Published in final edited form as:
J Immunol. 2009 June 1; 182(11): 6753–6762. doi:10.4049/jimmunol.0803241.

Hyper-costimulation Through 4-1BB Distorts Homeostasis of Immune cells

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Abstract

The deleterious side-effects associated with a recent clinical trial with anti-CD28 super-agonist antibodies have questioned the use of reagents to costimulatory molecules in human therapy. We now show that sustained signaling from an agonist antibody to 4-1BB, a member of the tumor necrosis factor receptor (TNFR) superfamily, results in detrimental effects on immune cell homeostasis. Repeated anti-4-1BB treatment during the reconstitution of hematopoietic cells in irradiated mice engrafted with bone marrow, or in mice infected with vaccinia virus, induced abnormal apoptosis of pre- and immature-B cells in the bone marrow, and led to peripheral B cell depletion. Inhibition of B cell development was indirect and due to costimulation of CD8 T cells and dependent on IFN-γ. Moreover, anti-4-1BB also suppressed the development of NK and NKT cells, but in this case independently of T cells and IFN-γ. The altered NK cell homeostasis resulted from activation-induced cell death triggered by anti-4-1BB. These results show that hyper-costimulation elicits strong T cell immunity, but it can simultaneously distort immune homeostasis, suggesting that careful attention to activity, dose, and periodicity of treatment will be needed in any immunotherapeutic strategy with agonist antibodies to costimulatory molecules.

Keywords

Costimulation; Hematopoiesis; T cells; B cells; Natural Killer cells

Introduction

The outcome of T cell immunity and tolerance is fine-tuned through secondary signals from costimulatory or coinhibitory molecules on T cells (1–3). Appropriate costimulation is essential for generating optimal immunity, controlling activation, division, survival, and functionality of T cells (4–6). In this regard, targeting T cell costimulatory molecules with agonistic antibodies has achieved a great deal of therapeutic activity in many disease models in the mouse, such as with infectious agents and tumors, where augmentation of the number and function of antigen-specific T cells is desirable. Naturally, these molecules have then gained attention in clinical circles as future targets for human immunotherapy. Whereas the potential of stimulatory reagents for promoting T cell immunity is great, for allowing effective

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Disclosures
The authors declare no competing financial interests.

immunotherapy of cancer, and as adjuvants for vaccination or therapeutic intervention against pathogens, there are possible side effects that need to be considered when discussing which reagent or target might be clinically applicable.

Recently, a phase 1 clinical trial involving a super agonist antibody to the costimulatory receptor CD28 resulted in almost fatal adverse reactions involving a type of systemic inflammatory response and “cytokine storm” (7). Whether this is the only type of adverse response that could result from excessive costimulatory signaling is not clear, but other clues are already in the literature that might not have been appreciated fully. For example, strains of mice that transgenically over-express costimulatory ligands, such as LIGHT (8) and OX40L (9), generate certain signs of autoimmunity associated with strong T cell activation. CD70 transgenic mice also exhibited a lethal T cell immunodeficiency via apparent chronic costimulation through its receptor CD27 (10). Moreover, one common trait of transgenic mice over-expressing T cell costimulatory ligands, including B7-1 (11), B7-2 (12), LIGHT (8), CD70 (13), and 4-1BBL (14), is an abnormal loss of B cells. Given that these phenotypes are seen in mice where expression of these molecules is often much higher than normal, and from birth, and that expression in some cases was forced on cell types that normally might not express the molecules, it is not clear whether such adverse reactions could occur with therapeutic targeting of a receptor that might be used within the clinic, or be a feature of targeting all costimulatory receptors.

4-1BB, a member of the tumor necrosis factor receptor (TNFR) superfamily (TNFRSF9), can play a costimulatory role for T cell immunity upon binding with 4-1BB ligand (4-1BBL), a member of the TNF superfamily (TNFSF9) (2,15). In particular, 4-1BB/4-1BBL interactions boost CD8 T cell responses, although the expression profile of 4-1BB is now known to be quite broad, being present or induced on various types of immune cells, and not solely restricted within T-lineage cells. Agonist antibodies against 4-1BB are very efficient reagents for eliciting a strong anti-tumor response, even against pre-established (16,17) or carcinogen-driven primary tumors (18), and in most cases CD8 T and NK cells have been found crucial for the therapeutic activity. In parallel, anti-4-1BB also has been shown to enhance T cell immunity against viruses, although the timing of antibody injection might be crucial for gaining positive effects (19,20).

In striking contrast, 4-1BB agonistic antibodies unexpectedly have been shown to ameliorate disease progression in many autoimmune and inflammatory models in mice (21), including experimental autoimmune encephalomyelitis (EAE) (22), lupus (23,24), collagen-induced arthritis (CIA) (25), graft-versus-host disease (GVHD) (26,27), and allergic asthma (28). Combined with the pro-inflammatory effects of anti-4-1BB seen in cancer and infectious disease models, this has suggested that stimulatory reagents to 4-1BB might be ideal for immunotherapy of multiple diseases.

Here, we show that sustained costimulation by repeated treatment with agonist antibody to 4-1BB can strongly alter the homeostasis of immune cells. Most notably, anti-4-1BB inhibited B cell development in the bone marrow by preventing maturation of pre- and immature B cells but not pro-B cells, which ultimately reduced the number of B cells in the periphery. This adverse activity of anti-4-1BB was indirect and a consequence of hyper-stimulating the CD8 T cell compartment, that through production of IFN-γ resulted in apoptosis of developing B cells. Furthermore, anti-4-1BB impeded the development of NK and NKT cells, an action that was independent of T cells and IFN-γ. Anti-4-1BB induced the activation of NK cells, followed by the induction of activation-induced cell death. These effects of targeting 4-1BB were seen in both recipients of bone marrow grafts and in virus infected animals, providing complementary data and extending the recent side effects noted in normal naïve animals treated with anti-4-1BB (29). Thus, although clinical targeting of costimulatory receptors has great

therapeutic potential and should not be discouraged, a great deal of caution should be exercised when considering the type and length of a therapeutic regimen that will use agonists to costimulatory molecules, as excessive stimulation can lead to an unwanted imbalance in immune stasis.

Materials and Methods

Mice

4-1BB$^{-/-}$, TCRα$^{-/-}$, IFN-γ$^{-/-}$, CD8α$^{-/-}$ and Rag-1$^{-/-}$ mice on a C57BL/6 background were bred at LIAI. C57BL/6J (CD45.2^{+}) and C57BL/6$^{Pep3b/BoyJ}$ (CD45.1^{+}) were purchased from the Jackson Laboratories. All experiments were in compliance with the regulations of the LIAI animal care committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

Immunofluorescence labeling

Spleens, lymph nodes, and thymus tissue were digested for 30 min at 37 °C with collagenase D (Roche) and DNase (Sigma-Aldrich). Bone marrow was extracted from the tibia and femur in culture medium, HEPES-buffered, RPMI 1640 medium (Irvine Scientific) containing 1% FBS (Omega Scientific), and erythrocytes lysed. Intracellular stainings of cytokines in T cells and phospho-STAT-1 (pSTAT-1) in B cells were performed as described previously (30). For intracellular cytokine staining cells from spleen and BM were restimulated with PMA/ionomycin or epitope peptides of vaccinia virus (VACV) in the presence of brefeldin for 5 hrs. VACV peptide epitopes used in this study were predicted and synthesized as described previously (31,32). For ex-vivo cytokine staining, BM cells were cultured with media alone in the presence of brefeldin for 2 hrs. For ex-vivo pSTAT-1 staining of BM-resident B cells, BM cells were fixed with paraformaldehyde followed by permeabilization with methanol and then stained with antibodies to B220, CD43, and pSTAT-1. All immunofluorescent staining was done after FcγR blocking by pre-incubation with 2.4G2. Cells were gated for flow cytometry as follows: pro-B cells, B220^{+}CD43^{+}IgM^{-}; pre-B cells, B220^{+}CD43^{-}IgM^{-}; immature/mature B cells, B220^{+}CD43^{-}IgM^{+}; NK cells, CD3^{-}NK1.1^{+}DX5^{+}; total T cells, CD3^{+}TCRβ^{+}; CD4 T cells, CD3^{+}CD4^{+}; CD8 T cells, CD3^{+}CD8^{+}; cDC, CD11c^{+}I-A/E^{hi}; pDC, CD11c^{lo}PDCA-1^{+}I-A/E^{med}; Vα14*i*NKT cells, B220^{-}CD1d-tetramer^{+}TCRβ^{+}; germinal center B cells, B220^{+}IgD^{-}Fas^{+}PNA^{+}. LSR II (Becton Dickinson) and FACS-Calibur (Becton Dickinson) flow cytometers were used with FlowJo software (TreeStar).

