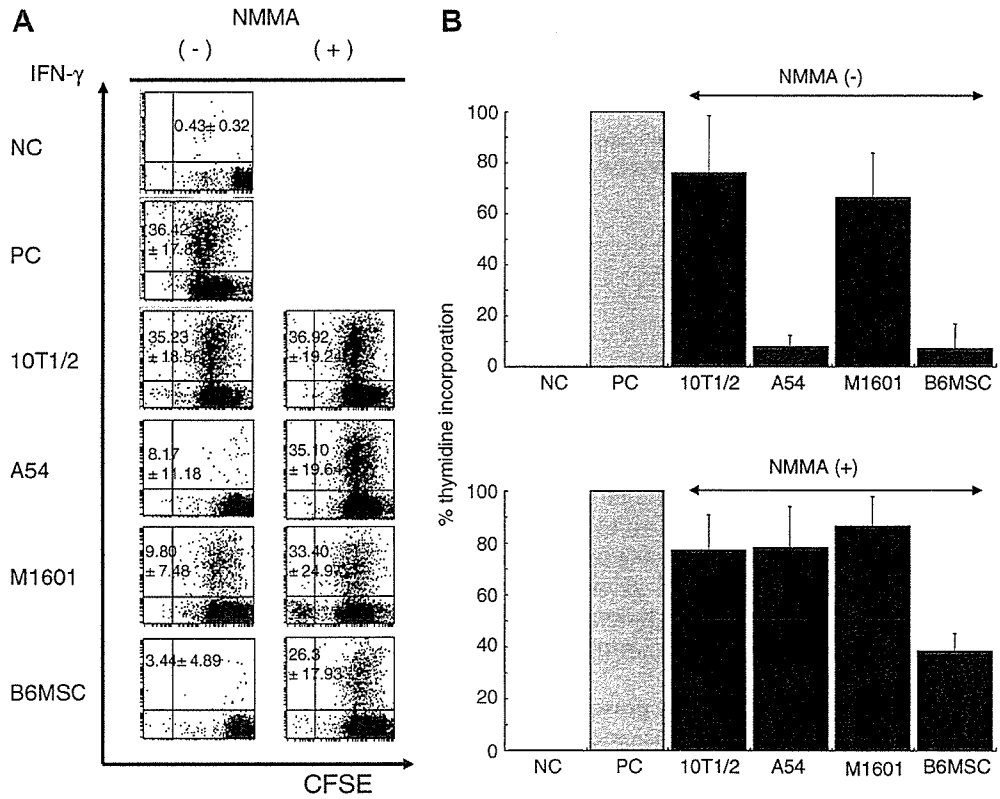

Fig. 3. A specific NO synthase inhibitor, L-NMMA, restores T cell proliferation and IFN-γ production in Th1 condition. (A) A simultaneous flow cytometric analysis of T cell differentiation and proliferation in Th1 condition as described in Fig. 1. $CD4^+$ T cells (1×10^6) were stimulated in Th1 condition in the presence of 10T1/2, A54, M1601, and primary MSCs (1×10^5) with or without 1 mM L-NMMA for 48 h. The numbers indicated are mean ± SD of the percentage of cells in upper right quadrant. (B) [^{3}H]Thymidine incorporation assay in the presence or absence of L-NMMA. $CD4^+$ T cells (1×10^5) were stimulated in Th1 condition in the presence of 10T1/2, A54, M1601, and primary MSCs (1×10^4) with or without 1 mM L-NMMA for 48 h. [^{3}H]Thymidine was pulsed for the last 6 h. 10T1/2, A54, M1601, and primary MSCs were pre-irradiated with 30 Gy to prevent [^{3}H]thymidine incorporation into MSCs. The percentage of proliferation is indicated compared to positive control, which is no MSCs. Shown are means ± SD from three independent experiments. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen and without MSCs.

IFN-γ and a factor that activates NF-κB are crucial for NO production from MSCs in the absence of T cells

To determine what inhibits the production of NO in Th2 condition, the two differentiation factors that mediate the Th2 pathway, anti-IFN-γ antibody and IL-4, were investigated. It is possible one or both of these factors is responsible for the minimum production of NO in Th2 condition. As shown in Fig. 4A, anti-IFN-γ antibody clearly inhibits the production of NO, whereas suppression by IL-4 was less evident. These results suggest that IFN-γ is a key regulator of NO production by MSCs.

Interestingly, cell supernatant collected from activated but not non-activated T cells had the ability to induce NO by MSCs (Supplementary Fig. 2A). What signal(s) are required for NO production by MSCs? As shown in Fig. 4A, IFN-γ is critical for NO production; however, in a T cell-free environment, IFN-γ alone does not induce production of NO from primary MSCs (Fig. 4B, both panels). IFN-γ in combination with LPS, but not IL-2, stimulates NO secretion from primary MSCs (Fig. 4B, left panel and data not shown), suggesting that both the IFN-γ and the signal from Toll-like receptor-4 (TLR4) are required for NO induction by MSCs. Could other TLR ligands substitute for LPS? The addition of flagellin induced NO production in combination with IFN-γ (Fig. 4B, left panel). While, synthetic double strand RNA, poly(I:C), and CpG-oligonucleotide did not induce NO (data not shown). Flagellin is a protein component of bacteria known to induce NO production from macrophages via TLR5 in the presence of either a TLR4 or IFN-γ signal [21,22]. In addition to these factors, IL-1β and TNF-α induce NO when provided in combination with IFN-γ (Fig. 4B, right panel and Supplementary Fig. 2B). As NF-κB is a downstream target of the signaling cascades activated by LPS, flagellin, IL-1β, and TNF-α, we hypothesized that activation of NF-κB is required for NO induction by MSCs. As shown in Fig. 4C, Bay 11-7085 [20], a specific inhibitor of NF-κB suppressed production of inducible NO synthase (iNOS) in MSCs, thus suggesting that NF-κB is involved in NO production by MSCs as well as IFN-γ.

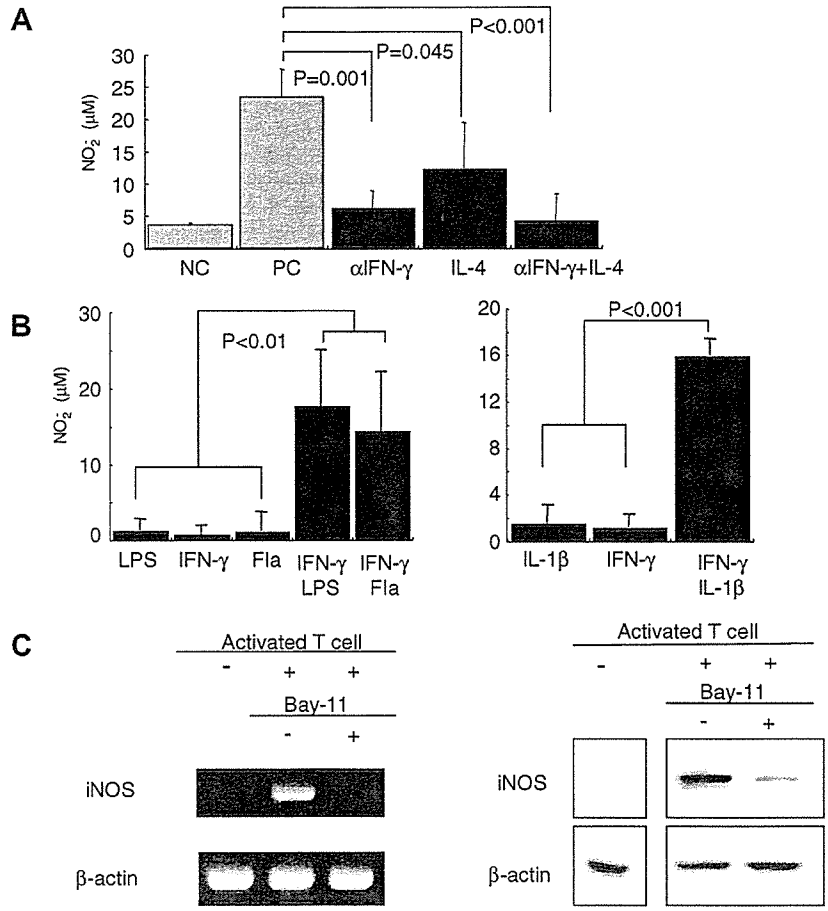

Fig. 4. IFN-γ and NF-κB are involved in NO production. (A) Anti-IFN-γ antibody inhibits NO production. $CD4^+$ T cells (1×10^6) were stimulated with anti-CD3/CD28 beads in the presence of primary MSCs (1×10^5) without any differentiation factors. A neutralizing antibody against IFN-γ (10 μg/ml), IL-4 (100 ng/ml), and a combination of both were added. NC, negative control; $CD4^+$ T cells alone; PC, positive control; $CD4^+$ T cells with mitogen. (B) LPS, flagellin, TNF-α, or IL-1β in a combination with IFN-γ induces NO production from primary MSCs in a T cell-free system. The primary MSCs (1×10^5) were treated with the indicated stimuli without T cells for 48 h and NO concentrations of supernatants were determined by the Greiss assay. (C) A NF-κB inhibitor, Bay11-7085, suppresses iNOS induction in MSCs. Unfractionated splenocytes (1×10^7) were stimulated with anti-CD3/CD28 beads for 48 h and put onto primary MSCs (1×10^6). After co-cultivation for 6 h, total RNA was harvested for RT-PCR and after 30 h, cell lysates were harvested for immunoblot analysis. In advance of co-cultivation, primary MSCs were pre-treated for 30 min with the irreversible NF-κB inhibitor, Bay11-7085 (5 μM), and washed twice with PBS. Just before harvesting, T cells were washed out extensively with PBS.

Discussion

This study demonstrates that mouse primary MSCs strongly suppress T cell proliferation and differentiation in Th1 condition, while the suppression in Th2 condition is weak. Moreover, it provided the first evidence, that NO production can elucidate the differential suppression in Th1/Th2 differentiation. We found a higher NO production in Th1 in comparison to Th2. Furthermore, L-NMMA, a specific inhibitor of NO synthases, restores not only T cell proliferation but also IFN-γ production in Th1 condition. In addition, the treatment with an anti-IFN-γ antibody resulted in a low level of NO production, suggesting that IFN-γ is critical for NO production. While the combination of IFN-γ and a NF-κB activating factor induces NO in a T cell-free system, and moreover, an inhibitor of NF-κB diminishes the induction of iNOS in MSCs, suggesting that NF-κB is also crucial for NO production by MSCs.

The suppression of IFN-γ production may be due to the suppression of cell proliferation, since differentiation is dependent upon cell division [23]. In fact, Fig. 1A, right panel shows that only proliferating cells can produce IFN-γ. However, MSCs were shown to suppress IFN-γ production, regardless of cell division [2,19].

Primary human MSCs induced a fivefold increase in IL-4 synthesis in Th2 condition [4], however, in this study, primary mouse MSCs in the Th2 pathway suppressed production of IL-4 as measured by intracellular staining and ELISA. The discrepancy may be due to species-specific difference. MSC-like cell lines also showed suppression in IL-4 production.

