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H．知的財産権の出願・登録状況
（予定を含む。）
なし。

次世代シーケンシングによる高 IgE 症候群の原因遺伝子の発見

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研究要旨

本研究では、先天性免疫不全症候群の中でも、高 IgE 症候群に特に焦点を当て、その疾患群の予後予測判定因子の同定を行い、本疾患の病態を解析する。それによって、単一遺伝子の異常によって生じる多様な病態の発症機序を探り、骨粗鬆症、アトピー性皮膚炎、高 IgE 血症などの新規の診断法、治療法とケア、予後予測に結びつけることを目指す。特にこの今年度の分担研究では、高 IgE 血症の臨床検体の全エクソン解析に集中し、全エクソン解析結果から各症例の候補変異の蓄積を進めた。

A. 研究目的

本研究では、先天性免疫不全症候群の中でも、高 IgE 症候群に焦点を当て、その疾患群の予後予測判定因子の同定を行うことが一つの目的であり、もう一つの目的は、本疾患の病態を解析することによって、単一遺伝子の異常によって骨粗鬆症、アトピー性皮膚炎、高 IgE 血症、肺嚢胞などの多様な病態の発症機序を探ることにある。最終的に、これらの解析によって、骨粗鬆症、アトピー性皮膚炎、高 IgE 血症の新規の診断法、治療法とケア、予後予測に結びつけることを目指す。特に今年度のこの分担研究では、高 IgE 血症の臨床検体の症例特異的に見られる遺伝的変異情報を蓄積し、新規な遺伝的素因を明らかにすることを最終目的とする。

B. 研究方法

難治疾患克服研究事業「原発性免疫不全症に関する研究」班と連携し、本邦の高IgE血症症例の既知遺伝子における変異の有無を検討し、原因の未知の高IgE症候群症例を蓄積する。

全エクソンシーケンシングを研究代表者から提供された検体について実施し、その情報解析パイプラインを稼働させ、遺伝子変異情報を構築したローカルに稼働する情報解析ツールを介して共有する。公的に利用できる１塩基多様性情報（特に日本人健常者の１塩基多様性情報）と研究分担者が蓄積した日本人１塩基多様性情報の両者を統合し、それらと得られた高IgE血症疾患症例に見られた変異情報を比較することで、発症原因となる候補変異を絞り込む。

（倫理面への配慮）

本研究のために、理化学研究所において既に承認を受けていた先天性免疫不全症原因探索のための倫理申請に、１）徳島大学の施設追加、２）次世代シーケンサーによる網羅的疾患原因探索の実施、の２点の追加の修正申請を行い、平成２４年１２月２６日付で承認を得た。

C. 研究結果

原発性免疫不全症研究班と連携して、４０件を超える高 IgE 血症症例について、Tyk2 もしくは Stat3 遺伝子の全コード領域配列解析を行った。しかし、これらの解析により変異が見られた症例はわずかに 20%以下であり、多数の症例については遺伝的素因

の特定ができなかった。

この既知遺伝子変異の検索と並行して、本研究を実施するため理化学研究所統合生命医科学研究センターに導入したイルミナ社 HiSeq1500 を用いて、研究代表者の峯岸博士から提供を受けた症例について、全エクソーム解析を実施した。第一グループとして、１４検体の既知遺伝子に変異が見られない症例群と１８検体と既知遺伝子に変異があることが確認済みであるが症状の重篤性に違いのある症例群を解析した。後者は、疾患発症の原因遺伝子だけでなく、他の病態修飾因子の候補を拾い上げるのが目的である。今回の検体は家族例を含まないため、公的なデータベース及び本研究グループ内に蓄積されている１塩基多型情報との比較によってのみフィルタリングを行った。これらのデータを、Mac mini 上に構築したデータベースに格納し、今後の検体間の変異情報の比較検討をローカルに実施できる環境を構築した。更に、峯岸博士から３４検体の遺伝的原因未同定の高 IgE 血症症例の DNA 検体の提供を受け、解析を継続中である。すべてのデータが揃った段階で、それぞれの症例に見られた症例特異的な変異群を相互に比較検討する。

D. 考察

１）高 IgE 血症の既知原因遺伝子の変異探索は第一に行われるべき解析ではあるが、それにより変異同定に至れる症例数はあまり多くなく、除外診断としての意味に留まるケースが多い。

２）全エクソン解析により、タンパク質コード領域にある遺伝的素因については網羅的に探索が可能であり、実際、現在利用できる健常者の１塩基多型情報によって、１症例当たり数 100 以下の変異候補まで絞り込むことが可能であった。

３）本邦のように集められる希少疾患の症例数に限りがある状況下では、候補変異を更に絞り込むためには時間をかけての継続的なデータ蓄積を進めることが重要であり、本研究成果はそのための重要な情報となる。

４）残念ながら本年度は解析できなかったが、より動的な血球細胞の遺伝子発現動態の計測を併用することで、今後原因遺伝子変異の更なる絞り込みと疾患発症機構解明の端緒が得られると考えられる。

E. 結論

１）高 IgE 血症の既知原因遺伝子である Tyk2 と Stat3 について、コード領域の配列解析を進めた。

２）研究代表の峯岸博士から提供された高 IgE 血症症例の検体（現時点、66 検体）について全エクソン解析を実施し、公的データベースなどからの情報を活用して、それぞれの検体における原因候補変異のリストを作成した。

