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# IL-32γ induces chemotaxis of activated T cells via dendritic cell-derived CCL5

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ABSTRACT

Interleukin (IL)-32 has been associated with a variety of inflammatory diseases including rheumatoid arthritis, vasculitis and Crohn's disease. We have previously reported that IL-32γ, the IL-32 isoform with the highest biological activity, could act as an immune modulator through regulation of dendritic cell (DC) functions in immune responses. Cell locomotion is crucial for induction of an effective immune response. In this study, we investigated the effect and underlying mechanisms of IL-32γ on recruitment of T cells. IL-32γ upregulated the expression of several chemokines including CCL2, CCL4, and CCL5 in the DCs. In particular, IL-32γ significantly increased CCL5 expression in a dose-dependent manner. Treatment with JNK and NF-κB inhibitors suppressed IL-32γ-induced CCL5 expression in DCs, indicating that IL-32γ induced CCL5 production through the JNK and NF-κB pathways. Furthermore, supernatants from IL-32γ-treated DCs showed chemotactic activities controlling migration of activated $CD4^+$ and $CD8^+$ T cells, and these activities were suppressed by addition of neutralizing anti-CCL5 antibody. These results show that IL-32γ effectively promotes migration of activated T cells via CCL5 production in DCs. The chemotactic potential of IL-32γ may explain the pro-inflammatory effects of IL-32 and the pathologic role of IL-32 in immune disorders such as rheumatoid arthritis.



## 1. Introduction

IL-32 acts on a variety of cells of the innate arm of immunity, including neutrophils, macrophages, dendritic cells and mast cells, and it has a role as an amplifier of inflammation [1]. IL-32 is produced by mitogen-activated lymphocytes, interferon-γ activated epithelial cells, and by IL-12-, IL-18-, and IL-32-activated NK cells [2]. Elevated levels of IL-32 expression have also been associated with chronic obstructive pulmonary disease, inflammatory bowel disease, and psoriasis. Over-expression of IL-32 in murine bone marrow transplantation results in increased levels of pro-inflammatory cytokines, exacerbation of collagen-induced arthritis, and more profound inflammation in sulfuric acid-induced colitis [3]. In addition, PBMCs from patients with rheumatoid arthritis display upregulated expression of IL-32 as compared to those from healthy controls [4]. Knockdown of expression of endogenous IL-32 by using siRNA in HIV-1-infected PBMC has been shown to reduce the expression of Th1 cytokines and chemokines, CD40L, and C5a [5]. IL-32γ is the longest isoform among 6 IL-32 splice variants (IL-32α, IL-32β, IL-32γ, IL-32δ, IL-32ε, and IL-32ξ), and it exhibits the highest biological activity of all these variants [6].

Dendritic cells (DCs) are the most potent antigen-presenting cells in the immune system. DCs are located in many different tissues and organs, where they act as sentinels [7]. Upon detecting a pathogen or other foreign object, immature DCs (iDCs) actively capture and process antigens, and then migrate to the peripheral lymphoid organs where they undergo profound phenotypical and functional changes. DC maturation in response to various ligands appears to involve a variety of receptors and several intracellular signaling pathways, including NF-κB, PI-3K, and MAPKs, which underlie induction of the phenotypic and functional characteristics typical of mature DCs [8].

Chemokines are a family of 8–10 kDa polypeptides that act as soluble extracellular chemoattractants playing vital roles in many biological processes [9]. Chemokines regulate the induction of adaptive immunity by controlling the trafficking of antigen-loaded DCs, T and B lymphocytes, and they directly influence antigen-specific immune responses. Cell locomotion is crucial to the induction of innate and adaptive immune responses and pivotal to the recruitment and migration of inflammatory cells into the site of inflammation [10]. Directed cell migration is a tightly regulated process, which is critical for numerous biological processes

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including tissue development, wound healing, and protection against pathogens. CCL5/RANTES is a member of β-chemokines and is chemotactic for immune cells, including Th1 cells, through expression of CCR1 and/or CCR5 [11]. Studies with PI-3K inhibitors such as Wortmannin and LY294002 have known that CCL5-mediated PI-3K activation is critical for chemotaxis [12].

