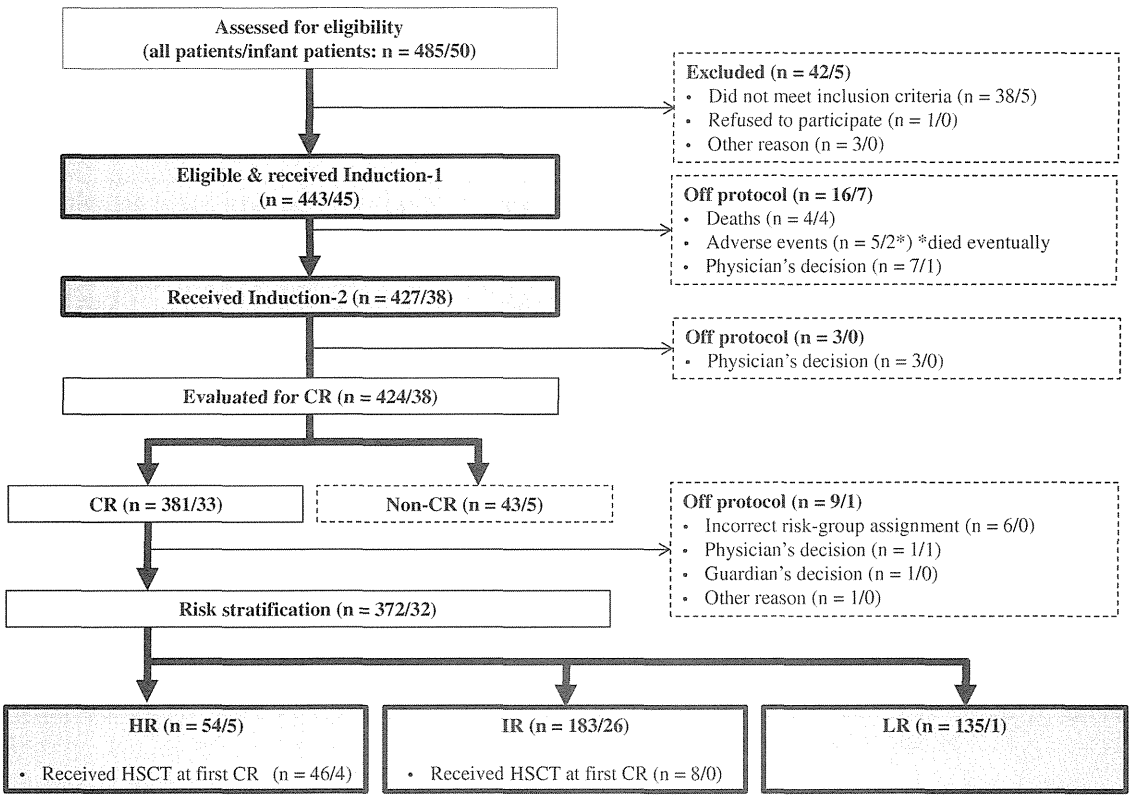

Fig. 2 Patient disposition in the AML-05 study

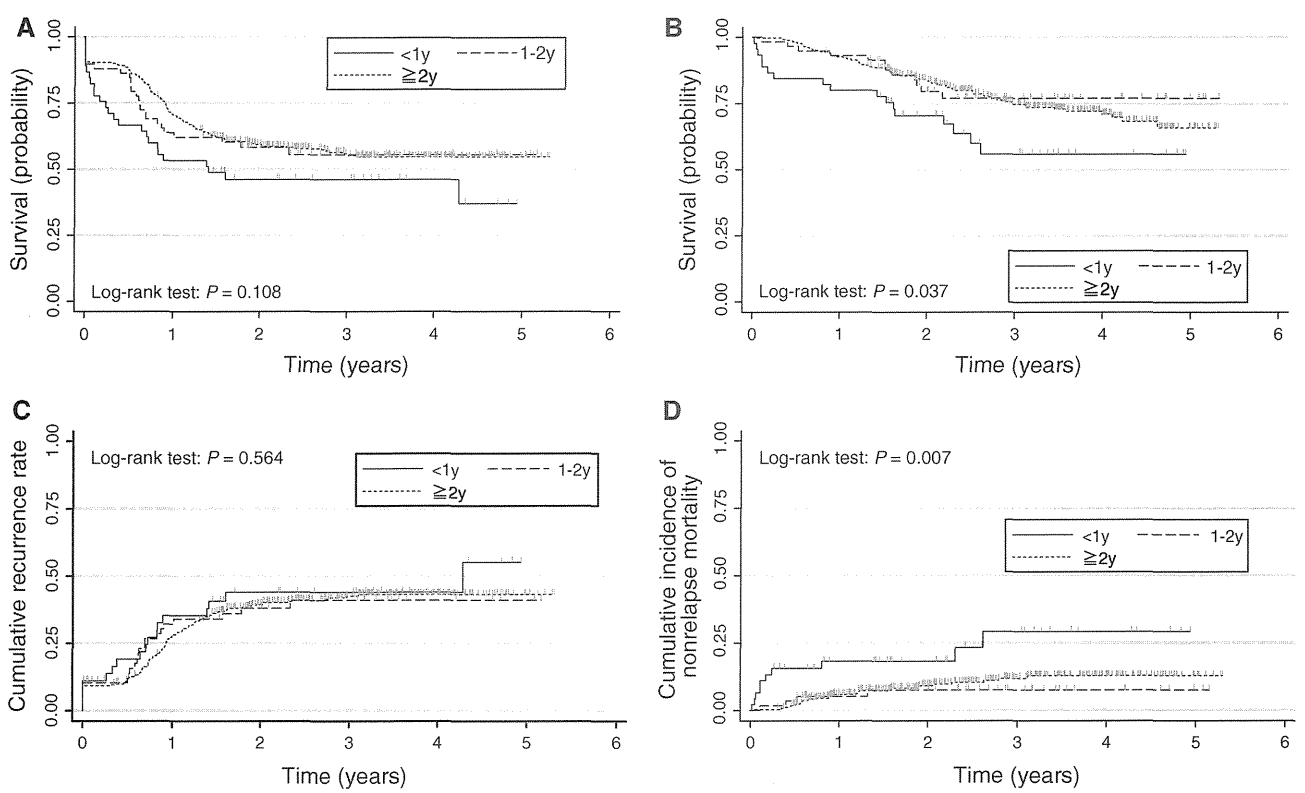

Fig. 3 Comparison of outcomes of patients according to age group. **a** Event-free survival (EFS). **b** Overall survival (OS). **c** Cumulative relapse rate. **d** Non-relapse mortality

Table 4 Incidence of grade ≥3 adverse events among infants

	Induction			Intensification		
	1: ECM		2: HCEI	1: HCE or HCM	2: HCI or HCEI	3: HC or HCM
	Before amendment	After amendment				
Number of patients assessed	28	17	38	32	30	24
Blood/bone marrow (%)						
Hemoglobin	100	100	94	100	86	91
Leukocytes	96	88	100	100	100	100
Neutrophils	96	100	100	100	100	100
Platelets	100	100	100	100	96	100
Cardiac (%)						
LV systolic dysfunction	7	0	0	0	0	0
Coagulation (%)						
DIC	17	5	2	0	0	0
Dermatology/skin (%)						
Rash/desquamation	3	5	0	0	0	0
Gastrointestinal (%)						
Vomiting	7	5	0	3	0	0
Diarrhea	21	11	2	12	6	4
Mucositis	3	0	0	0	3	0
Hemorrhage/bleeding (%)						
Hemorrhage, CNS	3	0	0	0	0	0
Hemorrhage, pulmonary	14	0	0	0	0	0
Infection (%)						
Febrile neutropenia	57	64	34	46	30	33
Infection (documented clinically)	42	17	18	21	23	25
Metabolic/laboratory (%)						
Creatinine	5	0	0	0	0	0
ALT	14	0	10	6	13	12
AST	21	0	10	0	13	12
Bilirubin	3	0	0	0	0	0
Neurology (%)						
Somnolence	5	0	0	0	0	0
Seizure	3	0	0	0	0	0
Pulmonary/upper respiratory (%)						
ARDS	5	0	0	0	0	0
Hypoxia	28	5	0	0	0	4
Syndromes (%)						
Tumor lysis syndrome	14	5	0	0	0	0

Treatment courses are described in Fig. 1

LV left ventricular, *DIC* disseminated intravascular coagulation, *CNS* central nervous system, *ALT* alanine aminotransferase, *AST* aspartate aminotransferase, *ARDS* acute respiratory distress syndrome

induction were due to rapid progression of leukemia in one patient, ARDS in four patients, and interstitial pneumonia in one patient. All five of the non-leukemic deaths were associated with infectious disease or febrile neutropenia. Notably, two of the patients had respiratory syncytial virus (RSV) infection. Concurrent hemophagocytic syndrome (HPS) was also found in three of the patients who died because of non-leukemic causes.