３）得られた大量の塩基配列データを比較閲覧するためのツールを構築し、今回得られたデータを搭載したローカルで稼働するシステムを実現した。

F. 健康危険情報　　なし

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H. 知的所有権の出願・取得状況（予定も含む）なし

高 IgE 症候群に関与する遺伝要因の探索

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研究要旨

高IgE症候群はアトピー性皮膚炎、高IgE血症などの病態を呈する先天性免疫不全症である。近年、その原因としてSTAT3、TYK2の遺伝子変異が同定されたが、その臨床経過は様々であり、さらなる遺伝要因の解明が待たれている。またしばしば高IgE症候群は重症アトピー性皮膚炎との鑑別は困難であり、両者に共通の遺伝要因が存在する可能性がある。本研究は高IgE血症の遺伝要因を詳細に明らかにするとともに、アトピー性皮膚炎関連遺伝子群を同定することを目的とする。本年度は日本人のゲノムワイド関連解析（ＧＷＡＳ）により同定された8つのゲノムワイド水準（$P<5x10^{-8}$）をみたす疾患関連領域について、重症アトピー性皮膚炎症例(血清IgE値>10000IU/ml) 149例について関連解析を行った。その結果、2ヶ所の領域、GLB1-CCR4領域(rs6780220、$P=1.6x10^{-4}$)、IL1RL1-IL18R1-IL18RAP領域(rs13015714、$P=2.1x10^{-4}$)で強い関連を認めた。

Ａ．研究目的

先天性疾患の遺伝要因の解明は、しばしばCommon diseaseの遺伝要因の解明につながる可能性がある。これまで先天性免疫不全症である高IgE症候群の原因としてSTAT3およびTYK2の遺伝子変異が同定されている。一方、大規模なGWASによりSTAT3の遺伝子多型が炎症性腸疾患、クローン病、多発性硬化症に、そしてTYK2の多型が炎症性腸疾患、I型糖尿病そして乾癬に関連することが示されている。本研究は高IgE血症の遺伝要因を詳細に明らかにするとともに、アトピー性皮膚炎の病態に関連する遺伝子群を同定することを目的とする。

Ｂ．研究方法

昨年度より重症アトピー性皮膚炎症例で、関連解析を行っているが、本年は症例数を増やし、これまで日本人のゲノムワイド関連解析(GWAS)により8つのゲノムワイド水準($P<5x10^{-8}$)をみたす疾患関連領域(IL1RL1-IL18R1-IL18RAP、MHC 、 OR10A3-NLRP10 、 GLB1-CCR4 、CCDC80、CARD11、ZNF365-EGR2、CYP24A1-PFDN4)のSNPsについて、アトピー性皮膚炎で高IgE(＞10000IU/ml)血症を伴う症例(149例)とコントロール(1474例)で関連解析を行なった。タイピングはTaqMan法およびInvader法を用いた。

(倫理面への配慮)

本研究は三省合同「ヒトゲノム・遺伝子解析研究に関する倫理指針」に準拠して行い、当該実施機関の倫理委員会の承認を受けたうえで研究を行っている。

Ｃ．研究結果

その結果、GLB1-CCR4領域のSNP(rs6780220)で$P=1.6x10^{-4}$、IL1RL1-IL18R1-IL18RAP領域のSNP(rs13015714)で$P=2.1x10^{-4}$と多重比較の有意水準(P=0.0063)を満たす強い関連を認めた。

Ｄ．考察

アトピー性皮膚炎患者のうち重症例は社会生

活に支障をきたすこともあり、そのメカニズムの解明が待たれている。IgE値は重症例で高く、症状の改善とともにIgE値が低下することも報告されている。アトピー性皮膚炎で高IgE(＞10000IU/ml)血症を伴う症例に特徴的な遺伝要因の解明は重症化のメカニズムの解明につながる可能性がある。また、乳幼児期から重症アトピー性皮膚炎として治療を受けていた患者が、高IgE症候群と診断される例も多い。今回強い関連を示したIL1RL1-IL18R1-IL18RAP領域にはIL33受容体(IL1RL1, ST2), IL18受容体(IL18R1,IL18RAP)遺伝子が含まれている。IL-33とアトピー性皮膚炎との関連において、IL-33を皮膚で過剰発現させたマウスにおいて、アトピー性皮膚炎様の皮膚の炎症が生じることが報告されている。STAT3はIL-33のシグナル伝達経路において重要な役割を果たしている可能性が示唆されており興味深い。CCR4はアトピー性皮膚炎の病勢と相関するTARCの受容体である。今後、先天性免疫不全症が疑われる高IgE症候群の症例についてはエクソーム解析を行い、新規の遺伝子変異の探索を行っていく。

E．結論

日本人の高IgE血症（＞10000IU/ml)を伴うアトピー性皮膚炎とGLB1-CCR4領域(rs6780220、$P=1.6x10^{-4}$)およびIL1RL1-IL18R1-IL18RAP領域(rs13015714、$P=2.1x10^{-4}$)の間に強い関連をみとめた。

F．健康危険情報

なし

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H. 知的財産権の出願・登録状況 伺います
(予定を含む。)

