AIMP1/p43 Protein Induces the Maturation of Bone Marrow-Derived Dendritic Cells with T Helper Type 1-Polarizing Ability[1]

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AIMP1 (ARS-interacting multifunctional protein 1) , previously known as p43, was initially identified as a factor associated with a macromolecular tRNA synthetase complex. Recently, we demonstrated that AIMP1 is also secreted and acts as a novel pleiotropic cytokine. In this study, we investigated whether AIMP1 induces the activation and maturation of murine bone marrow-derived dendritic cells (DCs). AIMP1-treated DCs exhibited up-regulated expression of cell-surface molecules, including CD40, CD86, and MHC class II. Additionally, microarray analysis and RT-PCR determinations indicated that the expression of known DC maturation genes also increased significantly following treatment with AIMP1. Treatment of DCs with AIMP1 resulted in a significant increase in IL-12 production and Ag-presenting capability, and it also stimulated the proliferation of allogeneic T cells. Importantly, AIMP1-treated DCs induced activation of Ag-specific Th type 1 (Th1) cells in vitro and in vivo. AIMP1-stimulated DCs significantly enhanced the IFN-γ production of cocultured CD4$^+$ T cells. Immunization of mice with keyhole limpet hemocyanin-pulsed AIMP1 DCs efficiently led to Ag-specific Th1 cell responses, as determined by flow cytometry and ELISA. The addition of a neutralizing anti-IL-12 mAb to the cell cultures that had been treated with AIMP1 resulted in the decreased production of IFN-γ, thereby indicating that AIMP1-stimulated DCs may enhance the Th1 response through increased production of IL-12 by APCs. Taken together, these results indicate that AIMP1 protein induces the maturation and activation of DCs, which skew the immune response toward a Th1 response. ***The Journal of Immunology,*** **2008, 180: 2894–2902.**

Dendritic cells (DCs)[3] are professional APCs with a key role in the induction of primary T cell responses. Tissue-resident immature DCs actively capture and process Ags, but they exhibit a low ability to stimulate naive T cells. Dendritic cells can be directed into fully mature immunostimulatory APCs by various stimuli, including allergens, bacterial (LPS and CpG DNA motifs) or viral (dsRNA) components, cytokines (IL-1β, TNF-α, IFN-α, GM-CSF), and cognate T cell interactions (1, 2). These stimuli occasionally occur at the time of infection by microorganisms or at the time of tissue damage. Upon activation, DCs migrate to the secondary lymphoid tissues to interact with naive T lymphocytes (3). Activated DCs are able to prime T cells through the increased expression of MHC molecules presenting Ag peptides (signal 1) and costimulatory molecules (signal 2). Moreover, this interaction can be regulated by chemokines and cytokines (signal 3) (4–7). Thus, the activation of DCs is critical in bridging the innate and adaptive immune responses.

T cell activation and proliferation may lead to immunity or to tolerance, to the generation/activation of effector T cells or regulatory T cells, and to T cells that secrete different patterns of cytokines, including the cytokine-polarized Th type 1 (Th1) and Th2 responses (8). Importantly, the primed Th cells can be divided into two distinct subsets of effector cells, Th1 and Th2 cells, based on their functional capabilities and on the profile of cytokines that they produce. The nature of the Th1/Th2 polarizing signals is not yet fully understood. However, the cytokines that are present in the environment of the CD4$^+$ T cell at the time that they encounter the Ag importantly regulates the differentiation of Th cells into either Th1 or Th2 subsets (9). In particular, secretion of Th1-driving cytokines, especially IL-12, IL-18, IL-23 and IL-27, induces a Th1 cell-mediated response. Th1 cells, which are characterized by a high level of IFN-γ secretion, are primarily responsible for activating and regulating the development and persistence of CTL (6). DCs that induce a Th1 response can therefore be valuable for cancer immunotherapy. DCs that secrete Th1-driving cytokines have been referred to as type-1 polarized dendritic cells, or DC1 (9, 10). Conversely, DC2s, which produce a lower level of IL-12, induce the polarization of Th cells toward a Th2 phenotype.

AIMP1 (ARS-interacting multifunctional protein 1) is 35-kDa polypeptide consisting of 312 amino acids. It has been identified as an auxiliary factor that is associated with the macromolecular aminoacyl-tRNA synthetase (ARS) complex, which contains nine different enzymes and three nonenzymatic factors, including AIMP1 (11, 12). In the cytoplasm, AIMP1 helps the catalytic reaction of arginyl-tRNA synthetase. Recently, it has been demonstrated that AIMP1 is secreted as a result of various stimuli, including TNF-α, heat shock, and hypoxia (13–15), and that it acts as a multifunctional cytokine on both endothelial and immune cells (16–19).

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Received for publication July 20, 2007. Accepted for publication December 21, 2007.

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[1] This work was supported by grants from the National R&D Program for Cancer Control, Ministry of Health and Welfare (070335) and the Science Research Center Program, Ministry of Science and Technology, Republic of Korea (R11-2005-017-02001-0 to T.S.K.). This work was also supported by a grant from the Korea Science and Engineering Foundation (R17-2007-020-01000-0 to S.H.K.).

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[3] Abbreviations used in this paper: DC, dendritic cell; AIMP1, ARS-interacting multifunctional protein 1; iDC, immature DC; DC1, type-1 polarized dendritic cell; DC2, type-2 polarized dendritic cell; LN, lymph node; MFI, mean fluorescence intensity; PMB, polymyxin B; SOCS, suppressor of cytokine signaling.

 0022-1767/08/$2.00

Previously we found that AIMP1 stimulated macrophages to secrete IL-12, and that AIMP1-activated macrophages induced the IFN-γ production from keyhole limpet hemocyanin (KLH)-primed Th cells (20). It is not clear at present whether AIMP1 is involved in the maturation and activation of DCs, which lead to regulate T cell responses.

In this report, we demonstrate that AIMP1 induces the phenotypic and functional maturation of DCs, leading to IFN-γ production by $CD4^+$ T cells via AIMP1-mediated IL-12 secretion. These data indicate that AIMP1 induces the maturation of DCs to functional APCs, which subsequently differentiate naive Th cells into IFN-γ-secreting Th1 cells.

Materials and Methods

Experimental animals

Female 8- to 10-wk-old C57BL/6 (H-2^b) and BALB/c (H-2^d) mice were purchased from OrientBio. The mice were maintained under specific viral pathogen-free conditions and were treated according to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

mAbs, cytokines, and reagents

Recombinant murine IL-4 and IFN-γ were obtained from BD Pharmingen, and recombinant murine IL-12 was generously provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA). Anti-IL-12 p40 (C17.8 and C15.6), anti-IL-4 (BVD4 and BVD6), and anti-IFN-γ (HB170 and XMG1.2) mAbs were purified from ascitic fluid by ammonium sulfate precipitation, followed by DEAE-Sephagel chromatography (Sigma-Aldrich). Anti-IL-12 p70 mAbs (9A5 and C17.8) were purchased from BD Pharmingen. LPS (from *Escherichia coli* 0111:B4) was purchased from Sigma-Aldrich.