The overall early treatment responses are summarized in Table 2. Although none of the parameters showed statistically significant differences because of the small number of patients analyzed, there were no early deaths after the protocol amendments. Additionally, the incidence of grade ≥3 non-hematological toxicities, as evaluated by the common terminology criteria for adverse events (3rd version), was lower in patients enrolled after versus before the

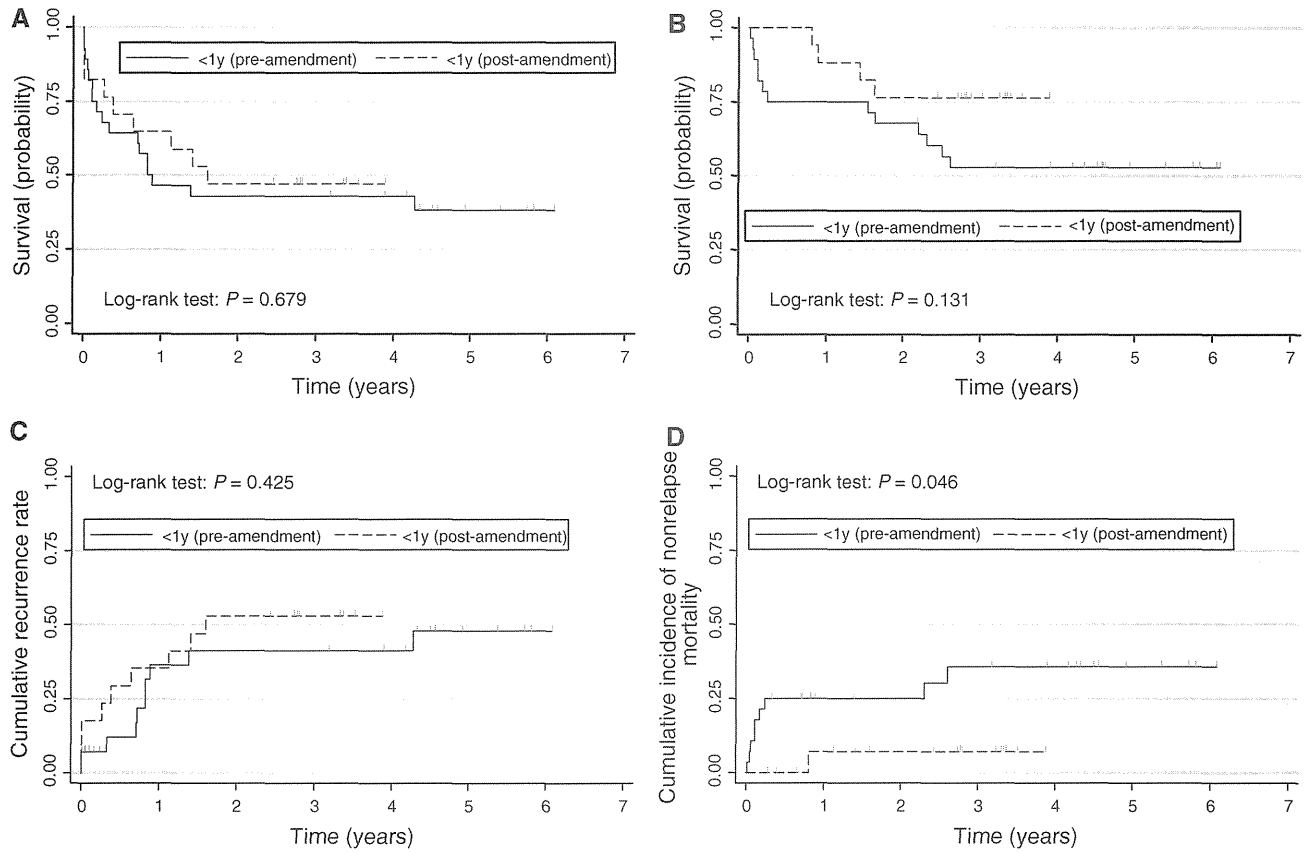

Fig. 4 Comparison of outcomes of infants enrolled before or after the protocol amendments. **a** Event-free survival (EFS). **b** Overall survival (OS). **c** Cumulative relapse rate. **d** Non-relapse mortality

protocol amendments (Table 4). Importantly, it seems that the dose reductions in the initial induction course did not compromise treatment efficacy, as 82.4 % of the infants enrolled after the protocol amendments achieved CR. Additionally, the percentage of patients receiving prophylactic G-CSF use seemed to be lower in infants enrolled after the protocol amendment compared to the pre-amendment cohort; 23.5 % (4/17) vs. 39.2 % (11/28). Median days for G-CSF use in these patients were also shorter in the post-amendment cohort; 9 days (range 5–19 days) vs. 14 days (range 2–25 days).

Overall outcomes of infants enrolled before and after the protocol amendments

There were no significant differences in 3-year pEFS [42.8 % (95 % CI 24.5–59.9 %) vs. 47.0 % (95 % CI 22.9–67.9 %); $P = 0.679$] and the cumulative relapse rate [41.3 % (95 % CI 23.8–64.8 %) vs. 52.9 % (95 % CI 32.0–77.0 %); $P = 0.425$] between infants enrolled before and after the protocol amendments (Fig. 4a, c). However, there was a significant improvement in the reduction of non-relapse mortality rate [35.7 % (95 % CI 19.9–58.3 %) vs. 7.1 % (95 % CI 1.0–40.9 %); $P = 0.046$; Fig. 4d] and achieved 3-year pOS of 76.4 % (95 % CI 48.8–90.4 %) for the post-amendment cohort [vs. 52.7 % (95 % CI 32.8–69.3 %) for the pre-amendment cohort; $P = 0.131$; Fig 4b]. The incidence of grade ≥3 adverse events by treatment courses in infants is described in Table 4. Besides hematologic toxicities and febrile neutropenia/infection that were frequent throughout the whole treatment courses, the other grade ≥3 adverse events that occurred in >10 % of infants beyond the initial induction course were gastrointestinal toxicity (diarrhea) and elevated liver function parameters (aspartate aminotransferase and alanine aminotransferase).

Discussion

Intensive combination chemotherapy with cytarabine and anthracyclines, together with optimal risk stratification based on the cytogenetic characteristics of leukemia cells and the response to the initial induction course, has led to a 70 % probability of survival in childhood AML [10, 11]. Infants with AML aged <1 year have features that are

usually associated with poor prognosis, including high WBC count and/or extramedullary involvement at diagnosis, higher frequencies of monocytic leukemia or megakaryocytic leukemia, and higher frequency of *MLL* gene rearrangement, and few infants have favorable cytogenetic characteristics, such as t(8;21)(q22;q22)/*RUNX1-RUNX1T1* and t(15;17)(q22;q12)/*PML-RARA* [1–3]. Thus, infants with AML are usually classified in the IR or HR groups and receive treatments appropriate for this level of risk.

Despite these 'unfavorable' characteristics, the outcomes of infants with AML are generally not much worse than those of older children with AML. For example, in the Japan Infant Leukemia Study Group, the 3-year pEFS and pOS were 72 and 76 %, respectively, for 35 infants with AML [12]. The AML Berlin–Frankfurt–Münster (BFM) Study Group reported that the 5-year pEFS and pOS were 44 and 61 %, respectively, in the AML-BFM 98 study ($n = 59$) and 51 and 75 %, respectively, in the AML-BFM 04 study ($n = 61$)[6]. The 5-year pEFS was 58 % in the United Kingdom (UK) Medical Research Council (MRC) AML-10 and AML-12 studies ($n = 151$) [7]. In other Japanese AML studies, similar results were reported for infant AML, with 5-year pEFS and pOS of 49.4 and 58.3 % in the Tokyo Children's Cancer Study Group (TCCSG) M91-13 and M96-14 studies ($n = 24$) [13] and 46.0 and 64.5 %, respectively, in the Japanese Childhood AML Cooperative Group AML99 study ($n = 27$) [10]. In the current study, pOS was lower in infants than in the other age groups. However, this disparity was due to the higher non-relapse mortality rate in infants and not the cumulative relapse rate, which was not significantly different among the three age groups.

