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The CD70-CD27 interaction during the stimulation with dendritic cells promotes naïve CD4$^+$ T cells to develop into T cells producing a broad array of immunostimulatory cytokines in humans

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Abbreviations

APC	antigen-presenting cell
CD40L	CD40 ligand
DC	dendritic cell
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
mAb	monoclonal antibody
mDC	myeloid dendritic cell
MFI	mean fluorescence intensity
MoDC	monocyte-derived dendritic cell
ODN	oligodeoxynucleotide
OX40L	OX40 ligand
PBMC	peripheral blood mononuclear cell
pDC	plasmacytoid dendritic cell
PE	phycoerythrin
PG	prostaglandin
PMA	phorbol myristate acetate
poly I:C	polyinosinic-polycytidylic acid
Th	T helper
TLR	Toll-like receptor
TNF	tumor necrosis factor
TSLP	thymic stromal lymphopoietin

Abstract

CD70 expressed on dendritic cells (DCs) has been shown to play a critical role in inducing effective $CD8^+$ T cell responses and a Th1 response in mice. However, it has not been extensively examined whether human primary DCs express CD70 and whether the CD70-CD27 interaction promotes naïve $CD4^+$ T cells to acquire the ability to produce effector cytokines during the DC-T cell interaction in humans. Here we show that human myeloid DCs (mDCs) and plasmacytoid DCs (pDCs) stimulated with CD40 ligand together with proinflammatory cytokines or Toll-like receptor ligands express CD70. Thymic stromal lymphopoietin plus prostaglandin E_2 also induced CD70 on mDCs. Naïve $CD4^+$ T cells stimulated with DCs but not with anti-CD3/CD28 microbeads expressed CD70. Stimulation with CD70 together with anti-CD3/CD28 microbeads imparted the ability to produce T helper (Th)1 (IFN-γ), Th2 (IL-4, IL-5, IL-13) cytokines, IL-2, and TNF-α to naïve $CD4^+$ T cells. The production of IFN-γ was associated with the induction of T-bet. Naïve $CD4^+$ T cells stimulated with mDCs acquired an enhanced ability to produce a broad array of immunostimulatory cytokines in a CD70-dependent manner. These data suggest that human CD70 expressed on mDCs and activated T cells transmits a “basal level” signal, rather than a “polarizing” signal, to naïve $CD4^+$ T cells, in that CD70 promotes the development of $CD4^+$ T cells that produce a variety of effector cytokines including both Th1 and Th2 types, thus contributing to enhancement of a broad spectrum of immune responses.

Introduction

Dendritic cells (DCs) play a pivotal role in the activation of naïve T cells as the most potent antigen-presenting cells (APCs) (1). In humans, DCs are composed of two subsets that have different functions: myeloid DCs (mDCs), which induce either T helper (Th)1 or Th2 responses depending on environmental stimuli, and plasmacytoid DCs (pDCs), which produce a large amount of interferon (IFN)-α in response to viruses and are also able to induce regulatory T cells(2). In order to fully activate naïve T cells, DCs need to be activated by proinflammatory mediators, Toll-like receptor (TLR) ligands, and CD40 ligand (CD40L) and to express various co-stimulatory molecules, which are instrumental in activating T cells quantitatively as well as qualitatively. Thus, it is important to elucidate (i) environmental factors that induce DCs to express a particular co-stimulatory molecule and (ii) the type of T cell responses induced by the molecule.

A tumor necrosis factor (TNF) family member CD70 represents an important co-stimulatory molecule that induces effective T cell responses(3). In the absence of CD27, the only known receptor of CD70, both primary and secondary T cell responses are impaired(4). Thus, it is important to clarify which cells express CD70. Particularly, it is a key question whether DCs express CD70, because in contrast to other members of the TNF receptor family, CD27 is expressed on naïve T cells(5), and DCs are the principal APCs that activate naïve T cells. Initially, human activated T and

B cells have been shown to express CD70(6-9). Subsequently, mouse CD70 has been found to be expressed on DCs in lymph nodes (10) or spleen (11), as well as bone marrow-derived DCs stimulated with TLR ligands and/or CD40L (12-14). In humans, it has been shown that monocyte-derived DCs (MoDCs) vigorously washed and stimulated with LPS and those stimulated with TNF-α and LPS express CD70 (15,16). Another recent study has shown that prostaglandin (PG) E_2 upregulates CD70 on human MoDCs and primary mDCs in blood(17). However, there is no study that extensively analyzed the expression of CD70 on human different primary DC subsets, mDCs and pDCs.

Studies of functions of CD70 have largely focused on the importance of the interaction between CD70 and its receptor CD27 in the enhancement of $CD8^+$ T cell responses. For example, the CD70-CD27 signal enhances $CD8^+$ T cell responses mainly by promoting these T cells to survive(4,18-21) and to acquire strong effector functions(18). Recent studies have also been investigating the role of CD70 in $CD4^+$ T cell responses. For example, expansion of influenza virus-specific $CD4^+$ T cells is impaired during primary and secondary infections in CD27-deficient mice, as observed in $CD8^+$ T cells (4). The CD70-CD27 signal has been shown to provide $CD4^+$ T cells the ability to help memory $CD8^+$ T cell responses(22). Intriguingly, constitutive stimulation of $CD4^+$ T cells through CD27 results in destruction of splenic architecture and impairment of antibody production due to the secretion of IFN-γ and TNF-α by the $CD4^+$ T cells (23,24). These studies

indicate that, on one hand, the CD70-CD27 interaction enhances the activity of $CD4^+$ T cells, and on the other hand, the interaction needs to be tightly regulated in order to avoid pathological immune responses. Thus, it is important to explore cellular interactions where naïve $CD4^+$ T cells are activated through the CD70-CD27 interaction, in order to understand physiological and pathological situations where CD70 is critically involved and to develop novel therapies by manipulating the CD70-CD27 interaction. In this regard, it has recently been shown that CD70 on murine DEC-205^+ DCs is important for the induction of interleukin (IL)-12-independent IFN-γ production by $CD4^+$ T cells(25). However, it has never been examined whether the CD70-CD27 interaction is involved in differentiation of naïve $CD4^+$ T cells into IFN-γ-producing cells during human DC-T interaction. In addition, it has not been shown that the CD70-CD27 interaction can promote naïve $CD4^+$ T cells to produce Th2 cytokines (IL-4, IL-5, and IL-13).

Here we examined (i) whether human mDCs and pDCs express CD70 after various stimuli and (ii) what types of $CD4^+$ T cell responses are induced by the CD70-CD27 interaction using naïve $CD4^+$ T cells and a CD70 transfectant, and (iii) whether the CD70-CD27 interaction promotes human naïve $CD4^+$ T cells to acquire the ability to produce effector cytokines during stimulation with DCs. This is the first study which (i) extensively analyzes CD70 expression on human primary DC subsets and (ii) reveals that the CD70-CD27 interaction enhances not only Th1 but also Th2 differentiation of naïve $CD4^+$ T cells.

Materials and Methods

Media and reagents

RPMI 1640 (Sigma-Aldrich, St. Louis, MO) supplemented with 10% heat-inactivated fetal calf serum (ThermoTrace, Victoria, Australia), 2 mM L-glutamine, penicillin G, streptomycin (Gibco BRL, Carlsbad, CA), and 10 mM HEPES (Nacalai tesque, Kyoto, Japan) was used for cell culture. Mouse anti-human CD70 monoclonal antibody (mAb) 2F11 (IgG1) (8) was used to block the interaction between CD70 and CD27. Mouse anti-human OX40 ligand (OX40L) mAb ik-1 (IgG1) (26) was used to block the interaction between OX40L and OX40. A murine pre-B-cell line 300-19 cells transfected with CD70 cDNA, 300-19/CD70(27) (irradiated with 5,500 rad), were used to stimulate T cells through CD27.

Isolation of DCs and T cells

Peripheral blood mononuclear cells (PBMCs) were obtained from buffy coat of healthy donors with written informed consent. $CD4^+CD11c^-lin^-$ cells and $CD4^+CD11c^+lin^-$ cells were isolated as pDCs and mDCs, respectively, as described(28), using FACSAria™ cell sorter (BD Biosciences, San Jose, CA). Reanalysis of the sorted cells confirmed a purity of more than 98%. PBMCs were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3, phycoerythrin (PE)-conjugated

anti-CD45RA, and PC5-conjugated anti-CD4 mAbs (Beckman Coulter Immunotech, Marseille, France), and $CD3^+CD4^+CD45RA^+$ cells were isolated using FACSAria™ cell sorter as naïve $CD4^+$ T cells. Reanalysis of the sorted cells confirmed a purity of more than 98%.

Culture and phenotypic analysis of DCs

mDCs were cultured for 3 days at 2 x 10^4/200 μl in round-bottom 96-well culture plates in the presence of 15 ng/ml thymic stromal lymphopoietin (TSLP) (R&D Systems, Minneapolis, MN), 50 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (sargramostim, Leukine®, Immunex, Seattle, WA), or 50 μg/ml polyinosinic-polycytidylic acid (poly I:C) (Pharmacia Biotech, Uppsala, Sweden) in the absence or presence of 1 μg/ml PGE_2 (MP Biomedicals, Solon, OH) or human CD40L-transfected L cells(29) (irradiated with 5,500 rads) at one L cell for five DCs. pDCs were cultured for 3 days at 2 x 10^4/200 μl in round-bottom 96-well culture plates in the presence of 10 ng/ml IL-3 (PeproTech, London, UK) or 5 μg/ml oligodeoxynucleotide (ODN)2006(30) (Hokkaido System Science, Sapporo, Japan) in the absence or presence of the irradiated CD40L-L cells or 1 μg/ml PGE_2. The expression of surface markers on DCs was analyzed by FACSCalibur™ (BD Biosciences), using FITC-conjugated mouse anti-human CD70 (clone Ki-24) or FITC-conjugated mouse anti-human CD86 mAbs (BD Biosciences). Dead cells were excluded by staining with propidium iodide. Data were analyzed with CellQuest™ software (BD Biosciences).

T cell culture with microbeads or DCs

$CD4^+CD45RA^+$ T cells were stimulated for 7 days with Dynabeads® CD3/CD28 T Cell Expander (Invitrogen Dynal, Oslo, Norway) at 1 bead for 10 T cells without IL-2, at 1 x 10^5 cells/200 μl/well in round-bottom 96-well culture plates in the presence of irradiated 300-19 or 300-19/CD70 at one 300-19 cell for 10 T cells. Mouse IgG1 isotype control mAb or anti-CD70 mAb 2F11 was added at 10 μg/ml. In some experiments, the stimulated T cells were harvested and restimulated for 7 days in the same condition as the first round.

mDCs and pDCs were cultured as indicated for 3 days and were washed. Then, allogeneic $CD4^+CD45RA^+$ T cells (1 x 10^5 cells) were cocultured with the DCs (1 x 10^4 cells) without IL-2 for 8 days in 200 μl of the medium in 96-well round-bottom microplates in the presence of 10 μg/ml anti-CD70 mAb 2F11, 50 μg/ml anti-OX40L mAb ik-1, 5 μg/ml human CTLA-4/Fc chimeric protein (R&D Systems), or 10 μg/ml mouse IgG1 isotype control.

Analysis of T cell proliferation by CFSE

$CD4^+CD45RA^+$ T cells were stained with CFSE (Invitrogen, Carlsbad, CA) and were stimulated for 5 days with Dynabeads® CD3/CD28 T Cell Expander or DCs as described above. The T cells were harvested and stained with PE-conjugated mouse anti-human CD70 mAb (BD Biosciences),

and were analyzed for proliferation and CD70 expression by FACSCalibur. Dead cells were excluded by staining with propidium iodide.

Analysis of cytokine production by ELISA or Cytometric Bead Array

Primed T cells were harvested, and viable cell numbers were counted by trypan blue exclusion. The T cells were restimulated at 1 x 10^6 cells/ml with 10 μg/ml plate-bound anti-CD3 (OKT3) and 1 μg/ml soluble anti-CD28 mAbs (BD Biosciences) for 24 h. The supernatants were harvested and analyzed for cytokine by ELISA. The following reagents were used for ELISA: Human IFNg MAb clone 2G1, Human IFNg MAb Biotin-Labeled, and horseradish peroxidase-conjugated Streptavidin (Endogen, Rockford, IL), OptEIA™ Human TNF, IL-4, and IL-10 ELISA Set (BD Biosciences), Human IL-5 CytoSets™ Kit (Biosource International, Camarillo, CA), and Human IL-13 ELISA Kit (Biosource International). In some experiments, supernatants were analyzed for IFN-γ, IL-13, TNF-α, and IL-10 by BD™ Cytometric Bead Array (CBA) and FCAP Array Software using a FACSCalibur™ flow cytometer.