Expression and purification of AIMP1

AIMP1 (312 aa) was expressed as a His tag fusion protein in *E. coli* BL21 (DE3) and purified by nickel affinity chromatography and Mono Q or Mono S ion-exchange chromatography. To remove endotoxins such as LPS, the protein solution was dialyzed in pyrogen-free buffer (10 mM of potassium phosphate buffer (pH 6.0), 100 mM of NaCl). After dialysis, the AIMP1 solution was loaded onto polymyxin resin (Bio-Rad) preequilibrated with the same buffer, incubated for 20 min, and then eluted. To further remove residual LPS, the protein solution was dialyzed against PBS containing 20% glycerol and filtered with a Posidyne membrane (Pall Gelman Laboratory). The concentration of the LPS in AIMP1 was <0.05 endotoxin U/10 μg of protein (1 endotoxin unit = 0.1 ng/ml *E. coli* LPS), as determined using the *Limulus* amebocyte lysate QCL-1000 kit (BioWhittaker).

Generation of bone marrow DCs

DCs were generated using a modification of the method that was originally described by Inaba et al. (21). Briefly, the femurs and tibiae of mice were removed and the marrows were flushed with ice-cold RPMI 1640 using a syringe that was equipped with a 26-gauge needle. Larger cell clusters were dissociated by gentle pipetting, and the cell suspension was then filtered through a 70-μm nylon cell strainer (BD Biosciences). RBCs were lysed with a lysing solution containing 0.15 M of NH_4Cl, 1 mM of $KHCO_3$, and 0.1 mM of EDTA. The bone marrow cells were then suspended in growth medium. The number of cells in the suspension was then adjusted to 5×10^5 cells/ml, and the cell suspension was added to culture dishes. The cells were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS (Biomeda), 50 μM of 2-ME (Sigma-Aldrich), 2 mM of glutamine, 1 mM of sodium pyruvate, 10 mM of HEPES, and 20 μg/ml gentamicin sulfate (Life Technologies) supplemented with 10 ng/ml GM-CSF (ProSpec). The culture medium containing cytokine was replaced at day 3. At day 5, nonadherent cells were removed, and fresh medium containing cytokine was added. At day 6 or 7 of culture, nonadherent cells and loosely adherent DC aggregates were harvested for use in the experiments.

Flow cytometric analysis of cell-surface phenotype

DCs (1×10^6 cells) were harvested, washed with PBS, and resuspended in FACS washing buffer (0.5% FBS and 0.05% sodium azide in PBS). The cells were then incubated with FITC-conjugated anti-CD11c (HL3) and one of the following PE-conjugated mAbs: anti-IA^b (AF6–120.1), anti-CD40 (3/23), and anti-CD86 mAbs (GL1). The stained cells were analyzed in a FACScan cytometer (BD Biosciences). Data were analyzed using CellQuest software (BD Biosciences).

Cytokine assay

The quantities of IL-12 p40, IFN-γ, and IL-4 in the culture supernatants were determined by a sandwich ELISA using mAbs specific for each cytokine, as previously described (22). The mAbs that were used to coat the plates and the biotinylated second mAbs were as follows: for IL-12 p40, C17.8 and C15.6; for IFN-γ, HB170 and XMG1.2; for IL-4, BVD4-11B11 and BVD6. Standard curves were generated using recombinant cytokines (purchased from BD Pharmingen). The lower limits of detection were 30 pg/ml for IL-12 p40, 125 pg/ml for IFN-γ, and 3 pg/ml for IL-4, respectively.

Microarray analysis

Total RNA was extracted from unstimulated immature DCs (iDCs) and AIMP1-stimulated DCs (AIMP1-DCs) using TRIzol (Sigma-Aldrich) according to the manufacturer's instructions. Total RNA was then quantified by UV spectroscopy, and the integrity was confirmed by gel electrophoresis. The fluorescence-labeled cDNA probes were reverse-transcribed from 30 μg of the total RNA using oligo$(dT)_{18}$-primed polymerization with SuperScript II reverse transcriptase (Life Technologies) in the presence of 15 mM of dATP, dTTP, and dGTP, 0.6 mM of dCTP, and 3 mM Cy3- or Cy5-labeled dCTP (NEN Life Science Products). Combinations of fluorescently labeled cDNAs were then hybridized onto mouse cytokine 145 (Genocheck) glass microarray slides containing 145 mouse cDNA spots including the Operon clones, housekeeping genes, and *Arabidopsis* DNA as the internal control. These slides were then scanned using an Axon Instruments GenePix 4000B scanner, and the images were analyzed using the software program GenePix Pro 5.1 (Axon Instruments). The housekeeping genes and *Arabidopsis* DNA were used to normalize the intensity of each spot and to determine the background signal intensity, respectively. To filter out unreliable data, spots with a signal-to-noise ratio (signal/background/background SD) < 100 were excluded. The genes in the iDC vs AIMP1-DC sample comparison with a mean fold change of >1.5 were considered to be significant. The results of the microarray analysis were confirmed by RT-PCR.

RT-PCR

Total RNA that was obtained from the cells was reverse-transcribed into cDNA, and then PCR amplification of the cDNA was performed using a thermal cycler from MJ Research. The sequences of the PCR primers used in this study were as follows: IL-6 (forward (F)), TGAACAACGATGAT GCACTT; IL-6 (reverse (R)), CGTAGAGAACAACATAAGTC; IL-1β (F), CTGAAGCAGCTATGGCAACT; IL-1β (R), ACAGGACAGGT ATAGATTC; IL-1α (F), CTCTAGAGCACCATGCTAC AGAC; IL-1α (R), TGGAATCCAGGGGAAACACTG; IL-12 p40 (F), CAGAAGCTA ACCATCTCCTGGTTTG; IL-12 p40 (R), TCCGGAGTAATTTGGT GCTTCACAC; IL-18 bp (F), GCCACTGTCTTAACTGGAAG; IL-18 bp (R), AGAATGATGTGATAC TGGGC; IL-13 (F), CAGTCCTGGCTCT TGCTTGC; IL-13 (R), GGCATTGCAATT GGAGATGT; TNF-α (F), GGCAGGTCTACTTTGGAGTCATTG; TNF-α (R), ACATT CGAG GCTCCAGTGAATTTCGG; IL-12 p35 (F), TCAGCGTTCCAACAGC CTC; IL-12 p35 (R), CGCAGAGTCTCGCCATTATG; CCR7 (F), GAGACAAGAACCAAA AGCAC; CCR7 (R), GGGAAGAATTAG GAGGAAAA; IL-23 p19 (F), CTGTGCCTA GGAGTAGCAGT; IL-23 p19 (R), TGCTTATAAAAAGCCAGACC; CCR2 (F), CTGTGTTTA ATTTTGGAGGC; CCR2 (R), TCCATTTTGCTTCTGATTCT; IL-27 p28 (F), TACCATCTTCCCAATGTTTC; IL-27 p28 (R), TCCTTTGAA CATTTG AATCC; CCR1 (F), TACCACCAACCTTAACAACC; CCR1 (R), ATGAGAGCTGA GCAGAATGT; β-actin (F), TGGAATCCTGTGG CATCCATGAAAC; β-actin (R), TAAAACGCAGCTCAGTAACA GTCCG. After amplification, the products were separated on 1.5% (w/v) agarose gels and stained with ethidium bromide.

