Winter 2-16-2005

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Massimo Morra

Robert A. Barrington

Ana C. Abadia-Molina

Susumo Okamoto

Aimee Julien

See next page for additional authors

Authors
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Defective B cell responses in the absence of SH2D1A

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*Division of Immunology, Beth Israel Deaconess Medical Center, Harvard Medical School, and ‡CBR Institute for Biomedical Research and Department of Pathology, Harvard University, Boston, MA 02215; §Edward Jenner Institute for Vaccine Research, Compton, Newbury, Berkshire RG20 7NN, United Kingdom; ¶Department of Pathology, Tufts University School of Medicine, 150 Harrison Avenue, Boston, MA 02111; ‖Laboratory of Molecular Immunology, National Heart, Lung, and Blood Institute, Bethesda, MD 20892; **Department of Molecular Microbiology and Immunology, Division of Biology and Medicine, Brown University, Providence, RI 02912; and ††Department of Microbiology, Ohio State University, Columbus, OH 43210

Edited by Douglas T. Fearon, University of Cambridge School of Medicine, Cambridge, United Kingdom, and approved February 16, 2005 (received for review December 1, 2004)

More than half of patients with X-linked lympho-proliferative disease, which is caused by a defect in the intracellular adapter protein SH2D1A, suffer from an extreme susceptibility to Epstein–Barr virus. One-third of these patients, however, develop dysgammaglobulenemia without an episode of severe mononucleosis. Here we show that in *SH2D1A*$^{-/-}$ mice, both primary and secondary responses of all Ig subclasses are severely impaired in response to specific antigens. Because germinal centers were absent in *SH2D1A*$^{-/-}$ mice upon primary immunization, and because SH2D1A was detectable in *wt* germinal center B cells, we examined whether *SH2D1A*$^{-/-}$ B cell functions were impaired. Using the adoptive cotransfer of B lymphocytes from hapten-primed *SH2D1A*$^{-/-}$ mice with CD4$^+$ T cells from primed *wt* mice into irradiated *wt* mice provided evidence that signal transduction events controlled by SH2D1A are essential for B cell activities resulting in antigen specific IgG production. Defects in naïve *SH2D1A*$^{-/-}$ B cells became evident upon cotransfer with non-primed *wt* CD4$^+$ cells into *Rag2*$^{-/-}$ recipients. Thus, both defective T and B cells exist in the absence of SH2D1A, which may explain the progressive dysgammaglobulinemia in a subset of X-linked lympho-proliferative disease patients without involvement of Epstein–Barr virus.

Epstein–Barr virus | germinal center | immunoglobulin | SLAM/CD150

X-linked lymphoproliferative disease (XLP) is a primary immunodeficiency that, in more than half of patients, results in an extreme susceptibility to Epstein–Barr virus (EBV), leading to fatal infectious mononucleosis or B cell lymphoma (1–6). XLP patients who are unaffected by an EBV infection develop dysgammaglobulinemia or agammaglobulinemia even at a very young age (4, 7–10). *SH2D1A*, the XLP gene, encodes a single SH2-domain adapter, which is primarily expressed in T lymphocytes and natural killer cells (6, 11–20). SH2D1A binds to a tyrosine motif in the cytoplasmic tail of six CD150-related surface receptors (12, 21) and in turn recruits the protein tyrosine kinase Fyn (16, 17, 22–24). Because the influence of EBV on B cell responses greatly complicates analyses of dysgammaglobulinemia of XLP patients, we examined whether dysgammaglobulinemia occurs in a mouse in which the XLP gene *SH2D1A* (or *SAP*) has been disrupted (25–28). Upon infection with the parasites *Leishmania major* and *Toxoplasma gondii*, impaired T helper 2 responses were observed in *SH2D1A*$^{-/-}$ mice (25–27). Memory antibody responses to lymphocytic choriomeningitis virus (LCMV) were impaired, because *SH2D1A*$^{-/-}$ mice had a severe defect in maintaining anti-LCMV IgG levels (28).

Here we show that SH2D1A is essential for both T and B cell responses to soluble T dependent (T-D) antigens. That SH2D1A-controlled signaling is pivotal in primary Ig responses is evident from impaired IgM and IgG responses to LCMV and to the murine Gamma Herpes virus 68 (MHV68), as well as to well defined protein antigens and haptens. Furthermore, hapten-specific class switching of all Ig isotypes and germinal center (GC) formation is defective in *SH2D1A*$^{-/-}$ mice. Using adoptive transfers of CD4$^+$ T cells together with B lymphocytes from *SH2D1A*$^{-/-}$ mice into *Rag2*$^{-/-}$ or *wt* recipients, we find that SH2D1A controls both T and B cell activities.

Materials and Methods

Mice. C57BL/6, BALB/c, and *Rag2*$^{-/-}$ mice were purchased from The Jackson Laboratory and were kept under specific pathogen-free conditions at the Beth Israel Deaconess Medical Center (BIDMC) Animal Facility. *SH2D1A*$^{-/-}$ C57BL/6 and *SH2D1A*$^{-/-}$ BALB/c mice were backcrossed seven times (25). All animal studies were approved by the BIDMC Institutional Animal Care and Use Committee.

Quantitation of Serum Ig by ELISA. Isotype-specific Igs were detected and quantitated by ELISA, as described (29).

4-Hydroxy-3-nitrophenylacetyl–Keyhole Lymphocyte Hemocyanin (NP-KLH)/2,4,6,-trinitrophenyl-KLH (TNP-KLH) Immunizations. Mice were injected i.p. with 300 μg of alum-precipitated NP-KLH (Biosearch). Imject Alum was purchased from Pierce. Mice were reinjected after 14–21 days with an i.p. injection of 100 μg of NP-KLH in PBS and killed 7 days later. For immunizations with TNP-KLH (Biosearch), 300 μg of alum-precipitated TNP-KLH plus pertussis toxin (300 ng per mouse) (Calbiochem) was used.

Infection with LCMV or MHV68. C57BL/6 *SH2D1A*-deficient mice and their *wt* littermates were infected i.p. with 2×10^4 plaque-forming units of the Armstrong strain (ARM) of LCMV-ARM, as described (25). In other experiments, C57BL/6 *SH2D1A*-deficient mice and controls were infected with MHV68 (30).

Histology and Immunofluorescence. Snap-frozen spleens in OCT media were cryosectioned and stained as described (29). Intracellular SH2D1A protein was detected by a combination of two antibodies: first, a rabbit anti-mouse-SH2D1A antibody (SH2D1A) was added (12). Subsequently, a Rhodamine-labeled donkey anti-rabbit IgG was used (Jackson ImmunoResearch).