Analysis of cell surface molecules, intracellular cytokines, granzymes, perforin, and Foxp3 in T cells by flow cytometry

T cells stimulated with DCs were stained with FITC-conjugated mouse anti-human CD62L (BD

Biosciences), CCR7 (R&D Systems), CXCR3 (R&D Systems mAbs, or with PE-conjugated mouse anti-human CCR4 (BD Biosciences) or CCR5 (R&D Systems) mAbs, and analyzed with a FACSCalibur™ flow cytometer. Dead cells were excluded by staining with propidium iodide. T cells stimulated with DCs were restimulated with 50 ng/ml phorbol myristate acetate (PMA) (Sigma-Aldrich) and 500 ng/ml A23187 (Calbiochem). T cells were harvested after 6 h of stimulation, while brefeldin A (10 μg/ml; Sigma) was added at 3 h. After fixed with 2% paraformaldehyde and permeabilized with saponin, T cells were incubated with PE-conjugated anti-IL-4, anti-IL-13, or isotype control, and FITC-conjugated anti-IFN-γ or isotype control mAbs. For analysis of granzyme B and perforin, T cells stimulated with DCs were fixed and permeabilized as described above, and were incubated with FITC-conjugated anti-granzyme B (clone GB11), anti-perforin (clone δG9), or an isotype control mAb. All the mAbs for intracellular staining were purchased from BD Biosciences. For staining of Foxp3, PE Anti-Human Foxp3 Staining Set (eBioscience, San Diego, CA) was used. Cells were analyzed using a FACSCalibur™ flow cytometer.

Real-time RT-PCR

$CD4^+CD45RA^+$ T cells were stimulated for 48 h with Dynabeads® CD3/CD28 T Cell Expander in the presence of 10 μg/ml isotype control mAb, 10 μg/ml mouse anti-human IFN-γ mAb (BD

Biosciences), 10 μg/ml anti-CD70 mAb 2F11, 10 ng/ml IFN-γ, 10 ng/ml IL-12 (PeproTech), 300-19 cells, or 300-19/CD70 cells. Total RNA was isolated from activated T cells using RNeasy Mini kit (Qiagen, Hilden, Germany). 0.5 μg of total RNA was reverse transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Tokyo, Japan) according to the manufacturer's protocol. Real-time PCR was performed using qPCR Mastermix Plus (Eurogentec, Belgium) and TaqMan Gene Expression Assays for T-bet and GATA-3 (Applied Biosystems) on the ABI PRISM 7700 Sequence Detection System. Relative quantitations of mRNA expressions were performed by the relative standard curve method and mRNA expression levels of each gene were normalized to those of β-glucronidase.

Results

mDCs and pDCs express CD70 upon stimulation with CD40 ligand together with DC-stimulating cytokines or TLR ligands

We first examined what kinds of stimulation induce mDCs and pDCs to express CD70. It has been shown that TSLP(31) and IL-3(32) strongly activate human mDCs and pDCs, respectively. It is also known that poly I:C and unmethylated CpG ODN stimulate mDCs and pDCs through TLR3 and TLR9, respectively(33). CD40 signaling together with the cytokines or TLR ligands is known to

further induce maturation of DCs. We thus stimulated freshly isolated mDCs and pDCs with the cytokines or TLR ligands in the absence or presence of CD40L-transfected L cells for 3 days, and examined expression of CD70 as well as CD86 for comparison. As negative controls, we kept mDCs and pDCs in culture without any stimulation for 3 days. As shown in Figure 1A, freshly isolated mDCs expressed a low level of CD86 and almost no CD70. Culture of mDCs without stimulation did not change the phenotype. In contrast, stimulation with TSLP or poly I:C upregulated the expression of CD86 and induced a low level of CD70. The addition of CD40L increased the expression of CD70. Similarly, freshly isolated pDCs expressed a low level of CD86 and almost no CD70. Almost all pDCs died after culture without stimulation for 3 days, and thus were not suitable for analysis. Stimulation with IL-3 or ODN 2006 upregulated the expression of CD86, and ODN 2006 but not IL-3 induced a low level of CD70. The addition of CD40L increased the expression of CD70 as well as CD86. Therefore, both mDCs and pDCs are capable of expressing CD70 upon stimulation with appropriate cytokines or TLR ligands, together with CD40 signaling derived from CD40L-expressing cells.

PGE_2 induces CD70 on TSLP-stimulated mDCs but not on IL-3-stimulated pDCs

Other than CD40L, PGE_2 represents a potent stimulus to induce maturation of MoDCs (34). Thus, we cultured mDCs and pDCs in the presence of basal cytokines, TSLP(31) or GM-CSF(35) for

mDCs and IL-3(32) for pDCs, together with PGE_2 and/or CD40L-transfected L cells for 3 days, and examined the expression of CD70 and CD86.

In the absence of the cytokines, PGE_2 or CD40L alone or in combination induced only marginal levels of CD70 on mDCs, although they upregulated CD86 (Figure 1B). Whereas TSLP alone upregulated CD86 and induced an only marginal level of CD70, the addition of PGE_2 or CD40L together with TSLP substantially upregulated CD70. PGE_2 and CD40L showed almost no additive effect on the upregulation of CD70 or CD86. In contrast to TSLP, GM-CSF in combination with PGE_2 and/or CD40L induced only a low level of CD70, whereas these stimuli upregulated CD86.

Although CD40L alone upregulated CD70 and CD86 on pDCs in the absence of IL-3 (Figure 1C), the viability was greatly reduced (propidium iodide-negative cells 17%), compared to IL-3-simulated pDCs (64%) and IL-3- and CD40L-stimulated pDCs (43%). Whereas the addition of CD40L together with IL-3 upregulated CD70 and CD86, the addition of PGE_2 did not have such an effect. There was no additive effect of PGE_2 and CD40L on the upregulation of CD70 or CD86 on IL-3-stimulated pDCs.

Taken together, PGE_2 upregulated CD70 on mDCs, when it was combined with TSLP but not with GM-CSF. PGE_2 did not upregulate CD70 on IL-3-stimulated pDCs. Thus, PGE_2 appears to be an important factor for the upregulation of CD70 on mDCs but not on pDCs.

Naïve CD4$^+$ T cells express CD70 after stimulation with DCs but not with anti-CD3/CD28 stimulation

It has been shown that CD70 is expressed on activated T cells(6,7). Thus, we examined whether naïve CD4$^+$ T cells gain the expression of CD70 after the stimulation with DCs. We stimulated CD4$^+$CD45RA$^+$ T cells for 5 days either with anti-CD3/CD28 microbeads or with allogeneic mDCs or pDCs that had been stimulated and washed, and examined the expression of CD70 on activated CD4$^+$ T cells. The majority of live CD4$^+$ T cells are activated as indicated by the dilution of CFSE staining (Figure 2). Whereas CFSE^{high} resting T cells did not express CD70, subpopulation of CFSE^{low} T cells activated by either poly I:C-stimulated mDCs, TSLP-stimulated mDCs, or IL-3-stimulated pDCs expressed CD70. In contrast, stimulation with anti-CD3/CD28 microbeads did not induce the expression of CD70 on activated T cells. The addition of TNF-α, IL-1β, and/or IL-12 to anti-CD3/CD28 microbeads did not induce CD70 on T cells (data not shown). CD4$^+$CD45RA$^+$ T cells cultured with freshly isolated mDCs or pDCs proliferate only weakly, and did not express CD70 (data not shown). Thus, naïve CD4$^+$ T cells cultured with activated DCs gain the expression of CD70, and DC-derived factors other than CD80/CD86 and the proinflammatory cytokines are necessary for the induction of CD70 on activated T cells.

CD70-CD27 signaling imparts the ability to produce a broad spectrum of effector cytokines to

naïve CD4$^+$ T cells

The above data indicate that during the interaction between DCs and naïve CD4$^+$ T cells, CD70 expressed on mature DCs and activated T cells may contribute to the activation and differentiation of naïve CD4$^+$ T cells that express CD27. The principal function of CD4$^+$ T cells is to produce immunostimulatory and regulatory cytokines and thus to orchestrate the effector phase of immune responses. Therefore, we examined whether stimulation of naïve CD4$^+$ T cells with CD70-expressing cells together with CD3/CD28 stimulation leads to the induction of effector CD4$^+$ T cells. We stimulated naïve CD4$^+$ T cells with anti-CD3/CD28 microbeads together with a parental 300-19 murine pre-B-cell line or 300-19 cells transfected with human CD70 (300-19/CD70), restimulated the T cells with anti-CD3 and CD28 mAbs, and measured the concentrations of various cytokines in the supernatants by ELISA. Whereas parental 300-19 cells did not express CD70, 300-19/CD70 expressed a high level of CD70 (Supplementary figure 1). Stimulation with CD70 increased the production of a broad array of effector cytokines by CD4$^+$ T cells: IFN-γ, IL-2, and TNF-α (Figure 3A). The increases in the production of these cytokines were abrogated by the addition of blocking anti-CD70 mAb. In contrast, stimulation with CD70 did not increase IL-10 production. Whereas a single round stimulation with anti-CD3/CD28 microbeads in the presence of parental 300-19 cells induced only a low level of IL-13, the presence of 300-19/CD70 increased the production of IL-13, and anti-CD70 mAb abrogated it. Stimulation with anti-CD3/CD28 microbeads

and 300-19/CD70 also induced the production of IL-4 to a lesser extent, and anti-CD70 mAb abrogated it. CD70-CD27 signaling did not increase the production of IL-5. Because a single round of stimulation with anti-CD3 mAb and CD80 has been shown to be insufficient to induce a high level of IL-4 production by human naïve $CD4^+$ T cells(36), we stimulated $CD4^+CD45RA^+$ T cells for two rounds. Thereafter, the production of Th2 cytokines (IL-4, IL-5, IL-13) was obviously increased by 300-19/CD70 cells, and the increases were abrogated by the addition of anti-CD70 mAb (Figure 3B). To examine whether the parental 300-19 cells by themselves have activity to increase the cytokine production, we compared stimulation with anti-CD3/CD28 microbeads alone to stimulation with anti-CD3/CD28 microbeads and the parental 300-19 cells. The parental 300-19 cells by themselves increased the production of IFN-γ and IL-13 by T cells probably through some helper effects, but 300-19/CD70 further increased the cytokine production and this effect was completely abrogated by anti-CD70 mAb (Supplementary figure 2). These data indicate that CD70-CD27 signaling imparts the ability to produce a variety of immunostimulatory cytokines including Th1 and Th2 types, but not a regulatory cytokine IL-10, to naïve $CD4^+$ T cells, and may thus represent an important signal that enhances a broad array of adaptive immune responses through the activation of naïve $CD4^+$ T cells. During the stimulation of T cells, the CD70-CD27 signal did not increase the cell number (Figure 3A). Thus, in the culture shown here, the CD70-CD27 signal promoted the functional development but not expansion of $CD4^+$ T cells.

It is well established that T-bet is a master transcription factor that induces a prototype Th1 cytokine IFN-γ, and that GATA-3 is important in inducing the production of Th2 cytokines by $CD4^+$ T cells in mice(37). Thus, we examined whether the induction of both Th1 and Th2 cytokines by CD70 is accompanied by the induction of T-bet and GATA-3. We stimulated naïve $CD4^+$ T cells with anti-CD3/CD28 microbeads with or without the 300-19/CD70 cells for 48 h, extracted RNA, and quantitated T-bet and GATA-3 mRNA by real-time RT-PCR. As shown in Figure 4, the addition of IFN-γ or IL-12 increased the expression of T-bet mRNA, as reported(38,39). The addition of 300-19/CD70 cells also increased the expression of T-bet mRNA to the same extent, and the increase was abrogated by the addition of anti-CD70 as well as anti-IFN-γ mAbs to a similar extent. Together with the finding that CD70 induces IFN-γ production by naïve $CD4^+$ T cells (Figure 3), these data suggest that CD70-CD27 signaling induces naïve $CD4^+$ T cells to express T-bet mRNA through the induction of IFN-γ. In contrast to the enhancement of T-bet expression by CD70, we observed a similar level of expression of GATA-3 mRNA in the presence or absence of the CD70 transfectant, whereas the addition of IL-4 enhanced the expression of GATA-3 mRNA (data not shown).

