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Aging Converts Innate B1a Cells into Potent CD8+ T Cell Inducers

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B cell dysregulation in aging is thought to mostly occur in conventional B2 cells without affecting innate B1 cells. Elderly humans and mice also accumulate 4-1BBL+ MHC class-I^Hi CD86^Hi B cells of unknown origin. In this article, we report that these cells, termed 4BL cells, are activated murine and possibly human B1a cells. The activation is mediated by aging human monocytes and murine peritoneal macrophages. They induce expression and activation of 4-1BBL and IFN-γR1 on B1a cells to subsequently upregulate membrane TNF-α and CD86. As a result, activated B1a/4BL cells induce expression of granzyme B in CD8+ T cells by targeting TNFR2 via membrane TNF-α and providing costimulation with CD86. Thus, for the first time, to our knowledge, these results indicate that aging affects the function of B1a cells. Upon aging, these cells lose their tumor-supporting activity and become inducers of potentially antitumor and autoimmune CD8+ T cells. *The Journal of Immunology*, 2016, 196: 3385–3397.

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he immune system becomes dysregulated upon aging in mammals, and this dysregulation affects both myeloid and lymphoid immune cell compartments (see reviews in Refs. 1, 2). Aging-associated intrinsic and extrinsic factors skew hematopoiesis toward myelopoiesis (3, 4), resulting in reduced B cell–committed progenitors in bone marrow (BM) (5–7) and changes in the prevalence and function of myeloid cells and lymphocytes in circulation and in tissues. For example, the elderly accumulate monocytes and macrophages expressing pro-inflammatory cytokines such as TNF-α and IFN-γ (8, 9) and,

in mouse peritoneum, M1 macrophages expressing reactive oxygen (10). Aging also impairs phagocytosis of human peripheral blood (PB) CD14+ monocytes, murine neutrophils, and peritoneal cavity (PC) macrophages (8, 11, 12). Compounded with a lifelong antigenic exposure, changes in T cells, and increased half-life of mature B cells (13, 14), B cells subsequently become enriched for Ag-experienced memory and mature conventional B2 cells at the expense of naive B cells (15–18). In contrast, little is known whether aging affects innate B1a cells, the key producers of natural Abs to pathogens and endogenous Ags. Although the capacity to generate B1 cells is reduced in BM after birth (19), aging does not appear to affect their generation in BM of mice (20) and even increases their frequency in PC and spleen of old mice (11, 21). B1 cells differ from B2 cells by a distinct cellular origin, such as the generation from specific fetal progenitors and a limited self-renewing capacity, and by their preferential localization in the coelomic cavity, Peyer's patches, and tonsils (22–25). They also represent <5% of B cells in circulation and in the peripheral lymphoid tissues of young adult mice (22, 25). Murine B1 cells consist of CD5+(B1a) and CD5−(B1b) CD11b+ B cells (25), whereas their human counterparts remain poorly characterized (26–28) but have been shown to be a subset of memory CD27+ CD43+CD69−CD20+ B cells (27). Aging mice also accumulate CD23− CD21/CD35−B cells (so-called aging-associated B cells, ABCs) refractory to BCR and CD40 engagement (29). Recently, we reported that aging humans, macaques, and mice also accumulate another type of CD19+ B cells of unknown origin. These cells, designated 4BL cells, express high levels of HLA-I, the costimulatory molecule CD86, and a TNF superfamily type 2 transmembrane protein 4-1BBL/CD137L (30). Although macrophages and dendritic cells (DC) express 4-1BBL (31), it can be transiently expressed on B cells upon a combined stimulation of BCR and CD40 (32). This cell-surface molecule receives and transmits signals upon engagement with its receptor 4-1BB/CD137 expressed on activated NK and T cells and splenic DCs (31, 32), resulting in activation of cytolytic CD8+ T cells and NK cells (33–36). In synergy with B7 and ICAM, 4-1BBL can also induce proliferation of naive CD4+ T cells when Ag is limiting (37). We have reported that, by using the 4-1BBL/4-1BB and

MHC class-I/TCR signaling pathways, 4BL cells also induce the generation of Ag-specific perforin$^+$ GrB$^+$ CD8$^+$ T cells (30). In fact, this feature of 4BL cells explains the enrichment of highly differentiated GrB$^+$CD45RA$^+$CD8$^+$ T cells in aging humans (15) and accounts for a paradoxical retarded growth of poorly immunogenic tumors in the elderly (30).

In this article, we report that murine B1a cells, and possibly human B1 cells, become converted into 4BL cells upon aging. Surprisingly, the conversion is not due to intrinsic changes in aging B cells, but instead is induced by myeloid cells of aging subjects, such as human PB monocytes and murine peritoneal macrophages. First, they induce BCR and CD40 signaling that leads to expression of 4-1BBL, IFN-γR1, and membrane TNF-α (mTNF-α) in B1a cells. Then, upon engagement of 4-1BBL and IFN-γR1 with their respective stimuli (4-1BB and IFN-γ also provided by aging myeloid cells), B1a cells further upregulate expression of mTNF-α and CD86. The resulting 4BL cells then use mTNF-α in the induction of GrB expression in CD8$^+$ T cells by targeting TNFR2 while providing costimulation with CD86. Overall, our results reveal for the first time, to our knowledge, a unique functional consequence of the cross talk between dysregulated myeloid and lymphoid cell compartments upon aging. This process converts innate B1a cells from presumably being immunosuppressive cells into the inducers of cytolytic GrB$^+$CD8$^+$ T cells.

Materials and Methods

PBMCs were collected from PB of elderly ($n = 19$, age ± SD: 79 ± 6.45 y) and young ($n = 11$, age ± SD: 41 ± 7.2 y) healthy humans from the Baltimore Longitudinal Study of Aging (National Institute on Aging [NIA]) under the Human Subject Protocol 2003054 and Tissue Procurement Protocol 2003-076. Young (5–8 wk) female C57BL/6j and BALB/c, congenic J$_H$T mice with B cell deficiency (B6.129P2-Igh-Jtm1Cgn/J) and GFR$^+$ mice [C57BL/6-Tg(UBC-GFP)30Scha/J] were from The Jackson Laboratory (Bar Harbor, ME), and 4-1BBL and 4-1BB–knockout (KO) mice (4-1BBL KO and 4-1BB KO) were reported elsewhere (33, 38). Old mice (18–22 mo old) were from Aged Rodent Colony, NIA. ARE-Del mice (39) and TNF-α, TNFR1, TNFR2, and IFN-γR KO mice were from the National Cancer Institute (NCI)-Frederick, MD.

Flow cytometry

All Abs were from Biolegend (San Diego, CA) except otherwise specified. Human cell Fc receptors were blocked using Human TruStain FcX 5 min before staining with Abs, such as anti-human Ab: TNF-α–allophycocyanin (BD Pharmingen, Piscataway, NJ) or –FITC (clone MAb11), IFN-γ–FITC (BD Pharmingen) or –Pacific blue (clone 4S.B3), CD19-PerCP/Cy5.5 or –Alexa Fluor 780 (clone HIB19), 4-1BBL-PE or -allophycocyanin (5F4), CD86-PE (clone FUN-1; BD Pharmingen), CD27-PerCP/Cy5.5 (clone M-T271), CD119 (IFN-γR α-chain)-PE or -Biotin (clone GIR-208), streptavidin-Pacific blue or –allophycocyanin, IL-10–PerCP/Cy5.5 (JES3-9D7), CD14–Pacific blue (clone M5E2) or -FITC (clone HCD14), CD3-PE (clone SP34; BD Pharmingen), CD8-allophycocyanin or -PE (clone HIT8a), GrB-FITC (clone GB11), CD11b-FITC or –Alexa Fluor 700 (clone M1/70), CD138-PECy7 (clone DL101; eBioscience), CD43-allophycocyanin (clone eBio 84-3C1; eBioscience), CD69-allophycocyanin-Cy7 (clone FN50; BD Pharmingen), and CD120b (TNFR2)-PE (clone 3G7A02). For murine cells phenotype analysis, cells were preincubated with Tru Stain FcX before immunostaining with different combinations of the following anti-mouse Ab: TNF-α–FITC (clone MP6-XT22), 4-1BBL–PerCP–eFluor710 (eBioscience) or -PE (clone TKS-1), CD5-PerCP/Cy5.5 (eBioscience), -FITC or -allophycocyanin (clone 53-7.3), CD119 (IFN-γR α-chain)-Biotin (clone 2E2), streptavidin-Pacific blue or -allophycocyanin, CD19-allophycocyanin–eFluor 780 (clone 1D3; eBioscience) or -PerCP/Cy5.5 or -allophycocyanin (clone 6D5), CD86-PerCP/Cy5.5 (clone GL-1), CD23-FITC (clone B3B4; BD Pharmingen), CD21/CD35-allophycocyanin (clone 7G6), CD93-PeCy7 or -FITC (clone AA4.1), CD1d-PE or –Pacific blue (clone 1B1), CD120b (TNFR2)-PE (clone TR75-89), CD8-allophycocyanin (clone 53-6.7). For intracellular cytokine staining, freshly isolated human PBMCs or murine cells were activated with PMA (5 ng/ml; R&D, Minneapolis, MN) + Ionomycin (500 ng/ml; R&D) and brefeldin A (1/1000; eBioscience) for 5 h at 37˚C. The cells were stained using the Intracellular Fixation and Permeabilization Buffers Kit (eBioscience) following the manufacturer's protocol. Appropriate isotype controls were used throughout all experiments.

In vitro assays

B cells from human PB and murine spleens were negatively isolated using B-Cell Isolation Kit II (≥98% purity; Miltenyi Biotec, Auburn, CA) and the EasySep Mouse B Cell Isolation Kit (≥95% purity; STEMCELL Technologies, Vancouver, BC), respectively. 4-1BBL–expressing B cells (named as 4BL cells) were magnetically sorted using anti–4-1BBL–PE (human: clone 5F4; mouse: clone TKS-1; both from Biolegend) + anti-PE MicroBeads (Miltenyi Biotec). CD8$^+$ T cells were isolated negatively using the Human CD8$^+$ T Cell Isolation Kit or Mouse CD8a$^+$ T Cells Isolation Kit (≥97% purity; Miltenyi Biotec). The B cell–mediated activation of CD8$^+$ T cells was performed as previously reported (30). In brief, young subject CD8$^+$ T cells were labeled with eFluor 450 cell proliferation dye (10 min at 37˚C, 5 μM; eBioscience) and mixed with respective syngeneic (murine) or allogeneic (human) B cells from young and aging subjects at 1:1 ration in the presence of 1.5 μg/ml anti-CD3 Ab (UCHT1 and HIT3a clones, respectively, for human and murine cells; BD Pharmingen) for 4–5 d in complete RPMI at 37˚C in humidified atmosphere with 5% CO2. To test the function of TNF-α/TNFR2 axis in induction of GrB$^+$CD8$^+$ T cells, we used B cells and CD8$^+$ T cells from TNF-α and TNFR2-deficient congenic mice, respectively. Alternatively, wild type (WT) cells were incubated with 5 μg/ml TNF-α blocking Ab with murine (clone MP6-XT22, Rat IgG1κ; eBioscience) or human cells (clone MAb-1, Mouse IgG1κ; eBioscience), or human CD8$^+$ T cells were pretreated 10 min at room temperature with 10 μg/ml anti-human TNFR2 blocking Ab (clone hTNFR-M1, Rat IgG2b; BD Pharmingen) before mixing with B cells. To test the importance of cell contact, we performed a transwell assay in a 24-well tissue plate (3-μm pore size; Corning Life Science, Acton, MA). To test the role of CD86 function, murine 4BL cells were pretreated with 10 μg/ml anti-CD86 Ab (clone PO3.1; eBioscience) or isotype control (Rat IgG2b, κ; eBioscience) at room temperature before mixing at 1:1 ratio with eFluor 450–labeled CD8$^+$ T cells and 1.5 μg/ml soluble anti-mouse CD3.

Induction of TNFR2 expression on human CD8$^+$ T cells was evaluated after 24-h activation with beads coupled with anti-CD3/CD28 or anti-CD3/CD28/CD137 Abs at 1:30 bead/cell ratio (Invitrogen), or with soluble anti-CD3 (1.5 μg/ml, clone HIT3a; BD Pharmingen) and anti-CD28 (1.5 μg/ml, clone 37.51; BD Pharmingen) and 4-1BB agonist 3H3 Ab as described elsewhere (40). Alternatively, 4-1BBL$^+$ B cells were cultured overnight with eFluor 450–labeled CD8$^+$ T cells from young mice at a 1:1 ratio in the presence of 1.5 μg/ml soluble anti-CD3 Ab. 4-1BBL and IFN-γR1 upregulation in murine splenic B cells (2 × 10^6/ml) was induced with 10 μg/ml IgM [AffiniPure F(ab′)$_2$ Fragment Goat anti-mouse IgM; Jackson Laboratories] and 5 μg/ml anti-mouse CD40 agonistic Ab (FGK45; ENZO Lifesciences) by treatment for 18 h. CD86 was induced by treating B cells with 100 U/ml IFN-γ (R&D) for 18 h. To evaluate the effect of 4-1BBL ligation on TNF-α induction, we stimulated cells with LPS (1 μg/ml), plate-bound 4-1BB:Fc fusion protein (R&D), or control Ab (human IgG1; R&D) in 96 flat-bottomed plates for 24 h at 37˚C.