Reagents

The following reagents were used; purified anti-CD16/CD32 (2.4G, BD Bioscience); biotin-conjugated anti-4-1BB (17B5) and hamster IgG1 (HTK888, all from BioLegend); fluorescein isothiocyanate-conjugated anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD11c (HL3), anti-CD44 (1M7), anti-CD122 (5H4), anti-CD69 (H1.2F3), anti-Ly49D (4E5), anti-Ly49C/I (SW5E6), anti-TCRβ (H57-597, all from BD Bioscience), anti-Gr-1 (RB6-8C5), anti-IgM (RMM-1), anti-DX5, anti-CD11b (M1/70), anti-CD94 (18d3, all from eBioscience); phycoerythrin-conjugated anti-CD43 (S7), anti-IL-2 (JES6-5H4), anti-IFN-γ(XMG1.2), anti-Fas (15A7, all from BD Bioscience), anti-CD24 (M1/69), anti-I-A/E (M5/114.15.2), anti-TNF (MP6-XT22), anti-DX5 (all from eBioscience), anti-NKG2D (C7), anti-IL-17 (TC11, all from BioLegend), anti-human granzyme-B (CALTAG) and Annexin-V (BD Bioscience); allophycocyanin-conjugated anti-NK1.1 (PK136), anti-CD8α (53–6.7, all from BD Bioscience), anti-B220 (RA3-6B2, BioLegend), anti-F4/80 (BM8, eBioscience) and; peridinin chlorophyll protein-conjugated anti-CD45.2 (104), anti-CD4 (L3T4), and anti-CD11b (M1/70, all from BD Bioscience); alexa fluor 488-conjugated anti-pSTAT-1 (4a, BD Bioscience); and 7-AAD (BioLegend). FITC-labeled peanut lectin agglutinin (PNA) was from Vector Laboratories, and phycoerythrin- or allophycocyanin-conjugated anti-mouse pDC antigen

(anti-PDCA-1), and anti-biotin microbeads were from Miltenyi Biotec. Anti-4-1BB (3H3) and anti-IFN-γ (XMG1.2) were purified as described (29). Rat IgG was purchased from Invitrogen.

Bone marrow chimeras

Recipient mice ($CD45.1^+$ or $CD45.2^+Rag1^{-/-}$) were irradiated with two rounds (separated by a 3-hour interval) of irradiation with 5.5 Gy (11 Gy total). BM cells ($5–10 \times 10^6$) were transferred i.v. one day after irradiation. Mice were administered antibiotics (1.0 mg/ml neomycin and 0.1 mg/ml of polymyxin-B sulfate; Sigma-Aldrich) in the drinking water for 2–3 weeks post-reconstitution. Anti-4-1BB or rat IgG antibody (100 μg) was injected i.p. once per week for 4 weeks starting from 2 weeks post BM-reconstitution, and where indicated anti-IFN-γ (400 μg) was injected i.p. every four days from 2 weeks post-reconstitution. The reconstitution of immune cells was analyzed after 6 or 7 weeks.

Vaccinia virus infection

VACV Western Reserve (VACV-WR) strain was purchased from the American Type Culture Collection (Manassas, VA), grown on HeLa cells. C57BL/6 mice were infected i.p. with 2×10^5 PFU of VACV. The injection of antibody (100 μg) was started 1 day after VACV infection and repeated once per week until the end of the study. For quantitation of VACV-specific IgG from serum, VACV antigen was prepared by UV inactivating stock VV-NYCBOH with trioxsalen-psoralen (4' aminomethyl-trioxsalen HCl; Calbiochem). The UV-inactivated virus was then used at a 1:25 dilution in phosphate-buffered saline (PBS) with bovine serum albumin supplemented to a final concentration of 0.1%. Direct ELISA was performed using Nunc Polysorp flat-bottomed 96-well plates coated overnight with 100 μl/well VACV antigen. The plates were washed, and serum samples were added to the plates and serially diluted (twofold dilutions) in PBS plus 0.05%Tween 20 plus 10% FCS. Caltag horseradish peroxidase-conjugated goat anti-mouse IgG diluted 1:1,000 in PBS plus 0.05% Tween 20 plus 10% FCS was used for detection. The plates were developed using o-phenylenediamine, and the optical density at 490 nm (OD490) was read on a SpectraMax 250 (Molecular Devices). Anti-VACV serum IgG antibody titers were determined as endpoint titers 0.1 OD unit more than background (no serum well).

Statistical analysis

Statistical significance was determined by two-tailed Student's t-test.

Results

Agonist anti-4-1BB inhibits B cell development in the bone marrow

To determine whether treatment with an agonist antibody to 4-1BB might alter immune homeostasis under conditions where hematopoiesis might be required, for example, in immuno-compromised individuals or those undergoing transplantation, we lethally irradiated mice and reconstituted the immune system with bone marrow. Anti-4-1BB (3H3) was injected once per week for four weeks, starting 2 weeks after BM reconstitution. Significantly, the number of mature B cells in the spleen (Fig. 1A), as well as in the lymph nodes (data not shown), was dramatically reduced by 4-1BB signaling. We then addressed whether this loss of mature B cells in the periphery was mirrored in the bone marrow where B cell development takes place. Interestingly, anti-4-1BB-treated mice showed a strong reduction in B-lineage cells ($B220^+$) in the bone marrow (Fig. 1B–C). Division of B-lineage cells into subsets (33), $CD43^+IgM^-$ (pre-pro- and pro-B cells), $CD43^-IgM^-$ (pre-B cells), and $CD43^-IgM^+$ (immature/mature B cells), revealed significant decreases in the $CD43^-IgM^-$ and $CD43^-IgM^+$ populations but not the $CD43^+IgM^-$ population (Fig. 1C).

Analysis of the percentage of cells undergoing apoptosis further suggested that anti-4-1BB was promoting death primarily in these pre- and immature B cells, and that the effect was specific in that no difference was observed on myeloid-lineage ($CD11b^+$) cells in the bone marrow (Fig. 1D). Moreover, no significant apoptosis of B cells was found in the spleen, implying that this 4-1BB-induced death of B-lineage cells occurred specifically within the bone marrow. As we did not visualize 4-1BB on developing B cells or mature B cells (data not shown) it was unlikely that the action of anti-4-1BB was direct. To show this, we generated mixed BM chimeras with equal numbers of wild type and 4-$1BB^{-/-}$ cells. Anti-4-1BB treatment reduced total B cell numbers in this situation (Fig. 1E), and this was seen in both wt and 4-$1BB^{-/-}$ populations, suggesting that 4-1BB-mediated B cell loss is independent of 4-1BB expression in this lineage. Collectively, these results show anti-4-1BB impedes B cell development in the bone marrow under conditions of strong lymphopoiesis by inducing apoptosis at the pre- and immature B cell stages. This in turn results in reduced output of immature B cells into the blood and reduced numbers of mature B cells residing in secondary lymphoid organs.

CD8 T cells and IFN–γ are crucial for the inhibition of B cell development by anti-4-1BB

Activated T cells can express 4-1BB and thus might be potential target cells to mediate the effects of anti-4-1BB. We found augmented numbers of effector/memory phenotype CD8 T cells ($CD44^{hi}$) in spleen (Fig. 2A) of antibody treated chimeras, which corresponds with a previous report showing that anti-4-1BB treatment or transgenic expression of 4-1BBL enhanced the accumulation of memory phenotype T cells (34). In contrast, reduced numbers of CD4 T cells were visualized in spleen (Fig. 2A). To assess whether T cells were required for impeding B cell development, we then generated chimeras with TCR$\alpha^{-/-}$ BM. In line with this, in the absence of T cells, anti-4-1BB treatment had no effect on developing or mature B-lineage cells in the bone marrow and spleen (Fig. 2B–C). To further dissect which T cell subset is crucial for the inhibition of B cell development, we made another chimera with CD8$\alpha^{-/-}$ BM. Strikingly, the inhibitory effects were totally abolished in the absence of CD8 T cells, although anti-4-1BB now increased the number of effector/memory CD4 T cells (Fig. 2D). This data then suggests that ligation of 4-1BB led to preferential expansion of CD8 T cells, which directly or indirectly resulted in apoptosis of pre- and immature B cells in the bone marrow.