The CD70-CD27 signal enhances the production of a broad spectrum immunostimulatory cytokines by naïve $CD4^+$ T cells during the stimulation with mDCs

We examined whether the CD70-CD27 interaction promotes naïve $CD4^+$ T cells to acquire the

ability to produce effector cytokines during the stimulation with two human primary DC subsets: mDCs and pDCs. We co-cultured naïve $CD4^+$ T cells with allogeneic mDCs stimulated with TSLP and PGE_2, which express a high level of CD70 (Figure 1B). We blocked the CD70-CD27 interaction with anti-CD70 mAb and the CD80/CD86-CD28 interaction with a CTLA-4/Fc chimeric protein, in order (1) to compare the effects of blockade of the two costimulations that transmit signals to naïve T cells and (2) to examine the additive effects of blockade of the two. Whereas CTLA-4/Fc reduced T cell expansion during stimulation with mDCs, anti-CD70 mAb did not (Figure 5A). The addition of anti-CD70 mAb reduced the ability of naïve $CD4^+$ T cells to produce both Th1 and Th2 cytokines IFN-γ, TNF-α, IL-4, IL-5, and IL-13. In contrast, anti-CD70 mAb did not reduce or rather increased the ability of the T cells to produce an immunoregulatory cytokine IL-10. The addition of CTLA-4/Fc reduced the ability of the T cells to produce the Th1 and Th2 cytokines to a greater extent than anti-CD70 mAb, whereas CTLA-4/Fc increased IL-10 production similarly to anti-CD70 mAb. Anti-CD70 mAb and CTLA-4/Fc showed an additive effect of reducing the production of the immunostimulatory cytokines. To exhibit the cytokine-producing profiles of T cells at a single cell level, we examined the production of a Th1 cytokine (IFN-γ) and Th2 cytokines (IL-4 and IL-13) by intracellular cytokine staining. Stimulation of naïve $CD4^+$ T cells with mDCs induced IFN-γ-, IL-4-, or IL-13-single producing cells as well as minor populations of IFN-γ/IL-4- or IFN-γ/IL-13-double producing cells (Figure 5B). Although the quantitative differences as shown by intracellular cytokine

staining are marginal compared to ELISA, CTLA-4/Fc and to a lesser extent anti-CD70 mAb had a tendency to reduce the fluorescence intensity of intracellular IFN-γ, IL-4, and IL-13 (Figure 5B, C). These data indicate that the CD70-CD27 interaction promotes naïve $CD4^+$ T cells to acquire the ability to produce both Th1 and Th2 immunostimulatory cytokines, as the CD80/CD86-CD28 interaction does, but does not promote T cell expansion, differently from the CD80/CD86-CD28 interaction, during the stimulation with $CD70^+$ mDCs. Both of the blockade of the CD70-CD27 and CD80/CD86-CD28 interactions appeared to diminish IFN-γ-, IL-4-, and IL-13-single producing cells as well as IFN-γ/IL-4-, and IFN-γ/IL-13-double producing cells at the single cell level.

The findings that $CD4^+$ T cells stimulated with mDCs in the presence of anti-CD70 mAb and/or CTLA-4/Fc produce less immunostimulatory cytokines whereas maintain the ability to produce IL-10 raise a question whether such $CD4^+$ T cells are particular types of conventional T cells or regulatory T cells. To examine this, we stained naïve $CD4^+$ T cells stimulated with mDCs with an array of mAbs that recognize surface or intracellular molecules characteristic of particular types of $CD4^+$ T cells(40): CD62L (naïve and central memory), CCR7 (naïve and central memory), CCR5 (effector memory), CXCR3 (Th1), CCR4 (Th2), Foxp3 (regulatory T cells), perforin, and granzyme B. $CD4^+$ T cells stimulated with mDCs had a phenotype $CD62L^+$ $CCR7^+$ $CCR5^-$ $CXCR3^+$ $CCR4^{low}$ $Foxp3^-$ $perforin^-$ granzyme B^{low} (Supplementary figure 3). Decreases in the slight expression of granzyme B were observed, but otherwise the profile did not change, when anti-CD70 mAb and/or

CTLA-4/Fc were added to the DC-T cell coculture. The high levels of expression of CD62L and CCR7 indicate that the T cells generated after the stimulation with $CD70^+$ mDCs are not effector cells but Th0 type central memory cells. The absence of Foxp3 argues against the possibility that the T cells developed without the CD27 and CD28 signals acquire regulatory activity.

Because it has been shown that TSLP-stimulated mDCs promote naïve $CD4^+$ T cells to develop into T cells with an proinflammatory Th2 profile (IL-4^+ IL-13^+ TNF-α^+ IL-10^-) through the OX40L-OX40 interaction (41), we compared anti-CD70 mAb with anti-OX40L mAb in terms of the effects on the production of IL-13, TNF-α, and IL-10. When anti-CD70 mAb and/or anti-OX40L mAb were added to the coculture of naïve $CD4^+$ T cells and allogeneic mDCs stimulated with TSLP and PGE_2, anti-OX40L mAb but not anti-CD70 mAb slightly diminished T cell expansion (Figure 5D). The addition of anti-CD70 mAb or anti-OX40L mAb diminished the production of IL-13 (Th2 cytokine) and TNF-α (proinflammatory cytokine). The addition of both mAb had an additive effect of diminishing the production of these cytokines. In contrast, the addition of anti-CD70 mAb or anti-OX40L mAb increased the production of IL-10 (immunoregulatory cytokine), and the addition of both mAb had an additive effect of increasing the production of IL-10. These data indicate that the CD70-CD27 as well as OX40L-OX40 interactions promotes naïve $CD4^+$ T cells to acquire the ability to produce Th2 and proinflammatory cytokines whereas inhibits the T cells from producing IL-10.

Finally, we co-cultured naïve $CD4^+$ T cells with allogeneic pDCs stimulated with ODN2006 and CD40L-transfected L cells, which express a substantial level of CD70 (Figure 1C). Whereas CTLA-4/Fc greatly reduced T cell expansion during stimulation with pDCs, anti-CD70 mAb did not have such an effect (Figure 6). In line with the marked reduction of T cell expansion by CTLA-4/Fc, this blockade markedly reduced the production of IFN-γ, IL-13, and IL-10. Anti-CD70 mAb exhibited a suppressive effect on IL-13 production, whereas the mAb did not have such an effect on the production of IFN-γ or IL-10. We concluded that CTLA-4/Fc has a dominant effect on T cell expansion and cytokine production during the stimulation with pDCs, and that the CD70-CD27 interaction plays a minor role.

Discussion

DCs play a pivotal role in inducing adaptive immune responses by triggering the activation and differentiation of naïve $CD4^+$ T cells into cytokine-producing effector $CD4^+$ T cells. Various factors derived from DCs determine the direction of differentiation of naïve $CD4^+$ T cells(42). Studies on $CD4^+$ T cell differentiation have been mainly focused on defining factors that selectively induce a particular type of $CD4^+$ T cell responses such as Th1 and Th2 types, and factors that enhance the production of a broad range of effector cytokines from naïve $CD4^+$ T cells are ill defined. Here we

showed that the CD70-CD27 signal functions as such a general cytokine inducer from naïve $CD4^+$ T cells, and may represent important costimulation to augment a basal level of adaptive immune responses during the elicitation with DCs in humans.

As it has not been comprehensively studied the expression of CD70 on human primary DCs, i.e. mDCs and pDCs, we first examined it. Here we showed that mDCs and pDCs in human peripheral blood express low levels of CD70 after stimulation with appropriate cytokines (TSLP for mDCs) or TLR ligands (poly I:C for mDCs and CpG ODN for pDCs), and that the expression is augmented by stimulation with CD40L. The combined effect of TLR and CD40 signaling on CD70 expression has also been shown with murine DCs in vivo(14), and indicates the importance of activated $CD4^+$ T cells expressing CD40L in augmenting adaptive immune responses through CD70-CD27 signaling(43-45).

Other than CD40L, PGE_2 potently induce maturation of MoDCs (34). Thus, we compared PGE_2 to CD40L in terms of the upregulation of CD70. PGE_2 as well as CD40L strongly upregulated CD70 on mDCs, when these were combined with TSLP. However, CD40L but not PGE_2 upregulated CD70 on pDCs, when these were combined with IL-3. Thus, PGE_2 appears to be an important factor for the upregulation of CD70 on mDCs but not pDCs stimulated with representative cytokines for each DC subset. The upregulation of CD70 on mDCs by PGE_2 is consistent with a recent study showing that PGE_2 induces CD70 on mDCs when it is combined with CD40L(17). However, CD40L was

sufficient for the upregulation of CD70 in the present study. This difference may due to different culture conditions in the two studies.

We previously showed that $CD4^+CD45RA^+$ T cells hardly express CD70 after stimulation with PHA and T cell-depleted PBMCs, in contrast to the induction of CD70 on ~30% of $CD4^+CD45R0^+$ T cells after the same stimulation(7). A study using total T cells have shown that CD70 is induced on activated T cells in an APC-dependent manner, but it was not shown whether the $CD70^+$ T cells were derived from naïve or memory T cells(46). Here we showed that whereas CD3 and CD28 signaling together with TNF-α, IL-1β, and/or IL-12 hardly induces CD70 on naïve $CD4^+$ T cells, both mDCs and pDCs efficiently induce CD70 on a substantial fraction of naïve $CD4^+$ T cells. Thus, DC-derived signals other than CD80/CD86 and the proinflammatory cytokines are required for the induction of CD70 on naïve $CD4^+$ T cells. These results suggest that $CD4^+$ T cells themselves as well as DCs may serve as a source of CD70 during the activation of naïve $CD4^+$ T cells by DCs. However, it remains to be determined whether CD70 on DCs and that on activated T cells have comparable functional significance in the DC-T cell coculture shown here. In this regard, on one hand, it has recently been reported that CD70 traffics together with MHC class II molecules to immunological synapses on murine DCs(47), indicating that CD70 on DCs is critically involved in stimulation of $CD4^+$ T cells by DCs. On the other hand, it has been shown that CD70 on activated T cells is capable of transmitting signals to CD27 on T and B cells during T-T and T-B interactions, respectively(8,48),

indicating that CD70 on activated T cells is functional. Thus, it is possible that CD70 on both DCs and T cells play a role in activating $CD4^+$ T cells during their interaction with DCs.

Although CD27 is broadly expressed on T cells, i.e. naïve and memory T cells, and $CD4^+$ as well as $CD8^+$ T cells, previous studies have largely focused on the role of the CD70-CD27 interaction in increases in memory $CD8^+$ T cells(13,18-21,43,49), and the role of CD70 in functional differentiation of naïve $CD4^+$ T cells has been studied only scarcely. Recent studies have shown that the CD70-CD27 signal is important for IFN-γ production by $CD4^+$ T cells and is not involved in the induction of Th2 cytokines, by using a murine in vivo system(25) as well as by briefly stimulating human naïve $CD4^+$ T cells in vitro(50). However, it has been shown that differentiation of human naïve $CD4^+$ T cells into Th2 cells occurs more slowly than differentiation into Th1 cells, when the T cells are stimulated with anti-CD3 mAb and CD28 signaling(36,51). Thus, we stimulated $CD4^+CD45RA^+$ T cells with the CD70 transfectant for one or two rounds. Whereas the production of IFN-γ, IL-2, TNF-α, IL-13, and to a lesser extent, IL-4 was augmented after one round of stimulation in the presence of the CD70 transfectant, augmentation of IL-5 production by CD70 was observed after two rounds of stimulation. The faster kinetics of IL-13 induction than IL-4 and IL-5 is consistent with the previous report where human naïve $CD4^+$ T cells were stimulated with anti-CD3/CD28 mAbs(51). In contrast to the upregulation of the immunostimulatory cytokines, an immunoregulatory cytokine IL-10 did not increase with CD70 stimulation. These results indicate

that the CD70-CD27 interaction promotes naïve $CD4^+$ T cells to acquire the ability to produce a broad spectrum of immunostimulatory cytokines, including Th1 and Th2 types.

The production of Th1 and Th2 cytokines is governed by the balance of activity of key transcription factors, particularly T-bet and GATA-3(37). Stimulation of naïve $CD4^+$ T cells with the CD70 transfectant upregulated T-bet mRNA, and anti-IFN-γ mAb inhibited the CD70-induced T-bet upregulation to the similar extent to anti-CD70 mAb. This suggests that the CD70-CD27 signal per se does not induce T-bet but enhances T-bet-independent initial production of IFN-γ, which induces T-bet(38), thus augmenting IFN-γ production. The lack of polarization to a Th1 type by the stimulation with CD70 in spite of the upregulation of T-bet implies that naïve $CD4^+$ T cells need to be stimulated with a sufficient amount of IL-12 to undergo optimal polarization to a Th1 type. Although CD70-CD27 signal also promoted the production of Th2 cytokines, it did not upregulate the expression of GATA-3 mRNA. This may be due to the lack of preferential induction of GATA-3 during human Th2 cell development(52).

Finally, we compared a role of the CD70-CD27 signal with that of the CD80/CD86-CD28 signal in the expansion and differentiation of naïve $CD4^+$ T cells stimulated with mDCs or pDCs. Blockade of the CD80/CD86-CD28 signal during the stimulation with mDCs diminished both expansion and differentiation of naïve $CD4^+$ T cells into T cell population producing a broad array of immunostimulatory cytokines (IFN-γ, IL-4, IL-5, IL-13, TNF-α). In particular, the blockade strongly

suppressed the acquisition of the capacity to produce Th2 cytokines (IL-4, IL-5, IL-13), consistent with more pronounced dependence of Th2 than Th1 differentiation on the CD80/CD86-CD28 signal(53). Whereas blockade of the CD70-CD27 signal did not diminish expansion, it diminished differentiation of naïve $CD4^+$ T cells into T cell population producing the broad array of immunostimulatory cytokines. Blockade of both CD80/CD86-CD28 and CD70-CD27 signals exhibited an additive inhibitory effect on the production of the cytokines. Intriguingly, blockade of these costimulations did not diminish but rather increased the production of IL-10. These data suggest that the CD70-CD27 signal promotes naïve $CD4^+$ T cells to acquire the ability to produce a broad array of immunostimulatory cytokines, including not only Th1 but also Th2 cytokines, during the interaction with mDCs in humans. The high levels of expression of CD62L and CCR7 indicate that the T cells generated after the stimulation with $CD70^+$ mDCs are not effector cells but Th0 type central memory cells. It has been shown that murine $DEC\text{-}205^+$ DCs induce IFN-γ but not IL-4 production by $CD4^+$ T cells in a CD70-dependent manner(25). It is not clear why there is the apparent difference in IL-4 induction by CD70 between the two studies, but it may be due to differences in species and/or experimental systems.

