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Cooperative action of IL-10 and IFN-γ to regulate dendritic cell functions

Short title: IL-10 and IFN-γ regulate DC functions

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Abbreviations: Ab, antibody; APC, antigen presenting cell; CM, complete medium; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte-macrophage colony-stimulating factor; IDO, indoleamine 2,3-dioxygenase; iNOS, inducible nitric synthase; IFN, interferon; IL, interleukin; JAK, Janus kinase; MACS, magnetic-activated cell-sorting; MHC, major histocompatibility complex; mAb, monoclonal Ab; NO, nitric oxide; ODN, oligonucleotide; PE, phycoerythrin; PBS, phosphate buffered saline; STAT, signal transducers and activators of transcription; Th1, T helper type 1; TNF, tumor necrosis factor; Treg, T regulatory; Tr1, T regulatory type 1;

Summary

Interleukin (IL)-10 and interferon (IFN)-γ double producer is found in a subpopulation of T regulatory type 1 (Tr1) cells and T helper type 1 (Th1). Thus, it is of interest how IL-10 and IFN-γ influence the immune system. However, few studies have addressed the cooperative action of these "immunosuppressive" and "immunostimulatory" cytokines. Here we examined the effect of IL-10 combined with IFN-γ on dendritic cell (DC) functions. Murine bone marrow-derived conventional DCs were stimulated with IL-10 and/or IFN-γ for 24 hr. Tumor necrosis factor (TNF)-α and IL-12 p40 productions by DCs treated with both IL-10 and IFN-γ were significantly lower than those by DCs treated with IL-10 or IFN-γ alone. Major histocompatibility complex class II expression on DCs treated with both cytokines was attenuated compared to that on DCs treated with either cytokine. In contrast, levels of inducible nitric oxide synthase and indoleamine 2,3-dioxygenase, which appear to suppress T-cell responses and promote tolerance, in DCs treated with both cytokines were higher than those in DCs treated with IL-10 or IFN-γ alone. Simultaneous treatment with IL-10 and IFN-γ significantly suppressed ability of DCs to activate $CD4^+$ T cells compared to treatment with either cytokine. Thus, IL-10 and IFN-γ cooperatively suppress immunostimulatory functions of DCs.

Introduction

Interferon (IFN)-γ plays a decisive role in T helper type 1 (Th1) polarization and promotes the cell-mediated immune responses involving NK cells and $CD8^+$ T cells, ultimately leading to the elimination of intracellular pathogens and tumor cells.[1] On the other hand, it has been reported that IFN-γ exhibits not only the immunostimulatory properties but also immunoregulatory functions in certain animal models of autoimmune disease and human autoimmune diseases. Indeed, susceptibility and severity of experimental autoimmune encephalomyelitis and collagen-induced arthritis were increased in susceptible mouse strains deficient in IFN-γ or the IFN-γ receptor.[2-4] However, thus far, the paradoxical roles of IFN-γ have not been taken into full consideration, but rather underestimated comparing with the prominent role as a Th1 cytokine.

Interleukin-10 (IL-10) is a potent immunoregulatory cytokine that inhibits undesirable innate and acquired immune responses.[5, 6] IL-10 can be produced by various cell types including B cells, monocytes/macrophages, DCs, Th2 cells and T regulatory (Treg) cells.[5-9] IL-10 is responsible for limitation and eventual termination of inflammatory responses, which reduces damage of self tissues in the process of pathogen eradication.[5, 6] In addition, IL-10 plays an important role in immune tolerance to protect self organs from autoimmunity.[5] IL-10 antagonizes Th1 responses by inhibiting the production of IFN-γ.[5, 6] IL-10 inhibits IFN-γ-induced monocytes/macrophage activation, subsequent cytokine production and upregulation of costimulatory molecules.[5, 6] On the contrary, it has been reported that IFN-γ suppresses Toll-like receptor-mediated induction of IL-10 expression.[10] Thus, it seems that IL-10 and IFN-γ are entirely adverse factors to regulate immune responses.

Treg cells are efficient regulators of excessive inflammation and autoimmunity. At least three distinct types of Treg cells have been identified; Foxp3 positive Treg cells, Foxp3 negative Th3 cell and T regulatory type 1 (Tr1) cells.[9] Tr1 cells mainly produce IL-10 and thereby suppress various inflammatory responses. Of interest, it has been shown that a subset of Tr1 cells produce substantial level of IFN-γ as well as IL-10.[11] However, the biological and immunological significance of the simultaneous productions of these apparently opposite, "immunosuppressive" and "immunostimulatory", cytokines has been never elucidated.

Dendritic cells (DCs) are potent professional antigen presenting cells (APCs) that are primarily responsible for the initiation and regulation of immune responses against various antigens.[12-14] It has been reported that a variety of extracellular stimuli such as cytokines, chemical mediators and pathogen associated molecular patterns modulate DC ability to induce T cell activation and Th1, Th2 and Th17 differentiation.[15-20] However, no report has demonstrated cooperative action of IL-10 and IFN-γ on DC functions. We examined the effect of IL-10 combined with IFN-γ on DC functions using murine bone marrow-derived conventional DCs. Here we demonstrate that these cytokines cooperatively suppress immunostimulatory functions of DCs.

Materials and Methods

Mice

C57BL/6 (B6) and BALB/c mice were purchased from Japan SLC Inc (Hamamatsu, Shizuoka, Japan) and maintained in a specific pathogen-free condition of our animal facility at Hokkaido University. All experiments were approved by regulations of Hokkaido University Animal Care and Use Committee.