4BL cell induction by myeloid cells

PB monocytes (isolated using the Human Monocyte Isolation Kit II; Miltenyi Biotec) or murine PC myeloid (depleted of B, T, and NK cells treated with mixture of anti-mouse CD19-PE, CD3-PE, and DX5-PE Abs and anti-PE MicroBeads; Miltenyi Biotec) were mixed with eFluor 450–labeled (5 μM; eBioscience) B cells from respective young subjects at a 1:1 ratio. To block BCR signaling, we pretreated B cells with Bruton's tyrosine kinase (Btk; 20 nM, PC1-32765; Selleckchem, Boston, MA) and SYK (4 μM, R406; Selleckchem) inhibitors for 30 min at 37˚C before mixing with myeloid cells. CD40 signaling was blocked with anti-mouse CD40L blocking Ab (10 μg/ml, clone MR1; eBioscience).

Ag-specific induction of CD8$^+$ T cells by 4BL cells

Young (10-wk-old) and old (21-mo-old) C57BL/6 mice were either s.c. challenged with B16 melanoma cells as described elsewhere (41) or i.p. injected with 3 mg/ml albumin from chicken egg white (Sigma-Aldrich, St. Louis, MO) in 200 μl PBS, to test whether naive 4BL cells can process and present self-Ag. After 4 d, the mice were euthanized and splenic B cells were isolated with the EasySep Mouse B Cell Isolation Kit (≥95% purity; STEMCELL Technologies). Cells were further separated into CD5$^+$ and CD5$^-$ B cells using anti-mouse CD5-PE (clone 53-7.3) and anti-PE MicroBeads. Alternatively, peritoneal cavity B cells were depleted of CD23$^+$ cells using anti-mouse CD23-PE (clone B3B4; Biolegend) and anti-PE MicroBeads. CD19$^+$ B cells were then positively selected using

anti-mouse CD19-PE (clone 6D5) and anti-PE MicroBeads (>98% purity). B cells were enriched with 70–90% B1a cells according to CD11b and CD5 staining. To prepare target CD8$^+$ T cells, we s.c. immunized 8- to 11-wk-old female pmel and OT-1 mice with gp100$_{25–33}$– or OVA$_{257–264}$–specific TCR-transgenic CD8$^+$ T cells, respectively, with 10 μg human gp100$_{25–33}$ peptide or 5 μg OVA$_{257–264}$ peptide (*SIINFEKL*; ANASpec) in IFA. After 4–5 d, splenic CD8$^+$ T cells from immunized mice were isolated using the EasyStepMouse CD8$^+$ T Cell Enrichment Kit (STEMCELL Technologies) and labeled with eFluor450 cell proliferation dye (eBioscience). Then, to test for B cell–induced activation of CD8$^+$ T cells, we cultured the eFluor450$^+$ CD8$^+$ T cells from pmel and OT-1 mice with B cells from young or old WT mice challenged with B16 melanoma or OVA protein, respectively, at 1:1 ratio for 5 d in complete RPMI without any stimulation.

In vivo manipulations

Animals were housed in a pathogen-free environment at the National Institute on Aging Animal Facility (Baltimore, MD) under the Guide for the Care and Use of Laboratory Animals (National Institutes of Health [NIH] Publication No. 86-23, 1985). For adoptive transfer experiments, 5×10^6 eFluor 450–labeled B cells from spleens and PC of young WT or GFP-transgenic mice were injected (i.v. or i.p.) into congenic old and young mice to evaluate PC B cells after 5–6 d. PC macrophages were depleted in old mice by two i.p. injections of 150 μl clodronate liposomes (Chlophosome) 2 d before B cell transfer. The generation of Old-restored mice (i.e., induction of new B cell lymphopoiesis in old mice treated with anti-CD20 Ab) was described elsewhere (30). B1a cells and follicular B (FOB) cells were magnetically or FACS sorted from C57BL/6 mice and i.v. injected (2×10^6 cells) into J$_H$T mice 1 d after s.c. challenge with B16-F10 melanoma cells (10^5 in 100 μl PBS; American Type Culture Collection).

Statistical analysis

The results are presented as the mean ± SEM, and significance was assessed by Mann–Whitney and nonparametric test (Prism 6; GraphPad Software, San Diego, CA). A p value <0.05 was considered statistically significant.

Results

Aging milieu activates innate B1a cells

We previously observed that the reappearance of 4BL cells after transient B cell depletion in old mice (Old-restored) is delayed for 1 mo compared with the rest of the B cells (M. Bodogai and A. Biragyn, personal communication, and Ref. 30), and that cancer patients also accumulate 4BL cells upon autologous hematopoietic stem cell transfer (30). These data suggest that the generation of 4BL cells could be induced by extrinsic factors. To test this possibility, we injected splenic B cells of young GFP-expressing mice into the PC of 18-mo-old and 8- to 10-wk-old congenic mice (old and young, respectively, $n = 6$/group; Supplemental Fig. 1Ai). After 6 d, mice were euthanized to evaluate PC B cells. The injected GFP$^+$ B cells markedly induced expression of 4-1BBL in old, but not young, mice ($p < 0.01$; Fig. 1Ai), indicating that aging milieu induces 4BL cells. The 4-1BBL$^+$GFP$^+$ B cells also upregulated TNF-α (both intracellular and membrane forms; Fig. 1Aii, 1iii) and surprisingly expressed CD5 ($p < 0.05$; Fig. 1Aiv). Because CD5 defines murine B1a cells (23–25), they could be the source of 4BL cells upon aging. To test this possibility, we repeated the experiment by i.p. injecting a separate group of young and old mice with GFP$^+$ B cells isolated from PC (instead of spleen) of young mice. Compared with splenic B cells, PC GFP$^+$ B cells induced markedly higher levels of 4-1BBL and mTNF-α in aging mice (Supplemental Fig. 1Aii–iv). We also detected 4-1BBL$^+$TNF-α$^+$CD5$^+$GFP$^+$ B cells in PC and spleens of old mice injected via tail vein (Supplemental Fig. 1B). Importantly, host PC B cells (GFP$^-$) were markedly enriched for 4-1BBL$^+$TNF-α$^+$ CD5^{High} B cells in old mice ($p < 0.005$ as compared with young mice; Fig. 1B). To further implicate B1a cells in the conversion, we analyzed PC B cell subsets and found that CD5$^+$ pro-B10 cells [which generate regulatory B10 cells (42)] and CD5$^-$ B cells were

only marginally positive (<2%) for 4-1BBL and TNF-α regardless of the age of mice (Fig. 1C, Supplemental Fig. 1C). We also tested whether the increase of 4-1BBL$^+$TNF-α$^+$ B cells is due to proliferation of pre-existing 4BL cells. Upon adoptive transfer of eFluor 450–labeled B cells into PC of old and young mice, we did not detect dilution of eFluor 450 even 6 d after injection (Supplemental Fig. 1D). Thus, the aging milieu mostly activates B1a cells without expanding their pre-existing 4BL cell subsets.

Next, to further link the activated B1a cells with 4BL cells, we tested whether they can induce the generation of cytolytic CD8$^+$ T cells expressing GrB [the key function of 4BL cells (30)]. Sort-purified B cells (B-total), pro-B10, and B1a cells from PC of old and young mice were in vitro–cocultured with young mouse eFluor 450–labeled CD8$^+$ T cells stimulated with anti-CD3 Ab for 5 d. Although anti-CD3 Ab stimulation induced proliferation of CD8$^+$ T cells, that is, the cells comparably diluted eFluor 450 regardless of the B cells or age of the mice (Supplemental Fig. 1E), old mouse B cells per se induced greater GrB expression in CD8$^+$ T cells compared with young mouse B cells (B-total: 22.8 ± 0.42 versus 10.5 ± 0.42% and pro-B10: 14 ± 0.27 versus 6.2 ± 0.29%, respectively, old versus young, $p \leq 0.05$; Fig. 1D). However, compared with young or old mouse B-total cells or even pro-B10 cells, B1a cells of old mice induced significantly higher levels of GrB$^+$CD8$^+$ T cells (45 ± 4.4 versus 12.8 ± 0.23%, respectively, old versus young, $p < 0.05$; Fig. 1D). Hence B1a cells can indeed become 4BL cells in aging mice. To test whether there is a similar conversion in humans, we compared PB of young (<50 y of age, $n = 11$) and old (>75 y old, $n = 19$) healthy donors. The frequency of 4-1BBL$^+$TNF-α$^+$ memory (CD27$^+$CD43$^-$) and B1 cells (CD27$^+$CD43$^+$CD69$^-$CD20$^+$) (27) was increased in the elderly (Supplemental Fig. 1F). (Note: The definition of human B1a cells remains unclear, and thus we evaluated only B1 cells usually found within memory B cells in PB.) As in mice, the 4BL cells of elderly people expressed higher levels of mTNF-α than 4-1BBL$^-$ B cells ($p < 0.01$) and 4BL cells of young donors ($p < 0.05$; Fig. 1E). In the MLR assay with allogeneic CD8$^+$ T cells of young people, 4BL cells within memory CD27$^+$ B cells of the elderly induced higher levels of GrB expression in CD8$^+$ T cells compared with unsorted B cells (B-total) or 4BL cells of young donors ($p \leq$ 0.05; Fig. 1F, Supplemental Fig. 1G).

Aging converts splenic B1a cells into 4BL cells and disables their tumor-supporting activity

