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IL-10 enhances the phenotype of M2 macrophages induced by IL-4 and confers the ability to increase eosinophil migration

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Abstract

M2 macrophages have been subdivided into subtypes such as IL-4-induced M2a and IL-10-induced M2c *in vitro*. Although it was reported that IL-10 stimulation leads to an increase in IL-4Rα, the effect of IL-4 and IL-10 in combination with macrophage subtype differentiation remains unclear. Thus, we sought to clarify whether IL-10 enhanced the M2 phenotype induced by IL-4. In this study, we showed that IL-10 enhanced IL-4Rα expression in M-CSF-induced bone marrow-derived macrophages (BMDMs). Global gene expression analysis of M2 macrophages induced by IL-4, IL-10 or IL-4 + IL-10 showed that IL-10 enhanced gene expression of M2a markers induced by IL-4 in M-CSF-induced BMDMs. Moreover, IL-4 and IL-10 synergistically induced CCL24 (Eotaxin-2) production. Enhanced CCL24 expression was also observed in GM-CSF-induced BMDMs and zymosan-elicited, thioglycolate-elicited and naive peritoneal macrophages. CCL24 is a CCR3 agonist and an eosinophil chemoattractant. *In vitro*, IL-4 + IL-10-stimulated macrophages produced a large amount of CCL24 and increased eosinophil migration, which was inhibited by anti-CCL24 antibody. We also showed that IL-4 + IL-10-stimulated (but not IL-4 or IL-10 alone) macrophages transferred into the peritoneum of C57BL/6J mice increased eosinophil infiltration into the peritoneal cavity. These results demonstrate that IL-4 + IL-10-simulated macrophages have enhanced M2a macrophage-related gene expression, CCL24 production and eosinophil infiltration-inducing activity, thereby suggesting their contribution to eosinophil-related diseases.

Keywords: CCL24, eosinophil infiltration, IL-4, IL-10, M2a macrophage

Introduction

Macrophages are innate immune cells with well-established roles in homeostasis, tissue remodeling and host defense (1). Depending on the microenvironment, differentiation and function of macrophages are governed by numerous cell-extrinsic factors, including cytokines, chemokines and TLRs (2). Differentiated macrophages have been broadly classified as M1 or M2. Classically activated macrophages or M1 are induced by IFNγ and TLR agonists. M1 macrophages express inducible nitric oxide synthase and secrete IL-12, TNFα, IL-1β, IL-23, IL-6, CXCL10 (IP-10) and CCL5 (RANTES) (3). In contrast, alternatively activated macrophages or M2 are proposed to be a heterogeneous population induced by a variety of stimuli *in vitro*: M2a, induced by IL-4 (4) or IL-13 (5); M2b, induced by immune complexes with IL-1 or LPS (6); and M2c, induced by IL-10 (7) or glucocorticoids (8). Transcriptome analysis showed gene expression signatures of M2 macrophages using microarrays: M1 macrophages (LPS + IFNγ) versus M2a macrophages (IL-4) (9); M-CSF-induced macrophages versus GM-CSF-induced macrophages (10); and decidual macrophages versus IL-10-induced macrophages (11, 12). Although there are many microarray analyses of macrophage subtypes induced by a single cytokine, only a few subtypes are induced by combination of cytokines (13).

Previous *in vitro* studies report that IL-4-induced M2a macrophages show enhanced Mrc1 (also known as CD206), arginase 1 (Arg1), Chi3l3 and Retnla expression (9) and produce CCL17, CCL22 and CCL24 (10) and that IL-10 stimulation leads to an increase in IL-4Rα expression through STAT3, resulting in enhanced Arg1 expression (14, 15). And *in vivo* differentiated M2 macrophages induce Th2 and eosinophil infiltration, which are related to allergy and helminth infections

(5, 16). Although these studies suggest that M2 macrophages play a role in eosinophil infiltration *in vivo*, it remains unclear whether IL-4 alone is enough to confer the ability of eosinophil infiltration on macrophages. On the other hand, it is reported that *in vivo* IL-10-induced M2c macrophages promote skeletal muscle repair (17, 18). However, there is no report showing the relationship between M2c and eosinophil infiltration. Recent studies suggest that a combination of cytokines, rather than a single cytokine, induces the Th2 response and eosinophil infiltration *in vivo* through M2 macrophages, such as IL-13 + thymic stromal lymphopoietin (TSLP) and IL-4/IL-13 + IL-33 (19, 20). These studies also show that IL-13 + TSLP- and IL-4/IL-13 + IL-33-induced M2 macrophages produce a large amount of CCL17 and CCL24 *in vitro*. However, it is unclear whether these M2 macrophages directly induce eosinophil infiltration *in vivo* because they did not show that, when transferred into animals, M2 macrophages induce eosinophil infiltration.

In this study, we sought to clarify whether IL-10 enhanced the M2 phenotype induced by IL-4. We confirmed that IL-10 induces IL-4Rα expression and enhances Arg1 expression. And we demonstrated that IL-10 enhances M2a macrophage-related gene expression, CCL24 production and the ability to increase eosinophil migration in IL-4-induced M2 macrophages *in vitro* and further demonstrated that, when transferred into mice, IL-4 + IL-10-induced M2 macrophages indeed induce eosinophil infiltration *in vivo*.

Methods

Reagents

Recombinant mouse cytokines were obtained from R&D Systems (Minneapolis, MN, USA). Recombinant human cytokines were obtained from Pepro Tech (Princeton, NJ, USA). LPS (L4391) was obtained from Sigma–Aldrich (St Louis, MO, USA). Antibodies were purchased from BD Biosciences (Franklin Lakes, NJ, USA), eBioscience (San Diego, CA, USA) or AbD Serotec (Oxford, UK). Mouse CCL24 was measured using commercially available ELISA kits (DY528, R&D Systems).

Mice

C57BL/6J mice and BALB/c mice were purchased from Charles River (Japan). All experiments were approved by the Ethics Committee for Animal Experiments of Asubio Pharma Co., Ltd.

Culture of macrophages

M-CSF- or GM-CSF-induced bone marrow-derived macrophages (BMDMs) were generated as described previously (21). Briefly, bone marrow cells from C57BL/6J mice were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and M-CSF (50 ng ml^{-1}) for 7 days to induce M-CSF-induced BMDMs. GM-CSF (10 ng ml^{-1}) was used instead of M-CSF to induce GM-CSF-induced BMDMs. Naive, thioglycolate-elicited and zymosan-elicited peritoneal macrophages were isolated as described previously (22). Briefly, naive peritoneal macrophages were obtained from peritoneal fluids in C57BL/6J mice. Thioglycolate-elicited macrophages were obtained from peritoneal fluids 3 days after intra-peritoneal (i.p.) injection of 2 ml 3% thioglycolate into C57BL/6J mice. Zymosan-elicited macrophages were obtained from peritoneal fluids 24 h after i.p. injection of 1 mg zymosan into C57BL/6J mice. To induce macrophage subtypes, macrophages were further stimulated for 24 h with LPS (10 ng ml^{-1}) + IFNγ (50 ng ml^{-1}), IL-4 (10 ng ml^{-1}), IL-1β (10 ng ml^{-1}), IL-10 (10 ng ml^{-1}), TGFβ (10 ng ml^{-1}) and IL-4 + IL-10, respectively. Control macrophages were prepared by incubating for 24 h without additional factors.