The high early mortality rate (17.8 %, 5/28) observed in infants enrolled before the protocol amendments was somewhat unexpected, because the induction regimen, which consisted of etoposide (150 mg/m^2 per day on days 1–5), cytarabine (200 mg/m^2/day via 12 h continuous intravenous infusion on days 6–12), and mitoxantrone (5 mg/m^2/day on days 6–10), together with an age-adjusted dose of triple intrathecal chemotherapy on day 6, was identical to the induction regimen used in prior Japanese studies, although the dose modification method differed among these studies [10, 12, 13]. In the TCCSG M91-13 and M96-14 studies, the doses were adjusted for body surface area, with an additional dose reduction of 33 %, and the early mortality rate was 12.5 % (3/24). In the AML99 study, the doses were adjusted for body weight, as in our study before the protocol amendments, and the early mortality rate was 3.7 % (1/27).

It is notable that all non-leukemic deaths during the initial induction course were due to pulmonary complications following infectious complications. Of note, three patients had HPS. These findings suggest that infections in infants are likely to induce hypercytokinemia and result in severe conditions, such as ARDS and/or HPS. Consequently, we also amended the protocol to avoid the prophylactic administration of G-CSF, because this may aggravate inflammatory cytokine production, is often observed in engraftment syndrome after HSCT, and G-CSF was reported to have no benefits in the treatment of children with AML [14–16]. The BFM Study Group reported that, although hemorrhage and leukostasis were the main causes of death within the first 15 days of initial therapy, fatal infections were more common between days 15 and 42. They also reported that acute toxicities during the induction course, particularly severe infection and pulmonary toxicity, were more frequent in infants than in older children [17]. Therefore, the management of infectious complications is vital to prevent early death when treating infants with AML.

To prevent fatal infectious complications, we decided to modify the doses of chemotherapeutic drugs during the initial induction course, by reducing the doses of etoposide, cytarabine, and mitoxantrone by 33 %, but not of intrathecal chemotherapy. However, appropriate dose adjustments for infants are not well documented because very few pharmacokinetic studies have been performed in infants [18, 19]. Many factors may affect drug metabolism in infants, including higher total body water content and extracellular water content, higher unbound active fraction of drugs because of lower affinity of drugs to serum proteins, lower p450 enzyme activity, which could reduce or increase the cytotoxic effects, and lower renal clearance, which could increase systemic exposure of drugs because of reduced tubular and glomerular function until about 6 months of age. In addition, the ratio of body weight to body surface area is lower in infants than in older children. Therefore, if the doses are calculated based on the body surface area, the infants would be exposed to greater amounts of each drug. Thus, prior studies have used arbitrary methods to modify the drug doses for infants, including adjustment for body weight in the BFM [6], CCG-2891 [20], and AML99 [10] studies, while the doses were reduced by 25 % in the MRC AML-10 and AML-12 studies [7].

In addition to reducing the doses of chemotherapeutic drugs, we have also revised the guidelines for general supportive care and infection prevention, by recommending bacterial and fungal prophylaxis, intravenous immunoglobulin therapy to maintain IgG levels $\geq$500 mg/dl, and the use of rooms with positive air pressure with high-efficacy particulate air (HEPA) filtration. Although the incidence of RSV infection is low among patients with AML, it is associated with high mortality in children with AML [21], and it is well known that infants, both

immunocompetent and immunocompromised, are particularly vulnerable to RSV. Although RSV infection may be prevented with palivizumab, a humanized monoclonal antibody that targets the RSV epitope, it was not approved for use in malignant disease in Japan until August 2013, so its use was at the physician's discretion. An aerosol formulation of the anti-viral agent ribavirin is also effective against RSV infection, but only oral agents have been approved in Japan.

With these aforementioned amendments, most of the grade ≥ 3 non-hematological adverse events decreased, especially, clinically documented infections and pulmonary complications (Table 4). No more early death was observed and only 7.1 % cumulative incidence of non-relapse mortality was documented among the 17 infants enrolled after the protocol amendments. Although this reduction in early and non-relapse death led to achievement of >70 % pOS for the post-amendment cohort, improvements in pEFS and pOS were not statistically significant. Naturally, there are limitations for the exact explanations due to a relatively small number of patients included in the current study, but one must consider the possibility of increased relapse led by treatment reduction in the post-amendment cohort, although cumulative relapse rate itself was not statistically different.

In conclusion, appropriate dose reduction of chemotherapeutic drugs, particularly in the induction phase, together with enhanced supportive care is essential to prevent non-relapse death when treating AML in infants. As the conventional dose-intensifying approach is difficult to apply for this age group, less toxic agents targeting specific biological features are needed to improve the outcomes of infants with AML.

Acknowledgments This work was supported by a Grant for Clinical Cancer Research and a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour, and Welfare of Japan.

Conflict of interest The authors declare that they have no conflict of interest.

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Immunity

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

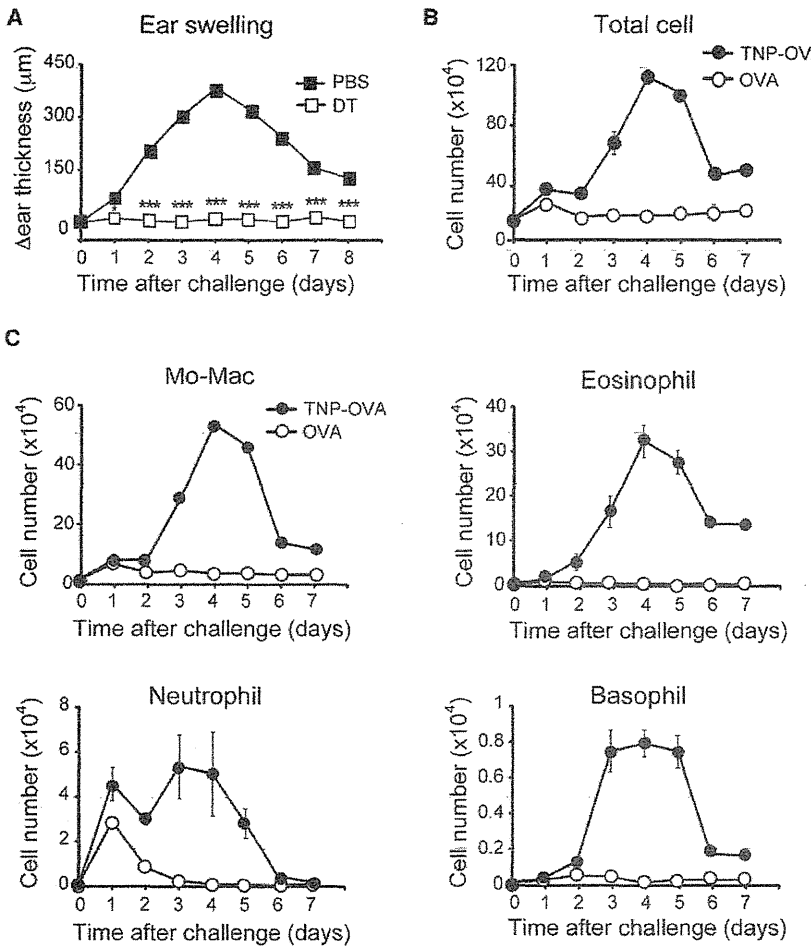

Figure 1. Cellular Components in the IgE-CAI Reaction that Is Elicited by Basophils

(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

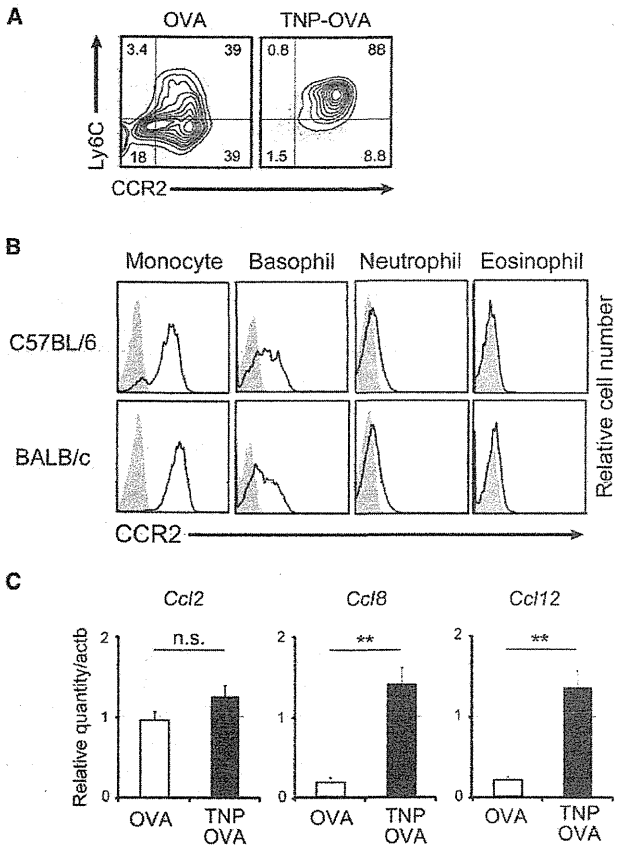

Figure 2. Monocytes-Macrophages Accumulating in the IgE-CAI Skin Lesions Display a Phenotype of Inflammatory Monocytes