We further compared the CD70-CD27 to the OX40L-OX40 interactions, because the latter has been shown to promote naïve $CD4^+$ T cells to develop into Th2 cells with a proinflammatory profile ($IL\text{-}4^+$ $IL\text{-}13^+$ $TNF\text{-}\alpha^+$ $IL\text{-}10^-$) during culture with TSLP-stimulated mDCs(41). Here we showed that

the CD70-CD27 interaction is equivalent to the OX40L-OX40 interaction in terms of the effects on the production of IL-13, TNF-α, and IL-10, and that the two interactions have an additive effect. This indicates that the two costimulations cooperate for the induction of Th2 and proinflammatory cytokines and the suppression of IL-10.

During the stimulation of naïve $CD4^+$ T cells with $CD70^+CD80^+CD86^+$ pDCs, the blockade of the CD80/CD86-CD28 interaction strongly inhibited both expansion and development of naïve $CD4^+$ T cells into cytokine-producing cells (IFN-γ, IL-13, IL-10), whereas the blockade of the CD70-CD27 interaction did not inhibit the expansion and diminished only IL-13 production. These data indicate that activation of naïve $CD4^+$ T cells with pDCs is highly dependent on the CD80/CD86-CD28 interaction, and that the CD70-CD27 interaction plays a minor role.

There are two types of DC-derived signals that induce differentiation of naïve $CD4^+$ T cells into cytokine-producing effector cells. One category is “basal level” signals, which augment the production of a broad spectrum of cytokines including Th1 and Th2 types, such as CD80/CD86(54). Another category is “polarizing” signals, which induce distinct types of T helper cell differentiation, such as IL-12 (for Th1) and OX40 ligand (for Th2)(41). CD70 appears to belong to “basal level” signals, thereby augmenting the effector function of $CD4^+$ T cells during the elicitation with DCs, in which CD70 is expressed on both DCs and activated $CD4^+$ T cells themselves. Thus, the CD70-CD27 signal during the interaction between DCs and naïve $CD4^+$ T cells may represent a

promising target to modulate a broad range of immune responses for the treatment of a variety of inflammatory disorders and cancers. Of note, TSLP is strongly expressed by epithelial cells, especially keratinocytes in patients with atopic dermatitis(31). PGE_2 is also present at a high concentration in atopic lesions(55). Thus, the strong induction of CD70 on mDCs by TSLP plus PGE_2 and the induction of not only Th1 but also Th2 cytokines by the CD70-CD27 interaction imply that CD70 on mDCs plays an important role in the pathogenesis of allergic disorders in epithelium.

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Figure legends

Figure 1. Expression of CD70 on mDCs and pDCs.

(A) Purified mDCs were stimulated with 15 ng/ml TSLP or 50 μg/ml poly I:C with or without human CD40L-transfected L cells for 3 days. Purified pDCs were stimulated with 10 ng/ml IL-3 or 5 μg/ml ODN 2006 with or without human CD40L-transfected L cells for 3 days. As negative controls, mDCs and pDCs were kept in culture without any stimulation for 3 days. Fresh mDCs and pDCs as well as the cultured DCs were analyzed for the expression of CD70 and CD86 by flow cytometry. Dead cells were excluded by staining with propidium iodide. Almost all pDCs died after culture without stimulation for 3 days, and are thus not shown. Open histograms represent cells stained with isotype-matched control mAbs. Data are representative of three experiments.

(B) Purified mDCs were cultured without cytokines or with 15 ng/ml TSLP or 50 ng/ml GM-CSF, with or without 1 μg/ml PGE_2 and/or human CD40L-transfected L cells for 3 days. DCs were analyzed for the expression of CD70 and CD86 by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms represent cells stained with isotype-matched control mAbs. Data are representative of three experiments.

(C) Purified pDCs were cultured without cytokines or with 10 ng/ml IL-3, with or without 1 μg/ml PGE_2 and/or human CD40L-transfected L cells for 3 days. As negative controls, mDCs and pDCs were kept in culture without any stimulation for 3 days. DCs were analyzed for the expression of

CD70 and CD86 by flow cytometry. Dead cells were excluded by staining with propidium iodide. Almost all pDCs died after culture with PGE_2 alone for 3 days, and are thus not shown. Open histograms represent cells stained with isotype-matched control mAbs. Data are representative of three experiments.

Figure 2. Naïve $CD4^+$ T cells stimulated with DCs expresses CD70.

mDCs and pDCs were stimulated with the indicated factors for 3 days, and were washed. Purified $CD4^+CD45RA^+$ T cells were stained with CFSE and were stimulated for 5 days with Dynabeads® CD3/CD28 T Cell Expander, mDCs, or pDCs. The T cells were harvested and stained with PE-conjugated mouse anti-human CD70 mAb, and were analyzed for proliferation, as detected by the decrease in the intensity of CFSE, and for CD70 expression by flow cytometry. Dead cells were excluded by staining with propidium iodide. Data are representative of three experiments.

Figure 3. Stimulation with CD70 induces naïve $CD4^+$ T cells to acquire the ability to produce a broad spectrum of immunostimulatory cytokines.

(A) $CD4^+CD45RA^+$ T cells were stimulated for 7 days with Dynabeads® CD3/CD28 T Cell Expander in the presence of irradiated 300-19 or 300-19/CD70. Isotype control mAb or mouse anti-human CD70 mAb 2F11 was added at 10 μg/ml. The stimulated T cells were harvested, and

viable cells were restimulated at 1 x 10^6 cells/ml with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 mAbs for 24 h. The supernatants were harvested and analyzed for cytokine by ELISA. Viable cell numbers after 7-day culture were counted by trypan blue exclusion, and were depicted as fold expansion compared to starting cell numbers. Data are representative of five experiments.

(B) The T cells stimulated for 7 days were harvested and restimulated for 7 days in the same condition as the first round. The supernatants were harvested after restimulation for 24 h as in (A), and were analyzed for cytokine by ELISA. Data are representative of five experiments.

Figure 4. T-bet mRNA expression in $CD4^+CD45RA^+$ T cells stimulated with CD70.

$CD4^+CD45RA^+$ T cells were stimulated for 48 h with Dynabeads® CD3/CD28 T Cell Expander in the presence of 10 ng/ml IFN-γ, 10 ng/ml IL-12, 10 μg/ml isotype control mAb, 10 μg/ml mouse anti-human IFN-γ mAb, 10 μg/ml anti-CD70 mAb 2F11, 300-19 cells, or 300-19/CD70 cells in the indicated combinations. Total RNA was isolated from fresh T cells and stimulated T cells, and real-time RT-PCR was performed for T-bet mRNA by the relative standard curve method and mRNA expression levels were normalized to those of β-glucronidase. Data are representative of three experiments.

Figure 5. Stimulation with CD70 promotes naïve $CD4^+$ T cells to acquire the ability to produce immunostimulatory cytokines during the stimulation with mDCs.

(A) $CD4^+CD45RA^+$ T cells were cocultured for 8 days with allogeneic mDCs stimulated with TSLP and PGE_2 in the presence of 10 μg/ml anti-CD70 mAb, 5 μg/ml human CTLA-4/Fc chimeric protein, or 10 μg/ml isotype control mAb (mouse IgG1). The stimulated T cells were harvested, and viable cells were restimulated at 1 x 10^6 cells/ml with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 mAbs for 24 h. The supernatants were harvested and analyzed for cytokine by ELISA. Viable cell numbers after 8-day culture were counted by trypan blue exclusion, and were depicted as fold expansion compared to starting cell numbers. Data are representative of five experiments.

(B) $CD4^+CD45RA^+$ T cells stimulated as in (A) were restimulated with PMA/A23187 and were analyzed by intracellular cytokine staining for IFN-γ, IL-4, and IL-13. The ratios of mean fluorescence intensity (MFI) of each cytokine to MFI of isotype control are indicated on the plot. Data are representative of three experiments.

(C) The ratios of MFI shown in (B) are depicted for each cytokine. The MFI ratios of IFN-γ are from the plots of staining for IFN-γ and IL-4 in (B).

(D) $CD4^+CD45RA^+$ T cells were cocultured for 8 days with allogeneic mDCs stimulated with TSLP and PGE_2 in the presence of 10 μg/ml anti-CD70 mAb, 50 μg/ml anti-OX40L mAb, or 10 μg/ml isotype control mAb (mouse IgG1). The stimulated T cells were harvested, and viable cells were

restimulated at 1 x 10^6 cells/ml with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 mAbs for 24 h. The supernatants were harvested and analyzed for cytokine by Cytometric Bead Array. Viable cell numbers after 8-day culture were counted by trypan blue exclusion, and were depicted as fold expansion compared to starting cell numbers. Data are representative of three experiments.

Figure 6. The CD80/CD86-CD28 interaction strongly promotes naïve $CD4^+$ T cells to proliferate and to acquire the ability to produce cytokines during the stimulation with pDCs, whereas the CD70-CD27 interaction has a minor effect.

$CD4^+CD45RA^+$ T cells were cocultured for 8 days with allogeneic pDCs stimulated with ODN2006 and CD40L-transfected L cells in the presence of 10 μg/ml anti-CD70 mAb, 5 μg/ml human CTLA-4/Fc chimeric protein, or 10 μg/ml isotype control mAb (mouse IgG1). The stimulated T cells were harvested, and viable cells were restimulated at 1 x 10^6 cells/ml with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 mAbs for 24 h. The supernatants were harvested and analyzed for cytokine by Cytometric Bead Array. Viable cell numbers after 8-day culture were counted by trypan blue exclusion, and were depicted as fold expansion compared to starting cell numbers. Data are representative of three experiments.

Supplementary figure 1. 300-19/CD70 but not parental 300-19 cells express CD70.

Parental 300-19 cells and the 300-19/CD70 transfectant were analyzed for the expression of CD70 by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms represent cells stained with isotype-matched control mAbs.

Supplementary figure 2. Effects of parental 300-19 cells on the cytokine production by naïve $CD4^+$ T cells stimulated with anti-CD3/CD28 microbeads.

$CD4^+CD45RA^+$ T cells were stimulated for 7 days with Dynabeads® CD3/CD28 T Cell Expander in the absence of 300-19 cells or in the presence of irradiated 300-19 or 300-19/CD70. Isotype control mAb or mouse anti-human CD70 mAb 2F11 was added at 10 μg/ml. T cells were stimulated for one or two rounds as in Figure 3. The stimulated T cells were harvested, and viable cells were restimulated at 1 x 10^6 cells/ml with 10 μg/ml plate-bound anti-CD3 and 1 μg/ml soluble anti-CD28 mAbs for 24 h. The supernatants were harvested and analyzed for cytokine by Cytometric Bead Array. Data are representative of two experiments.

Supplementary figure 3. Expression profiles of cell surface or intracellular molecules on $CD4^+$ T cells stimulated with mDCs.

$CD4^+CD45RA^+$ T cells were cocultured for 8 days with allogeneic mDCs stimulated with TSLP and

PGE_2 in the presence of 10 μg/ml anti-CD70 mAb, 5 μg/ml human CTLA-4/Fc chimeric protein, or 10 μg/ml isotype control mAb, and were analyzed for the expression of surface or intracellular molecules characteristic of particular types of $CD4^+$ T cells. Dead cells were excluded by staining with propidium iodide. Open histograms represent cells stained with isotype-matched control mAbs. Data are representative of two experiments.