In the present study we investigated the chemotactic potential of IL-32γ on T cells and its underlying mechanism. We demonstrate here that IL-32γ exhibits chemotactic activity on activated T cells via CCL5 produced from IL-32γ-treated DCs.

## 2. Materials and methods

### 2.1. Experimental animals

Female 8- to 10-week old C57BL/6 mice were purchased from OrientBio Inc. (Kapyong, Korea). The mice were maintained under specific viral pathogen-free conditions and were treated according to the Korea University Guidelines for the Care and Use of Laboratory Animals (Approval No. KLG 08-011).

### 2.2. Cytokines, antibodies, and chemicals

Recombinant human IL-32γ was purchased from YbdY Biotechnology (Seoul, Korea). Anti-CCL5 Ab was purified from ascitic fluids by ammonium sulfate precipitation, followed by DEAE-Sephagel chromatography (R&D system, St. Louis, MO). Rat anti-mouse CD3 (145-2C11) and rat anti-mouse CD28 (37.51) were purchased from eBioscience (San Diego, CA). FITC- anti-mouse CD4 and anti-mouse CD8 were purchased from BD Pharmingen (San Diego, CA). Anti-CCL5, anti-GAPDH, HRP-conjugated mouse anti-goat lgG, and rat anti-mouse lgG1 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The ERK inhibitor 2-(2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one (PD98059) was purchased from Tocris Cookson Ltd. (Bristol, UK). LPS (from *Escherichia coli* 0111:B4), p38 inhibitor 4-[4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-1*H*-imidazol-5-yl] pyridine (SB203580), JNK inhibitor anthrapyrazol-one (SP600125), and other reagents were purchased from Sigma–Aldrich (St. Louis, MO).

### 2.3. Generation of bone marrow DCs

DCs were generated using a modification of the method that was originally described by Inaba et al. [13]. 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. Red blood cells were lysed with a lysing solution containing 0.15 M $NH_4Cl$, 1 mM $KHCO_3$, and 0.1 mM EDTA. The bone marrow cells ($5 \times 10^5$ cells/ml) were cultured in growth medium supplemented with 10 ng/ml GM-CSF (ProSpec, Rehovort, Israel). The culture medium containing cytokine was replaced every other days. At day 6 of the culture, loosely adherent DC aggregates were harvested for use in the experiments.

### 2.4. Treatment of DCs with IL-32γ and inhibitors

iDCs were incubated for 24 h in the presence of various concentrations of IL-32γ (0–200 ng/ml), or 100 ng/ml of LPS. In case of the experiments using inhibitors, the cells were pretreated for 1 h with each specific inhibitors at the indicated concentrations (0.5–10 μM), after which the cells were stimulated with 200 ng/ml of IL-32γ. To demonstrate whether IL-32γ-mediated effects were not due to the endotoxin contamination, IL-32γ or LPS was boiled for 30 min at 95 °C for 3 and used in the experiments.

### 2.5. Flow cytometric analysis

DCs ($1 \times 10^6$ 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 incubated for 20 min with FITC-conjugated anti-CD11c, anti-CD4 (L3T4), and anti-CD8 (LY-2). For detection of CCL5 expression, DCs were fixed and permeabilized and then stained with PE-conjugated anti-CCL5 Ab. The data were analyzed using Cell Quest software (BD Biosciences).

### 2.6. Microarray analysis

Total RNA was extracted from unstimulated iDCs and IL-32γ-stimulated DCs (IL-32γ-DCs) using TRIzol (Sigma–Aldrich), and microarray analysis was performed according to the manufacturer's instructions (Genocheck Co., Ansan, Korea). The microarray 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 versus IL-32γ-DC sample comparison with a mean fold change of >1.5 were considered to be significant.

### 2.7. Semiquantitative RT-PCR

Total RNA was isolated from the cells and reverse-transcribed into cDNA, and then PCR amplification of the cDNA was performed. The sequences of the PCR primers used in this study were as following: mouse CCL2: forward, 5′-AAGCTTTGAGACCACACCCTATG-3′; and reverse, 5′-GACCCCAGCTCTTTGATTCTGA-3′, mouse CCL4: forward, 5′-AGGCCCAGTGGGAGTTCAC-3′; and reverse, 5′-TCTTCCA CTGCTTCAGGCTCTT-3′, mouse CCL5: forward, 5′-TGCCCACGTCAA GGAGTATTTC-3′; and reverse, 5′-AACCCACTTCTTCTCTGG GTTG-3′, mouse GAPDH: forward, 5′-TGGAATCCTGTGGCATCCAT-3′; reverse, 5′-TAAAACGCAGCTCAGTAACA-3′. Relative expression was determined by normalizing the expression of each band to GAPDH.