Allostimulatory activity

Unstimulated or AIMP1-stimulated DCs (I-A^b) were harvested on day 8, washed, gamma-irradiated (2000 rad), and used as stimulators. T cells (I-A^d) were isolated using MACS with CD4 microbeads (Miltenyl Biotec), and then used as responder cells. Purified allogeneic T cells were distributed at a density of 1×10^5 cells/well. Various numbers of gamma-irradiated DCs were then added to the wells, and the cell mixtures were cultured at 37°C in a 5% CO_2/air atmosphere. After being cultured for 72 h, 1 μCi of [^{3}H]thymidine was added to each well, and the cells were incubated for an additional 18 h. Next, the cells were harvested on glass-fiber filters, and the cell-associated radioactivity was assessed using a Wallac MicroBeta TriLux liquid scintillation counter (PerkinElmer), and the results were expressed as counts per minute.

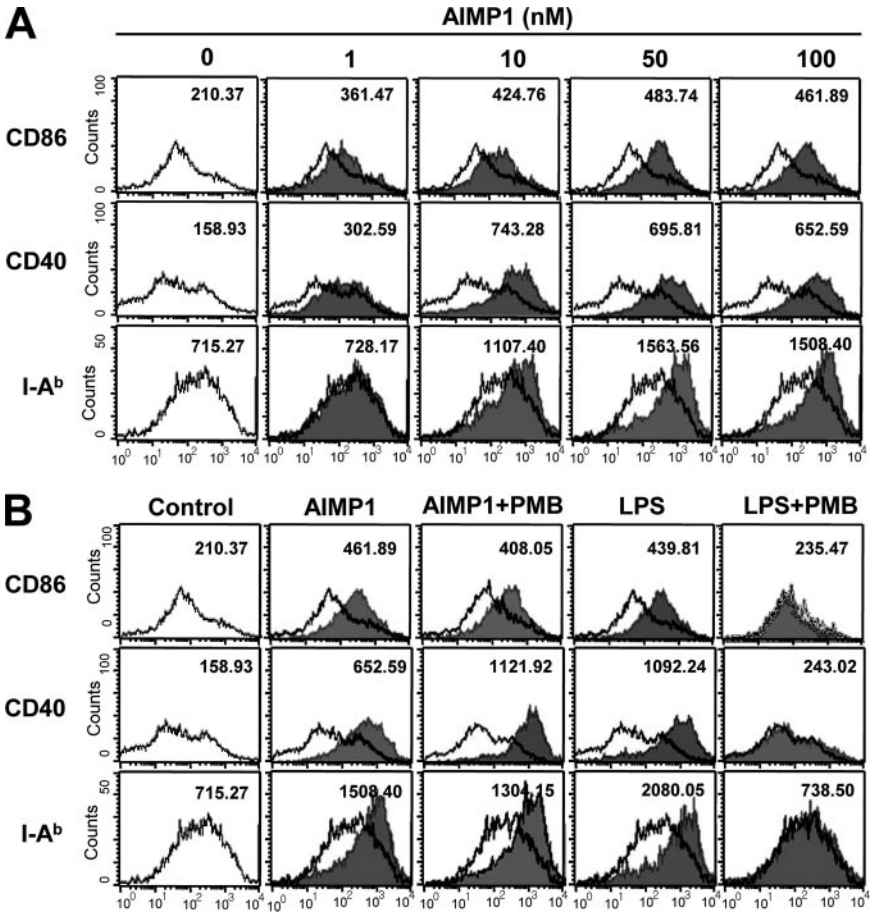

FIGURE 1. AIMP1-induced expression of surface molecules on mouse bone marrow-derived myeloid DCs. Bone marrow cells were isolated from C57BL/6 mice, and iDCs were obtained, as described in the *Materials and Methods*. *A*, iDCs were cultured for 18 h in the absence and presence of the indicated concentrations of AIMP1. *B*, iDCs were pretreated for 2 h with PMB (10 μg/ml), followed by a further incubation for 18 h with either AIMP1 (100 nM) or LPS (0.5 μg/ml). The expression of CD86, CD40, and I-A^b molecules on CD11c$^+$ cells was determined by cytofluorometric analysis using PE-conjugated anti-CD86, anti-CD40, and anti-IA^b mAbs, respectively (shaded areas), or with a PE-conjugated isotype control mAb (unshaded areas). The iDCs that were stained with each specific mAb in the absence of AIMP1 (control) are shown as unshaded histograms. The values in the histograms represent the MFI. The data are representative of three independent experiments.

Ag presentation assay

Unstimulated or AIMP1-stimulated DCs were generated from C57BL/6 mice (I-A^b). The DCs that were harvested on day 8 were irradiated and cocultured with CD4$^+$ T cells from mice (I-A^b) that had been primed with 100 μg of keyhole limpet hemocyanin (KLH) absorbed to aluminum hydroxide adjuvant for 7 days. After incubation for 72 h, 1 μCi of [^{3}H]thymidine was added to each well, and the cells were incubated for an additional 18 h. The cells were then harvested on glass-fiber filters, and the cell-associated radioactivity was measured in a Wallac MicroBeta TriLux liquid scintillation counter.

Migration assay

For the in vitro migration assay, recombinant mouse MIP-3β (ProSpec) was diluted to 300 ng/ml and placed in 24-well plates (CoStar). DCs (5×10^5 cells/well) were added to the upper migration chamber and allowed to migrate through a polycarbonate mesh (pore size 5 μm). After incubation for 2 h at 37°C, the cells that had migrated to the lower chamber were harvested and counted by light microscopy. The results were expressed as a migration index that was calculated by dividing the number of DCs migrating in each test by the number of DCs migrating in the medium control. A migration index >2.0 was defined as significant.

For the in vivo migration assay, DCs were labeled with 1.25 μM of CFSE (Invitrogen: Molecular Probes) in PBS at 37°C for 10 min and washed twice. Mice were injected s.c. with 2×10^6 labeled DCs. At the indicated numbers of hours after inoculation, popliteal and brachial LNs were removed, and LN cells were stained with PE-conjugated anti-CD11c Ab. The stained cells were analyzed with a FACScan cytometer using CellQuest software.

In vitro and in vivo polarization of T cells

Naive C57BL/6 mice were immunized by footpad/haunch injections of 1×10^6 DCs per immunization on day 1. The cells obtained by draining the LNs were collected on day 7 and cultured for 72 h. Cell supernatants were then harvested and the IFN-γ levels were determined by ELISA.