Figure 1A

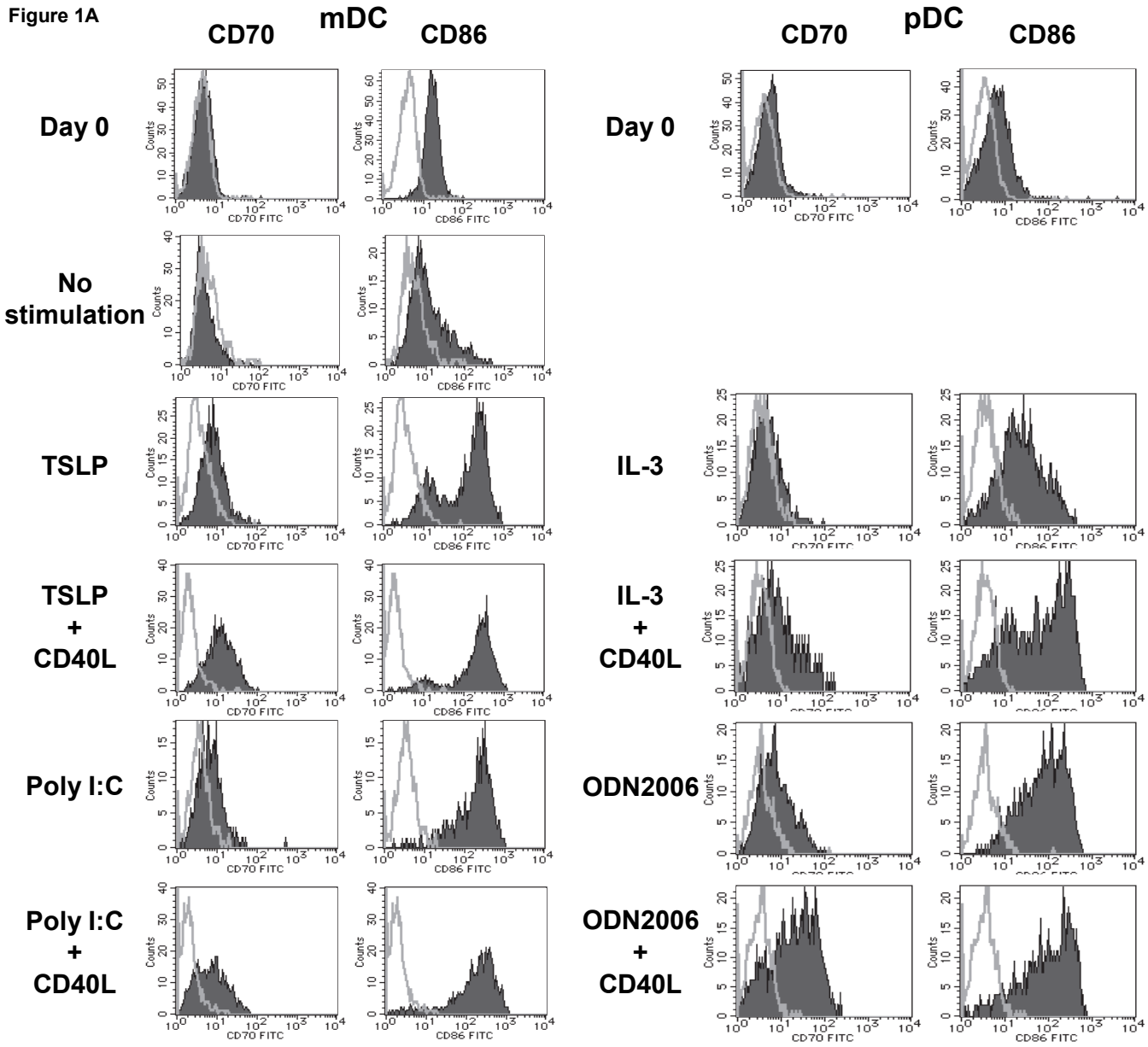

mDC
CD70
CD86
pDC
CD70
CD86
Day 0
Day 0
No stimulation
TSLP
IL-3
TSLP + CD40L
IL-3 + CD40L
Poly I:C
ODN2006
Poly I:C + CD40L
ODN2006 + CD40L
Counts
CD70 FITC
CD86 FITC

Figure 1B

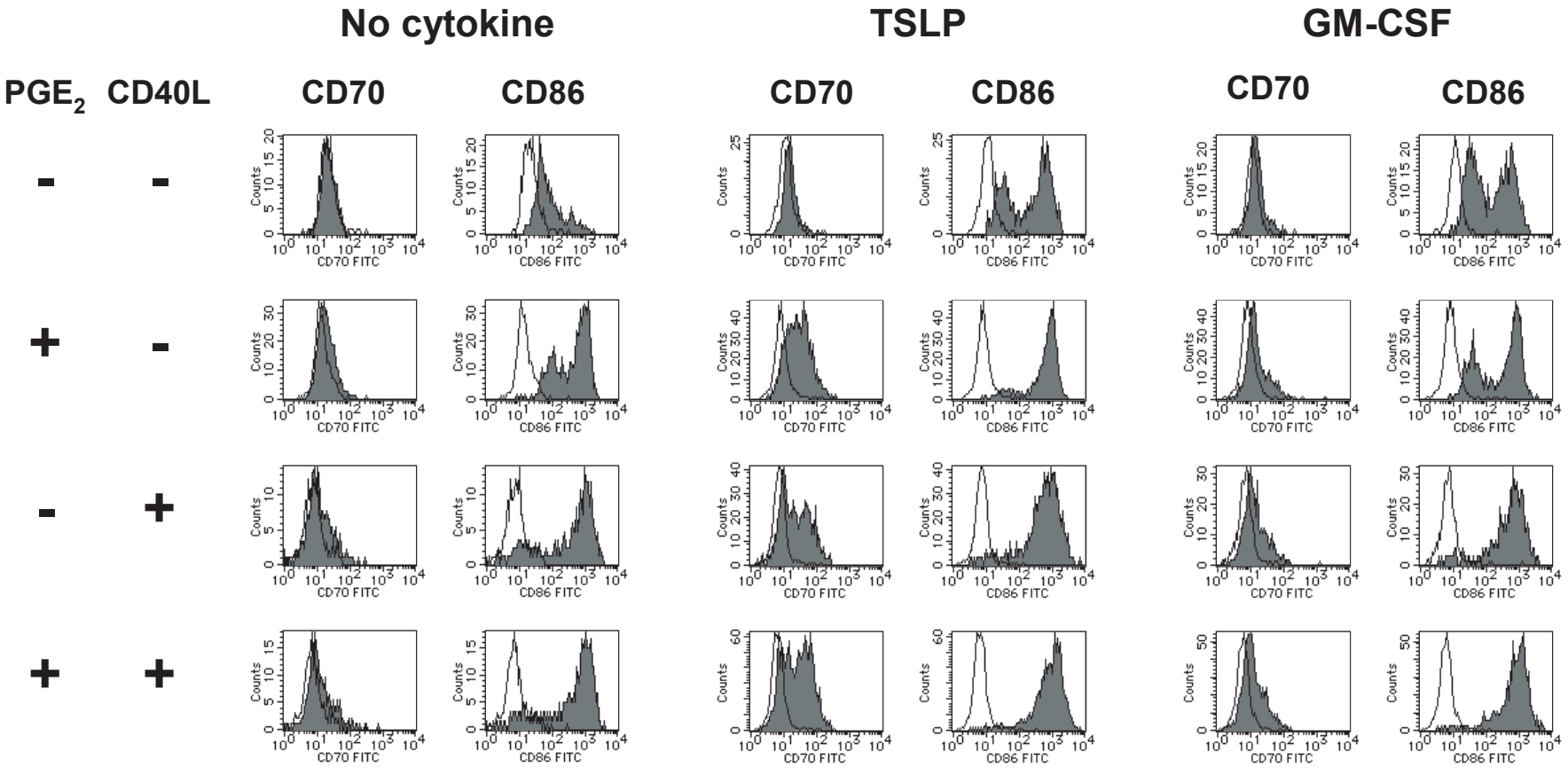

No cytokine
TSLP
GM-CSF
PGE2
CD40L
CD70
CD86
CD70 FITC
CD86 FITC
Counts

Figure 1C

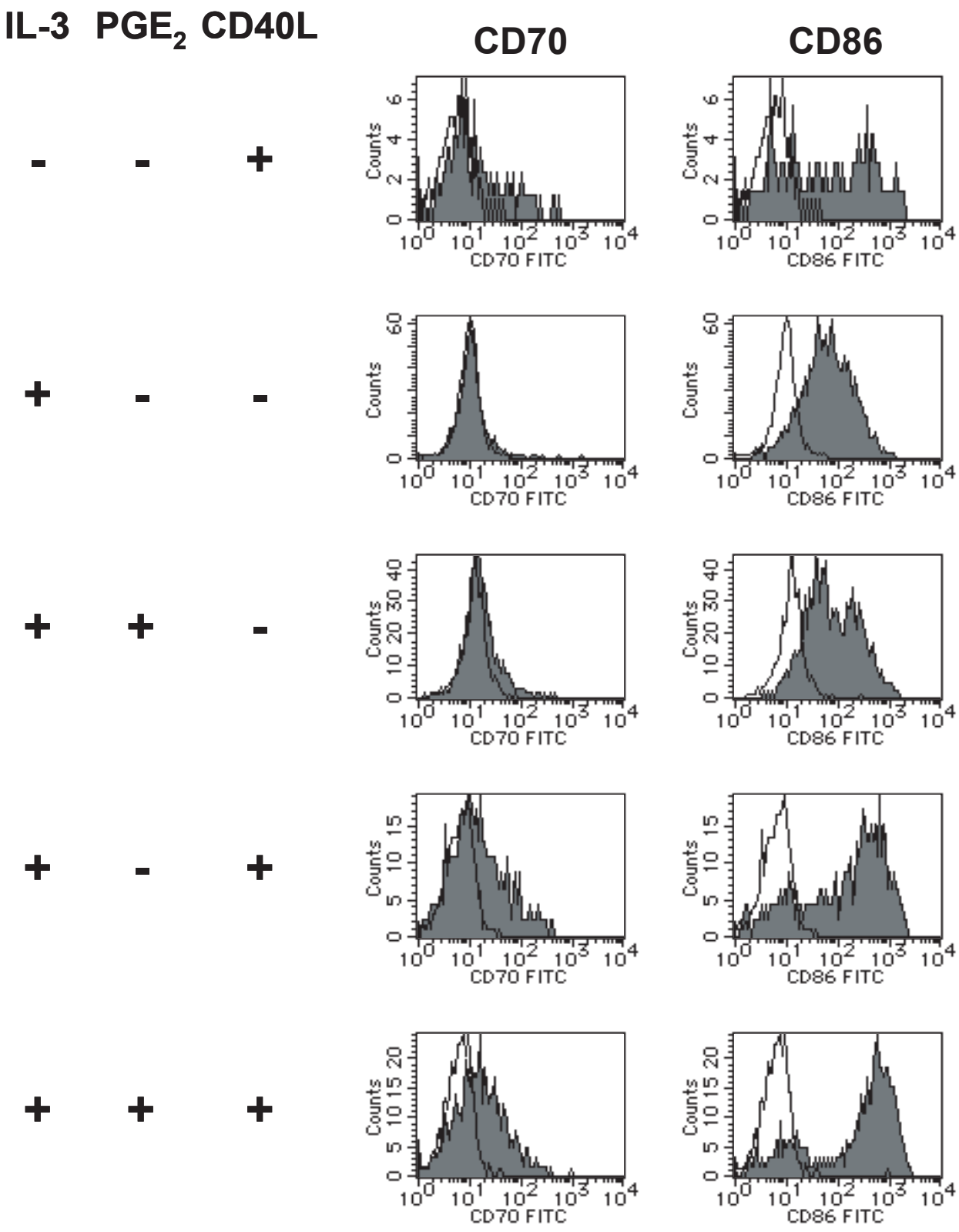


Figure 2

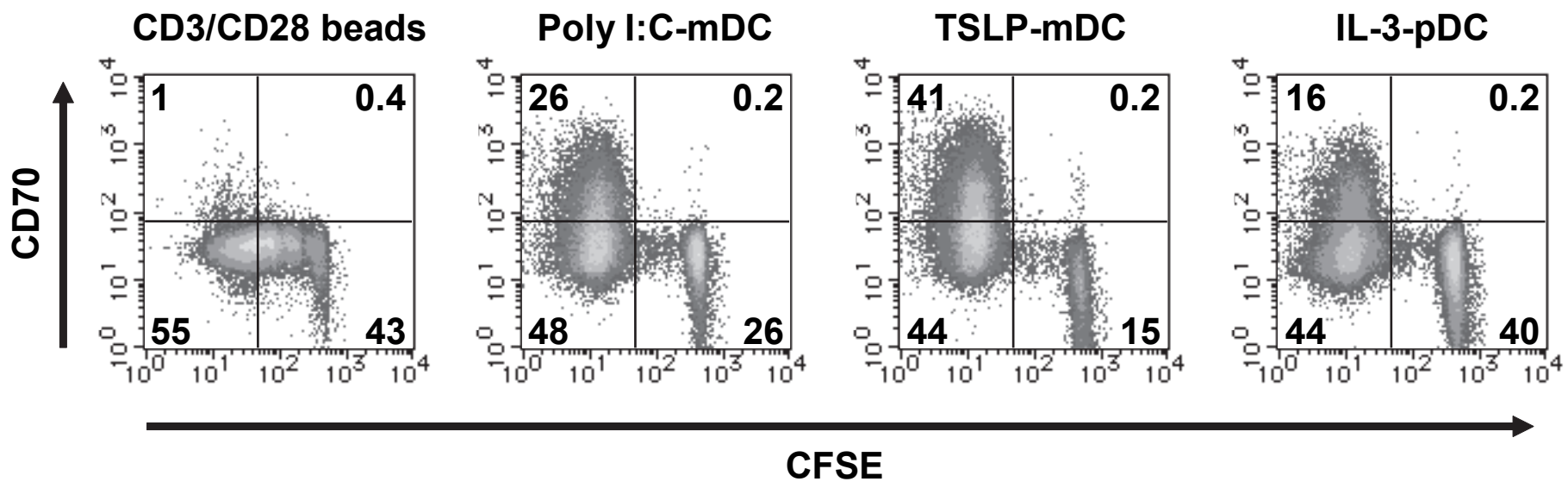

CD3/CD28 beads
Poly I:C-mDC
TSLP-mDC
IL-3-pDC
CD70
CFSE
1
0.4
55
43
26
0.2
48
26
41
0.2
44
15
16
0.2
44
40