Reagents and antibodies (Abs)

Murine recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF) was purchased from PeproTech (London, United Kingdom). Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD86 monoclonal Ab (mAb) (GL1), FITC-conjugated anti-mouse CD69 mAb (H1.2F3), biotin-conjugated anti-I-A^b mAb (AF6-120.1) and streptavidin Per-CP™ were obtained from BD Biosciences (San Diego, CA). As a control IgG, FITC-conjugated rat IgG2a and PE-conjugated hamster IgG were purchased from BioLegend (San Diego, CA). Biotin-conjugated mouse IgG2a was purchased from Immunotech (Marseille, France). Low endotoxin and azide-free purified anti-mouse CD210 (IL-10R) mAb (1B1.3a) and rat IgG1 (isotype control) were obtained from BioLegend. Purified anti-phospho-signal transducers and activators of transcription (STAT1) (Tyr701) Ab, anti-phospho-STAT3 (Tyr705) mAb (3E2) and anti-GAPDH mAb (14C10) were purchased from Cell Signaling Technology (Beverly, MA). Purified anti-inducible nitric synthase (iNOS) mAb (6/iNOS/NOS Type II) was obtained from BD Biosciences. Purified anti-indoleamine 2,3-dioxygenase (IDO) mAb (mIDO-48) was obtained from BioLegend.

DC culture

Murine bone marrow-derived DCs (BMDCs) were generated as previously described [21,22] with a minor modification. Bone marrow cells were prepared from femur and tibial bone marrow of B6 mice. After lysis of erythrocytes, major histocompatibility complex (MHC) class II, CD45R (B220), CD4, and CD8 positive cells were removed by killing with mAbs (1E4, RA3-6B2, GK1.5, and 53.4.9) and rabbit complement. The cells were

cultured in complete medium (CM), RPMI-1640 medium (Sigma Chemical) containing 5% fetal calf serum, 20 ng/ml GM-CSF, 50 μM 2-melcaptoethanol, 100 IU/ml penicillin and 100 μg/ml streptomycin, at a density of 1 x 10^6 cells/ml/well using a 24 well plate (tissue culture treated, FALCON®, Becton Dickinson Labware, Franklin Lakes, NJ). On day 2, the medium was gently exchanged to fresh CM. On day 4, nonadherent granulocytes were removed without dislodging clusters of developing DCs and fresh CM was added. On day 6, free-floating and loosely adherent cells were collected and cultured in CM at a density of $1{\cdot}5 \times 10^6$ cells/3 ml/35-mm dish (non treated, NUNC™, Nalge Nunc International, New York, USA). On day 7, suspended cells and adherent cells detached with 3 mM ethylenediaminetetraacetic acid (EDTA) were collected and used as conventional DCs (>97% $CD11c^+$ $B220^-$). DC culture was performed using CM in the whole experiments.

Measurement of tumor necrosis factor (TNF) and IL-12

DCs were cultured with IL-10 (20 ng/ml) and/or IFN-γ (20 ng/ml) for 24 hr at a density of 1×10^5 cells/200 μl/well using a 96 well plate (tissue culture treated, FALCON®) and the culture supernatants were subjected to quantification of the protein level of TNF-α and IL-12 p40 by enzyme-linked immunosorbent assay (ELISA) using OptEIA™ Set (BD Biosciences Pharmingen). The dose of each cytokine was determined on the basis of our preliminary dose-response study (data not shown).

Measurement of cell surface expression of CD86 and I-A^b

DCs were cultured with IL-10 (20 ng/ml) and/or IFN-γ (20 ng/ml) for 24 hr at a density of $1{\cdot}5 \times 10^6$ cells/3 ml/35-mm dish (non treated, NUNC™). The cells were detached with 3

mM EDTA, collected, and washed 3 times. The cells were incubated with 2.4G2 (rat anti-mouse Fcγ III/II receptor, CD16/CD32) supernatant to prevent binding of specific mAb to Fcγ III/II receptor and then stained with FITC- or biotin-conjugated mAb and streptavidin-PerCP™. Expression of cell surface markers was analyzed by flow cytometry on EPICS® XL (Beckman Coulter. Miami, FL). Mean fluorescence intensity (MFI) subtracted from the level of isotype-matched control Ab was shown.

Nitric oxide (NO) measurement

NO production was assayed by measurement of the nitrite concentration with the Griess-Romijin assay as previously described [23]. DCs were cultured with IL-10 (20 ng/ml) and/or IFN-γ (20 ng/ml) for 24 hr at a density of 1×10^5 cells/200 μl/well using a 96 well plate (tissue culture treated, FALCON®). Supernatants (100 μl) were added to 100 μl of 6 mg/ml Griess-Romijin reagent and incubated at room temperature for 10 min. Absorbance at 540 nm was measured with a microplate reader. Nitrite concentrations were calculated with a sodium nitrite standard curve as reference.

Immunoblotting

DCs were cultured with IL-10 (20 ng/ml) and/or IFN-γ (20 ng/ml) for the indicated time period at a density of 1×10^6 cells/2 ml/well or 5×10^5 cells/1 ml/well using a 24 or 48 well plate (tissue culture treated, FALCON®). Reactions were halted by rapidly cooling on ice and these DCs were washed by ice-cold phosphate buffered saline (PBS). The whole cell lysates were prepared using cell lysis buffer (Cell Signaling Technology). The cell lysates were separated by SDS-PAGE, then blotted onto a PVDF membrane (Immobilon™-P transfer membrane, Millipore). The membrane was probed with

primary Ab, and developed with horseradish-peroxidase conjugated secondary Ab by enhanced chemiluminescence. The luminescence intensity was quantified using a luminescent image analyzer LAS 1000 (Fujifilm, Tokyo, Japan) and a software program Image Gauge version 3.4 (Fujifilm) as descried elsewhere.[24] The intensity relative to the mean of all band intensities in each experiment was shown as a relative intensity.