### 2.8. Western blot analysis

The cells ($4 \times 10^6$ cells) were treated with IL-32γ in the presence or absence of MAPK inhibitors or NF-κB inhibitors, after which the cells were harvested and lysed with a lysis buffer. Cell lysates were centrifuged for 15 min at 20,000*g* and separated by 10% SDS–PAGE before being transferred to nitrocellulose membrane. The membrane was blocked with blocking buffer (PBS with 0.1% Tween 20 plus 5% skim milk) and incubated with anti-CCL5 or anti-GAPDH Ab, followed by incubation with HRP-conjugated, anti-goat IgG, or anti-rabbit IgG (1/1000 dilution).

### 2.9. Chemotaxis assay

For the in vitro chemotaxis assay, $CD4^+$ or $CD8^+$ T cells ($1 \times 10^6$ cells/well) were added to the upper chamber and allowed to migrate through a polycarbonate mesh (pore size, 3.5 μm). After incubation for 2 h at 37 °C, the cells that had migrated to the lower chamber were harvested and counted under a light microscope. The results were expressed as the migration index that was calculated by dividing the number of T cells migrating in each test by

the number of T cells migrating in the medium control. A migration index >2.0 was defined as significant.

### 2.10. Statistical analysis

The Student's *t*-test and one-way analysis of variance followed by Dunnett's method were used to determine the statistical differences between various experimental and control groups. A *P* value less than 0.05 was considered significant.

## 3. Results

### 3.1. IL-32γ upregulates CCL5 expression in bone marrow-derived DCs

To assess whether IL-32γ regulates the expression of chemokines, a DNA microarray analysis was used to determine the changes in chemokine expression during stimulation with IL-32γ for 6 h. In DCs treated with 200 ng/ml IL-32γ, expression of CCL2, CCL4, and CCL5 was significantly induced with the highest increase of CCL5, whereas expression of CCL12 and CXCL14 was decreased (Fig. 1A). Genes that showed significantly upregulated expression in the microarray were selected and their expression was confirmed using RT-PCR. As shown in Fig. 1B, mRNA levels of CCL4 and CCL5 were induced by IL-32γ in a concentration-dependent manner. CCL2 expression was increased at a low IL-32γ concentration and maintained at similar levels even at high IL-32γ concentrations.

To determine whether IL-32γ increases expression of CCL5 protein, the levels of intracellular CCL5 protein in IL-32γ-treated DCs was measured by flow cytometry using PE-conjugated anti-CCL5 Ab. As shown in Fig. 1C, DCs treated with IL-32γ increasingly stained with PE-conjugated anti-CCL5 Ab in a time-dependent manner of IL-32γ treatment. This increase of CCL5 protein in IL-32γ-treated DCs was also confirmed by the results of Western blot analysis (Fig. 1D). These results indicated that IL-32γ induces expression of CCL5 from DCs in a concentration- and time-dependent manner.

### 3.2. IL-32γ induces CCL5 production from DCs, which is not due to LPS contamination

To test whether IL-32γ induces secretion of CCL5 from DCs, iDCs were incubated for 24 h with IL-32γ, and CCL5 production was determined using ELISA. IL-32γ significantly induced CCL5 production in a concentration-dependent manner (Fig. 2A). To rule out the possibility that endotoxin contamination in the preparation of recombinant IL-32γ was responsible for the IL-32γ-mediated expression of CCL5, IL-32γ and LPS (a heat-resistant endotoxin) were boiled for 30 min at 95 °C. The boiled IL-32γ or LPS was added to the DCs cultures, and the levels of CCL5 production were determined after 24 h. As shown in Fig. 2B, boiled LPS induced CCL5 production at high levels. In contrast, DCs treated with boiled IL-32γ produced CCL5 to a significantly lesser extent than did the DCs treated with native IL-32γ.