(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

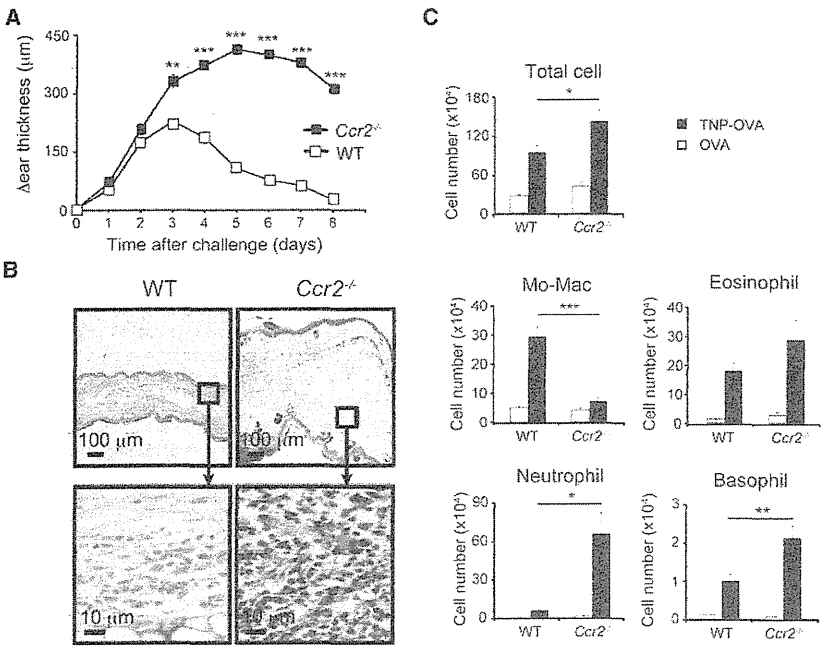

Figure 3. IgE-CAI Is Exacerbated rather than Ameliorated in *Ccr2*$^{-/-}$ Mice

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

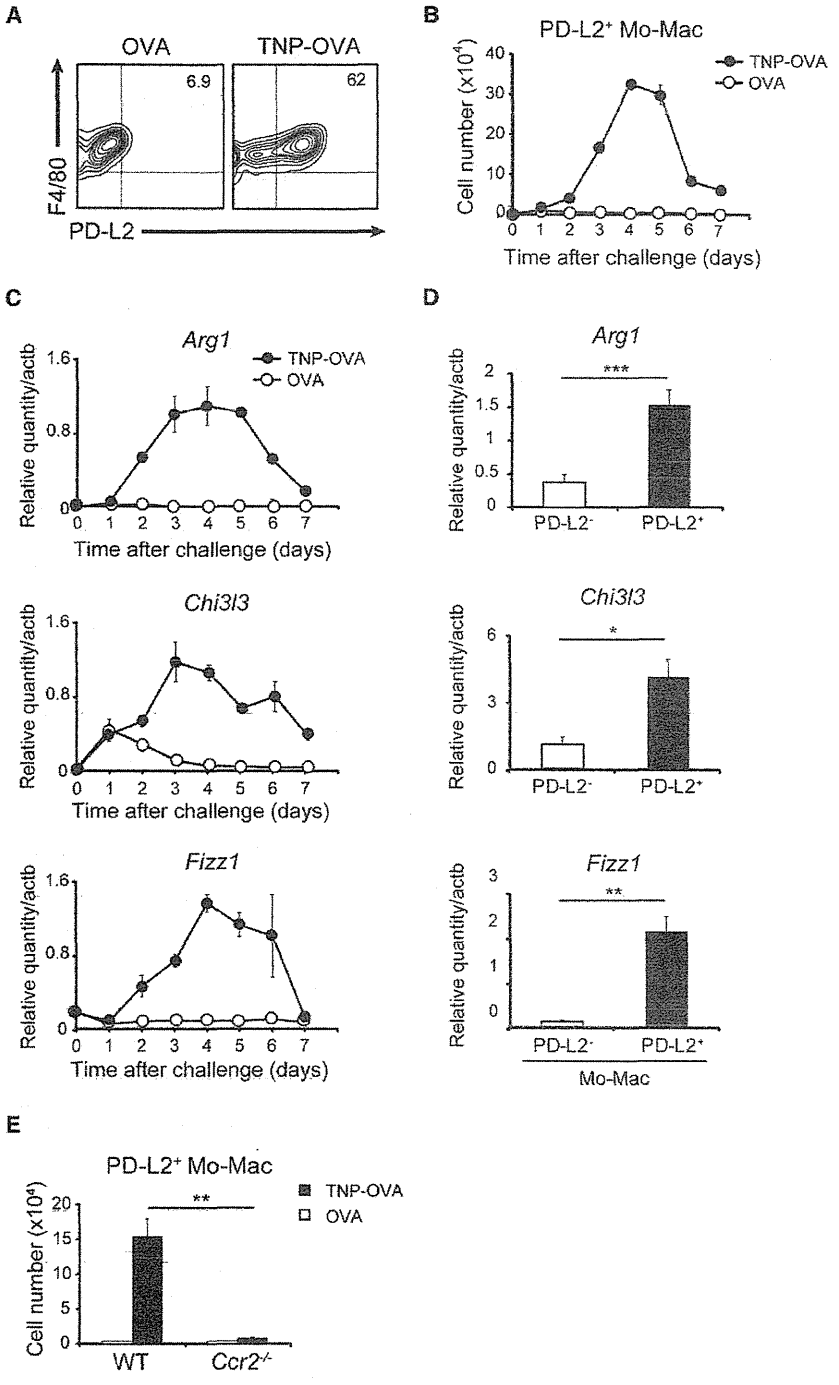

Figure 4. Monocytes-Macrophages Accumulating in the IgE-CAI Skin Lesions Display a Phenotype of M2-type Macrophages

(A–D) C57BL/6 mice were treated as in Figure 1 to induce IgE-CAI.

(A) The expression of PD-L2 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 post-challenge.

(B and C) Time course of the PD-L2$^+$ monocytes-macrophage number (B, mean ± SEM, n = 3 each) and the expression of indicated mRNAs (C, mean ± SEM, n = 3 each) in the skin lesions of mice challenged with TNP-OVA (closed circles) or control OVA (open circles).

(D) The expression of indicated mRNAs in PD-L2$^-$ and PD-L2$^+$ monocytes-macrophages that were isolated on day 3 postchallenge from the ear skin of mice challenged with TNP-OVA (mean ± SEM, n = 6 each).

(E) Wild-type and *Ccr2*$^{-/-}$ BALB/c mice were treated as in Figure 3 to induce IgE-CAI. Data show the numbers of PD-L2$^+$ monocytes-macrophages that were isolated on day 4 postchallenge from the ear skin of mice challenged with TNP-OVA or control OVA (mean ± SEM, n = 4–5 each). Data shown are representative of at least three independent experiments. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. See also Figures S1–S5.

of inflammatory monocytes in *Ccr2*$^{-/-}$ mice resulted in the exacerbation rather than alleviation of allergic inflammation, and adoptive transfer of CCR2$^+$ inflammatory monocytes normalized it. Thus, M2-like monocytes-macrophages derived from inflammatory monocytes appear to negatively regulate allergic inflammation in IgE-CAI.