DC activation of allogeneic $CD4^+$ T cells

DC activation of allogeneic $CD4^+$ T cells was performed using purified $CD4^+$ T cells from BALB/c mouse lymph nodes. $CD4^+$ T cells were purified from axillary, mesenteric and inguinal lymph node cells using anti-CD4 (L3T4) MicroBeads and a magnetic-activated cell-sorting (MACS) column (Miltenyi Biotec, Bergisch Gladbach, Germany). Purity of the $CD4^+$ T cells ($CD4^+$ $TCR\beta^+$ cells) was more than 98%.

BMDCs from B6 mice were cultured with IL-10 (20 ng/ml) and/or IFN-γ (20 ng/ml) for 24 hr at a density of $1{\cdot}5 \times 10^6$ cells/3 ml/35-mm dish (non treated, NUNC™). The cells were detached with 3 mM EDTA, collected, and extensively washed 3 times. The cytokine primed DCs (2×10^4 cells) were co-cultured with $CD4^+$ T cells (2×10^5 cells) for 4 or 5 days on a U bottom 96-well plate (FALCON®) in triplicate wells. The number of $CD69^+$ activated T cells in the culture of each group was counted by flow cytometry using comparison to a known number of beads as an internal standard (Flow-Count, Beckman Coulter).

Statistical analysis

The Student's *t*-test was used to analyze data for significant differences. *P* values less than 0·05 were regarded as significant.

Results

STAT3 and STAT1 activation in DCs upon IL-10 and IFN-γ stimulation

Hematopoietic cytokine receptor signaling is largely mediated via Janus kinase (JAK)-STAT pathway.[25] STAT3 is a key mediator of IL-10 responses, while STAT1 plays a major role in IFN-γ signaling.[25] We first examined effects of IL-10 and IFN-γ on STAT1 and STAT3 activity in murine BMDCs that were positive for CD11b and negative for CD8 and B220 (data not shown), a pattern typical of conventional DCs.

DCs were stimulated with IL-10, IFN-γ, or both for 15 or 30 min and intracellular protein levels of active form of STAT1 and STAT3, phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3), were determined (Fig. 1 A and B). Neither pSTAT1 nor pSTAT3 was detected in unstimulated DCs. IL-10 markedly increased pSTAT3 in DCs at 15 and 30 min after the stimulation, while showing no influence on the level of pSTAT1. IFN-γ induced substantial level of pSTAT1 and slight level of pSTAT3 at 15 and 30 min. These findings are consistent with previous studies.[25] Simultaneous stimulation of DCs with IL-10 and IFN-γ resulted in marked activation of both STAT1 and STAT3. It should be noted that neither IFN-γ nor IL-10 showed significant effect on the IL-10-induced STAT3 activation or IFN-γ-induced STAT1 activation, respectively.

The bands of pStat1 and pStat3 at 30 min appeared to be slightly weak after the double cytokine culture compared to that after culture with IFN-γ alone (pSTAT1) and with IL-10 alone (pSTAT3), respectively, in the immunoblot shown in Figure 1A. However, these differences were not observed in other two separate experiments and the mean of intensities was not different among these three groups (Fig. 1B).

Effects of IL-10 and IFN-γ on cytokine production by DCs

Cytokine production by DCs was crucial in regulating innate and adoptive immunity. We thus examined effects of IL-10 and IFN-γ on cytokine production by DCs. BMDCs were treated with IL-10, IFN-γ, or both cytokines for 24 hr and TNF-α and IL-12 levels in the supernatants were quantified. Treatment with IL-10 alone slightly attenuated spontaneous DC production of TNF-α, while significantly decreased that of IL-12 p40 compared to untreated control DCs (Fig. 2). It has been reported that IFN-γ alone shows little or no effect on cytokine production by DCs unlike macrophages.[26] As reported previously, IFN-γ alone showed little or no effect on TNF-α and IL-12 p40 productions by DCs. This low IL-12 p40 production stands marked contrast to that by TLR ligands-stimulated DCs.[27, 28] Treatment with IL-10 combined with IFN-γ more significantly decreased DC production of TNF-α and IL-12 p40 than IL-10 alone. This finding demonstrates that IFN-γ augments suppressive function of IL-10 in cytokine production by DCs.

Although we attempt to quantitate production of biologically active IL-12p70, no detectable IL-12 p70 was obtained in any cultures tested (data not shown). Increased cell concentration and potent stimulators may be required to detect IL-12p70 production by DCs.[29]

Effects of IL-10 and IFN-γ on cell surface expression of CD86 and MHC class II on DCs

We next evaluated the expression of cell surface markers on DCs. BMDCs were treated with IL-10, IFN-γ, or both cytokines for 24 hr, and cell surface expression of CD86 and MHC class II ($I\text{-}A^b$) were analyzed by flow cytometry. IL-10 alone showed little effect

on CD86 expression on DCs. IFN-γ markedly increased CD86 expression on DCs compared to that on untreated (control) DCs. IL-10 completely blocked the IFN-γ-induced elevation of CD86 expression on DCs (Fig. 3). Treatment with IL-10 alone showed little effect on I-A^b expression on DCs, while IFN-γ slightly increased the I-A^b expression on DCs compared to control DCs. Of note, IL-10 combined with IFN-γ significantly decreased I-A^b expression on DCs compared to IL-10, IFN-γ, or medium alone treated group.