1. 特許取得

なし

2. 実用新案登録

なし

3.その他

なし

Ⅳ　研究成果の刊行に関する一覧表

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Ⅴ　代表的な研究成果の刊行物

Immunity
Article

Inflammatory Monocytes Recruited to Allergic Skin Acquire an Anti-inflammatory M2 Phenotype via Basophil-Derived Interleukin-4

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http://dx.doi.org/10.1016/j.immuni.2012.11.014

SUMMARY

Monocytes and macrophages are important effectors and regulators of inflammation, and both can be divided into distinct subsets based on their phenotypes. The developmental and functional relationship between individual subsets of monocytes and those of macrophages has not been fully elucidated, although Ly6C$^+$CCR2$^+$ inflammatory and Ly6C$^-$CCR2$^-$ resident monocytes are generally thought to differentiate into M1 (classically activated) and M2 (alternatively activated) macrophages, respectively. Here we show that inflammatory monocytes recruited to allergic skin acquired an M2-like phenotype in response to basophil-derived interleukin-4 (IL-4) and exerted an anti-inflammatory function. CCR2-deficient mice unexpectedly displayed an exacerbation rather than alleviation of allergic inflammation, in spite of impaired recruitment of inflammatory monocytes to skin lesions. Adoptive transfer of inflammatory monocytes from wild-type but not IL-4 receptor-deficient mice dampened the exacerbated inflammation in CCR2-deficient mice. Thus, inflammatory monocytes can be converted from being proinflammatory to anti-inflammatory under the influence of basophils in allergic reactions.

INTRODUCTION

Monocytes are circulating leukocytes that can differentiate into macrophages and dendritic cells after their migration to peripheral tissues (Auffray et al., 2009; Domínguez and Ardavín, 2010; Geissmann et al., 2010; Shi and Pamer, 2011). Monocytes, macrophages, and dendritic cells are essential components of the innate immune system and participate in clearance of dead cells and pathogens, tissue healing, and initiation and regulation of the adaptive immunity. They can also contribute to the pathogenesis of inflammatory disorders. Accumulating evidence indicates that those cell types can be further divided into phenotypically distinct subsets, and each subset might have particular function in the steady state and inflammation (Auffray et al., 2009; Geissmann et al., 2010; Gordon and Taylor, 2005; Mosser and Edwards, 2008; Shi and Pamer, 2011).

Circulating monocytes commonly express CD115 (CSF1 receptor) on their surface and are divided into subsets on the basis of the expression of particular surface molecules including chemokine receptors (Auffray et al., 2009; Gordon and Taylor, 2005). In humans, differential expression of CD14 and CD16 allowed monocytes to be divided into two subsets: CD14$^+$ CD16$^-$ and CD14$^+$CD16$^+$ monocytes (Passlick et al., 1989). The former cells represent 80%–90% of blood monocytes, express high amounts of the chemokine receptor CCR2 and low amounts of CX3CR1, and are often called classical monocytes. By contrast, the latter (nonclassical) cells express high amounts of CX3CR1 and low amounts of CCR2 and can be further divided into at least two populations based on the expression of CD14 and CD64. Also in mice, two subsets of monocytes have been described (Auffray et al., 2009; Geissmann et al., 2003). The main subset of murine monocytes expresses Ly6C, CCR2, and low amounts of CX3CR1, suggesting that they are phenotypically equivalent to human CD14$^+$CD16$^-$ monocytes. Ly6C$^+$CCR2$^+$ monocytes are readily recruited to affected tissues where they produce inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and IL-1 during infection and inflammation, and they were therefore termed "inflammatory" monocytes. The second subset of murine monocytes is characterized by high expression of CX3CR1 and the lack of Ly6C and CCR2 expression and were termed "resident" monocytes because they have a longer half-life and are found in both resting and inflamed tissues. They adhere to and migrate along the luminal surface of endothelial cells that line small blood vessels and therefore appear to patrol the endothelium in the steady state (Auffray et al., 2007).

Macrophages are also heterogeneous in their phenotype and function, depending on the signals they receive (Biswas and

Mantovani, 2010; Gordon and Taylor, 2005; Mosser and Edwards, 2008; Murray and Wynn, 2011). Classically activated M1-type macrophages are generated by stimulation with bacterial moieties such as lipopolysaccharide (LPS) and the Th1 cell cytokine interferon-γ (IFN-γ), whereas alternatively activated M2-type macrophages are typically elicited by stimulation with the Th2 cell cytokines such as IL-4 and IL-13. M1 macrophages produce proinflammatory cytokines including IL-1 and destroy intracellular pathogens such as *M. tuberculosis* by means of an increased oxidative burst and NO production. Although the in vivo roles of M2 macrophages have been less well characterized, several functions are ascribed to them, including those in protection from parasitic infections, promoting Th2 cell-type immune responses, damping excessive inflammation, tumor progression, angiogenesis, wound healing, tissue remodeling, and fibrosis (Kreider et al., 2007; Martinez et al., 2009; Murray and Wynn, 2011).