Assessment of cytokine expression by peripheral T cells revealed that IFN-γ production by both CD4 and CD8 subsets was augmented after anti-4-1BB treatment (data not shown). The secretion of other cytokines, including IL-17, IL-2, and TNF, in CD4 and CD8 T cells was largely comparable between both groups of mice. When we calculated the actual numbers of cells in the spleen that were capable of expressing individual cytokines, we found that anti-4-1BB strongly upregulated the number of IFN-γ-secreting CD8 T cells and moderately upregulated the number of IFN-γ-secreting CD4 T cells (Fig. 3A). Anti-4-1BB also promoted IL-2-secreting CD8 cells, but led to reduced IL-17-, IL-2-, and TNF-secreting CD4 cells due to the diminution in the total number of CD4 T cells.

Few T cells are normally found in the bone marrow. Anti-4-1BB treatment dramatically increased the number of T cells in the bone marrow (Fig. 3B), and these were capable of making IFN-γ, IL-2 and TNF (Fig. 3C), but not Th2 cytokines (IL-4 or IL-10, not shown). However, the most pronounced effect was on the number of IFN-γ-producing CD8 T cells that were 2-fold more prevalent than IFN-γ-producing CD4 T cells, and at a frequency 12-fold higher than in control bone marrow. Further, ex vivo direct staining of IFN-γ implied that 4-1BB ligation of CD8 T cells in the BM can induce IFN-γ in situ (Fig. 3D). We did not observe in situ IFN-γ secretion from CD4 T cells (not shown). These results suggested that T cell production of IFN-γ in response to 4-1BB signals might be involved in suppressing B-cell development, potentially correlating with a prior study in CD70 transgenic animals where CD27-CD70

interactions resulted in progressive depletion of peripheral B cells that was IFN-γ-dependent (13).

To assess this, we first neutralized IFN-γ by injection of a blocking antibody during the whole course of anti-4-1BB treatment. Anti-IFN-γ did not strongly alter the activity of anti-4-1BB on T cells (Fig. 4A), but it prevented the deletion of B cells, almost fully restoring the numbers of splenic B cells as well as pre- and immature B cells in the bone marrow (Fig. 4B–C). Further in line with this, anti-4-1BB had no significant effect on B cells numbers in chimeras made with IFN-$\gamma^{-/-}$ bone marrow (Fig. 4D), indicating that 4-1BB-induced IFN-γ is required for the suppression of B cell development. To determine whether there was active signaling triggered by IFN-γ in developing B-lineage cells in the bone marrow, we performed ex-vivo staining for phosphorylated STAT-1, an indicator of IFN-γR signaling. We found phospho-STAT-1 (Y701) was limited to the $B220^{+}CD43^{-}$ population that includes pre- and immature B cells, and that anti-4-1BB treatment greatly augmented expression (Fig. 4E). It is unlikely that anti-4-1BB induced direct signaling events in B cells, since both bone marrow and peripheral B-lineage cells do not express 4-1BB (35) and anti-4-1BB treatment in purified bone marrow B cells in vitro could not induce apoptosis (data not shown). Overall, these results suggest that anti-4-1BB treatment inhibits B-lineage differentiation by inducing apoptosis in the bone marrow, albeit indirectly through IFN-γ secreted from T cells activated by 4-1BB signals.

Anti-4-1BB depletes NK and NKT cell populations

During the time of active hematopoiesis in bone marrow chimeras, we also observed a profound effect of anti-4-1BB on NK and NKT cells (Fig. 5A). Anti-4-1BB led to a complete reduction in the population of NK cells and Vα14 *i*NKT cells in the spleen as well as a slight reduction in the number of DC in both conventional (cDC) and plasmacytoid (pDC) lineages. We also found a similar reduction of NK cells in the BM, blood, LN, and liver, and of Vα14 *i*NKT cells in the thymus and liver (data not shown). These results are partially consistent with a previous report that anti-4-1BB injection in naïve mice reduced NK/NKT cell numbers in the spleen, albeit cells were not depleted in liver (29). In contrast, anti-4-1BB had no significant effect on repopulation of granulocytes and monocyte/macrophages. In contrast to the action on B cells, NK depletion by anti-4-1BB did not rely on T cells and IFN-γ, since it was observed in the absence of T cells (Fig. 5B) and IFN-γ (Fig. 5C–D). Since the expression of 4-1BB has been observed on NK and NKT cells previously (35,36), we further tested whether NK depletion was dependent upon 4-1BB expression. In a mixed chimera with wild type and 4-$1BB^{-/-}$ BM, we found that the level of NK depletion was significantly less in the population derived from 4-$1BB^{-/-}$ mice (Fig. 5E), suggesting that 4-1BB expression on NK cells or progenitors of NK cells is required for at least some of the action of anti-4-1BB on depletion of this subset of cells.

To gain further insights by which anti-4-1BB depletes NK cells, we first examined expression of NK cell surface markers (37,38) after anti-4-1BB treatment. More than 60% of splenic NK cells constitutively expressed 4-1BB in the BM chimeras (Fig. 6A) similar to expression in normal mice (not shown). Most NK markers were expressed equivalently in both 4-1BB+ and 4-1BB− populations except CD69 whose expression was enhanced in 4-1BB+ NK cells, which suggests that 4-1BB expression correlates with the activation status of NK cells similar to T cell subsets. To determine whether anti-4-1BB modulated surface marker expression we injected anti-4-1BB once during the last week during BM reconstitution, since repeated injection of antibody depleted most NK cells. This single injection of anti-4-1BB depleted NK cells by ~70% when compared to control IgG-injected mice (data not shown). Expression levels of most NK markers appeared normal after anti-4-1BB treatment except CD11b and CD69 whose expression were significantly reduced and augmented, respectively (Fig. 6A, bottom histograms). Of note, both Ly49D and Ly49C/I positive populations were diminished

after anti-4-1BB injection. Moreover, anti-4-1BB greatly augmented IFN-γ expression although granzyme-B was comparable to controls (Fig. 6B). Finally, we found that anti-4-1BB induced more apoptosis of NK cells determined by annexin-V staining (Fig. 6B). Overall, these results suggest that anti-4-1BB activates NK cells followed by the induction of cell death.

Detrimental activity of anti-4-1BB on B cell development during a virus infection

Lastly, we addressed whether 4-1BB hyper-costimulation is also determintal to B cell development under conditions of infection where hematopoiesis does occur but at a slower rate than in the irradiation chimeras. We used a virus model since anti-4-1BB agonistic antibody has been shown to augment T cell immunity against several viruses. Anti-4-1BB treatment starting 1 day after acute vaccinia virus (VACV) infection augmented the number of VACV-specific effector CD8 and CD4 T cells (Fig. 7A), as well as promoting increased numbers of total polyclonal memory/effector T cells, but it decreased the number of NK cells in the spleen (data not shown). However, anti-4-1BB-treatment strongly inhibited B cell development in the bone marrow in VACV-infected mice, again targeting $CD43^{-}IgM^{-}$ and $CD43^{-}IgM^{+}$ B cells but not $CD43^{+}IgM^{-}$ B cells (Fig. 7B). Decreased numbers of B cells were also found in the spleen (Fig. 7C), although the effect of anti-4-1BB in this case was more modest, presumably because of slower peripheral repopulation during virus infection compared to that in irradiated animals. Strikingly, anti-4-1BB additionally prohibited germinal center (GC) development as shown by analysis of GC B cells ($B220^{+}IgD^{-}PNA^{+}FAS^{+}$) in the spleen (Fig. 7D), and significantly reduced titers of VACV-specific IgG found in the serum (Fig. 7E). The latter action was unlikely due to B cell depletion, but correlates with prior published results that showed that anti-4-1BB can result in diminished follicular dendritic cell (FDC) networks in the B cell follicles, which are required for GC formation and T-dependent IgG responses (39). Thus, during viral infection, although targeting 4-1BB can strongly boost anti-viral T cell responses, detrimental effects are seen on B cell development and antibody formation.