Although B1a cells represent <5% of B cells in spleens of young adult mice (22, 25), their conversion into 4BL cells probably explains the induction of GrB$^+$CD8$^+$ T cells that we previously linked with the splenic B cells of aging mice (30). To test this possibility, we analyzed expression of 4-1BBL in various subsets of splenic B cells. 4-1BBL was increased in aging marginal zone B, FOB cells, and ABCs, but the increase only reached the level of sB1a cells of young mice (Fig. 2A). In contrast, aging markedly upregulated 4-1BBL in sB1a cells (17.23 ± 0.67 versus 5.39 ± 1.62%, respectively, old versus young, $p < 0.001$; Fig. 2A), suggesting that they become 4BL cells. To further confirm this possibility, we tested the function of sB1a cells and FOB cells of young and old mice in a 5-d GrB$^+$CD8$^+$ T cell induction assay (as used earlier). Note that ABCs were not tested here because they do not function as 4BL cells (30). Aging mouse sB1a cells strongly induced expression of GrB in CD8$^+$ T cells, whereas young mouse sB1a cells or FOB cells of young or old mice failed to do so (Fig. 2B). To confirm this finding, we also tested sB1a cells from Old-restored mice, the old mice whose 4BL cells were reduced despite the restoration of the B cell compartment after depletion of B cells with anti-CD20 Ab (30). Compared with control old mice
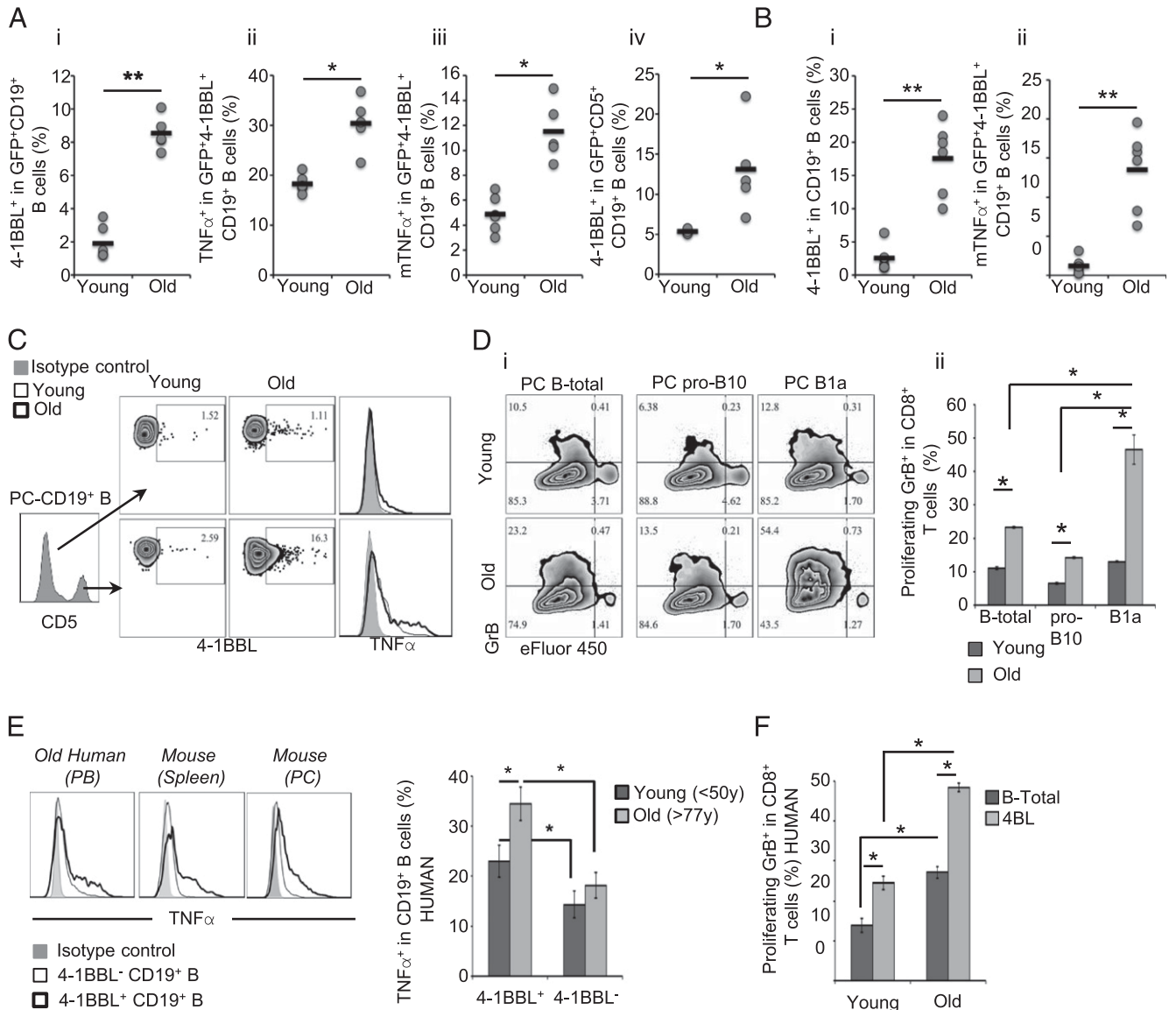
FIGURE 1. The aging milieu activates B1a cells. (**A** and **B**) Young and old C57BL/6 WT mice were i.p. injected with 5×10^6 splenic B cells from congenic GFP$^+$ young mice. Frequency (dots) and mean value (lines) of 4-1BBL$^+$ in GFP$^+$ B cells (**Ai** and **Aiv**) and host GFP$^-$ B cells (**Bi**) coexpressing TNF-α (**Aii**), mTNF-α (**Aiii** and **Bii**), and CD5 (**Aiv**) in PC of each mouse after 6 d, as indicated in the y-axis. Results are from three to four mice/group, and experiments were reproduced three times. (**C**) PC CD5$^+$ B cells of old mice mostly express 4-1BBL and TNF-α. Arrows indicate the B cell population tested (*center panel*). (**D**) Aging PC B1a cells mostly induce GrB$^+$CD8$^+$ T cells. Unsorted B cells (B-total) and FACS-sorted pro-B10 (pro-B10) and B1a B (B1a) cells from PC of young and old mice were cultured for 4–5 d with eFluor 450–labeled CD8$^+$ T cells stimulated with 1.5 μg/ml anti-mouse CD3 Ab. A representative dot plot (**Di**) of a summary graph [mean ± SEM, (**Dii**)] of two independent experiments performed in triplicate. (**E**) Elderly human 4-1BBL$^+$ B cells express high levels of TNF-α. Frequency mean ± SEM of TNF-α^+ in 4-1BBL$^-$ and 4-1BBL$^+$ B cells in young and elderly people PB after PMA/ Ionomycin/brefeldin A activation. (**F**) 4-1BBL$^+$ B cells (4BL cells) from elderly people induce higher levels of GrB$^+$CD8$^+$ T cells. Frequency mean ± SEM of a representative result reproduced with five old and five young human cells. $*p < 0.05$, $**p < 0.01$.

treated with an irrelevant IgG (Old-IgG), B cells of Old-restored mice contained fewer 4-1BBL$^+$sB1a cells (Fig. 2A) and could not efficiently induce GrB$^+$CD8$^+$ T cells ($p < 0.05$; Fig. 2B).

A consequence of 4BL cell enrichment in mice is that it retards growth of poorly immunogenic B16 melanoma cells (30). Thus, to test the in vivo function of sB1a cells, we adoptively transferred B cell–deficient J$_H$T mice (J$_H$T mice) with sB1a or FOB cells sort-purified from congenic naive young or old mice. Then the mice were s.c. challenged with a lethal dose of B16 melanoma cells (Fig. 2C). Transfer of FOB cells and, in particular, sB1a cells of young mice significantly increased growth of the B16 melanoma ($p < 0.05$; Fig. 2C, 2D), confirming their tumor-supporting functions (43, 44). In contrast, the transfer of sB1a cells of old

mice failed to do so and instead retarded tumor growth ($p < 0.05$ compared with mock treatment; Fig. 2C, 2D). This response was lost if sB1a cells were from Old-restored mice, as the tumor grew as fast as after the transfer of young sB1a cells (Fig. 2C, 2D). The J$_H$T mice replenished only with aging sB1a cells contained high levels of GrB$^+$CD8$^+$ T cells in the circulation (Fig. 2E) and in the secondary lymphoid organs (Supplemental Fig. 1H), whereas transfer of sB1a cells of Old-restored or young mice failed to increase those cells (Fig. 2E, Supplemental Fig. 1H). This result corroborates our previous finding that 4BL cells retard B16 melanoma growth by inducing antitumor GrB$^+$CD8$^+$ T cells (30), suggesting that aging B1a cells became potent inducers of Ag-specific GrB$^+$CD8$^+$ T cells. We tested this possibility using TCR-
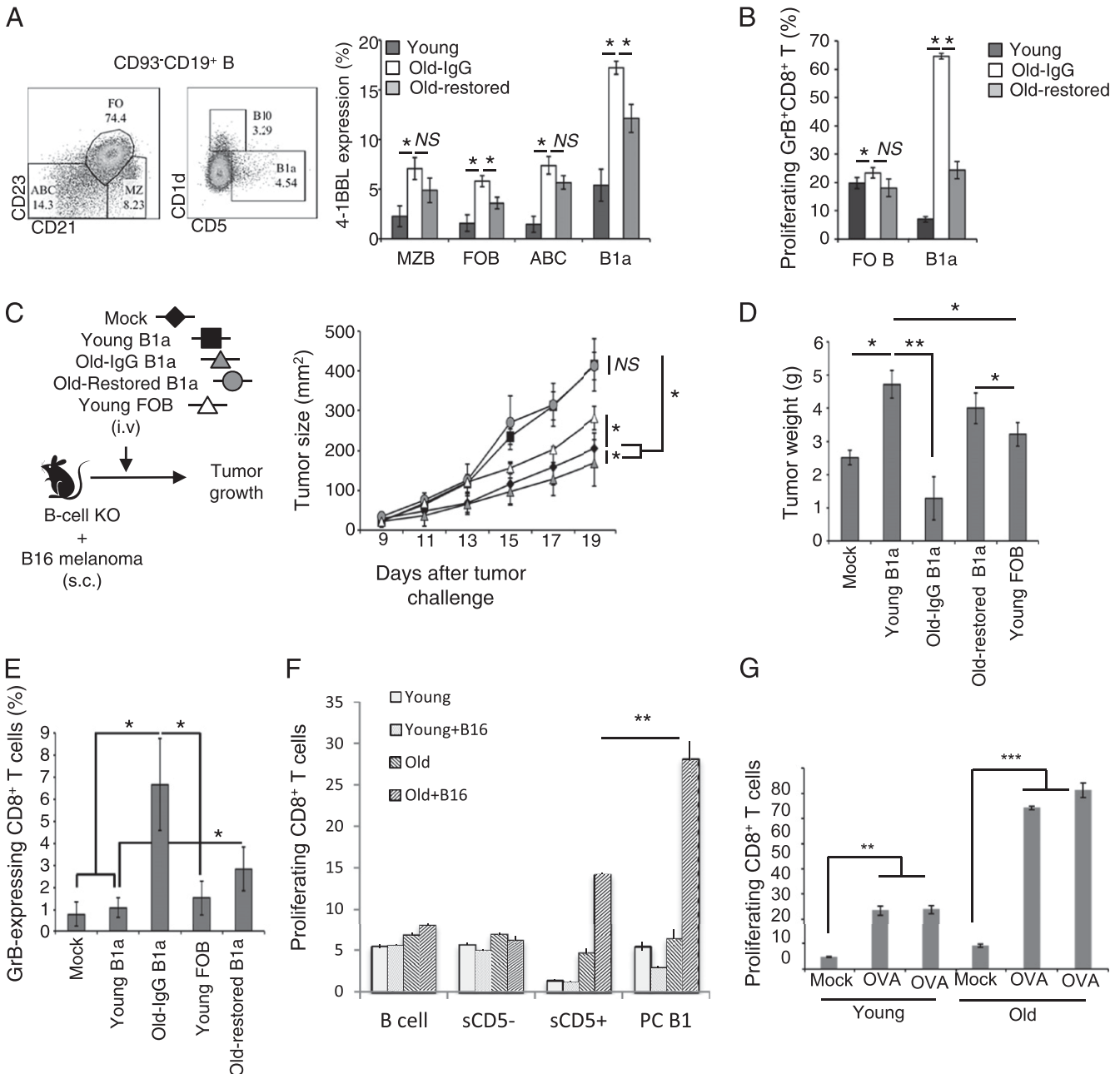
FIGURE 2. Aging splenic B1a cells express 4-1BBL (**A**), induce GrB⁺CD8⁺ T cells (**B**), and reduce tumor growth (**C**). The 4-1BBL⁺ subset (*left panel*) within splenic mature B-cell subsets ($CD93^-CD19^+$) and their ability to induce GrB^+CD8^+ T cells (B) as in Fig. 1. Y-axis, frequency mean ± SEM of three independent experiments performed in triplicate with B cells sort-purified from young (dark bars), old (Old-IgG, open bars), and old mice with a restored B cell compartment (Old-restored, light gray bars). Sort-purified FOB cells and sB1a cells of young, Old-restored mice (Old-restored), or old mice pretreated with control IgG (Old-IgG) were transferred into B cell–deficient J_HT mice with B16 melanoma to test their ability to affect growth (C) and weight (**D**) of tumor and generate GrB^+CD8^+ T cells at day 20 after tumor challenge (**E**). Experiment was reproduced twice with four to five mice per group. (**F** and **G**) Aging B1a cells induce Ag-specific GrB^+CD8^+ T cells by presenting endogenous Ags. Sort-purified $CD5^-$ and $CD5^+$ splenic B cells or CD23-depleted PC B cells (B1) from old and young C57BL/6 mice either with B16 melanoma or i.p. injected OVA protein were cultured for 5 d with TCR transgenic $CD8^+$ T cells from young pmel (F) or OT-1 mice (G), respectively. (F and G) No exogenous Ag or anti-CD3 Ab stimulation was used during B cell and T cell coculture; the TCR transgenic T cells were preactivated in pmel and OT-1 mice for 5 d by s.c. injection of 5 μg of a respective peptide in IFA. Mean (%) ± SEM of three mice per group experiment (F) and individual mice (G).*$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

transgenic $CD8^+$ T cells from pmel and OT-1 mice, which recognize $gp100_{25–33}$ peptide [of a pigment synthesis Ag expressed by B16 melanoma cells (45)] and $OVA_{257–264}$ peptide, respectively. First, we in vivo–primed B cells with an Ag by challenging young and old WT C57BL/6 mice with B16 melanoma or i.p. injecting 200 μl OVA (intact protein, 3 mg/ml). Then, after 4–5 d, B cells were isolated and cocultured with respective TCR-transgenic $CD8^+$ T cells for 5 d without any cognate Ag or anti-CD3 Ab