Quantitative PCR

Cells were lysed and total RNA was purified using the Qiagen microRNeasy system (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Total RNA was reverse transcribed using the SuperScript Vilo cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA) and quantitative PCR was carried out using Taqman-based detection methods (universal probe library, Roche, Penzberg, Germany). β-Actin levels were used as normalization controls and fold induction was calculated using the ΔCT methods. The primers used were Arg1 (NM_007482.3; forward, ggcaaggtgatggaagagac; reverse, aggtgaatcggccttttctt; probe, #3), CCL24 (NM_019577.4; forward, cctctgtccctgaacttgga; reverse, tcccagctggtctgtcaaa; probe, #64), Retnla (NM_020509.3; forward, ttgggagatccagagtggag; reverse, cagtggtccagtcaacgagtaa; probe, #96), IL-4Rα (NM_001008700.3; forward, gagaggacaaccctgcagaa; reverse, caggatgttgatcgggaag; probe, #64), Mrc1 (NM_008625.2; forward, caacccaagggctcttctaa; reverse, ggcacctatcacaatcaggag; probe, #18), β-actin (NM_007393.3; forward, tcaacaccccagccatgta; reverse, gtggtacgaccagaggcatac; probe, #64).

Flow cytometry

Cells were washed in ice-cold flow cytometry buffer (00-4222-26, eBioscience), then incubated with Fc block (CD16/CD32 antibody) for 5 min. Each antibody was added, incubated for 30 min and washed twice with flow cytometry buffer. Data were acquired using MACSquant (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using FlowJo (Tree Star, Ashland, OH, USA). The gating strategy is described in Supplementary Figure 1, available at *International Immunology* Online.

Microarrays

RNA was isolated using the RNeasy Mini Kit (Qiagen). The transcriptional profile was evaluated using the Mouse Whole Genome Ver. 2.0 arrays (G4846A, Agilent Technologies, Santa Clara, CA, USA). Microarrays were scanned, and data extraction was conducted using Feature Extraction software Version 9.5.1 and then analyzed using Genespring GX software, Version 12.6 (Agilent Technologies). Following 75th percentile shift normalization, the probe list was filtered based on signal intensity value to exclude genes that were expressed at very low levels in all sample conditions (the lower cut-off was set to 20%). Principal component analysis was conducted to assign the general variability in the data to a reduced set of variables called principal components. Genes were defined

Table 1. List of the 10 most highly upregulated genes in IL-10-induced M2c macrophages

Gene name	LPS + IFNγ	IL-4	IL-1β	IL-10	TGFβ1
Saa3 (serum amyloid A 3)	8023.2	1.4	251.4	234.0	0.6
Lrg1 (leucine-rich α-2-glycoprotein 1)	0.3	0.4	0.8	31.6	0.6
Flrt3 (fibronectin leucine rich transmembrane protein 3)	10.4	1.5	3.5	25.0	0.5
Nrg1 (neuregulin 1)	3.8	1.1	1.3	19.0	1.8
Tug1 (taurine upregulated gene 1)	2.3	1.0	1.2	17.8	0.5
IL4ra (IL-4 receptor α chain)	2.7	0.3	1.0	14.2	0.4
Socs3 (suppressor of cytokine signaling 3)	43.3	0.2	4.7	13.5	0.3
Gm7676 (Gm7676 predicted gene 7676)	14.4	0.1	1.1	12.6	0.1
Itfitm1 (interferon induced transmembrane protein 1)	14.0	0.1	1.1	12.5	0.1
Clec1b (C-type lectin domain family 1, member b)	2.4	0.3	0.8	11.1	0.4

The numbers in the table show fold change in gene expression compared with non-stimulated BMDMs.

