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Mast Cells Enhance T Cell Activation: Importance of Mast Cell Costimulatory Molecules and Secreted TNF[1]

Susumu Nakae,* Hajime Suto,*† Motoyasu Iikura,* Maki Kakurai,* Jonathon D. Sedgwick,[2]‡ Mindy Tsai,* and Stephen J. Galli[3]*

We recently reported that mast cells stimulated via FcεRI aggregation can enhance T cell activation by a TNF-dependent mechanism. However, the molecular mechanisms responsible for such IgE-, Ag- (Ag-), and mast cell-dependent enhancement of T cell activation remain unknown. In this study we showed that mouse bone marrow-derived cultured mast cells express various costimulatory molecules, including members of the B7 family (ICOS ligand (ICOSL), PD-L1, and PD-L2) and the TNF/TNFR families (OX40 ligand (OX40L), CD153, Fas, 4-1BB, and glucocorticoid-induced TNFR). ICOSL, PD-L1, PD-L2, and OX40L also are expressed on APCs such as dendritic cells and can modulate T cell function. We found that IgE- and Ag-dependent mast cell enhancement of T cell activation required secreted TNF; that TNF can increase the surface expression of OX40, ICOS, PD-1, and other costimulatory molecules on $CD3^+$ T cells; and that a neutralizing Ab to OX40L, but not neutralizing Abs to ICOSL or PD-L1, significantly reduced IgE/Ag-dependent mast cell-mediated enhancement of T cell activation. These results indicate that the secretion of soluble TNF and direct cell-cell interactions between mast cell OX40L and T cell OX40 contribute to the ability of IgE- and Ag-stimulated mouse mast cells to enhance T cell activation. *The Journal of Immunology,* **2006, 176: 2238–2248.**

Mast cells represent important effector cells in Th2- and IgE-associated immune responses to parasites and allergic diseases (1–3). However, several lines of evidence have indicated that mast cells also can contribute to the sensitization phase of acquired immune responses and can interact with T cells to amplify the magnitude of the immune responses elicited in sensitized hosts at sites of Ag challenge.

For example, mast cells can produce several product that can promote dendritic cell (DC) migration, maturation, or function (4, 5). Moreover, studies using genetically mast cell-deficient mice have shown that mast cells can promote the migration of Langerhans cells from the epidermis upon hapten sensitization in a model of oxazolone-induced contact hypersensitivity (6) and that the IgE- and Ag-dependent activation of skin mast cells can promote the migration of skin DCs to local draining lymph nodes by a mechanism that is in part H_2 histamine receptor-dependent (7).

In addition to their interactions with DCs, mast cells can influence the function of T cells and vice versa. For example, purified populations of mast cells can present Ags to T cells by either MHC class I- or class II-restricted mechanisms in vitro (8–11), and contact with activated T cells in vitro can induce some mast cell populations to secrete histamine, TNF, and metalloproteinase 9 and to exhibit enhanced levels of IL-4 mRNA (12–15). Besides representing a major potential source of TNF (16, 17), which can have several effects that influence T cell recruitment, activation, and function (18–21), at least some mast cells can produce many other factors, including CCL2, CCL3, CCL4, CCL5, XCL1, IL-16, and leukotriene B_4, which also have the potential to enhance T cell recruitment to local inflammatory sites (5, 22–25).

However, certain mast cell populations, in addition to having the capacity to produce soluble factors that can influence T cell biology, can express costimulatory molecules such as OX40 ligand (OX40L) and 4-1BBL (22, 26). Although such costimulatory molecules are generally thought to have roles in DC-associated T cell activation, Kashiwakura et al. (26) reported that human tonsil mast cell-OX40L can contribute to the T cell proliferation induced when human T cells are incubated with mast cells that have undergone FcεRI-dependent activation in vitro.

We recently reported that mouse mast cells can significantly enhance T cell proliferation and cytokine production by at least two mechanisms, one of which is dependent on the activation of mast cells via the FcεRI and requires mast cell-derived TNF (27). Although we showed that this pathway of mast cell-dependent enhancement of T cell activation is favored by proximity between mast cells and T cells, the molecular mechanisms underlying this mast cell-T cell interaction were not identified, nor was it clear to what extent soluble TNF, as opposed to membrane-associated TNF, contributed to the important role of TNF in mast cell-dependent T cell activation.

In the present study we found that mouse bone marrow-derived cultured mast cells (BMCMCs)[4] can express several costimulatory molecules, including members of the B7 family (ICOS ligand (ICOSL), PD-L1, and PD-L2) and the TNF/TNFR families (OX40L, CD153, Fas, 4-1BB, and glucocorticoid-induced TNFR (GITR). We also found that mouse BMCMCs activated by IgE and

*Department of Pathology, Stanford University School of Medicine, Stanford, CA 94305; †Atopy Research Center, Juntendo University School of Medicine, Tokyo, Japan; and ‡Department of Immunology, DNAX Research, Palo Alto, CA 94304

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[2] Current address: Lilly Research Laboratories, Eli Lilly & Co., Indianapolis, IN 46285.

[3] Address correspondence and reprint requests to Dr. Stephen J. Galli, Department of Pathology, L-235, Stanford University School of Medicine, 300 Pasteur Drive, Stanford, CA 94305-5324. E-mail address: sgalli@stanford.edu

[4] Abbreviations used in this paper: BMCMC, bone morrow-derived cultured mast cell; DC, dendritic cell; DNP-HSA, 2,4-dinitrophenyl-conjugated human serum albumin; ESCMC, embryonic stem cell-derived mast cell; FLCMC, fetal liver-derived cultured mast cell; GITR, glucocorticoid-induced TNFR; ICOSL, ICOS ligand; OX40L, OX40 ligand; PMC, peritoneal mast cell; rm, recombinant mouse.

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Ag can enhance T cell proliferation by a mechanism involving soluble TNF, cell contact, and mast cell expression of OX40L.

Materials and Methods

Mice

Completely TNF-deficient mice ($TNF^{-/-}$ mice) as well as mice expressing only membrane TNF and lacking the ability to release secreted TNF ($memTNF^{\Delta/\Delta}$ mice), were generated from C57BL/6 ES cells as described previously (28, 29). C57BL/6J-Rag-$1^{-/-}$, C57BL/6J, and WB × B6 F_1 mice were purchased from The Jackson Laboratory. BALB/cKa mice were obtained from the Veterinary Service Center, Stanford University. All mice were housed at the animal care facilities at Stanford University Medical Center; were kept under standard temperature, humidity, and timed lighting conditions; were provided mouse chow and water ad libitum; and were treated in a humane manner in compliance with the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources, National Research Council, and the Stanford Institutional animal care and use committee.

Preparation of BMCMCs

Mouse BMCMCs were obtained by culturing mouse femoral BM cells in 20% WEHI-3-conditioned medium (containing IL-3) for 6–12 wk, at which time the cells were >98% c-$Kit^{high}Fc\epsilon RI\alpha^{high}$ by flow cytometric analysis (data not shown).

Preparation of T cells

A single-cell suspension of spleen cells was prepared, and RBC were lysed in RBC lysing buffer (Sigma-Aldrich). For $CD3^+$ T cell purification, spleen cells were incubated with biotinylated anti-mouse B220 (RA3-6B2), Gr-1 (RB6-8C5), CD11b (M1/70), CD11c (N418), CD49b (DX5), Ter119, and c-Kit/CD117 (2B8) for 20 min at 4°C. All Abs were obtained from eBioscience. The cells were then washed and incubated with streptavidin-beads (Miltenyi Biotec) for 20 min at 4°C, washed again, and passed through a MACS column (Miltenyi Biotec), yielding >95% $CD3^+$ T cells.