Synergistic effect of IL-10 and IFN-γ on NO production and iNOS expression by DCs

iNOS induction and subsequent NO production by macrophages are responsible for efficient eradication of microbes.[30] On the other hand, it has been reported that DC-produced NO negatively regulates T cell proliferation during the antigen presentation.[31] Thus, NO functions appear to be different depending on the cell type and assay system used. Although mechanism underlying iNOS induction and NO production has been extensively investigated in monocytes/macrophages, regulatory system of NO production and iNOS induction in DCs has not been well documented.

We then examined effects of IL-10 and IFN-γ on NO production and iNOS induction in DCs. IFN-γ significantly increased NO production and iNOS expression by DCs in agreement with a previous study (Fig. 4).[31] IL-10 alone showed no effects on NO production and iNOS expression by DCs. Of note, treatment with both IFN-γ and IL-10 considerably augmented iNOS expression and NO production. Thus, IL-10 acted in synergy with IFN-γ in NO production and iNOS expression by DCs. The IL-10-mediated synergy in IFN-γ-induced NO production was completely blocked by

treatment with anti-IL-10R (CD210) mAb (Fig. 4).

We also performed dose-response study in NO production by DCs (Fig. 4C). IFN-γ alone increased NO production by DCs in a dose-dependent manner and the effect reached a peak at 20 ng/ml. In the presence of IL-10 (20 ng/ml), IFN-γ synergistically augmented the NO production in a dose-dependent manner similar to that produced by DCs stimulated with IFN-γ alone. On the other hand, IL-10 alone showed no effects on NO production by DCs up to 80 ng/ml (Fig. 4C). In contrast, IL-10 combined with IFN-γ (20 ng/ml) markedly increased NO production by DCs in a dose-dependent manner and the effect reached a peck at 20 ng/ml IL-10. Thus, it was demonstrated that the maximum effect of these cytokines was obtained at 20 ng/ml concentration.

Effects of IL-10 and IFN-γ on indoleamine 2,3-dioxygenase (IDO) expression by DCs

IDO is an enzyme that degrades the essential amino acid tryptophan. IDO produced by DCs appears to suppress T-cell responses and promote tolerance.[32] We next compared effects of IFN-γ, IL-10 and IFN-γ plus IL-10 on IDO expression in DCs. Certain level of IDO was detected in untreated (control) DCs (Fig. 5). Treatment with IL-10 alone never affected the IDO expression. By contrast, IFN-γ treatment markedly upregulated IDO expression in DCs. Of note, IL-10 significantly enhanced the IFN-γ-induced IDO expression.

Ability of DCs treated with IFN-γ and IL-10 to activate $CD4^+$ T cells

To evaluate effects of IFN-γ and IL-10 on APC ability of DCs to activate $CD4^+$ T cells, DCs from B6 mice were pretreated with IFN-γ and/or IL-10, extensively washed and then

co-cultured with $CD4^+$ T cells (>98%) from BALB/c mouse. On day 4 and 5, the number of $CD69^+$ activated T cells in the culture was counted by flow cytometry. Treatment with IFN-γ or IL-10 alone showed no significant effect on DC ability to activate $CD4^+$ T cells (Fig. 6). In contrast, simultaneous treatment of DCs with IL-10 and IFN-γ significantly suppressed the APC ability to activate $CD4^+$ T cells.

Discussion

Tr1 cells produce large amounts of IL-10. It has been reported that a subpopulation of the Tr1 cells also produces substantial level of IFN-γ but no IL-4.[11] Tr1 cells suppress T cell responses *in vitro* and *in vivo*. In a colitis model, administration of Tr1 cells has been shown to ameliorate the disease manifestation.[33] Although it seems clear that the IL-10 produced by Tr1 cells is responsible for their immunosuppressive activity, the role of IFN-γ simultaneously produced by a certain subset of Tr1 cells remains elusive. The IL-10 and IFN-γ double producer cells have also been found in T-bet^+ $Foxp3^+$ Th1 cells from *Toxoplasma gondii* or *Leishmania major* infected mice.[34] The IL-10 produced by the Th1 cells in these infected mice appeared to moderate the inflammatory responses. It seems that Th1 cells control themselves by producing IL-10 to minimize the tissue damage. However, coordinated action of IL-10 and IFN-γ produced by certain subsets of Tr1 and Th1 cells has not been taken into consideration in these previous studies.

In the present study, we examined influences of simultaneous signaling from these "immunosuppressive" and "immunostimulatory" cytokines on DC functions. As previously reported, IL-10 treatment suppressed the DC production of TNF-α and IL-12. Of note, treatment of DCs with both IL-10 and IFN-γ cooperatively induced a

considerable down-modulation of not only these inflammatory cytokines but also MHC class II expression. On the contrary, expressions of iNOS and IDO, which can suppress T-cell responses,[31, 32] were significantly increased by the simultaneous treatment of DCs with IL-10 and IFN-γ. In addition, APC ability of these DCs to activate $CD4^+$ T cells was markedly suppressed. A single treatment with either cytokine exhibited no such suppression. From these findings, we concluded that IL-10 and IFN-γ cooperatively suppressed immunostimulatory functions of murine conventional DCs.

It has been reported that IFN-γ markedly increased TNF-α and NO productions by macrophages and IL-10 decreased the IFN-γ-induced NO production.[35-37] However, IL-10 showed little or no effect on the IFN-γ-induced NO production in the presence of excessive amount of exogenous TNF-α. These findings suggest that IL-10 decreases the macrophage NO production by inhibiting of TNF-α synthesis following IFN-γ stimulation. Thus, the autocrine production of TNF-α appears to be responsible for accelerating NO production by macrophages.