Our results demonstrate that LPS, flagellin, TNF-α, and IL-1β can induce NO from MSCs in a combination with

IFN-γ. These ligands are reported to induce NO from monocytes or macrophages in the presence of IFN-γ [24–28]. Monocytes and macrophages have been assumed to be prominent sources of NO. This implies, MSCs have several features in common with monocytes and macrophages. However, a combination of IFN-γ with IL-2 [27], flagellin alone [21], LPS alone [29], and IFN-γ alone [27], which induce NO from macrophages, do not induce NO from MSCs under the conditions we tested here, suggesting that MSCs also have features that make them different from monocytes and macrophages.

What is the factor required for NO induction in combination with IFN-γ? Cell supernatant from activated T cells induces NO, thus suggesting that activated T cells produce the NO inducer. Neither a soluble TNF-α receptor, a naturally occurring decoy receptor, nor a neutralizing antibody against IL-1β diminished NO production (data not shown). Our results do not define the factor from T cells but they do suggest that the factor may be a ligand that activates NF-κB in MSCs, such as LPS, flagellin, TNF-α or IL-1β.

This study demonstrate that NO is responsible for the preferential suppression of T cell proliferation in Th1 condition by MSCs, and that IFN-γ and NF-κB are involved in NO production in primary MSCs. This appears to be the first study to elucidate the molecular mechanism for preferential Th1 suppression by MSCs. Therefore, these results are considered to enhance the understanding and investigation in this field.

Acknowledgments

This work was supported in part by grants from the Ministry of Health, Welfare, and Labor of Japan and Grants-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Technology of Japan. We also like to thank Dr. Motohiro Matsuura (Jichi Medical University) for providing flagellin, CpG, and poly(I:C).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.02.054.

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Nitric oxide plays a critical role in suppression of T-cell proliferation by mesenchymal stem cells

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The molecular mechanisms by which mesenchymal stem cells (MSCs) suppress T-cell proliferation are poorly understood, and whether a soluble factor plays a major role remains controversial. Here we demonstrate that the T-cell–receptor complex is not a target for the suppression, suggesting that downstream signals mediate the suppression. We found that Stat5 phosphorylation in T cells is suppressed in the presence of MSCs and that nitric oxide (NO) is involved in the suppression of Stat5 phosphorylation and T-cell proliferation. The induction of inducible NO synthase (NOS) was readily detected in MSCs but not T cells, and a specific inhibitor of NOS reversed the suppression of Stat5 phosphorylation and T-cell proliferation. This production of NO in the presence of MSCs was mediated by CD4 or CD8 T cells but not by CD19 B cells. Furthermore, inhibitors of prostaglandin synthase or NOS restored the proliferation of T cells, whereas an inhibitor of indoleamine 2,3-dioxygenase and a transforming growth factor–β–neutralizing antibody had no effect. Finally, MSCs from inducible NOS$^{-/-}$ mice had a reduced ability to suppress T-cell proliferation. Taken together, these results suggest that NO produced by MSCs is one of the major mediators of T-cell suppression by MSCs. (Blood. 2007;109:228-234)

Introduction

Because mesenchymal stem cells (MSCs) differentiate into osteocytes, chondrocytes, myotubes, and adipocytes,[1-3] they are expected to become a source of cells for regenerative therapy. Also, MSCs support hematopoietic stem cell engraftment[4-9] and modulate immunologic responses by unknown mechanisms.[9-14] Here, we investigated the molecular mechanisms by which MSCs suppress T-cell proliferation.

Transforming growth factor–β (TGF-β), hepatocyte growth factor, indoleamine 2,3-dioxygenase (IDO), and prostaglandin E2 (PGE_2) have been reported to mediate T-cell suppression by MSCs.[13-15] Specifically, neutralizing antibodies against TGF-β or hepatocyte growth factor,[13] an inhibitor of IDO,[14] or an inhibitor of prostaglandin production reverse the inhibition of T-cell proliferation by MSCs.[15] In addition, some reports have shown that a soluble factor is the major mediator of suppression,[13-17] whereas some reports have demonstrated that T-cell–MSC contact is required for this suppression.[12-14,16,17] In the current study, we sought to resolve these conflicting results by using a mouse bone marrow–derived MSC system.

One candidate soluble factor for T-cell suppression is nitric oxide (NO) because it is known to inhibit T-cell proliferation.[18-25] NO is produced by NO synthases (NOSs), of which there are 3 subtypes: inducible NOS (iNOS), endothelial NOS, and neuronal NOS. Like MSCs, it has been known that macrophages suppress T-cell proliferation. This suppression was reported to be mediated by NO inhibition of Stat5 phosphorylation.[18,19] Also, MSCs were reported to produce NO when they differentiate into chondrocytes.[26] We therefore investigated whether MSCs can produce NO and whether NO is involved in their ability to suppress T-cell proliferation.

Materials and methods

Materials

N-nitro-L-arginine methyl ester (L-NAME), indomethacin, and concanavalin A (Con A) were purchased from Wako (Osaka, Japan). Con A was used at 5 μg/mL. Indomethacin was used at 5 μM. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were from Sigma (St Louis, MO) and were used at concentrations of 50 ng/mL and 1 μg/mL, respectively. Antimouse CD3/CD28 beads (Dynal Biotech ASA, Oslo, Norway) were used at 10 μL per 10^6 cells. The transwell system with 1-μm pores for 12-well dishes was from BD Falcon (Franklin Lakes, NJ). Monoclonal antibodies for CD4, CD8, CD11b, CD25, CD29, CD44, CD45, CD69, Sca-1, B220, Gr-1, and interferon-γ (IFN-γ) were from BD Pharmingen (San Diego, CA). An inhibitor of IDO, 1-methyl-DL-tryptophan (1-MT), was purchased from Sigma. An antibody for TGF-β was purchased from Peprotech (Rocky Hill, NJ). Lipopolysaccharide was from Sigma.

MSCs

MSCs were obtained from wild-type or iNOS$^{-/-}$ C57BL/6 mice. Bone marrow cells were harvested from femurs and tibias by a standard flushing method[1] and then cultivated in a plastic dish in Iscove modified Dulbecco medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 U/mL penicillin G (Invitrogen) or in MF medium (Toyobo, Tokyo, Japan).

All primary MSCs were characterized at least once by flow cytometry and an in vitro differentiation assay. All MSCs were positive for CD29, CD44, and Sca-1, negative for CD11b, Gr-1, and CD45, and able to

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differentiate into adipocytes and osteoblasts (Figure S1, available at the *Blood* website; see the Supplemental Figures link at the top of the online article). We used at least 2 independently isolated batches of MSCs. These cells can be propagated for a long time and retain their surface phenotype and capacity to differentiate for at least 4 months.

Flow cytometric analysis

Cells were incubated with Fc block (BD Pharmingen) to inhibit nonspecific binding of antibodies to Fc receptors. Next, cells were stained in FACS buffer (phosphate-buffered saline [PBS] supplemented with 10% fetal bovine serum) with antibodies for 30 minutes on ice, washed with FACS buffer, and analyzed on a BD LSR flow cytometer (Becton Dickinson, Franklin Lakes, CA). Collected data were analyzed with CELLQUEST software (Becton Dickinson).

Enzyme-linked immunosorbent assay (ELISA)

ELISA kits for mouse IFN-γ and mouse interleukin-2 (IL-2; BD Pharmingen) were used according to the manufacturer's instructions.

Selection of CD4+, CD8+, and CD19+ cells

$CD4^+$, $CD8^+$, and $CD19^+$ cells were selected using mouse CD4, CD8, and CD19 MACS beads and an autoMACS system (Miltenyi Biotech, Auburn, CA). The purity of the cells as determined by flow cytometry with antibodies against CD4, CD8, and B220 was more than 80%.

Thymidine incorporation

Splenocytes were grown in 96-well plates containing RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum (Sigma), 2 mM L-glutamine (Invitrogen), 50 μM 2-mercaptoethanol (Sigma), 0.1 mg/mL streptomycin, and 100 U/mL penicillin G (Invitrogen). During the cultivation, the cells were pulsed for the last 8 hours of culture with 1 mCi (3.7×10^7 Bq) of [^{3}H]-thymidine (Amersham Biosciences, Piscataway, NJ). After 48 hours of growth, cells were harvested with a Packard FilterMate harvester (Perkin Elmer Life Sciences, Boston, MA), transferred to a UniFilter plate (Perkin Elmer Life Sciences), and analyzed using a TopCount microplate scintillation counter (Perkin Elmer Life Sciences). In the coculture system, MSCs were γ-irradiated (30 Gy) prior to cultivation to prevent thymidine incorporation. When using the transwell system (BD Falcon), one tenth of the cells were harvested and counted because the system includes 12-well culture dishes, which contain 10-fold more cells than the wells of a 96-well plate.

Western blot analysis

Polyclonal antibodies to phosphorylated Stat5 (Cell Signaling Technology, Danvers, MA), Stat5 (Santa Cruz Biotechnology, Santa Cruz, CA), cyclin D2 (Cell Signaling Technology), and Kip1 (Cell Signaling Technology) were used for Western blotting. Cells were lysed on ice for 15 minutes with a buffer consisting of 50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% NP40, Complete proteinase inhibitor cocktail (Roche Diagnostics, Mannheim, Germany), and 1 mM Na_3VO_4. Lysates were centrifuged at 13 000*g* for 15 minutes, and supernatants were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred from the gel to a PVDF membrane (Invitrogen), and Western blotting was performed using enhanced chemiluminescence reagents (Pierce, Rockford, IL) to visualize the immunoreactive proteins.

Assay for NO production

NO is quickly converted to NO_2 and NO_3 in culture medium. Because of the presence of NO_3 in RPMI medium, we measured NO_2 production using a Griess reagent kit (Wako).

Detection of iNOS expression

Total RNA was prepared using an RNeasy kit (Qiagen, Valencia, CA) and 1 μg was reverse-transcribed using a First Strand Synthesis Kit (Invitrogen), and one tenth of the product was subjected to PCR using the following primers: for iNOS, 5′-GAGATTGGAGTTCGAGACTTC-3′ and 5′-TGGCTAGTGCTTCAGACTTC-3′; and for β-actin, 5′-CCATCATGAAGTGTGACGTTG-3′ and 5′-GTCCGCCTAGAAGCACTTGCG-3′.

Western blotting was performed using a polyclonal antibody for iNOS (BD Transduction Laboratories, Lexington, KY) or a monoclonal antibody for β-actin (Sigma) to confirm equal loading. Immunofluorescence was carried out using the polyclonal iNOS antibody (BD Transduction Laboratories) followed by Alexa Fluor 488 goat anti–rabbit IgG (Molecular Probes, Eugene, OR). To distinguish splenocytes from MSCs, cells were simultaneously stained with phycoerythrin-conjugated anti-CD45 monoclonal antibody (BD Pharmingen). Cells were fixed with ProLong Gold antifade reagent (Molecular Probes) and were visualized by confocal microscopy (Nikon, Tokyo, Japan), and images were analyzed with the accompanying confocal image analysis software (Bio-Rad, Hercules, CA).

Intracellular staining

Intracellular staining was performed using a BD Cytofix/Cytoperm kit (BD Pharmingen) according to the manufacturer's instructions.

Mice

Wild-type mice (C57BL/6) were purchased from Clea Japan (Tokyo). The $iNOS^{-/-}$ mice (C57BL/6 background) were purchased from Jackson Laboratory (Bar Harbor, ME).

Cell lines

RAW264.7 mouse macrophage cells were a generous gift from Dr Matsuura (Jichi Medical University, Tochigi, Japan). HeLa human cervical carcinoma cells were used as negative control for iNOS expression.