Flow cytometry

For analysis of costimulatory molecules/activation markers, BMCMCs were incubated with 5 μg/ml anti-DNP IgE mAb (H1-ϵ-26) (30) at 37°C overnight. After washing, BMCMCs were cultured with 5 ng/ml DNP-conjugated human serum albumin (DNP-HSA; Sigma-Aldrich) at 37°C for 6 h, then harvested and incubated with anti-mouse CD16/CD32 (2.4G2; BD Pharmingen) on ice for 15 min. After FcR blocking, cells were incubated on ice for 40 min with FITC-conjugated anti-mouse FcϵRIα (MAR-1; eBioscience), allophycocyanin-conjugated anti-mouse c-Kit/CD117 (2B8; BD Pharmingen), and a PE-conjugated Ab against a specific costimulatory molecule/activation marker, as described below. PE-anti-mouse $I\text{-}A^b$ (AF6-120.1), $I\text{-}A^d$ (AMS-32.1), CD40 (3/23), CD70 (FR70), CD80 (16-10A1), CD86 (GL1), CD95 (Jo2), CD120b/TNFRII (TR75-89), and CD153 (RM153) were obtained from BD Pharmingen. PE-anti-mouse 4-1BB/CD137 (17B5), CD40L/CD154 (MAR1), Fas ligand/CD178 (MLF3), OX40L (RM134L), 4-1BBL (TKS-1), ICOSL (HK5.3), PD-L1 (MIH6), PD-L2 (TY25), B7-H3 (M3.2D7), B7x (clone 9), and TNF-related activation-induced cytokine (IK22/5) were obtained from eBioscience. PE-anti-mouse CD120a/TNFRI (55R-170) and PE anti-mouse GITR (108619) were obtained from Santa Cruz Biotechnology and R&D Systems, respectively. All isotype-matched control IgGs for Abs against specific costimulatory molecules/activation markers were purchased from BD Pharmingen.

For analysis of costimulatory molecules/activation markers on T cells, DCs, or BMCMCs in the absence or the presence of TNF, C57BL/6-$CD3^+$ T cells, C57BL/6-Rag-$1^{-/-}$ spleen cells, or C57BL/6-BMCMCs sensitized with anti-DNP IgE as described above (2×10^6 cells/well in a 12-well plate) were cultured with 0.3 μg/ml plate-coated anti-CD3 with or without recombinant mouse TNF (rmTNF; PeproTech) for 72 h, with rmTNF alone for 72 h, or with 5 ng/ml DNP-HSA with or without rmTNF for 6 or 72 h, respectively, then harvested. After FcR blocking, cells were incubated on ice for 40 min with allophycocyanin-anti-mouse CD4 (RM4-5; BD Pharmingen), FITC-anti-mouse CD8 (53-6.7) plus biotin anti-mouse OX40/CD134 (OX-86; BD Pharmingen), PE-anti-mouse 4-1BB/CD137, PE-anti-mouse GITR, PE-anti-mouse ICOS (C398.4A; eBioscience), or PE-anti-mouse PD-1 (J43; eBioscience) for T cells; with allophycocyanin-anti-mouse CD11c (N418, eBioscience) plus PE anti-mouse OX40L for DCs; or with FITC-anti-mouse FcϵRIα and allophycocyanin-anti-mouse c-Kit plus PE anti-mouse OX40L for BMCMCs. For OX40 expression, cells were incubated with PE-streptavidin (BD Pharmingen). After incubation with Abs, cells were washed and suspended in Hanks' buffer (Cellgro) containing 2% FCS and 1 μg/ml 7-aminoactinomycin D (Sigma-Aldrich). The expression of cell surface markers was analyzed on a FACSCalibur (BD Biosciences) using Cell Quest software (BD Biosciences).

T cell-mast cell coculture

T cells were cocultured with BMCMCs as described previously (27). BMCMCs were sensitized with 1 μg/ml H1-ϵ-26 IgE anti-DNP mAb (30) at 37°C overnight. After IgE sensitization, BMCMCs were treated with mitomycin C (Sigma-Aldrich; 50 $\mu g/10^7$ cells) for 15 min at 37°C. BMCMCs and T cells were suspended in RPMI 1640 medium (Cellgro)

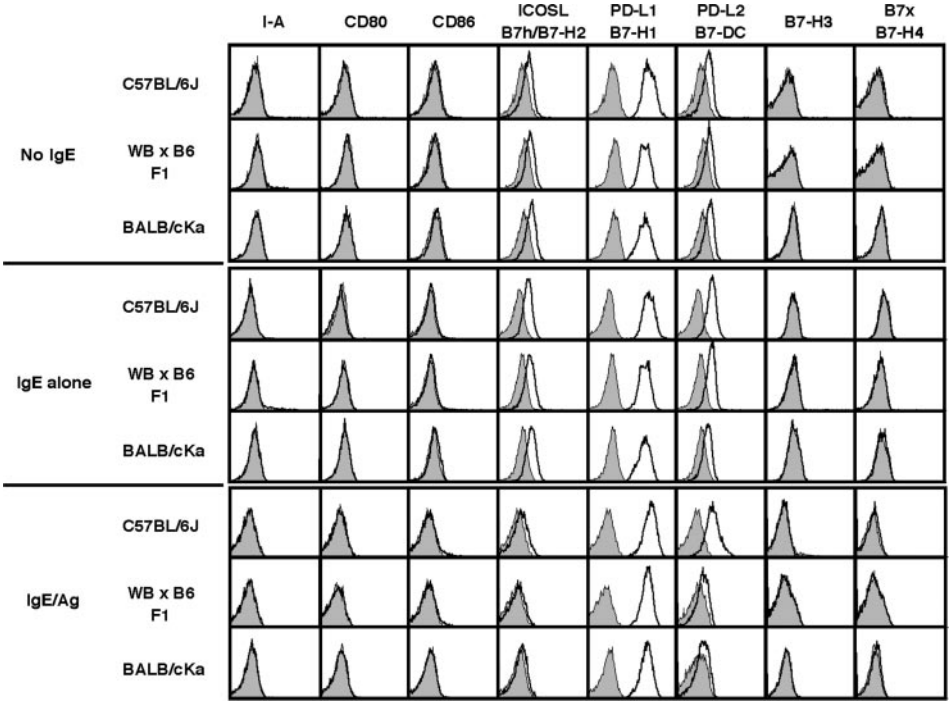

FIGURE 1. Expression of members of the B7 family of molecules on BMCMCs from C57BL/6J, WB × B6 F_1, and BALB/cKa mice. BMCMCs were incubated without IgE (No IgE) or were sensitized with 10 μg/ml anti-DNP IgE overnight, then some of these IgE-sensitized BMCMCs (IgE/Ag), but not others (IgE alone), were incubated with 20 ng/ml DNP-HSA for 6 h. The expression of cell surface molecules on c-$Kit^+Fc\epsilon RI\alpha^+$ BMCMCs was determined by flow cytometry. Shaded areas show staining with isotype-matched control Ab; bold lines show specific Ab staining. Figures show representative results from at least three independent experiments using at least two or three different batches of BMCMCs.

including 50 μM 2-ME (Sigma-Aldrich), 50 μg/ml streptomycin (Invitrogen Life Technologies), 50 U/ml penicillin (Invitrogen Life Technologies), and 10% heat inactivated FCS (Sigma-Aldrich). T cells (0.25×10^5 cells/well) were plated on a 96-well, flat-bottom plates (Falcon; BD Biosciences) coated with 1 μg/ml hamster anti-mouse CD3 mAb (145-2C11) or with an isotype-matched hamster IgG mAb (eBioscience; in some experiments, "anti-CD3 (−)" means the substitution of control IgG for anti-CD3), with mitomycin C-treated, IgE-sensitized or nonsensitized BMCMCs (0.25×10^5 cells/well) in the presence or the absence of 5 ng/ml DNP-HSA at 37°C for 72 h. In some experiments, anti-OX40L (RM134L; BD Pharmingen), anti-ICOSL (HK5.3; eBioscience), anti-PD-L1 (MI5; eBioscience), or rat IgG2a (eBioscience) was added. For pretreatment of T cells with rmTNF, C57BL/6-CD3$^+$ T cells (2×10^6 cells/well in a 12-well plate) were cultured with 0.3 μg/ml plate-coated anti-CD3 with or without rmTNF (PeproTech) at 37°C for 72 h. Then T cells pretreated with or without TNF were washed, and 0.25 or 0.03×10^5 T cells/well were cocultured with mitomycin C-treated, IgE-sensitized or nonsensitized BMCMCs (0.25 or 0.03×10^5 cells/well) in the presence or the absence of 1.0 or 0.03 μg/ml anti-CD3 mAb and in the presence or the absence of 5 ng/ml DNP-HSA, but without additional rmTNF, at 37°C for 72 h, as described above. In some coculture experiments, T cells and mast cells were separated by a Transwell membrane as described previously (27). Proliferation was assessed by pulsing with 0.25 μCi [^{3}H]thymidine (Amersham Biosciences) for 6 h, harvesting the cells using Harvester 96 Mach IIIM (Tomtec), and measuring incorporated [^{3}H]thymidine using the Micro β System (Amersham Biosciences).

ELISAs for cytokine measurements

Cytokine levels in culture supernatants were measured using mouse TNF, IL-2, IL-4, IL-6, IL-17, and IFN-γ BD OptEIA ELISA sets (BD Pharmingen).