Adoptive Transfers. CD4$^+$ T and B220$^+$ B cells were purified from the spleen of primed or unprimed *SH2D1A*$^{-/-}$ BALB/c or *wt* BALB/c mice by using negative selection, as described (31). Cell purity was assessed by FACS analysis. Naïve CD4$^+$ cells (5×10^6)

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: XLP, X-linked lymphoproliferative disease; EBV, Epstein–Barr virus; LCMV, lymphocytic choriomeningitis virus; T-D, T dependent; MHV68, murine Gamma Herpes virus 68; KLH, keyhole lymphocyte hemocyanin; TNP, 2,4,6,-trinitrophenyl; NP, 4-hydroxy-3-nitrophenylacetyl; GC, germinal center; PNA, peanut agglutinin.

†To whom correspondence may be addressed. E-mail: mmorra@bidmc.harvard.edu or terhorst@bidmc.harvard.edu.

from *SH2D1A*$^{-/-}$ and *wt* mice (>99% of the cells were CD4$^+$, and <1% of the cells were B220$^+$) were transferred into *Rag2*$^{-/-}$ mice together with 10×10^6 *SH2D1A*$^{-/-}$ or *wt* B cells (cell purity >99%, with <1% of CD4$^+$ cells). Mice were then rested for 1 week before standard i.p. immunization with NP-KLH plus alum. For transfer experiments of hapten-carrier primed cells, KLH primed and boosted CD4$^+$ cells (5×10^6 cells) of *SH2D1A*$^{-/-}$ and *wt* mice were transferred into irradiated *wt* recipients (600 rad) together with NP-hen egg lysozyme primed B cells (10×10^6) and 100 μg of NP-KLH.

Flow Cytometry. Red cell-depleted single-cell suspensions were stained with fluorescence- or biotin-conjugated antibodies, as described (25, 29). Cell sorting was performed on a FACSCalibur cytometer (Becton Dickinson).

Results

Impaired Primary and Secondary Ig Responses in *SH2D1A*$^{-/-}$ Mice. Because dysgammaglobulinemia is one of the major manifestations of XLP (7, 32), we assessed B cell functions and phenotypes in *SH2D1A*$^{-/-}$ mice in the absence of immunization. Whereas the basal serum concentrations of IgG1 were lower in *SH2D1A*$^{-/-}$ C57BL/6 mice than in age-matched *wt* C57BL/6 mice kept under specific pathogen-free conditions, the serum IgG2a concentration was consistently increased during 6 months (data not shown). Although B cell subsets in bone marrow, lymph nodes, and spleens of 10-week-old *SH2D1A*$^{-/-}$ and *wt* C57BL/6 mice were comparable to those of *wt* mice, the number of Transitional-2 B lymphocytes (CD21^{bright} IgM^{bright} B220$^+$) in the lymph nodes of *SH2D1A*$^{-/-}$ mice was lower than in *wt* mice (Table 1, which is published as supporting information on the PNAS web site). An increase in the number of CD21-IgM-B220$^+$ and CD21-CD23-B220$^+$ B lymphocytes was also observed (Table 1). Taken together, our results indicate that minor B cell abnormalities are detectable in nonimmunized *SH2D1A*$^{-/-}$ mice.

Because reduced primary antigen-specific IgM and IgG responses were detected after infection of *SH2D1A*$^{-/-}$ mice with LCMV at day 8 (Fig. 1*a*) or MHV68 (day 15) (Fig. 1*b*), we also determined responses to a better-defined antigen. To this end, we immunized mutant and *wt* mice with TNP-KLH precipitated by alum. Although this protocol primarily elicits a Th2 response and promotes IgG1 production, the serum titers of all TNP-KLH-specific IgG isotypes and IgM were severely reduced in *SH2D1A*$^{-/-}$ BALB/c mice even 5 days after NP-KLH immunization (Fig. 1*c* and data not shown). Similar results were obtained after immunization of *SH2D1A*$^{-/-}$ C57BL/6 mice with alum-precipitated TNP-KLH (data not shown).

Secondary IgG1, IgG2a, IgG2b, and IgG3 responses to TNP-KLH were severely impaired in *SH2D1A*$^{-/-}$ BALB/c and C57BL/6 mice (Fig. 6, which is published as supporting information on the PNAS web site, and data not shown). IgG, IgG1, and IgG2a serum titers were also significantly reduced in *SH2D1A*$^{-/-}$ BALB/c mice that were immunized with ovalbumin and Freund's adjuvant, conditions that do not favor Th2 responses (data not shown). No defect in T independent antibody responses was found (data not shown). Thus, *SH2D1A*$^{-/-}$ mice have a defective early IgM and IgG antigen-specific antibody response against viruses and T-D antigens.

Requirement of SH2D1A for GC Formation. GCs, the anatomical sites of T–B cell cooperation (33–35), were almost completely absent in the spleen of *SH2D1A*$^{-/-}$ mice that had been immunized with NP-KLH/alum (Fig. 7, which is published as supporting information on the PNAS web site). Fewer than 2% of the splenic follicles contained GCs in immunized *SH2D1A*-deficient animals, whereas >50% of the follicles in identically treated *wt* mice contained one or more GC (Fig. 2*a*). Because of the almost complete absence of GC formations in immunized *SH2D1A*$^{-/-}$ mice and reports indicating the expression of *SH2D1A* by human GC B cells (13, 14, 20, 36, 37), splenic sections of immunized *wt* BALB/c mice were costained for SH2D1A. Fig. 2*b* shows that the SH2D1A protein is expressed by follicular GC B cells. Thus, an inability to form GCs correlates with defective primary IgM and IgG antibody responses in the absence of SH2D1A.