Statistical analysis

We used the Student *t* test for statistical analysis. Differences were considered statistically significant at *P* values less than .05.

Results

Characteristics of T-cell suppression by MSCs

Although many reports[9-17,27] have shown that MSCs suppress T-cell proliferation, the molecular mechanisms and the signaling molecules inhibited by MSCs have not been defined. We therefore investigated the status of activated T cells in the presence of MSCs. The expression of activation markers and the production of IL-2 and IFN-γ were evaluated by flow cytometry, ELISA, and intracellular staining. The expression of the activation markers CD25 and CD69 on CD4 or CD8 T cells was not changed by the presence of MSCs (Figure 1A). In addition, MSCs suppressed the production of IFN-γ but not IL-2 (Figure 1B, upper panel). Also, IFN-γ production was diminished after 24 hours in the presence of MSCs (Figure 1B, lower panel). These findings are in agreement with previous results,[27] but they do not explain the strong suppression of T-cell proliferation by MSCs; for example, thymidine incorporation by T cells is reduced more than 10-fold in the presence of MSCs (data not shown).

We next induced T-cell proliferation using a combination of PMA and ionomycin, which act downstream of the T-cell–receptor complex by activating protein kinase C and inducing Ca^{2+} influx, respectively. This proliferation was suppressed by MSCs (Figure 1C), suggesting that the T-cell receptor complex is not a target for the suppression and that MSCs influence signals downstream of protein kinase C and Ca^{2+} influx. As demonstrated in Figure 1C, the proliferation of both purified CD4 and CD8 T cells as well as unfractionated splenocytes was suppressed by MSCs.

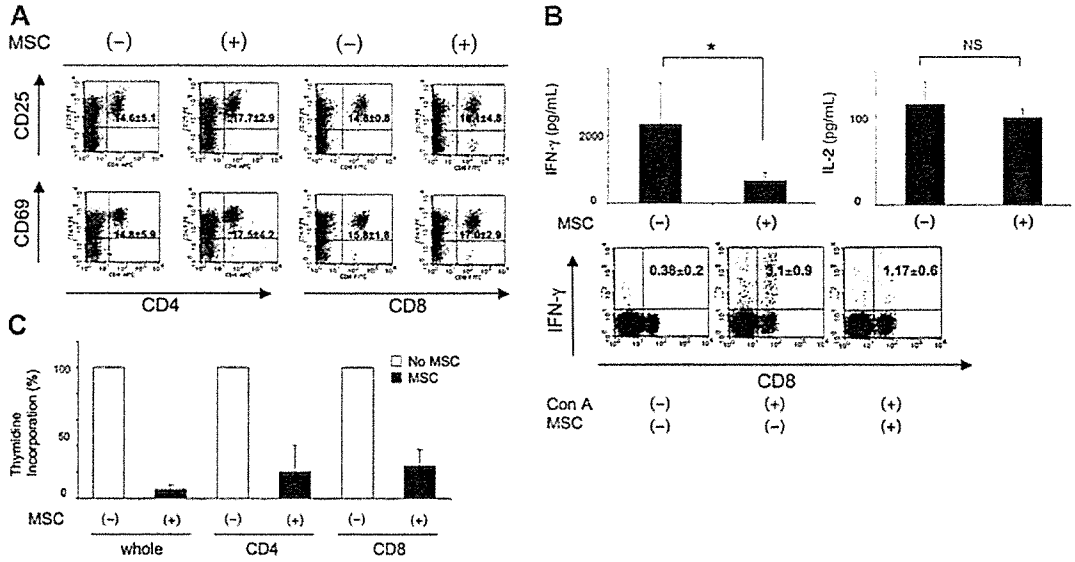

Figure 1. Status of activated T cells in the presence of MSCs. (A) Expression of the T-cell activation markers CD25 and CD69 on CD4 or CD8 cells 24 hours after stimulation of splenocytes (1×10^6 cells) in a 12-well dish with anti-CD3/CD28 beads (10 μL) in the presence or absence of 1×10^5 MSCs. The numbers in the top right quadrants indicate the percentage ± the standard deviation (SD). (B) Top panel, cytokine production in the same condition as in panel A. Concentrations of IL-2 and IFN-γ were determined at 48 hours by ELISA. The values are the means ± SD from 3 independent experiments. Bottom panel, intracellular staining of IFN-γ at 24 hours. GolgiStop (monensin) was used for the last 8 hours. The values are the mean percentages of CD8/IFN-γ–positive cells ± SD from 3 independent experiments. (C) MSCs suppress the induction of $CD4^-$ and $CD8^+$ T-cell proliferation by PMA and ionomycin. Splenocytes (1×10^5), $CD4^+$ cells (1×10^5), or $CD8^+$ cells (1×10^5) were stimulated in the presence or absence of irradiated MSCs (1×10^4) in the wells of a 96-well plate. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. NS indicates $P > .05$.

Stat5 phosphorylation is inhibited by MSCs

Although T cells from Stat5ab$^{-/-}$ mice do not proliferate upon stimulation with anti-CD3, they up-regulate CD25.[28] Because this phenotype is similar to the status of activated T cells in the presence of MSCs (Figure 1A), we hypothesized that they suppress Stat5 phosphorylation. Indeed, as shown in Figure 2, Stat5 phosphorylation was diminished in activated T cells in the presence of MSCs despite equivalent IL-2 production (Figure 1B). The reported changes in cell-cycle–related proteins, including the down-regulation of cyclin D2 and up-regulation of p27 Kip1 in the presence of MSCs,[27] were observed less consistently; in most cases, we found that MSCs up-regulated cyclin D2 and down-regulated p27 Kip1 in activated splenocytes compared with freshly isolated splenocytes (data not shown).

Dose dependency and time course of NO production

Macrophages have been reported to suppress T-cell proliferation[19-23] due to the production of NO and its inhibition of Stat5 phosphorylation.[18,19] This prompted us to examine the production of NO in our mouse MSC system. We found that MSCs caused a significant and dose-dependent production of NO (Figure 3A). NO could be first detected approximately 12 hours after the activation of T cells in the presence of MSCs. In the transwell system, in which the T cells were separated from the MSCs by a 1-μm-pore membrane, NO production was initially detected approximately 24 hours after the activation of T cells (Figure 3B).

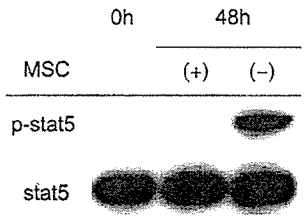

Figure 2. Inhibition of Stat5 phosphorylation in the presence of MSCs. Western blot analysis of Stat5 phosphorylation. Splenocytes (2×10^6) were activated with anti-CD3/CD28 beads in the presence or absence of 1×10^5 MSCs. After 48 hours, splenocytes were collected, lysed, and analyzed by Western blotting. Each lane contains 20 μg protein. Western blotting with anti-Stat5 is shown as a loading control. Shown are representative results from more than 5 experiments.

T-cell suppression and NO

Significant amounts of NO were not produced by MSCs cocultured with T cells in the absence of Con A or by Con A–treated MSCs or T cells (Figure 4A). In the presence of a direct interaction between T cells and MSCs, there was a high level of NO production accompanied by a strong suppression of T-cell proliferation (Figure 4A-B). In contrast, both NO production and T-cell suppression were reduced in a transwell system (Figure 4A-B). We further examined whether such a difference is observed using the RAW264.7 macrophage cell line, a well-characterized producer of NO. As with MSCs, T-cell suppression and NO production were inhibited in the transwell system using the RAW264.7 cells (Figure 4B, right side, and data not shown), suggesting that the difference reflects common aspects of T-cell suppression by NO.

T cells but not B cells induce NO

We next asked which cell type causes the NO production. We found that purified $CD4^+$ and $CD8^+$ T cells induce similar degrees of T-cell suppression as unfractionated splenocytes (Figure 1C). Therefore, it is not surprising that they also produce NO in the presence of MSCs (Figure 4C). Although MSCs suppress B-cell proliferation (~50%; data not shown), purified $CD19^+$ B cells did not appear to induce NO production in the presence of MSCs, suggesting that the mechanisms of B-cell and T-cell suppression are different.

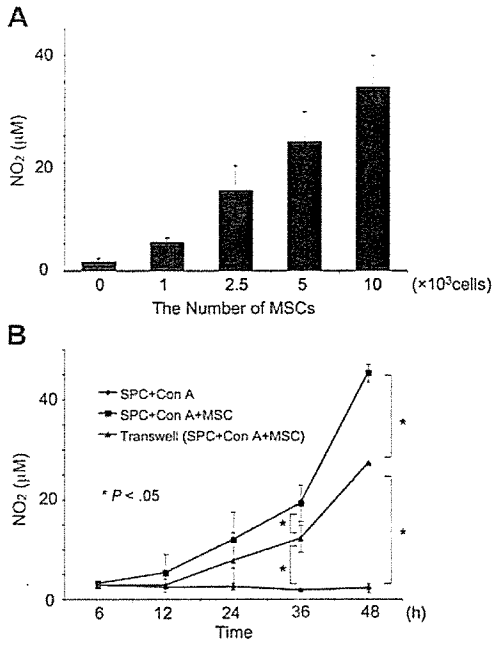

Figure 3. NO production in the presence of MSCs. (A) Dose-dependent effect of MSCs on NO production. Splenocytes (1×10^6) were activated with Con A (5 μg/mL) in the presence of the indicated number of MSCs for 48 hours in a 12-well dish. The concentrations of NO were determined by Griess assay. (B) Time course of NO production. MSCs (1×10^5/well) were treated as in panel A for the indicated amount of time. "Transwell" indicates experiments performed in 12-well dishes in which the T cells were separated from MSCs by a 1-μm-pore membrane. Values represent the means ± SD from 3 independent experiments. *$P < .05$.

MSC–T-cell interaction and NO production

There are 2 possible explanations for the difference in NO production in the presence and absence of the transwell system. First, it is possible that there is a difference in the time course of NO production in the 2 systems. In the transwell system, a significant level of NO was typically detected after 24 hours, whereas NO production was detected after 12 to 18 hours in the presence of a direct interaction. Thus, the amount of NO produced in the transwell system was always lower than that in the presence of a direct interaction (Figure 3B). These findings suggest that a direct interaction is critical for the early and efficient production of NO as well as for the strong suppression of T-cell proliferation. A second possible explanation for the different results obtained in the transwell and direct interaction systems is that, because NO is highly unstable, in the transwell system it can lose its activity before it influences T cells.

MSCs are a producer of NO

If this second explanation is correct, MSCs should be the main producer of NO. Therefore, we examined whether MSCs can produce NO. It is known that there are 3 NO synthases (iNOS, endothelial NOS, and neuronal NOS), and only one of these, iNOS, can be induced by cell stimulation.[29] Therefore, we suspected that iNOS is induced in either T cells or MSCs. Reverse transcriptase–polymerase chain reaction (RT-PCR) (Figure 5A), Western blot analysis (Figure 5B), and immunofluorescence (Figure 5C) detected the induction of iNOS in MSCs cocultured with activated splenocytes but not in MSCs alone, splenocytes alone, or HeLa cells. The immunofluorescence studies showed that iNOS was exclusively expressed by large adherent $CD45^-$ cells, which correspond to MSCs (Figure 5C). In addition, iNOS appeared to be expressed throughout the cytoplasm as previously found in Kupffer cells and hepatocytes (Figure 5C).[30]

Specific inhibitor of NOS restores T-cell proliferation and Stat5 phosphorylation

Next, we investigated the effects of L-NAME, a specific inhibitor of NOS. As expected, L-NAME dose-dependently inhibited the production of NO by MSCs in the presence of activated T cells (Figure S2). Importantly, L-NAME restored T-cell proliferation (Figure 6A, left panel). The effect of L-NAME was dose dependent and more efficient when lower numbers of MSCs were used (Figure 6A, right panel). Using 2.5×10^3 MSCs, 1 mM L-NAME resulted in up to an approximately 80% recovery compared with the positive control (Figure 6A, left panel), suggesting that NO is one of the most important factors for T-cell suppression under the stringent conditions of our assays. On the other hand, even under these conditions, 100% recovery was not achieved, implying that other factors also contribute to the suppression of T-cell proliferation by MSCs.