Statistics

Student's *t* test (two-tailed) was used for statistical evaluation of the results.

Results

Expression of members of the B7 and CD28 families of costimulatory molecules on BMCMCs

It is known that mast cells are capable of Ag presentation to T cells in certain settings (31). Moreover, our group recently reported that mRNA expression of several costimulatory molecules, including OX40L and 4-1BBL, which are important for T cell activation, was increased in human umbilical cord blood-derived mast cells after stimulation with IgE and anti-IgE (22). We therefore examined mouse BMCMCs by flow cytometry for the surface expression members of the B7 molecule family, which are regarded as potent costimulatory molecules for T cell activation.

We tested three populations of BMCMCs by FACS analysis: nonsensitized (naive), IgE-sensitized, and IgE- and Ag-stimulated (IgE/Ag), because we and other investigators have reported evidence that occupancy (and perhaps cross-linking) of the high affinity IgE receptor, FcϵRI, by IgE alone can enhance mast cell survival and/or function (6, 32, 33). We found that naive BMCMCs from C57BL/6J, WB × B6 F_1 hybrid, or BALB/cKa mice did not express detectable levels of MHC class II (I-A) molecules on their cell surface (No IgE cells in Fig. 1). The expression of I-A was not increased after IgE sensitization alone or 6 h after Ag stimuli (Fig. 1). Similarly, CD80, CD86, B7-H3, and B7x (B7-H4) expression was not observed on BMCMCs from the three different strain backgrounds under any conditions tested (Fig. 1). Weak surface expression of ICOSL (B7h/B7-H2) and PD-L2 (B7-DC) was observed on naive, IgE-sensitized or IgE/Ag-stimulated BMCMCs derived from mice of the three backgrounds tested (Fig. 1). In addition, we report for the first time that a high intensity and constitutive expression of PD-L1 is detectable on BMCMCs from mice of the three genetic backgrounds examined. PD-L1 surface expression was slightly increased on BMCMCs from all three backgrounds after IgE/Ag stimulation (Fig. 1).

CD28 family members, including CD28, ICOS, CTLA-4, and PD-1, are receptors for members of the B7 family. CD28 family members are expressed on T cells, but it has been reported that CD28 expression can also be observed on murine BMCMCs and a mouse mast cell line, and that CD28 engagement can induce TNF production by mast cells (34, 35). However, to date we have not

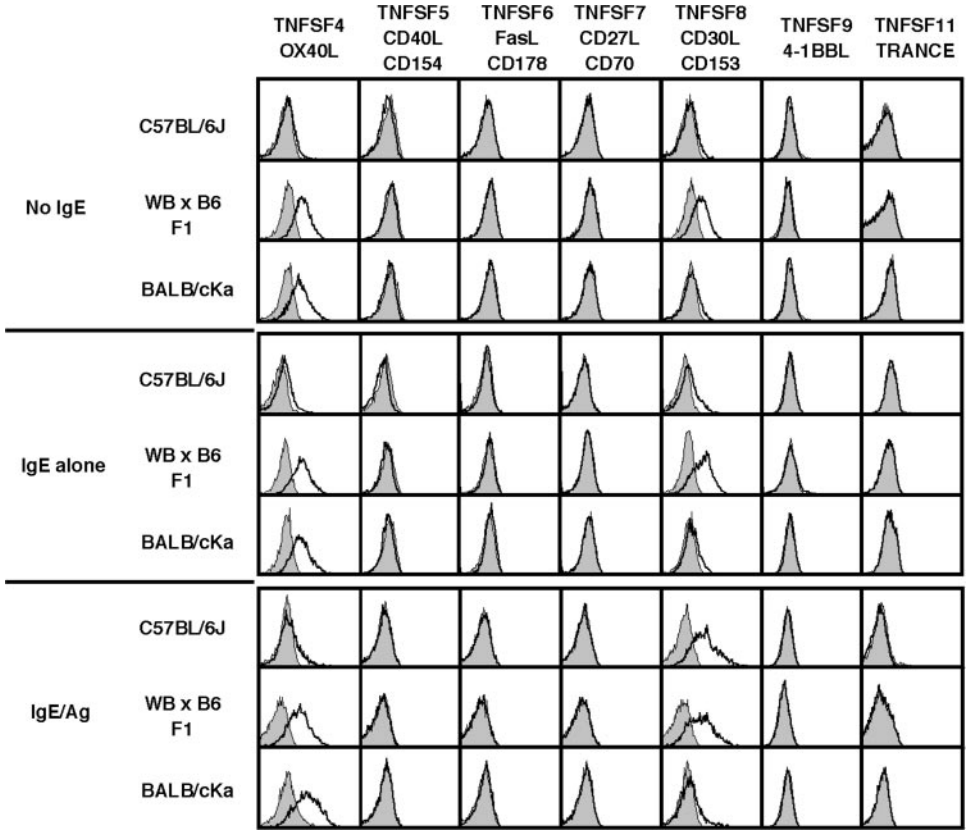

FIGURE 2. Expression of members of the TNF superfamily of molecules on BMCMCs from C57BL/6J, WB × B6 F_1, and BALB/cKa mice. BMCMCs were incubated without IgE (No IgE) or were sensitized with 10 μg/ml anti-DNP IgE overnight, then some of these IgE-sensitized BMCMCs (IgE/Ag), but not others (IgE alone), were incubated with 20 ng/ml DNP-HSA for 6 h. The expression of cell surface molecules on c-Kit$^+$FcϵRIα^+ BMCMCs was determined by flow cytometry. Shaded areas show staining with isotype-matched control Ab; bold lines show specific Ab staining. Figures show representative results from at least three independent experiments using at least two or three different batches of BMCMCs.

observed detectable surface expression of CD28, ICOS, CTLA-4, or PD-1 on mouse BMCMCs derived from any of the genetic backgrounds or in any conditions of culture tested in our experiments (data not shown). It is possible that the different results obtained by us and others reflect differences in the strains of mice used to generate BMCMCs and/or in the culture conditions or the methods of Ab staining of mast cells.

Expression of members of the TNF and TNFR families of molecules on BMCMCs

In addition to B7-CD28 interactions, interactions between TNF and TNFR family molecules represent an important mechanism of costimulation for T cell activation (36, 37). We therefore also examined the expression of TNF-TNFR family molecules on BMCMCs. It has been reported that human mast cell lines and tissue mast cells can express CD40L (CD154), and mast cell-derived CD40L is thought to contribute to IgE production by B cells (38, 39). However, we did not detect any CD40L expression on BMCMCs in our experiments (Fig. 2). We also detected no surface expression by BMCMCs of Fas ligand (CD178), CD70, or TNF-related activation-induced cytokine (also called receptor activator of NF-κB ligand; Fig. 2).

We and other investigators recently showed that OX40L and 4-1BBL are expressed on human umbilical cord blood-derived mast cells or tissue mast cells (22, 26). Consistent with these observations in human mast cells, we observed some OX40L surface expression on naive BMCMCs from all three genetic backgrounds tested (Fig. 2). However, the expression intensity was quite different among the different mast cell populations; OX40L expression by C57BL/6J-derived naive BMCMCs was barely detectable, whereas that of BALB/c- or WB $\times$ B6 F_1-derived naive BMCMCs was much stronger. The expression of OX40L was not increased by IgE sensitization alone, whereas it was slightly enhanced by IgE/Ag stimulation (Fig. 2). In contrast to the findings with human mast cells, we did not observe surface expression of 4-1BBL on our BMCMCs (Fig. 2).

The expression of CD153 (CD30L) was observed on mast cells from patients with Hodgkin's diseases (40), suggesting the contribution of mast cell-derived CD153 to the pathology associated with the disorder. In this study we report for the first time the expression of CD153 on mouse BMCMCs (Fig. 2). CD153 expression appeared to be only minimally increased after IgE sensitization alone, but expression increased substantially on C57BL/6J or WB $\times$ B6 F_1 BMCMCs after IgE/Ag stimulation (Fig. 2). As with OX40L, the expression of CD153 was influenced by the mouse genetic background: constitutive expression of CD153 was highest in WB $\times$ B6 F_1 BMCMCs, whereas IgE/Ag-dependent up-regulation was highest in C57BL/6J-derived BMCMCs (Fig. 2).