Previous studies have shown two distinct modes of M2 generation. One is based on the observation during infection with *Listeria monocytogenes*. Ly6C$^{lo/-}$ resident monocytes recruited to the infection site turn on the expression of typical M2 marker genes, including *Arg1* and *Fizz1*, indicating their differentiation into M2 macrophages that probably contribute to tissue repair (Auffray et al., 2007). In contrast, Ly6C$^+$ inflammatory monocytes differentiate into dendritic cells that produce inflammatory mediators (Kurihara et al., 1997; Serbina et al., 2003). The other mode of M2 generation has been demonstrated in infection with helminth *Litomosoides sigmodontis*, in that M2 macrophages are generated through proliferation and alternative activation of tissue-resident macrophages rather than the recruitment of circulating monocytes (Jenkins et al., 2011). Our study on IgE-CAI has identified the third mode of M2 generation, in that Ly6C$^+$ inflammatory monocytes give rise to M2-type macrophages. The differentiation of inflammatory monocytes to M2 macrophages may not be restricted to allergic responses. A similar differentiation was suggested in experimental autoimmune encephalomyelitis, even though no direct evidence for this in vivo was provided (Denney et al., 2012).

The in vivo function of M2 macrophages has been less well characterized, compared to that of M1 macrophages, but has

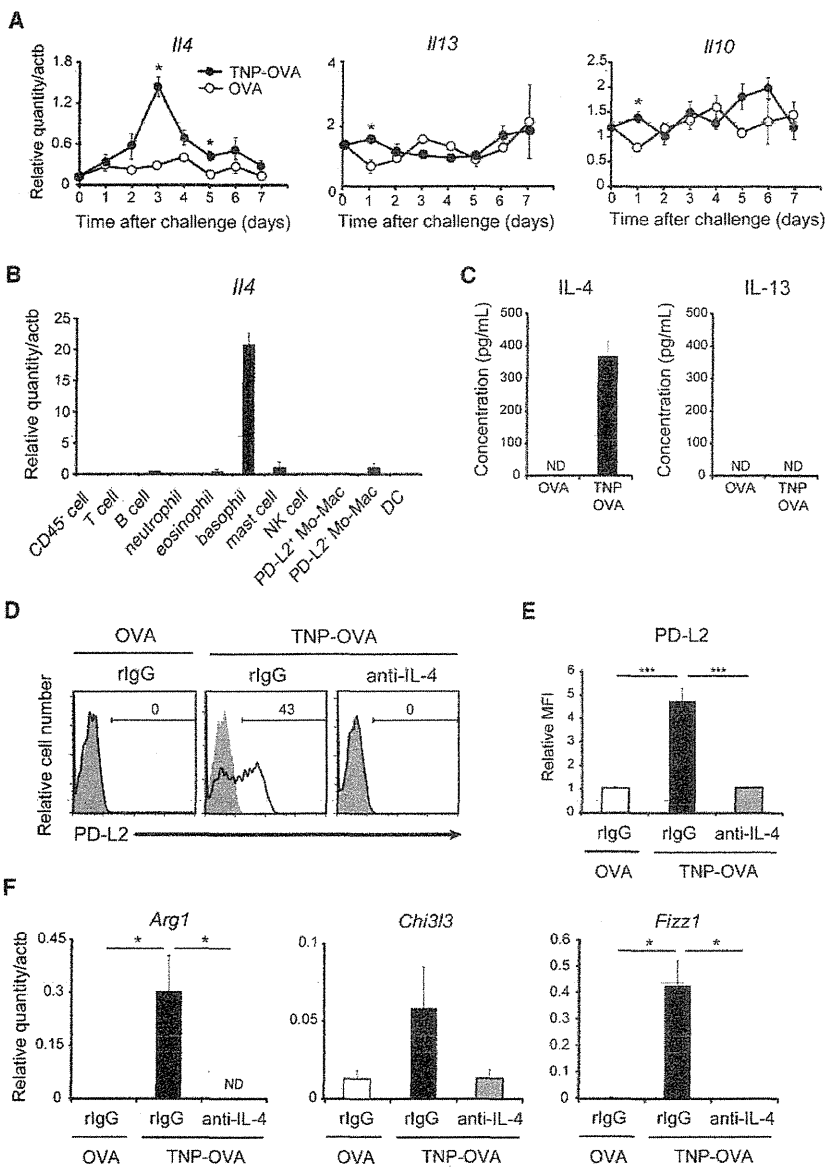

Figure 5. Basophil-Derived IL-4 Confers an M2-type Phenotype on Ly6C$^+$ Inflammatory Monocytes Ex Vivo

(A and B) C57BL/6 mice were treated as in Figure 1 to induce IgE-CAI. Time course of the expression of indicated mRNAs in the skin lesions of mice challenged with TNP-OVA (closed circles) or control OVA (open circles) is shown in (A) (mean ± SEM, n = 3 each). *p < 0.05. In (B), the indicated cell lineages were isolated on day 3 postchallenge from the skin lesions of mice challenged with TNP-OVA and subjected to quantitative RT-PCR for the analysis of *Il4* expression (mean ± SEM, n = 3 each).

(C) Basophils (2 × 10^5 cells/ml) enriched from *bone marrow cells were sensitized ex vivo* with anti-TNP IgE and then stimulated with TNP-OVA or control OVA at 37°C for 10 hr, and the concentration of IL-4 and IL-13 in their culture supernatants was determined by ELISA (mean ± SEM, n = 5 each). ND, not detectable.

(D–F) Ly6C$^+$ inflammatory monocytes were purified from C57BL/6 bone marrow cells and incubated at 37°C for 24 hr in the presence of anti-IL-4 or control rat IgG (rIgG), with the culture supernatants of basophils that had been stimulated as in (C).

(D and E) The cultured monocytes were subjected to flow cytometric analysis for PD-L2 expression. Representative staining profiles are shown in (D). Shaded histograms show control staining with isotype-matched antibody. All the data are summarized in (E) (mean ± SEM, n = 5–7 each), in that the relative mean fluorescence intensity (MFI) was calculated as MFI (PD-L2 staining)/MFI (control staining).

(F) The cultured monocytes were subjected to quantitative RT-PCR analysis for expression of indicated mRNAs (mean ± SEM, n = 5 each).

Data shown are representative of at least three independent experiments. *p < 0.05, ***p < 0.001.

been implicated in a variety of processes, including protection against parasitic infection, promoting Th2 cell-type immune responses, wound healing, tissue fibrosis, metabolic regulation, angiogenesis, and tumorigenesis (Kreider et al., 2007; Martinez et al., 2009; Murray and Wynn, 2011). The role of M2 macrophages in allergy and asthma has not been well understood, in contrast to the extensive study on its role in parasitic infections. Of note, in mice infected with helminth *Schistosoma mansoni*, M2 macrophages and their products have been shown to suppress rather than promote Th2 cell-type inflammation (Nair et al., 2009; Pesce et al., 2009). No such anti-inflammatory property of M2 macrophages was definitely demonstrated in allergic responses, as far as we aware. Instead, in mouse models of airway allergic inflammation, M2 macrophages reportedly contribute to the pathogenesis of disease, including promotion of inflammation (Ford et al., 2012; Kim et al., 2008; Kurowska-Stolarska et al., 2009; Melgert et al., 2010; Moreira et al., 2010; Nagarkar et al., 2010) and angiogenesis (Sun et al., 2008). Intriguingly, *Ccr2*$^{-/-}$ mice were reported to display enhanced airway allergic inflammation, but the underlying mechanism remains to be determined (Kim et al., 2001).

In the present study, we clearly demonstrated that M2-like monocytes-macrophages derived from inflammatory monocytes exert an anti-inflammatory function in IgE-CAI. Their absence or the failure in their conversion to M2 type resulted in the exacerbation of allergic inflammation. Thus, M2-like monocytes-macrophages appear to dampen excessive inflammation in IgE-CAI. It remains to be definitely demonstrated how they exert an anti-inflammatory function. Treatment of mice with an inhibitor of arginase-1 showed no apparent effect on IgE-CAI (data not show), although arginase-1 has been shown to suppress Th2 cell-type inflammation in helminth

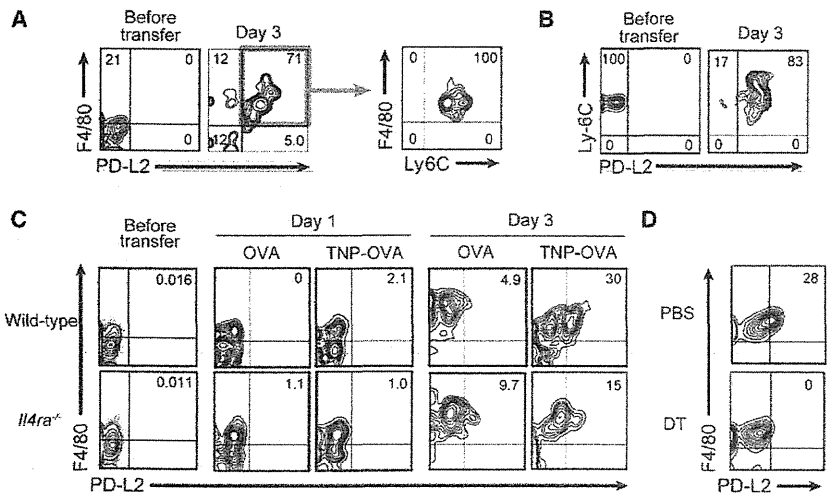

Figure 6. CD115$^+$ Monocytes Acquire PD-L2 Expression after Their Infiltration into the Skin Lesions, in a Manner Dependent on IL-4R and Basophils