L-NAME restored not only T-cell proliferation but also Stat5 phosphorylation (Figure 6B), indicating that NO inhibits Stat5 phosphorylation. Because Stat5 is required for T-cell division,[28] we suspect that NO first inhibits Stat5 phosphorylation, which then results in arrest of the cell cycle.

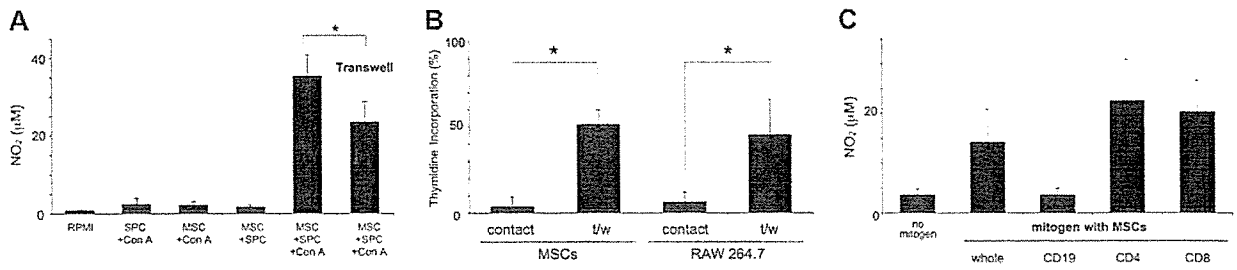

Figure 4. Relationship between NO production and T-cell suppression. (A) Production of NO. Splenocytes (1×10^6) were incubated with or without Con A in the presence or absence of MSCs for 48 hours. "Transwell" indicates experiments performed in 12-well dishes in which the T cells were separated from MSCs by a 1-μm-pore membrane. The values are the means ± SD from 3 independent experiments. *$P < .05$. (B) T-cell proliferation in the presence or absence of the transwell system. Splenocytes (1×10^6) were activated with Con A in the presence or absence of 1×10^5 MSCs or RAW264.7 cells for 48 hours with or without the transwell. The incorporation of $[^3H]$-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. (C) T cells but not B cells induce NO production. Purified CD4 or CD8 T cells (1×10^5; ~80% purity) induce NO in the presence of MSCs (1×10^4), whereas purified CD19 B cells (1×10^5; ~95% purity) do not induce significant NO production. The mitogen for T cells was Con A, and for B cells, it was lipopolysaccharide (1 μg/mL). The values are the means ± SD from 3 independent experiments. *$P < .05$.

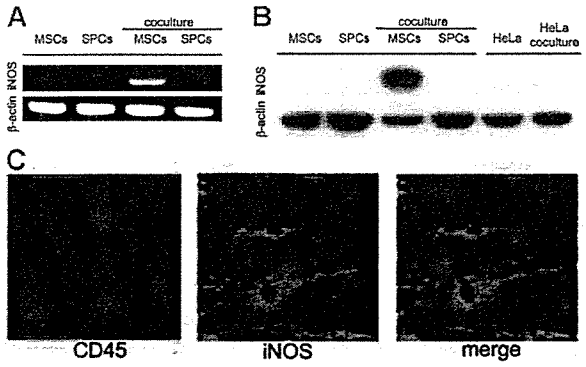

Figure 5. Induction of iNOS in MSCs. Total RNA and cell lysates were collected from MSCs alone, splenocytes alone, MSCs cocultured with activated T cells, or activated splenocytes cocultured with MSCs. MSCs were harvested just after washing out activated T cells with PBS. (A) RT-PCR analysis of iNOS mRNA. β-actin is shown as a control. (B) Western blot analysis of iNOS protein. Each lane contains 20 μg protein. β-actin is shown as a loading control. HeLa cells were used as negative control because the antibody also reacts with human iNOS protein. (C) Immunofluorescence of iNOS protein. Left panel, confocal immunofluorescent image of CD45 protein. Middle panel, confocal immunofluorescent image of iNOS protein. Right panel, merged confocal immunofluorescent images of CD45 protein and iNOS protein. Splenocytes (1×10^6) were activated with Con A in the presence of 1×10^5 MSCs for 48 hours. Images were visualized using a Nikon Eclipse TE300 microscope (Nikon, Tokyo, Japan) equipped with a 100×/1.40 numerical aperture oil objective lens, Nikon CFI Plan APO (Nikon). Images were acquired using Lasersharp software version 2.1 (Bio-Rad).

Other candidates as mediators of suppression by MSCs

Because TGF-β, IDO, and PGE_2 were reported as mediators of T-cell suppression by MSCs,[13-15] we further compared the effects of L-NAME with inhibitors of each mediator. Indomethacin (inhibitor of PGE_2 production) but not 1-MT (inhibitor of IDO) or an anti–TGF-β–neutralizing antibody restored T-cell proliferation as effectively as L-NAME (Figure 7A); however, the effects of L-NAME and indomethacin were not additive, suggesting that the NO and PGE_2 share signaling pathways leading to T-cell suppression (Figure 7A).

MSCs from iNOS$^{-/-}$ mice have reduced activity in T-cell suppression

Finally, we used MSCs from iNOS$^{-/-}$ mice to confirm that NO is produced by MSCs and that NO suppresses T-cell proliferation. MSCs from iNOS$^{-/-}$ mice were less effective than MSCs from wild-type mice at suppressing T-cell proliferation, suggesting that NO produced by MSCs is a major mediator of this effect (Figure 7B, left panel). We also confirmed that MSCs from iNOS$^{-/-}$ mice do not produce NO even in the presence of activated T cells (Figure 7B, right panel).

Discussion

Here, we demonstrate for the first time that the production of NO is involved in the suppression of T cells by MSCs. We also showed that NO inhibits Stat5 phosphorylation. Although NO was already known to suppress T-cell proliferation, NO has not been previously reported to mediate T-cell suppression by MSCs.[29] Our hypothesis that NO is produced by MSCs and that it suppresses T-cell proliferation in part through Stat5 inhibition was supported by the following facts: (1) NO was readily detected in the medium in the presence of MSCs; (2) L-NAME restored T-cell proliferation as well as Stat5 phosphorylation; and (3) MSCs from iNOS$^{-/-}$ mice had markedly reduced abilities to suppress T-cell proliferation. This hypothesis was further confirmed by the finding that iNOS was detected only in MSCs.

Compared with experiments in which cells were in direct contact, experiments performed in transwells showed a lag in NO production, suggesting that T-cell–MSC contact is critical for the early and efficient production of NO and, thus, T-cell suppression. Whether a soluble factor is a main mediator of T-cell suppression by MSCs has been controversial because results from transwell systems have been inconsistent.[12,14,16-17] Our finding that the transwell reduces but does not abolish T-cell suppression (Figure 4B) may help explain these conflicting reports. Although we could not define the mechanism by which NO production is suppressed in the transwell system, the amount of NO production appears to correspond with the extent of T-cell suppression.

Under stringent conditions, in which a lower number of MSCs was used, the restoration of T-cell proliferation by L-NAME reached up to approximately 80%, suggesting that NO is one of the major mediators; however, 100% restoration was never attained, suggesting that other factor(s) contribute to the suppression. Because TGF-β, IDO, and PGE_2 have been considered as possible mediators of T-cell suppression by MSCs,[13-15] we examined the effect of specific inhibitors of each. We found that indomethacin (inhibitor of PGE_2 production) restores T-cell proliferation as previously reported[15] but that neither 1-MT (inhibitor of IDO) nor the TGF-β antibody had an effect. Also, the effects of indomethacin

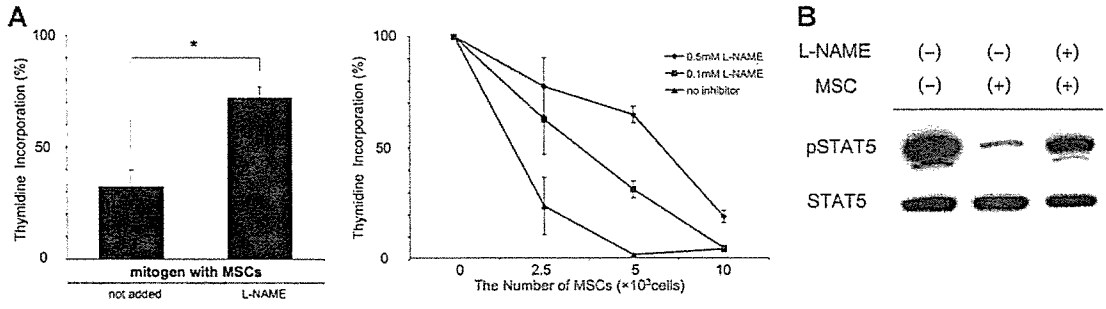

Figure 6. A specific inhibitor of NOS restores T-cell proliferation and Stat5 phosphorylation. (A) Effect of L-NAME on thymidine incorporation. Top panel, splenocytes (1×10^5) were activated with Con A in the presence or absence of 2.5×10^3 irradiated MSCs and in the presence or absence of 1 mM L-NAME. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. The values are the means ± SD from 3 independent experiments. *$P < .05$. Bottom panel, dose-dependent restoration of T-cell proliferation by L-NAME. Splenocytes (1×10^5) were activated with Con A in the presence of the indicated number of irradiated MSCs for 48 hours. The concentrations of L-NAME are shown. Shown is a typical result of 3 independent experiments. (B) L-NAME restores Stat5 phosphorylation. Splenocytes (2×10^6) were activated with anti-CD3/CD28 beads in the presence or absence of 0.5×10^5 to 1×10^5 MSCs for 48 hours and in the presence or absence of 1 mM L-NAME. Western blotting for phosphorylated and total Stat5 was performed as described in Figure 2. Shown is a representative result from 5 independent experiments.