The developmental and functional relationship between individual subsets of monocytes and those of macrophages has not been fully elucidated. It is generally thought that $Ly6C^+CCR2^+$ inflammatory monocytes exit the bone marrow in a CCR2-dependent manner and are recruited to inflamed tissues where they can differentiate to inflammatory M1 macrophages (Auffray et al., 2009; Dunay et al., 2008; Ingersoll et al., 2011; Serbina and Pamer, 2006; Tsou et al., 2007). In contrast, the differentiation of monocytes toward M2 macrophages remains ill defined. It has been suggested that $Ly6C^-CCR2^-$ resident monocytes are also recruited to sites of inflammation and then differentiate into M2 macrophages, contributing to wound healing (Auffray et al., 2007, 2009; Geissmann et al., 2010). Alternatively, recent study with a mouse model of helminth infection demonstrated that M2 macrophages are generated through IL-4-mediated proliferation and alternative activation of tissue-resident macrophages rather than the recruitment of blood monocytes (Jenkins et al., 2011). Thus, the origin of M2 macrophages and their mode of generation under homeostatic and pathological conditions remain obscure.

Basophils, the least common granulocyte, represent ~0.5% of peripheral blood leukocytes (Galli, 2000). Owing to their phenotypic similarities to mast cells and their small numbers, basophils had long been neglected in immunological studies. However, recent studies have defined previously unrecognized roles for basophils, including those in allergic responses, protection against parasitic infections, and regulation of acquired immunity (Karasuyama et al., 2011a; Min et al., 2012; Siracusa et al., 2011; Voehringer, 2011). Basophils readily generate large quantities of Th2 cell cytokines such as IL-4 and IL-13 (Piccinni et al., 1991; Seder et al., 1991), which contribute to initiation of Th2 cell differentiation (Perrigoue et al., 2009; Sokol et al., 2008, 2009; Yoshimoto et al., 2009) and to activation of B cells for the enhancement of humoral memory responses (Chen et al., 2009; Denzel et al., 2008). It remains to be investigated whether basophils and their products have any impact on the activation and differentiation of innate immune cells, including monocytes and macrophages.

In the present study, we analyzed the fate, polarization, and function of monocytes after their recruitment to skin lesions of immunoglobulin E (IgE)-mediated chronic allergic inflammation (IgE-CAI), a model where basophils rather than mast cells and T cells play a critical role for the elicitation of allergic response (Mukai et al., 2005). We found that $Ccr2^{-/-}$ mice unexpectedly displayed an exacerbation rather than alleviation of IgE-CAI, and ultimately identified a previously unappreciated mode of M2 generation, in that inflammatory monocytes can differentiate into anti-inflammatory M2-type macrophages via basophil-derived IL-4, which in turn dampen allergic inflammation.

RESULTS

$Ly6C^+CCR2^+$ Inflammatory Monocytes Are Recruited to Allergen-Exposed Skin in IgE-CAI

We previously showed that an intradermal administration of allergen induces three consecutive waves of ear swelling in mice sensitized with allergen-specific IgE, with peaks of swelling 30 min, 10 hr, and 3–4 days after the allergen challenge (Mukai et al., 2005). The delayed-onset (third) ear swelling with prominent inflammation was designated IgE-CAI (Mukai et al., 2005). Diphtheria toxin (DT)-mediated basophil ablation before the antigen challenge abolished the development of IgE-CAI in $Mcpt8^{DTR}$ mice (Wada et al., 2010) as shown in Figure 1A. This confirmed the conclusion in our previous studies that basophils play a pivotal role in the initiation of IgE-CAI, based on the results of experiments via the cell transfer and antibody-mediated basophil depletion (Mukai et al., 2005; Obata et al., 2007). Flow cytometric analysis revealed that the cell number in the skin lesions increased during the progress of IgE-CAI (Figure 1B). Monocyte- and macrophage-lineage cells (referred to here as monocytes-macrophages) and eosinophils were the major cell types among the cellular infiltrates whereas neutrophils and basophils were much less abundant (Figure 1C).

The vast majority of monocytes-macrophages isolated from the IgE-CAI skin lesions expressed Ly6C and CCR2, in contrast to those isolated from the control ear skin (Figure 2A and Figure S1A available online). Although resident macrophages in ear skin of naive mice barely express Ly6C, substantial numbers of $Ly6C^+CCR2^+$ monocytes-macrophages were detectable in the skin lesions even at 1 day after challenge (Figure S1A, top). These results suggested that monocytes-macrophages accumulating in the skin lesions were derived from $Ly6C^+CCR2^+$ inflammatory monocytes circulating in the peripheral blood (Figure S1B). Among the skin-infiltrating cells examined, basophils also expressed relatively high amounts of CCR2 on their surface in both C57BL/6 and BALB/c mice (Figure 2B). The expression of mRNAs encoding CCR2 ligands CCL8 and CCL12 (but not CCL2) was upregulated in the IgE-CAI skin lesions (Figure 2C). Various types of cells in the skin lesions expressed the CCR2 ligands, but basophils showed little or no expression of any of them (Figure S2A). Based on these observations, we assumed that CCR2 could contribute to the recruitment of both basophils and inflammatory monocytes to the skin lesions and hence the development of IgE-CAI.