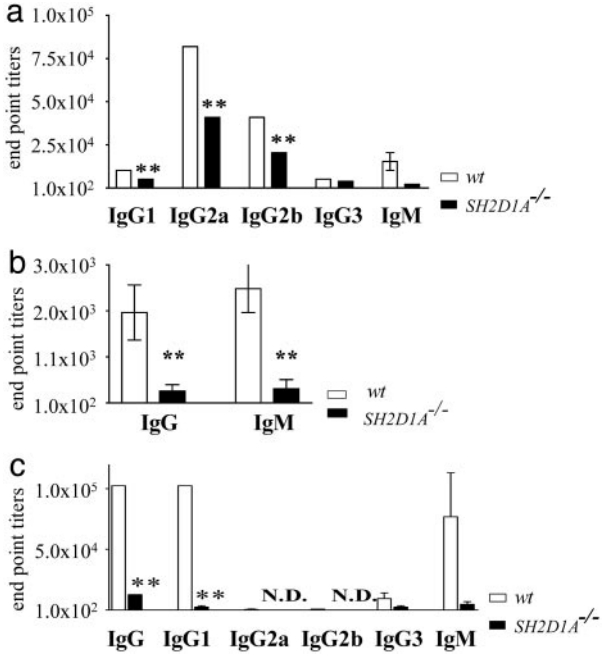

Fig. 1. Impaired primary antibody responses in *SH2D1A*$^{-/-}$ mice. (*a*) LCMV-specific antibody responses in *SH2D1A*$^{-/-}$ mice. *SH2D1A*$^{-/-}$ (filled bars) and *wt* (open bars) C57BL/6 mice were infected with LCMV. LCMV-specific IgM, IgG1, IgG2a, IgG2b, and IgG3 antibody end-point titers were determined at day 8 after infection, as indicated. *y* axis, end-point titers (**, $P < 0.001$; $n = 3$; n = number of mice tested per experiment). Results are representative of two independent experiments. (*b*) MHV68-specific antibody responses in *SH2D1A*$^{-/-}$ mice. *SH2D1A*$^{-/-}$ (filled bars) and *wt* (open bars) C57BL/6 mice were infected with MHV68. MHV68-specific IgM and IgG antibody end-point titers were determined at day 15 after infection, as indicated. *y* axis, end-point titers (**, $P < 0.01$; seven *SH2D1A*$^{-/-}$ and four *wt* mice were tested). (*c*) Primary antibody responses to T-D antigens in *SH2D1A*$^{-/-}$ BALB/c mice. Primary TNP-specific antibody titers in the serum of *SH2D1A*$^{-/-}$ BALB/c mice ($n = 4$) were determined 10 days after i.p. immunization with alum-precipitated TNP-KLH. TNP-specific IgG, IgM, IgG1, IgG2a, IgG2b, and IgG3 antibody titers of *SH2D1A*$^{-/-}$ (filled bars) and *wt* (open bars) mice were determined by ELISA. Serum dilutions start at 1:100; N.D., nondetectable titers (<1:100) (*y* axis = end-point titers) (**, $P < 0.001$; $n = 4$; n = number of mice tested per experiment). Results are representative of three independent experiments.

Defective B Cell Responses in the Absence of SH2D1A. The observation that early antibody responses of all isotypes (including IgM) are impaired in *SH2D1A*-deficient mice prompted us to focus on a potential contribution of *SH2D1A*$^{-/-}$ B cells to the impaired humoral responses in absence of SH2D1A. To this end, carrier-specific memory CD4$^+$ T helper cells were generated by immunizations of *SH2D1A*$^{-/-}$ or *wt* mice with KLH (Fig. 3*a*). Hapten-specific memory B cells were generated by immunization with NP-hen egg lysozyme. Cotransfer of CD4$^{-/-}$ and B$^{+/+}$ cells confirmed that a functional impairment of CD4$^{-/-}$ cells contributes to defective T-D responses by *SH2D1A*$^{-/-}$ mice (Fig. 3*b*), and a very limited NP-specific antibody response was evident upon antigen recall of recipients reconstituted with B$^{-/-}$ cells (Fig. 3*b*). Thus, the results of this hapten-carrier experiment show that primed SH2D1A-deficient B cells are unable to function efficiently in

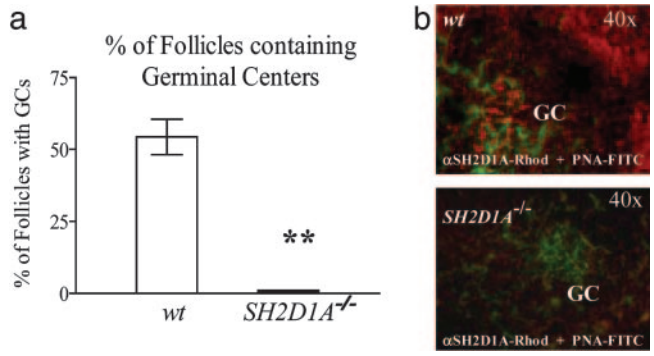

Fig. 2. Follicular GC formation. (*a*) Reduced number of GC-containing follicles in the spleens of *SH2D1A*$^{-/-}$ mice. The number of GC-containing follicles was determined from at least three different consecutive stained cryosections taken from the spleens of the TNP-KLH mice, described in Fig. 1*c*. B cell follicles were identified by anti-CD45R/B220-phycoerythrin, and GCs were stained with PNA-FITC. *y* axis, percentage of follicles containing one or more GCs. Filled bar, *SH2D1A*$^{-/-}$ BALB/c; open bar, *wt* BALB/c (**, $P < 0.001$; $n = 4$; n = number of mice tested per experiment). Results are representative of three independent experiments. (*b*) Expression of the SH2D1A protein in follicular GCs. Cryosections of the spleen of *wt* BALB/c mice 12 days after immunization with NP-KLH were stained with immunofluorescent antibodies, as indicated. Fluorescence was recorded in a Nikon fluorescent microscope. To detect SH2D1A, acetone-fixed and permeabilized spleen sections were first stained with a rabbit anti-mouse anti-SH2D1A antibody (SH2D1A). In a second step, the sections were stained with Rhodamine-labeled donkey anti-rabbit IgG (red). Shown are FITC-labeled-PNA- (green) identified GCs. (*Lower*) One of the few GCs identified on spleens of *SH2D1A*$^{-/-}$ injected mice. Antibodies used for staining are indicated at the bottom; magnification is indicated at top right.