We did not detect surface expression of OX40, CD40, CD27, or CD30, which are receptors for OX40L, CD40L, CD70, or CD153, respectively, on any of our BMCMCs (Fig. 3). A small amount of TNFR1 expression was observed on naive and IgE-sensitized BMCMCs from three different genetic backgrounds, whereas expression was decreased on IgE/Ag-stimulated BMCMCs (Fig. 3). By contrast, TNFR2 and 4-1BB (CD137) expression were hardly observed on naive and IgE-sensitized BMCMCs, whereas expression was increased on IgE/Ag-stimulated BMCMCs (Fig. 3). Low levels of Fas (CD95) expression were observed on naive and IgE-sensitized BMCMCs, and expression was increased on IgE/Ag-stimulated BMCMCs (Fig. 3). Finally, we report for the first time that mouse mast cells can constitutively express GITR, and that such expression was not changed by IgE sensitization or IgE/Ag stimulation of BMCMCs (Fig. 3).

TNF can enhance T cell expression of costimulatory molecules

The findings shown in Figs. 1–3 suggest that mast cells may be able to act as accessory cells to promote T cell activation through the expression of several different costimulatory molecules. However, we recently reported that mast cells can promote T cell activation, such as proliferation and cytokine production, by at least one mechanism that requires proximity between T cells and mast cells as well as by the production of soluble factors (27). We also showed that one pathway by which IgE- and Ag-activated mast cells could stimulate T cells was by production of TNF (27). However, the mechanism by which mast cell-derived TNF promotes T cell activation was not clarified.

When APCs, such as DCs, present Ags to T cells, soluble factors produced by APCs, such as IL-1, promote both T cell proliferation

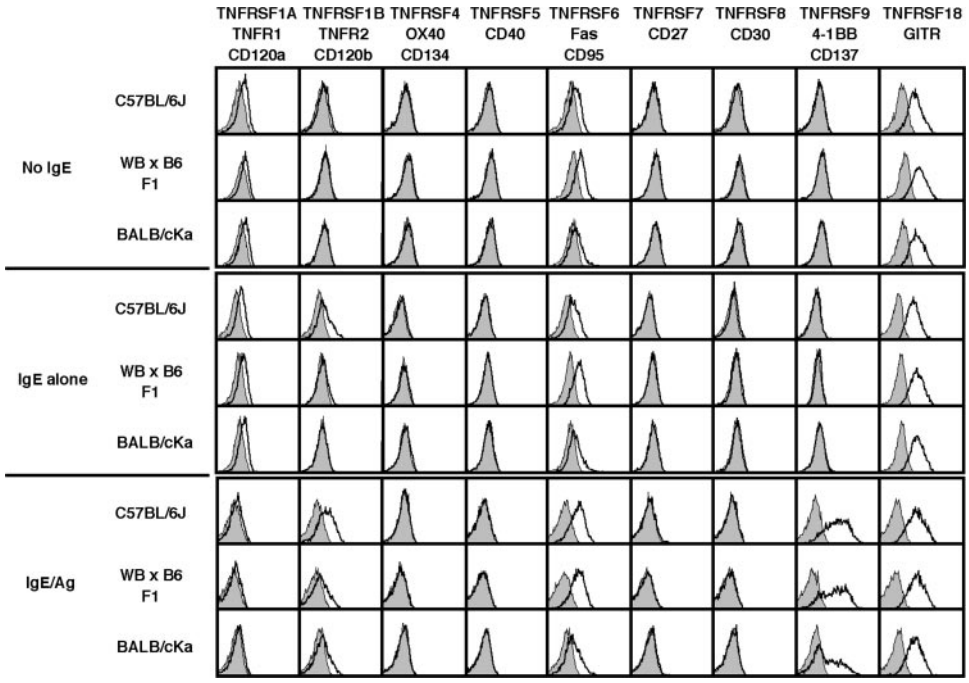

FIGURE 3. Expression of members of the TNFR superfamily of molecules on BMCMCs from C57BL/6J, WB $\times$ B6 F_1, and BALB/cKa mice. BMCMCs were incubated without IgE (No IgE) or were sensitized with 10 μg/ml anti-DNP IgE overnight, then some of these IgE-sensitized BMCMCs (IgE/Ag), but not others (IgE alone), were incubated with 20 ng/ml DNP-HSA for 6 h. The expression of cell surface molecules on c-Kit$^+$FcϵRIα^+ BMCMCs was determined by flow cytometry. Shaded areas show staining with isotype-matched control Ab; bold lines show specific Ab staining. Figures show representative results from at least three independent experiments using at least two or three different batches of BMCMCs.

and the expression of some costimulatory molecules, such as CD40L and OX40, on T cells (41). TNF can have pathophysiological functions similar to those of IL-1 in various host defense responses (42). We therefore assessed whether TNF can promote the expression of costimulatory molecules on T cells. Splenic T cells were stimulated with plate-coated anti-CD3 mAb or an isotype-matched control hamster IgG mAb (0.3 μg/ml) in the presence of various concentrations of rmTNF. We found that the expression levels of OX40, 4-1BB, GITR, ICOS, and PD-1 on C57BL/6J CD4$^+$ T cells were markedly enhanced by rmTNF (10 or 100 ng/ml; Fig. 4*A*). Notably, the expression of 4-1BB, GITR, and PD-1 at the highest dose of rmTNF tested (100 ng/ml) was about the same or less than that observed at 10 ng/ml rmTNF, suggesting that the optimal concentration of rmTNF for enhancing the expression of costimulatory molecules may be between 10 and 100 ng/ml. The surface expression of CTLA-4, CD40L, or CD30 was not affected by culture with rmTNF (data not shown). Similar effects of rmTNF on OX40, 4-1BB, ICOS, PD-1, and GITR