stimulation. Control B cells from naive or mock-treated mice did not activate $CD8^+$ T cells, verifying that their activation requires a cognate Ag presentation (Fig. 2F, 2G). In contrast, splenic $CD5^+$ B1 cells and, in particular, PC B1 cells of old mice with B16 melanoma, but not splenic $CD5^-$ B cells of old mice or B cells and B1a cells of young mice, markedly induced proliferation of pmel $CD8^+$ T cells (Fig. 2F). Similarly, B1 cells of OVA-injected WT mice also activated OT-1 $CD8^+$ T cells, which was more
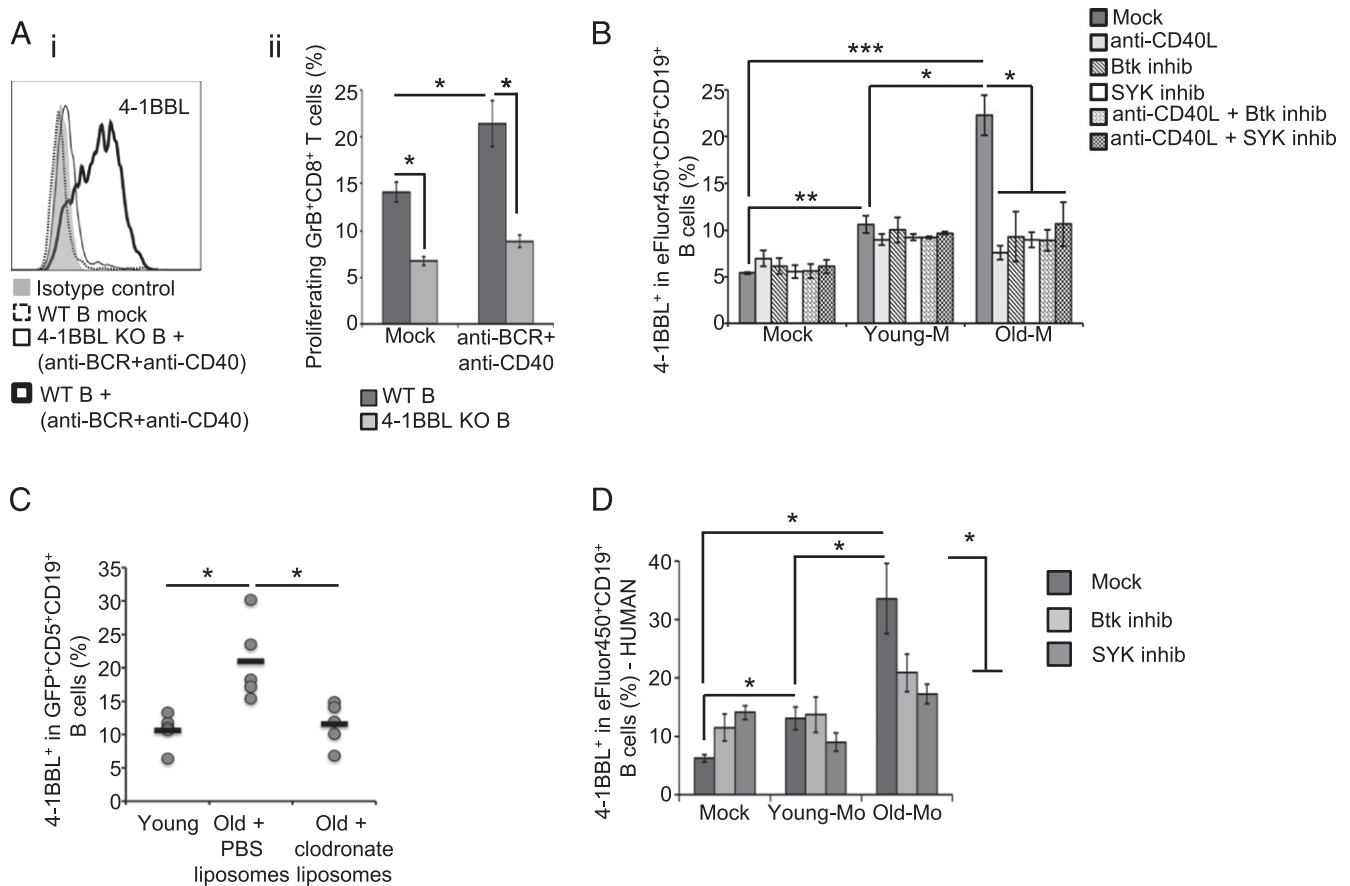
FIGURE 3. PC myeloid cells induce the generation of 4BL cells. Resting B cells of young mice upregulate 4-1BBL upon overnight stimulation with anti-mouse IgM (Fab, 10 μg/ml) and CD40 agonistic Ab (**Ai**, 5 μg/ml) or with myeloid cells from PC of old, but not young, WT mice (**B**). Upregulation was associated with the ability to induce GrB$^+$CD8$^+$ T cells if B cells were from WT but not 4-1BBL KO mice (**Aii**). Myeloid cells cannot induce 4-1BBL$^+$ B cells if B cells were pretreated with Btk (20 nM) or SYK (4 μM) inhibitors, or stimulated in the presence of CD40 blocking Ab (B). (**C**) Clodronate liposome–induced depletion of phagocytic cells in PC of old mice abrogates the induction of 4-BBL expression in i.p. injected congenic young mouse GFP$^+$ B cells. Individual mouse results (dots) and mean value (lines) of five to six representative mice per group. This experiment was reproduced twice. (**D**) Unlike young (Young-Mo), elderly human PB monocytes (Old-Mo) also convert young human B cells into 4BL cells. The induction was lost if B cells were pretreated with Btk or SYK inhibitors. Mean ± SEM of 4-1BBL$^+$ in eFluor 450-labeled B cells treated with myeloid cells from three young and five elderly people. *$p < 0.05$, **$p < 0.01$, ***$p < 0.005$.

pronounced for aging B1 cells (Fig. 2G). Thus, aging B1a cells can induce Ag-specific CD8$^+$ T cell responses.

Myeloid cells induce the generation of 4BL cells

To understand the mechanism of the 4BL cell induction, we treated B cells of young mice with PC lavage of old mice. It did not induce 4BL cells (not depicted). Thus, given that BCR/CD40 stimulation upregulates (albeit transiently) expression of 4-1BBL on B cells (32), the induction is probably mediated by PC cells. To test this possibility, we first confirmed the upregulation of 4-BBL on young mouse B cells upon stimulation with anti-IgM/CD40 Ab (Fig. 3Ai). The stimulated B cells also induced higher levels of GrB$^+$CD8$^+$ T cells in our in vitro induction assay ($p < 0.05$; Fig. 3Aii). However, this induction was lost if B cells were from 4-1BBL–deficient (KO) mice (Fig. 3Aii), underscoring the importance of 4-1BBL (30). Because the BCR/CD40 signaling can be triggered by macrophages (46, 47) dysregulated upon aging (8), we also evaluated CD11b$^+$ myeloid cells and macrophages in PC of old and young mice. These cells expressed higher levels of CD40L (needed for CD40 signaling) and IFN-γ in PC of aging mice compared with young mice (Supplemental Fig. 2A–F). To test their function, we in vitro–cocultured CD11b$^+$ cells purified from PC of young or old mice (respectively, Young-M and Old-M, >70% pure after depletion of T, NK, and B cells; Supplemental

Fig. 3A) with eFluor 450–labeled PC CD5$^+$ B cells of young mice. Upon overnight coculture, the Old-M, but not Young-M, cells strongly upregulated expression of 4-1BBL in B1a cells ($p < 0.005$; Fig. 3B). Because the CD5$^+$ B cells failed to dilute eFluor450 even after 5–6 d of coculture (Supplemental Fig. 3B), Old-M cells induced conversion of 4BL cells without expansion of pre-existing 4-1BBL$^+$B1a cells. Next, to link the fact that induction requires BCR/CD40 signaling, we repeated the experiment by coculturing Old-M cells with B1a cells in the presence of anti-CD40L blocking Ab or with B1a cells pretreated with spleen tyrosine kinase (Syk) or Btk inhibitors, which inactivate BCR signaling (48). Both treatments completely disabled the induction of 4-1BBL$^+$B1a cells ($p < 0.05$; Fig. 3B), indicating the importance of the BCR/CD40 signaling axis. To confirm the importance of aging myeloid cells in vivo, we modified the experiment shown in Fig. 1A by transferring young mouse GFP$^+$ B cells into congenic old mice treated with clodronate liposomes to deplete macrophages. Although control macrophage-sufficient old mice significantly increased the frequency of 4-1BBL–expressing CD5$^+$GFP$^+$ B cells ($p = 0.02$; Fig. 3C, see also Fig. 1A), the macrophage-depleted old mice failed to do so just as observed in young mice (Fig. 3C). Thus, the conversion of B1a cells into 4BL cells is, at least in part, mediated by macrophages of aging mice. To test whether a similar induction occurs in human 4BL cells, we cocultured overnight PB
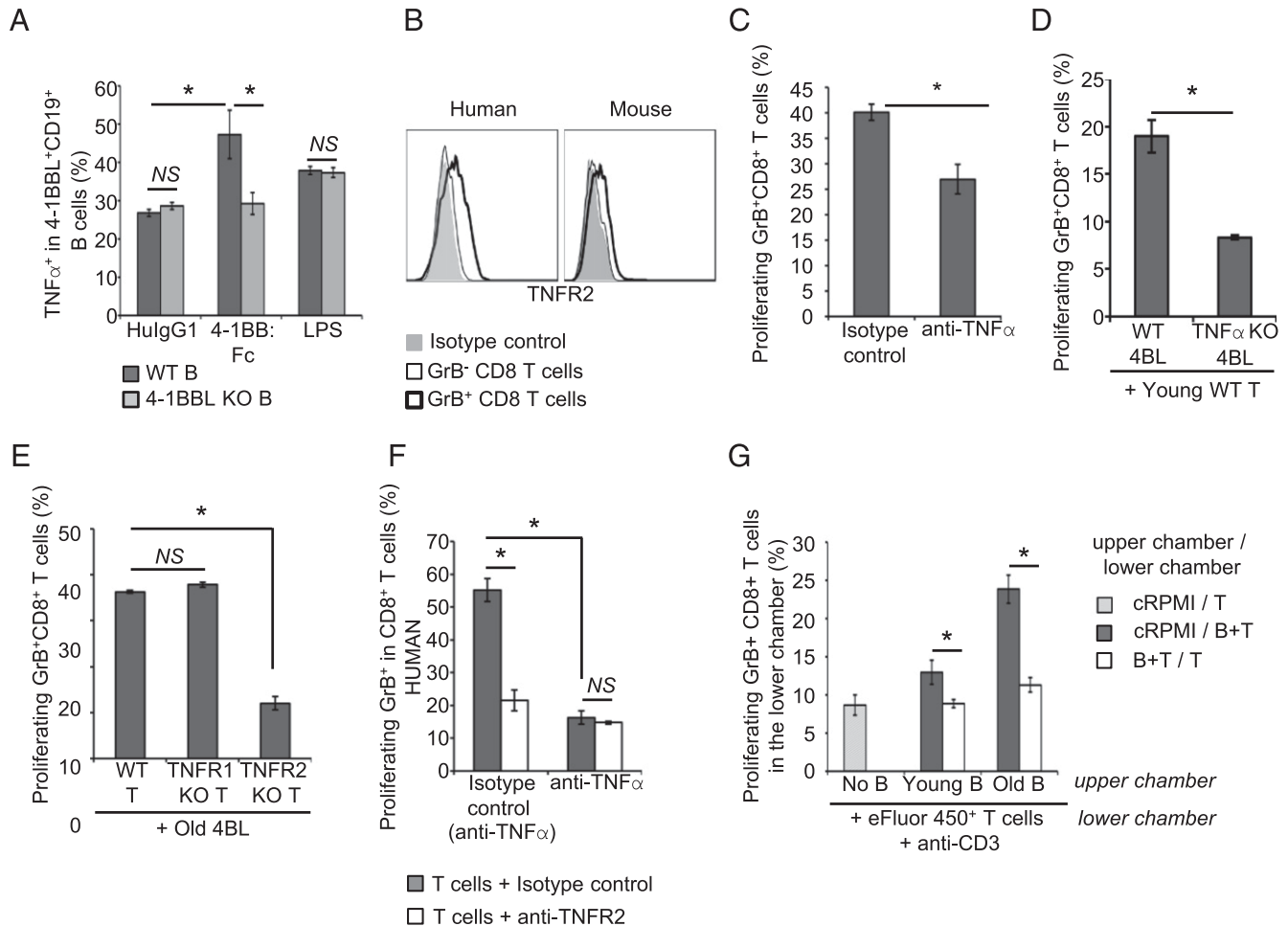
FIGURE 4. 4BL cells induce GrB$^+$CD8$^+$ T cells using TNF-α. (A) The 4-1BBL expression in B cells induced with anti-mouse IgM/anti-mouse CD40 Ab is further upregulated upon overnight stimulation with plate-bound 4-1BB:Fc protein, but not control human IgG1 (HuIgG1, 10 μg/ml). Upregulation is lost in 4-BBL KO B cells. Control cells were treated with LPS (1 μg/ml) as a nonspecific stimulator. (B) Representative histogram shows that TNFR2 is mostly expressed on GrB$^+$ CD8$^+$ T cells. Thick and thin lines are for GrB$^+$ and GrB$^-$CD8$^+$ T cells, respectively. Murine (C–E and G) and human (F) 4BL cells lose the ability to induce GrB$^+$ CD8$^+$ T cells in the presence of TNF-α neutralizing Ab (C and F, 5 μg/ml), anti-TNFR2 Ab (F, 10 μg/ml), or if B cells are TNF-α KO (D) or T cells are from TNFR2 KO, but not TNFR1 KO, mice (E). No GrB$^+$CD8$^+$ T cells were induced if B cells and T cells were physically separated in a transwell assay (G). Frequency mean $\pm$ SEM of GrB$^+$CD8$^+$ T cells of a representative experiment repeated at least three times with two to four mice and human cells per group. *$p < 0.05$.

B cells of young humans with PB monocytes of young or elderly humans. As in mice, only elderly human monocytes strongly induced expression of 4-1BBL in B cells ($p < 0.05$; Fig. 3D), and this induction was lost if human B cells were pretreated with Btk and Syk inhibitors ($p < 0.05$, Btk or Syk versus Mock; Fig. 3D).