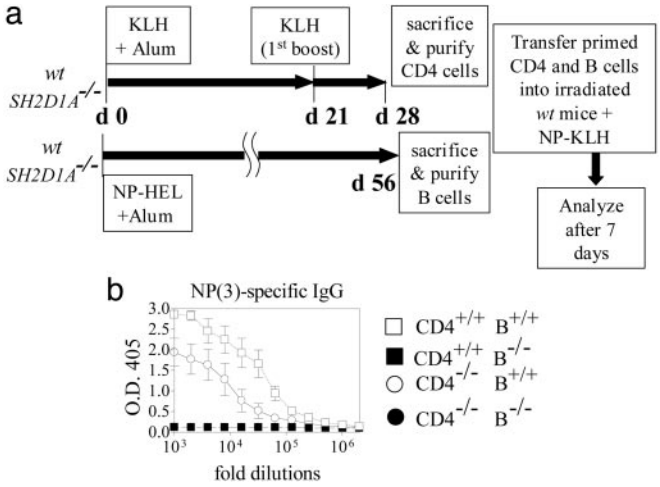

Fig. 3. Reduced hapten-specific antibody responses after cotransfers of primed *SH2D1A*$^{-/-}$ CD4$^+$ T cells with *wt* B cells or primed *wt* CD4$^+$ T cells with *SH2D1A*$^{-/-}$ B cells into irradiated *wt* recipient mice. (*a*) Outline of the experiment. CD4$^+$ T cells were purified from the spleens of *SH2D1A*$^{-/-}$ BALB/c or *wt* BALB/c mice that had been immunized with KLH attached to alum on day 0 (d 0) and boosted on day 21. B cells were purified from the spleens of *SH2D1A*$^{-/-}$ BALB/c or *wt* BALB/c mice that had been immunized 56 days earlier with NP-hen egg lysozyme (*Materials and Methods*). The purified CD4$^+$ (5×10^6/recipient) and B cells (10×10^6/recipient) were then cotransferred into irradiated *wt* BALB/c recipients. At the same time, 100 μg of NP-KLH was injected. Four combinations of CD4 and B cells were used to reconstitute the irradiated recipients: [CD4$^{+/+}$ B$^{+/+}$]; [CD4$^{+/+}$ B$^{-/-}$]; [CD4$^{-/-}$ B$^{+/+}$] and [CD4$^{-/-}$ B$^{-/-}$], in which [$^{-/-}$] represented *SH2D1A*$^{-/-}$ and [$^{+/+}$], *wt*. Reconstituted animals were killed and analyzed 7 days after transfer. (*b*) Hapten-specific antibody responses. High-affinity NP-specific IgG antibody titers in the serum of recipient mice ($n = 4$) were determined, as described in *Materials and Methods* (n = number of mice tested per experiment). Results are representative of two independent experiments. Results of ELISAs are shown as follows: *y* axis, OD 405 units; *x* axis, fold dilutions. $^{-/-}$, cells derived from *SH2D1A*$^{-/-}$ mice; $^{+/+}$, cells derived from *wt* mice; open squares, mice reconstituted with CD4$^{+/+}$ B$^{+/+}$ cells; filled squares, mice reconstituted with CD4$^{+/+}$ B$^{-/-}$ cells; open circles, mice reconstituted with CD4$^{-/-}$ B$^{+/+}$ cells; filled circles, mice reconstituted with CD4$^{-/-}$ B$^{-/-}$ cells.

recipient
mice even in the presence of *wt* primed T helper cells, as judged by T-D antigen responses. Although this apparent B cell defect was likely to be cell intrinsic, because the effect was determined upon adoptive transfer in a *wt* host, it could not be excluded that the T cell environment of the donor *SH2D1A*$^{-/-}$ mouse had permanently affected the donor memory B$^{-/-}$ cell. This prompted us to examine whether naive *SH2D1A*$^{-/-}$ B cells were functionally impaired by using adoptive transfers of naïve B and T cells. Four combinations of CD4$^+$ and B cells were adoptively transferred into *Rag2*$^{-/-}$ recipient mice: [CD4$^{+/+}$ B$^{+/+}$], [CD4$^{+/+}$ B$^{-/-}$], [CD4$^{-/-}$ B$^{+/+}$], and [CD4$^{-/-}$ B$^{-/-}$] (outlined in Fig. 4*a*). Recipient animals were immunized with NP-KLH at day 7 after the transfer and were boosted twice with NP-KLH (Fig. 4*a*). Seven days after the last antigen challenge, the [CD4$^{+/+}$ B$^{+/+}$] chimeras had the highest-titer NP(2)-specific high-affinity antibodies (the number in brackets indicates the NP:BSA conjugation ratio), whereas the [CD4$^{-/-}$ B$^{-/-}$] chimeras had the lowest titers (Fig. 4*b Left*). Consistent with an intrinsic B cell defect, [CD4$^{+/+}$ B$^{-/-}$] chimeras almost had no detectable NP(2)-specific IgG (Fig. 4*b Left*). As expected, *Rag2*$^{-/-}$ mice reconstituted with [CD4$^{-/-}$ B$^{+/+}$] cells had intermediate levels of NP(2)-specific IgG (Fig. 4*b Left*). Although the low-affinity responses by the [CD4$^{+/+}$ B$^{-/-}$] chimeras were more robust than their high-affinity antibody response, they were still defective compared with those of the [CD4$^{+/+}$ B$^{+/+}$] chimeras (Fig. 4*b Right*). As judged by the total IgG serum concentrations (Fig. 8, which is published as supporting information on the PNAS web site), reconstitution of CD4$^+$ and B cells was comparable in all four chimeras. To examine whether the impaired hapten-specific antibody production in the [CD4$^{+/+}$ B$^{-/-}$] and [CD4$^{-/-}$ B$^{+/+}$] mice correlated with defects in the organization of GCs, the spleens of all four chimeras were stained with anti-CD4, anti-B220, and peanut agglutinin (PNA). Whereas chimeras that had received CD4$^{+/+}$ and B$^{+/+}$ cells had follicular GCs with normal follicular and T cell areas, [CD4$^{-/-}$ B$^{-/-}$] chimeras had no GCs (Fig. 4*c*, CD4$^{-/-}$ B$^{-/-}$). Importantly, the organization of GCs was disrupted in [CD4$^{+/+}$ B$^{-/-}$] chimeras, and large numbers of extrafollicular PNA$^+$ cells were detected (Fig. 4*c*, CD4$^{+/+}$ B$^{-/-}$). Consistent with a defect in *SH2D1A*$^{-/-}$ T helper cells, [CD4$^{-/-}$ B$^{+/+}$] chimeras had smaller follicular GCs and an increased relative number of extrafollicular PNA-positive cells (Fig. 4c, CD4$^{-/-}$ B$^{+/+}$). Thus, the presence of extrafollicular GC B cells, which are known to produce low-affinity antibodies, might explain low-affinity hapten-specific responses detected in [CD4$^{+/+}$ B$^{-/-}$] and [CD4$^{-/-}$ B$^{+/+}$] chimeras. It was conceivable that during the week after transfer into the *Rag2*$^{-/-}$ recipients, the *wt* and mutant cells multiplied at a different rate before immunization. To address this issue, a second set of adoptive transfer experiments into *Rag2*$^{-/-}$ recipient mice was done in which the recipient mice received a single immunization at time of cell transfer (outlined in Fig. 5*a*). At day 10 after the transfer, NP-specific IgM antibody responses (Fig. 5*b*) were defective in the [CD4$^{+/+}$ B$^{-/-}$] and [CD4$^{-/-}$ B$^{+/+}$] chimeras (Fig. 5*b Left*). Total IgM serum levels were lower in chimeras reconstituted with B$^{-/-}$ cells, when compared with mice reconstituted with B$^{+/+}$ cells (Fig. 9*a*, which is published as supporting information on the PNAS web site). Furthermore, high- and low-affinity NP-specific IgG antibody responses at day 10 (Fig. 5*b Right*; see also Fig. 9*b*) and at day 20 (data not shown) were markedly lower in [CD4$^{+/+}$ B$^{-/-}$] and [CD4$^{-/-}$ B$^{+/+}$] chimeras than in [CD4$^{+/+}$ B$^{+/+}$] mice. The IgG concentrations in the serum of all recipients were comparable (Fig. 9*c*). Taken together, the early antibody responses to one injection of antigen given at the day of the adoptive transfer confirm that both