On the other hand, regulation system of NO production by DCs has not been well characterized compared to that by macrophages. In the present study, we found that IFN-γ increased NO production and iNOS expression by DCs, while showing no effects on TNF-α production. It was also shown that IL-10 markedly enhanced the IFN-γ-induced NO production by DCs, but significantly decreased TNF-α production. Thus, it seems that TNF-α is not involved in the IL-10-induced enhancement of NO production by DCs. This finding stands marked contrast to that obtained with macrophage analysis.

It has been reported that IFN-γ-induced iNOS expression was synergistically enhanced

by IL-22, a newly described member of the IL-10 cytokine family, in human DLD-1 colon carcinoma cells.[38] IL-22 induced marked phosphorylation of STAT3 in these cells and short interfering RNA technology identified that STAT3 was crucial for the synergy in iNOS expression by DLD-1 cells. In the present study, IL-10 induced marked phosphorylation of STAT3 and synergistically enhanced IFN-γ-induced iNOS expressions in DCs. The IL-10-induced STAT3 activation may be responsible for the synergistic augmentation of IFN-γ-induced iNOS expression. Thus far, we have been unable to apply short interfering RNA technology for STAT3 knockdown in our DC culture system.

IDO degrades the essential amino acid tryptophan and shows immune suppressive properties via tryptophan starvation in the microenvironment.[32] IDO also induces production of pro-apoptotic metabolites, kynurenines, from the tryptophan catabolism. Indeed, IDO produced by DCs during the antigen presentation suppresses the T cell proliferation and promotes T cell tolerance. IDO expression by DCs is induced by IFN-γ via STAT1 pathway. However, definitive role of IL-10 in the IFN-γ-induced IDO expression by DCs has been unclear. We could demonstrate herein that IL-10 significantly enhanced the IFN-γ-mediated IDO expression by DCs without apparent augmentation of STAT1 activation. It seems that IL-10 cooperatively act with IFN-γ for the expression of IDO, an immunosuppressive factor in DCs, by a STAT1-independent pathway.

It has been reported that IL-10 suppresses DC functions including cytokine production, cell surface marker expression and ability to induce T cell proliferation. However, IL-10 alone showed no significant effects on the cell surface marker expression on DCs and DC ability to induce T cell proliferation. In the previous studies, IL-10 was added during the

DC development from the precursor cells [39] or DC were treated with IL-10 for a long time (i.e. 2 days) [40,41] compared to the present study (24 hr). It seems that short term treatment with IL-10 alone is not sufficient to modify DC functions.

On the other hand, it has been reported that IL-10 clearly suppressed DC activation by simultaneous or subsequent stimulation with potent stimulators such as LPS and a cytokine cocktail, but IL-10 alone showed limited effects on DC functions.[42-44] Similarly, IL-10 alone showed limited effects in most responses in our present study. However, a considerable effects of IL-10 on DC functions was observed upon simultaneous treatment with IFN-γ. Thus, the effects of IL-10 on DC functions were clearly revealed upon simultaneous or subsequent stimulation with another factor(s).

In the present study, we showed a novel regulation system via cooperative action of IFN-γ and IL-10 in DC functions. This regulation may contribute to suppress excessive undesirable immunity or terminate the immune responses after pathogen eradication in response to IFN-γ and IL-10 double producer of Tr1 or Th1 cells. Elucidation of the complex pathways of DC regulation via IFN-γ and IL-10 may lead to the development of clinical applications exploiting this new regulation system for the treatment of various infectious diseases and immune disorders.

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Figure legends

Figure 1. STAT1 and STAT3 activation in DCs upon IL-10 and IFN-γ stimulation. BMDCs were treated with IL-10 and IFN-γ for 15 or 30 min. Levels of phospho-STAT1 (pSTAT1) and phospho-STAT3 (pSTAT3) were determined by immunoblotting. A, Representative immunoblot from three independent experiments is shown. B, The relative intensity of the specific band is shown. Each column represents the mean ± SE of three independent experiments.

Figure 2. Effects of IL-10 and IFN-γ on cytokine production by DCs. BMDCs were treated with IL-10 and/or IFN-γ for 24 hr. The amounts of TNF-α and IL-12 p40 in the culture supernatants were measured by ELISA. Each column represents the mean ± SE of three independent experiments. Statistical significance was calculated by Student's *t*-test (*: $p<0{\cdot}05$).

Figure 3. Effects of IL-10 and IFN-γ on CD86 and I-A^b expressions on DCs. BMDCs were treated with IL-10 and/or IFN-γ for 24 hr. A, Representative histogram from three independent experiments. B, Each column represents the mean ± SE of three independent experiments. Statistical significance was calculated by Student's *t*-test (*: $p<0{\cdot}05$, **: $p<0{\cdot}01$).

Figure 4. Effects of IL-10 and IFN-γ on NO production and iNOS expression by DCs. BMDCs were treated with IFN-γ (IFN) and IL-10 (IL10) for 24 hr in the presence or absence of anti-IL-10R mAb (αIL10R) or isotype matched control IgG (IgG). A, NO

production. Cytokines were treated at 20 ng/ml. Each column represents the mean ± SE of three independent experiments. B, Levels of iNOS and GAPDH were determined by immunoblotting. Cytokines were treated at 20 ng/ml. Representative immunoblot from three independent experiments is shown (upper). The relative intensity of the specific band is shown (lower). Each column represents the mean ± SE of three independent experiments. Statistical significance was calculated by Student's *t*-test (**: $p<0{\cdot}01$). C, Dose-response study in NO production. DCs were treated with IFN-γ (top) or IL-10 (bottom) at 0.078, 0.313, 1.25, 5, 20, or 80 ng/ml in the presence or absence of IL-10 (20 ng/ml) (top) or IFN-γ (20 ng/ml) (bottom). Each symbol represents the mean of triplicate wells. The analysis was repeated twice with essentially the same results.