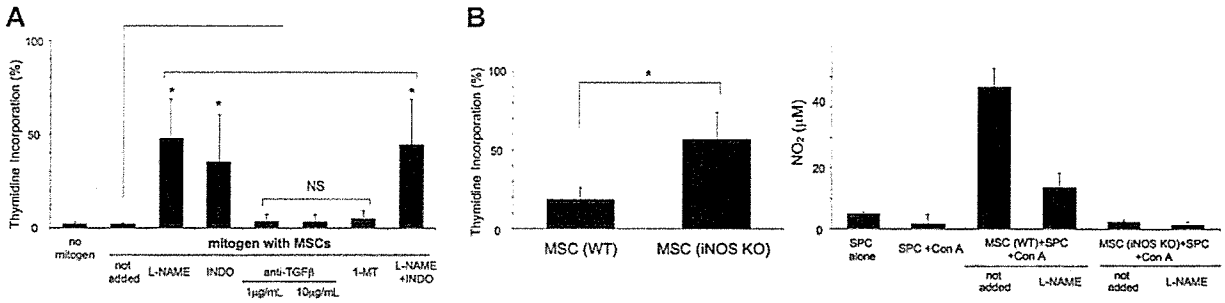

Figure 7. Effect of inhibitors and T-cell suppression by MSCs from iNOS$^{-/-}$ mice. (A) L-NAME and indomethacin (INDO) restored T-cell proliferation, but TGF-β antibody and 1-MT had no effect. Splenocytes (1×10^5) were activated with Con A in the presence or absence of irradiated MSCs (2.5×10^3), 1 mM L-NAME, 5 μM indomethacin,[14] 1 μg/mL or 10 μg/mL TGF-β antibody, and 1 mM 1-MT for 48 hours. The incorporation of [^{3}H]-thymidine is shown relative to that in the absence of MSCs. Shown is the mean ± SD of 3 independent experiments. *$P < .05$. NS indicates $P > .05$. (B) MSCs from iNOS$^{-/-}$ mice have a reduced ability to inhibit T-cell proliferation. Splenocytes (1×10^5) were activated with Con A in the presence or absence of MSCs (2.5×10^3) from either wild-type or iNOS$^{-/-}$ mice. Left panel, incorporation of [^{3}H]-thymidine relative to that in the absence of MSCs. Right panel, production of NO. The values are the means ± SD from 3 independent experiments. *$P < .05$.

were not additive with those of L-NAME. These results, combined with previous reports,[31,32] suggest that NO acts upstream of PGE_2. Furthermore, our results imply that NO may be the central mediator of T-cell proliferation.

Under our standard conditions (1:10 ratio of MSCs to splenocytes), 1 mM L-NAME restored T-cell proliferation to approximately 25%. Similarly, 5 μM indomethacin also produced an approximately 25% recovery (data not shown). These results suggest that the restoration by L-NAME or indomethacin is not specific to the more stringent conditions (1:40 ratio of MSCs to splenocytes).

Although most of the results from our mouse MSC system are consistent with previous reports,[12,15,27] we did not find a clear correlation between T-cell suppression and the up-regulation of Kip1 or the down-regulation of cyclin D2. Instead, our results suggest that the inhibition of Stat5 phosphorylation is more important for T-cell suppression, at least under the conditions of our experiments. In the conditions studied here, after coculture with MSCs, T cells could respond to a second mitogenic stimulation (data not shown), whereas they could not respond to a second stimulation in a previous report,[27] suggesting that the status of T cells in our experiments is different than that in the previous report.

Our results provide new insight into how MSCs modulate immune function. Although it is known that the NO-Stat5 pathway is important for T-cell suppression by macrophages, this is the first report demonstrating that the NO-Stat5 pathway is also critical for T-cell suppression by MSCs. The physiologic role of NO produced by MSCs is unknown, and we are currently investigating the possibility that MSCs in bone marrow protect hematopoietic stem cells from T-cell–mediated destruction by inhibiting T-cell proliferation.

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Authorship

Contribution: K.S. performed the research and analyzed data; K. Ozaki designed the research and wrote the paper; K.H. performed Western blotting; I.O. carried out experiments regarding PMA plus ionomycin; T.N. provided technical advice; A.M. and K.M. provided some reagents and analyzed data; and K. Ozawa organized the research project.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

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Overexpression of Interleukin 21 Induces Expansion of Hematopoietic Progenitor Cells

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Abstract

The interleukin 21 (IL-21) receptor is expressed on T-cells, B-cells, and natural killer cells, and IL-21 is critical for regulating immunoglobulin production in vivo in cooperation with IL-4. So far, however, little is known about a role for IL-21 outside the immune system. We investigated the effect of IL-21 on hematopoiesis in vivo by using the hydrodynamics gene-delivery method. Overexpression of IL-21 increases Sca-1$^+$ cells in the periphery and spleen. It also increases the numbers of c-Kit$^+$, Sca-1$^+$, and lineage$^{-/low}$ (KSL) cells and colony-forming units–granulocyte-macrophage (CFU-GM) in the spleen, indicating the expansion of hematopoietic progenitor cells. We found that even in RAG2$^{-/-}$ mice, which lack mature T-cells and B-cells, IL-21 induced an increase in KSL cells and CFU-GM in the spleen. These results demonstrate that IL-21 can induce the expansion of hematopoietic progenitor cells in vivo, even in the absence of mature T-cells and B-cells.
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Key words: Interleukin 21; Hematopoietic progenitor cells; Overexpression; Expansion; CFU-GM

1. Introduction

Interleukin 21 (IL-21) is a cytokine produced by CD4$^+$ T-cells [1] that acts on T-cells, B-cells, and natural killer (NK) cells [1,2]. Like the receptor complexes for IL-2, IL-4, IL-7, IL-9, and IL-15, the receptor for IL-21 contains the common cytokine receptor γ chain (γc) [3,4]. IL-21 augments T-cell proliferation in response to T-cell receptor signaling, augments B-cell proliferation by anti-CD40, and induces the expansion, differentiation, and apoptosis of NK cells [1,5]. IL-21, together with IL-4, plays a critical role in regulating immunoglobulin production [6]. IL-21 can also promote B-cell apoptosis but nevertheless drives terminal B-cell differentiation to plasma cells [7]. IL-21 overexpression exhibits antitumor activity in vivo [8], and this effect is greater when IL-21 is combined with IL-15 [9,10]. A role for IL-21 has also been implicated in autoimmune disease [11].

The effect of IL-21 on hematopoietic cells is unknown. Previously, we established an IL-21–overexpression system in vivo [8], and we have used this system in the present study to investigate the action of IL-21 in the hematopoietic system. The hematopoietic stem cell compartment is known to express many kinds of cytokine receptors [12]. The common cytokine receptor γc is one such receptor, and this protein and the IL-21 receptor (IL-21R) comprise the functional IL-21 heterodimeric receptor. Here we describe our investigation of the potential role of IL-21 in hematopoiesis.

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2. Materials and Methods

2.1. Cytokines and Cell Culture

All cytokines were from R&D Systems (Minneapolis, MN, USA). Cells were cultured in RPMI 1640 complete

medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum, sodium pyruvate, nonessential amino acids, and penicillin-streptomycin (Invitrogen/Life Technologies, Rockville, MD, USA).

2.2. Mice

C57BL/6J (CD45.2) and congenic C57BL/6-Ly5.1 (CD45.1) were from CLEA Japan (Tokyo, Japan) and Sankyo Laboratory (Tokyo, Japan), respectively. $RAG2^{-/-}$ mice were a kind gift from Dr. Satoshi Takaki (The University of Tokyo).

2.3. Bone Marrow Mononuclear Cell Preparation

5-Fluorouracil (5-FU) (Sigma-Aldrich) at 150 mg/kg was administered via intraperitoneal injection. Three or four days later, the mice were sacrificed, and femurs and tibias were excised and flushed. If necessary, mononuclear cells were isolated with Lymphoprep (Axis-Shield, Oslo, Norway).

2.4. Splenocyte Preparation

Spleens were crushed with nylon mesh in erythrocyte-lysis buffer (ACK buffer: 8.29 g NH_4Cl, 1.00 g $KHCO_3$, and 0.0372 g EDTA in 1 L). After the addition of medium and centrifugation, splenocytes were counted and used for the colony-forming unit (CFU) assay and fluorescence-activated cell-sorting (FACS) analysis.

2.5. CFU Assay

For the CFU–granulocyte-macrophage (CFU-GM) assay, we used methylcellulose containing IL-3, IL-6, and stem cell factor (SCF) (M3534; StemCell Technologies, Vancouver, British Columbia, Canada). For the CFU-granulocyte (CFU-G), CFU-macrophage (CFU-M), CFU-GM, burst-forming unit–erythroid (BFU-E), and CFU–granulocyte, erythrocyte, megakaryocyte, macrophage (CFU-GEMM) assays, we used methylcellulose containing IL-3, IL-6, SCF, and erythropoietin (M3434; StemCell Technologies).

2.6. Peripheral Blood Cell Preparation

For FACS analysis, blood was collected, lysed with erythrocyte-lysis buffer, and analyzed. Complete blood counts were determined with an automated counter (Sysmex Corporation, Tokyo, Japan).

2.7. Flow Cytometric Analysis

Fc Block (blocking antibody against CD16 and CD32; BD Biosciences, San Jose, CA, USA) was used to eliminate nonspecific binding of the antibody to Fc receptors. Propidium iodide at 5 μg/mL was added to gate out dead cells. Antibodies conjugated with fluorescein isothiocyanate (FITC), phycoerythrin (PE), or allophycocyanin were used for analysis on a FACSCalibur flow cytometer (BD Medical Systems, Franklin Lakes, NJ, USA). All antibodies were from BD Pharmingen (San Diego, CA, USA) or eBioscience (San Diego, CA, USA), except for the biotin-conjugated antibodies, which were components of a Lineage Cell Depletion kit from Miltenyi Biotec (Auburn, CA, USA).

2.8. IL-21 Overexpression In Vivo

Murine IL-21 complementary DNA was subcloned into a pORF expression vector (InvivoGen, San Diego, CA, USA), and an empty pORF expression vector was used as a negative control. Briefly, 20 μg of the empty vector or the IL-21 vector in 2 mL phosphate-buffered saline was administered intravenously into the tail vein, as previously described [7,8]. This method of protein expression in vivo is known as hydrodynamics-based transfection [13,14].

2.9. Cell Sorting

Bone marrow (BM) and spleen cells were stained with antibodies as described above and sorted with a FACSVantage instrument (BD Medical Systems). We first depleted differentiated cells with a Lineage Cell Depletion kit containing biotin-conjugated antibodies for CD5, B220, CD11b, Gr-1, 7-4, and Ter-119 (Miltenyi Biotec), stained them with FITC–Sca-1, PE–c-Kit, and lineage marker again, and sorted them for $c\text{-}Kit^+$ and $Sca\text{-}1^+$ double-positive cells with a gating out of the $lineage^+$ cells.

2.10. Reverse Transcriptase–Polymerase Chain Reaction Analysis

Total RNA or polyA RNA was obtained from sorted cells, KSL cells ($c\text{-}Kit^+$, $Sca\text{-}1^+$, and $lineage^{-/low}$ cells), cells positive for both Gr-1 and Mac-1, $B220^+$ cells, and $CD3^+$ cells by using RNAeasy (Qiagen, Valencia, CA, USA) or QuickPrep (GE Healthcare, Piscataway, NJ, USA). Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control. Briefly, after the amount of GAPDH RNA was measured by reverse transcriptase–polymerase chain reaction (RT-PCR) analysis, the same amount of RNA was subjected to RT-PCR for the IL-21R. The primers used were as follows: GAPDH, 5′-CTCAACTAC ATGGTTTACATGTTCC-3′ and 5′-GCCAGTGGACTC CACGACGTAC-3′; IL-21R, 5′-ATGCCCCGGGGCCCA GTGGCTG-3′ and 5′-CACAGCATAGGGGTCTCTGAG GTTC-3′; ANG-1, 5′-CAGTGGCTGCAAAAACTTGA-3′ and 5′-ACGAGAAACCAAGCCTTGAA-3′.

2.11. BM Transplantation

Unless otherwise stated, recipient mice were irradiated with 9.50 Gy by means of an x-ray irradiation machine (Hitachi, Tokyo, Japan). Donor cells were injected into the tail vein.