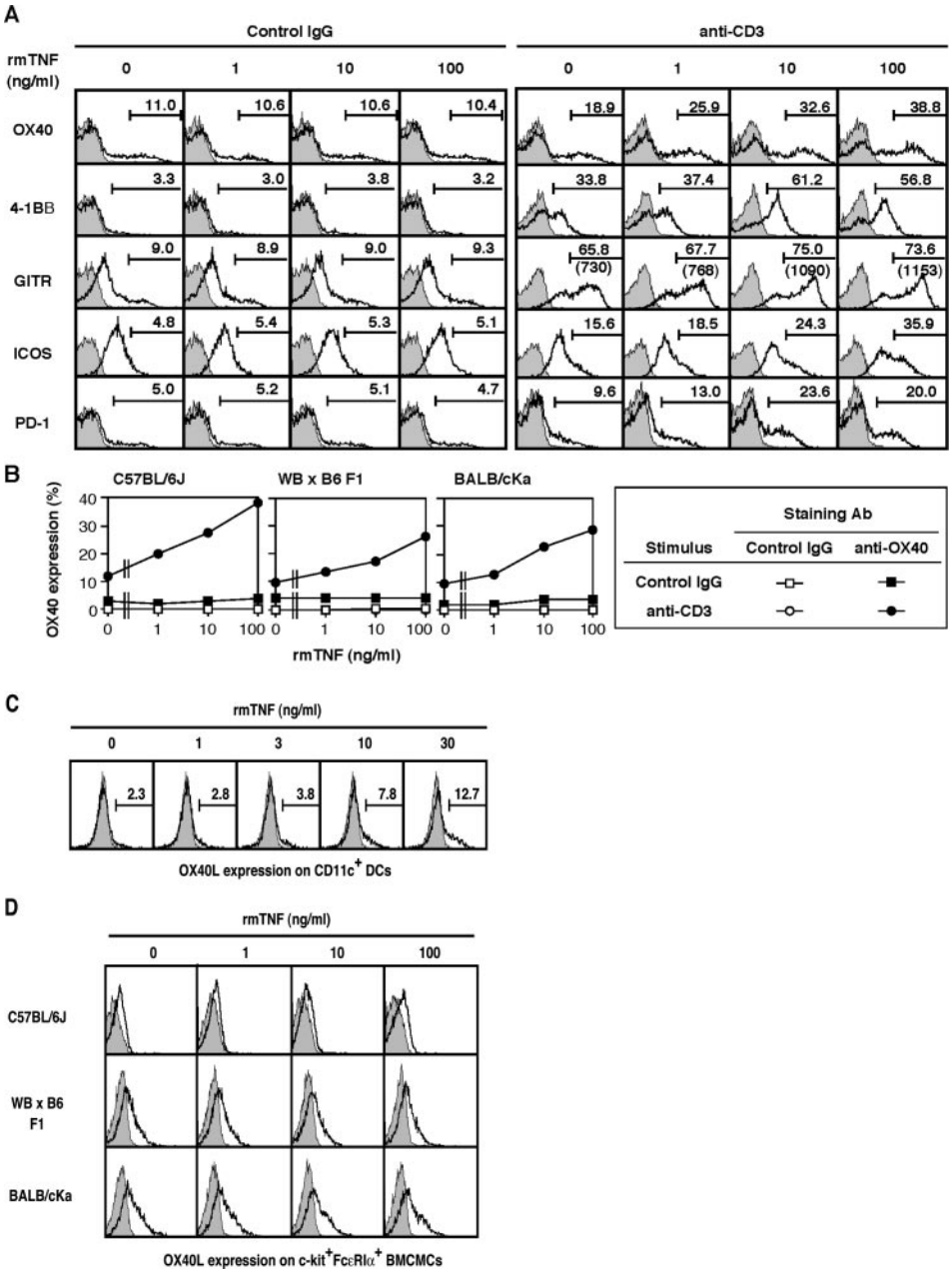

FIGURE 4. Effect of exogenous TNF on the expression of activation markers on T cells, DCs, or BMCMCs. *A* and *B*, Purified splenic CD3$^+$ T cells from C57BL/6J mice (*A* and *B*), WB $\times$ B6 F_1 mice (*B*), or BALB/cKa mice (*B*) were stimulated with plate-coated anti-CD3 mAb (0.3 μg/ml) or isotype-matched control hamster IgG in the presence of various concentrations of rmTNF for 72 h. The expression of costimulatory molecules on CD3$^+$CD4$^+$ cells was examined by flow cytometry. *B*, Symbols show the mean of triplicate measurements, but the error bars (to show the mean $\pm$ SD) are so small that they are contained within the symbols in the graphs. *C*, Spleen cells from Rag-1$^{-/-}$ mice on the C57BL/6J background were cultured with various concentrations of rmTNF for 72 h. The expression of OX40L on CD11c$^+$ cells was examined by flow cytometry. *D*, Anti-DNP IgE-sensitized C57BL/6J, WB $\times$ B6 F_1, or BALB/cKa BMCMCs were cultured with 20 ng/ml DNP-HSA in the presence of various concentrations of rmTNF for 72 h. The expression of OX40L on c-Kit$^+$FcϵRIα^+ cells was examined by flow cytometry. Shaded areas in *A*, *C*, and *D* show staining with an isotype-matched control Ab; bold lines show specific Ab staining. Numbers in *A* and *C* are the percentage of expression, and numbers in parentheses in *A* represent the fluorescence intensity. *A–D* show representative results from at least two or three independent experiments.

expression were observed in C57BL/6J $CD8^+$ T cells (data not shown). We found that rmTNF enhanced the expression of OX40 in WB × B6 F_1 or BALB/cKa $CD3^+CD4^+$ T cells that were stimulated with plate-coated anti-CD3 mAb as well as identically treated T cells that were isolated from C57BL/6J mice (Fig. 4*B*)

In both $CD4^+$ and $CD8^+$ T cells, rmTNF enhanced the expression of these costimulatory molecules in the presence of low concentrations of anti-CD3 mAb (0.1 and 0.3 μg/ml), but not when high concentrations of anti-CD3 mAb (1, 3, and 10 μg/ml) were used (data not shown). Similar observations were obtained using rmIL-1 (41) or anti-CD28 mAb (data not shown). These results indicate that exogenous TNF can promote the expression of some costimulatory molecules (including OX40, ICOS, and PD-1) on T cells when stimulation via the TCR/CD3 complex is relatively weak, but TNF is not required for high levels of expression of these molecules when T cells are strongly activated via the TCR/CD3 complex.

TNF also has an important role in modulating certain aspects of APC biology, i.e., DC migration and functional maturation (43). When spleen cells from Rag-$1^{-/-}$ mice were cultured in the presence of rmTNF for 72 h, OX40L expression was markedly increased on $CD11c^+$ DCs (Fig. 4*C*). We also assessed whether rmTNF influenced the expression of OX40L on BMCMCs from wild-type C57BL/6J, WB × B6 F_1, or BALB/cKa mice. BMCMCs were sensitized with anti-DNP IgE overnight, then cultured with DNP-HSA (10 ng/ml) in the presence of various concentrations of rmTNF (0, 1.0, 10, or 100 ng/ml) for 6 or 72 h. However, the expression of OX40L on C57BL/6J, WB × B6 F_1 or BALB/cKa BMCMCs was not increased by rmTNF (Fig. 4*D* and data not shown), nor did rmTNF enhance the expression of these molecules on C57BL/6-$TNF^{-/-}$ BMCMCs (data not shown). In C57BL/6J or WB × B6 F_1 BMCMCs, incubation with rmTNF for 72 h did not alter the expression of any of the other costimulatory molecules shown in Figs. 1–3 (data not shown). These results indicate that neither endogenous mast cell-derived TNF nor exogenous TNF can significantly influence the expression of any of the costimulatory molecules examined in Figs. 1–3 on BMCMCs from C57BL/6J or WB × B6 F_1 mice, nor can TNF significantly influence the expression of OX40L on BMCMCs from BALB/cKa mice.

Mast cell-derived soluble TNF, but not membrane-bound TNF, is required for optimal T cell activation by mast cells

In accord with results we recently reported (27), we confirmed that culturing low concentrations of purified splenic $CD3^+$ T cells (0.25×10^5 cells/well in 96-well plates) in the presence of plate-bound anti-CD3 mAb for 72 h did not induce significant T cell proliferation, as determined by incorporation of [^{3}H]thymidine (T cells alone in Fig. 5*B*). However, when such T cells (0.25×10^5 cells/well in 96-well plates) were cocultured with mitomycin C-treated, wild-type BMCMCs (0.25×10^5 cells/well in 96-well plates) that had been stimulated with IgE/Ag, T cell proliferation was markedly increased (Fig. 5*B*) (27). Consistent with our previous observations, we confirmed that $TNF^{-/-}$ BMCMCs did not enhance T cell proliferation efficiently compared with wild-type BMCMCs (Fig. 5*B*).

TNF consists of two biologically active forms, secreted and membrane-bound. The membrane-bound form is processed into the cleaved, secreted form by TNF-converting enzyme (44). As shown in Fig. 5*A*, $memTNF^{\Delta/\Delta}$ BMCMCs derived from mutant mice (C57BL/6 $memTNF^{\Delta/\Delta}$), which cannot generate soluble TNF from the membrane-bound form, were not able to secrete TNF into the culture supernatants 3 h after stimulation with either PMA and ionophore or IgE/Ag, whereas we observed by FACS analysis that $memTNF^{\Delta/\Delta}$ BMCMCs did contain the presumably uncleaved form of TNF intracellularly (data not shown). In contrast, like $TNF^{-/-}$ BMCMCs, $memTNF^{\Delta/\Delta}$ BMCMCs exhibited normal BMCMC morphology by light microscopy as well as a normal ability to release β-hexosaminidase and IL-6 6 h after PMA and ionophore or IgE/Ag stimulation (Fig. 5*A* and data not shown). Notably, neither $memTNF^{\Delta/\Delta}$ BMCMCs nor $TNF^{-/-}$ BMCMCs