For the in vitro coculture assay, CD3$^+$ or CD4$^+$ T cells that had been isolated from naive C57BL/6 mice (I-A^b) were cultured at a 10:1 ratio with AIMP1-stimulated DCs (I-A^b). Five days later, the cell supernatant was collected and the secreted levels of cytokines (IL-12, IFN-γ, IL-4) were measured by ELISA.

Statistical analysis

Student's *t*-test was used to determine the statistical differences between various experimental and control groups. A p value of <0.05 was considered to be significant.

Results

AIMP1 cytokine increases the expression of MHC and costimulatory molecules of bone marrow-derived DCs

Matured DCs have the capacity to activate T cells via signals from MHC and costimulatory molecules (3–5). Immature DCs, however, are not able to present their Ags to the TCR because they express low levels of MHC molecules. Although an efficient interaction exists between Ag-loaded MHC molecules and TCRs, primed T cells rarely proceed to effector T cells in the absence of a costimulation signal. To investigate whether AIMP1 influences the expression of these cell-surface molecules, iDCs were incubated for 18 h with AIMP1, and the expression of cell-surface molecules was determined by cytofluorometric analysis. As shown in Fig. 1*A*, AIMP1 up-regulated the expression of CD40, CD86, and I-A^b on CD11c$^+$ DCs in a dose-dependent manner. The mean fluorescence intensity (MFI) values of cell-surface molecules on DCs that had been treated with 10 nM of AIMP1 was comparable with MFIs of DCs that were stimulated with 500 ng/ml LPS. The MFI values of AIMP1-treated DCs slightly increased when they were treated with higher concentrations of AIMP1.

To exclude the possibility that endotoxin contamination was responsible for the recombinant AIMP1-mediated expression of cell-surface molecules, iDCs were pretreated for 2 h with polymyxin B

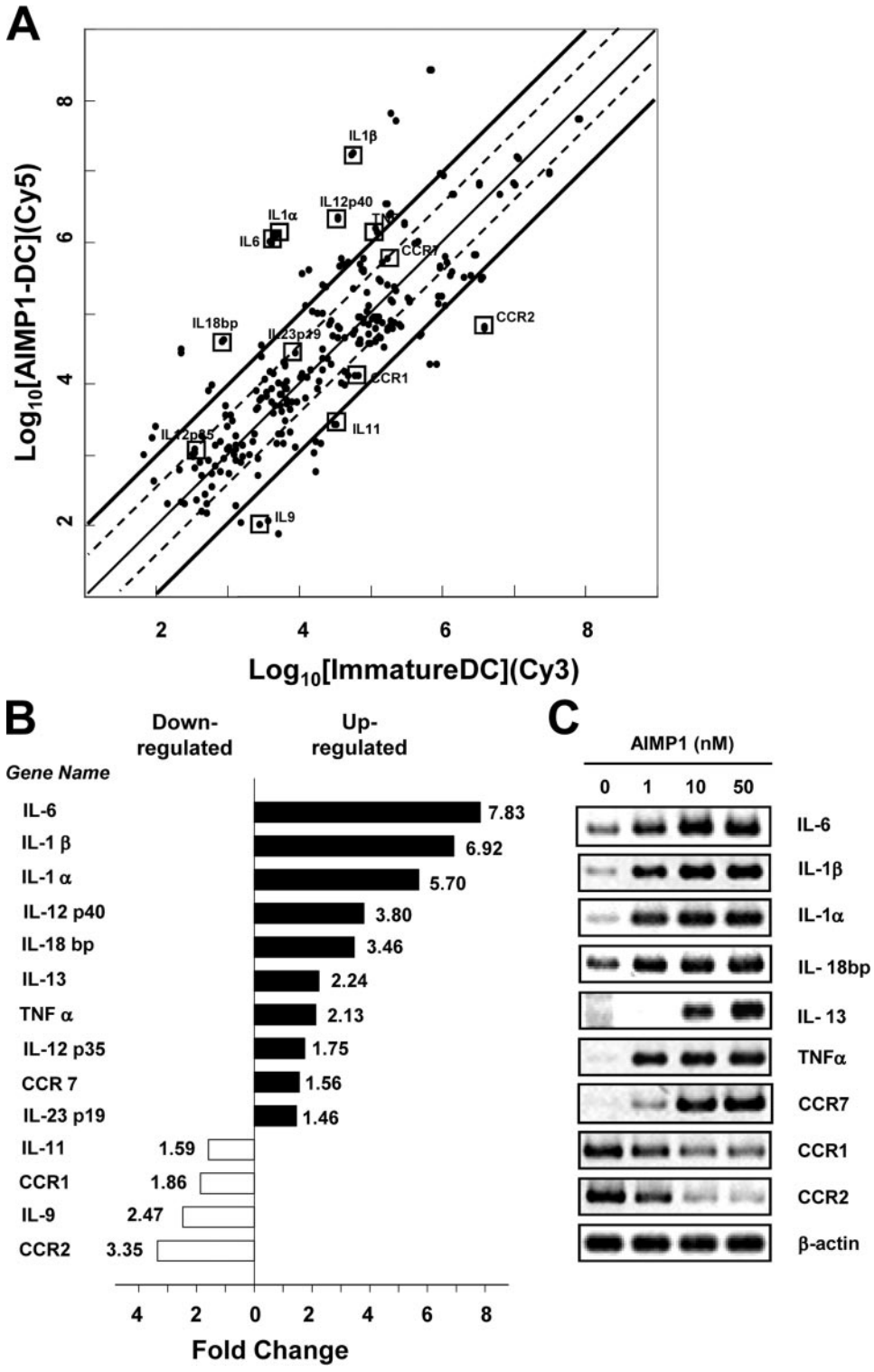

FIGURE 2. Increased expression of known DC maturation genes by AIMP1. Immature DCs from C57BL/6 mice were incubated for 6 h with AIMP1 (10 nM), and cytokine mRNA levels were then measured by microarray analysis. *A*, Log-scale scatter plot of 145 cytokine and cytokine-related genes with probes of the two platforms. The expression profiles from AIMP1-treated DCs were compared with those of the untreated DCs. Genes in which the detected signal showed more than a 2-fold difference between samples fall outside the thick line near the center of the scatter plot. *B*, Altered expression of representative cytokine and cytokine-related genes in AIMP1-treated DCs was shown as the fold-change intensity compared with that of untreated DCs. The results are representative of four similar experiments. *C*, The effect of AIMP1 on the expression of representative cytokine and cytokine-related genes at the mRNA level was confirmed by RT-PCR. The iDCs were treated for 6 h with various concentrations of AIMP1 (0–50 nM), and total RNA was prepared from the cells. RT-PCR products for various cytokines, chemokine receptors, and β-actin were analyzed on 1.5% agarose gels.

(PMB, 10 μg/ml) to inhibit LPS signaling, followed by incubation with either AIMP1 or LPS for 18 h. As shown in Fig. 1*B*, pretreatment of DCs with PMB significantly inhibited the expression levels of all three surface molecules that were increased by 500 ng/ml LPS. However, PMB treatment did not affect the AIMP1-mediated expression of cell-surface molecules, indicating that the increasing effect of AIMP1 on the expression of cell-surface molecules was not due to LPS contamination. Less than 0.05 endotoxin U/10 μg of protein (1 endotoxin unit = 0.1 ng/ml *E. coli* LPS) was detected, as determined by the *Limulus* amebocyte lysate assay. These endotoxin concentrations were insufficient to activate immune cells, including bone marrow-derived DCs.