Figure 5. Effects of IL-10 and IFN-γ on IDO expression in DCs. BMDCs were treated with IL-10 and/or IFN-γ for 24 hr. IDO level was determined by immunoblotting. Representative immunoblot from three independent experiments is shown (left). The relative intensity of the specific band is shown (right). Each column represents the mean ± SE of three independent experiments. Statistical significance was calculated by Student's *t*-test (*: $p<0{\cdot}05$, **: $p<0{\cdot}01$).

Figure 6. Ability of cytokine primed DCs to activate allogeneic $CD4^+$ T cells. BMDCs (B6) were untreated (control DC) or treated with IL-10 and/or IFN-γ for 24 hr (IL-10 DC, IFN DC, or IL10 + IFN DC). Each group of DCs (2×10^4 cells) was co-cultured with $CD4^+$ T cells (2×10^5 cells) (BALB/c) and the number of expanded $CD69^+$ activated T cells was counted on day 4 and 5. Each symbol represents the mean ± SE of three (day 4) or five (day 5) independent experiments (*: $p<0{\cdot}05$, **: $p<0{\cdot}01$ vs. control DC).

Figure 1A

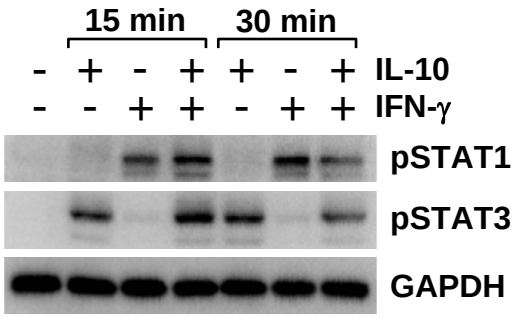


Figure 1B

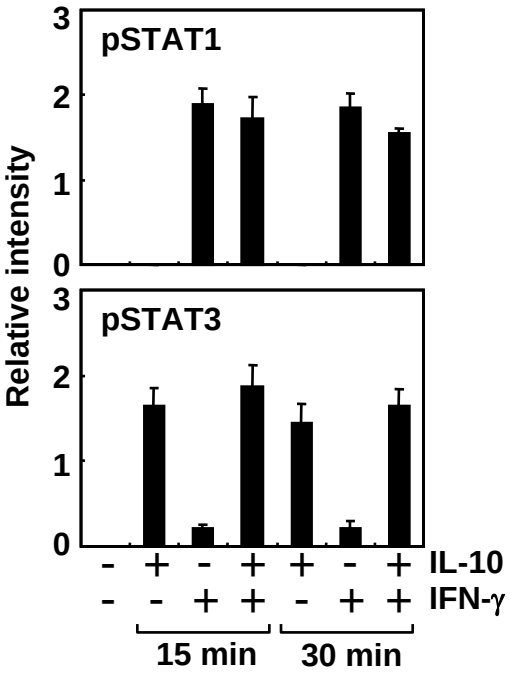


Figure 2

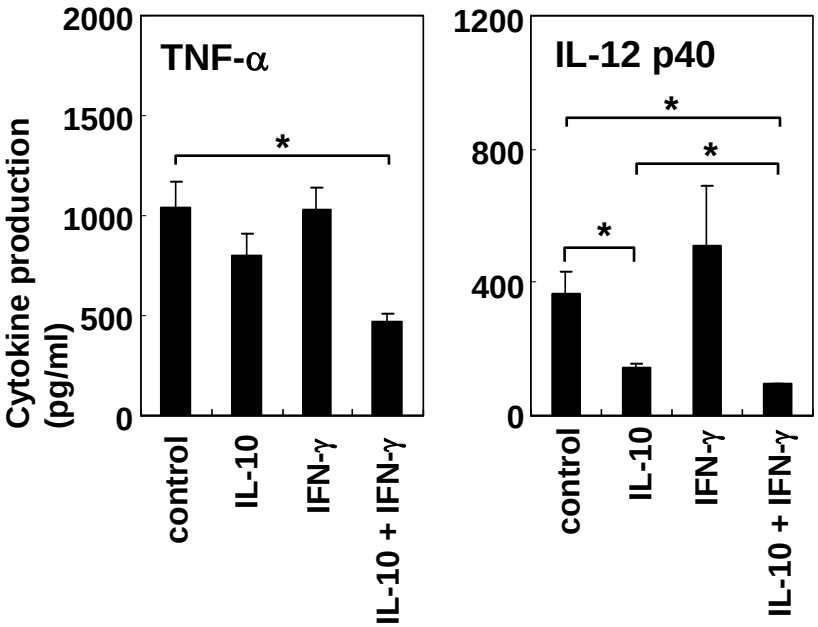

TNF-α
IL-12 p40
Cytokine production (pg/ml)
2000
1500
1000
500
0
1200
800
400
0
*
*
*
*
control
IL-10
IFN-γ
IL-10 + IFN-γ
control
IL-10
IFN-γ
IL-10 + IFN-γ