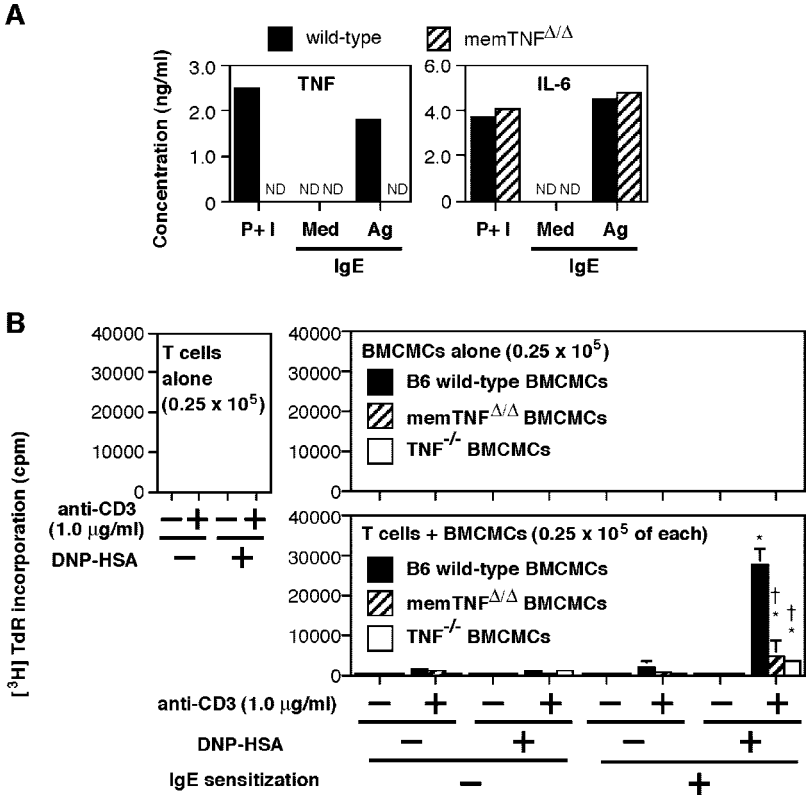

FIGURE 5. Soluble TNF, but not membrane-bound TNF, is important for T cell proliferation mediated by IgE/Ag-stimulated BMCMCs. *A*, BMCMCs were incubated with or without 10 μg/ml anti-DNP IgE overnight. After washing, BMCMCs were cultured in the presence (Ag) or the absence (medium alone (Med)) of 20 ng/ml DNP-HSA or PMA and ionophore (P + I) for 3 h (for TNF) or 6 h (for IL-6). TNF and IL-6 levels in the supernatants were determined by ELISA. ■, Wild-type C57BL/6J BMCMCs; ▨, $memTNF^{\Delta/\Delta}$ BMCMCs. Data are the mean ± SD (triplicate wells) and show representative results from at least two independent experiments using two different batches of BMCMCs. ND, not detected. *B*, Proliferation of C57BL/6J $CD3^+$ T cells (0.25×10^5 cells) cocultured with mitomycin C-treated C57BL/6J (B6) wild-type, $memTNF^{\Delta/\Delta}$, or $TNF^{-/-}$ BMCMCs (0.25×10^5 cells). [^{3}H]Thymidine incorporation was measured at 72 h. ■, Wild-type BMCMCs; ▨, $memTNF^{\Delta/\Delta}$ BMCMCs; □, $TNF^{-/-}$ BMCMCs. *B*: *, $p < 0.05$ vs any other values for T cells plus BMCMCs of that genotype that were not stimulated with IgE, Ag (DNP-HSA), and anti-CD3; +, $p < 0.05$ vs corresponding values for T cells plus wild-type BMCMCs. Data are the mean ± SD (triplicate wells) and show representative results from at least three independent experiments using at least two different batches of BMCMCs.

promoted T cell proliferation efficiently compared with wild-type BMCMCs (Fig. 5*B*). These findings indicate that secreted TNF, rather than membrane-bound TNF, is required for the optimal enhancement of T cell proliferation by IgE/Ag/FcεRI-stimulated BMCMCs.

OX40L is important for optimal T cell activation by IgE/Ag-stimulated BMCMCs

Based on these findings, we hypothesized that TNF secreted by mast cells can induce or enhance the expression of certain costimulatory molecules on T cells, and that interactions between the costimulatory molecules expressed by such T cells and the complementary mast cell costimulatory molecules can enhance T cell proliferation.

TNF can enhance OX40 expression on $CD4^+$ and $CD8^+$ T cells (Fig. 4*A* and data not shown), and BMCMCs express OX40L (Fig. 2), suggesting that mast cell-derived TNF may contribute to T cell activation by promoting the engagement of T cell OX40 with mast cell OX40L. Indeed, we found that OX40 expression on T cells that had been cocultured with $TNF^{-/-}$ BMCMCs under conditions similar to those used for the experiments shown in Fig. 5 was decreased compared with that in T cells that had been cocultured with wild-type BMCMCs (data not shown). Moreover, when T cells were cocultured with IgE/Ag-stimulated BMCMCs for 72 h under conditions in which the two cell types could be in close proximity or contact ("in proximity" in Fig. 6), T cell proliferation was significantly greater than that when the T cells were cocultured with IgE/Ag-stimulated BMCMCs from which they were separated by a transmembrane ("separated" in Fig. 6). These findings support the conclusion that cell-cell proximity between T cells and mast cells can significantly contribute to the ability of mast cells to enhance T cell activation.

We found that in cells from BALB/cKa mice, but not from C57BL/6J mice, a neutralizing anti-OX40L mAb significantly decreased the T cell proliferation and IFN-γ and IL-17 production induced by coculture of T cells with IgE/Ag-stimulated BMCMCs (Fig. 7*A*). By contrast, levels of IL-2 and IL-4 in such supernatants were below the limits of detection of ELISA in any of the culture conditions tested (data not shown). These results correlate quite well with our observation of much higher levels of expression of OX40L on BALB/cKa vs C57BL/6J BMCMCs (Fig. 2).

It is known that costimulatory molecules in the B7 and CD28 families also can have important roles in T cell activation. ICOSL was originally identified as a molecule whose expression is up-regulated by TNF (45). ICOSL is weakly expressed on IgE/Ag-stimulated BMCMCs, and PD-L1, which is known to have both positive and negative regulatory effects on T cell activation (46, 47), is constitutively expressed on BMCMCs (Fig. 1). Moreover, the expression of ICOS and PD-1, which are receptors for ICOSL and PD-L1/2, respectively, was enhanced by rmTNF treatment of T cells (Fig. 4). However, the surface expression of ICOSL and PD-L1 was not different between wild-type and $TNF^{-/-}$ BMCMCs (data not shown) The addition of neutralizing anti-ICOSL or anti-PD-L1 mAbs had no detectable effect on mast cell-dependent enhancement of T cell proliferation using cells from either the C57BL/6J or BALB/cKa background (Fig. 7*B*). As a control to establish the effectiveness of these neutralizing Abs, we determined that the same concentrations of anti-ICOSL or anti-PD-L1 mAbs that were used in our mast cell-T cell coculture experiments were able to significantly reduce (anti-ICOSL) or increase (anti-PD-L1) the proliferation of OVA-specific TCR expressing OTII transgenic mouse T cells when these cells were cocultured with APCs in the presence of OVA peptides (data not shown).

These results suggest that IgE/Ag/FcεRI- and TNF-dependent enhancement of T cell proliferation by mast cells can be influenced by T cell OX40 and mast cell OX40L interactions in BALB/cKa mice, but probably is mediated by molecules other than OX40L, ICOSL, or PD-L1 in C57BL/6J mice. Alternatively, the response may reflect the synergistic effects of several costimulatory molecules and may not depend on a single costimulatory pathway. However, at least on the BALB/cKa mouse background, surface expression of OX40L on BMCMCs can contribute significantly to the T cell activation mediated through mast cell-T cell contact.

As shown in Fig. 4, *A* and *B*, exogenous TNF can promote the expression of OX40 and other costimulatory molecules on T cells. Therefore, we examined whether pretreatment of T cells with rmTNF might be able to compensate for the role of mast cell-derived TNF in the enhancement of T cell proliferation by mast cells. As shown previously (27), once primary T cells have been stimulated with anti-CD3 mAb, T cell proliferation can be promoted by exposure to a very small amount of anti-CD3 mAb during the period of secondary stimulation. We found that when anti-CD3-stimulated T cells (0.25×10^5 cells, incubated with or without rmTNF for 72 h) were then cocultured with IgE-sensitized or nonsensitized BMCMCs (0.25×10^5 cells) in the presence of 1 μg/ml anti-CD3 (or control hamster IgG) with or without DNP-HSA, strong secondary T cell proliferation in was observed in response to anti-CD3 regardless of pretreatment with rmTNF or

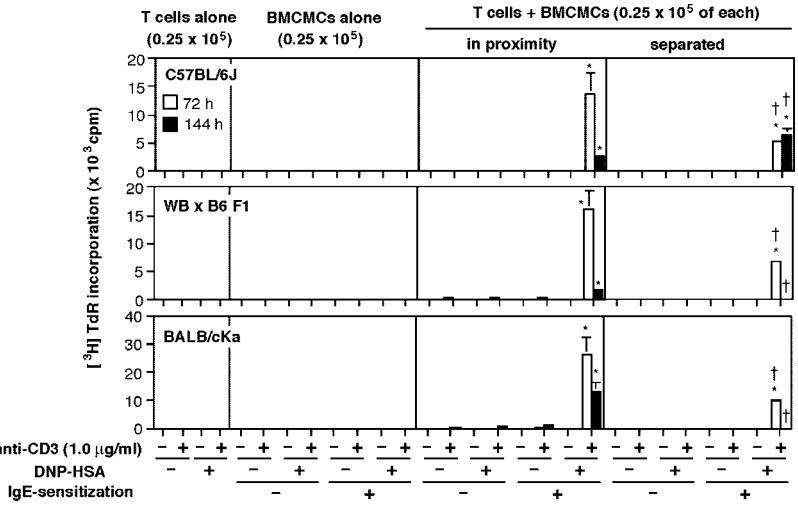

FIGURE 6. Importance of cell-cell proximity for T cell proliferation by IgE/Ag-stimulated BMCMCs. Proliferation of C57BL/6J, WB × B6 F_1, or BALB/cKa $CD3^+$ T cells (0.25×10^5 cells) cocultured together with (in proximity) mitomycin C-treated C57BL/6J, WB × B6 F_1, or BALB/cKa BMCMCs (0.25×10^5 cells) or separated from them by a Transwell membrane (separated). [^{3}H]thymidine incorporation was measured at 72 h (□) or 144 h (■). Data are the mean ± SD (triplicate wells) and show representative results from two independent experiments using two different batches of BMCMCs. *, $p < 0.05$ vs any other values for cells at that time point cultured under the same conditions (e.g., T cells and BMCMCs in proximity or separated), but that were not stimulated with IgE, Ag (DNP-HSA), and anti-CD3; +, $p < 0.05$ vs corresponding values for cells cocultured in proximity.