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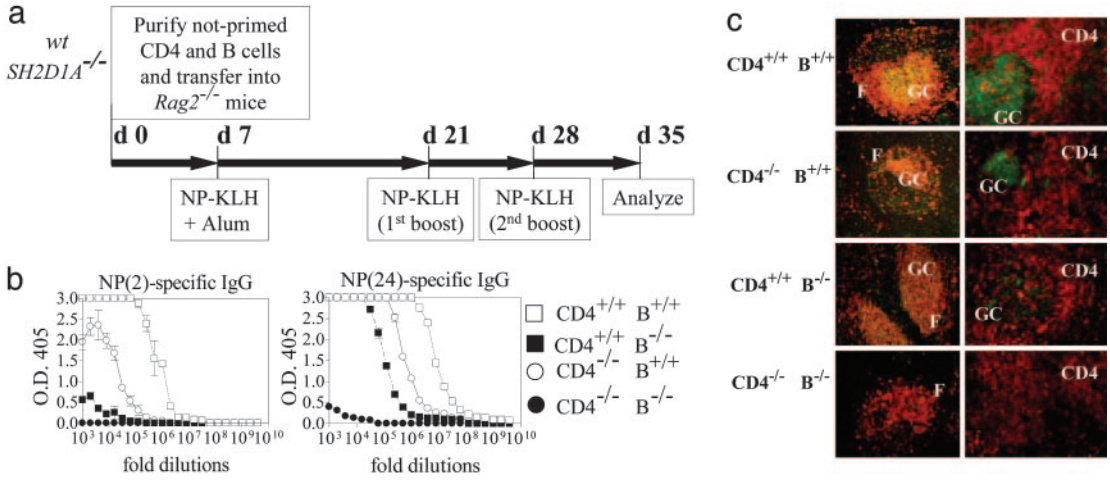

Fig. 4. Defective hapten-specific antibody responses after cotransfer of naïve *SH2D1A*$^{-/-}$ B cells with naïve *wt* CD4$^+$ cells. (*a*) Outline of the experiment. CD4$^+$ cells (5×10^6) together with 10×10^6 B cells from unprimed *SH2D1A*$^{-/-}$ BALB/c or *wt* BALB/c mice were transferred into *Rag2*$^{-/-}$ mice at day 0 (d 0). Four combinations of CD4 and B cells were used to reconstitute the *Rag2*$^{-/-}$ recipients: [CD4$^{+/+}$ B$^{+/+}$], [CD4$^{+/+}$ B$^{-/-}$], [CD4$^{-/-}$ B$^{+/+}$], and [CD4$^{-/-}$ B$^{-/-}$], in which [$^{-/-}$] represents *SH2D1A*$^{-/-}$ and [$^{+/+}$], *wt*. At day 7 (d 7), mice were immunized with NP-KLH in alum. Reconstituted *RAG2*$^{-/-}$ mice were then boosted twice with NP-KLH (at d 21 and d 28), and serum antibody levels responses were determined at d 35. (*b*) Analysis of hapten-specific antibody responses. High- [NP(2)-specific, *Left*] and low-affinity [NP(24)-specific, *Right*] IgG antibody titers in the serum of recipient mice ($n = 4$) were determined as described in *Materials and Methods* (n = number of mice tested per experiment). Results are representative of three independent experiments. Results of ELISAs are shown (*y* axis, OD 405 units; *x* axis, fold dilutions). $^{-/-}$, cells derived from *SH2D1A*$^{-/-}$ mice; $^{+/+}$, cells derived from *wt* mice; open squares, mice reconstituted with CD4$^{+/+}$ B$^{+/+}$ cells; filled squares, mice reconstituted with CD4$^{+/+}$ B$^{-/-}$ cells; open circles, mice reconstituted with CD4$^{-/-}$ B$^{+/+}$ cells; filled circles, mice reconstituted with CD4$^{-/-}$ B$^{-/-}$ cells. (*c*) Cryosections prepared from the spleens of mice reconstituted with naïve *SH2D1A*$^{-/-}$ or *wt* B and CD4 cells (as indicated on the left), 7 days after the last immunization with NP-KLH. Sections were stained with immunofluorescent antibodies, and fluorescence was recorded in a Nikon fluorescent microscope. Anti-CD45R/B220–phycoerythrin (PE) (red) (*Left*) detects follicle areas (*F*), anti-CD4-PE (red) (*Right*) detects T cell areas (CD4), and PNA-FITC (green) (*Left* and *Right*) identifies GC.