(A and B) CD115$^+$ bone marrow cells isolated from BALB/c mice (A) or Ly6C$^+$Ly6G$^-$ inflammatory monocytes (purity > 99%) sorted from CD115$^+$ bone marrow cells (B) were labeled with CFSE and intravenously transferred (2 × 10^6 cells/mouse) into BALB/c mice that had been sensitized with anti-TNP IgE 1 day earlier. Recipient mice were challenged with intradermal administration of TNP-OVA immediately after the cell transfer. Flow cytometric analysis was performed for the surface expression of F4/80, PD-L2, and Ly6C in CFSE-labeled cells before the transfer and in those isolated from the TNP-OVA-injected skin on day 3 posttransfer.

(C) CD115$^+$ bone marrow cells were prepared from wild-type or *Il4ra*$^{-/-}$ mice, labeled with CFSE, and intravenously transferred (3 × 10^6 cells/mouse) into IgE-sensitized BALB/c mice, followed by the antigen challenge (TNP-OVA or control OVA) as in (A) and (B). Flow cytometric analysis was performed for the surface expression of F4/80 and PD-L2 in CFSE-labeled cells before the transfer (left) and in those isolated from the ear skin on day 1 and day 3 posttransfer (middle and right, respectively).

(D) *Mcpt8*^{DTR} C57BL/6 mice were sensitized with anti-TNP IgE and challenged with TNP-OVA as in Figure 1A to induce IgE-CAI. On day 2 postchallenge, the mice were treated with intravenous injection of CFSE-labeled CD115$^+$ bone marrow cells (1 × 10^7 cells/mouse) derived from wild-type mice, in conjunction with DT or control PBS. On day 4 postchallenge, the expression of F4/80 and PD-L2 on CFSE-labeled cells isolated from the ear skin was examined.

Data shown are representative of three independent experiments.

infection (Pesce et al., 2009). Notably, M2-like monocytes-macrophages infiltrating skin lesions of IgE-CAI express PD-L2, a ligand for the inhibitory receptor PD-1 (Loke and Allison, 2003). Blockade of PD-L2 with a specific antibody enhanced a Th2 cell-type response in helminth infection (Huber et al., 2010). However, our preliminary experiments with PD-L2 antibody suggested no apparent contribution of PD-L2 to the damping of allergic inflammation in IgE-CAI. In skeletal muscle injury, Ly6C$^+$ inflammatory monocytes are recruited and converted into "anti-inflammatory" macrophages that express IL-10 and transforming growth factor-β (TGF-β) (Arnold et al., 2007). In IgE-CAI, however, neither IL-10 nor TGF-β seems to be involved in damping inflammation in IgE-CAI (not all data shown). Of note, PD-L2$^+$ monocytes-macrophages accumulating in the IgE-CAI skin lesions expressed high amounts of a mannose receptor CD206 (Figure S6A), suggesting that they might have enhanced endocytic activity (Montaner et al., 1999). Indeed, PD-L2$^+$ monocytes-macrophages in the skin lesions showed a much higher extent of antigen uptake compared to PD-L2$^-$ monocytes-macrophages or other cell lineages including eosinophils and neutrophils (Figure S6B). This suggests that the failure in the generation of M2-like monocytes-macrophages may lead to the insufficient clearance of antigens in the skin lesions. Considering the fact that the extent and duration of the IgE-CAI reaction correlate well with the dose of antigens (Sato et al., 2003), the anti-inflammatory property of M2-like monocytes-macrophages could be attributed, at least in part, to their efficient uptake and clearance of antigens, making antigens unavailable for basophil activation.

Alternatively activated M2-type macrophages are typically generated by stimulation with the Th2 cell cytokines, IL-4 and IL-13, that can be produced by Th2 cells, natural killer T (NKT) cells, mast cells, eosinophils, basophils, and innate-type lymphoid cells (Paul and Zhu, 2010). Memory Th2 cells are the major source of Th2 cell cytokines for M2 generation in helminth infection (Anthony et al., 2006), whereas NKT cell-derived IL-4 is important for M2 generation in experimental autoimmune encephalomyelitis (Denney et al., 2012). In adipose tissues, eosinophil-derived IL-4 and IL-13 are crucial for M2 generation to maintain glucose homeostasis (Wu et al., 2011). In the present study, we demonstrated that basophil-derived IL-4 can act on inflammatory monocytes and convert them to anti-inflammatory M2-like monocytes-macrophages. Thus, basophils can contribute to the activation and differentiation of monocytes and macrophages, in addition to those of T and B cells as reported previously (Perrigoue et al., 2009; Sokol et al., 2008, 2009; Yoshimoto et al., 2009; Chen et al., 2009; Denzel et al., 2008). Of note, when stimulated ex vivo with IL-4, human CD14$^+$ monocytes display a phenotype characteristic for human M2-type macrophages, including upregulated expression of PD-L2 on their surface (Semnani et al., 2011). Given the fact that human basophils produce large quantities of Th2 cell cytokines as do murine basophils (Piccinni et al., 1991), it is plausible that basophils contribute to the generation of M2-type monocytes-macrophages in humans as observed in mice.

In conclusion, the present study demonstrated a previously unappreciated mode of monocyte-to-macrophage transition, that is, a conversion from inflammatory monocytes to anti-inflammatory M2-type monocytes-macrophages in an allergic response. In repeated infections with parasites, host animals often raise IgE against parasite antigens, and hence basophils can be stimulated with IgE plus antigens as seen in IgE-CAI (Karasuyama et al., 2011b; Voehringer, 2009). Moreover, basophils can be directly activated in an IgE-independent manner,

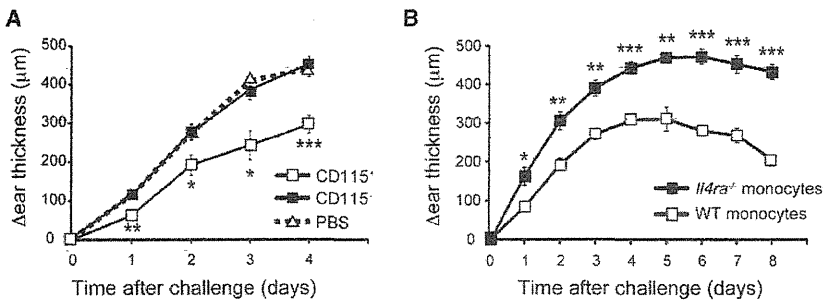

Figure 7. Adoptive Transfer of CCR2$^+$Ly6C$^+$ Inflammatory Monocytes from Wild-Type but Not *Il4ra*$^{-/-}$ Mice Normalizes the Exacerbated IgE-CAI in *Ccr2*$^{-/-}$ Mice

Ccr2$^{-/-}$ mice were treated as in Figure 3A to induce IgE-CAI.

(A) CD115$^+$ (open squares) or CD115$^-$ (closed squares) bone marrow cells from BALB/c mice or control PBS (open triangles) were intravenously administered to the mice four times (1 × 10^6 cells/injection/mouse), on days 0, 1, 2, and 3 post-challenge. Time course of ear swelling (Δear thickness) is shown (mean ± SEM, n = 3–5 each).

(B) Ly6C$^+$Ly6G$^-$ inflammatory monocytes (purity > 99%) sorted from bone marrow cells of wild-type (open squares) or *Il4ra*$^{-/-}$ (closed squares) mice were intradermally administered once (1 × 10^6 cells/site) in the ear of *Ccr2*$^{-/-}$ mice, in conjunction with administration of TNP-OVA or control OVA. Time course of ear swelling (Δear thickness) is shown (mean ± SEM, n = 4 each).