Figure 3A

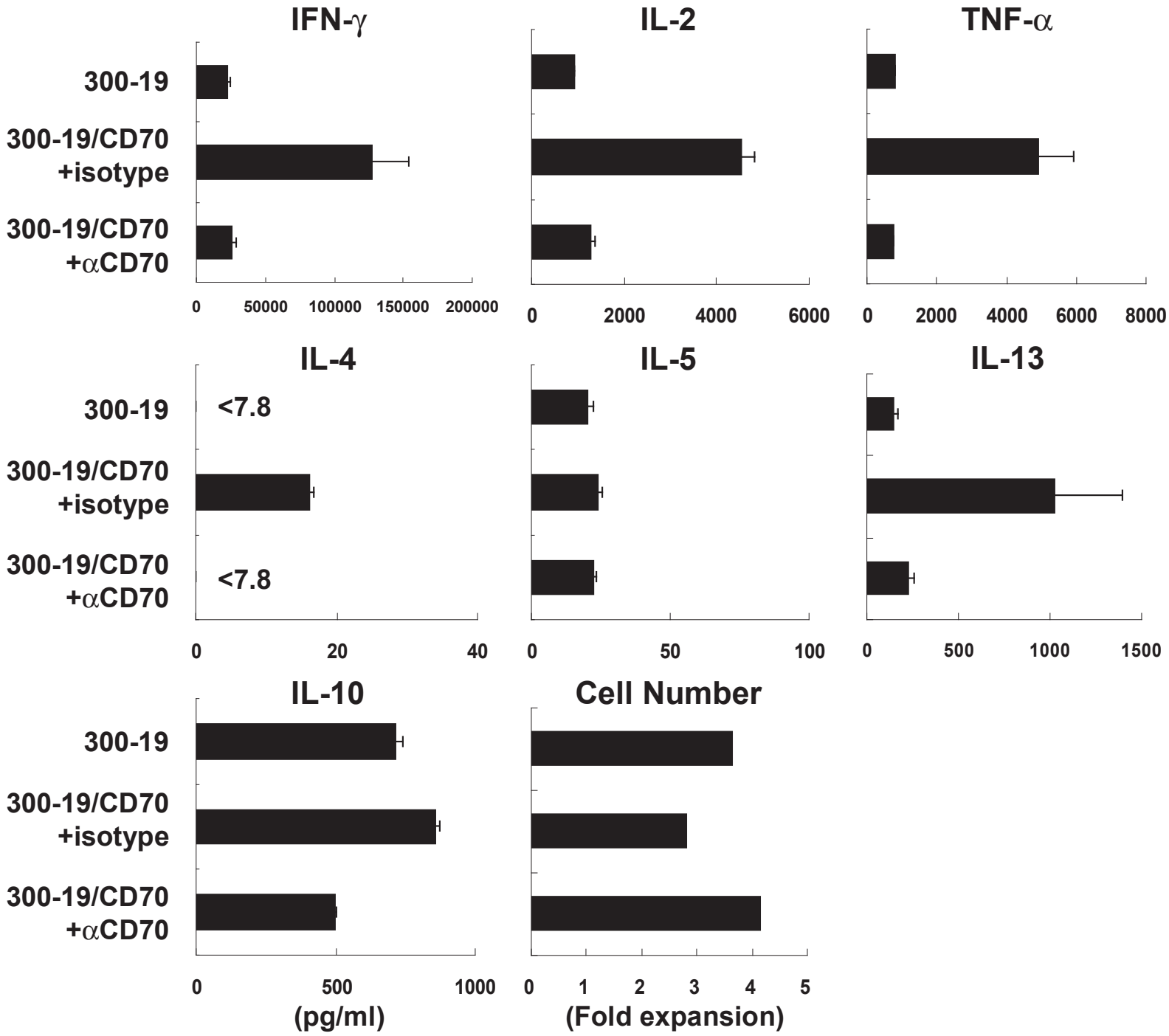

IFN-γ
IL-2
TNF-α
300-19
300-19/CD70 +isotype
300-19/CD70 +αCD70
0
50000
100000
150000
200000
0
2000
4000
6000
0
2000
4000
6000
8000
IL-4
IL-5
IL-13
<7.8
<7.8
0
20
40
0
50
100
0
500
1000
1500
IL-10
Cell Number
500
1000
1
2
3
4
5
(pg/ml)
(Fold expansion)

Figure 3B

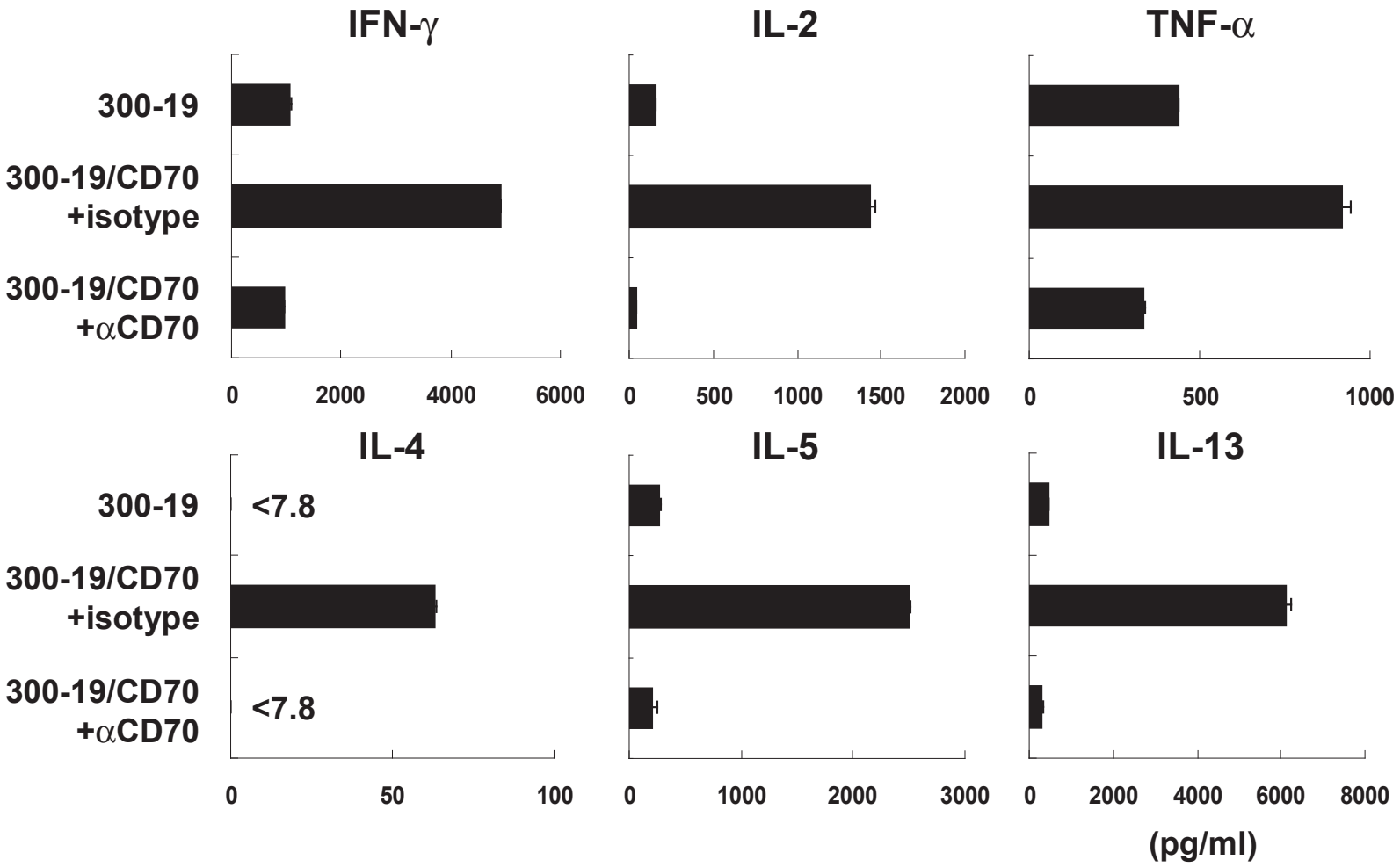

IFN-γ
IL-2
TNF-α
300-19
300-19/CD70
+isotype
300-19/CD70
+αCD70
0
2000
4000
6000
0
500
1000
1500
2000
0
500
1000
IL-4
IL-5
IL-13
300-19
<7.8
300-19/CD70
+isotype
300-19/CD70
+αCD70
<7.8
0
50
100
0
1000
2000
3000
0
2000
4000
6000
8000
(pg/ml)

Figure 4

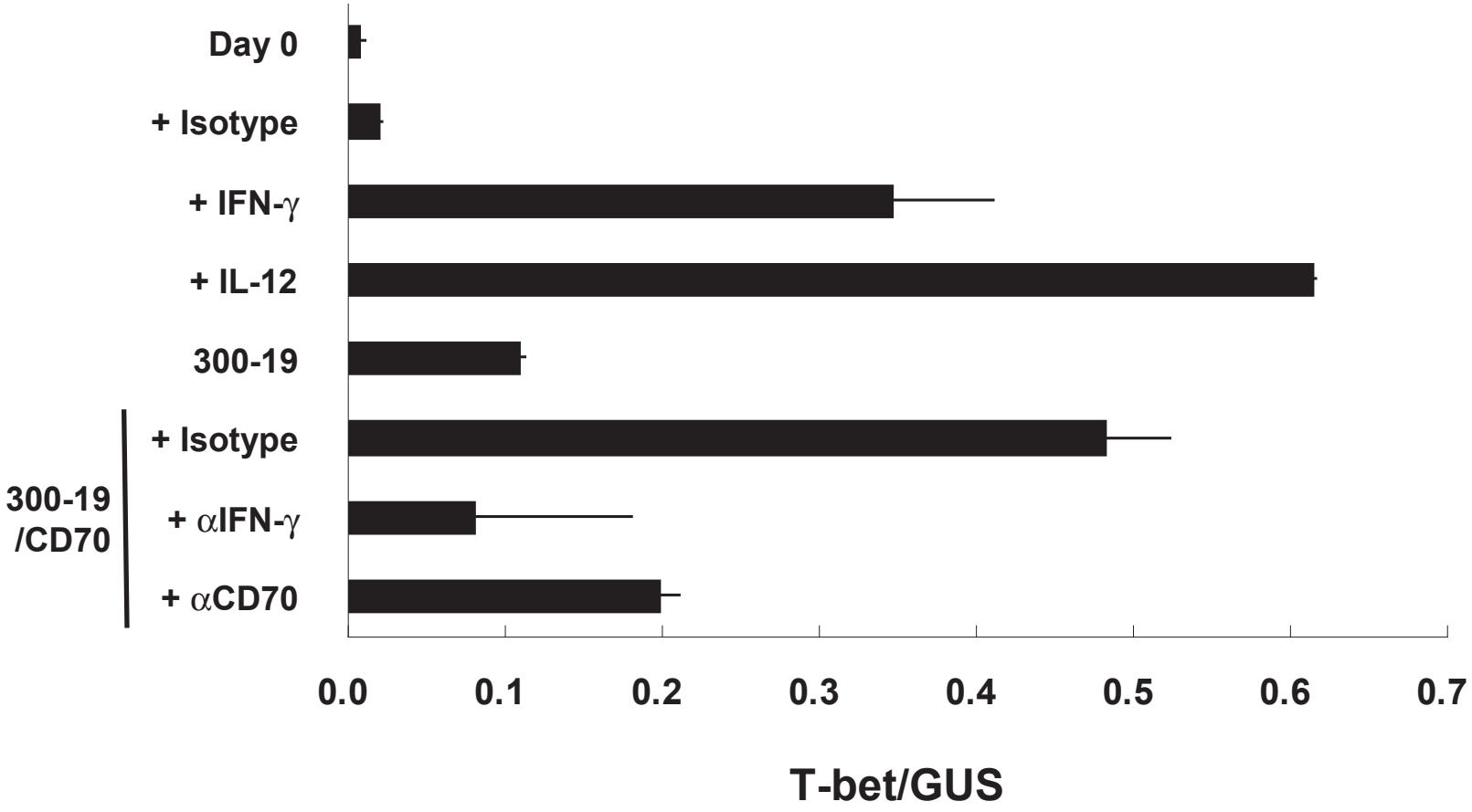

Day 0
+ Isotype
+ IFN-γ
+ IL-12
300-19
300-19 /CD70
+ Isotype
+ αIFN-γ
+ αCD70
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
T-bet/GUS