AIMP1 is able to induce the expression of known DC maturation genes

DC maturation influences the coordinated regulation of several genes. Therefore, it may be possible to conduct a comprehensive assessment of the DC maturation status by monitoring the expression of these transcripts. We used a DNA microarray technique to analyze the specific changes in gene expression that occurred when

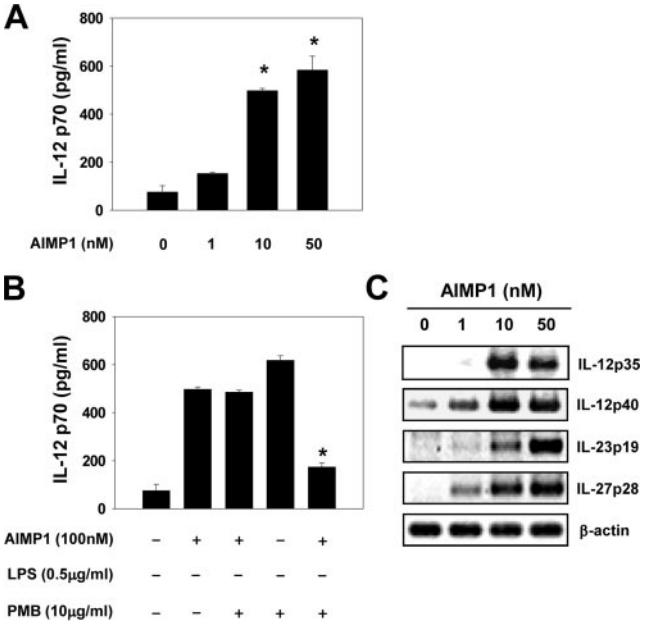

FIGURE 3. Increased IL-12 production in AIMP1-stimulated DCs. Immature DCs were treated for 18 h with the indicated concentrations of AIMP1 (0–100 nM) (*A*), or iDCs were pretreated with PMB (10 μg/ml), followed by treatment with AIMP1 or LPS (0.5 μg/ml) for 18 h (*B*). IL-12 levels in the culture supernatants were determined by an IL-12 ELISA. The results are presented as the means ± SEM ($n = 3$). *, $p < 0.001$, relative to a group of unstimulated DCs (*A*) or LPS-stimulated DCs (*B*). *C*, Effect of AIMP1 on the expression of IL-12 family genes at the mRNA level. The iDCs were treated for 6 h with various concentrations of AIMP1 (0–50 nM), and total RNA was prepared from the cells. RT-PCR products for IL-12 p35, IL-12 p40, IL-23 p19, IL-27 p28, and β-actin were analyzed on 1.5% agarose gels.

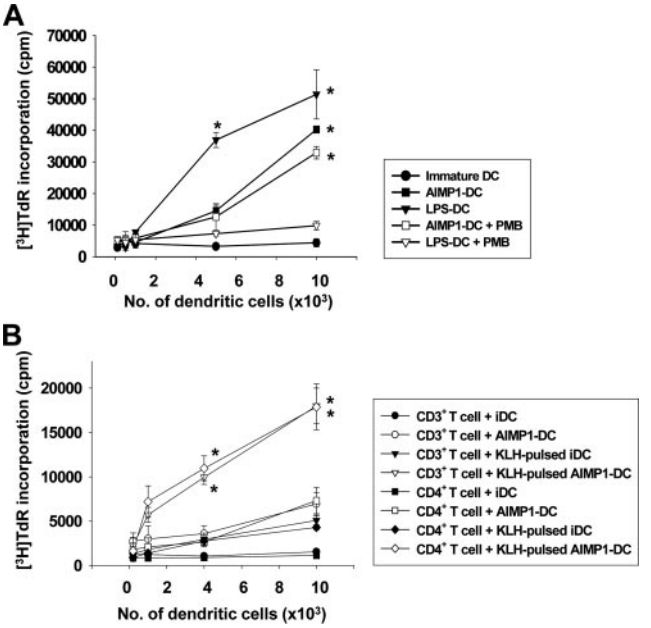

FIGURE 4. AIMP1 increases the allostimulatory and Ag-presenting activity of DCs. *A*, Immature DCs (H-2K^b) were treated with AIMP1 (10 nM) or LPS (0.5 μg/ml). Some iDCs were pretreated with PMB, followed by treatment with AIMP1 or LPS. The DCs were gamma-irradiated (2000 rad) and then the indicated number of DCs was cocultured for 24 h with purified CD4$^+$ T cells (H-2K^d). *, $p < 0.001$, relative to a group of unstimulated DCs cocultured with allogeneic CD4$^+$ T cells. *B*, DCs (H-2K^b) were cultured for 8 h in the presence or absence of KLH (100 μg/ml). At 2 h, AIMP1 (10 nM) was added to the culture. At 8 h, DCs were washed and cultured with freshly isolated KLH-primed T cells (H-2K^b) for 72 h. Proliferation of responding T cells was evaluated by [^{3}H]thymidine incorporation into CD4$^+$ T cells or CD3$^+$ T cells. A background level of [^{3}H]thymidine uptake was determined by measuring reactions without stimulators. The data indicate means ± SEM ($n = 3$) of three independent experiments. *, $p < 0.001$, relative to a group of unstimulated DCs cocultured with syngeneic CD3$^+$ or CD4$^+$ T cells.

immature DCs were stimulated for 6 h with AIMP1. The microarray data were also subjected to scatter plot analysis (Fig. 2*A*). Genes that showed remarkable specificity among the DNA clones that were included in the microarray were then selected and confirmed by RT-PCR. As shown in Fig. 2*B*, the expression of IL-6, IL-1β, IL-1α, TNF-α, and IL-12 family genes was significantly increased in the DCs that were treated with 10 nM of AIMP1. Although AIMP1 stimulation for 6 h may be insufficient to cause distinct changes in chemokines, the expression of CCR1 and CCR2, which are representative chemokine receptors on immature DCs, was significantly decreased. In contrast, the expression of CCR7, a pivotal chemokine receptor involved in DC migration, was increased. Additionally, to confirm the differential gene expression, competitive RT-PCR analysis was performed using a subset of differentially expressed cDNAs (Fig. 2*B*). The results of this analysis demonstrated a similar expression pattern to that of the DNA microarray analyses (Fig. 2*C*).