$Ccr2^{-/-}$ Mice Show Exacerbated IgE-CAI in Spite of Impaired Recruitment of Inflammatory Monocytes

In sharp contrast to our expectation, the ear swelling in IgE-CAI was greatly augmented and prolonged in $Ccr2^{-/-}$ mice compared to that in wild-type mice (Figure 3A). Histopathological

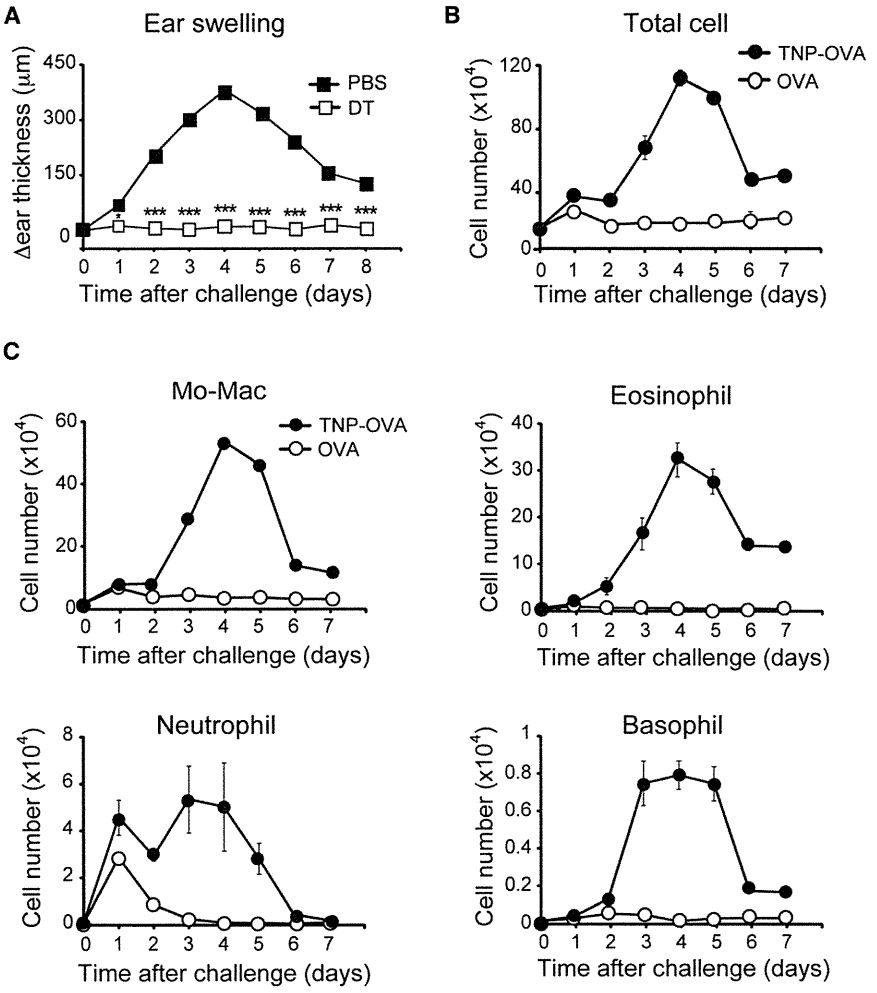

Figure 1. Cellular Components in the IgE-CAI Reaction that Is Elicited by Basophils

(A) *Mcpt8*^{DTR} C57BL/6 mice were sensitized with anti-TNP IgE and challenged with intradermal administration of TNP-OVA (or control OVA) in their ears to induce IgE-CAI. The mice were treated with either DT (open squares) or control PBS (closed squares) twice, 1 day before and 3 days after the antigen challenge. Time course of ear swelling (Δear thickness) is shown (mean ± SEM, n = 5 each). *p < 0.05, ***p < 0.001.

(B and C) C57BL/6 mice were sensitized with anti-TNP IgE and challenged with TNP-OVA (closed circles) or control OVA (open circles). The number of total cells (B) and indicated cell types (C) isolated from the ear skin at each time point postchallenge is shown (mean ± SEM, n = 3 each).

Data shown are representative of at least three independent experiments. Note that error bars are displayed in all figures, but often are hidden behind symbols such as squares and circles.

examination revealed many more cellular infiltrates in the skin lesion of *Ccr2*$^{-/-}$ mice (Figure 3B). Flow cytometric analysis demonstrated that the accumulation of monocytes-macrophages in the skin lesions was almost completely abolished in *Ccr2*$^{-/-}$ mice, as expected (Figure 3C). By contrast, the infiltration of basophils was enhanced rather than reduced in *Ccr2*$^{-/-}$ mice (Figure 3C), indicating that CCR2 was dispensable for the basophil recruitment, unlike for the monocyte recruitment. The accumulation of neutrophils in the skin lesions was also augmented in *Ccr2*$^{-/-}$ mice (Figure 3C). Thus, the IgE-CAI reaction was exacerbated rather than alleviated in *Ccr2*$^{-/-}$ mice, in spite of the fact that the recruitment of Ly6C^{+} inflammatory monocytes was abolished.

Monocytes-Macrophages in the Skin Lesions Display a Combined Phenotype of Inflammatory Monocytes and M2 Macrophages

To clarify the reason for this unexpected observation, we further examined the phenotype of monocytes-macrophages infiltrating the IgE-CAI skin lesions of wild-type mice. Approximately two-thirds of them expressed programmed death 1 ligand 2 (PD-L2) on their surface, whereas few cells isolated from the control skin did so (Figures 4A, 4B, and S1A, bottom). Because PD-L2 is a marker of M2-type macrophages (Loke and Allison, 2003), we examined the expression of other M2 markers in the skin lesions during the IgE-CAI reaction. The *Arg1*, *Chi3l3*, and *Fizz1* expression was upregulated and then downregulated, in parallel with the number of PD-L2^{+} monocytes-macrophages in the skin lesions (Figures 4B and 4C). Moreover, PD-L2^{+} monocytes-macrophages expressed significantly higher amounts of these mRNAs compared to PD-L2^{-} monocytes-macrophages and other cell lineages in the IgE-CAI skin lesions (Figures 4D and S2B), demonstrating that PD-L2^{+} monocytes-macrophages indeed displayed an M2 phenotype.