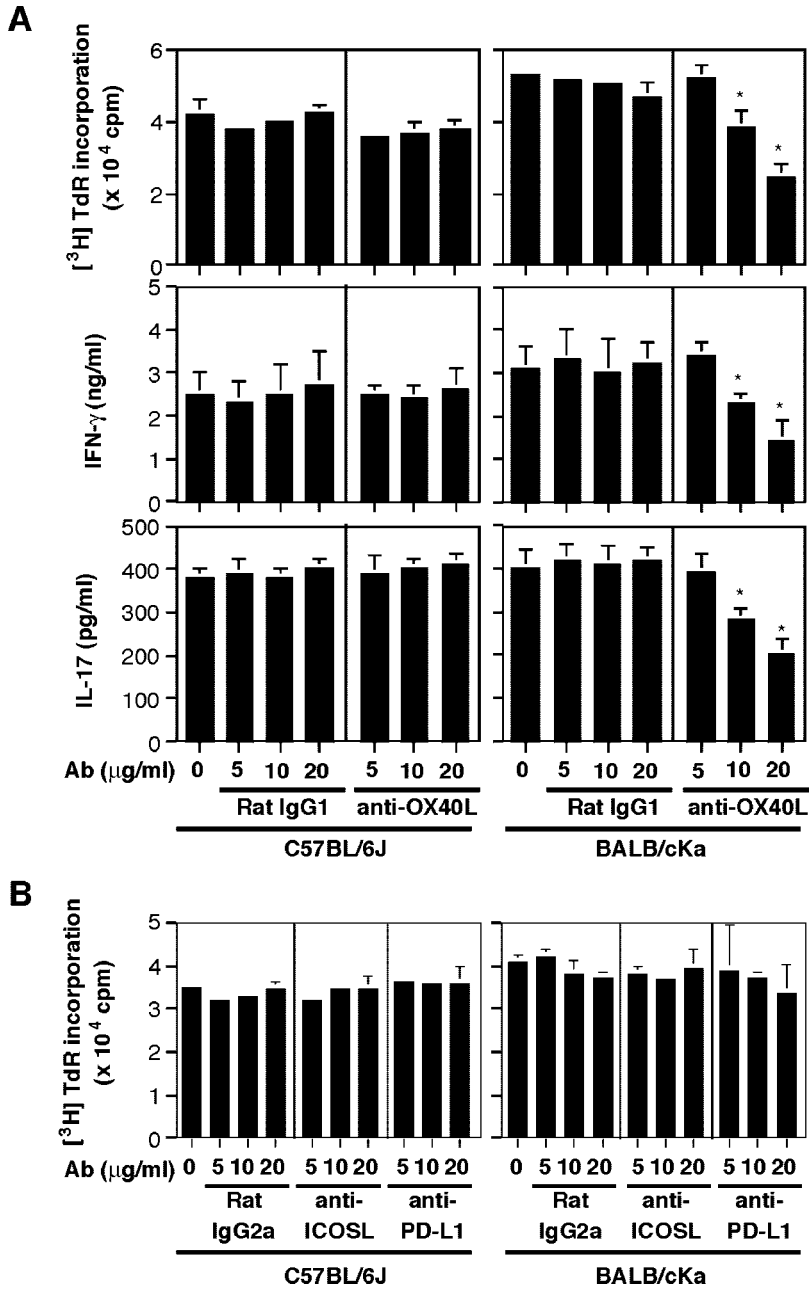

FIGURE 7. The effects of anti-OX40, anti-ICOS, or anti-PD-L1 neutralizing Abs on the enhancement of T cell proliferation by IgE/Ag-stimulated mast cells. *A*, Proliferation and IFN-γ and IL-17 production of C57BL/6J or BALB/cKa $CD3^+$ T cells (0.25×10^5 cells) cocultured with mitomycin C-treated IgE/Ag-stimulated wild-type BMCMCs (0.25×10^5 cells) of the same genetic background as the T cells in the presence of isotype-matched control IgG (rat IgG1) or anti-OX40L mAb. IL-2 and IL-4 levels were below the limit of detection in all of the culture conditions. *, $p < 0.05$ vs corresponding values for treatment with rat IgG1. *B*, Proliferation of C57BL/6J or BALB/cKa $CD3^+$ T cells (0.25×10^5 cells) cocultured with mitomycin C-treated IgE/Ag-stimulated wild-type BMCMCs (0.25×10^5 cells) of the same genetic background as the T cells in the presence of isotype-matched control IgG (rat IgG2a), anti-ICOSL mAb, or anti-PD-L1 mAb. Data are the mean ± SD (triplicate wells). Data in *A* and *B* show representative results from at least two independent experiments using at least two different batches of BMCMCs.

the presence or the absence of BMCMCs, IgE/Ag stimulation, or mast cell-derived TNF (Fig. 8*A*).

However, when we reduced the concentration of anti-CD3 mAb during the period of secondary stimulation from 1.0 to 0.03 μg/ml and also reduced the concentration of Th/Tc cells and BMCMCs from 0.25×10^5 to 0.03×10^5 cells, optimal enhancement of T cell proliferation required BMCMCs, IgE/Ag stimulation, and mast cell-derived TNF, but did not seem to be affected by pretreatment of the T cells with rmTNF (Fig. 8*B*). Nevertheless, some BMCMC- and IgE/Ag-dependent enhancement of T cell proliferation was induced under these conditions even by $TNF^{-/-}$ BMCMCs (Fig. 8*B*).

Although different factors may influence the extent of primary vs secondary T cell activation, our data indicate that pretreatment of T cells with TNF during the period of primary stimulation cannot compensate for the role of mast cell-derived TNF in promoting the IgE/Ag- and mast cell-dependent enhancement of secondary T cell proliferation. These findings taken together with the results of our studies of $TNF^{-/-}$ vs $memTNF^{\Delta/\Delta}$ BMCMCs (Fig. 5*B*) and our cell separation experiments (Fig. 6) suggest that optimal IgE/Ag- and mast cell-dependent enhancement of T cell proliferation requires both cell-cell proximity and effects of soluble TNF derived from mast cells.

Discussion

Our findings clarify some of the mechanisms by which mast cells activated via FcεRI aggregation can enhance T cell activation. However, they also reveal considerable complexity in this potentially important mast cell-T cell interaction. Thus, we showed that the mast cell TNF-dependent component of this interaction required that the mast cells are able to produce soluble TNF; mast cells derived from mice that expressed only the membrane-associated form of the molecule, such as BMCMCs which completely lacked the ability to make soluble TNF, had essentially no activity in our mast cell-T cell coculture assay (Fig. 5*B*). Similarly, we showed that a neutralizing Ab