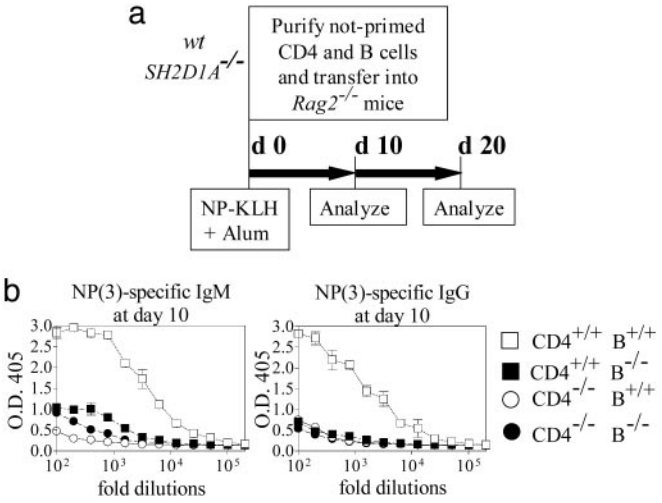

Fig. 5. Defective early hapten-specific antibody responses after cotransfer of naïve *SH2D1A*$^{-/-}$ B cells with naïve *wt* CD4$^+$ cells. (*a*) Outline of the experiment. CD4$^+$ cells (5×10^6) together with 10×10^6 B cells from unprimed *SH2D1A*$^{-/-}$ BALB/c or *wt* BALB/c mice were transferred into *Rag2*$^{-/-}$ mice and immunized with NP-KLH in alum at day 0 (d 0). Four combinations of CD4 and B cells were used to reconstitute the *Rag2*$^{-/-}$ recipients: [CD4$^{+/+}$ B$^{+/+}$], [CD4$^{+/+}$ B$^{-/-}$], [CD4$^{-/-}$ B$^{+/+}$], and [CD4$^{-/-}$ B$^{-/-}$], in which [$^{-/-}$] represented *SH2D1A*$^{-/-}$ and [$^{+/+}$], *wt*. Serum antibody levels responses were determined at day 10 (d 10), and mice were killed at day 20 (d 20) for analysis. (*b*) Analysis of IgM and IgG hapten-specific antibody responses at day 10. High-affinity [NP(3)-specific] IgM (*Left*) and IgG (*Right*), NP-specific antibody titers in the serum of recipient mice ($n = 3$) were determined as described in *Materials and Methods* (n = number of mice tested per experiment). Low-affinity [NP(24)-specific] IgM and IgG NP-specific antibody titers are shown in Fig. 9*b*. Results are representative of two independent experiments. Results of ELISAs are shown (*y* axis, OD 405 units; *x* axis, fold dilutions). $^{-/-}$, cells derived from *SH2D1A*$^{-/-}$ mice; $^{+/+}$, cells derived from *wt* mice; open squares, mice reconstituted with CD4$^{+/+}$ B$^{+/+}$ cells; filled squares, mice reconstituted with CD4$^{+/+}$ B$^{-/-}$ cells; open circles, mice reconstituted with CD4$^{-/-}$ B$^{+/+}$ cells; filled circles, mice reconstituted with CD4$^{-/-}$ B$^{-/-}$ cells.

B and CD4$^+$ T cell defects contribute to severely impaired primary and secondary humoral immune responses in *SH2D1A*$^{-/-}$ mice.

Discussion

Because dysgammaglobulinemia develops in a subset of XLP patients without fatal infectious mononucleosis, we hypothesized that progressively impaired humoral responses might occur in *SH2D1A*-deficient individuals and mice, as a consequence of normal immune insults. Without specific immunization of *SH2D1A*$^{-/-}$ mice, the levels of serum IgG1 are consistently lower than in age-matched *wt* mice, whereas serum IgG2a is increased (data not shown), and serum IgE is almost undetectable (25, 26). Upon exposure to hapten, protein, or viral antigens (LCMV and MHV68), both primary and secondary humoral responses, however, are severely impaired in *SH2D1A*$^{-/-}$ mice. Concomitantly, GC formation and hapten-specific Ig class switching in *SH2D1A*$^{-/-}$ mice are impaired. Staining of GC with an anti-SH2D1A antibody shows that the adapter protein is expressed in a subset of B cells that are present in the GC. Indeed, using the adoptive transfer of B lymphocytes from unprimed *SH2D1A*-deficient mice together with either *SH2D1A*-deficient or *wt* CD4$^+$ cells into *Rag2*$^{-/-}$ recipients, we demonstrate that both *SH2D1A*$^{-/-}$ B and *SH2D1A*$^{-/-}$ CD4$^+$ T cells contribute to defective antigen-specific IgM and IgG production. Not unexpectedly, these defects are exacerbated upon adoptively transferring antigen-primed B and T cells. Thus, SH2D1A controls both CD4$^+$ T cell and B cell functions that are implicated in T-D humoral immune responses.

We observed a reduction in primary antibody responses after infection of *SH2D1A*$^{-/-}$ mice with two unrelated viruses, LCMV and MHV68. Late humoral responses to infection with LCMV (28) or MHV68 (data not shown) are more severely affected in *SH2D1A*$^{-/-}$ mice, because these animals fail to generate long-lived virus-specific plasma cells and memory B cells (28, 38). Whereas the failure of naive and memory *SH2D1A*$^{-/-}$ CD4$^+$ cells to produce IL-4 (refs. 25 and 26 and data not shown) could

in part explain these defective early and late responses, the underlying cause of the low levels of antigen-specific IgM responses required further investigation. Obvious T cell-dependent factors that could have contributed to the *SH2D1A*$^{-/-}$ phenotype, such as up-regulation of CD40L or secretion of IL-21 (39), were not impaired in *SH2D1A*$^{-/-}$ mice (data not shown). We therefore focused first on examining the possibility of a B cell defect. Our adoptive transfer experiments indeed demonstrate that *SH2D1A* is essential for B cell activities that partake in T cell-dependent IgG and IgM production. The presence of endogenous *wt* B cells in the irradiated *wt* recipients could readily explain why Crotty *et al.* (28) did not detect a defect in *SH2D1A*$^{-/-}$ B cells. Because no endogenous B cells exist in the *Rag2*$^{-/-}$ recipients used in the current experiments, the defect in *SH2D1A*$^{-/-}$ B cells could be observed.

The finding that SH2D1A is expressed in a B cell subset is consistent with the results of the adoptive transfer experiments. SH2D1A has also been detected in human GC B cells and B cell tumors (13, 19, 20). EBV-positive Burkitt's lymphoma lines, which resemble B cells at the GC stage of differentiation, are mostly SH2D1A-positive (18). Moreover, a contraction of the CD19^{+} CD27^{+} B cell memory compartment has been observed in XLP patients (40). Taken together, the data strongly suggest that expression of SH2D1A by GC or memory B cells controls early and memory antibody responses. In the absence of SH2D1A, the contribution of a small B cell subset to the process of GC formation is likely to be affected. Because GC B cells express high levels of the CD95/Fas molecule (33), it is tempting to speculate that the absence of *SH2D1A* might result in exaggerated cell death and inefficient GC formation in *SH2D1A*$^{-/-}$ mice. This notion was indirectly supported by the observation that the CD150/SH2D1A receptor adapter complex modulates CD95-mediated apoptosis (20). In general, antibody production by mature B cells outside the GC is intact, and T-independent responses are normal in the absence of SH2D1A (data not shown).

Conclusion

The present study unequivocally demonstrates that dysgammaglobulinemia in *SH2D1A*-deficient mice takes place in the absence of a viral infection. This observation is consistent with dysgammaglobulinemia in XLP patients in the absence of a detectable infection with EBV or any other virus previously observed by us and others (4, 7–10). Because SH2D1A controls signal transduction pathways in both T and B cells initiated by at least six cell-surface receptors belonging to the CD150 family of costimulatory adhesion molecules, a dissection of the contribution of each of these receptors to humoral responses to T-D antigens will be required for an understanding of the molecular underpinnings of these biochemical events.