Figure 5A

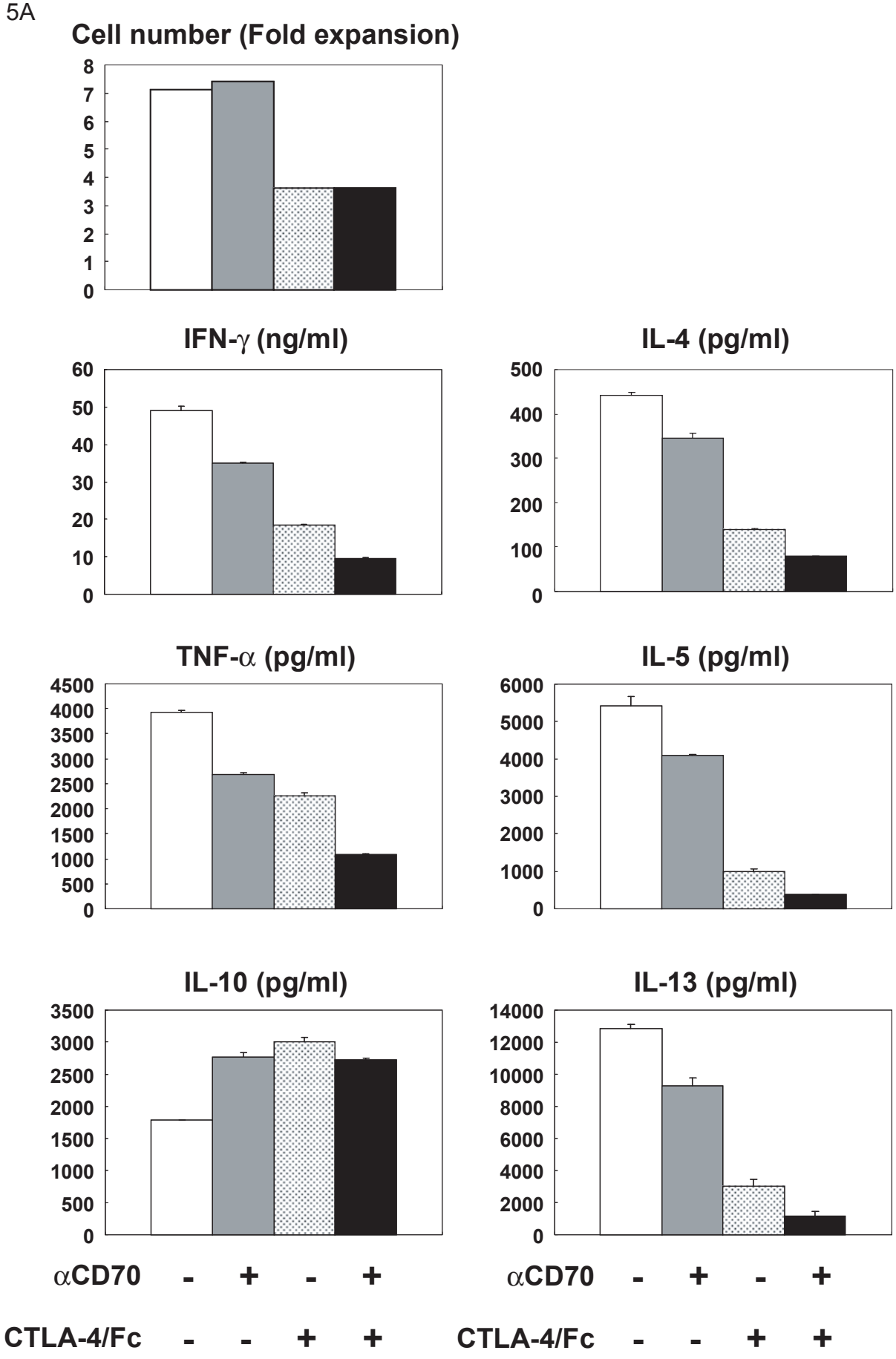

Cell number (Fold expansion)
8
7
6
5
4
3
2
1
0
IFN-γ (ng/ml)
60
50
40
30
20
10
0
IL-4 (pg/ml)
500
400
300
200
100
0
TNF-α (pg/ml)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
IL-5 (pg/ml)
6000
5000
4000
3000
2000
1000
0
IL-10 (pg/ml)
3500
3000
2500
2000
1500
1000
500
0
IL-13 (pg/ml)
14000
12000
10000
8000
6000
4000
2000
0
αCD70 - + - +
CTLA-4/Fc - - + +
αCD70 - + - +
CTLA-4/Fc - - + +

Figure 5B

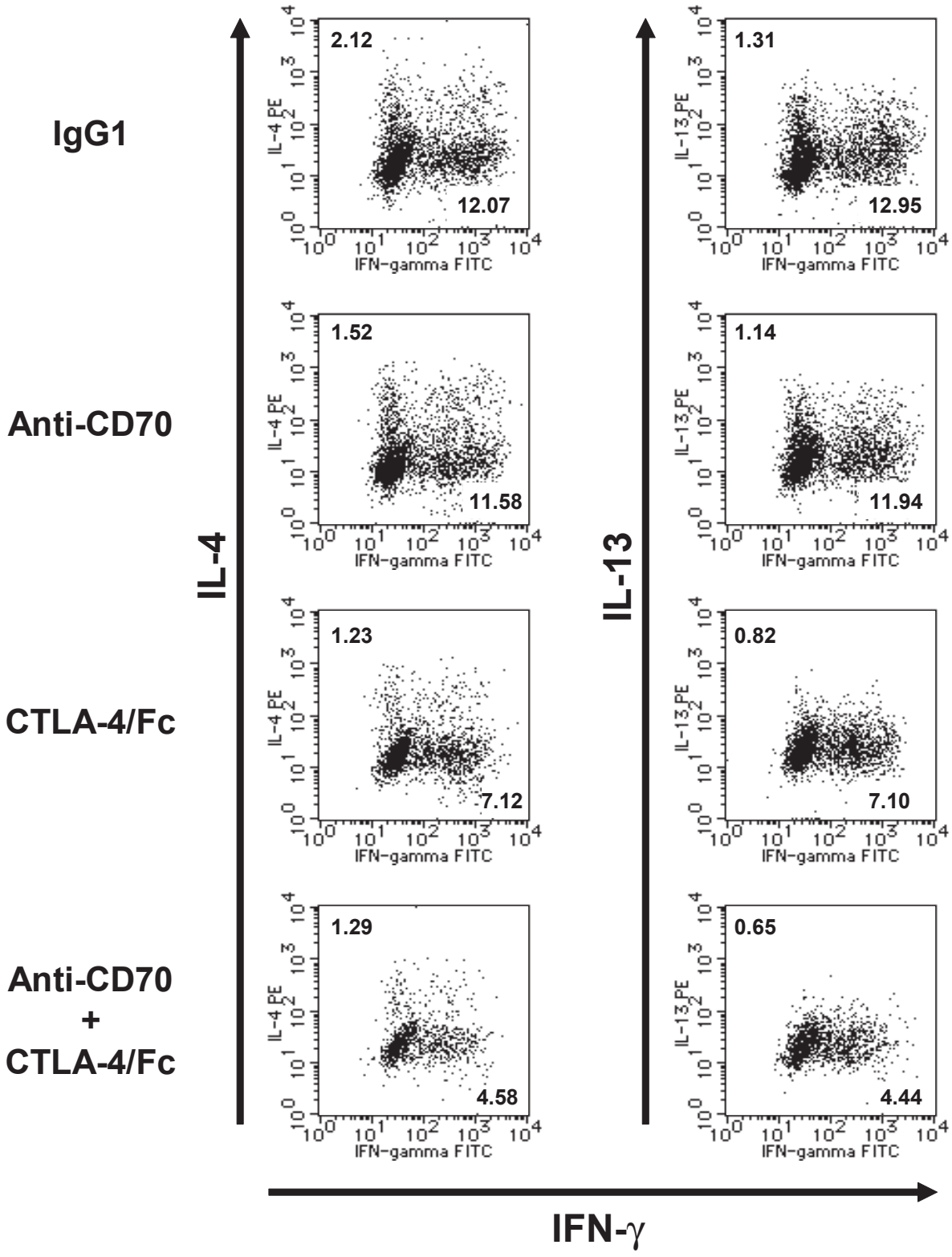

IgG1
Anti-CD70
CTLA-4/Fc
Anti-CD70
+
CTLA-4/Fc
IL-4
IL-13
IFN-γ
IL-4 PE
IL-13 PE
IFN-gamma FITC
2.12
12.07
1.31
12.95
1.52
11.58
1.14
11.94
1.23
7.12
0.82
7.10
1.29
4.58
0.65
4.44

Figure 5C

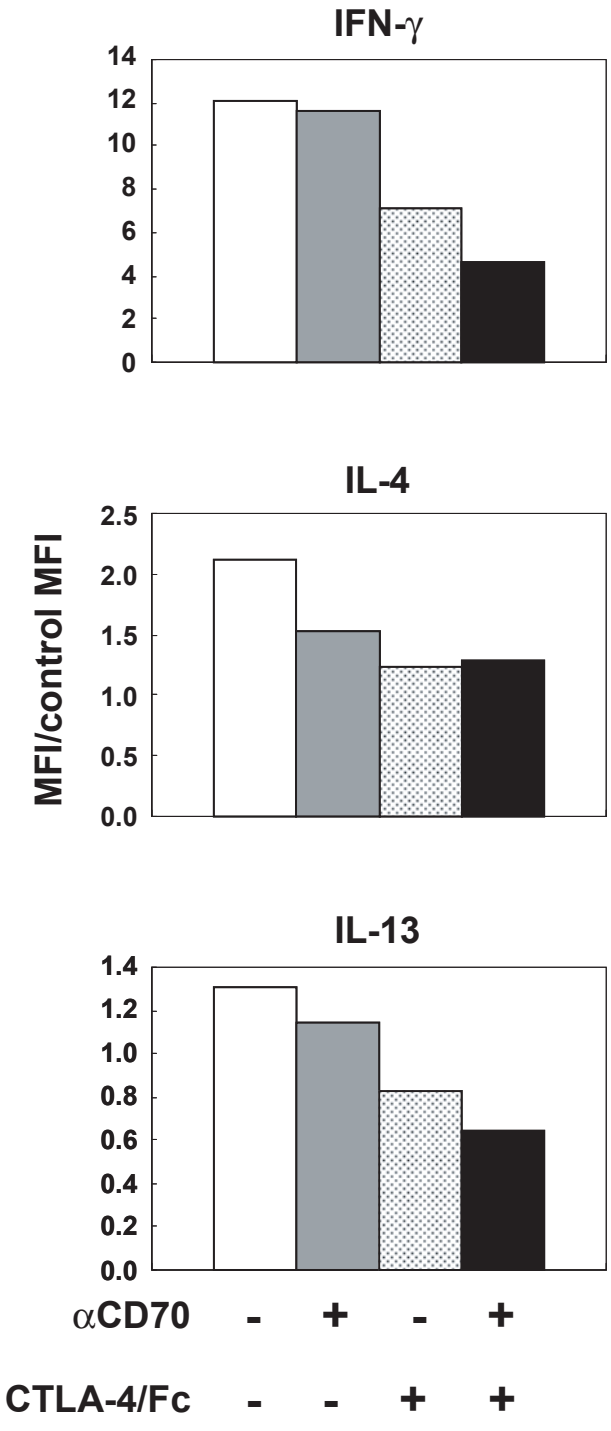

IFN-γ
14
12
10
8
6
4
2
0
IL-4
MFI/control MFI
2.5
2.0
1.5
1.0
0.5
0.0
IL-13
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
αCD70 - + - +
CTLA-4/Fc - - + +