4BL cells upregulate mTNF-α via signaling with 4-1BBL

Because the conversion of 4BL cells leads to upregulation of mTNF-α (Fig. 1A, 1B), we next wanted to understand the mechanism of this induction. Macrophages require 4-1BBL signaling to sustain expression of TNF-α (49), suggesting that a similar mechanism could also be used in 4BL cells. To test this possibility, we stimulated B cells from WT and 4-1BBL KO mice with anti-BCR/CD40 Ab alone or together with plate-bound 4-1BB–Fc protein [4-1BBL agonist (49)] or with a nonspecific B cell mitogen, LPS (50). Although LPS or anti-IgM/CD40 Ab induced comparable expression of TNF-α in B cells regardless of the presence or absence of 4-1BBL (i.e., WT and 4-1BBL KO; Fig. 4A), a combined treatment with BCR/CD40 Ab and 4-1BB–Fc strongly upregulated TNF-α in WT B cells, but not in 4-1BBL KO B cells (Fig. 4A). Hence BCR/CD40 signaling initiates expression of 4-1BBL and TNF-α (as also discussed for Fig. 3B, 3C); then 4-1BBL is needed to further upregulate TNF-α in 4BL cells upon engagement with 4-1BB.

Because TNF-α can be involved in expression of GrB (51), we evaluated expression of its receptors on CD8$^+$ T cells. Unlike TNFR1, TNFR2 was upregulated on GrB$^+$CD8$^+$ cells of aging mice and humans (Fig. 4B). Thus, considering high levels of expression of its ligand mTNF-α on 4BL cells (Fig. 1A, 1B), we next tested whether the mTNF-α/TNFR2 axis is used to induce expression of GrB in CD8$^+$ T cells. First, we included TNF-α neutralizing Ab or control IgG1 in our in vitro GrB$^+$CD8$^+$ T cell induction assay with 4BL cells, and we observed that the depletion of TNF-α significantly retarded the induction of GrB expression in CD8$^+$ T cells (Fig. 4C). The induction of GrB$^+$ CD8$^+$ T cells was also markedly retarded if we used 4BL cells from TNF-α KO mice (Fig. 4D). Moreover, WT 4BL cells failed to efficiently induce expression of GrB in CD8$^+$ T cells from TNFR2 KO mice (Fig. 4E), whereas the induction was unimpaired in TNFR1 KO CD8$^+$ T cells (Fig. 4E). Similarly for human cells, TNFR2 or TNF-α neutralizing Abs also abrogated the induction of GrB$^+$CD8$^+$ T cells in an MLR assay using elderly human 4BL cells (Fig. 4F). Because the mTNF-α/TNFR2 axis probably requires a cell contact, we also cultured 4BL cells with CD8$^+$ T cells either in the same well or separated with a porous membrane in transwell assay. The separation of the two cells completely abrogated the induction of GrB$^+$CD8$^+$ T cells
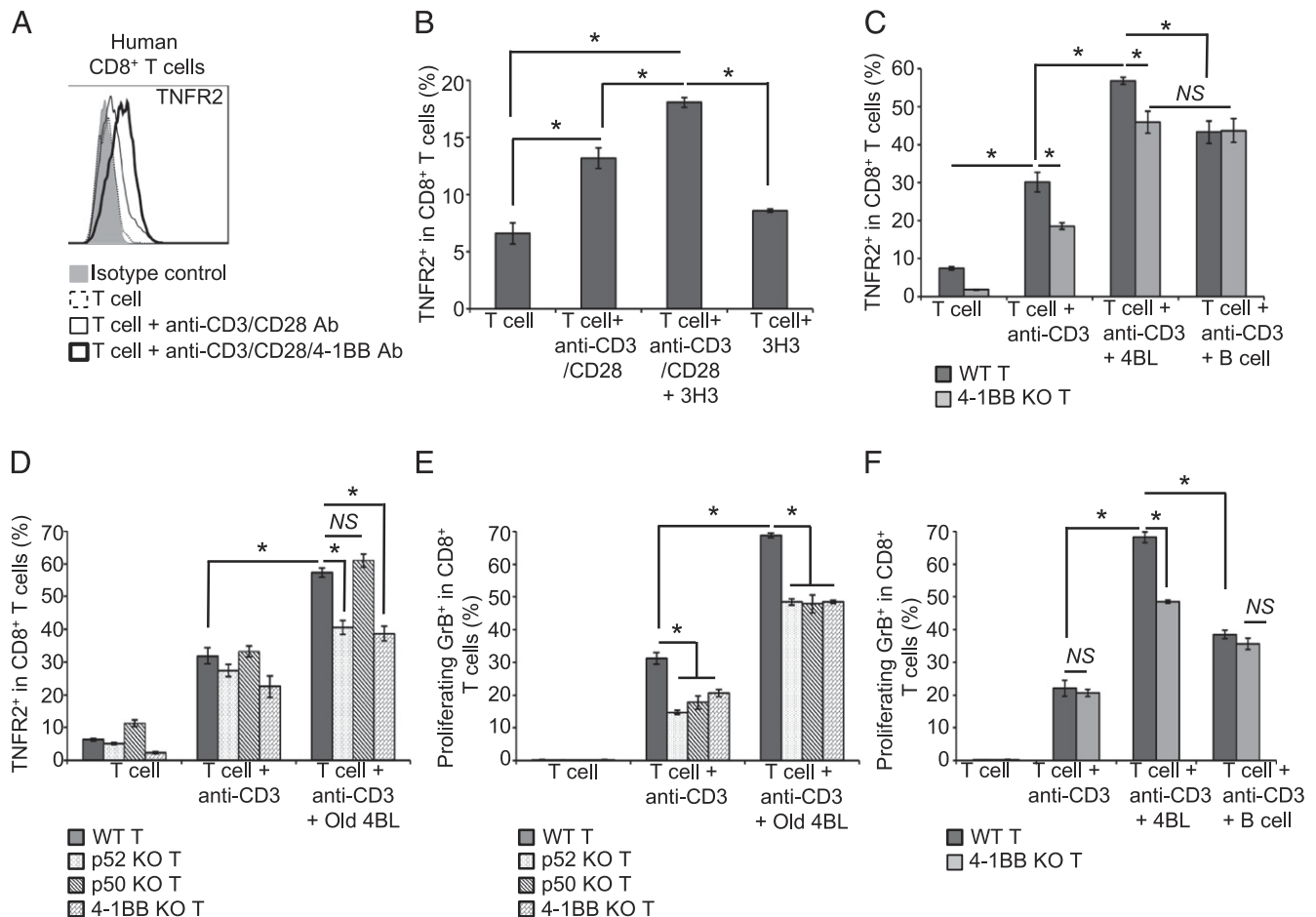
FIGURE 5. 4BL cells upregulate TNFR2 in CD8$^+$ T cells via the 4-1BB/4-1BBL axis. Although TNFR2 is induced on resting human (**A**) and murine (**B**) CD8$^+$ T cells after stimulation with anti-CD3/CD28 Ab (thin line) for 24 h, it is further upregulated upon 4-1BB engagement [anti-CD3/CD28/4-1BBL Ab beads, thick line in (A), and anti-CD3/CD28 Ab with 3H3 Ab, 5 μg/ml in (B)]. Dotted line is for nonstimulated T cells. Unlike 4-1BBL$^-$B cells (B cell, **C**), aging 4BL cells induce TNFR2 in CD8$^+$ T cells (C and **D**). Upregulation is lost in T cells from 4-1BB KO (C and D) and p52, but not p50, NF-κB KO (D) mice. Before mixing with B cells, T cells were pretreated with anti-CD3 Ab (1.5 μg/ml) for 24 h to upregulate 4-1BB. CD8$^+$ cells shown in (C) and (D) also express GrB after 5-d culture (**E** and **F**). Mean ± SEM of a representative experiment repeated at least twice with three to four mice per group. *$p < 0.05$.

(Fig. 4G). Together, these data demonstrate that BCR/CD40 signaling induces expression of 4-1BBL and mTNF-α on 4BL cells. Furthermore, upon activation of 4-1BBL, mTNF-α is further upregulated to induce GrB$^+$CD8$^+$ T cells by targeting TNFR2.

4BL cells upregulate TNFR2 in CD8$^+$ T cells by targeting 4-1BB

Next, we wanted to understand how CD8$^+$ T cells upregulate TNFR2. Because TCR stimulation induces expression of 4-1BB (32, 33, 36), we tested whether it can also upregulate TNFR2. Indeed, CD8$^+$ T cells stimulated with anti-CD3 or anti-CD3/CD28 Ab not only induced expression of 4-1BB (Supplemental Fig. 3C) but also TNFR2 (Fig. 5A–D). However, expression of TNFR2 was further and considerably upregulated if the stimulation also contained agonistic 4-1BB Ab (3H3; Fig. 5A, 5B). Because treatment with 3H3 alone did not induce expression of TNFR2 (Fig. 5B) (presumably because of low levels of 4-1BB on resting T cells; Supplemental Fig. 3C) (33), these results suggest that activated T cells first need to induce expression of 4-1BB, which upon receiving signaling from 4-BBL expressed on 4BL cells can further upregulate expression of TNFR2. In support of this model, the anti-CD3/CD28 Ab–induced expression of TNFR2 in CD8$^+$ T cells was markedly augmented upon coculture with sort-purified