2.12. Competitive Repopulation Assay

One thousand BM KSL cells from a Ly5.1 mouse with 2.5×10^5 Ly5.2 BM cells were injected into irradiated C57BL6/J mice. Repopulation of the peripheral blood was assessed by using antibodies to CD45.1 and/or lineage markers.

Table 1.
Flow Cytometric Analysis of Splenocytes and Peripheral Blood Cells from Control and Interleukin 21 (IL-21)-Overexpressing Mice*

	pORF, %	mIL-21/pORF, %
Spleen		
Sca-1	39.38 ± 3.01	62.41 ± 4.42†
c-Kit	2.53 ± 0.89	3.76 ± 1.17
CD11b	8.91 ± 2.11	9.87 ± 1.34
Gr-1	2.5 ± 1.39	2.28 ± 0.25
Ter-119	9.98 ± 3.21	16.52 ± 2.9
Peripheral blood		
Sca-1	36.33 ± 1.71	62.57 ± 3.61†
c-Kit	0.13 ± 0.02	0.16 ± 0.05
CD11b	18.13 ± 1.91	24.31 ± 1.10†
Gr-1	7.11 ± 2.27	7.96 ± 0.84

*Data are expressed as the percentage of cells expressing the indicated marker and are presented as the mean ± SD. pORF indicates mice injected with empty pORF expression vector; mIL-21/pORF, mice injected with pORF vector expressing the murine IL-21 gene.
†$P < .05$, Student t test.

2.13. Enzyme-Linked Immunosorbent Assay for IL-21

A mouse IL-21 enzyme-linked immunosorbent assay kit was purchased from R&D Systems and used per the manufacturer's instructions.

2.14. Statistical Analysis

We used the Student t test for the determination of P values. A P value of <.05 was considered statistically significant.

3. Results

3.1. IL-21 Increases Sca-1$^+$ Cells in the Spleen and Peripheral Blood

Previously, we used a hydrodynamics-based transfection method [13,14] to establish an in vivo system of transient IL-21 overexpression [8], which resulted in serum IL-21 concentrations as high as 6 ng/mL for a period of several days [8]. The primary site of expression has been reported to be the hepatocyte [14], and the secreted IL-21 most likely circulates in the blood. Spleens from mice overexpressing IL-21 were larger than the spleens of control mice and contained 1.5- to 2.0-fold more cells [7]. This larger spleen size and the decreased expression of CD21 and CD23 on B-cells [7] were used as markers for IL-21 overexpression. Using the same system in the present study, we observed an increased percentage of Sca-1$^+$ cells in the spleen and peripheral blood (Table 1). In these particular experiments, the concentration of murine IL-21 in the sera of mice injected with murine IL-21 vector was 494 ± 360 pg/mL (mean ± SD; n = 6) at day 6, whereas the concentration of murine IL-21 in sera from mice injected with empty vector was, as expected, at approximately the lower limit of detection (24 ± 4 pg/mL; n = 6).

3.2. IL-21 Increases CFU-GM in the Spleen

What does an increase of Sca-1$^+$ cells in the spleen imply? Because Sca-1 is a hematopoietic stem/progenitor cell marker, we performed CFU-GM assays with BM and spleen cells. The number of CFU-GM per spleen increased approximately 5-fold, whereas the increase in the CFU-GM in the femur (BM) did not achieve statistical significance (Figure 1A), indicating IL-21 induction of progenitor cell expansion in the spleen. To investigate what kinds of progenitors increased, we evaluated the numbers of CFU-G, CFU-M, CFU-GM, BFU-E, and CFU-GEMM. The main increase was in granulocyte-macrophage lineages in the spleen (Figure 1B).

3.3. IL-21 Increases KSL Cells in BM and Spleen

The increases in CFU-GM and Sca-1$^+$ cells in the spleen led us to investigate whether there was an increase in the phenotypic hematopoietic stem cell compartment, as defined by KSL cells in the BM and spleen. The percentages of KSL cells in the BM and spleen were induced by IL-21 (Figure 1C: ii versus i; iv versus iii). The cellularity in the spleen increased, whereas that in the BM either did not change or slightly decreased, resulting in a dramatic increase in total KSL cells in the spleen (Figure 1C, vi) and a more modest increase in total KSL cells in the BM (Figure 1C, v).

3.4. IL-21 Augments Proliferation of KSL Cells In Vitro

We next assessed whether IL-21 augments the proliferation of hematopoietic stem cells in vitro. We used a combination of murine SCF (c-Kit ligand), human Flt-3 ligand (Flt-3L), and murine IL-7 as a cytokine "cocktail." BM cells from 5-FU–treated mice were incubated with the cytokine cocktail for 1 week in the presence or absence of IL-21. The total number of BM cells was greater when IL-21 was added (Figure 2A, upper panel). To eliminate any effects from differentiated cells, we used sorted KSL cells from untreated BM cells instead of 5-FU–treated BM cells. From 1000 KSL cells, cells treated with IL-21 increased to 30,000 cells after 1 week, but cells without IL-21 treatment expanded to only 10,000 cells (Figure 2A, lower panel). Regardless of the presence or absence of IL-21, KSL cells gave rise to B-lymphoid and myeloid cells, according to the results of B220, Mac-1, and Gr-1 staining. These results suggest that IL-21 was able to augment the proliferation of hematopoietic progenitor cells, at least in the presence of SCF, Flt-3L, and IL-7.

3.5. KSL Cells Express IL-21R

Given that IL-21 augments the proliferation of KSL cells, KSL cells should express IL-21R. To determine if KSL cells express IL-21R, we used semiquantitative RT-PCR analysis for KSL cells, Gr-1$^+$Mac-1$^+$ BM cells, B220$^+$ spleen cells, and CD3$^+$ spleen cells. IL-21R was expressed on KSL cells but at a lower level than on T-cells and B-cells (Figure 2B). Five independently sorted KSL cell preparations demonstrated IL-21R expression, as confirmed by DNA sequencing of the PCR fragments. Gr-1$^+$Mac-1$^+$ cells from

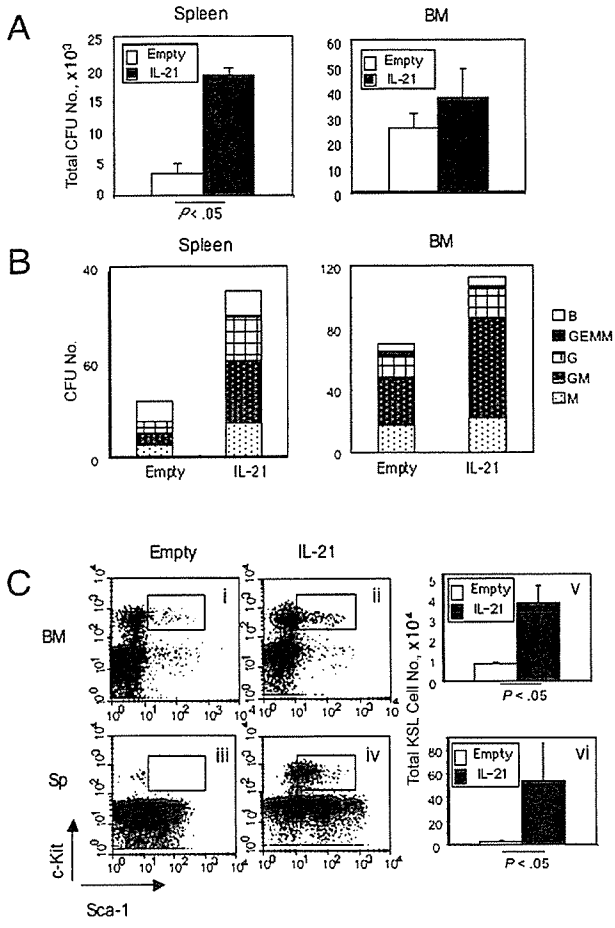

Figure 1. Numbers of colony-forming units (CFU) and c-Kit$^+$, Sca-1$^+$, and lineage$^{-/low}$ (KSL) cells in bone marrow (BM) and spleen (Sp). A, Evaluation of CFU–granulocyte-macrophage (CFU-GM). Splenocytes (2.5×10^5 cells) or BM cells (5×10^4 cells) were plated in methylcellulose medium (MethoCult GF M3534) containing interleukin 3 (IL-3), IL-6, and stem cell factor (SCF) (StemCell Technologies). The numbers of colonies were counted after 5 to 7 days and expressed as the number of CFU-GM per spleen or femur (BM). Data are expressed as the mean ± SD for 3 mice in each group, and the data presented are representative of more than 3 independent experiments. B, Evaluation of CFU-granulocyte (G), CFU-macrophage (M), CFU-GM (GM), burst-forming unit–erythroid (B), and CFU–granulocyte, erythrocyte, megakaryocyte, macrophage (GEMM). Splenocytes (2.5×10^5 cells) or BM cells (5×10^4 cells) were plated in methylcellulose medium (MethoCult GF M3434) containing IL-3, IL-6, SCF, and erythropoietin (StemCell Technologies), and the numbers of colonies were counted after 5 to 7 days. The data are presented as the mean of 5 mice in each group. C, Overexpression of IL-21 increases the number of KSL cells. Typical KSL staining of BM cells (i, ii) and splenocytes (iii, iv) from mice injected with either empty vector or IL-21–expression plasmid, and the numbers of KSL cells in a femur (BM) (v) or spleen (vi). The result shown is a representative result from 2 independent experiments with 5 mice in each group. The difference is partly due to the elevated expression of Sca-1, as shown in Table 1, but is not due to the compensation conditions, because these mice were compared at the same time, with the same machine, and under identical conditions.

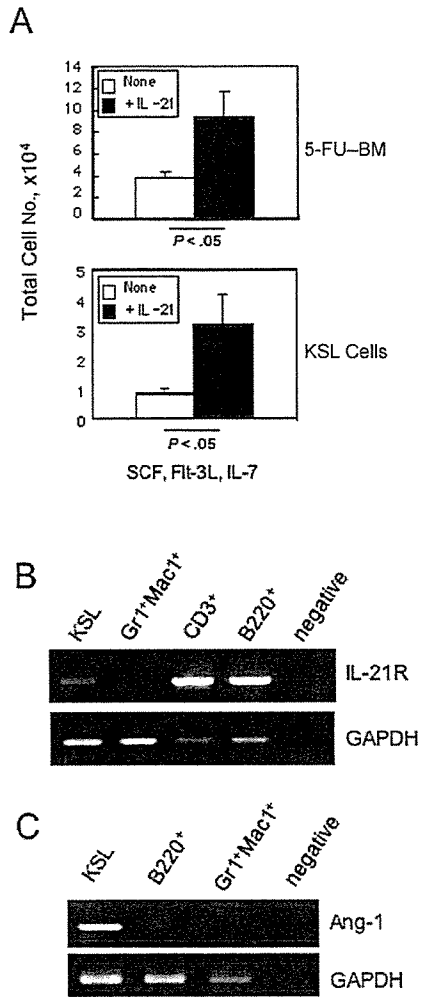

Figure 2. Augmentation of c-Kit$^+$, Sca-1$^+$, and lineage$^{-/low}$ (KSL) cell proliferation Interleukin 21 (IL-21) and expression of IL-21 receptor (IL-21R) on KSL cells. A, Total cell numbers after incubation with or without IL-21 using 5-fluorouracil–treated bone marrow cells (5-FU–BM) (upper panel) or purified KSL cells from untreated BM (lower panel). BM mononuclear cells (1×10^6) from 5-FU–treated mice or 1000 sorted BM KSL cells were incubated in a cytokine cocktail (20 ng/mL murine stem cell factor [SCF], 20 ng/mL human Flt-3 ligand [Flt-3L], and 50 ng/mL murine IL-7) with or without murine IL-21 (20 ng/mL), and cell numbers were counted after 1 week. We cultured cells under normal conditions and used no supporting cell lines, such as stromal cells. Shown is a representative result from 2 independent experiments. Error bars represent the SD of triplicate measurements. B, Semiquantitative reverse transcriptase–polymerase chain reaction (RT-PCR) analysis of IL-21R. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as a loading control. The same amount of RNA was subjected to the RT-PCR for IL-21R. Shown is a representative result from 5 independently sorted KSL cell populations. C, RT-PCR analysis of Ang-1, which has been reported to be expressed on hematopoietic stem cells as well as on stromal cells [15]. GAPDH was used as a loading control. The same amount of RNA was subjected to the RT-PCR for Ang-1.

the BM did not express IL-21R messenger RNA, suggesting that differentiated myeloid cells in the BM do not express IL-21R or that the expression level is diminished. Even though cell purity after sorting was >98%, we verified the cell quality further by checking the expression of Ang-1 (the ligand for the Tie-2 receptor), which is known to be expressed on hematopoietic stem cells [15] as well as stromal cells and which is critical for angiogenesis [15]. Among the cell populations we examined, only KSL cells expressed Ang-1 (Figure 2C).