Figure 3A

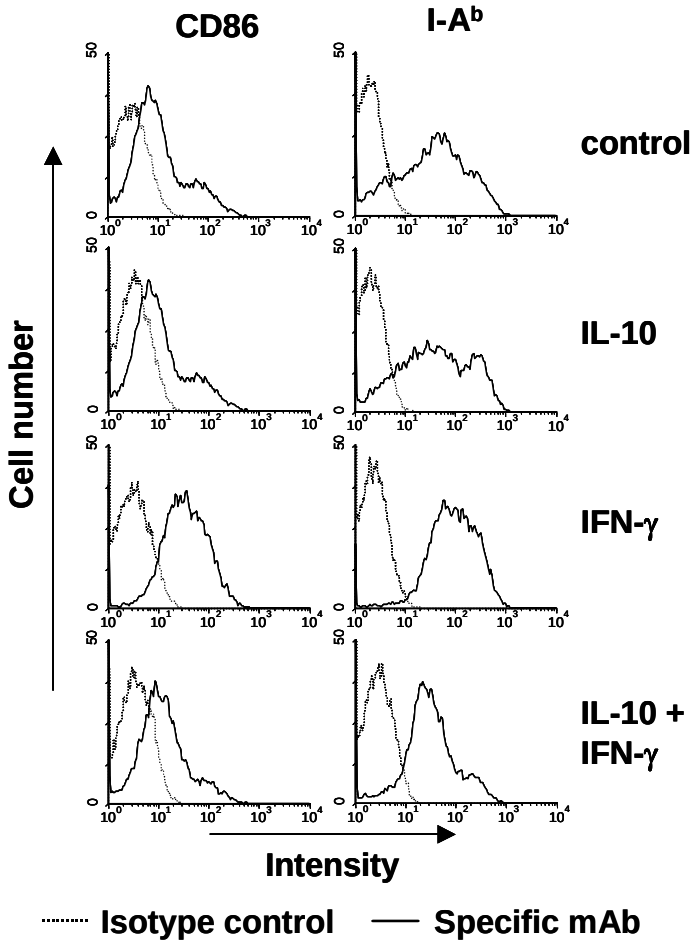


Figure 3B

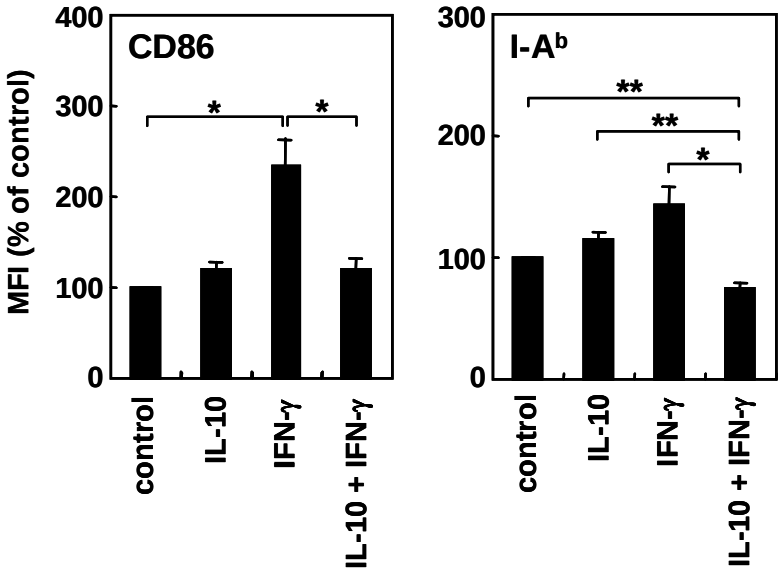


Figure 4A

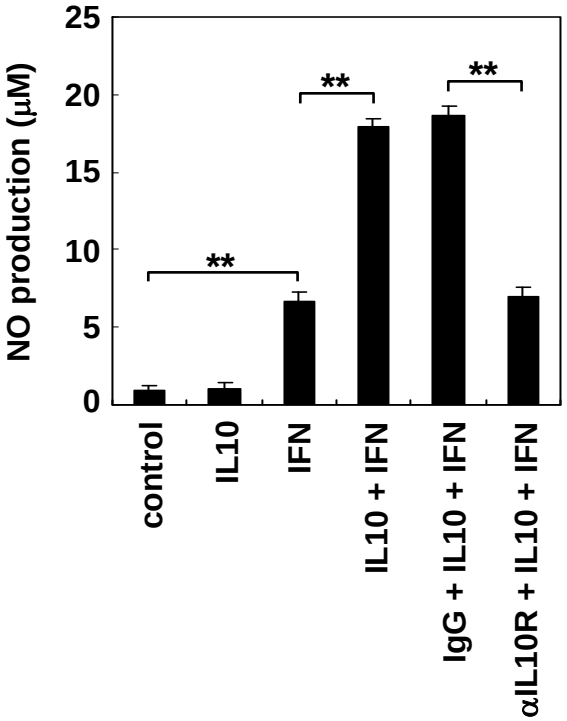


Figure 4B

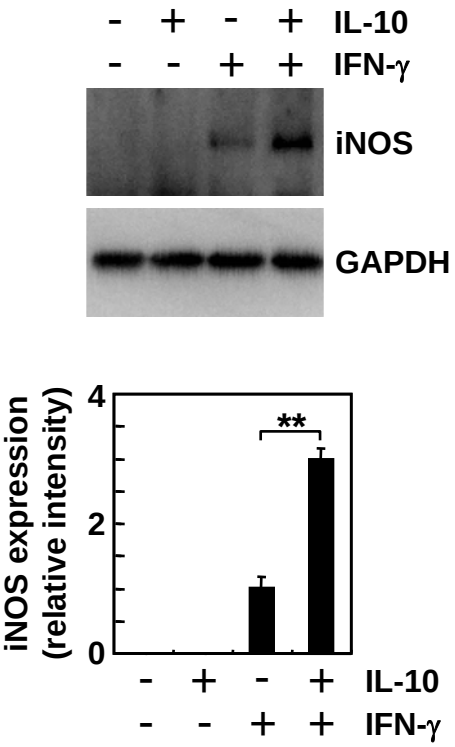


Figure 4C

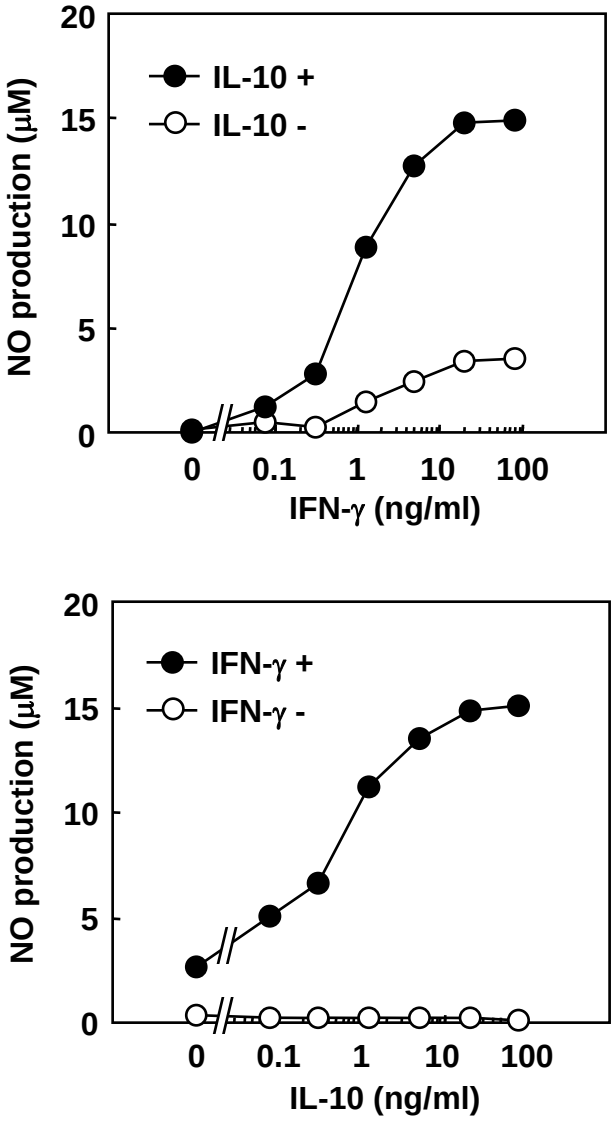
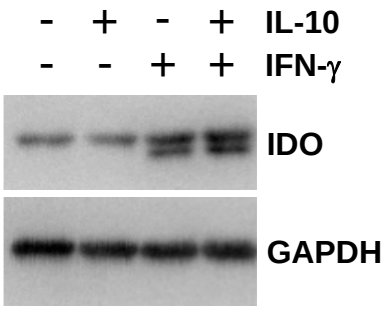

20
15
10
5
0
NO production (μM)
IL-10 +
IL-10 -
0
0.1
1
10
100
IFN-γ (ng/ml)
20
15
10
5
0