4-1BBL$^+$, but not 4-1BB$^-$, B cells of old mice (4BL and B cells, respectively; Fig. 5C). However, if CD8$^+$ T cells were from 4-1BB KO mice, the 4BL cell–induced upregulation of TNFR2 was lost, because its expression failed to increase beyond the levels induced by anti-CD3/CD28 Ab stimulation alone (Fig. 5C). Thus, the induction of TNFR2 expression in CD8$^+$ T cells is bimodal because its expression induced via TCR signaling is further upregulated upon 4-1BB signaling induced by 4BL cells. Because 4-1BB can signal via both the canonical and the noncanonical NF-κB pathway (52), we also compared the induction of TNFR2 in CD8$^+$ T cells from mice deficient in classical (p50) and noncanonical (p52) NF-κB. Even though 4BL cells in the presence of anti-CD3 Ab upregulated TNFR2 in p50 KO CD8$^+$ T cells as efficiently as in WT T cells (Fig. 5D), they failed to do so in p52 KO T cells (Fig. 5D) as if the T cells were 4-1BB KO (Fig. 5C, 5D). Hence 4BL cells use 4-1BBL to upregulate expression of TNFR2 in CD8$^+$ T cells via inducing noncanonical NF-κB signaling from 4-1BB. As discussed earlier, the TNFR2 is then targeted to induce expression of GrB in CD8$^+$ T cells. Indeed, the TNFR2 upregulation mirrored the induction of GrB$^+$CD8$^+$ T cells; that is, the induction of GrB expression in p52 KO (Fig. 5E) and 4-1BB KO (Fig. 5F) CD8$^+$ T cells was reduced to the levels induced by anti-CD3 Ab stimulation alone despite the presence of old mouse 4BL cells. Notably, GrB induction was also reduced in p50 KO CD8$^+$ T cells (Fig. 5E),
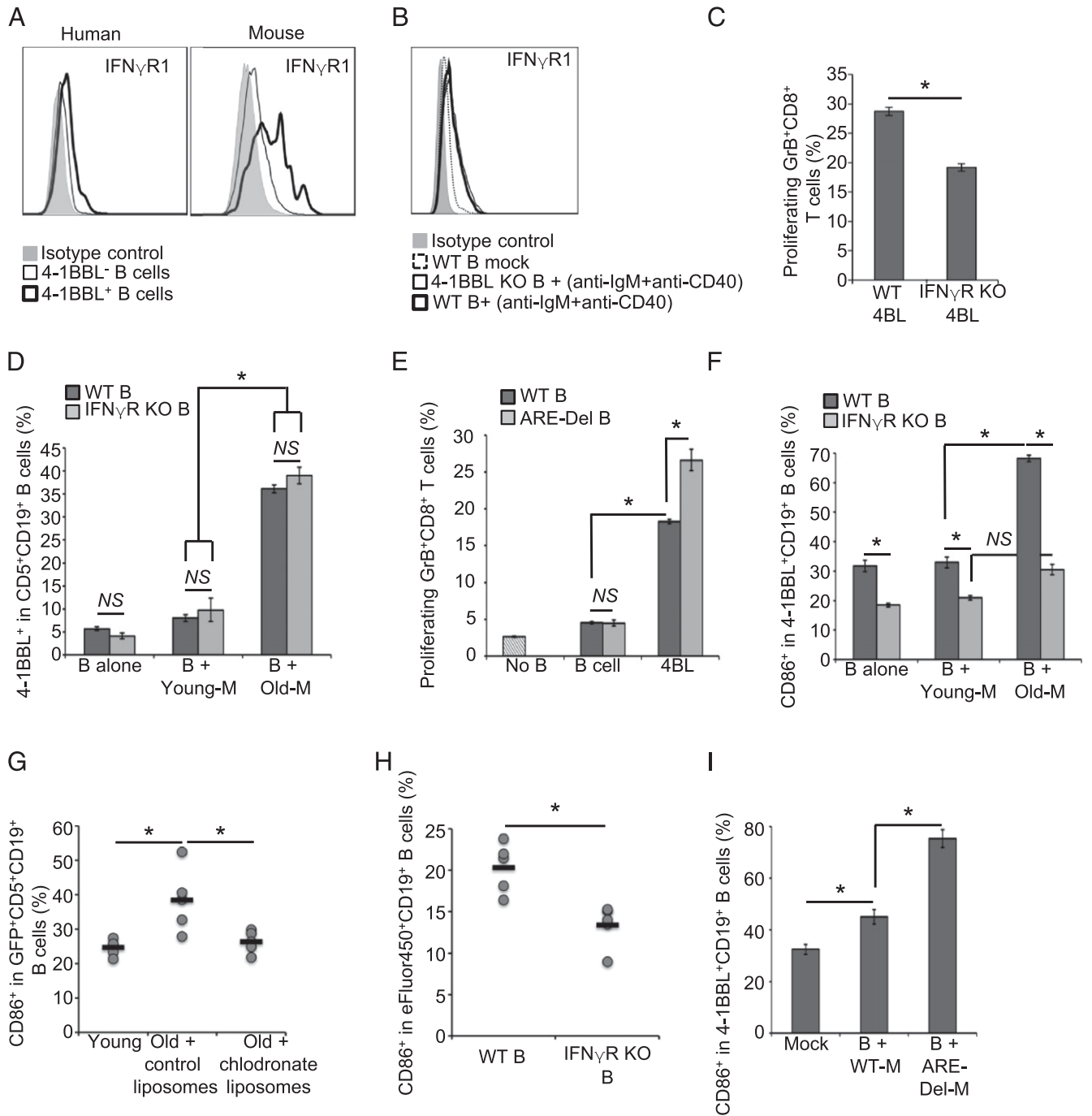
FIGURE 6. Myeloid cells also enhance the costimulatory function of 4BL cells via IFN-γ. (A) Although human and murine 4BL cells mostly express IFN-γR1 (A, thick line), both 4-BBL KO and WT B cells upregulate its expression upon anti-IgM/CD40 stimulation (B, red and black thick lines). IFN-γR1 KO 4BL cells less efficiently induce GrB$^+$CD8$^+$ T cells in vitro (C). Unlike 4-BBL that is induced on both IFN-γR KO and WT CD5$^+$ B cells stimulated with myeloid cells of old mice (Old-M) but not young mice (Young-M, D), CD86 requires IFN-γ signaling, because it was not induced on IFN-γR1 KO B (F). Compared with WT 4BL cells, 4BL cells from ARE-Del mice induce a higher frequency of GrB$^+$CD8$^+$ T cells (E). Mean ± SEM of a representative experiment repeated at least twice in triplicate experiments with three to four mice per group is shown. PC macrophages of old mice in vivo induce CD86 (G). B1a cells from young GFP$^+$ mice (G) were i.p. injected into congenic young and old mice pretreated with control liposome or clodronate liposomes (G), as in Fig. 3C. (H) eFluor 450–labeled B cells from WT or IFN-γR1 KO mice were i.p. injected into old WT mice. Frequency of CD86$^+$ in GFP$^+$ B1a (G) or eFlur450$^+$B1a (H) from individual mice (dots) and mean value (lines) of a representative five to six mice per group experiment repeated twice are shown. Ex vivo, myeloid cells of young ARE-Del mice (B+ARE-Del-M) also strongly upregulate CD86 on B cells of young WT mice compared with WT myeloid cells (B+WT-M, I). *$p < 0.05$.

suggesting that, unlike TNFR2, its expression requires canonical NF-κB (53).

Myeloid cells enhance costimulatory function of 4BL cells via IFN-γ

Interestingly, besides being high expressers of 4-1BBL, MHC class-I and CD86 (30), as well as mTNF-α, 4BL cells also

expressed IFN-γR1 (Fig. 6A). Its expression appears to also depend on BCR/CD40 signaling, because resting murine and human B cells markedly upregulated IFN-γR1 upon anti-IgM/CD40 Ab stimulation (Fig. 6B). Because IFN-γ is a potential inducer of CD86 (54), we hypothesized that 4BL cells probably enhance their T cell costimulatory functions by receiving IFN-γ expressed
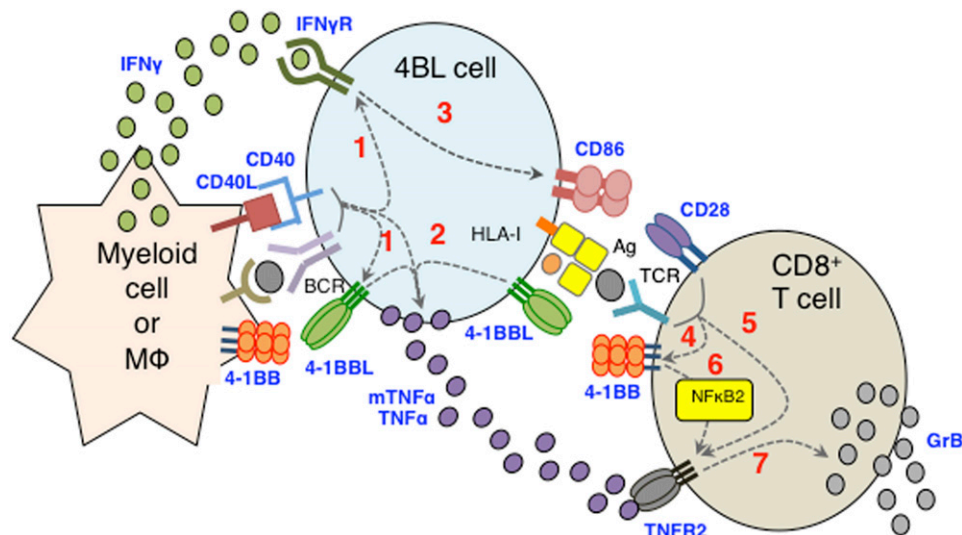
FIGURE 7. Summary schema. Aging CD11b^{+} myeloid cells initiate a chain of events that convert B1a cells into 4BL cells and concurrently induce GrB^{+} CD8^{+} T cells. First, they convert B1a cells into 4BL cells by inducing expression of 4-1BBL, mTNF-α, and IFN-γR (**1**). Then 4BL cells further upregulate mTNF-α and CD86 via activating 4-1BBL (**2**) and IFN-γR1 (**3**) signaling from 4-1BB and IFN-γ provided by aging myeloid cells, respectively. 4BL cells activate CD8^{+} T cells to express 4-1BB (**4**) and TNFR2 (**5**). This activation enables 4BL cells to trigger noncanonical NF-κB signaling from 4-1BB and further increase expression of TNFR2 on CD8^{+} T cells (**6**). The TNFR2 is targeted with mTNF-α to induce expression of GrB in CD8^{+} T cells while providing costimulation with CD86 (**7**).

in aging CD11b^{+}myeloid cells (Supplemental Fig. 2E, 2F). In concordance, 4BL cells from IFN-γR1 KO mice failed to efficiently induce the generation of GrB^{+}CD8^{+} T cells in vitro (Fig. 6C). Conversely, the presence of blocking CD86 Ab (but not control Ab) was sufficient to abrogate the induction of GrB^{+} CD8^{+} T cells with WT 4BL cells (Supplemental Fig. 4A). Although IFN-γ alone upregulated CD86 on resting B cells (Supplemental Fig. 4B), it was not sufficient to convert B cells into 4BL cells. IFN-γ and IFN-γR expression status (IFN-γ KO and IFN-γR1 KO) did not affect the low frequency of 4BL cells present in young mice (Supplemental Fig. 4C–E, and B-alone in Fig. 6D), which is usually around 5% of B cells (30). Similarly, the proportion of 4BL cells was not increased in ARE-Del mice (Supplemental Fig. 4F), which constitutively express IFN-γ (39). Although B cells of ARE-Del mice demonstrate highly upregulated CD86 compared with WT mice (Supplemental Fig. 4G), their sort-purified 4-1BBL^{−} B cells failed to induce GrB^{+} CD8^{+} T cells stimulated with anti-CD3 Ab (Fig. 6E) despite their chronic exposure to IFN-γ. In contrast, ARE-Del 4-1BBL^{+} (4BL cells) markedly induced GrB^{+}CD8^{+} T cells with a significantly higher potency than WT 4BL cells ($p < 0.05$; Fig. 6E). Hence these results suggest that 4BL cells use IFN-γR1 to upregulate CD86, and thereby contribute to the generation of GrB^{+}CD8^{+} T cells.

To test the role of myeloid cells in this process, we cocultured purified CD5^{+} B cells (B-alone) from young WT and IFN-γR1 KO mice with congenic PC Young-M and Old-M cells. Although Old-M upregulated 4-1BBL on both WT and IFN-γR1 KO B cells (Figs. 3, 6D), they failed to induce CD86 in IFN-γR1 KO B cells (Fig. 6F, Supplemental Fig. 4E). Young mouse B cells also significantly upregulated CD86 upon injection into PC of congenic and macrophage-sufficient old mice (Fig. 6G), but failed to do so in old mice depleted of macrophages (Fig. 6G) or if B cells were from IFN-γR1 KO mice (Fig. 6H). Control B cells both in vitro and in vivo exposed to Young-M cells did not induce expression of CD86 and 4-1BBL (see B-alone or mock with B + Young-M, Fig. 6D, 6F, Supplemental Fig. 4E, and Young in Fig. 6G). Furthermore, CD11b^{+} myeloid cells from PC of young ARE-

Del mice [which constitutively express IFN-γ (39)] also strongly upregulated CD86 upon their in vitro coculture, whereas WT CD11b^{+} cells of young mice failed to do so (Fig. 6I), probably accounting for the enhanced ability of 4-1BBL^{+} B cells from ARE-Del mice to induce GrB^{+}CD8^{+} T cells (Fig. 6E). Together, these results indicate that aging myeloid cells, such as macrophages, use the IFN-γ/IFN-γR1 signaling axis to enhance the costimulatory function of 4BL cells by upregulating CD86 (Fig. 7).

Discussion

Aging significantly dysregulates the function and distribution of B cells in the circulation (15–18), resulting in impaired humoral responses to new Ags and vaccines in the elderly (55) (see reviews in Refs. 1, 2). We recently reported that elderly humans, macaques, and mice accumulate 4-1BBL^{+} B cells (4BL cells) of unknown origin (30). They phenotypically and functionally differ from other B cells enriched in aging, such as mature and Ag-experienced memory B2 cells of mammals (15–18) and exhausted mature B2 cells (ABCs) in mice (29). In this study, we demonstrate that, at least in mice, 4BL cells are B1a cells converted upon aging, whereas in human PB they appear to be memory CD43^{−}CD27^{+} B cells. The failure to link 4BL cells with human memory CD43^{+}CD27^{+} B cells that contain B1 cells (27) could be because of reduced CD43 expression in aging or the species difference. Although aging is not known to affect the function of innate B1 cells (11, 20, 21), it increases the numbers of both B1a cells (as well as B2 cells) in PC of mice (11), presumably because of their enhanced longevity (13), self-renewal capabilities (21), and homing in response to elevated production of CXCL13 (56–58). Aging B1a cells increase 3-fold in mice (21, 59) and, being CXCR5[High], infiltrate into spleen and PC with a higher efficiency than B2 cells (56–58). Functionally, aging B1 cells are implicated in autoimmune diseases, such as Sjögren syndrome in humans (60) and lupus erythematosus in mice (61), and are primarily considered the producers of natural Abs, mediators of tissue homeostasis, and involved in the clearance of apoptotic cells (62). In contrast, our data indicate that aging dramatically affects the function of B1a cells, because they lose

tumor-supporting (43) and possibly immunosuppressive activities but gain a new property: the induction of GrB$^+$CD8$^+$ T cells. For example, whereas young B1a cells strongly enhance the growth of B16 melanoma in congenic B cell–deficient mice, aging B1a cells fail to do so. Instead, they retard tumor growth by inducing antitumor GrB$^+$CD8$^+$ T cells, the function of which we previously reported for 4BL cells in old mice with poorly immunogenic tumors and in breast cancer patients after autologous hematopoietic stem transplantation (30). Thus, aging renders B1a cells/4BL cells into APCs that can uptake endogenous Ags in vivo (e.g., B16 melanoma or i.p. injected OVA protein) and induce Ag-specific CD8$^+$ T cell responses such as TCR-transgenic CD8$^+$ T cells from pmel and OT-1 mice, respectively. This is in line with the fact that 4BL cells highly express an array of surface molecules needed for the induction of CD8$^+$ T cells, including MHC class-I, CD86, CD40, and 4-1BBL (30).