3.6. IL-21 Also Increases CFU-GM and KSL Cells in RAG2$^{-/-}$ Mice

There are 2 possible mechanisms, a direct mechanism and an indirect mechanism, for the effect of IL-21 on the expansion of progenitor cells. To date, the cells known to express IL-21R are T-cells, B-cells, NK cells, and dendritic cells. In terms of cell numbers, NK and dendritic cells are much less abundant than mature T-cells and B-cells. To investigate the possibility of a secondary effect from mature T-cells and B-cells, we used RAG2 knock-out mice, which lack mature T-cells and B-cells. In these mice, IL-21 overexpression induced a dramatic increase of KSL cells in the spleen (Figure 3A: iv versus iii; vi), a more modest increase of KSL cells in the BM (Figure 3A: ii versus i; v), and a corresponding increase of CFU-GM in the spleen (Figure 3B), analogous to results for normal mice. Thus, mature T-cells and B-cells are not required for the increase in KSL cells and CFU-GM induced by IL-21. The degree of induction in the number of KSL cells by IL-21 in normal mice is much greater than that in the number of CFU-GM (Figure 1C [v and vi] versus Figure 1A), but the difference in the magnitudes of the inductions is not as evident in RAG2$^{-/-}$ mice (Figure 3A [v and vi] versus Figure 3B), suggesting that secondary signals from mature T-cells and B-cells augment the increase in KSL cells but not in CFU-GM. Previously, we confirmed that IL-21 overexpression did not induce an elevation in the serum levels of various cytokines, including IL-1β, IL-2, IL-4, IL-5, IL-12, and tumor necrosis factor α [8]. Consistent with this result, no IL-21–induced elevation of interferon γ, granulocyte-macrophage colony-stimulating factor (GM-CSF), or IL-4 has been demonstrated [9]. These results suggest a direct rather than an indirect effect of IL-21; however, we could not exclude the possible contribution of dendritic, NK, or other unknown cells to the expansion of hematopoietic progenitor cells induced by IL-21.

3.7. Spleen KSL Cells Increased by IL-21 in Wild-Type Mice Have Less CFU-GM Activity than BM KSL Cells

The result with RAG2$^{-/-}$ mice suggests that mature T-cells and B-cells are responsible for the further increase in KSL cells in wild-type mice. The additional degree of induction in KSL cells but not in CFU-GM suggests the presence of less functional KSL cells. To observe the relationship between the induced KSL cells and CFU-GM numbers directly, we performed a CFU-GM assay with the same numbers of sorted

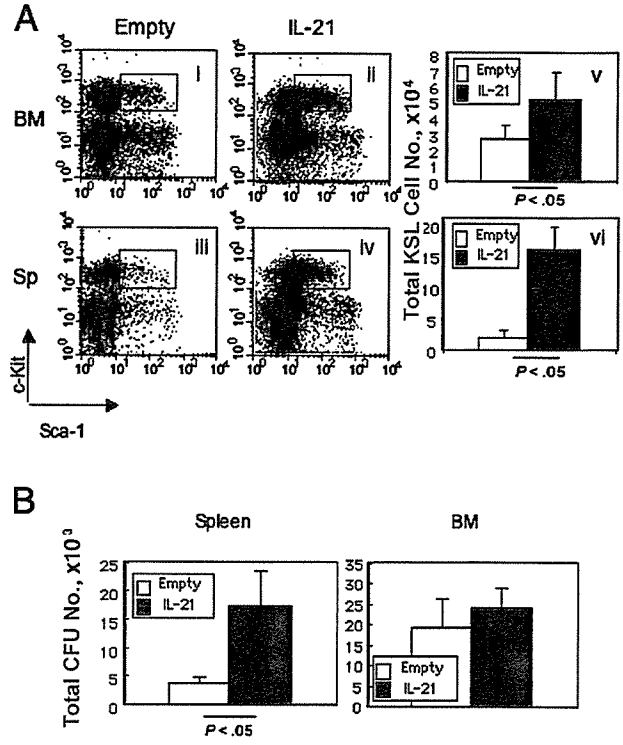

Figure 3. Interleukin 21 (IL-21) induces c-Kit$^+$, Sca-1$^+$, and lineage$^{-/low}$ (KSL) cells and colony-forming units–granulocyte-macrophage (CFU-GM) in RAG2$^{-/-}$ mice. A, KSL cell staining and the number of KSL cells. Typical KSL cell staining of bone marrow (BM) cells (i, ii) or splenocytes (Sp) (iii, iv) from RAG2$^{-/-}$ mice injected with either empty vector or IL-21 vector and the total number of KSL cells in a femur (BM) (v) or spleen (vi). The result shown is the combined results from 2 sets of 6 mice injected with either empty vector or IL-21 expression vector. Error bars indicate ±SD. The difference is partly due to the elevated expression of Sca-1, as shown in Table 1, but is not due to the compensation conditions, because these mice were compared at the same time, with the same machine, and under identical conditions. B, The number of CFU-GM in the spleen and BM (femur) from RAG2$^{-/-}$ mice injected with either empty vector or IL-21 vector. Shown is a combined result for 7 mice for each experimental condition. Error bars indicate ±SD.

KSL cells from wild-type mice injected with empty vector and mice injected with IL-21 vector. BM KSL cells from both types of mice yielded similar numbers of CFU-GM (Figure 4A, left and middle bars). The increased spleen KSL cells also formed CFU-GM colonies (Figure 4A, right bar), but the frequency was lower than in the BM KSL cells, suggesting that the increased splenic KSL cells have less colony-forming activity than BM KSL cells. These results account for the discrepancy in the magnitudes of the inductions between the CFU-GM and KSL cells in the spleen but not in the BM.

Next, we transplanted 1000 BM KSL cells and found that the reconstitution abilities after 3 months for BM KSL cells from the 2 types of mice were indistinguishable (Figure 4B). However, reconstitution after 1 month was lower in mice injected with BM KSL cells induced by IL-21, suggesting that

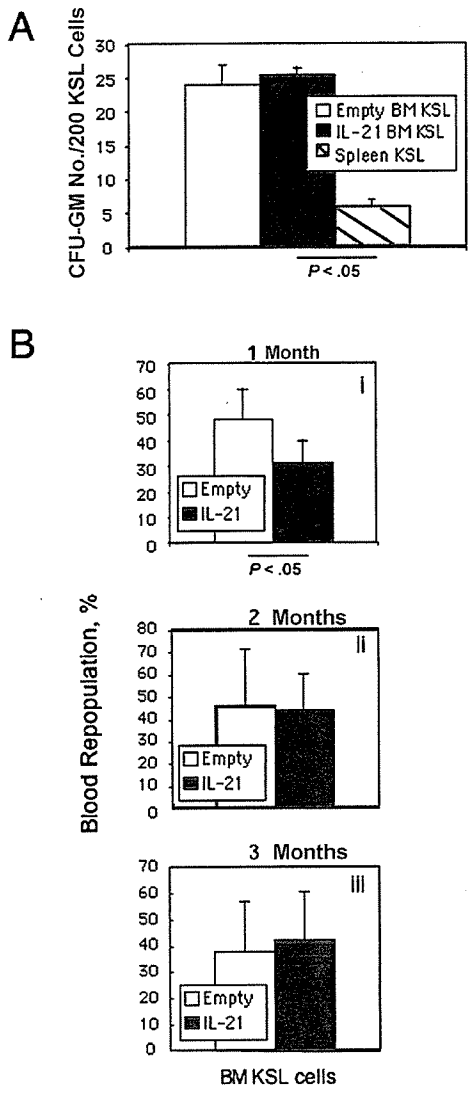

Figure 4. Analysis of induced c-Kit$^+$, Sca-1$^+$, and lineage$^{-/low}$ (KSL) cells. A, The number of colony-forming units–granulocyte-macrophage (CFU-GM) from 1000 bone marrow (BM) KSL cells from mice injected with either empty vector or IL-21 vector and from 1000 spleen KSL cells from mice injected with IL-21 vector. Shown is a representative result from 2 independent experiments. Six mice from each treatment were analyzed. B, Competitive repopulation analysis of the induced BM KSL cells. One thousand BM KSL cells from mice injected with either empty vector or IL-21 vector were transplanted, and peripheral blood engraftment was analyzed according to the percentage of CD45.1$^+$ cells after 1 (i), 2 (ii), and 3 (iii) months. Shown is a representative result from 2 independent experiments. Eight mice from each treatment were analyzed. Error bars indicate ± SD.

BM KSL cells sorted from mice injected with the IL-21 vector contain equal numbers of long-term hematopoietic stem cells but fewer short-term hematopoietic stem cells, compared with BM KSL cells sorted from the mice injected with the control vector.

4. Discussion

We report that overexpression of IL-21 induces an expansion of hematopoietic progenitor cells in the spleen. To our knowledge, this report is the first to show that IL-21 affects hematopoiesis in vivo by either a direct or an indirect mechanism. KSL cells express IL-21R, but the level of expression is low. Further experiments are needed to determine whether this expression is functional, which components of the stem cell compartment express this receptor, and whether a direct or an indirect effect of IL-21 induces the expansion of hematopoietic progenitor cells. We hypothesize that IL-21 has a costimulatory effect on progenitor cell proliferation in the presence of other cytokines, similar to the effect of IL-21 on T-cells or NK cells [1].

We have reported that overexpression of IL-21 induces an increase in immature B-cells [11] in the spleen, although the molecular mechanism remains unknown. The results reported here suggest that this increase might be derived from the induction of progenitor cells in the spleen. It is interesting that human IL-6/soluble human IL-6R double-transgenic mice, but not human IL-6 transgenic mice, exhibit extramedullary hematopoiesis and show elevated numbers of KSL cells, CFU-GM, granulocytes, macrophages, and Sca-1$^+$ cells in the spleen [16], similar to results of our mice overexpressing IL-21. These double-transgenic mice do not exhibit an increase of peripheral white blood cells until 8 weeks of age [16]. An overall similar phenotype suggests that the cell population on which IL-21 and IL-6/soluble IL-6R act may be the same or a closely similar population. In this regard, it is interesting that both IL-6 and IL-21 activate Stat3.