Gene profiling of monocytes-macrophages accumulating in the skin lesions revealed that M2 markers (*Arg1*, *Chi3l3*, and *Fizz1*) but not M1 markers (*Il1b*, *Nos2*, and *Tnfa*) were significantly upregulated during the IgE-CAI progression (Figure S3A). By contrast, the expression of the M2 markers and PD-L2 in blood monocytes, regardless of Ly6C expression, remained undetectable or very low during the IgE-CAI progression (Figures S3C and S3D). Importantly, the expression of genes involved in the macrophage differentiation (*Maf* and *Mafb*) but not those involved in the dendritic cell differentiation (*Sfpi1* and *Relb*) was upregulated in monocytes-macrophages in the skin lesions during the IgE-CAI progression (Figure S3B). These results strongly suggested that inflammatory monocytes recruited to the skin lesions differentiated into M2- but not M1-type macrophages during the IgE-CAI reaction. In contrast, monocytes-macrophages accumulating in skin lesions of delayed-type hypersensitivity (DTH) to the same antigen displayed an M1 phenotype with little or no expression of M2 markers including PD-L2 (Figure S4). Thus, the phenotype of monocytes-macrophages in skin lesions, either M1 or M2, appeared to be

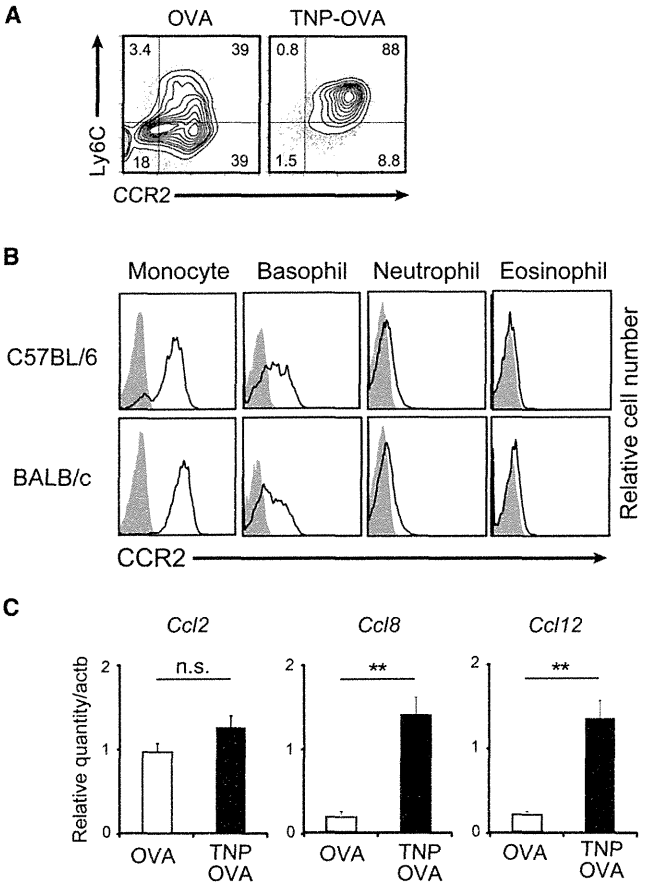

Figure 2. Monocytes-Macrophages Accumulating in the IgE-CAI Skin Lesions Display a Phenotype of Inflammatory Monocytes

(A) C57BL/6 mice were treated as in Figure 1 to induce IgE-CAI. The expression of Ly6C and CCR2 on F4/80$^+$CD11b$^+$SSC^{lo} monocytes-macrophages in the skin lesions of mice challenged with TNP-OVA or control OVA was examined on day 4 postchallenge.

(B) The expression of CCR2 on indicated cell lineages isolated from the bone marrow of C57BL/6 and BALB/c mice. Shaded histograms show control staining with isotype-matched antibody.

(C) The expression of indicated mRNAs in the skin lesions of mice challenged with TNP-OVA or control OVA was examined on day 3 postchallenge (mean ± SEM, n = 5 each).

Data shown are representative of three independent experiments. NS, not significant; **p < 0.01. See also Figures S1 and S2.

associated with the type of immune responses rather than the nature of antigens.

A previous study with a mouse model of helminth infection reported that M2 macrophages are generated through the proliferation and alternative activation of tissue-resident macrophages without any requirement of the blood monocyte recruitment (Jenkins et al., 2011). Therefore, we examined whether this mode of M2 generation could also take place in IgE-CAI. Although tissue-resident macrophages, mostly negative for Ly6C, were detected in ear skin of naive *Ccr2*$^{-/-}$ mice to an extent comparable to that observed in wild-type mice (Figure S5A), PD-L2$^+$ monocytes-macrophages were barely detected in the IgE-CAI skin lesions of *Ccr2*$^{-/-}$ mice (Figure 4E). Moreover, few monocytes-macrophages in the skin lesions of wild-type mice were positive for a proliferation marker Ki-67, regardless of the PD-L2 expression (Figure S5B). Thus, the proliferation and M2 conversion of tissue-resident macrophages appear to have little, if any, contribution to the M2 generation during the IgE-CAI reaction.