In our previous report, we hypothesized that the generation of 4BL cells is induced by extrinsic factors, because these cells also accumulate in cancer patients after autologous hematopoietic stem transplantation and their reappearance after B cell depletion in Old-restored mice was significantly delayed compared with the rest of the B cells (30). In this study, we experimentally confirm this hypothesis by demonstrating the importance of aging myeloid cells, such as human monocytes and murine PC macrophages, in the conversion of 4BL cells. This is in line with a plethora of changes reported for monocytes and macrophages upon aging in humans and rodents. For example, old rats enrich for CD11b$^+$ monocytes expressing IFN-γ (63), whereas elderly human PB accumulates so-called intermediate and nonclassical monocytes producing high levels of TNF-α (8). Aging also impairs phagocytosis of myeloid cells, such as human neutrophils and CD14$^+$ monocytes and murine PC macrophages (8, 11), and the responses of monocytes and macrophages to IFN-γ and TLR stimulations (64–66). We also found that old mice show an increase in CD11b$^+$ myeloid cells and macrophages expressing IFN-γ and CD40L. Importantly, our in vitro and in vivo experiments indicate that aging mouse PC CD11b$^+$ myeloid cells can directly convert young mouse B1 cells into 4BL cells. Because control myeloid cells from young mice or old mice depleted of phagocytic cells such as macrophages failed to do so, we conclude that the conversion is primarily mediated by aging macrophages. In humans, PB monocytes of the elderly appear to be the inducers of 4BL cells. Thus, by corroborating the importance of cross talk between myeloid cells/macrophages and B cells (67–69), our data suggest that the dysregulation of macrophages in the elderly can initiate a chain of events including the conversion of B1a cells into 4BL cells and concurrent induction of cytolytic CD8$^+$ T cells, as summarized in Fig. 7. First, they trigger BCR and CD40 signaling, and thereby induce expression of 4-1BBL, mTNF-α, and IFN-γR1 in B1a cells, converting them into 4BL cells (step 1, Fig. 7). This initial step can be abrogated by blocking BCR or CD40 signaling, indicating the importance of these pathways in the conversion of 4BL cells, as well as in the development and function of B1a cells (25, 48). Because human and murine monocytes and macrophages express CD40L (70) and CD40L$^+$CD11b$^+$ myeloid cells that are enriched in PC of aging mice, they could provide CD40L to activate CD40 on B1a cells. In contrast, we do not know the mechanism of the BCR signaling. Because macrophages can stimulate BCR during exchange of Ags (46, 47) via FcγRIIB-captured and recycled immune complexes (71), we can only speculate that myeloid cells of aging subjects activate B1a cells by using autoreactive Ab that is increased upon aging (72). As a result, 4BL cells express 4-1BBL to further upregulate expression of mTNF-α and

IFN-γR1 upon engagement with 4-1BB (step 2, Fig. 7). This is consistent with the fact that signaling of 4-1BBL is required for sustained production of TNF-α in macrophages (49, 73) and that its receptor is also expressed on myeloid cells such as splenic DCs (32). By expressing IFN-γR1, 4BL cells also upregulate CD86 in response to IFN-γ (step 3, Fig. 7). Using neutralizing Ab or cells with impaired expressions of IFN-γ or IFN-γR1, we demonstrate that aging CD11b$^+$ myeloid cells and macrophages provide IFN-γ, although in vivo it can probably be supplied by IFN-γ–producing cells such as Th1 T cells and NK cells. In addition, considering its importance in MHC class-I expression and Ag presentation (74), IFN-γ is probably responsible for the enhanced expression of MHC class-I on 4BL cells (30). Thus, aging myeloid cells induce high levels of expression of 4-BBL, mTNF-α, CD86, and MHC-I on B1a cells. As a result, the activated B1a cells/4BL cells become APCs that induce Ag-specific GrB$^+$CD8$^+$ T cell responses requiring the 4-1BBL/4-1BB signaling axis (30). First, aging B1a cells induce expression of 4-1BB (step 4) and TNFR2 (step 5, Fig. 7) in CD8$^+$ T cells. Then they target 4-1BB via 4-1BBL to further upregulate expression of TNFR2 in CD8$^+$ T cells (step 6, Fig. 7). Confirming the use of the noncanonical NF-κB signaling by 4-1BB (52), 4BL cells failed to upregulate TNFR2 in CD8$^+$ T cells deficient in the noncanonical, but not canonical, NF-κB signaling pathway. Then, using mTNF-α, 4BL cells target TNFR2 and induce expression of GrB in CD8$^+$ T cells (step 7, Fig. 7), while providing costimulation with CD86. In summary, our data suggest that aging converts B1a cells into potentially pathogenic and autoimmune 4BL cells via dysregulation of the myeloid cell compartment.

Acknowledgments

Disclosures

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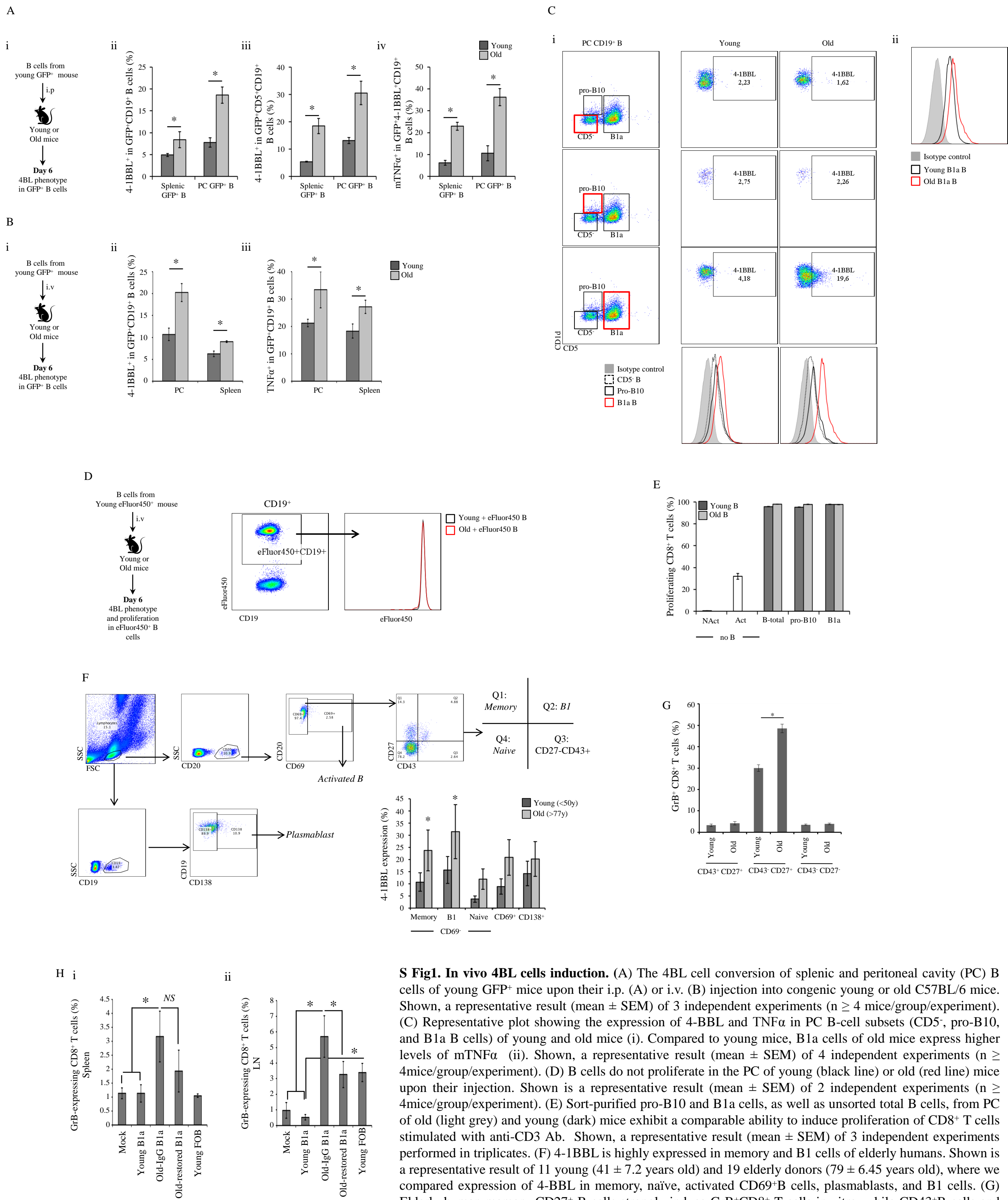
S Fig1. In vivo 4BL cells induction. (A) The 4BL cell conversion of splenic and peritoneal cavity (PC) B cells of young GFP+ mice upon their i.p. (A) or i.v. (B) injection into congenic young or old C57BL/6 mice. Shown, a representative result (mean ± SEM) of 3 independent experiments (n ≥ 4 mice/group/experiment). (C) Representative plot showing the expression of 4-BBL and TNFα in PC B-cell subsets (CD5-, pro-B10, and B1a B cells) of young and old mice (i). Compared to young mice, B1a cells of old mice express higher levels of mTNFα (ii). Shown, a representative result (mean ± SEM) of 4 independent experiments (n ≥ 4mice/group/experiment). (D) B cells do not proliferate in the PC of young (black line) or old (red line) mice upon their injection. Shown is a representative result (mean ± SEM) of 2 independent experiments (n ≥ 4mice/group/experiment). (E) Sort-purified pro-B10 and B1a cells, as well as unsorted total B cells, from PC of old (light grey) and young (dark) mice exhibit a comparable ability to induce proliferation of CD8+ T cells stimulated with anti-CD3 Ab. Shown, a representative result (mean ± SEM) of 3 independent experiments performed in triplicates. (F) 4-1BBL is highly expressed in memory and B1 cells of elderly humans. Shown is a representative result of 11 young (41 ± 7.2 years old) and 19 elderly donors (79 ± 6.45 years old), where we compared expression of 4-BBL in memory, naïve, activated CD69+B cells, plasmablasts, and B1 cells. (G) Elderly human memory CD27+ B-cells strongly induce GrB+CD8+ T cells in vitro, while CD43+B cells and CD27- B cells failed to do so. Shown is a representative result (mean ± SEM) of 3 independent experiments (n ≥ 2 human cells/group/experiment). (H) Adoptively transferred sB1a cells of old mice, but not FOB or young mouse sB1a cells, increase frequency of GrB+CD8+ T cells in B-cell deficient J_HT mice with B16 melanoma. Shown is the mean (%) ± SEM of GrB in CD8+T cells in spleens (i) and LN (ii) at day 20 post tumor challenge. The experiment was reproduced twice with 4-5 mice/group. P-value significance is shown as *p<0.05, **p<0.01, ***p<0.005, and NS = not statistically significant.
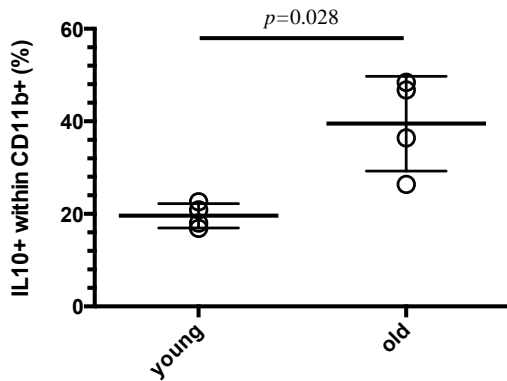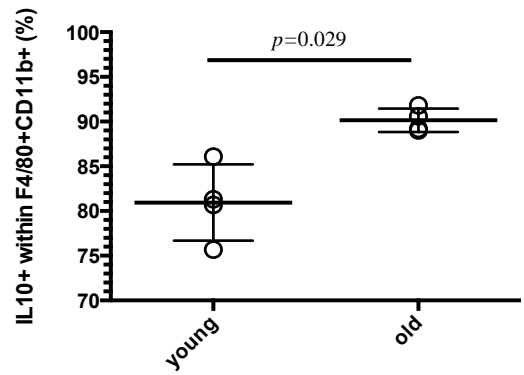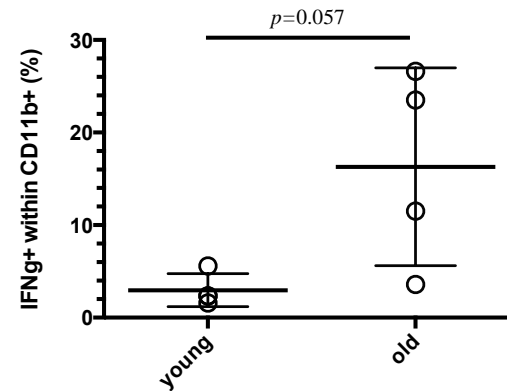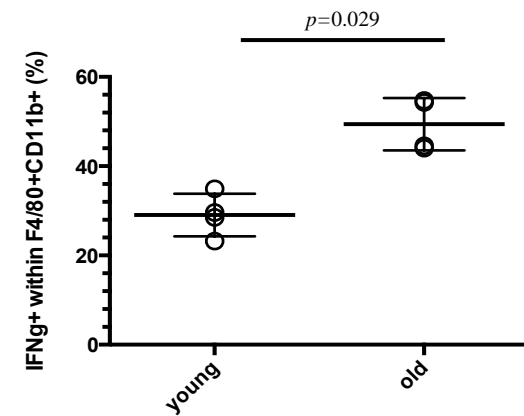
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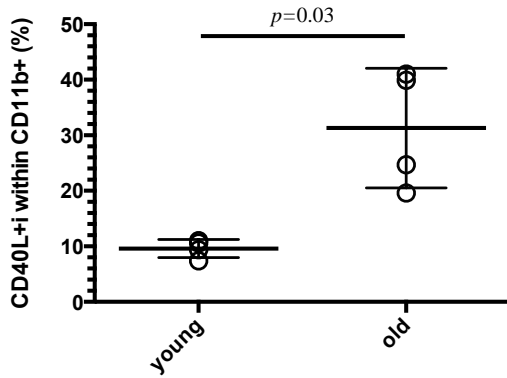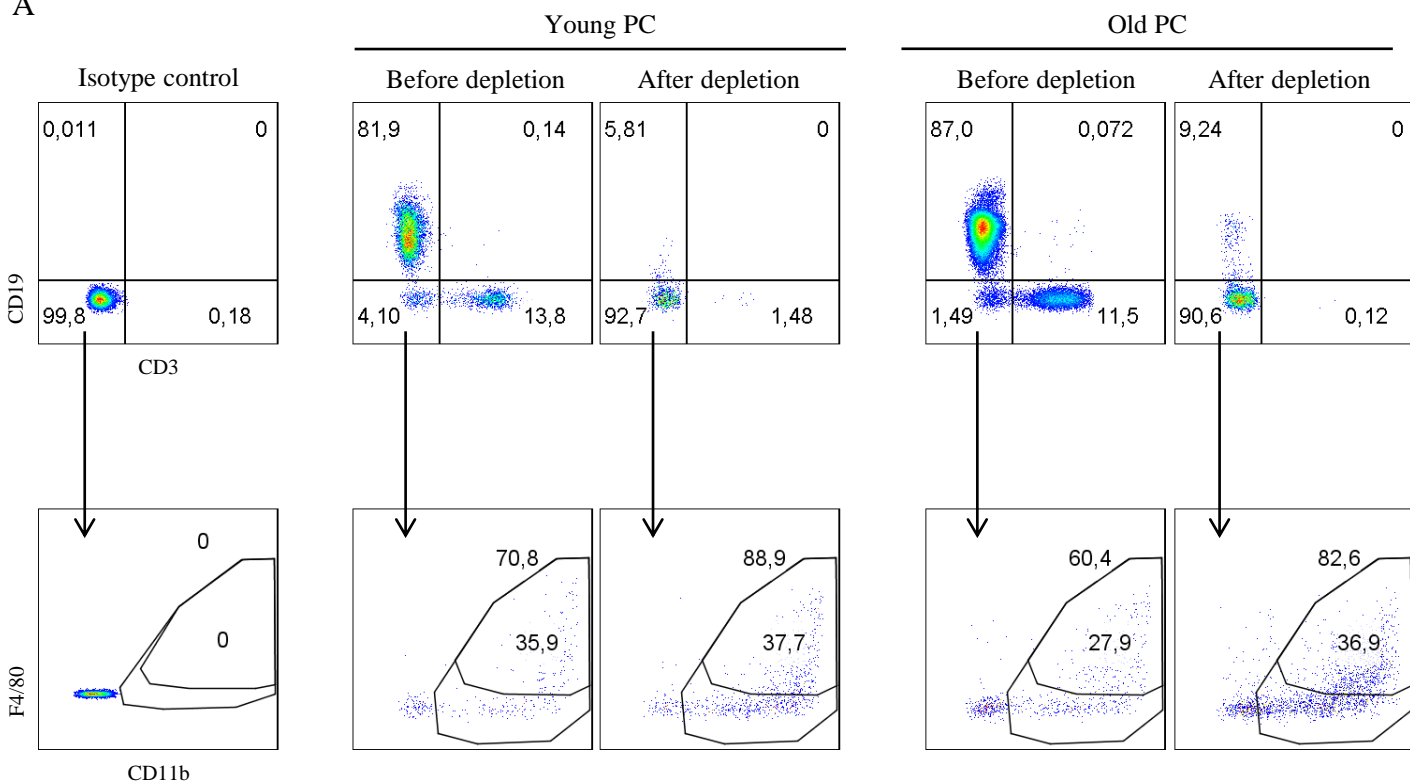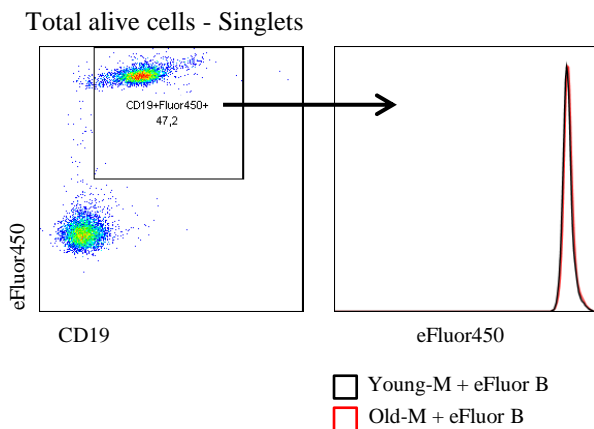