Next, to confirm that the observed IL-32γ-mediated CCL5 induction was indeed attributable to recombinant IL-32γ, DCs were treated with IL-32γ in the presence of increasing concentrations of anti-IL-32γ-neutralizing Ab and CCL5 levels were determined. Addition of the IL-32γ-neutralizing Ab to the IL-32γ-treated DCs significantly suppressed CCL5 production, whereas an isotype Ab did not affect CCL5 production (Fig. 2C), indicating that the observed induction of CCL5 in DCs was due to the IL-32γ protein.

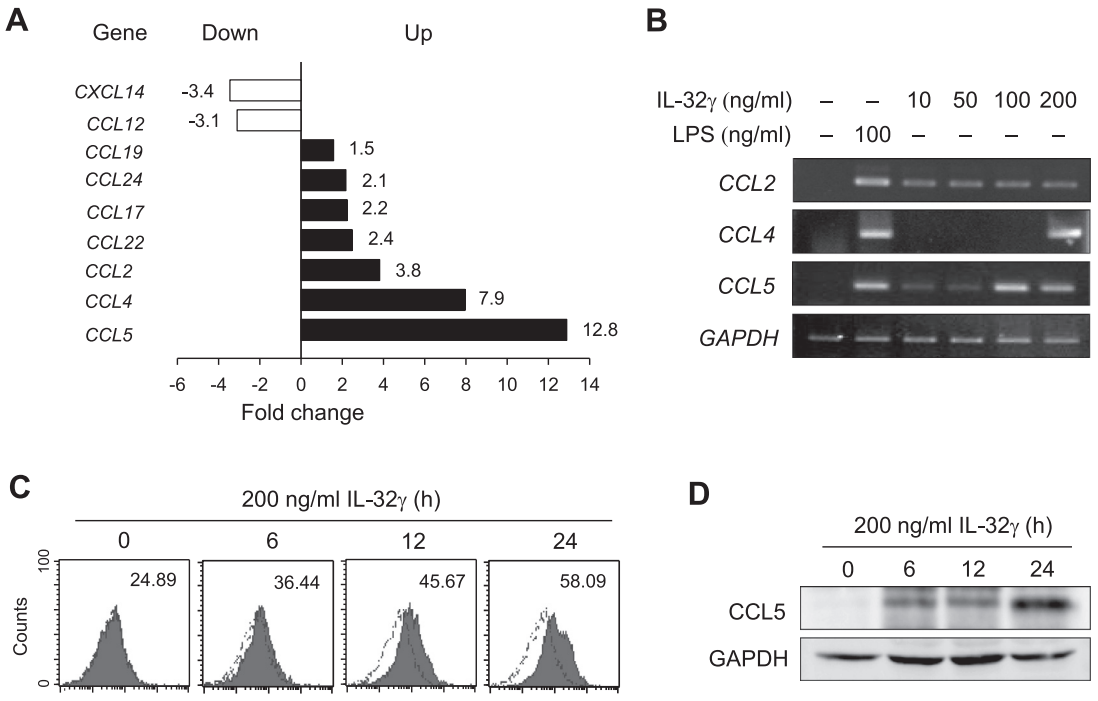


**Fig. 1.** Effects of IL-32γ on the expression of CCL2, CCL4, and CCL5 in bone marrow-derived DCs. (A) Immature DCs (iDCs) from C57BL/6 mice were incubated for 6 h with IL-32γ (200 ng/ml), after which chemokine mRNA levels were measured by microarray analysis. The expression profiles of various chemokines from IL-32γ -treated DCs were compared with those from the untreated DCs. (B) iDCs were treated for 6 h with increasing concentrations (10, 50, 100, and 200 ng/ml) of IL-32γ or LPS (100 ng/ml) and RNA was extracted from the cells. The expression of CCL2, CCL4, and CCL5 was confirmed by RT-PCR. (C) iDCs were treated with 200 ng/ml of IL-32γ for indicated times. The expression of CCL5 on $CD11c^+$ cells was determined by cytofluorometric analysis using PE-conjugated anti-CCL5 (filled histogram), or isotype-matched control Ab (dotted line). The values shown in the histograms represent mean fluorescence intensity (MFI). (D) iDCs were either left untreated or treated with 200 ng/ml of IL-32γ for the times indicated. Cells were lysed and immunoblotted with anti-CCL5 Ab. The data are representative of 3 independent experiments.