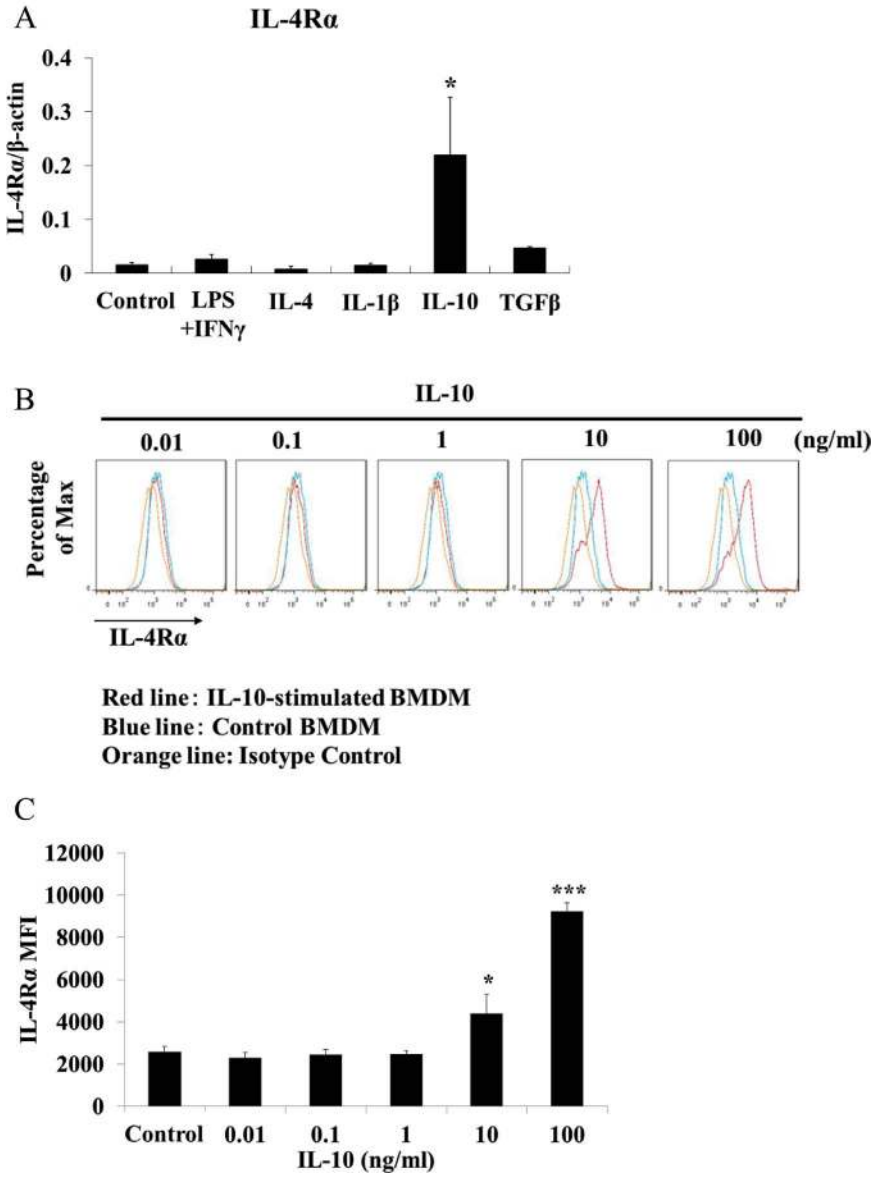

Fig. 1. IL-10 enhances IL-4Rα expression in BMDMs. BMDMs were stimulated with LPS (10 ng ml^{-1}) + IFNγ (50 ng ml^{-1}), IL-4 (10 ng ml^{-1}), IL-1β (10 ng ml^{-1}), IL-10 (10 ng ml^{-1}) or TGFβ (10 ng ml^{-1}) for 24 h. IL-4Rα gene expression in BMDMs was determined using real-time PCR (A). The IL-4Rα protein level in IL-10 (0.01–100 ng ml^{-1})-stimulated BMDMs was determined using flow cytometry (B). One of three representative experiments, in which similar results were obtained, is shown. The histogram shows IL-4Rα mean fluorescence intensity (MFI) in BMDMs (C). Data are presented as the mean ± SEM (n = 3–4). Statistically significant differences compared with control: $^{*}P \leq 0.05$ and $^{***}P \leq 0.001$.

as differentially regulated when the mean fold change regulation was at least 2.

When lists of upregulated genes were made, probe lists were sorted in the descending order by fold change of gene expression compared with non-stimulated BMDMs. If there was more than one probe for a gene, the probe having a higher value of fold change was used to make the lists.

In vitro eosinophil migration assay

BALB/c mice were sensitized by subcutaneous injection of 10 times diluted ragweed pollen extract (Torii Pharmaceutical, Tokyo, Japan) on days 0, 1, 6, 8 and 14. On day 20, 10 times diluted ragweed pollen extract was injected i.p. Peritoneal fluids were collected 48 h after the i.p. injection. Eosinophils were isolated by negative selection toward Thy1.2 and B220 MACS beads from peritoneal fluids. Eosinophil migration in response to CCL24 and macrophage culture supernatants was assayed using the ChemoTx System (106-5, Neuro Probe, Gaithersburg, MD, USA) according to the manufacturer's protocol with a slight modification. Briefly, CCL24 or macrophage culture supernatants with or without 10 μg ml^{-1} of isotype control IgG (14-4321-81, eBioscience) or anti-CCL24 antibody (MAB528, R&D Systems) (20) were added (29 μl per well) to the lower chamber of the ChemoTx System and served as chemoattractants. Eosinophils (1×10^6 cells ml^{-1}) were re-suspended in RPMI 1640 including 1% BSA, added (65 μl per well) to the upper chamber of the ChemoTx System (5-μm pore size filter) and incubated for 1 h at 37°C and 5% CO_2. Cells that migrated into the bottom chamber were measured using CelltiterGlo (Promega, Madison, WI, USA).

In vivo macrophage transfer

C57BL/6J mice were injected i.p. with 5×10^6 syngeneic macrophages cultured for 24 h with PBS (vehicle control), 10 ng ml^{-1} IL-4, 10 ng ml^{-1} IL-10 and IL-4 + IL-10, respectively, and labeled by 5-chloromethyl fluorescein diacetate (CMFDA; Life Technologies) at 1 μM for 0.5 h. Mice injected i.p. with saline were used as a no transfer control. After 12 h, peritoneal fluids were obtained and analyzed using flow cytometry. Peritoneal cells were analyzed according to the gating strategy (Supplementary Figure 1, available at *International Immunology* Online). CMFDA$^+$ (described as FITC$^+$) cells

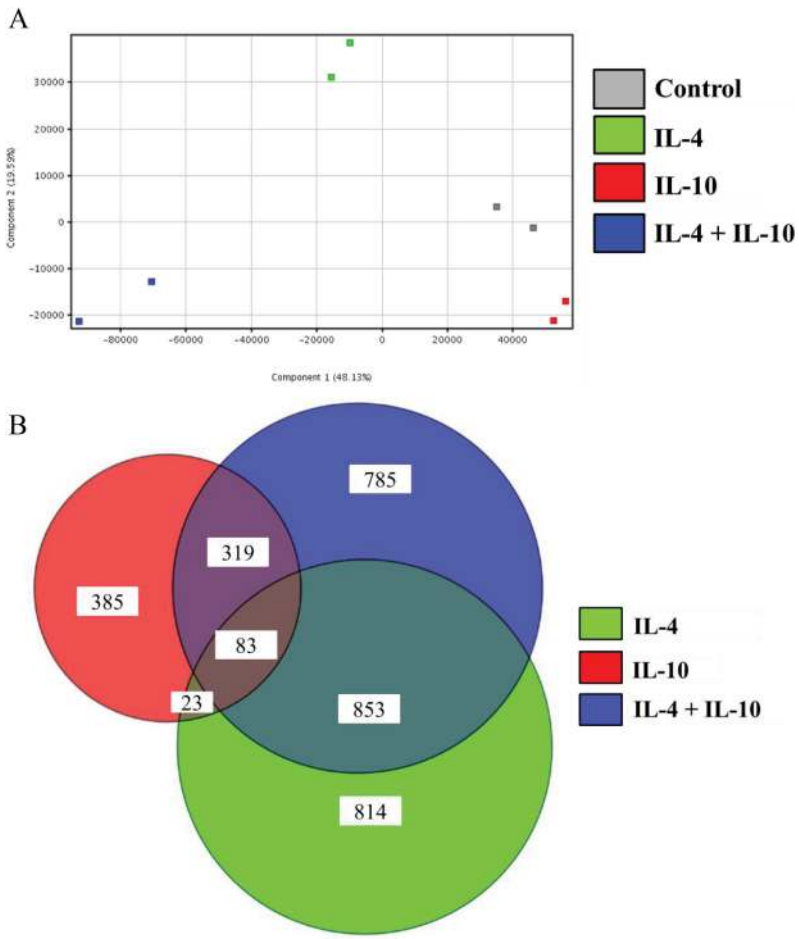

Fig. 2. Microarray analysis of macrophage subtypes showed the characteristic gene signature between the subtypes. BMDMs were stimulated with IL-4 (10 ng ml^{-1}), IL-10 (10 ng ml^{-1}) and IL-4 + IL-10. RNA was extracted from stimulated BMDMs and analyzed using microarrays. Principal component analysis was carried out on all genes under investigation to determine expression trends within the data set. The sample trend between the subtypes is shown in a scatter plot (A). The number of differentially expressed genes compared with control (more than 2-fold) in each portion of a Venn diagram is shown (B).

were removed from analysis to eliminate the transferred cells. Ratios of eosinophils (Siglec-F$^+$/CD115$^-$) and monocytes/macrophages (CD115$^+$) to total cells in peritoneal fluid were analyzed using FlowJo, and total eosinophils and monocytes/macrophages in peritoneal fluid were calculated by multiplying the ratio and the total cells in peritoneal fluid.