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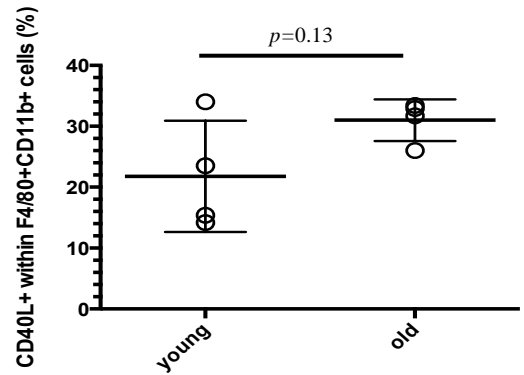
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S Fig2. PC myeloid cells and macrophages are dysregulated in old mice, as shown by enrichment for CD40L, IL10, and IFNγ –expressing CD11b$^+$ PC myeloid cells (A,C,E) and CD11b$^+$F4/80$^+$macrophages (B, D, F) of young and old mice (n=4 mice/group).
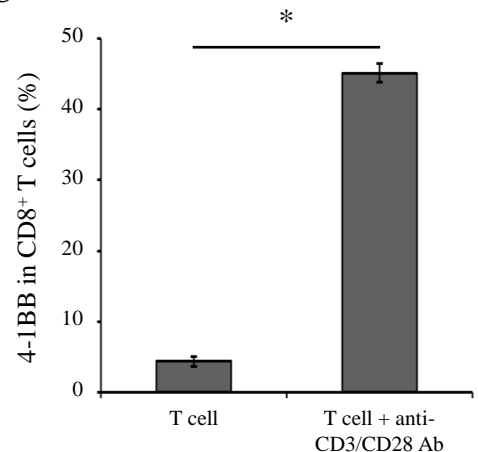
S Fig3. Aging myeloid cells induce 4BL cells (A,B)**.** CD11b^{+} myeloid cells isolated from PC of young and old mice (depleted of B cells, T cells, and NK cells with CD19-PE+CD3-PE + anti-PE MicroBeads and DX5-PE + anti-PE MicroBeads, which yielded >80% enrichment in CD11b^{+} cells, (A) were cultured with eFluor® 450-labeled B cells (from naïve young mice) at 1:1 ratio (B) for 24 h. As shown in a representative experiment performed ≥ 4 times (B), B cells did not proliferate. (C) **Murine CD8^{+} T cells express 4-1BB upon CD3/CD28 activation**. Murine CD8^{+} T cells were activated with 1.5 μg/ml of soluble anti-CD3 Ab + 1.5 μg/ml of soluble anti-CD28 for 24 h. Shown, a representative result (mean ± SEM) of 2 independent experiments performed in triplicates. P-value significance is shown as *p<0.05.
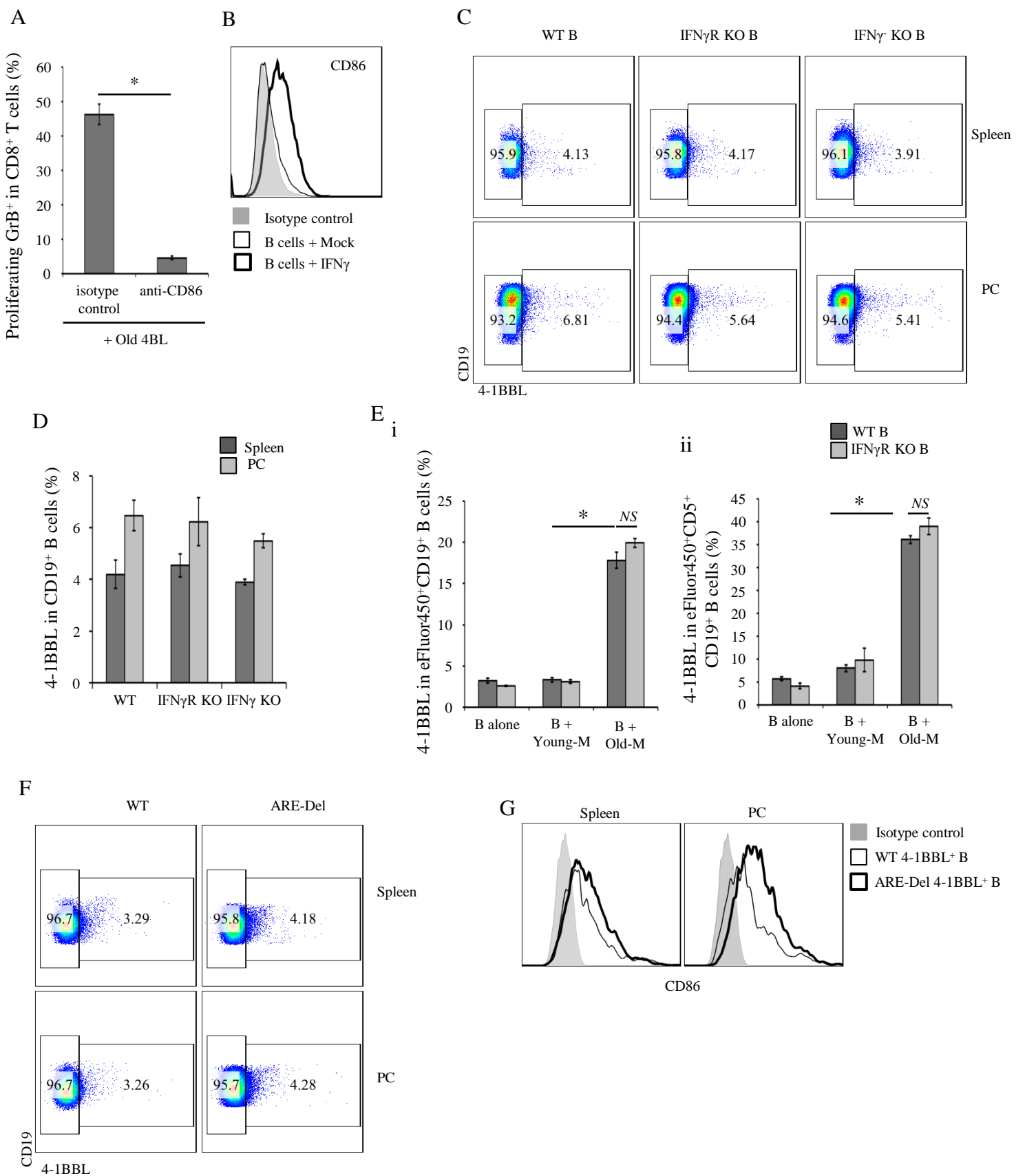
S Fig4. Importance of IFNγ provided by myeloid cells in induction of 4BL cells. (A) 4BL cells use CD86 in induction of GrB$^+$CD8$^+$ T cells, as it was lost in the presence of an anti-CD86 blocking Ab. (B) Young mouse splenic B cells up-regulate CD86 upon treatment with IFNγ (100 U/ml, 24 h). (C, D) Expression of 4-BBL in splenic and PC B cells is not affected by the loss of IFNγ signaling, such as in IFNγR1 KO or IFNγ KO mice. (E) Despite the loss of IFNγR (light grey), B cells up-regulate 4-1BBL in response to aging myeloid cells of mice (Old-M vs Young-M) as well as WT B cells (dark bars). (F) Compared to WT mice, B cells from young ARE-Del mice, which constitutively produce IFNγ, express comparable levels of 4-1BBL in splenic and PC B cells. (G) However, only ARE-Del mouse B cells express higher levels of CD86. Shown is a representative result (mean ± SEM) of 3 independent experiments (n ≥ 3 mice/group) performed in triplicates. P-value significance is shown as *p<0.05 and NS = not statistically significant.