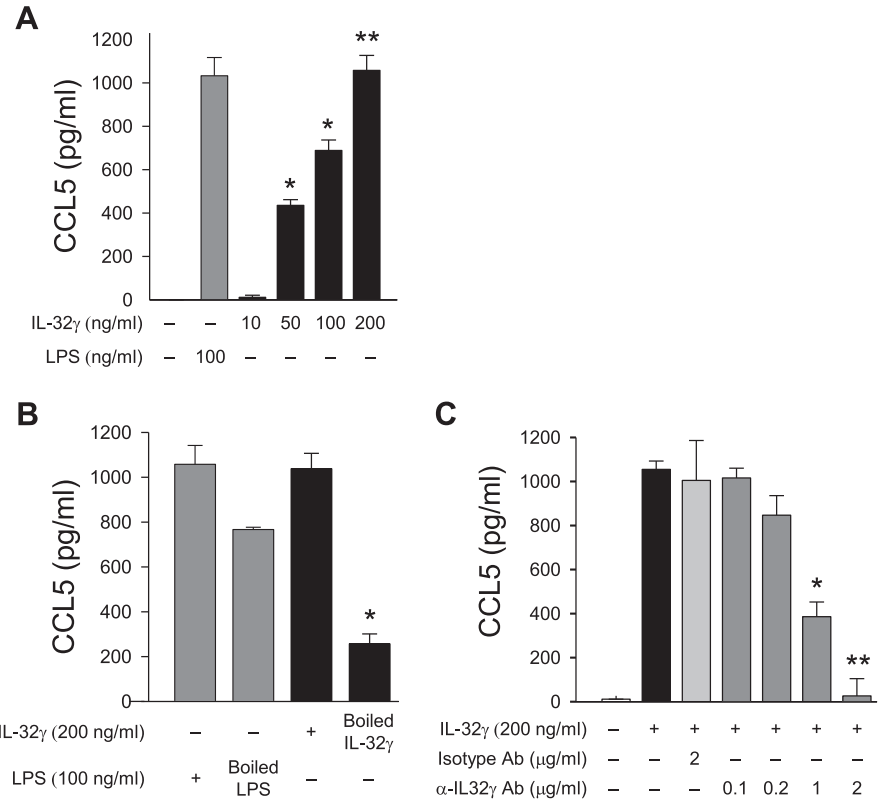


**Fig. 2.** IL-32γ induces CCL5 secretion from DCs. (A) Immature DCs (iDCs) were treated for 24 h with various concentrations of IL-32γ (10, 50, 100, or 200 ng/ml) or LPS (100 ng/ml), and levels of CCL5 in the culture supernatants were determined by using sandwich ELISA. $^{*}P < 0.05$; $^{**}P < 0.001$, versus a group of iDCs. (B) iDCs were treated for 24 h with IL-32γ (200 ng/ml), boiled IL-32γ, LPS (100 ng/ml), or boiled LPS, and levels of CCL5 in the culture supernatants were determined via sandwich ELISA. $^{*}P < 0.01$, relative to a group of IL-32γ-stimulated DCs. (C) iDCs were pretreated for 1 h with increasing concentrations (0.1, 0.2, 1, and 2 μg/ml) of anti-IL-32γ neutralizing antibody (A11-C9) or isotype control antibody (2 μg/ml), after which the cells were stimulated for 24 h with IL-32γ. The production of CCL5 was measured via ELISA. $^{*}P < 0.05$; $^{**}P < 0.001$, compared with the group treated with IL-32γ alone.

### *3.3. JNK and NF-κB are involved in the IL-32γ-stimulated CCL5 production by DCs*

To investigate intracellular signaling mechanisms in response to IL-32γ in DC, the effects of specific inhibitors for 3 major MAPKs and NF-κB on IL-32γ-mediated CCL5 expression were analyzed. As shown in Fig. 3A, addition of the specific inhibitors for JNK (SP600125) and NF-κB (CAPE) clearly suppressed IL-32γ-mediated CCL5 expression. However, inhibitors for ERK (PD985059) and p38 kinases (SB203580) had only small effects on CCL5 expression. As determined by ELISA and Western blot analysis shown in Fig. 3B and C, SP600125 and CAPE reproducibly and significantly inhibited CCL5 secretion from IL-32γ-treated DCs in a dose-dependent manner, whereas SB203580 affected CCL5 expression only marginally. These results indicated that IL-32γ-induced CCL5 production in DCs is mediated mainly through JNK and NF-κB signaling pathways.