Statistical analysis

Data were analyzed using unpaired two-tailed Student's *t*-tests with significance between groups represented as $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.001$.

Results

IL-10 induces IL-4Rα expression in BMDMs

To clarify characteristics of M2 macrophage subtypes at the transcriptional level, M-CSF-induced BMDMs (hereafter, unless otherwise noted, BMDMs means M-CSF-induced BMDMs) were stimulated with LPS + IFNγ, IL-4, IL-1β, IL-10 or TGFβ for 24 h and analyzed using microarrays. As reported previously (14, 15), we also found an increase in IL-4Rα expression induced by IL-10. IL-4Rα was 14.2-fold higher in IL-10-stimulated BMDMs than in non-stimulated BMDMs. IL-4Rα was at most 2.7-fold higher in LPS + IFNγ-stimulated BMDMs and others (Table 1). On the other hand, IL-10Rα expression was not changed in all groups (data not shown).

We confirmed that IL-4Rα gene expression was higher in IL-10-stimulated BMDMs, compared with stimulation in other cytokines, using real-time PCR (Fig. 1A). Consistently, flow cytometric analysis showed that IL-10 increased IL-4Rα protein expression in a dose-dependent manner in BMDMs (Fig. 1B and C).

Enhanced induction of M2a-related genes in IL-4 + IL-10-stimulated BMDMs

Roles of IL-4 and IL-4Rα in M2a differentiation have been established (2) and we confirmed that IL-10 induced IL-4Rα expression as reported previously (14, 15). Therefore, we evaluated whether IL-10 would enhance M2a-related gene expression induced by IL-4. The transcriptional events associated with IL-4-, IL-10- and IL-4 + IL-10-stimulated macrophages were investigated using microarrays. Principal component analysis revealed that macrophage polarization was also associated with significant changes at the transcriptional level and that the expression profiles were different from each other (Fig. 2A). However, a significant number of differentially expressed genes in IL-4 + IL-10-stimulated macrophages compared with control were shared with those of IL-4-stimulated macrophages (Fig. 2B).

Next, we further analyzed each of M2a-related genes in the microarray data. As shown in Table 2, the 25 most highly upregulated genes in IL-4 + IL-10-stimulated BMDMs included 13 genes of the 25 most highly upregulated genes in IL-4-stimulated BMDMs, whereas they included only 4 genes of the 25 most highly upregulated genes in IL-10-stimulated BMDMs. Among them, M2a macrophage-related genes (Retnla, Arg1 and Chi3l3) were induced in IL-4-stimulated BMDMs as reported previously (9). IL-10 alone did not strongly induce the genes, but in the presence of IL-4, IL-10 further induced

Table 2. List of the 25 most highly upregulated genes in IL-4 + IL-10-stimulated BMDMs

Gene name	IL-10	IL-4	IL-4 + IL-10
Retnla (resistin-like α)	96.0[a]	10274.4[b]	34490.4
Ccl8 [chemokine (C-C motif) ligand 8]	16.4[a]	8.4	4679.6
Edn1 (endothelin 1)	1.1	148.1[b]	1526.4
Gm15056 (predicted gene 15056)	12.7[a]	0.9	1498.5
Arg1 (arginase 1)	2.5	409.3[b]	1388.5
Ccl24 [chemokine (C-C motif) ligand 24]	1.6	4.8	648.2
Scn3a (sodium channel, voltage-gated, type III, α subunit)	0.8	29.2[b]	643.4
Tuba8 (tubulin, α 8)	0.7	46.6[b]	508.2
Pdcd1lg2 (programmed cell death 1 ligand 2)	2.2	41.9[b]	495.9
Cdh1 (cadherin 1)	1.1	168.4[b]	490.9
Ccl22 [chemokine (C-C motif) ligand 22]	0.6	95.4[b]	382.2
Rbp4 (retinol-binding protein 4, plasma)	0.4	11.6	275.2
Cd209e (CD209e antigen)	2.2	1.5	250.3
Car2 (carbonic anhydrase 2)	0.5	20.2	239.0
Cish (cytokine-inducible SH2-containing protein)	1.5	64.9[b]	207.1
LOC620515 (similar to Chitinase 3-like protein 3 precursor)	1.4	65.4[b]	205.0
Ddx4 [DEAD (Asp-Glu-Ala-Asp) box polypeptide 4]	0.9	18.5	187.9
Ccl17 [chemokine (C-C motif) ligand 17]	0.6	326.9[b]	181.9
Chi3l3 (chitinase 3-like 3)	1.5	93.9[b]	173.8
Kif1a (kinesin family member 1A)	5.0	6.3	170.0
Slamf1 (signaling lymphocytic activation molecule family member 1)	0.8	5.1	159.1
Nrg1 (neuregulin 1)	107.7[a]	3.1	142.9
Msx3 (msh homeobox 3)	0.9	7.6	137.9
Rasgrp1 [RAS guanyl releasing protein 1 (calcium and diacylglycerol-regulated)]	2.0	15.0	121.1
Chi3l4 (chitinase 3-like 4)	0.8	30.9[b]	120.8

The numbers in the table show fold change in gene expression compared with non-stimulated BMDMs.
[a]The 25 most highly upregulated genes in IL-10-stimulated BMDMs.
[b]The 25 most highly upregulated genes in IL-4-stimulated BMDMs.

the M2a macrophage-related genes. Interestingly, CCL8, CCL22 and CCL24 were synergistically induced by IL-4 and IL-10. On the other hand, CCL17 expression was not increased in IL-4 + IL-10-stimulated BMDMs compared with IL-4-stimulated BMDMs.