The transient overexpression of IL-21 induces the expansion of hematopoietic progenitor cells in the spleen but appears to be insufficient for the survival and differentiation of these cells into granulocytes or monocytes, because the number of white blood cells in the periphery did not increase. The lack of IL-21R expression on BM-derived myelomonocytes suggests that IL-21 is able to augment the proliferation of primitive cells but not differentiated myelomonocytes.

Overexpression of IL-21 in vivo induced CFU-GM in the spleen (Figures 1A and 1B) and reduced short-term hematopoietic stem cells in the BM (Figure 4B, i), suggesting that the simplest explanation for this phenomenon is a conversion of short-term hematopoietic stem cells into more differentiated colony-forming cells. More detailed experiments are needed to establish evidence for this hypothesis.

A discrepancy between the increases in KSL cells and CFU-GM occurred only in wild-type mice. The presence of less functional "phenotypic" KSL cells in the spleen accounts for the discrepancy between the increases in KSL cells and CFU-GM in the spleen (Figure 4A). In the case of BM, in contrast to the spleen, we could not explain the discrepancy by the less functional "phenotypic" KSL cells (Figure 4A). However, in the absence of mature T-cells and B-cells, the discrepancy disappeared (Figure 3), suggesting that these discrepancies are due to modification by secondary signals from mature T-cells and B-cells and are not a direct effect of IL-21.

CD34$^-$ KSL cells have been demonstrated to express γc [12]. Recently, the hematopoietic hierarchy has been determined, and the expression of γc was reported to be progres-

sively up-regulated from hematopoietic stem cells to committed lymphoid progenitors to pro–T-cells [17]. These reports provide supporting evidence that the presence of the IL-21R on hematopoietic stem/progenitor cells enables them to respond to IL-21 stimulation.

From investigations of IL-21R–deficient mice, it is clear that IL-21 is dispensable for hematopoiesis under normal conditions [5,6]. Our data nevertheless suggest that IL-21 might contribute to hematopoiesis in a redundant fashion and moreover suggest that IL-21 could be useful in inducing differentiated hematopoietic cells in combination with another, yet undetermined cytokine, such as G-CSF, GM-CSF, or IL-7. In such a combination, IL-21 could have potentially therapeutic uses related to its ability to promote the expansion of hematopoietic progenitor cells.

Acknowledgments

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LPS-induced ROS generation and changes in glutathione level and their relation to the maturation of human monocyte-derived dendritic cells

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Abstract

Lipopolysaccharide (LPS)-induced reactive oxygen species (ROS) generation and the concomitant decline in the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) were demonstrated in human monocyte-derived dendritic cells (DC). Further, their relation to the maturation of DC, characterized by the production of cytokines, up-regulation of cell surface molecules and allo-stimulatory capacity, was examined. The LPS-induced ROS generation was demonstrated using electron paramagnetic resonance spectroscopy in intact cells, and was also confirmed using laser scanning confocal microscopy. The GSH/GSSG was assesed using a glutathione assay kit. When the DC were treated with α-phenyl-*tert*-butylnitrone, the ROS generation was attenuated, but the declined GSH/GSSG was not attenuated, and only cytokine production was suppressed among the above-mentioned maturation characteristics. When the DC were treated with glutathione monoethyl ester, both the ROS generation and the declined GSH/GSSG were attenuated, and the maturation characteristics were all suppressed. These findings suggest that the LPS-induced ROS generation and the concomitant decline in GSH/GSSG occur in human monocyte-derived DC and that the former is involved in cytokine production, while the latter is involved in the up-regulation of cell surface molecules and allo-stimulatory capacity. Since the cytokine production and the allo-stimulatory capacity of DC play an important role in inflammatory and immune responses, differential regulation of the ROS generation and the declined GSH/GSSG may be useful as therapeutic tools in diseases where both responses become entangled, such as sepsis and graft-versus-host disease.

Keywords: Dendritic cell; Maturation; Lipopolysaccharide; Reactive oxygen species; Glutathione

Introduction

Dendritic cells (DC) are the most potent antigen-presenting cells and play a critical role in initiating primary T cell responses (Banchereau and Steinman, 1998). Once exposed to foreign pathogens, immature DC are rapidly activated and become mature. Mature DC show strong expressions of HLA-DR, costimulatory molecules (CD40, CD80, CD86) and a specific maturation marker CD83 (Cella et al., 1997; Sallusto and Lanzavecchia, 1994; Zhou and Tedder, 1996). Among foreign pathogens, bacterial lipopolysaccharide (LPS) fully activates DC and induces DC maturation, characterized by the production of cytokines, up-regulation of cell surface molecules and allo-stimulatory capacity (Verhasselt et al., 1997). Recently, some reports showed that reactive oxygen species (ROS) were involved in the LPS-induced maturation of DC by using antioxidants such as, *N*-acetyl-L-cystein (NAC) and ebselen (Matsue et al., 2003; Verhasselt et al., 1999), and other reports showed that the intracellular glutathione level affected the LPS-induced DC maturation by using redox regulators, such as glutathione monoethyl ester (GSH-OEt) and L-buthionine-sulfoximine (BSO) (Kuppner et al., 2003; Murata et al., 2002). However, to our knowledge, there were no reports in which LPS-induced ROS generation and changes in glutathione level were directly observed in DC, except for a report in which the LPS-induced ROS generation was observed in a murine DC line using flow cytometry (Matsue et al., 2003).

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Therefore, in human DC, it is still unclear as to whether LPS-induced ROS generation and changes in glutathione level actually occur or not. In the present study, to elucidate this phenomenon, using electron paramagnetic resonance (EPR) spectroscopy and laser scanning confocal microscopy, we attempted to directly observe the LPS-induced ROS generation in intact cells of human monocyte-derived DC. Then, the LPS-induced changes in the glutathione level were verified by measuring the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG) of the whole cell extract. Finally, the relationship of the LPS-induced ROS generation and changes in the glutathione level with DC maturation, characterized by the production of cytokines, up-regulation of cell surface molecules and allo-stimulatory capacity, was examined using α-phenyl-*tert*-butylnitrone (PBN), a radical stabilizer (Ho et al., 2000), and GSH-OEt, a supplement of intracellular glutathione (Anderson et al., 1985).

Materials and methods

Reagents and antibodies

Roswell Park Memorial Institute (RPMI) medium, fetal calf serum (FCS) and penicillin–streptomycin liquid were purchased from GIBCO; fluorescein isothiocyanate (FITC)-conjugated Annexin V was from Roche Diagnostics (Indianapolis, IN); propidium iodide (PI) and diethylenetriaminepentaacetic acid (DETAPAC) were from Nacalai Tesque; 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) was from Labotec Co. (Tokyo, Japan); GSH-OEt was from Bachem AG (Bubendorf, Switzerland); and recombinant human interleukin-4 (rhIL-4) was from PeproTech (Rocky Hill, NJ). Recombinant human granulocyte macrophage-colony stimulating factor (rhGM-CSF) was kindly provided by Dainippon Pharmaceutical (Osaka, Japan). FITC-labeled anti-HLA-DR monoclonal antibody (mAb) was purchased from Beckman Coulter; phycoerythrin (PE)-labeled anti-CD40, FITC-labeled anti-CD80 and PE-labeled anti-CD83 mAb were from Immunotech; FITC-labeled anti-CD86 was from Dako; and FITC-and PE-labeled mouse IgG1 were from Becton Dickinson. Other chemicals, such as PBN and LPS, were purchased from Sigma.

Cell preparation

Human peripheral blood mononuclear cells (PBMC) were isolated from the blood of healthy adult volunteers by sedimentation through two-step Percoll (Amersham Biosciences, Uppsala, Sweden) gradients, as previously described (Takahashi et al., 1992). After the isolation of PBMC, monocytes and T lymphocytes were further magnetically selected using murine anti-human CD14 microbeads and Pan T cell isolation kit (Miltenyi Biotec, Auburn, CA), respectively, according to the manufacturer's recommendations. The purity of CD14 positive monocytes and that of CD3 positive T cells were always greater than 93% and 97%, respectively, as determined by flow cytometry with FITC-conjugated anti-CD14 and anti-CD3 antibodies (Becton Dickinson).

DC cultures

Freshly isolated monocytes were cultured at a concentration of 5×10^5/mL in RPMI-1640 supplemented with 10% heat-inactivated FCS containing 50 ng/ml rhGM-CSF and 15 ng/ml rhIL-4 for 7 days. On days 3 and 5, one tenth of the original medium was removed and replaced by fresh medium containing rhGM-CSF and rhIL-4. On day 7, immature DC were resuspended at a concentration of 2×10^5/mL, and stimulated for 48 h with 100 ng/ml LPS after pretreatment with PBN and GSH-OEt for 1 h.

Detection of ROS formation with EPR and laser scanning confocal microscopy

To examine if the LPS-stimulated DC generate ROS, EPR spectroscopy with a spin trap, DMPO, was used to directly measure oxygen radicals. The DC were stimulated for 4 h with 100 ng/ml LPS after pretreatment with or without PBN and GSH-OEt for 1 h. The mixture of the DC with DETAPAC (final concentration: 1 mM), DMPO (10 mM) and $FeSO_4$ (50 μM) was prepared and transferred to a flat quartz EPR aqueous cell, which was fixed in the cavity of the EPR spectrometer. The EPR spectrum recording was started exactly 30 s after adding $FeSO_4$. The EPR spectra were recorded on a spectrometer (Model JES-TE300, JEOL, Ltd., Tokyo, Japan). The EPR settings were as follows: microwave power: 10 mW; field: 329.3±5 mT (9.2535 GHz); modulation: 0.125 mT; time constant: 0.03 s; amplitude: 790; sweep time: 2 min. The intensity of the DMPO spin adducts and the hyperfine splitting constants (hfsc's) were calculated, based on the Mn^{2+} marker, which was inserted into the cavity of the EPR spectrometer.

The cells were stained with RedoxSensor Red CC-1 (Molecular Probes, Eugene, OR) to detect ROS accumulation and the mitochondrial-selective dye, MitoTracker Green FM (Molecular Probes). The DC were stimulated for 4 h with 100 ng/ml LPS after pretreatment with or without PBN and GSH-OEt for 1 h. After stimulation, 1 μM Red CC-1 and 500 nM MitoTracker Green were added and incubated for 10 min at 37 °C. Cells were washed twice in phosphate-buffered saline (PBS). Localizations of MitoTracker Green (488 nm) and Red CC-1 (543 nm) were examined using a laser scanning confocal microscope, a Nikon Digital Eclipse C1.

Quantitative determination of GSH/GSSG

The GSH/GSSG was assessed using a glutathione assay kit (Cayman Chemical Company, Ann Arbor, MI) based on the enzymatic recycling method with glutathione reductase (Griffith, 1980). The DC were stimulated for 4 h with 100 ng/ml LPS after pretreatment with or without PBN and GSH-OEt for 1 h. The cells were harvested and the whole cell extract was prepared according to the manufacturer's instructions. The contents of GSH and GSSG were photometrically determined using a microplate reader at 405 nm, and the GSH/GSSG was calculated.