### *3.4. CCL5 is a critical factor for the chemotactic effect of IL-32γ*

DCs are important for interactions with T cells. Chemokines, which are produced and secreted by DCs, are involved in one of the mechanisms for controlling migration of the T cells. To investigate whether soluble factors secreted from IL-32γ-treated DCs have chemotactic activity on T cells, iDCs were treated with or without native or boiled IL-32γ. The cultured supernatants were then tested for chemotactic activity against naïve and activated T cells. As shown in Fig. 4A, a supernatant of IL-32γ-treated DCs had chemotactic activity on both naïve and activated $CD4^+$ T cells, and also on $CD8^+$ T cells (Fig. 4B). The chemotactic effects of IL-32γ were much greater on the activated T cells than on the naïve cells. A supernatant from boiled IL-32γ-treated DCs did not exhibit chemotactic activity even on activated T cells (Fig. 4A and B), indicating that the chemotactic effects of IL-32γ were due to the IL-32γ protein, and not due to LPS contamination.

Since we observed that CCL5 production in DCs is highly induced by IL-32γ, we thought that CCL5 secreted from the IL-32γ-treated DCs is closely associated with T cell chemotaxis. To test this hypothesis, a chemotaxis assay was performed in the presence of anti-CCL5 neutralizing Ab. Neutralization with anti-CCL5 Ab significantly suppressed the chemotactic activities of the supernatants of IL-32γ-treated DCs on activated $CD4^+$ and $CD8^+$ T cells in a dose-dependent manner (Fig. 4C). These results demonstrated that activated $CD4^+$ and $CD8^+$ T cells can be effectively recruited through IL-32γ-induced CCL5 production.

## 4. Discussion

The present study has demonstrated that IL-32γ exhibits chemotactic activity on activated T cells through DC-derived CCL5. CCL5-induced chemotactic activity was mediated by JNK and NF-κB activation. One important observation of this study was that IL-32γ was able to increase the expression of several chemokines, including CCL2, CCL4, and CCL5 in DCs. In particular, IL-32γ strongly induced CCL5 chemokine production in DCs (Fig. 1). As CCL5 is a chemokine to recruit T cells, we hypothesized that IL-32γ might exhibit chemotactic activity via CCL5 produced from IL-32γ-treated DCs. As expected, addition of CCL5 blocking Ab into