Increases in expression of the well-known M2a markers Arg1, Retnla and Mrc1 were confirmed using real-time PCR (Fig. 3A–C). CD206 (Mrc1) protein expression was also higher in IL-4 + IL-10-stimulated BMDMs (Fig. 3D and E). Taken together, these data suggested that IL-4 and IL-10 synergistically enhance expression of Arg1, Retnla and Mrc1.

Enhanced induction of CCL24 and Arg1 in IL-4 + IL-10-stimulated BMDMs and macrophages from different sources

CCL24 was highly expressed in M-CSF-induced BMDMs stimulated by IL-4 and IL-10 compared with either IL-4 or IL-10 alone from the microarray (Table 2) and PCR (Fig. 4A) results. Therefore, we measured CCL24 protein in the culture medium. Figure 4B indicates that CCL24 was secreted into the culture medium from IL-4 + IL-10-stimulated BMDMs in a synergistic manner.

We examined whether this synergism was common among BMDMs induced by M-CSF and GM-CSF. In GM-CSF-induced BMDMs, CCL24 gene expression was induced slightly by IL-4 alone and not by IL-10 alone, but more strongly by IL-4 and IL-10 in combination than in M-CSF-induced BMDMs (Fig. 4A). We next sought to determine whether the combination of IL-4 and IL-10 stimulation had similar effects on macrophages from different sources. The combination of IL-4 and IL-10 also induced higher CCL24 gene expression in thioglycolate-elicited, zymosan-elicited and naive peritoneal macrophages than either IL-4 or IL-10 alone (Fig. 4C and D).

Arg1, a typical M2a signature gene, was then measured because we hypothesized that IL-10 might enhance not only CCL24 but also the M2a-related gene in macrophages

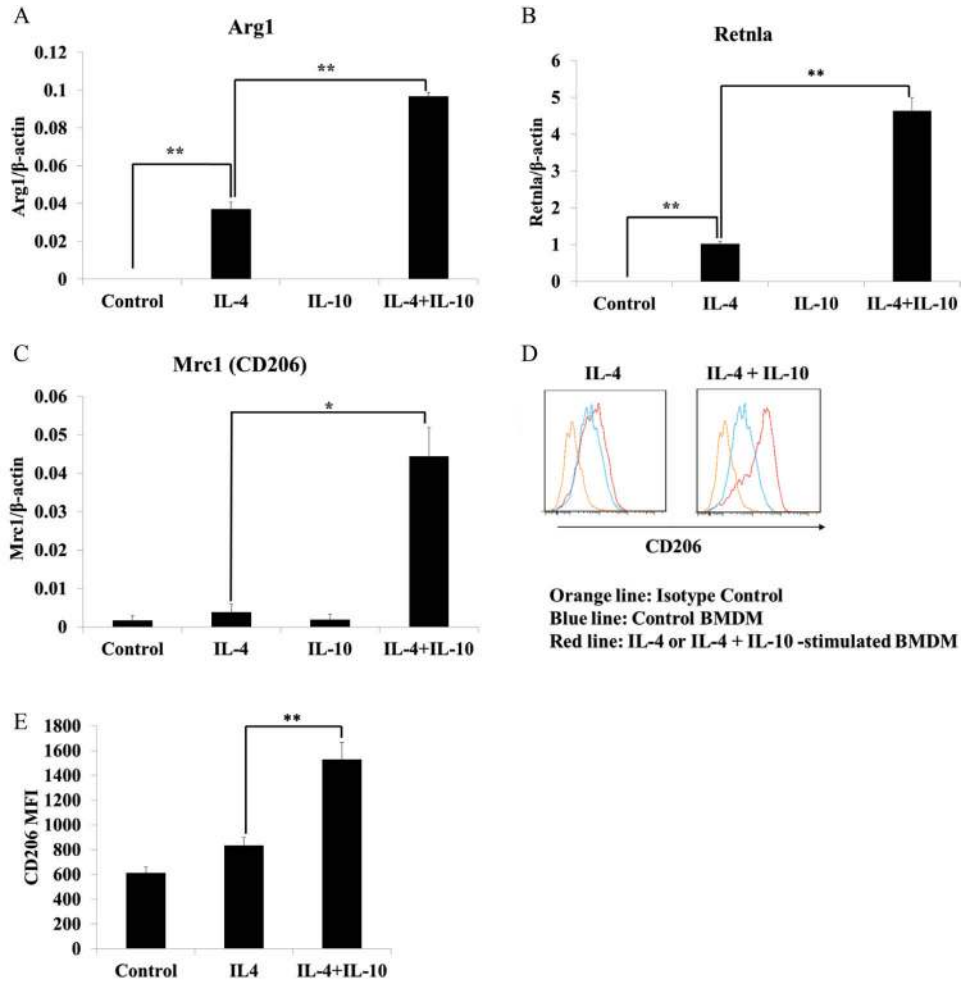

Fig. 3. IL-10 enhanced M2a macrophage marker expression in IL-4-stimulated BMDMs. BMDMs were stimulated with IL-4 (10 ng ml^{-1}), IL-10 (10 ng ml^{-1}) and IL-4 + IL-10 for 24 h. Gene expression of the M2a macrophage markers—Arg1 (A), Retnla (B) and Mrc1 (C)—was determined using real-time PCR in IL-4-, IL-10- and IL-4 + IL-10-stimulated macrophages. The CD206 (Mrc1) protein level was determined by flow cytometry in IL-4- and IL-4 + IL-10-stimulated BMDMs (D). One of three representative experiments, in which similar results were obtained, is shown. The histogram shows the mean fluorescence intensity (MFI) of CD206 (E). Data are presented as the mean ± SEM (n = 3–4). Statistical analyses were conducted between control and IL-4-stimulated macrophages and between IL-4- and IL-4 + IL-10-stimulated macrophages. Statistically significant differences: *$P \leq 0.05$ and **$P \leq 0.01$.

other than M-CSF-induced BMDMs. IL-4 and IL-10 in combination induced higher Arg1 gene expression in GM-CSF-induced BMDMs, thioglycolate- and zymosan-elicited macrophages than either IL-4 or IL-10 alone (Fig. 4E and F).

Taken together, our data suggest that IL-10 enhances IL-4-induced gene expression of CCL24 and the M2a-related gene, Arg1, in BMDMs and also in macrophages from different sources.

IL-4 + IL-10-stimulated BMDMs increased eosinophil migration in vitro

CCL24 is known as a chemoattractant for eosinophils through its receptor, CCR3 (23, 24). CCL24 increased eosinophil migration dose dependently and the EC_{50} was ~10 ng ml^{-1} (Fig. 5A). BMDMs produced ~10 ng ml^{-1} CCL24 in the presence of 10–100 ng ml^{-1} IL-4 and IL-10 (Fig. 4B). Therefore, we tested whether CCL24 in culture supernatant could increase eosinophil migration.