Discussion

We show that sustained costimulation through repeated targeting of the receptor 4-1BB with an agonist antibody can alter the homeostasis of immune cells, in particular B, NK, and NKT cells. This data then replicates prior results where costimulatory ligands have been transgenically and constitutively over-expressed and also led to peripheral B cell depletion (8,11–14), and shows that hyper-costimulatory effects can be brought about during a therapeutic treatment-like regimen with a normal agonist reagent. Thus, although targeting costimulatory molecules might strongly promote T cell immunity, excessive costimulatory signaling can lead to strong adverse effects that need to be considered within the overall therapeutic goal.

IFN-γ was found to be a crucial mediator for hindering B cell development following anti-4-1BB treatment. IFN-γ was a major cytokine produced from hyper-costimulated T cells in the spleen. In addition, T cells recruited into the bone marrow were capable of producing IFN-γ, and most notably, some CD8 T cells, but not CD4 T cells, expressed IFN-γ in situ, suggesting that continued costimulation of bone marrow located CD8 T cells by anti-4-1BB induces the local secretion of IFN-γ within the bone marrow itself. This data may correspond with previous results that found that transgenic expression of IFN-γ in B-lineage cells resulted in a severe reduction in B cell numbers (40), and that unexpected secretion of IFN-γ from bone marrow stromal cells in TC-$PTP^{-/-}$ mice also hindered B cell development in the bone marrow (41). Although the latter results were gathered in artificial situations, together with our data, they underscore the conclusion that excessive production of IFN-γ within the bone marrow from physiological sources such as hyper-costimulated migrating T cells could have serious adverse effects on the output of B cells. It is likely that IFN-γ directly induces the apoptosis of

developing B-lineage progenitors. IFN-γ was shown to arrest proliferation and cause apoptosis of IL-7-dependent pre-B cell lines and WEHI 279 cells in vitro, through disrupting mitochondria membrane potential via an action on DIABLO that regulates cytochrome c release, and also by down-regulating the anti-apoptotic molecules Bcl-2 and Bcl-xL (42). Alternatively, IFN-γ might inhibit IL-7R-mediated survival through down-regulation of IL-7Rα (41,43). We found enhanced phosphorylation of STAT-1, but no difference in IL-7Rα expression (data not shown), within $CD43^{-}B220^{+}$ cells following anti-4-1BB treatment, suggesting that IFN-γR-mediated signaling most likely directly promoted apoptosis in our experiments independently of IL-7R.

Whether inhibition of B cell development in the bone marrow and periphery is an adverse effect of hyper-stimulation of all costimulatory receptors is not clear. Mice transgenic for B7-1 (11), B7-2 (12), LIGHT (8), CD70 (13), and 4-1BBL (14), all showed a similar peripheral B cell loss, and in some strains a loss of B lineage cells was also found in bone marrow although the latter analysis was not performed in all cases. Each of the receptors for these molecules has been described to costimulate CD8 T cells in varying situations, and therefore it might be argued that any molecule that could strongly expand the number of IFN-γ-producing CD8 T cells, and keep them actively producing this cytokine for a long period due to repetitive signaling, could then adversely affect the B cell compartment. Trafficking might also play a role, along with the extent of cosignaling over time. Recently, a complementary study to the one here showed that repetitive treatment with anti-4-1BB (once per week for three weeks) in naïve unimmunized mice induced the loss of $CD19^{+}$ B cells in the bone marrow and spleen, which was dependent on IFN-γ and type-I IFN (29). In this scenario, B cells may not have been deleted, but rather displayed altered trafficking since dramatic increases in B cell numbers were found in the lymph nodes after antibody treatment. In our studies, however, we found a similar decrease of B cells in lymph nodes as well as spleen, suggesting that under the antibody treatment regimen used within the context of strong hematopoiesis and viral infection, the loss of B cells was not due to altered trafficking and fully dependent on apoptosis within the bone marrow.

It is likely that inhibition of B cell development is dependent on repetitive or sustained signaling through 4-1BB (anti-4-1BB was given once per week for four weeks in most experiments) which then promotes activated CD8 T cells secreting IFN-γ to traffic and accumulate in the bone marrow. We observed milder effects on B cell development, although still statistically significant, when animals were treated with antibody for less time (once per week for two weeks, data not shown). A loss of B cells and/or antibody production was also found in several autoimmune disease models when animals were treated with anti-4-1BB repetitively (once per week for three to five weeks) (23–25,44). Our data suggest that this reflected impaired B cell development in the bone marrow, and at least in some cases, for example in models of lupus, this might explain why anti-4-1BB suppressed disease in these studies. Furthermore, we observed that anti-4-1BB almost completely abolished the GC reaction in the spleen during VACV infection, which was accompanied with a severe loss of anti-VACV IgG responses. This action is likely the result of a separate adverse reaction independent of effects on B cell development in the bone marrow, and corresponds with previous results showing that anti-4-1BB inhibited GC development in a lupus model (24), and during immunization with SRBC and KLH (39). In the latter study, GC formation was disrupted with a single treatment of anti-4-1BB in a T cell-dependent manner, through an apparent action on follicular dendritic cells (39), suggesting that certain side-effects may be more sensitive than others to the level or periodicity of 4-1BB signaling events. However, in most scenarios where anti-4-1BB might be used for immunotherapy, a combined loss of B cells and IgG responses would not be a desirable effect during clinical treatment.

Anti-4-1BB also disrupted the development of NK and Vα14 *i*NKT cells, resulting in few cells found in most organs including the spleen, lymph nodes, liver, thymus, and bone marrow. It is unlikely that this altered development was due to an effect on early hematopoietic stem cells and progenitors, since no significant changes were found in myeloid-lineage cells, such as dendritic cells, macrophage/monocytes, and granulocytes. A similar depletion of NK/NKT cells was shown in naïve mice repetitively treated with anti-4-1BB (once per week for five weeks), but interestingly it only occurred in the spleen and lymph node but not in the liver and lung (29), perhaps reflecting a difference between steady state homeostasis and actively regulated homeostasis that occurred in our models. The mechanism of depletion of NK and Vα14 *i*NKT cells was not dependent on T cells or IFN-γ, but partially relied on the expression of 4-1BB, which implies that anti-4-1BB may have depleted these cells directly. It is unlikely that the mechanism was antibody-dependent cell-mediated cytotoxicity (ADCC), through complement activation, since CD8 effector T cells expressing high levels of 4-1BB were expanded rather than deleted. Instead, anti-4-1BB enhanced expression of molecules related with NK cell activation, for example, CD69 and IFN-γ, and more importantly it induced apoptosis of NK cells. These results imply that anti-4-1BB depletes NK cells through the activation-induced cell death. Given that a half of 4-1BB-deficient NK cells in the mixed BM chimera were still depleted after anti-4-1BB treatment, there might also be a 4-1BB-independent mechanism for NK cell depletion. Of note, treatment of purified NK cells with anti-4-1BB in vitro failed to enhance apoptosis (not shown), which suggests that inflammatory mediators from non-NK cells, produced after 4-1BB signaling, might be required together with a direct action of anti-4-1BB to result in NK cell depletion. No significant defects in NK and NKT cell development have been reported in transgenic mice that over-express costimulatory ligands where a B cell loss was found, although it is possible that the development or homeostasis of these subsets was overlooked.

In summary, the current study shows a number of potential side-effects that might result with excessive costimulatory signaling. This raises further caution when considering the use of agonist antibodies to costimulatory receptors for immunotherapy, particularly in situations where rapid hematopoiesis might occur, for example, in immuno-compromised individuals or with bone marrow transplantation. In order to minimize these undesirable effects, the periodicity of treatment with agonist reagents will need to be considered, as well as the pharmacokinetics of the individual reagents, including their affinity and binding characteristics to their receptors. Short term and single dosing regimes may prove extremely effective for boosting T cell immunity, as shown by many studies with reagents to molecules like OX40 and 4-1BB in basic mouse models (2,15,45), as well as in models of cancer and responses against viruses. It is likely that adverse side-effects will be minimized with such protocols, especially when targeting these types of inducible costimulatory receptors that are highly regulated in expression. However, any long-term and repetitive treatment may have the potential to lead to an imbalance in immune homeostasis reported here, and needs to be considered seriously before any clinical effort is initiated.

Acknowledgments

We thank X. Tang, Y. Adams, and H. Choi for technical assistance. We also thank Y. Kinjo, G. Kim, Y. Huang and M. Kronenberg for the gift of TCRα, IFN-γ, CD8α-deficient mice and CD1d-tetramer, and for helpful discussion.