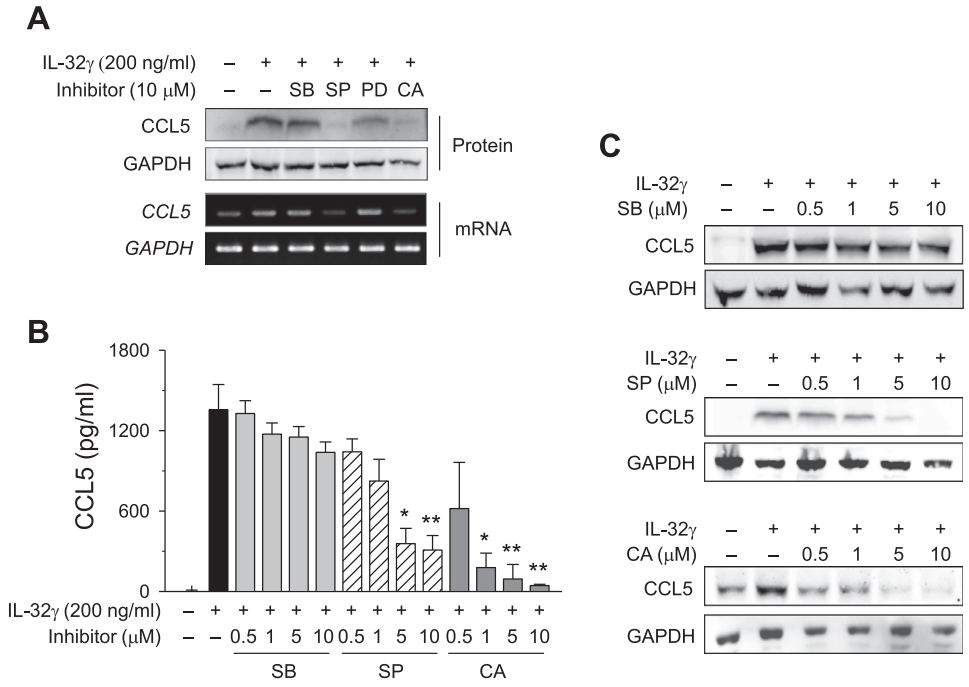


**Fig. 3.** JNK and NF-κB pathways are involved in CCL5 production by IL-32γ-stimulated DCs. (A) Immature DCs (iDCs) were either untreated or pretreated for 1 h with the chemical inhibitors, SB (SB203580, p38 inhibitor), SP (SP600125, JNK inhibitor), and CA (CAPE, NF-κB inhibitor) for 1 h, and then exposed to 200 ng/ml of IL-32γ for 24 h. The level of CCL5 protein was measured in whole-cell lysates (*upper panel*) using immunoblot assay. The expression of CCL5 was analyzed by RT-PCR (*lower panel*). (B) iDCs were pretreated for 1 h with increasing concentrations (0.5, 1, 5, and 10 μM) of indicated inhibitors, followed by the treatment with IL-32γ for 24 h. The production of CCL5 from the cells was measured by ELISA. $^{*}P < 0.05$; $^{**}P < 0.001$, versus a group of the IL-32γ-treated DCs in the absence of inhibitors. (C) iDCs were pretreated for 1 h with the indicated inhibitors, after which the cells were stimulated for 24 h with 200 ng/ml of IL-32γ. The expression of CCL5 was determined by Western blot analysis.

