Data are representative of two or more independent experiments and results are shown as means ± SEs of the means for at least five mice in each group. Significance determined by two-way ANOVA. *p < 0.05, **p < 0.01.
there may be reason to reconsider vaccine design for many challenging organisms. For example, in malaria, the leading but suboptimal vaccine candidate, RTS,S, was designed to include T cell epitopes from the coat protein of the parasite to augment Ab responses to the B cell epitope (41). Those T cell epitopes are limited and polymorphic in the organism (42), and as such, it may have been better to use more potent, non–parasite-derived T cell epitopes in the vaccine.

In conclusion, we have demonstrated that J8-DT–induced B cell responses are long lasting and result in the development of Ag-specific MBC and LLPC. Activation of MBC with antigenic stimulus or GAS infection results in a quick and effective Ab...

**FIGURE 8.** A schematic of experimental protocol employed to investigate the synergistic effect of J8-DT B and T cells in protection against GAS.

**FIGURE 9.** Adoptive transfer and assessment of protective efficacy of J8-DT memory and naive B and/or T cells. To investigate synergistic effect of memory and naive B and T cells in protection against GAS, SCID mice (n = 5/group) were transfused with combinations of B and T cells from J8-DT–I-R or naive BALB/c mice. As illustrated in schematic Fig. 8, cohorts of mice were transfused with J8-DT B cells/naive T cells or naive B/naive T cells (A), J8-DT B cells/J8-DT T cells or naive B/naive T cells (B), J8-DT–B cells or J8-DT–T cells (C), and J8-DT splenocytes or naive splenocytes (D). Twenty-four hours postadoptive transfer, the mice were given an i.v. infection with 50,000 CFU of GAS M1 strain. The bacterial bioburden in the blood (left y-axis) and serum J8-specific IgG titers (right y-axis) were monitored at indicated time points as shown. Data are representative of three or more independent experiments, and results are shown as means ± SEs of the means for at least five mice in each group. Significance determined by two-way ANOVA. *p < 0.05, **p < 0.01, ***p < 0.001.
response that is capable of clearing systemic GAS and protecting mice. Most importantly, our data demonstrate that activation of MBC does not require memory T cells. These findings not only have significant importance regarding the efficacy of the J8-DT vaccine but they also hold significance for vaccine design against other pathogens.

Acknowledgments

We thank Graham Magor for technical assistance in animal experiments and Karen Phillips for help with flow cytometry experiments.

Disclosures

The authors have no financial conflicts of interest.

References