This work was supported by NIH grants AI42944 and AI067341 to M.C. and fellowship from the Diabetes and Immune Disease National Research Institute (DIDNRI) to S.-W.L. This is manuscript #1023 from the La Jolla Institute for Allergy and Immunology.

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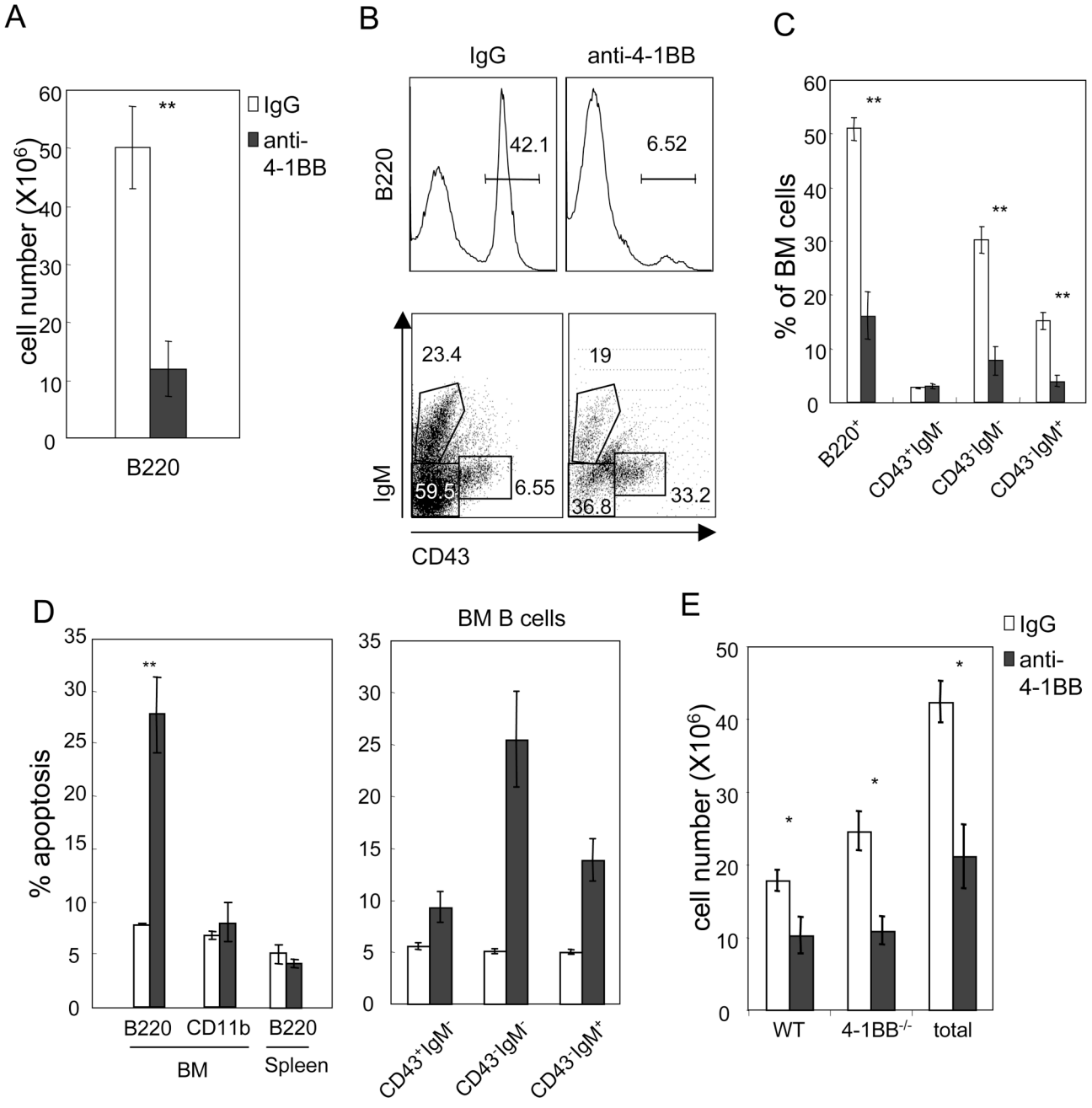

Figure 1. Hyper-costimulation with anti-4-1BB agonist antibody inhibits B cell development

(A–D) Wild type BM cells (CD45.1$^+$) were transferred to irradiated recipients (CD45.2$^+$) and then mice were treated with rat IgG or anti-4-1BB (3H3) once per week for 4 weeks, starting 2 weeks after BM reconstitution. Analyses were performed at 7 weeks. (A) The number of B220$^+$ cells in the spleen. (B) Percentage of B220$^+$ cells (top) and B cell subsets (bottom) in representative mice in the BM. (C) Mean percentage of B cells in the BM. (D) Percentage of apoptotic B cells (Annexin-V$^+$/7-AAD$^+$) in the BM and spleen. (E) A 1:1 mixture of wild type (CD45.1$^+$) and 4-1BB$^{-/-}$ (CD45.2$^+$) BM cells were transferred into irradiated Rag-1$^{-/-}$ mice (CD45.2$^+$). Antibody treatments were as above. The numbers of CD45.1$^+$ and CD45.2$^+$

$B220^+$ cells are shown from the spleen. Each bars represent mean value ± s.e.m. from three to four mice. * $P < 0.5$, ** $P < 0.1$.

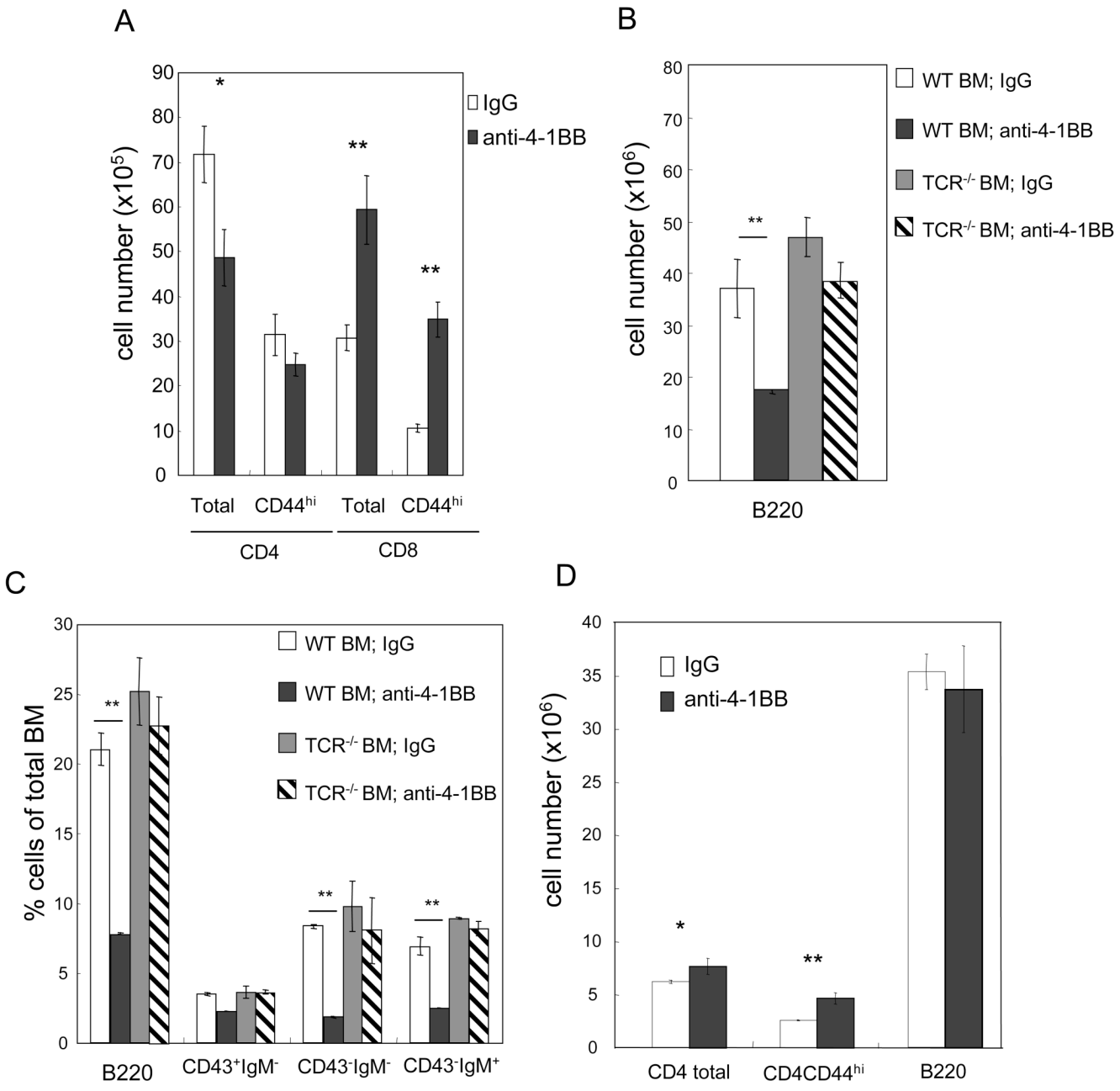

Figure 2. CD8 T cell activation is required for inhibition of B cell development promoted by anti-4-1BB treatment