We thank Drs. Stephen Laroux and Duncan Howie for critically reviewing the manuscript; Drs. Klaus Rajewsky and Stefano Casola for advice; Drs. Michael Grusby and Andrea Wurster for help with the IL-21 PCR; Drs. Khuong B. Nguyen and Gary C. Pien for help with LCMV infections; and Drs. John Kearney; Herbert C. Morse III; Max D. Cooper; and Goetz Ehrhardt for sharing PCR analysis data. M.M. is a Special Fellow of the Leukemia and Lymphoma Society. This work was supported by National Institutes of Health Grant AI-35714 (to C.T.).

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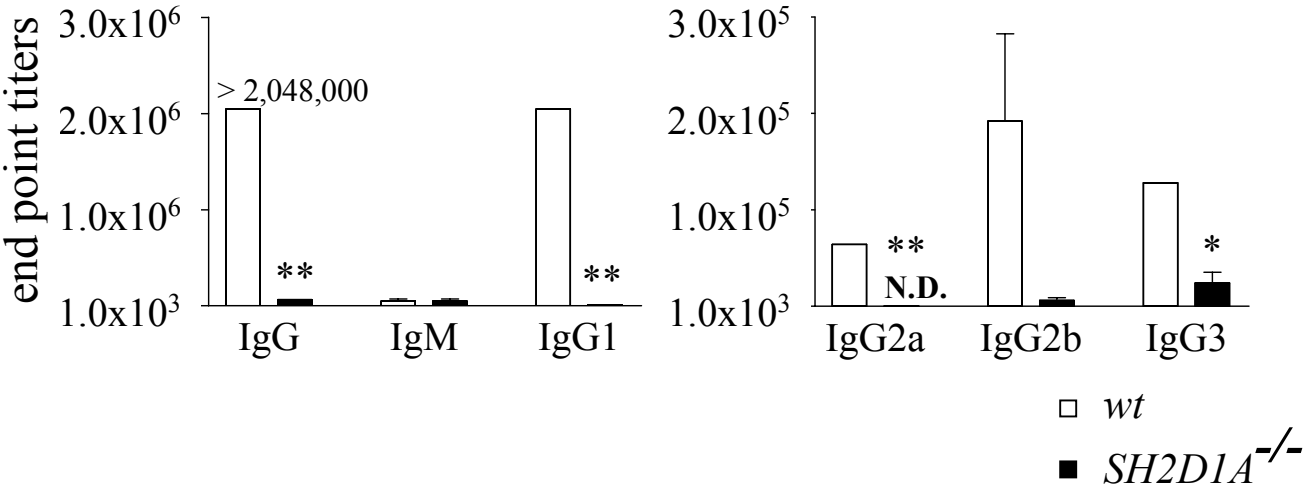
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3.0x10^5
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wt
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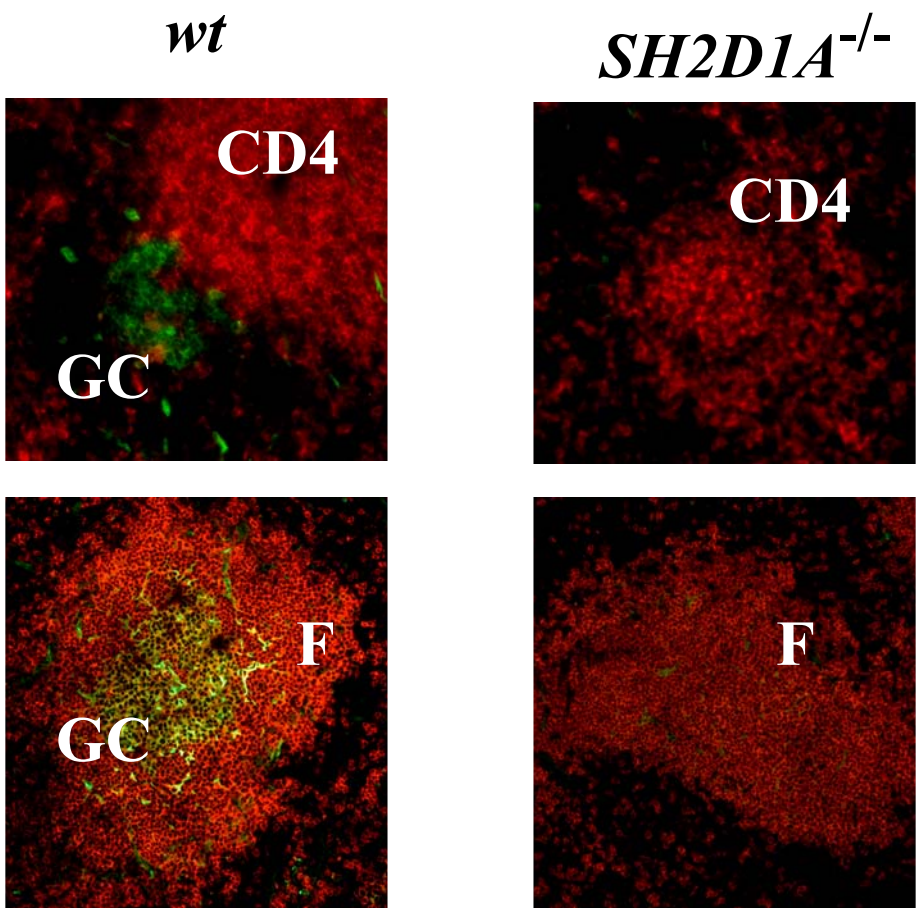
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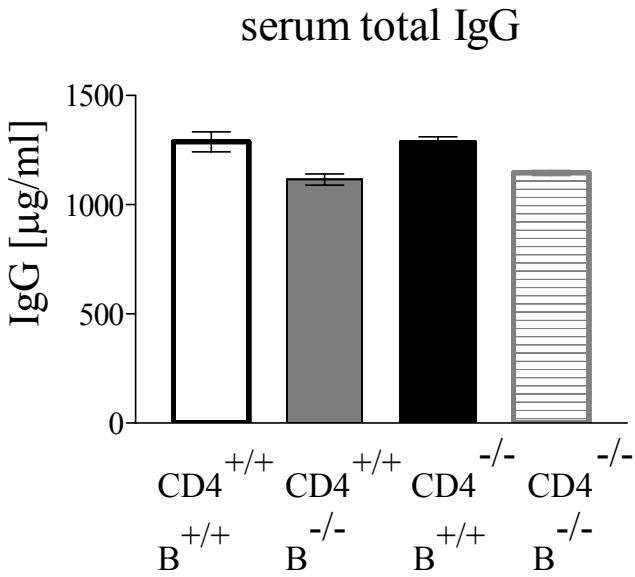
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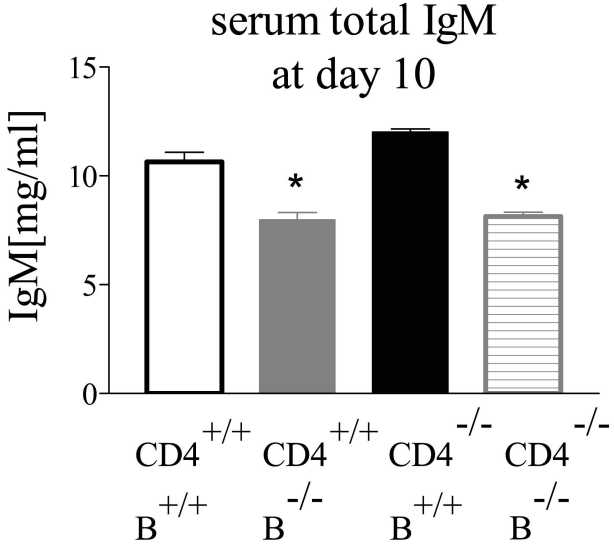