IL-4 + IL-10-stimulated BMDMs culture supernatant increased eosinophil migration while that of either IL-4- or IL-10-stimulated BMDMs did not (Fig. 5B). Next, we examined the contribution of CCL24 in this eosinophil migration. Anti-CCL24 antibody at 10 μg ml^{-1} inhibited eosinophil migration induced by culture supernatant of IL-4 + IL-10-stimulated BMDMs (Fig. 5C). On the other hand, IL-4, IL-10 or IL-4 + IL-10 itself did not affect eosinophil migration (Fig. 5D). These results suggested that IL-4 + IL-10 increased eosinophil migration activity in BMDMs through induction of CCL24.

Eosinophil infiltration induced by transfer of IL-4 + IL-10-stimulated BMDMs in vivo

Because IL-4 + IL-10-stimulated BMDMs produced a large amount of CCL24 and had the ability to increase eosinophil migration *in vitro*, we investigated their potential to increase eosinophil infiltration *in vivo*. To test this possibility, BMDMs, which were stimulated with IL-4, IL-10 or IL-4 + IL-10 and labeled with CMFDA, were transferred into the peritoneal

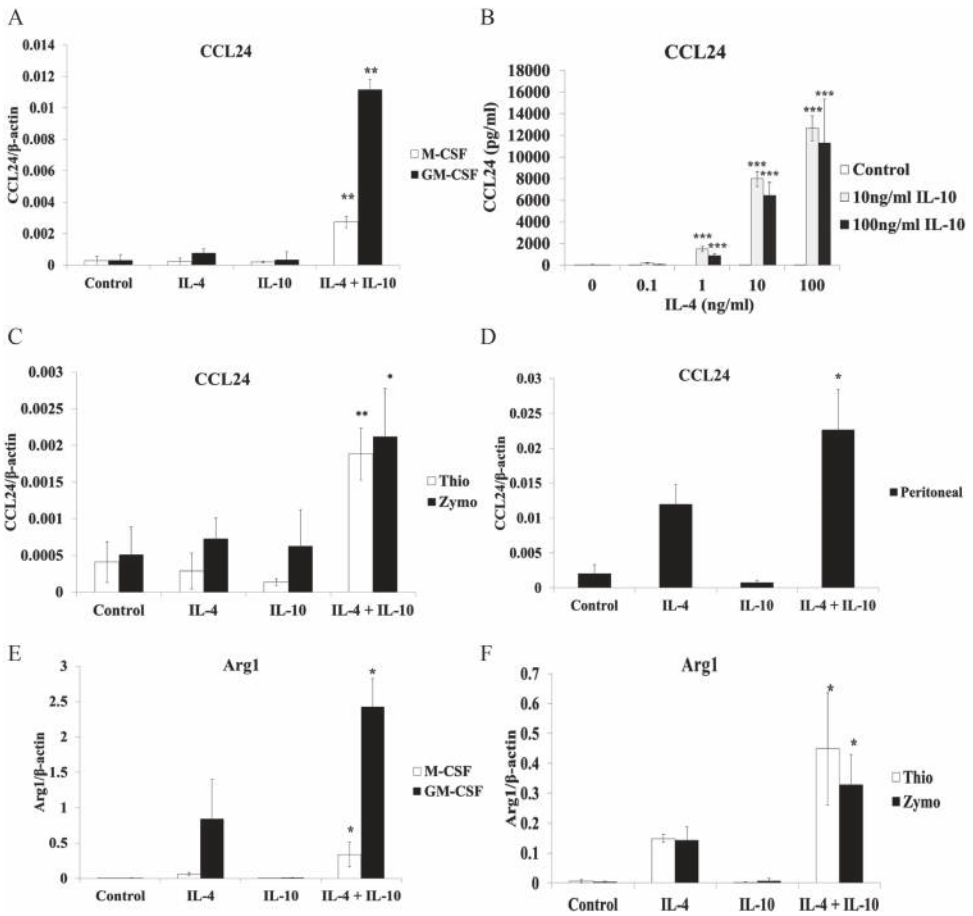

Fig. 4. IL-10 enhanced CCL24 and Arg1 expression in IL-4-stimulated BMDMs and macrophages from different sources. BMDMs and macrophages from different sources were stimulated with IL-4 (10 ng ml^{-1}), IL-10 (10 ng ml^{-1}) and IL-4 + IL-10 to measure gene expression for 24 h and CCL24 production for 72 h. Gene expression of the M2a macrophage markers CCL24 (A, C, D) and Arg1 (E, F) in M-CSF- and GM-CSF-stimulated BMDMs (A, E); thioglycolate- and zymosan-elicited peritoneal macrophages (shown as Thio and Zymo) (C, F) and naive peritoneal macrophages (D) was determined using real-time PCR. CCL24 in culture supernatant from M-CSF-induced BMDMs was determined using ELISA (B). Data are presented as the mean ± SEM (n = 3–4). Statistical analyses were conducted between IL-4- and IL-4 + IL-10-stimulated macrophages. Statistically significant differences: *$P \leq 0.05$, **$P \leq 0.01$ and ***$P \leq 0.001$.