Basophil-Derived IL-4 Confers an M2-like Phenotype on Ly6C$^+$ Inflammatory Monocytes Ex Vivo

Th2 cell cytokines such as IL-4 and IL-13 as well as IL-10 have been shown to induce the differentiation of macrophages toward M2. Quantitative RT-PCR analysis revealed that the expression of *Il4* but not *Il13* or *Il10* mRNAs in the IgE-CAI skin lesions was upregulated in parallel with the accumulation of PD-L2$^+$ monocytes-macrophages (Figure 5A). *Il4* mRNAs were almost exclusively expressed by basophils among various cell types isolated from the skin lesions (Figure 5B). Indeed, primary basophils isolated from the bone marrow produced substantial amounts of IL-4 but not IL-13 when stimulated ex vivo with IgE plus antigens (Figure 5C).

Ly6C$^+$Ly6G$^-$ inflammatory monocytes freshly isolated from the bone marrow expressed no detectable PD-L2 on their surface (Figure 5D). Of note, they upregulated the PD-L2 expression when incubated ex vivo with the culture supernatants of primary basophils that had been stimulated with IgE plus antigens. This upregulation of PD-L2 was abolished when IL-4 antibody was included during the incubation (Figures 5D and 5E), indicating that basophil-derived IL-4 was responsible for the PD-L2 upregulation in inflammatory monocytes. The expression of *Arg1*, *Chi3l3*, and *Fizz1* mRNAs in inflammatory monocytes was also upregulated when incubated with the culture supernatants of activated basophils in an IL-4-dependent manner (Figure 5F). These results demonstrated that basophil-derived IL-4 can confer an M2-like phenotype on monocytes even before they differentiate into macrophages.

Skin-Infiltrating Monocytes Acquire an M2-like Phenotype in an IL-4R- and Basophil-Dependent Manner

We next examined whether the basophil IL-4-mediated acquisition of an M2-like phenotype by inflammatory monocytes indeed occurs in vivo. First, CD115$^+$ bone marrow monocytes were prepared from wild-type mice, labeled with CFSE, and adoptively transferred into IgE-sensitized wild-type mice, simultaneously with the challenge with allergens. On day 3 postchallenge, many of CFSE-labeled cells infiltrating the skin lesions became positive for PD-L2, concomitantly with F4/80 upregulation (Figure 6A), indicating their differentiation into M2-type macrophages. Of note, virtually all of the CFSE$^+$PD-L2$^+$F4/80$^+$ cells expressed Ly6C (Figure 6A), suggesting that they were derived from Ly6C$^+$ inflammatory but not Ly6C$^-$ resident monocytes. Indeed, when CD115$^+$Ly6C$^+$Ly6G$^-$ inflammatory monocytes were purified from the bone marrow and adoptively transferred, most of them became positive for PD-L2 in the skin lesions on day 3 postchallenge (Figure 6B).

Second, to examine the IL-4 dependency of M2 differentiation, CD115$^+$ bone marrow monocytes were prepared from wild-type or *Il4ra*$^{-/-}$ mice, labeled with CFSE, and adoptively transferred into wild-type mice, followed by IgE-CAI induction (Figure 6C). On day 1 postchallenge, when few basophils were recruited to the skin lesions (Figure 1C), little or no expression of PD-L2

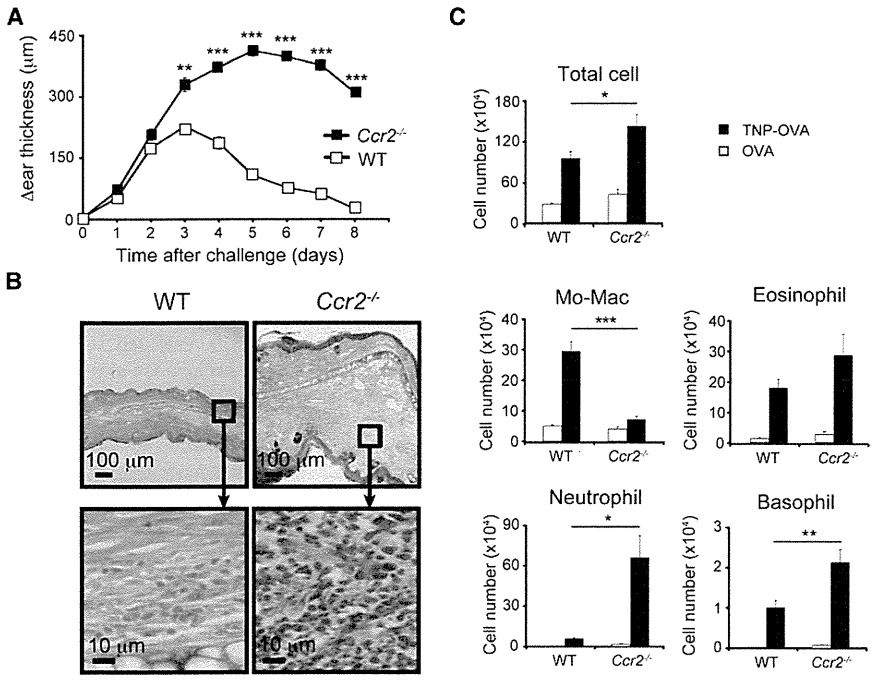

Figure 3. IgE-CAI Is Exacerbated rather than Ameliorated in *Ccr2*$^{-/-}$ Mice

Wild-type and *Ccr2*$^{-/-}$ BALB/c mice were treated as in Figure 1 to induce IgE-CAI.