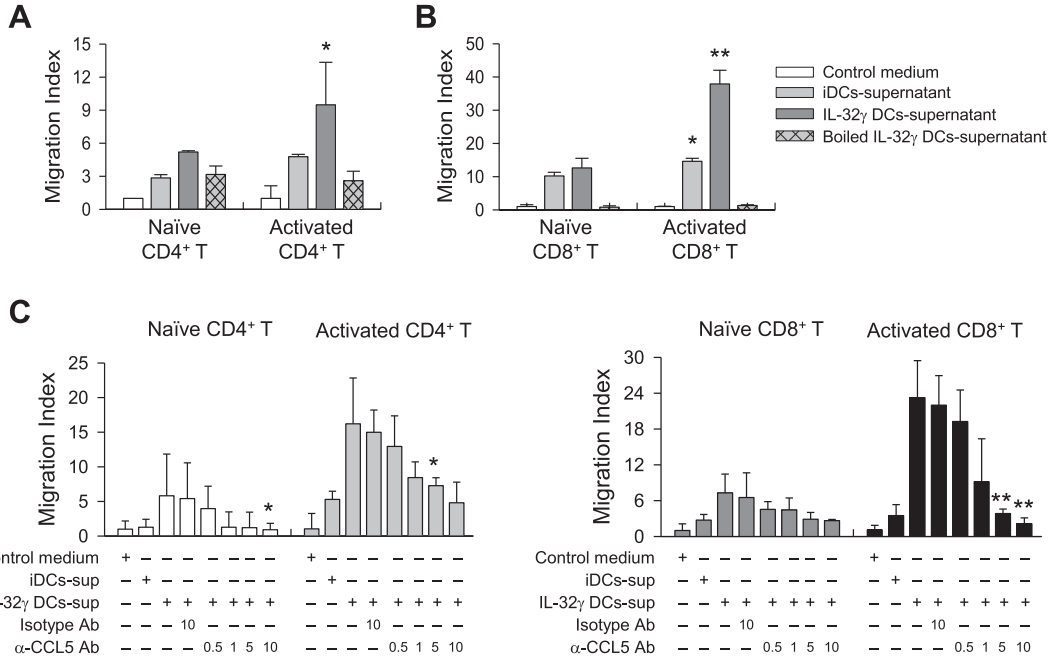


**Fig. 4.** IL-32γ-treated DCs have chemotactic activity on activated T cells. $CD4^+$ and $CD8^+$ T cells isolated from C57BL/6 were cultured for 3 days in the presence of anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) mAbs. Cell migration was measured by a transwell filter assay. Naïve and activated $CD4^+$ T cells (A), or naïve and activated $CD8^+$ T cells (B) at $2 \times 10^6$ cells/ml were added to the upper well of the chamber. The lower chambers were loaded with supernatant from IL-32γ-treated DCs, supernatant of iDCs, or incubated medium. After incubation for 2 h, the cells migrating to the lower chambers were counted by using a microscope using a hemocytometer. Bars and error bars depict the mean ± SD of data collected in duplicate in 3 separate experiments. $^{*}P < 0.05$; $^{**}P < 0.001$, versus a group of iDCs-supernatant with T cells. (C) Naïve and activated $CD4^+$ cells or naïve and activated $CD8^+$ T cells were subjected to the transwell assay. Cells were suspended ($1 \times 10^6$ cells/well) in the upper chamber, and the supernatant of IL-32γ-treated DCs, supernatant of iDCs, or incubated medium was loaded into the lower chamber. Supernatants were incubated with various concentrations of anti-CCL5 Ab (0.5, 1, 5, or 10 μM). Isotype-matched Ab (10 μM) was added to either the supernatant of IL-32γ-treated DCs or the supernatant of iDCs as control. After 2 h, the cells migrating to the lower chambers were counted. Bars and error bars depict the mean ± SD of data collected in duplicate in 3 separate experiments. $^{*}P < 0.05$; $^{**}P < 0.001$, versus a group of IL-32γ DCs-supernatant with T cells.

the supernatants of IL-32γ-treated DCs inhibited the migration of activated T cells (Fig. 4C). These observations suggest a critical role for CCL5 in the migration of T cells to the site of inflammation. In addition, CCL2 is a potent chemoattractant for T cell recruitment to inflamed tissues. Although we did not examine the effects of CCL2 in this study, CCL2 and CCL5 may act synergistically on the chemotactic activity inducing T cell migration. In this study we used IL-32γ among 6 IL-32 splice variants, because it is the most active although all variants are biologically active [6], and its levels are reported to be detectable (approximately 5–100 ng/ml) in granulomatosis with polyangiitis patients, patients with rheumatoid arthritis, or asthmatic patients [14–16]. Furthermore, recombinant IL-32γ has been used to investigate effects of IL-32 on several biological activities in vitro and in vivo [3,14,17].

It has been established that IL-32γ induces many different types of proinflammatory cytokines and that it acts itself as an inflammatory cytokine. IL-32γ promotes and regulates the evolution of functionally distinct T cell subsets, driving these cells toward either a type 1 or a type 17 functional status [17]. It has previously been shown that interactions between chemokines and chemokine receptors are crucial for T cell migration. CCL5 is of great importance in T cell migration to inflamed synovium both in in vitro experiments and in animal models [18,19]. CCR1, CCR3 and CCR5 have been reported as receptor for CCL5. While both CCR1 and CCR5 promote transendothelial chemotaxis towards CCL5, CCR5 is more involved in spreading into tissues. Shadidi et al. reported that Th1 clones expressed more of the CCL5 receptor, CCR5, than any other types of T cells [20]. It will be important to determine whether IL-32γ-induced T cell recruitment is mediated by other chemokine receptor-mediated pathways, in particular, by those operating via the putative Th1 cell marker CCL5.

These results have demonstrated that endogenously secreted IL-32γ may regulate a variety of immune responses via the CCL5 expression from DCs. DCs are key mediators of the immune response during the inflammation process. In response to pathogens and micro-environmental conditions, DCs secrete specific cytokines and chemokines involved in the polarization of T cell subsets that mediate distinct types of inflammation. Our results further indicate that IL-32γ contributes to T cell recruitment to inflammatory sites by promoting chemotactic responses. Furthermore, interaction of IL-32 with TNF-α is reported to contribute to the exacerbation of immune-inflammatory diseases [21]. This makes IL-32γ an attractive therapeutic target in treatments of several important inflammatory diseases.

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