NO production (μM)
IFN-γ +
IFN-γ -
0
0.1
1
10
100
IL-10 (ng/ml)

Figure 5


- + - + IL-10
- - + + IFN-γ
IDO
GAPDH

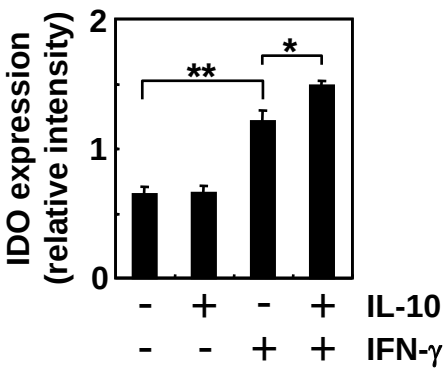
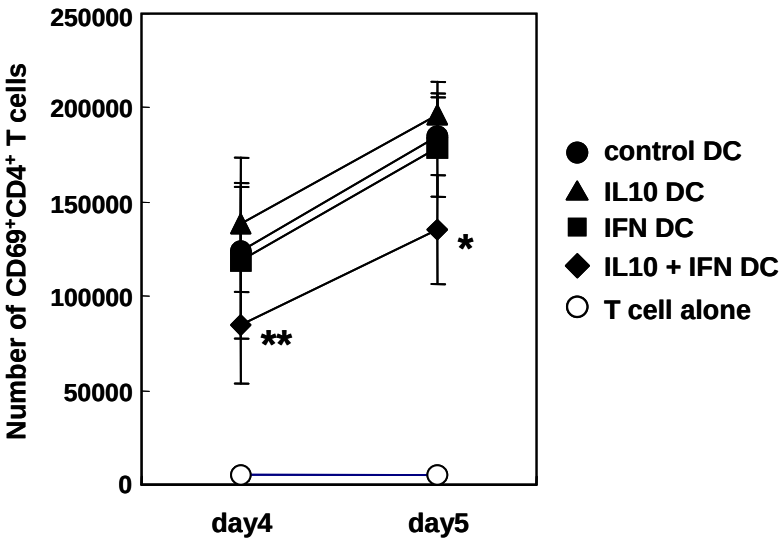

IDO expression
(relative intensity)
2
1
0
**
*
- + - + IL-10
- - + + IFN-γ

Figure 6


250000
200000
150000
100000
50000
0
Number of CD69+CD4+ T cells
day4
day5
**
*
control DC
IL10 DC
IFN DC
IL10 + IFN DC
T cell alone