Wild type (A), TCR($^{-/-}$ (B–C), or CD8($^{-/-}$ (D) BM cells were transferred into irradiated Rag1$^{-/-}$ mice, which were then treated with antibodies as in Fig. 1. (A) Numbers of total or activated (CD44^{hi}) CD4 and CD8 T cells in the spleen at 7 weeks. (B) Number of B220^{+} cells in the spleen. (C) Percentage of B cell subsets in BM. (D) Number of CD4 T cells and B cells in the spleen. Each bars represent mean value ± s.e.m. from four mice. Similar results were reproduced in a separate experiment. * $P < 0.5$, ** $P < 0.1$.

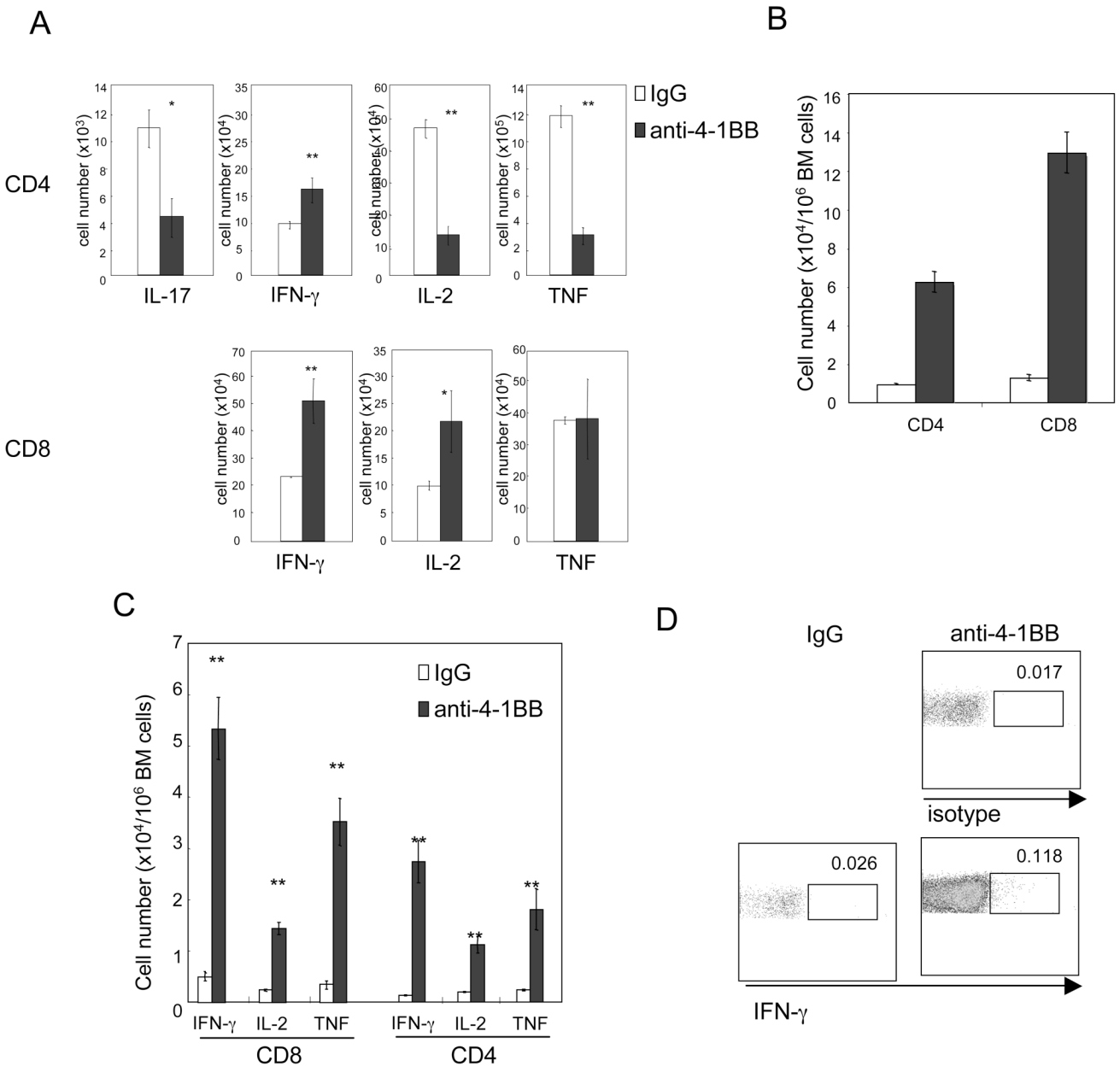

Figure 3. IFN-γ represents a major cytokine secreted from hyper-costimulated T cells

Wild type BM cells were transferred into irradiated Rag1$^{-/-}$ recipients and then mice were treated with antibodies as in Fig. 1. (A) Number of cytokine secreting T cells in spleen after in vitro PMA/ionomycin restimulation. (B–C) Numbers of total (B) and cytokine-secreting (C) CD4 and CD8 T cells in the BM after in vitro restimulation. (D) Ex-vivo secretion of IFN-(from BM CD8 T cells, without in vitro restimulaton. Bars represent mean value ± s.e.m. from three to four mice. Similar results were reproduced in separate experiments. * $P < 0.5$, ** $P < 0.1$.