Data shown are representative of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001. Note that error bars are displayed in all figures, but often are hidden behind symbols. See also Figures S6.

for example with certain proteases and pathogen products (Schroeder et al., 2001). Therefore, basophil-elicited M2 generation might be widely observed in various settings. Further studies will clarify their functional significance in each setting.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 and BALB/c mice were purchased from CLEA Japan. *Mcpt8*^{DTR} C57BL/6 (Wada et al., 2010), *Ccr2*$^{-/-}$ (Kuziel et al., 1997), and *Il4ra*$^{-/-}$ BALB/c (Noben-Trauth et al., 1997, 1999) mice were as described previously and maintained under specific-pathogen-free conditions in our animal facilities. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University.

Induction of IgE-CAI

IgE-CAI was elicited as described previously (Mukai et al., 2005). In brief, mice were sensitized with intravenous injection of 300 μg of anti-TNP IgE, and on the following day challenged with an intradermal injection of 10 μg TNP_{12}-conjugated ovalbumin (OVA) and control OVA into the right and left ear, respectively. The value of Δear thickness, the differences in ear thickness (right − left) was calculated for the evaluation of inflammation.

Isolation of Bone Marrow Basophils and Monocytes

Basophils and monocytes were enriched from bone marrow cells via IMag system with biotinylated anti-CD49b and anti-CD115, respectively, followed by streptavidin-conjugated magnetic particles (BD PharMingen). Inflammatory monocytes were purified by sorting Ly6C^{hi}Siglec-F$^-$CD11c$^-$Ly6G$^-$ cells from the CD115$^+$ bone marrow cell population with FACSAria (BD Biosciences).

In Vitro Stimulation of Basophils and Monocytes

Basophils were stimulated for 10 hr with TNP_{12}-OVA or control OVA (300 ng/ml) after sensitization with TNP-specific IgE. The concentration of cytokines in culture supernatants was determined with Mouse IL-4 ELISA MAX Standard (Biolegend) for IL-4 and mouse Ready-Set-Go! ELISA kit (eBioscience) for IL-13. Monocytes were incubated for 24 hr with the culture supernatant of activated basophils in the presence or absence of anti-IL-4 or control rat IgG (20 μg/ml).

Ablation of Basophils

Mcpt8^{DTR} mice were treated once or twice with intravenous injection of diphtheria toxin (DT, Sigma-Aldrich, 500 ng/injection).

Statistical Analysis

Statistical analysis was performed with unpaired Student's t test. A p value <0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.11.014.

ACKNOWLEDGMENTS

We thank W.A. Kuziel (External Scientific Affairs, Daiichi Sankyo Group, Edison, NJ) for providing *Ccr2*$^{-/-}$ mice, D. Yamanaka and R. Matsunaga for technical support, and M. Miki for secretary assistance. This work was supported by a research grant from JST, CREST (to H.K.).

Received: August 4, 2012
Accepted: November 19, 2012
Published: February 21, 2013

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The skin is an important bulwark of acquired immunity against intestinal helminths

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Once animals have experienced a helminthic infection, they often show stronger protective immunity against subsequent infections. Although helminthic infections are well known to elicit Th2-type immune responses, it remains ill-defined where and how acquired protection is executed. Here we show that skin-invading larvae of the intestinal helminth *Nippostrongylus brasiliensis* are surrounded by skin-infiltrating cells and are prevented from migrating out of infected skin during the second but not the first infection. B cell– or IgE receptor FcεRI–deficient mice showed impaired larval trapping in the skin. Selective ablation of basophils, but not mast cells, abolished the larval trapping, leading to increased worm burden in the lung and hence severe lung injury. Skin-infiltrating basophils produced IL-4 that in turn promoted the generation of M2-type macrophages, leading to the larval trapping in the skin through arginase-1 production. Basophils had no apparent contribution to worm expulsion from the intestine. This study thus reveals a novel mode of acquired antihelminth immunity, in which IgE-armed basophils mediate skin trapping of larvae, thereby limiting lung injury caused by larval migration.

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Abbreviations used: BEC, S-(2-boronoethyl)-L-cysteine; DT, diphtheria toxin; DTR, DT receptor; Hp, *Heligmosomoides polygyrus*; ILC2, group 2 innate lymphoid cell; Nb, *Nippostrongylus brasiliensis*; nor-NOHA, Nω-hydroxy-nor-L-arginine.

Helminths are the most common infectious agents of humans in developing countries (Hotez et al., 2008). The major helminthiases are those caused by intestinal helminths including large roundworms, whipworms, and hookworms, followed by schistosomiasis and lymphatic filariasis. More than two billion people in worldwide populations are infected with intestinal helminths, suffering from deleterious outcomes such as malnutrition, growth stunting, and intellectual retardation. For the development of effective antihelminth vaccines, we need to understand both the helminth biology and the host immune response to helminthic infections (Anthony et al., 2007; Hotez et al., 2010; Allen and Maizels, 2011). Most helminths, unlike many other types of pathogens such as bacteria, protozoa, fungi, and viruses, do not replicate in the mammalian host, showing a complex multistage life cycle. Once host animals have experienced a helminthic infection, they often show a stronger protective immunity against subsequent infections with the same type of helminth (Africa, 1931; Valdivieso and Tamsitt, 1969; Love et al., 1974). This is the rationale for the development of antihelminth vaccines. However, it remains ill-defined how host animals manifest an acquired resistance to reinfection, even though it is well known that infection with intestinal helminths typically elicits a type 2 immune response that is characterized by high levels of serum IgE and increased numbers of type 2 helper T (Th2) cells, eosinophils, mast cells, and basophils (Finkelman et al., 2004; Anthony et al., 2007; Allen and Maizels, 2011; Pulendran and Artis, 2012).

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The Rockefeller University Press $30.00
J. Exp. Med. 2013 Vol. 210 No. 12 2583-2595
www.jem.org/cgi/doi/10.1084/jem.20130761