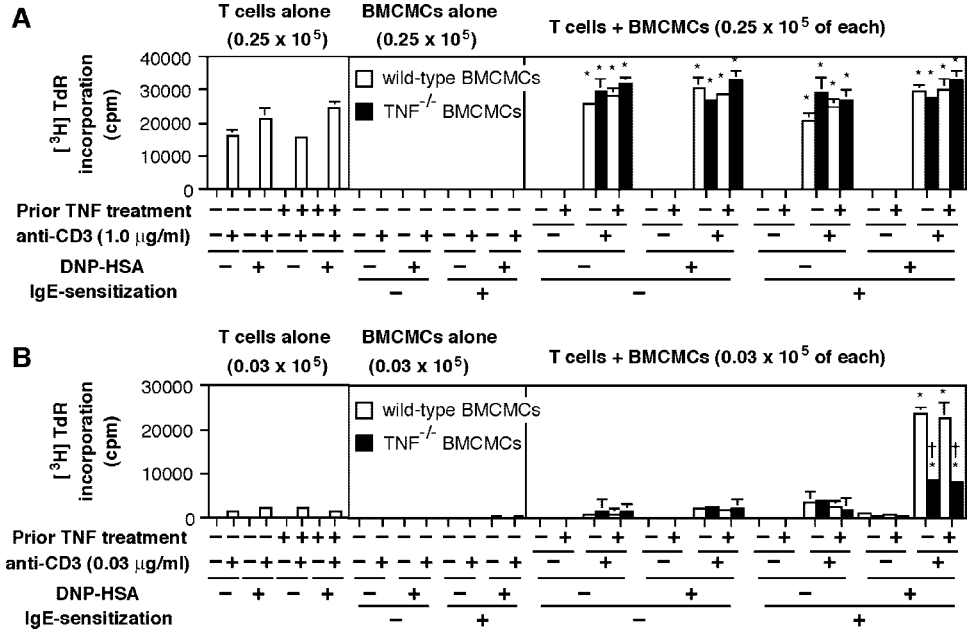

FIGURE 8. Pretreatment of T cells with TNF did not compensate for the effect of mast cell-derived TNF on the enhancement of T cell proliferation by mast cells. *A*, T cells pretreated for 72 h with or without 100 ng/ml rmTNF (0.25×10^5 cells) were cocultured with IgE-sensitized or nonsensitized wild-type or $TNF^{-/-}$ BMCMCs (0.25×10^5 cells) in the presence or the absence of anti-CD3 mAb (1 μg/ml) with or without DNP-HSA for an additional 72 h. *B*, T cells pretreated for 72 h with or without 100 ng/ml rmTNF (0.03×10^5 cells) were cocultured with IgE-sensitized or nonsensitized wild-type or $TNF^{-/-}$ BMCMCs (0.03×10^5 cells) in the presence or the absence of anti-CD3 mAb (0.03 μg/ml) with or without DNP-HSA for an additional 72 h. Data are the mean ± SD (triplicate wells) and show representative results from two independent experiments using two different batches of BMCMCs. *A*: *, $p < 0.05$ vs the corresponding values for T cells alone. *B*: *, $p < 0.05$ vs any other values for T cells and BMCMCs not stimulated with IgE, Ag (DNP-HSA), and anti-CD3; +, $p < 0.05$ vs corresponding values for T cells cocultured with wild-type BMCMCs.

to one mast cell-expressed costimulatory molecule, OX40L, significantly reduced (by ~50%) the ability of IgE- and Ag-stimulated BALB/cKa mast cells to enhance T cell proliferation and cytokine (IFN-γ and IL-17) secretion (Fig. 7*A*). Notably, we also showed that the surface expression of OX40 on T cells was markedly enhanced by incubation of the cells in TNF (Fig. 4, *A* and *B*) and that IgE- and Ag-dependent stimulation of BALB/cKa BMCMCs modestly enhanced their surface expression of OX40L (Fig. 2).

Taken together, these data strongly suggest a model in which 1) IgE- and Ag-dependent mast cell activation both enhances OX40L expression by mast cells and induces mast cells to secrete soluble TNF; 2) in response to such mast cell-derived TNF (and perhaps other mast cell- or T cell-derived factors, including T cell-derived TNF) (27), the T cells up-regulate surface expression of OX40; and 3) interactions between mast cell-associated OX40L and T cell-associated OX40 then contribute to enhancement of T cell proliferation and cytokine secretion.

However, that simple model cannot fully explain the ability of mouse mast cells stimulated via FcεRI aggregation to enhance T cell activation. The clearest illustration of this point is the fact that C57BL/6J BMCMCs are fully capable, when activated by IgE and Ag, of enhancing T cell activation. Yet C57BL/6J BMCMCs express only very low levels of OX40L on their surface, even after activation by FcεRI aggregation (Fig. 2). Moreover, in accord with these findings, in cocultures of cells derived from C57BL/6J mice, a neutralizing Ab to OX40L had no significant effect on the ability of mast cells stimulated with IgE and Ag to enhance T cell activation (Fig. 7*A*).

OX40L is not the only costimulating molecule whose surface expression varied considerably among mast cells derived from the three genetic backgrounds. For example, IgE- and Ag-challenge provoked a much stronger up-regulation of PD-L2 (B7-DC) in C57BL/6J BMCMCs than in either WB × B6 F_1 or BALB/cKa BMCMCs (Fig. 1), and TNFSF8 (CD30L, CD153) was more highly expressed at baseline in WB × B6 F_1 BMCMCs than in BMCMCs from the other two strains (Fig. 2).

All BMCMCs tested in these experiments were derived under identical conditions of culture. However, it is possible that the pattern of expression of costimulatory molecules by mast cells might vary in cells that had been cultured under other conditions, in mast cells derived from various tissues or anatomical sites of origin, or according to the stage of mast cell maturation. Indeed, Kashiwakura et al. (26) reported that human mast cells derived from tonsils and lungs expressed OX40L and 4-1BBL after stimulation via FcεRI aggregation. In addition, we have observed different expression patterns of activation markers among C57BL/6J peritoneal mast cells (PMCs), BMCMCs, embryonic stem cell-derived cultured mast cells (ESCMCs; generated as described previously (48)), and fetal liver cell-derived cultured mast cells (FLCMCs). We detected constitutive expression of PD-L1 and Fas/CD95 on c-Kit^+FcεRIα^+ PMCs from C57BL/6J mice without stimulation, whereas the expression of OX40L, CD153, and GITR was not observed (data not shown). No expression of I-A, CD80, CD86, or CD40 was observed on BMCMCs with or without IgE/Ag stimulation (as shown in Figs. 1 and 3), whereas low level expression of these molecules was observed on PMCs without stimulation (data not shown). After IgE/Ag stimulation, the expression of CD153 and Fas and low levels of OX40L were observed on C57BL/6-BMCMCs (Fig. 2), whereas the expression of CD153 and Fas, but not OX40L, was detected on C57BL/6J-FLCMCs, and none of these costimulatory molecules (CD153, Fas or OX40L) was detected on C57BL/6J-ESCMCs (data not shown). Thus, even in a single strain of mouse, mast cells derived from different sources in vitro or in vivo can exhibit differences in their patterns of expression of costimulatory molecules.

Our findings with BMCMCs derived from BALB/cKa mice are in accord with those reported by Kashiwakura et al. (26) for

human tonsil mast cells; in both cases, FcεRI-dependent stimulation of the mast cells resulted in enhanced surface expression of OX40L, and experiments with neutralizing Abs showed that mast cell OX40L:T cell OX40 interactions contributed to the T cell proliferation observed in these cell coculture systems. One difference between the two studies is that Kashiwakura et al. (26) used an allogeneic system, in which mast cells from one human donor were cocultured with T cells from another. In such a system, one cannot formally rule out the possibility that a mixed lymphocyte culture-type interaction between genetically distinct mast cells and T cells might have influenced the results. In our studies both mast cells and T cells were always derived from mice of the same inbred genetic backgrounds. Therefore, none of our results could have been influenced by such mixed lymphocyte culture effects.

In addition to OX40L, we detected the expression of a wide panel of costimulatory molecules on mouse BMCMCs, and in some cases these molecules have not previously been reported on mouse (or any) mast cells. Such newly reported mouse mast cell-associated costimulatory molecules include PD-L1 and GITR (not before reported on any mast cells) and CD30L (CD153) (not before reported on mouse mast cells). The potential roles of these molecules and of other mast cell-associated costimulatory molecules remain to be determined. However, we found that both PD-L1 and the mouse Th2 cell- (49) and mast cell- (50) associated surface molecule, T1/ST2/IL-IR4 were constitutively expressed at relatively high levels on the surface of C57BL/6 PMCs, BMCMCs, ESCMCs, and FLCMCs (Fig. 1 and data not shown). Accordingly, it is possible that PD-L1 will be useful as an additional lineage marker for mast cells.

In summary, we find that the IgE-, Ag-, and TNF-dependent mechanism by which mast cells can enhance T cell activation depends on soluble TNF, not membrane-associated TNF. Moreover, in BALB/cKa mice, the optimal expression of this mast cell-dependent mechanism of T cell activation requires interactions between mast cell OX40L and T cell OX40. Mast cells and T cells can occur in close proximity during a variety of examples of acquired immune response, including those involved in host defense, autoimmunity, and allergic disorders (27, 51). Accordingly, we speculate that the mechanism of mast cell- and TNF-dependent T cell activation investigated in this study may contribute to the progression or intensity of many different immune responses and immunological diseases.

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Disclosures

The authors have no financial conflict of interest.

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