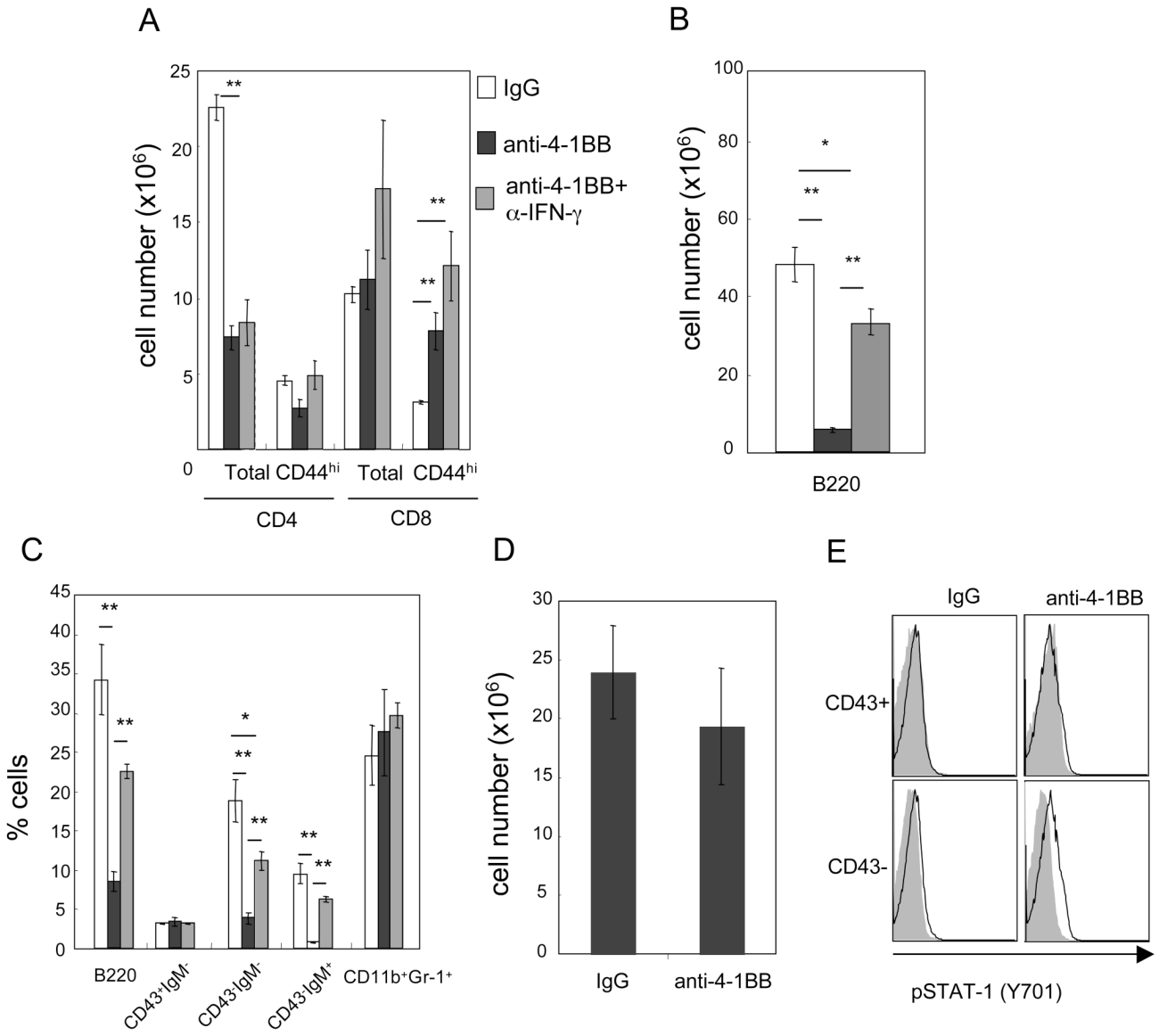

Figure 4. Blockade of IFN-(reverses inhibition of B cell development by anti-4-1BB

Wild type (A–C) or IFN-($^{-/-}$ (D) BM cells were transferred into irradiated Rag1$^{-/-}$ recipient and then mice were treated with antibodies as in Fig. 1. (A–C) Mice were injected with anti-IFN-((400 (g) every four days. (A) Number of total or activated (CD44^{hi}) CD4 and CD8 T cells in the spleen. (B) Number of B220^{+} cells in the spleen. (C) Percentage of B cell subsets in the BM. (D) Number of B220^{+} cells in the spleen. Bars represent mean value ± s.e.m. from four mice. (E) Enhancement of phospho-STAT-1 in developing B cells after anti-4-1BB treatment. BM cells were fixed, permeabilized, then stained with pSTAT-1 (open) or isotype (shaded) antibody together with anti-B220 and anti-CD43.

Data are representative of two or three experiments. * P < 0.5, ** P < 0.1.

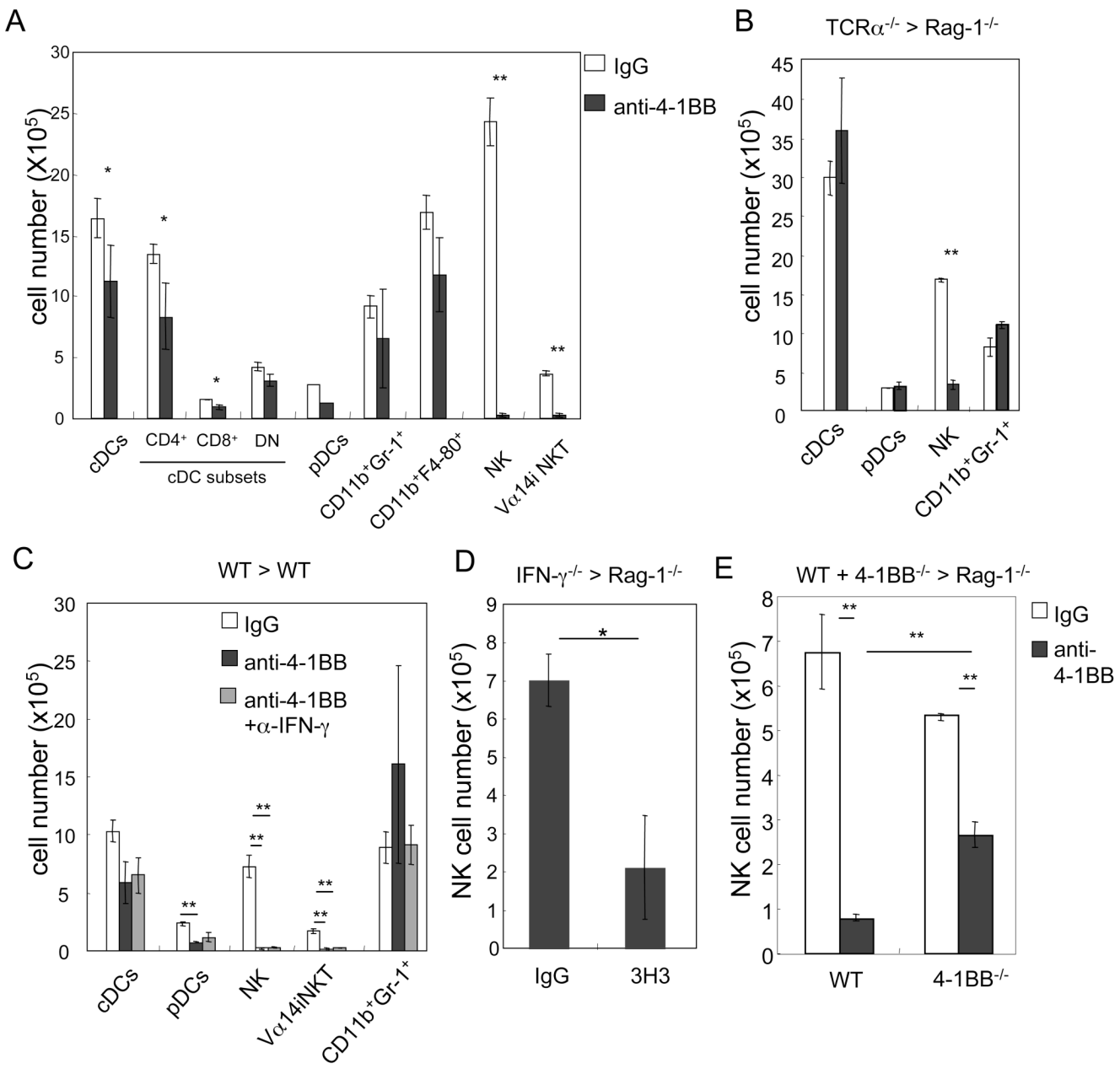

Figure 5. Inhibition of NK and NKT cell development following anti-4-1BB treatment

Wild type BM cells were transferred into irradiated wild type recipients (A and C). TCR($^{-/-}$ (B), IFN-($^{-/-}$ (D), or a 1:1 mixture of wild type and 4-1BB$^{-/-}$ (E) BM cells were transferred into irradiated Rag-1$^{-/-}$recipients. All mice were treated with control and anti-4-1BB antibodies as in previous Figs. (C) Mice were also injected with anti-IFN-((400 (g) every four days. The number of each cell type indicated is shown in the spleens. Each bars represent mean value ± s.e.m. from four mice. Data are representative of two or three experiments. * $P < 0.5$, ** $P < 0.1$.