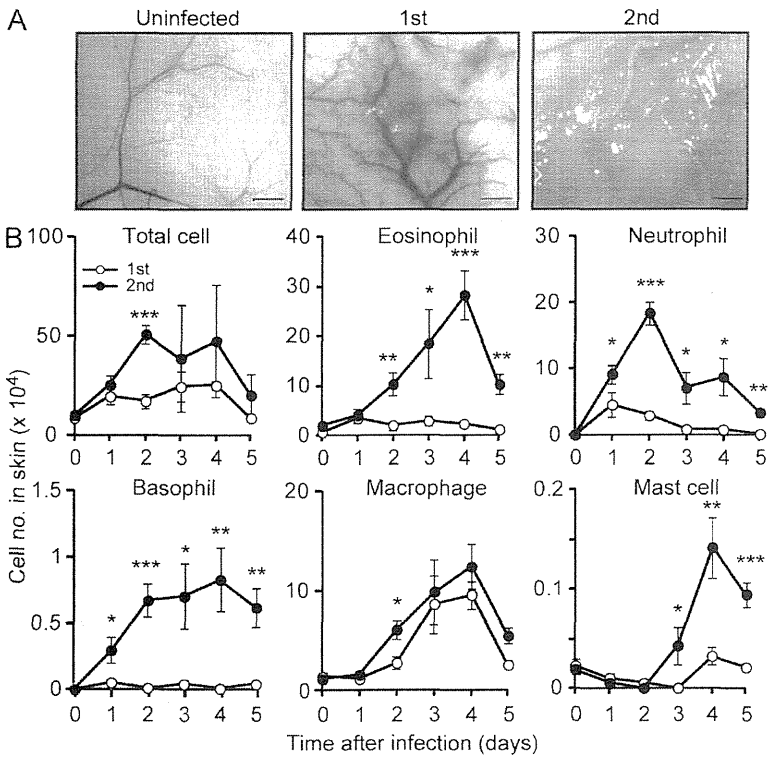

Figure 1. Prominent infiltration of proinflammatory cells in the skin of the larva inoculation site during the second but not first Nb infection. (A) BALB/c mice were left uninfected (left) or infected once (middle) or twice (right) with Nb larvae. Photographs of the subcutaneous tissue of the larva inoculation site were taken 2 d after the first or second inoculation. Bars, 8 mm. (B) Single-cell suspensions were prepared from the skin of the larva inoculation site at the indicated time points after the first and second inoculation and subjected to the flow cytometric analysis to identify the nature of skin-infiltrating cells. The numbers of total and individual cell lineages at each time point are plotted (mean ± SEM; $n = 3$ each). Data shown in A and B are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Nippostrongylus brasiliensis (Nb) is a well-studied helminth in rodents and shows a life cycle similar to that of human hookworms *Necator americanus* and *Ancylostoma duodenale* (Finkelman et al., 1997; Gause et al., 2003). Infective larvae enter host animals through skin penetration and migrate to the lung within 2 d after invasion. They further migrate to the small intestine starting from day 3 and develop into mature worms to produce eggs. Adult worms are then expelled from the intestine by 10 d. Recent studies have illustrated that group 2 innate lymphoid cells (ILC2s) play an important role in worm expulsion from the intestine, through the production of IL-13 that in turn induces goblet cell hyperplasia in the intestine to increase mucus production for the "weep and sweep" response (Moro et al., 2010; Neill et al., 2010; Price et al., 2010). In contrast, it remains uncertain how host animals acquire and manifest the more efficient protective immunity against the subsequent infections. Of note, the number of worms recovered from the intestine on day 5 after infection is significantly lower in the second infection than in the first infection (Love et al., 1974; Knott et al., 2007). This suggests that worms may be efficiently expelled from the intestine in a shorter period of time during the second infection compared with the first one. Alternatively or in addition, the acquired anti-Nb immunity may exert its action at the preintestinal stage. Supporting this, the number of mobile larvae recovered from the lung on day 2 after infection was reported to be lower in the second infection than in the first (Knott et al., 2007; Harvie et al., 2010), implying that some larvae might be damaged within the lung or at the prelung stage. Previous studies failed to detect larval retention in the skin during the second infection, suggesting the lung rather than the skin as an important site for the acquired protection (Knott et al., 2007; Harvie et al., 2010). In contrast, leukocyte accumulation in the lung becomes prominent only on day 6 or later when larvae have already left the lung for the intestine (Knott et al., 2007; Harvie et al., 2010), making protection in the lung questionable. Although one study reported the contribution of basophils to worm clearance from the small intestine during the second Nb infection (Ohnmacht et al., 2010), the other failed to reproduce it (Sullivan et al., 2011). Thus, the site and cellular components of acquired protection remain elusive.

In the present study, we addressed these unsolved issues and found that, in the second but not first Nb infection, larvae are prevented from migrating out of infected skin, and are surrounded by skin-infiltrating proinflammatory cells. This larval trapping was dependent on high-affinity IgE receptor FcεRI and was abolished in mice depleted of basophils but not mast cells. Basophil-derived IL-4 promoted the generation of M2-type (alternatively activated) macrophages that in turn contributed to the larval trapping via their expression of arginase 1 (Arg1). Importantly, basophil-mediated larval trapping in the skin reduced the worm burden in the lung and hence protected mice from severe lung injury caused by the larval migration through the lung. Thus, basophils provide acquired protection against intestinal helminth Nb by means of restraining skin-invading larvae from migration toward the lung and intestine, rather than expelling adult worms from the small intestine.

RESULTS

Migratory larvae are retained within cellular infiltrates in the infected skin during the second but not first Nb infection

Multiple small hemorrhagic petechiae were detected in the skin of the larva inoculation site in the first Nb infection, reflecting that larvae penetrated and migrated into the blood vessels toward the lung (Fig. 1 A, middle). We noticed that an abscess-like white bump developed in the subcutaneous tissue of the larva inoculation site during the second but not first Nb infection (Fig. 1 A, right), suggesting that severe inflammation occurred in the skin lesion during the second infection. Indeed, flow cytometric analysis revealed that large numbers of eosinophils and neutrophils as well as a small number of basophils were recruited to and accumulated in the skin lesions during the second but not first infection (Fig. 1 B). The number of ILC2 cells in the skin lesions remained unaltered during the first and second infection (not depicted). Histopathological examination of skin sections prepared on day 2 after inoculation identified many nodule-like aggregates of infiltrating cells in dermis and subcutaneous tissue in the second but not first infection (Fig. 2 A). Notably, high magnification view of the tissue section revealed a cross section of larva-like structure within the cell aggregate (Fig. 2 B), suggesting that some larvae might be trapped by infiltrating cells within the skin of the inoculation site during the second Nb infection. To explore this possibility, fluorescent dye–labeled larvae were intradermally inoculated and subjected to multiphoton microscopic analysis to track them. 2 d after the inoculation, few larvae were detected within the skin around the inoculation site in the first infection, whereas a substantial number of larvae were visible within the skin in the second infection (Fig. 2 C). This did not seem to be an artifact because of the labeling of larvae. When mice were infected with unlabeled larvae, the retention of larvae within the skin was also suggested by significantly higher expression of Nb *Actin* mRNAs at the inoculation site in the second infection than in the first one (Fig. 2 D).

We then sought to isolate and enumerate larvae that were retained within the skin. We first tried to recover larvae from the infected skin by using the traditional method, in that infected tissues such as the lung are placed in PBS at 37°C to let viable larvae migrate out from the tissues (Camberis et al., 2003), but few or no larvae migrated out from the infected skin. We assumed that this could be a reason why previous studies failed to detect larvae retained within the skin in the second infection. To overcome this problem, infected skin was isolated and incubated with simulated gastric juice so that mouse tissues but not larvae were digested (Jin et al., 2008), and released larvae were enumerated under a microscope. This allowed us to examine the time course of larval migration out of the skin (Fig. 2 E). In the first infection, virtually all larvae left the skin of inoculation site by day 2 after inoculation. In contrast, in the second infection, as many as 100 larvae were retained in the skin on day 2 and even on day 5 (Fig. 2 E). The larval retention in the skin was also observed when larvae were inoculated for the second infection in ear skin in place of flank skin (not depicted), suggesting that the occurrence of larval retention may not be restricted to a certain anatomical district in the skin.

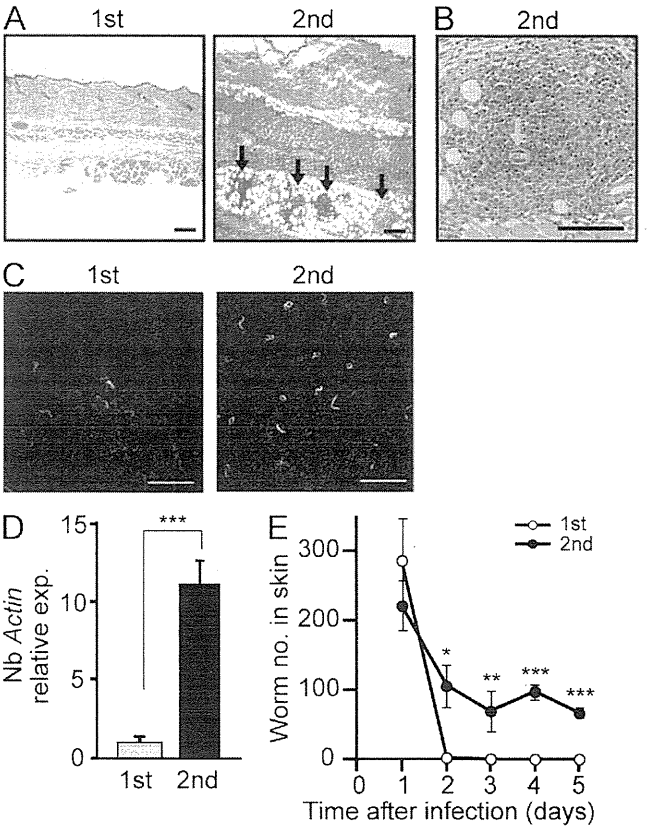

Figure 2. Retention of Nb larvae within cellular infiltrates in the infected skin during the second but not first infection. (A and B) The skin of the larva inoculation site was isolated 2 d after the first or second inoculation and subjected to histopathological examination by hematoxylin and eosin staining. Black arrows in A indicate clusters of cells surrounding larvae, and the yellow arrow in B indicates a section of larva surrounded by infiltrating cells. (C) PKH-labeled larvae were inoculated into the skin of naive (left) or previously infected (right) BALB/c mice and subjected to in vivo imaging analysis to examine their retention in the skin of the inoculation site. Photographs were taken 2 d after the larva inoculation. Bars: (A) 500 µm; (B) 200 µm; (C) 1 mm. (D) The skin of the inoculation site was isolated 2 d after the first and second inoculation and subjected to RT-PCR analysis. The relative expression of Nb *Actin* mRNA is shown (mean ± SEM; $n = 3$ each), and the level of expression in first infection was set as 1. (E) Larvae were isolated from the skin of the inoculation site at the indicated time points after the first and second inoculation. The number of isolated larvae at each time point is plotted (mean ± SEM; $n = 3$ each). Data shown in A–E are representative of at least three independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Antibodies and FcεRI are required for the larval retention in the skin

We next examined the possible mechanisms underlying larval retention in the skin during the second but not first Nb infections. A previous study has demonstrated that Nb infection elicits the production of anti-Nb antibodies (Lebrun and