AIMP1 induces IL-12 production by bone marrow-derived DCs

To assess whether AIMP1 can also induce functional maturation of DCs, immature DCs were treated for 18 h with AIMP1, and the levels of IL-12 p70 protein in the culture supernatants were determined using a sandwich ELISA. It has been reported that expression of IL-12 is a specific marker of functionally matured DCs to activate T cells, especially Th1 cell-mediated response (6, 7). As shown in Fig. 3*A*, AIMP1 strongly induced IL-12 production in a dose-dependent manner. Additionally, increased IL-12 production by AIMP1-treated DCs was independent of LPS contamination because PMB completely blocked IL-12 production in LPS-stimulated DCs, but not in AIMP1-treated DCs (Fig. 3*B*).

To determine whether the induction of IL-12 secretion by AIMP1 at the protein level is associated with IL-12 mRNA expression, we conducted RT-PCR in the AIMP1-treated DCs for IL-12 family genes, such as IL-12 p40, IL-12 p35, IL-23 p19, and IL-27 p28, which are all known to regulate Th1 cell responses (23). The levels of RT-PCR products for IL-12 family genes were significantly increased in a dose-dependent manner when DCs were treated for 6 h with AIMP1 (1–50 nM), indicating that AIMP1-induced IL-12 expression occurred at the transcriptional level (Fig. 3*C*). Treatment with AIMP1 did not influence β-actin mRNA expression in DCs, suggesting that the inductive effect on IL-12 p40 production by AIMP1 was not the result of a general phenomena associated with cellular activation.

AIMP1 enhances the allostimulatory activity of DCs

To further test whether AIMP1 induces the maturation of DCs to fully functional APCs, DCs that had been obtained from C57BL/6 mice (I-A^b) were incubated for 24 h with AIMP1 and then tested for their capacity to stimulate allogeneic CD4$^+$ T cells from BALB/c mice (I-A^d). As indicated in Fig. 4*A*, AIMP1-treated DCs stimulated proliferative responses in allogeneic CD4$^+$ T cells more effectively than did the control immature DCs, which is similar to the results observed when DCs were treated with LPS. Additionally, PMB treatment decreased the allostimulatory effect induced by LPS-stimulated DCs, but it had no effect on the allostimulatory activity of AIMP1-stimulated DCs (Fig.

4*A*). The data indicate that AIMP1-stimulated DCs are effective in priming and activating T cells.

AIMP1 facilitates the Ag-presenting activity of DCs

Next, we investigated whether AIMP1 could enhance the Ag-presenting activity of DCs. KLH-pulsed DCs (H-2^b) that had been stimulated for 8 h with AIMP1 were irradiated and cocultured with KLH-primed $CD3^+$ or $CD4^+$ T cells (H-2^b). As controls, immature DCs with or without pulsed KLH were incubated with KLH-primed $CD3^+$ or $CD4^+$ T cells. AIMP1-treated DCs without pulsed KLH were incubated with KLH-primed $CD3^+$ or $CD4^+$ T cells and used as additional controls. As shown in Fig. 4*B*, KLH-pulsed, AIMP1-stimulated DCs strongly augmented Ag-specific T cell proliferation, whereas AIMP1-stimulated DCs without pulsed KLH stimulated little or no proliferative response in $CD3^+$ or $CD4^+$ T cells. Thus, AIMP1-stimulated DCs have the ability to present their Ag to T cells, which in turn leads to the induction of Ag-specific immune responses.

In vitro and in vivo migration activity of AIMP1-stimulated DCs

To determine whether AIMP1 can affect the migration of DCs, DCs were treated with AIMP1 for 3, 8, and 24 h, and in vitro migration in response to MIP-3β was assayed, as described in the *Materials and Methods*. We chose MIP-3β as the inducer of cell migration because it is an important chemokine that is involved in the migration of several immune cells and is also the ligand of CCR7, which was found to be up-regulated on AIMP1-treated DCs (Fig. 2). As shown in Fig. 5*A*, AIMP-treated DCs migrated more efficiently toward MIP-3β than do immature DCs.

To further test whether the AIMP1-treated DCs had a capacity to migrate into peripheral lymphoid tissues in vivo, AIMP1-treated DCs and immature DCs were, respectively, labeled with CFSE and s.c. injected into the mice. After 24 h, popliteal and brachial LN cells were harvested, and $CFSE^+CD11c^+$ cells were counted using a flow cytometer. As shown in Fig. 5*B*, AIMP1-DC exhibited higher migratory activity than did iDC. However, 24 h AIMP1-DCs did not efficiently migrate in vivo to the LNs, although they migrated very well in vitro (Fig. 5). This result indicates that AIMP1-DCs have the capacity to migrate into the LNs, and this in vivo migratory activity may be influenced by the maturation state of DCs and also by any possibility of DCs to interact with T cells during the migration.

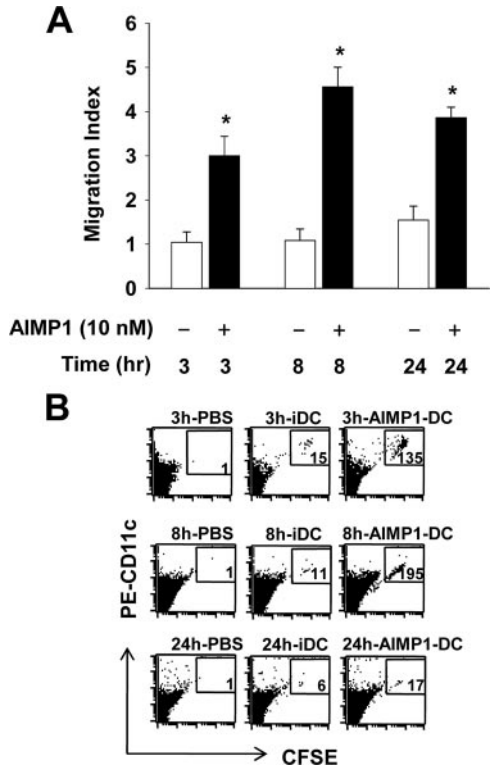

FIGURE 5. In vitro and in vivo migration activity of AIMP1-stimulated DCs. *A*, The iDCs were stimulated with AIMP1 (10 nM) for the indicated times. DCs (5×10^5 cells/well) were then added to the upper migration chamber and allowed to migrate through a polycarbonate mesh (pore size 5 μm) to the lower chamber, which contained recombinant MIP-3β (300 ng/ml). After incubation at 37°C for 2 h, the cells that had migrated to the lower chamber were harvested and counted by light microscopy. *, $p < 0.05$, relative to a group of unstimulated DCs. *B*, AIMP1-treated DCs or untreated DCs were labeled for 10 min with 2.5 μM of CFSE in PBS at 37°C and washed twice. CFSE-labeled DCs were s.c. injected into mice at a concentration of 2×10^6 labeled DCs per mouse. Popliteal and brachial LNs were removed 24 h later, and LN cells were stained with PE-conjugated anti-CD11c mAb and analyzed using a flow cytometer. A total of 2×10^6 cells were analyzed for each sample. Each frame consists of 200,000 cells.