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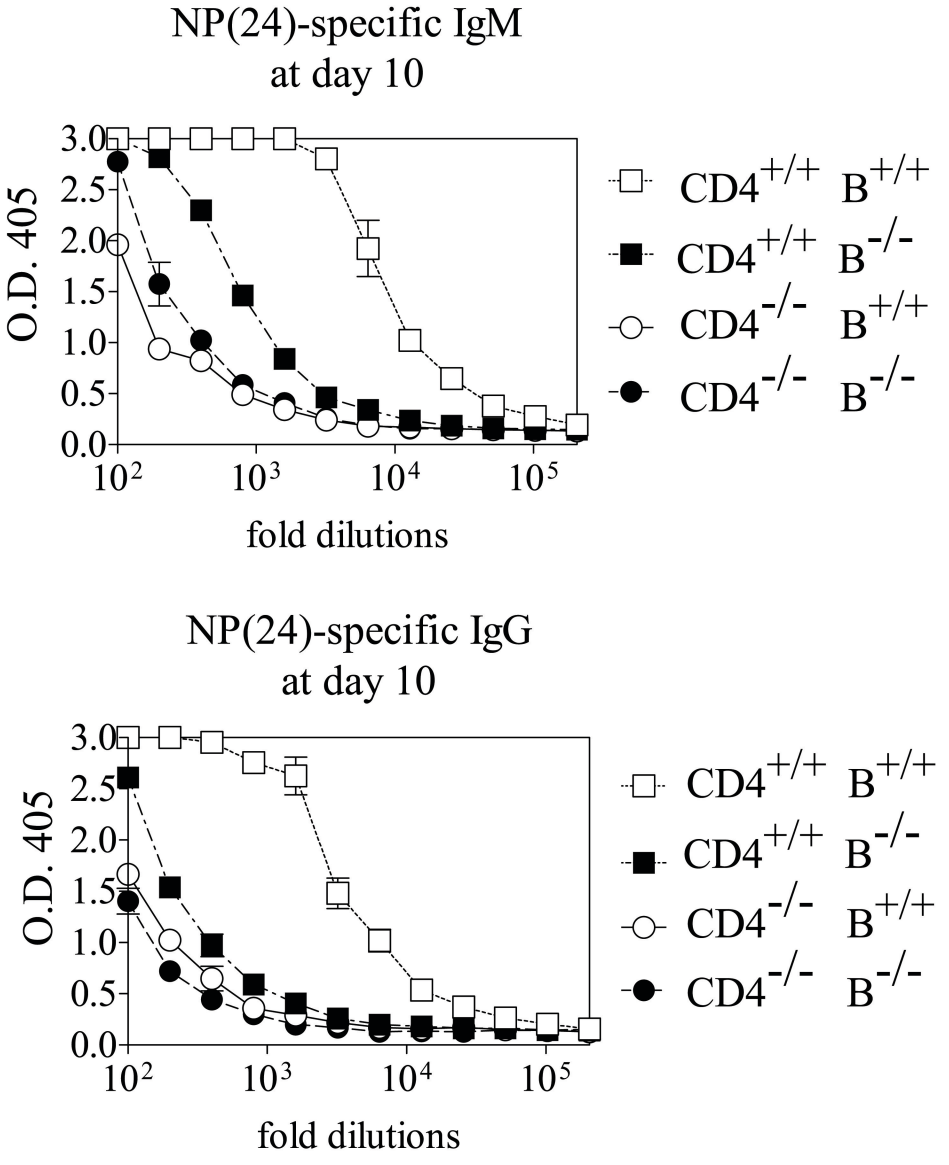
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2.0
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10^3
10^4
10^5
fold dilutions
$CD4^{+/+}$ $B^{+/+}$
$CD4^{+/+}$ $B^{-/-}$
$CD4^{-/-}$ $B^{+/+}$
$CD4^{-/-}$ $B^{-/-}$
NP(24)-specific IgG
at day 10
O.D. 405
3.0
2.5
2.0
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10^2
10^3
10^4
10^5
fold dilutions
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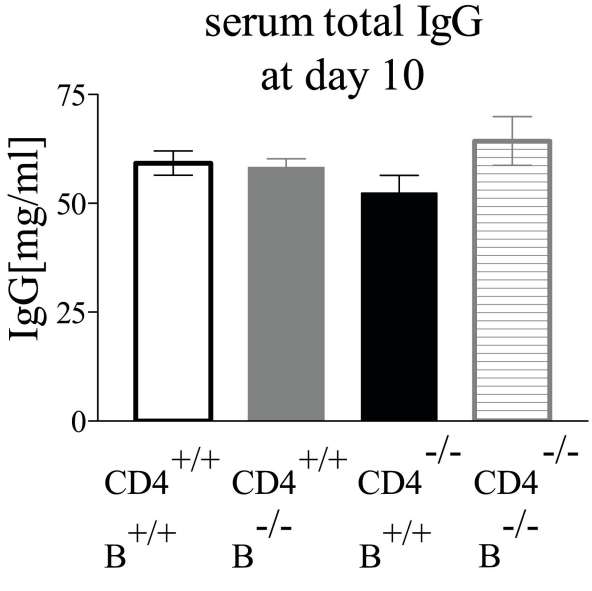
C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