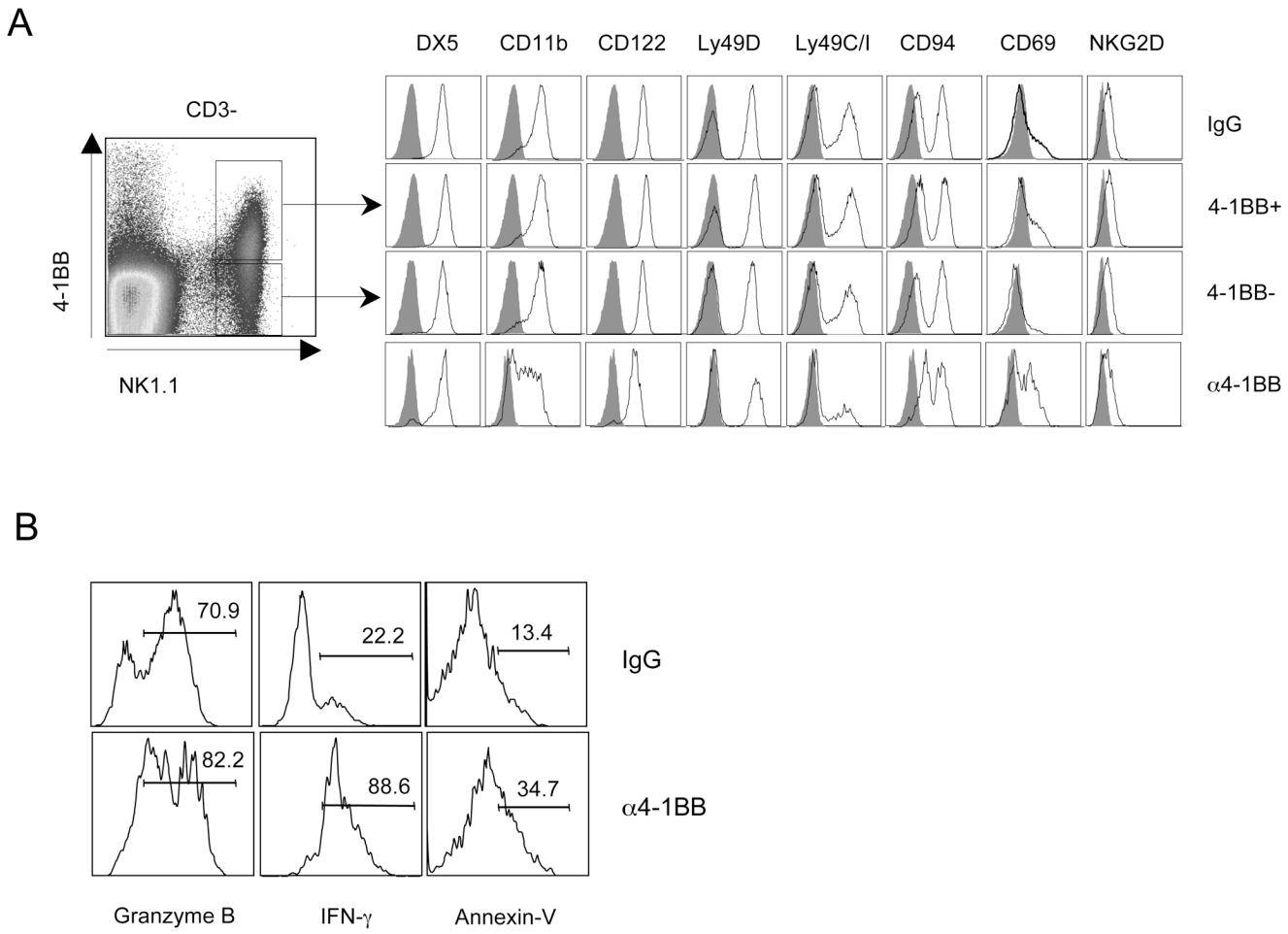

Figure 6. Activation-induced cell death in NK cells following anti-4-1BB treatment

Wild type BM cells were transferred into irradiated $Rag1^{-/-}$ recipients that were treated with control and anti-4-1BB antibodies once in the last week. (A) Expression of surface markers in splenic NK cells ($CD3^{-}NK1.1^{+}$, pooled from 2 mice) after control (top) and anti-4-1BB (bottom) antibody treatment (right histograms). NK cells isolated from mice treated with control antibody were divided into 4-1BB positive and negative populations (left). (B) Expression of granzyme-B and IFN-γ after stimulation of NK cells with PMA and ionomycin for 4 h. Apoptosis of NK cells was measured by annexin-V staining without stimulation (right). Data are representative of two experiments.

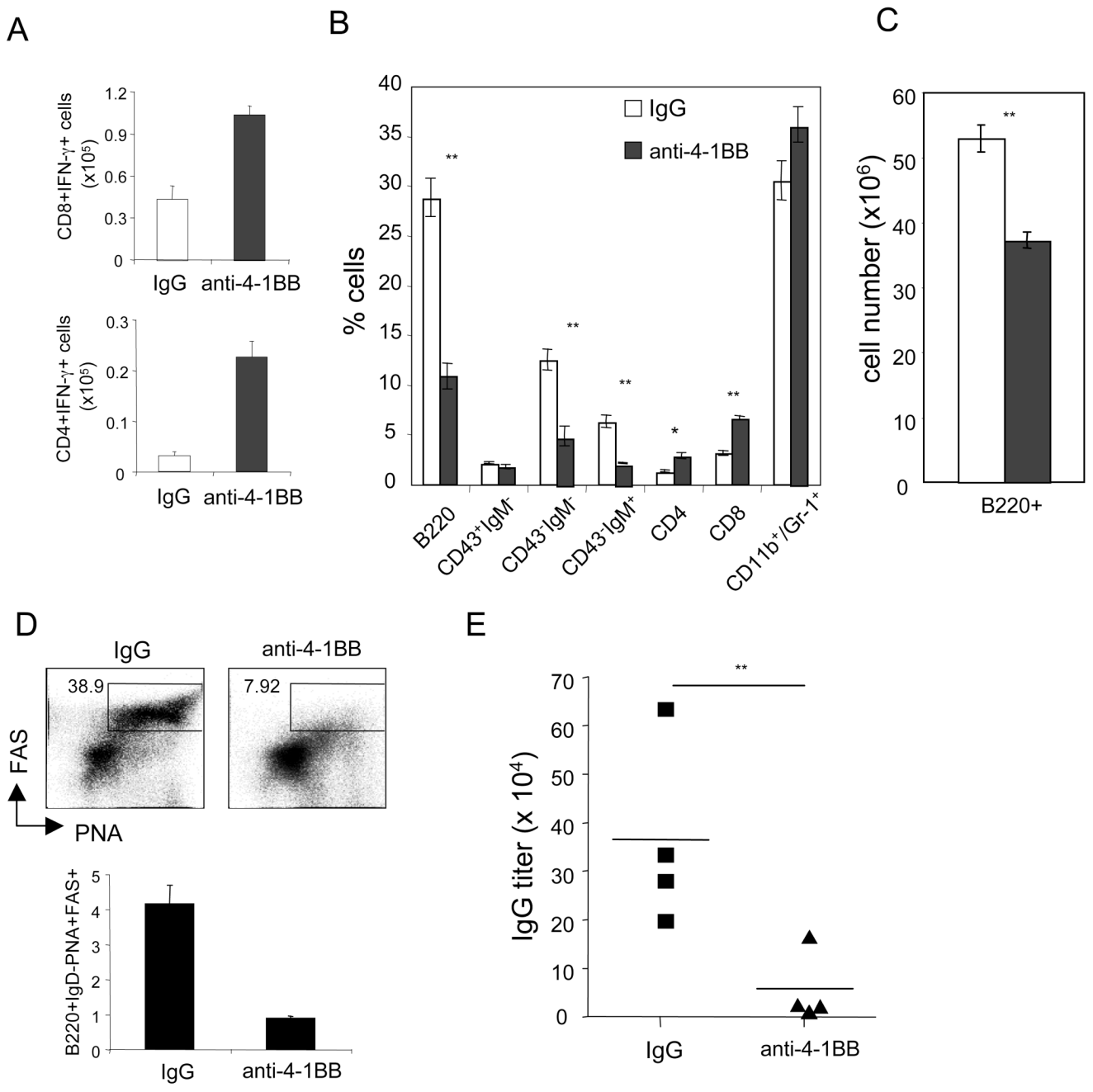

Figure 7. Inhibition of B cell development following hyper-costimulation with anti-4-1BB during VACV infection

Wild type mice were infected with VACV-WR i.p., then mice were treated with rat IgG or anti-4-1BB (100 (g, i.p.) one day after infection. Subsequently, antibodies were injected once per week until the end of the experiment. (A) The number of IFN-(-secreting CD8 (top) and CD4 (bottom) T cells in the spleen after in vitro restimulation with VACV peptides, B8R and B2R, respectively. (B) Percentage of indicated cell subsets in the BM after 2 weeks. (C) The number of $B220^+$ cells in the spleen at 5 weeks. (D) The percentage (top) and number (bottom) of GC B cells in the spleen at 2 weeks. (E) Titer of VACV-specific serum IgG at 2 weeks. Bars represent mean value ± s.e.m. from three to four mice. * $P < 0.5$, ** $P < 0.1$.