AIMP1-stimulated DCs enhance IFN-γ production in T cells

Mature DCs induce the polarization of Th cells toward either the Th1 or Th2 phenotype. To investigate whether AIMP1-treated DCs can regulate the Th cell-mediated response, KLH-pulsed, AIMP1-stimulated DCs were cocultured at a ratio of 1:10 with $CD4^+$ or $CD3^+$ T cells. After incubation for 5 days, the supernatants were collected and the levels of IL-12, IFN-γ, and IL-4 were determined using a sandwich ELISA. As shown in Fig. 6*A*, AIMP1-stimulated DCs that were cocultured with T cells significantly induced IL-12 production, compared with unstimulated immature DCs. Additionally, AIMP1-treated DCs strongly induced IFN-γ production from both $CD3^+$ and $CD4^+$ T cells. The levels of IFN-γ production in cocultured $CD3^+$ and $CD4^+$ T cells induced by AIMP1-treated DCs were similar, suggesting that $CD4^+$ T cells were the major producers in T cell populations affected by AIMP1-treated DCs. In contrast, AIMP1 did not affect the production of IL-4, a Th2 cytokine, in T cells.

IL-12 has been found to strongly enhance IFN-γ production in $CD4^+$ T cells. Therefore, we would expect the levels of IFN-γ production to be altered in response to the reduced levels of IL-12 in the cultures of T cells and DCs. The addition of anti-IL-12 p40 mAb (0.01–10 μg/ml) to the cocultures of AIMP1-stimulated DCs with $CD4^+$ T cells significantly inhibited IFN-γ production, whereas the IgG2a isotype control Ab did not affect IFN-γ production (Fig. 6*B*). These results suggest that IL-12 secretion by AIMP1-stimulated DCs was a major factor in the enhancement of IFN-γ production by $CD4^+$ T cells.

To further assess whether AIMP1-stimulated DCs induce polarization of T cells in vivo, naive C57BL/6 mice were immunized by footpad/haunch injections on day 1 with a total of 1×10^6 KLH-pulsed DCs that were treated with AIMP1 for 3, 8, or 24 h. Cells from draining LNs were collected on day 7 and then incubated for 72 h in the presence of KLH. As shown in Fig. 6*C*, immunization of mice with AIMP1-DC strongly induced IFN-γ, a Th1 cytokine, although IFN-γ was marginally induced in the immunized mice with 24 h iDC and AIMP1-DC. These results indicate that the inducing effect of AIMP1-DCs on the Th1 response in vivo might depend on in vivo migratory activity and the length of incubation time for which DCs were stimulated with AIMP1.

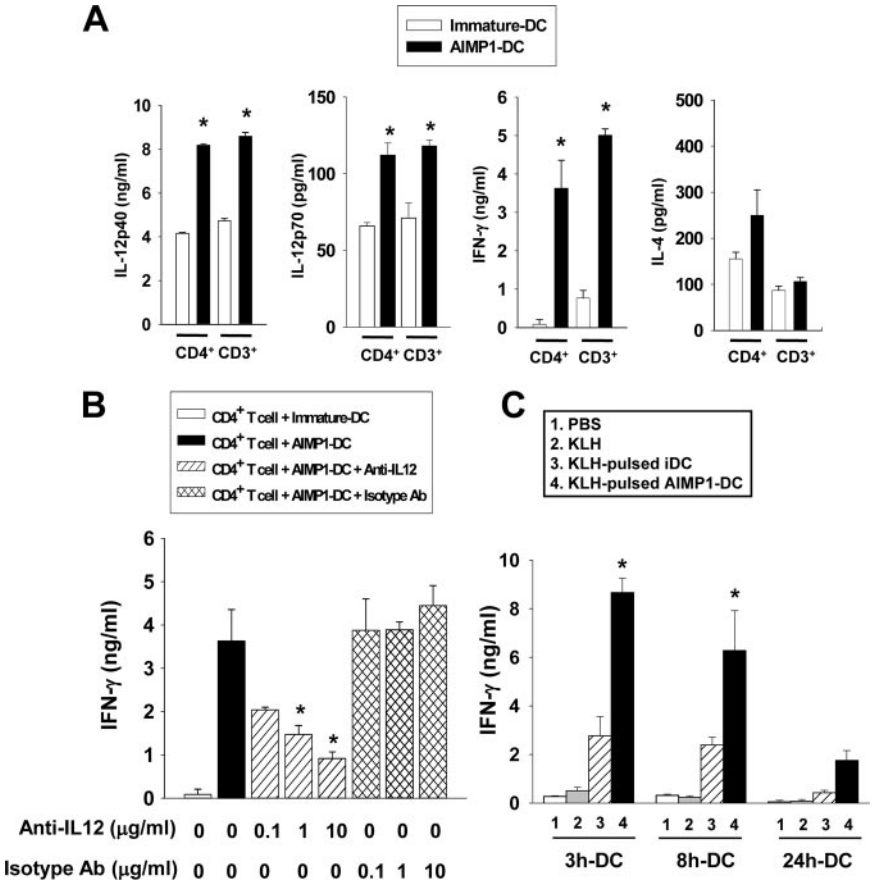

FIGURE 6. AIMP1-stimulated DCs enhance IFN-γ production in $CD4^+$ T cells in vitro and in vivo. *A*, KLH-pulsed, AIMP1-treated DCs were cultured for 4 days with freshly isolated KLH-primed T cells ($CD4^+$ or $CD3^+$ T cells), and the levels of IL-12 p40, IL-12 p70, IFN-γ, and IL-4 in the culture supernatants were then measured by cytokine ELISAs. *, $p < 0.01$, relative to a group of unstimulated DCs that were cocultured with $CD3^+$ or $CD4^+$ T cells. *B*, KLH-pulsed, AIMP1-treated DCs were cultured with freshly isolated KLH-primed $CD4^+$ T cells in the presence of anti-IL-12 Ab (C17.8: 0.1–10 μg/ml) or isotype Ab (JES3–19F1: 0.1–10 μg/ml). The results are presented as the means $\pm$ SEM ($n = 3$). *, $p < 0.01$, relative to a group of AIMP1-stimulated DCs cocultured with $CD4^+$ T cells. *C*, DCs were pulsed with KLH and stimulated with AIMP1, and then used to immunize naive mice via footpad injections on day 1 with a total of 1×10^6 DCs per mouse. At day 7, the LN cells were collected and incubated for 72 h. The cell culture supernatants were harvested, and the IFN-γ levels were determined by IFN-γ ELISA. *, $p < 0.001$, relative to an immunized group with unstimulated DCs.

Discussion

AIMP1 is secreted by several different cell types, including prostate cancer and immune cells (14, 17). Secreted AIMP1 itself functions as a multifunctional cytokine and may play an important role in angiogenesis as well as in the inflammatory process (16). It was previously reported that TNF-α induced AIMP1 expression and secretion from macrophages, which had been recruited to wounded regions of skin. As AIMP1 can induce the expression of TNF-α in macrophages, AIMP1 and TNF-α appear to form a positive feedback loop with one another that amplifies the inflammatory response to tissue injury (13). According to extracellular functions of AIMP1, endogenously secreted AIMP1 can be a natural mediator, which can initiate many immune responses, including spontaneous tumor rejection, and some forms of autoimmunity (24).