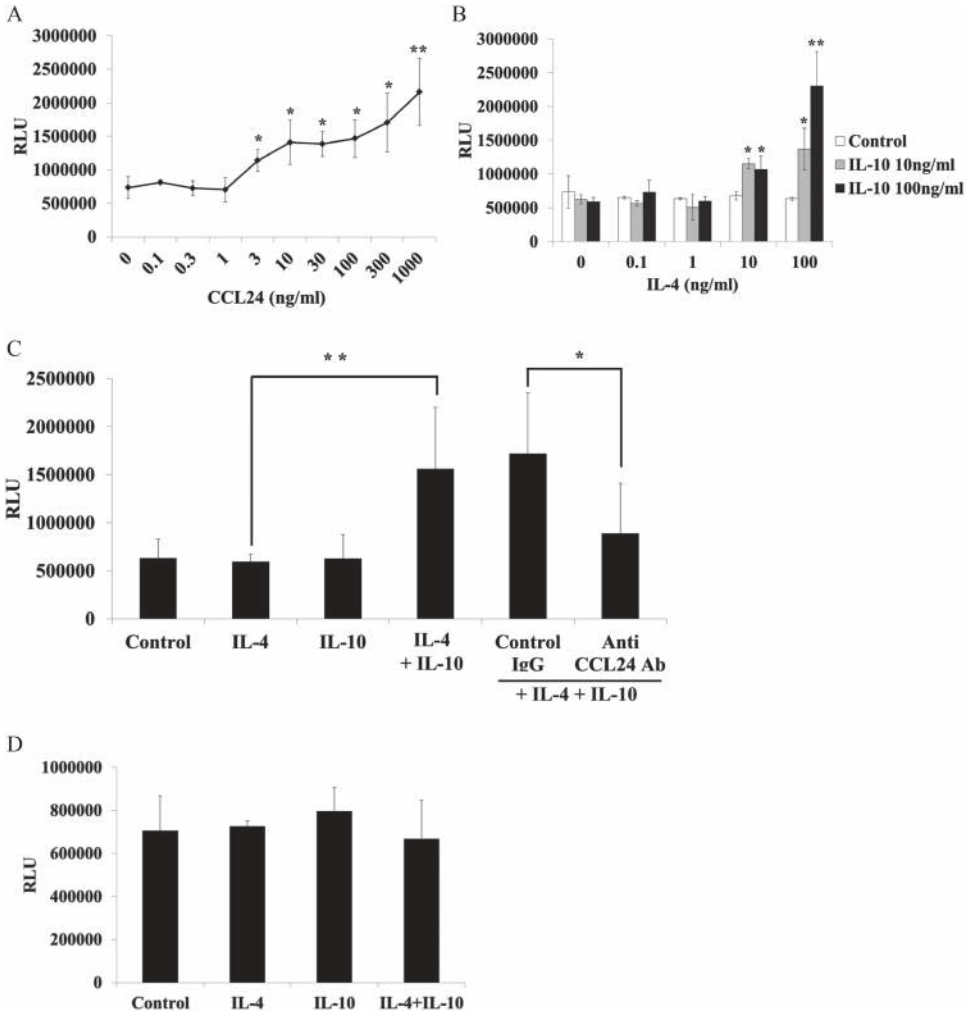

Fig. 5. IL-4 + IL-10-stimulated macrophages increased eosinophil migration *in vitro*. Eosinophils were isolated through negative selection of Thy1.2 and B220 MACS from peritoneal fluids in a mouse model of ragweed-induced peritonitis. Eosinophils were added into the upper side of a chemotaxis chamber. CCL24 (A), culture supernatant of BMDMs stimulated for 72 h with IL-4, IL-10 or IL-4 + IL-10 (B), culture supernatant of BMDMs stimulated for 72 h with 10 ng ml^{-1} of IL-4, IL-10 or IL-4 + IL-10 with or without isotype control or anti-CCL24 antibody (C) or only cytokine (10 ng ml^{-1} of IL-4, IL-10 or IL-4 + IL-10) (D) was added to the lower side of the chamber. After 1 h incubation, migrated eosinophils were quantified using the Celltiter Glo Luminescent Cell Viability Assay. The *y*-Axis shows relative luminescence unit (RLU), which reflects the number of live cells and is used as an index of the number of eosinophils. Data are presented as the mean ± SEM (n = 3–4). Statistically significant differences compared with control: $^{*}P \leq 0.05$ and $^{**}P \leq 0.01$.

cavity of C57BL/6J mice. Siglec-F was used as a marker for eosinophils in peritoneal cells because it is not expressed in peritoneal macrophages (25, 26). CD115 was used as a marker for monocytes/macrophages (27, 28), and it was also used to eliminate potential contamination of monocytes/macrophages in the eosinophil fraction. Eosinophil and monocyte/macrophage infiltration into the peritoneal cavity was analyzed 24 h after transfer (Fig. 6A). Only mice that received the transferred IL-4 + IL-10-stimulated BMDMs showed an increase in the Siglec-F^{+}/$CD115^{-}$ eosinophil population in peritoneal fluids (Fig. 6B and C), whereas there was no difference in the $CD115^{+}$ monocyte/macrophage population (Fig. 6D and E). Transfer of *in vitro* stimulated macrophages did not show a significant effect on total cells (Fig. 6F). These results suggested that IL-4 + IL-10-stimulated BMDMs, but neither IL-4- nor IL-10-stimulated BMDMs, increased eosinophil infiltration *in vivo*.

Discussion

The present study demonstrated that the combination of IL-4 and IL-10 enhanced the expression of the M2a-related genes compared with either IL-4 or IL-10 alone *in vitro* and conferred the ability to increase eosinophil infiltration *in vivo*. In previous studies, IL-4- and IL-10-stimulated M2 macrophages, respectively, have been widely studied (9, 12, 13). They showed that IL-4-stimulated M2a macrophages highly expressed Arg1 and Retnla, which are related to parasitic infections (16) and produced CCL17 and CCL22 (7, 10), which are known as Th2 chemokines. And it was reported that IL-10 stimulation leads to an increase in IL-4Rα expression through STAT3, resulting in enhanced Arg1 expression (14, 15). However, these studies did not show the effects of IL-4 and IL-10 in combination on expression of other M2a-related genes in the context of M2 macrophage differentiation. Therefore, the effects

of combination of IL-4 and IL-10 on the phenotype of macrophages were unclear.

IL-10-stimulated M2c macrophages were shown to express IL-4Rα using microarray in another study (13). Similarly, our microarray analysis showed that IL-10 enhanced the expression of IL-4Rα in BMDMs. Our results further demonstrated that IL-4Rα expression was enhanced at the mRNA and protein level using quantitative PCR and FACS, respectively. Although the expression profiles of IL-4 + IL-10-stimulated macrophages were different from those of IL-4- or IL-10-stimulated macrophages, there is a tendency for IL-10 to enhance the expression of M2a-related genes including Retnla, Arg1, Chi3l3 and Mrc1. Interestingly, CCL24 and CCL8 were synergistically induced in IL-4 + IL-10-stimulated macrophages. The enhancement of CCL24 and Arg1 was also shown not only in BMDMs but also in macrophages from different sources. These results strongly suggested the functional significance of the phenomenon.

We sought to examine whether CCL24 produced by IL-4- or IL-10-stimulated macrophages induced eosinophil migration *in vitro* because CCL24 is known as a chemoattractant for eosinophils through its receptor, CCR3 (23, 24). We focused on CCL24 among various eosinophil