Figure 5D

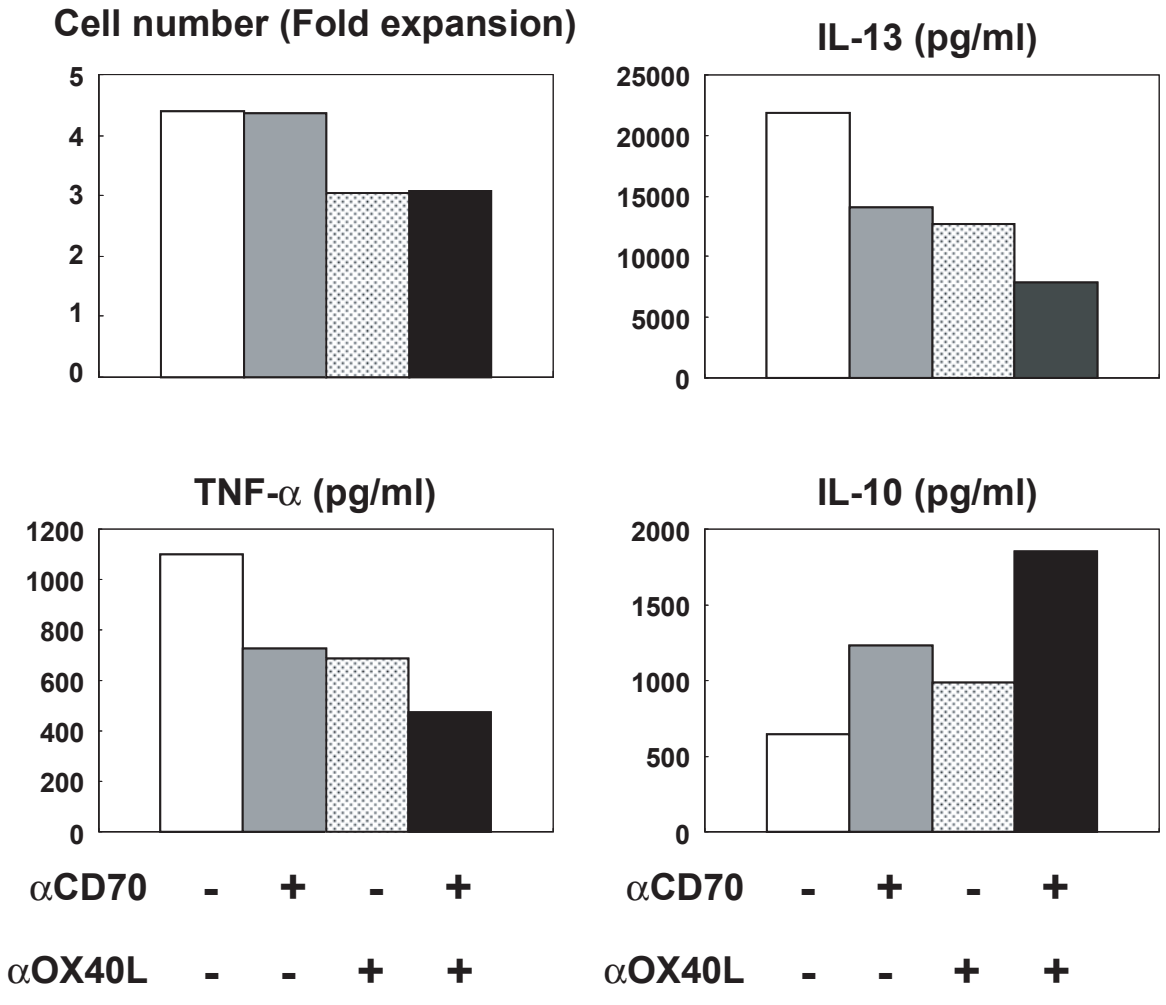
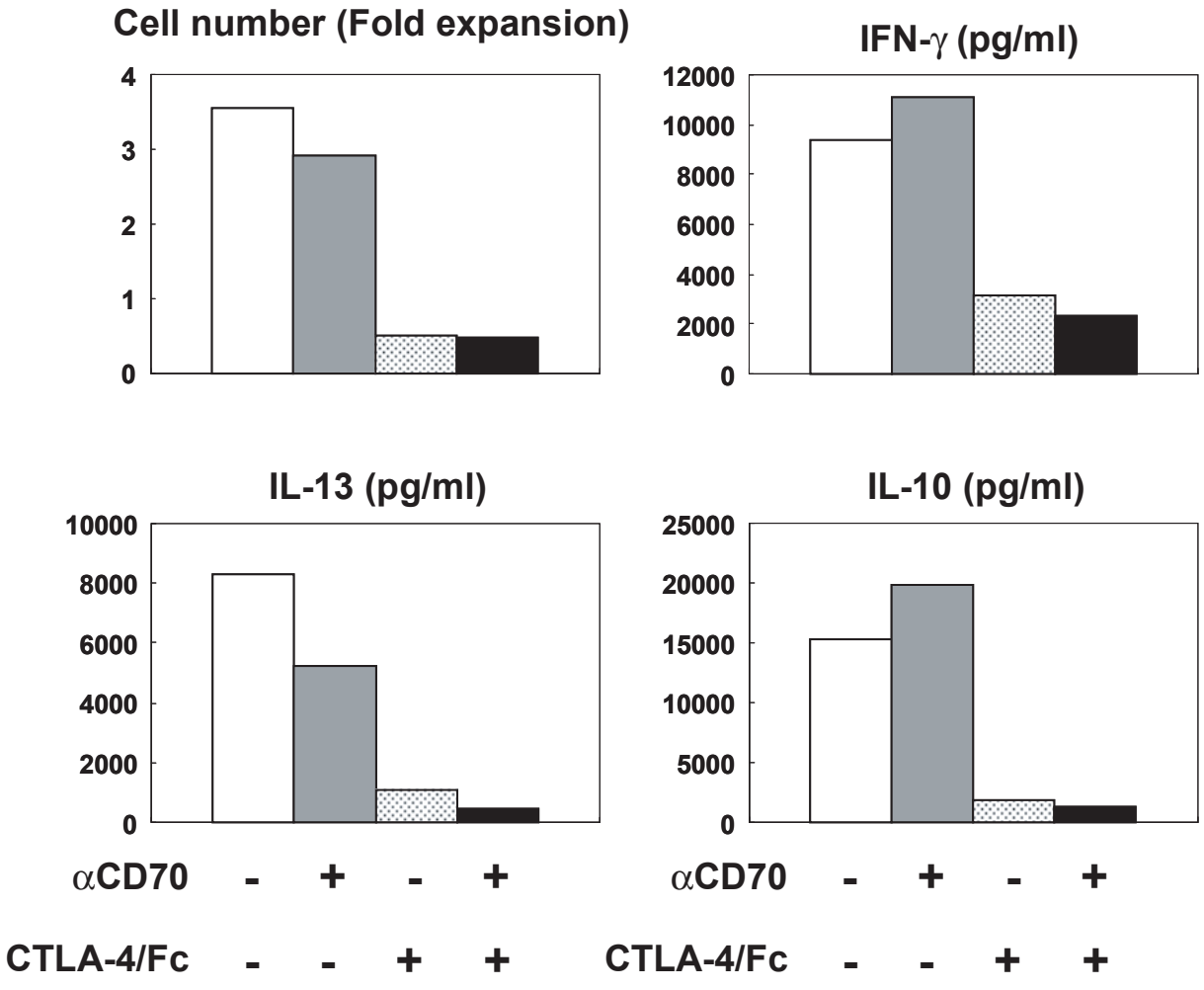
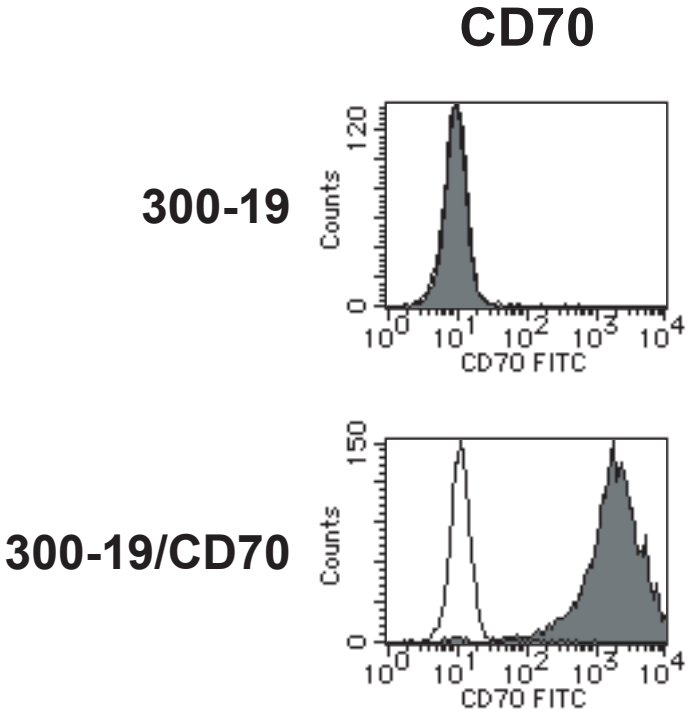

Cell number (Fold expansion)
5
4
3
2
1
0
IL-13 (pg/ml)
25000
20000
15000
10000
5000
0
TNF-α (pg/ml)
1200
1000
800
600
400
200
0
IL-10 (pg/ml)
2000
1500
1000
500
0
αCD70 - + - +
αOX40L - - + +
αCD70 - + - +
αOX40L - - + +

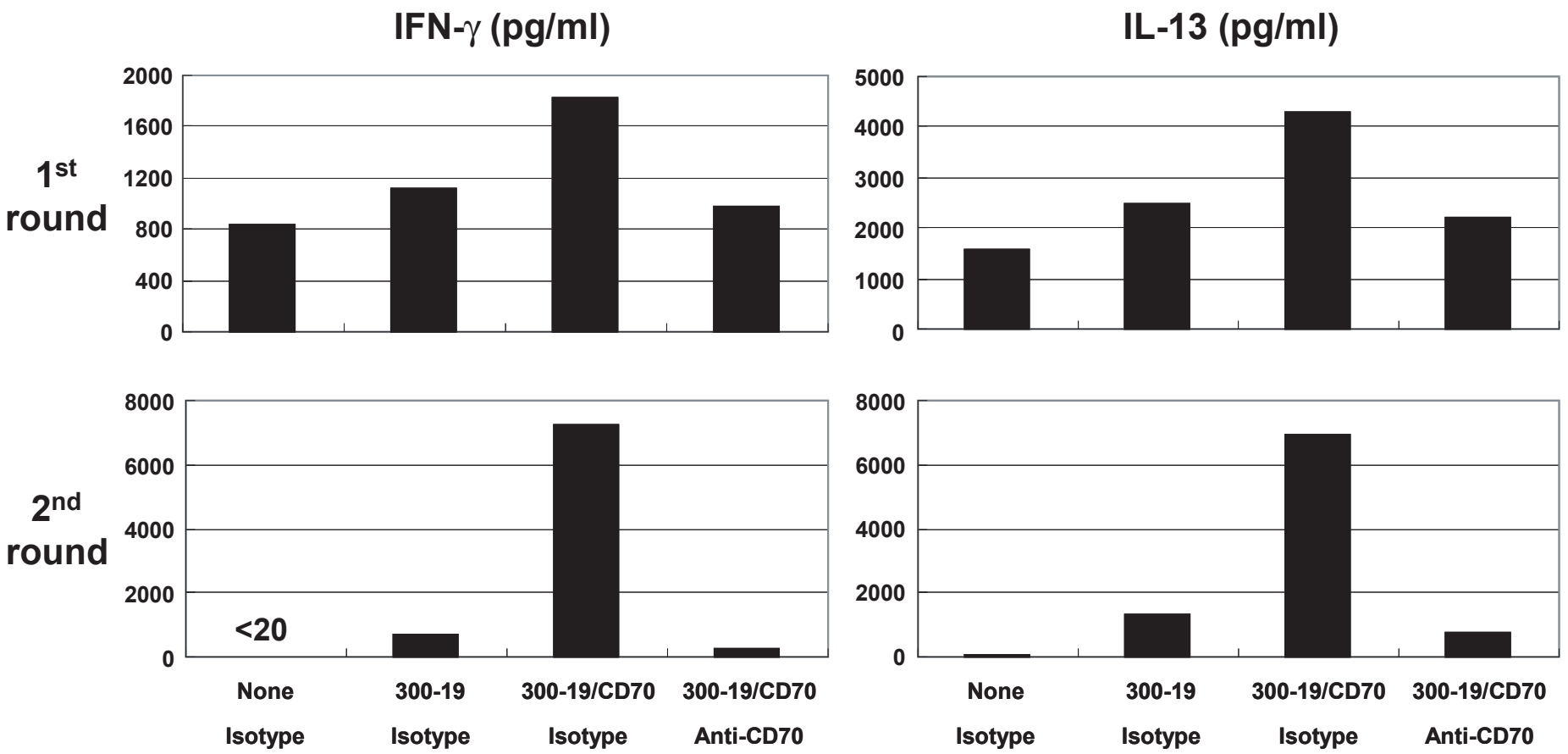
Figure 6


Cell number (Fold expansion)
4
3
2
1
0
IFN-γ (pg/ml)
12000
10000
8000
6000
4000
2000
0
IL-13 (pg/ml)
10000
8000
6000
4000
2000
0
IL-10 (pg/ml)
25000
20000
15000
10000
5000
0
αCD70 - + - +
CTLA-4/Fc - - + +
αCD70 - + - +
CTLA-4/Fc - - + +

Supplementary figure 1



Supplementary figure 2



Supplementary figure 3