This study is the first to demonstrate that the AIMP1 cytokine induces the phenotypic and functional maturation of bone marrow-derived DCs. AIMP1 increased the expression of MHC and costimulatory molecules, as well as IL-12 production by DCs. Additionally, AIMP1-stimulated DCs had enhanced activity of the allostimulation and Ag presentation to T cells. Furthermore, AIMP1-stimulation of DCs resulted in an increase of IFN-γ production by the Ag-specific $CD4^+$ T cells. Therefore, AIMP1 may induce the maturation of DCs into the DC1 type, as indicated by the inductive effects of IL-12 production in DCs and the enhanced IFN-γ production in $CD4^+$ T cells by AIMP1 treatment. These results suggest that endogenously secreted AIMP1 may act as a stimulus for the induction of Th1 cell-mediated immune responses.

Th1-skewed immune responses correlate with effector mechanisms that are important for immunity against tumors and some infectious microorganisms. DCs also influence the type of immune response by expressing a selective set of T cell-polarizing molecules that determine the balance between Th1, Th2, or regulatory T cell development (25). Methods to polarize DCs for the preferential induction of Th1-skewed immune responses have been developed, and polarized DCs are being evaluated in preclinical and clinical studies of DC-based immunotherapy (26, 27). DCs are generally polarized into DC1 using maturation inducers such as poly(I:C), CpGs, LPS, and GM-CSF (28). It has been reported that DC1 producing high levels of IL-12 family members can rescue patient Th1-type antimelanoma $CD4^+$ T cell responses in vitro (29). In this study we suggest that the AIMP1 cytokine is a maturation inducer of DC1 with Th1-polarizing ability.

The timing of the DC immunization in vivo is important because the duration of the DC stimulation determines the migratory activity of DCs and the capacity of the DCs to regulate T cell responses. We observed that AIMP1-DCs exhibited a higher migratory activity than did iDCs in vivo and in vitro. However, 24 h AIMP1-DCs did not efficiently migrate to the LNs in vivo, although they were demonstrated to migrate very well in vitro (Fig. 5). Recently it was reported that the duration of stimulation through CD40 influences migratory activity in vivo (30). CCR7 expression was increased when DCs were stimulated with anti-CD40 for 3 h, whereas it was decreased when DCs were stimulated with anti-CD40 for 24 h. Because DCs have a chance to interact with T cells in vivo, migratory activity of DCs in vivo could be different from in vitro migratory activity. Additionally, immunization of mice with AIMP1-DCs strongly induced IFN-γ production in vivo, although IFN-γ production was marginally induced in immunized mice with 24 h-cultured iDCs and AIMP1-DCs, as shown in Fig. 6*C*. Recently, it was suggested that as the time after stimulation increases, DCs become "exhausted," stop producing cytokines, and become refractory to further stimulation (31, 32).

Moreover, prolonged exposure of DCs to maturation signals can induce the type-2 T cell response and simultaneously dampen the type-1 T cell response (33). Additionally, DC maturation and DC functional activity can be down-regulated by inducible negative regulators, such as the suppressor of cytokine signaling (SOCS) family (34, 35). It has been reported that persistent TLR stimulation in response to DC immunization was tolerated by the restriction of positive cytokine feedback loops by SOCS1 (36). The mechanism by which AIMP1 treatment of DCs for prolonged periods results in differential effects in vivo needs to be elucidated.

Although AIMP1 may affect cytokine production in $CD4^+$ T cells in several ways, IL-12 secreted by DCs is a critical cytokine for the enhanced induction of IFN-γ production by Th1 cells. In an experiment evaluating the inhibition of AIMP1-stimulated DCs with anti-IL-12 mAb, IFN-γ production was remarkably decreased in $CD4^+$ T cells that were cocultured with AIMP1-stimulated DCs in the presence of a neutralizing anti-IL-12 mAb. Therefore, IL-12 production by AIMP1-stimulated DCs is a pivotal mechanism that leads to Th1 cell response.

The mechanism(s) by which AIMP1 induces the maturation of DCs is not known. Although it is still unclear how AIMP1 initiates signaling to APCs, one possibility is that AIMP1 may induce intracellular signaling through cell-surface receptors. It has been reported that AIMP1 bound to the cell surface corresponding to the distribution of lipid rafts, and that AIMP1 showed profound binding activity for lipid rafts that were isolated from endothelial cells, indicating that a potential AIMP1 receptor might be presented in the lipid rafts (37). More recently, the chemokine receptor 3 (CXCR3) was considered as a newly described possible candidate for the AIMP1 receptor (38). CXCR3, which is the receptor for CXCL9/MIG, CXCL10/IP10, and CXCL11/I-TAC, is expressed on activated T cells, NK cells, monocytes, dendritic cells, and microglia. Importantly, CXCR3 is preferentially expressed on activated Th1 cells, and it plays a role in their trafficking (39). These findings suggest that the CXCR3 would be involved in the mechanism of interaction between AIMP1 and immune cells.

Additionally, AIMP1 may induce the maturation of DCs via the up-regulation of NF-κB activity. We have previously demonstrated that AIMP1 induced IL-12 production in mouse macrophages via the activation of NF-κB (20). Consistent with these results, AIMP1 significantly increased the binding of the NF-κB transcription factor to the κB site in DCs, as measured by an electrophoretic mobility assay (data not shown). Further work is required to elucidate the mechanism(s) by which AIMP1 activates NF-κB in DCs. One known mechanism underlying the regulation of NF-κB activation is its binding to members of a family of ankyrin-containing molecules, the IκBs, in the cytoplasm. In most cells, IκBα is the predominant inhibitory molecule, and the activation and translocation of NF-κB are therefore contingent upon its release from IκB. In vivo, IκB is rapidly degraded in reponse to a variety of stimuli, including phorbol ester, bacterial LPS, and TNF-α. In short, it is plausible that increased NF-κB binding activity is derived from IκB phosphorylation and subsequent degradation in the cytoplasm, as well as activation of other upstream signals (40).

In conclusion, AIMP1 may be a critical mediator to induce the maturation and activation of DCs. As AIMP1 is secreted in inflammatory or cancerous regions, it may be a central link between innate and adaptive immune responses. Additionally, the pleiotropic effects of AIMP1, which control angiogenesis by a dual mechanism involving the migration and death of endothelial cells (16) and are involved in wound repair (13), are sufficient to confirm that AIMP1 is a natural stimulus for the immune response. Because AIMP1 induces IL-12 production in DCs, and because AIMP1-treated DCs have the ability to polarize $CD4^+$ T cells into IFN-γ-secreting Th1 cells, it may be possible for AIMP1-stimulated DCs to be used as a cell vaccine in immunotherapy against cancer.

Acknowledgments

We thank S. G. Park and K. Kim for many helpful insights and discussions, and Edward P. Cohen for critical review of the manuscript. We appreciate G. Trinchieri, S. Wolf, Y. K. Choe, and J. W. Lee for providing valuable reagents.

Disclosures

The authors have no financial conflicts of interest.

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