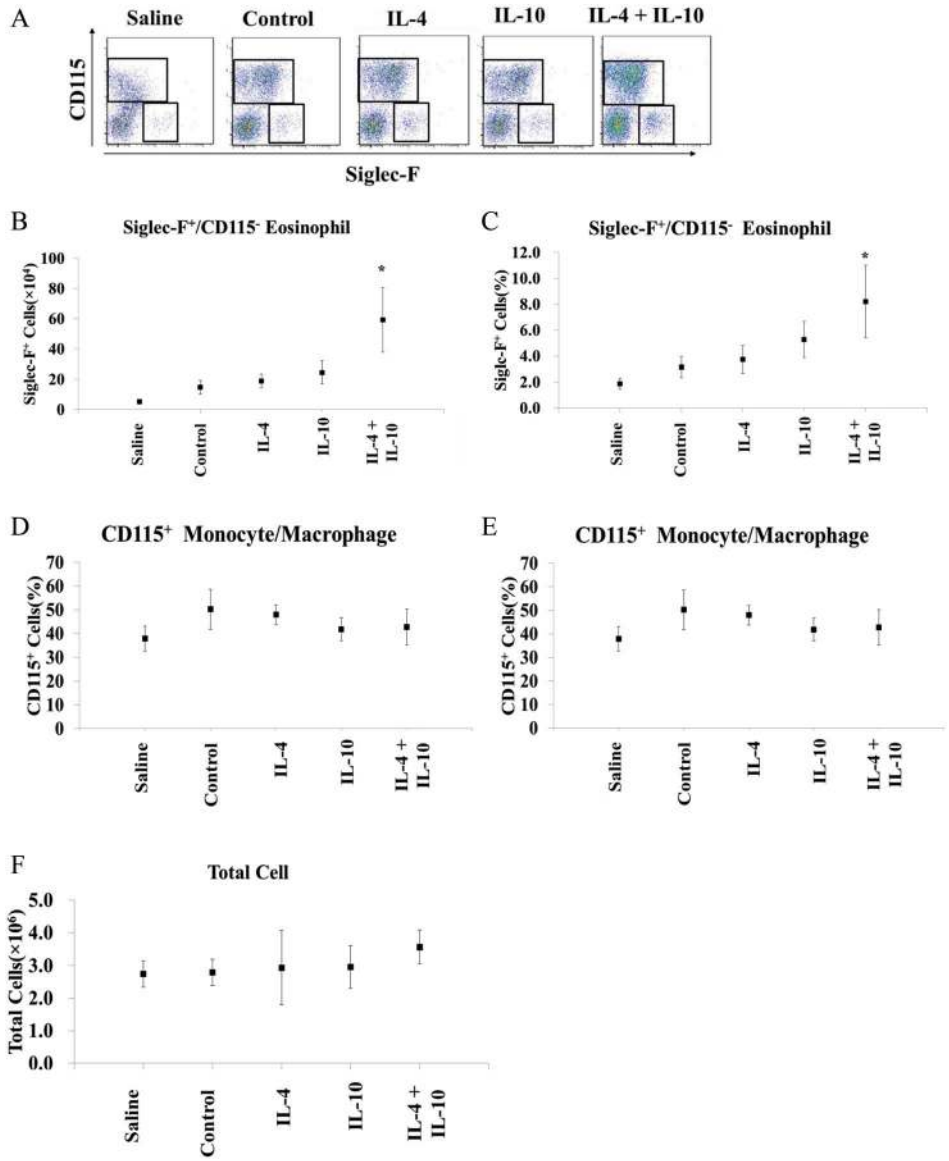

Fig. 6. Transfer of IL-4 + IL-10-stimulated BMDMs into the peritoneum increased eosinophil infiltration *in vivo*. BMDMs were unstimulated (control) or stimulated with IL-4 (10 ng ml^{-1}), IL-10 (10 ng ml^{-1}) and IL-4 + IL-10 for 24 h. These BMDMs (5×10^6 cells per head) were labeled with CMFDA and transferred into the peritoneum of C57BL/6J mice. Saline (no transfer) was injected to obtain a naive peritoneal cell population. After 24 h, peritoneal fluids were collected and analyzed using FACS (A). The percentage of Siglec-F$^+$/CD115$^-$ cells (C) and CD115$^+$ cells (E) (cells in the boxes shown in the dot plots in A) were measured. The number of Siglec-F$^+$/CD115$^-$ cells (B) and CD115$^+$ cells (D) was calculated by multiplying the number of total cells (F) and their ratio. Data are presented as the mean ± SEM (n = 4–6). Statistically significant differences compared with control: $^*P \leq 0.05$.

migration factors, such as chemokines (CCL11, CCL24 and CCL26) and lipid mediators (PGD_2, PAF, LTB_4, LTC_4, LTD_4 and LTE_4) (29, 30) because our microarray experiment showed synergistic induction of CCL24. CCL24 was an important factor in eosinophil migration induced by IL-4 + IL-10-stimulated macrophages from the result of neutralization of CCL24 (Fig. 5C). Although we demonstrated the importance of CCL24, it needs further study to clarify the contribution of other factors to the eosinophil migration.

In vivo, M2 macrophages in an ovalbumin-induced allergic airway disease model highly express CCL24 and CCL8 (24). Moreover, CCL24 is involved in eosinophil recruitment in allergic reactions (31, 32). CCL8 is known as a CCR8 ligand, which is related to Th2 infiltration into skin in skin inflammation (33). Therefore, M2 macrophages are expected to induce eosinophil and Th2 infiltration at local sites. However, these studies did not show *in vitro* eosinophil migration activity by M2 macrophage and *in vivo* eosinophil infiltration into local sites by M2 macrophage transfer. Our results showed that IL-4 + IL-10-induced (but not IL-4 or IL-10 alone) M2 macrophages indeed increased *in vivo* eosinophil infiltration, suggesting that the combination of IL-4 and IL-10 actually plays a role in allergic reactions, or at least eosinophil infiltration.

IL-10 was recently shown to be an anti-inflammatory cytokine (34), but it was first shown to be a Th2 cytokine (35). Although some studies showed that IL-10 suppressed allergic disease (36, 37), other studies showed that IL-10 led to allergic disease and parasitic infection (38, 39). These contradictions are thought to be caused by IL-10 function which suppresses T-cell activation through dendritic cells (40) and increases eosinophil infiltration through macrophages with IL-4 as shown in this study.

In regard to cytokine combinations, a combination of TSLP and IL-13 is important for induction of M2 macrophages, CCL24 production and eosinophil infiltration *in vivo* (19). However, it is not known whether it has direct or indirect effects on macrophages because the combination of TSLP and IL-13 treatment for macrophages *in vitro* has not been examined to see if it can directly induce CCL24 production and confer the ability to increase eosinophil infiltration. A combination of IL-4/IL-13 and IL-33 also induced M2 macrophages and CCL24 production (20). IL-4 + IL-10-stimulated BMDMs induced CD206 (a well-known M2 macrophage marker *in vivo*) (16), but IL-4/IL-13 + IL-33-stimulated macrophages did not induce CD206 expression compared with IL-4 alone (data not shown). These data suggested that IL-4 + IL-10-stimulated M2 macrophages were more similar to *in vivo* M2 macrophages than IL-4/IL-13 +IL-33-stimulated M2 macrophages.

In summary, these results define a previously unrecognized role for IL-10, acting with IL-4 as an important enhancer for differentiation of M2 macrophages, chemokine production and eosinophil infiltration.

Supplementary data

Supplementary data are available at *International Immunology* Online.

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