(A) Time course of ear swelling (Δear thickness) in wild-type (open squares) and *Ccr2*$^{-/-}$ (closed squares) mice is shown (mean ± SEM, n = 4–5 each). Note that error bars are displayed, but often are hidden behind symbols.

(B) Giemsa-stained specimens of IgE-CAI skin lesions isolated 4 days postchallenge.

(C) The numbers of total cells and indicated cell types isolated from the ear skin on day 4 postchallenge are shown (mean ± SEM, n = 4–5 each). Data shown are representative of four independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001.

was detected on CFSE-labeled cells infiltrating the skin lesions, regardless of the source of transferred cells (Figure 6C). On day 3 postchallenge, when the basophil infiltration reached a plateau (Figure 1C), a significant fraction of CFSE-labeled cells infiltrating the skin lesions expressed PD-L2 in mice that had received cells derived from wild-type but not *Il4ra*$^{-/-}$ mice (Figure 6C). Thus, monocytes recruited to the skin lesions acquired the PD-L2 expression in an IL-4 receptor (IL-4R)-dependent manner.

We then investigated whether basophils could contribute to this process. IgE-CAI was elicited in *Mcpt8*^{DTR} mice, and on day 2 postchallenge, CFSE-labeled CD115$^+$ bone marrow monocytes from wild-type mice were adoptively transferred to them, in conjunction with or without DT-mediated basophil ablation. The basophil ablation completely abolished the acquisition of PD-L2 expression by transferred monocytes infiltrating the skin lesions (Figure 6D). These results strongly suggested that blood-circulating monocytes acquire an M2-like phenotype after their recruitment to the IgE-CAI skin lesions, in response to basophil-derived IL-4.

Adoptive Transfer of Ly6C$^+$CCR2$^+$ Inflammatory Monocytes Dampens the Exacerbated IgE-CAI in *Ccr2*$^{-/-}$ Mice in an IL-4R-Dependent Manner

We next examined the functional consequence of the monocyte recruitment to the IgE-CAI skin lesions by means of adoptive transfer of wild-type monocytes to *Ccr2*$^{-/-}$ mice that display the exacerbated IgE-CAI. A single transfer of CD115$^+$ bone marrow monocytes at the time point of the antigen challenge, as shown in Figure 6A, showed no apparent impact on the ear swelling (data not show). We assumed that repeated transfer might be needed to reproduce the recruitment and accumulation of monocytes in the IgE-CAI skin lesions. Of note, four consecutive transfers of CD115$^+$ monocytes but not CD115$^-$ bone marrow cells dampened the exacerbated IgE-CAI in *Ccr2*$^{-/-}$ mice to the level observed in wild-type mice (Figure 7A), suggesting that CD115$^+$ bone marrow monocytes manifest an anti-inflammatory property after their recruitment to the skin lesion.

We then asked two questions. Are Ly6C$^+$Ly6G$^-$ inflammatory monocytes (rather than Ly6C$^-$ resident monocytes) that are recruited to and accumulate in the skin lesions indeed responsible for the negative regulation of IgE-CAI? Is the IL-4R-mediated acquisition of the M2-like phenotype by inflammatory monocytes associated with the regulation? To address these issues, Ly6C$^+$Ly6G$^-$ inflammatory monocytes were further purified from CD115$^+$ bone marrow cells, derived from either wild-type or *Il4ra*$^{-/-}$ mice, and directly transferred once into the ear dermis of *Ccr2*$^{-/-}$ mice where the antigens were administered (Figure 7B). The adoptive transfer of Ly6C$^+$Ly6G$^-$ inflammatory monocytes derived from wild-type but not *Il4ra*$^{-/-}$ mice dampened the exacerbated IgE-CAI. This strongly suggests that after the recruitment to the IgE-CAI skin lesions, CCR2$^+$Ly6C$^+$Ly6G$^-$ inflammatory monocytes acquired an M2-like phenotype through IL-4R and exerted an anti-inflammatory function to regulate the allergic inflammation.

DISCUSSION

Activated M2-type macrophages have been observed in a range of physiological and pathological processes, including Th2 cell-type immune responses (Kreider et al., 2007; Martinez et al., 2009; Murray and Wynn, 2011). However, the origin, differentiation pathway, and function of M2 macrophages have been ill defined, compared to those of M1 macrophages. In the present study, we have demonstrated a previously unappreciated cascade of monocyte-to-macrophage transition toward M2, being from proinflammatory to anti-inflammatory to dampen an allergic reaction. After recruitment to allergen-exposed skin, Ly6C$^+$CCR2$^+$ "inflammatory" monocytes acquired an M2-like phenotype and exerted an anti-inflammatory function in IgE-CAI, in response to IL-4 produced by antigen- and IgE-stimulated basophils. Accordingly, the failure in the recruitment