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CD8 α ⁺ Dendritic Cells Improve Collagen-Induced Arthritis In CC Chemokine Receptor (CCR)-2 Deficient Mice

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Abstract

Objective—Dendritic cells (DCs) have long been recognized as potential therapeutic targets of rheumatoid arthritis (RA). Increasing evidence has showed that DCs are capable of suppressing autoimmunity by expanding FoxP3⁺ regulatory T cells (T_{reg}), which in turn exert immunosuppression by increasing TGF β -1. In the SKG mice, activated DC prime autoreactive T cells causing autoantibody production and an inflammatory arthritic response. Recently, we reported that CC-chemokine receptor-2 deficient (*Ccr2*^{−/−}) mice had impaired DCs migration and reduced CD8 α ⁺ DCs in the C57Bl/6J mice strain and that these mice were more susceptible to collagen antibody-induced arthritis (CAIA), compared to wild type mice. To examine the mechanism by which DCs contribute to the increased susceptibility of arthritis in *Ccr2*^{−/−} mice, we tested the hypothesis that CD8 α ⁺ DCs are protective (tolerogenic) against autoimmune arthritis by examining the role of CD8 α ⁺ DCs in *Ccr2*^{−/−} and SKG mice.

Methods—To examine the mechanism by which DCs defects lead to the development of arthritis, we used two murine models of experimental arthritis: collagen-induced arthritis (CIA) in DBA1/J mice and zymosan-induced arthritis in SKG mice. DBA1/J mice received recombinant Flt3L-injections to expand endogenous DCs populations or adoptive transfers of CD8 α ⁺ DCs.

Results—Flt3L-mediated expansion of endogenous CD8 α ⁺ DCs resulted in heightened susceptibility of CIA. In contrast, supplementation with exogenous CD8 α ⁺ DCs ameliorated arthritis in *Ccr2*^{−/−} mice and enhanced TGF β 1 production by T cells. Furthermore, SKG mice with genetic inactivation of CCR2 did not affect the numbers of DCs nor improve the arthritis phenotype.

Conclusion—CD8 α ⁺ DCs were tolerogenic to the development of arthritis. CD8 α ⁺ DCs deficiency heightened the sensitivity to arthritis in *Ccr2*^{−/−} mice. *Ccr2* deficiency did not alter the arthritic phenotype in SKG mice suggesting the arthritis in *Ccr2*^{−/−} mice was T cell-independent.

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Keywords

CCR2; dendritic cells; CD8 α ⁺ DC; arthritis; SKG

Introduction

Dendritic cells (DCs) constitute a heterogeneous population of professional, bone marrow-derived antigen presenting cells (APCs) (Morelli *et al.* 2007). Specifically, CD8 α ⁺ DC present antigen to antigen-specific T-cells leading to T cell death, T cell anergy, expansion or generation of regulatory T cells (T_{reg}) (Morelli *et al.* 2007). Interestingly, DCs have been shown to suppress experimental autoimmunity (Morelli *et al.* 2007). Recent studies including work from our laboratory suggests that the effect of Ccr2, the receptor for monocyte chemoattractant protein-1, is crucial for recruiting monocytes and DCs to areas of inflammation (Bruhl *et al.* 2004; Healy *et al.* 2008; Quinones *et al.* 2004; Quinones *et al.* 2005).

Several lines of evidence point to the role of DCs in autoimmunity. First, non-arthritic prone C57Bl/6J *Ccr2*^{-/-} mice have impaired DCs migration, lower numbers of CD8 α ⁺ DCs and increased susceptibility to collagen antibody induced arthritis (CAIA) (Quinones *et al.* 2004; Quinones *et al.* 2005). Second, CD8 α ⁺ DCs are important in maintaining tolerance therefore loss of these cells can lead to the development of autoimmunity (O’Keeffe *et al.* 2005). Third, administration of the cytokine fms-like tyrosine kinase 3 ligand (Flt3L) increased the population of CD8 α ⁺ DCs and reduced incidence of autoimmune diabetes in mice (O’Keeffe *et al.* 2005; O’Keeffe *et al.* 2002). Lastly, DCs directly induce T_{reg} and thus could contribute to the suppression of autoimmunity (Li *et al.* 2008; Swee *et al.* 2009; Taylor *et al.* 2008; Yamazaki *et al.* 2009). We surmised that if DCs play a central role in autoimmunity, specifically increasing the quantity of CD8 α ⁺ DCs could result in tolerance and protection against the arthritis.

In this study, we utilized the collagen-induced arthritis (CIA) mouse model whereby autoimmune arthritis is induced by immunization with type II collagen (CII) emulsified in complete Freund’s adjuvant (CFA) (Rosloniec *et al.* 2010). In this widely used model, immunization with CII and CFA leads to the development of autoimmune-mediated polyarthritis that shares many features with human autoimmune disease RA (Rosloniec *et al.* 2010).

We previously showed that genetic inactivation of *Ccr2* in C57Bl/6J mice and DBA1/J mice were associated with enhanced susceptibility to CAIA and CIA, respectively (Quinones *et al.* 2004; Quinones *et al.* 2007). This finding was in complete contrast to our initial hypothesis that inactivation of *Ccr2* would reduce arthritis in these mice. Given that *Ccr2*^{-/-} mice had a reduced level of a specific subset of DCs, we next asked the question if increasing the number of these dendritic cells could protect against the development of arthritis.

To determine if DCs were capable of suppressing autoimmunity by expanding FoxP3⁺ regulatory T cells (T_{reg}), made use of the SKG mouse model of experimental arthritis. In this T cell-dependent mouse model, chronic autoimmune arthritis develops from a point mutation in the T cell receptor-signaling molecule (ZAP-70 mutation) (Sakaguchi *et al.* 2003). The mutation manifests in thymic positive selection and failure in negative selection of highly self-reactive T cells including potentially arthritogenic T cells (Wakasa-Morimoto *et al.* 2008). Autoimmune disease in SKG mice mimics the clinical and immune pathologies of RA including the development of joint inflammation, inflammatory cell infiltration, extra-articular lesions, cartilage and bone destruction, and autoantibodies (Wakasa-Morimoto *et*

al. 2008). Consequently, SKG mice serve as a good model to investigate the T cell contribution to arthritis in *Ccr2*^{-/-} mice.

Here we show that CD8α⁺ DCs play a central role in modulating RA using the two models of RA described above. We hypothesized that a numeric deficiency in DCs was responsible for enhanced arthritis seen in *Ccr2*^{-/-} mice. Examination of SKG mice and CIA in DBA/1J mice showed that the arthritis in these mice was not T cell-dependent but rather due to a quantitative defect of DCs.

Materials and Methods

Materials

RPMI 1640, antibiotics, FCS, PBS, were obtained from Invitrogen (Carlsbad, CA). Conjugated antibodies CD11b, CD8α, CD11c, CD25, CD4 and isotype-matched controls were obtained from BD Biosciences (San Diego, CA). FoxP3 antibody was from eBioscience (San Diego, CA). Collagenase D was purchased from (Roche, Indianapolis, IN). Collagen type II was from Elastin Products Co. (Owensville, MO). All other chemicals used were from Sigma (St. Louis, MO).

Mice

The generation of *Ccr2* gene knockout mice was previously described (Kuziel *et al.* 1997). Male *Ccr2*^{+/+} (wild type) and *Ccr2*^{-/-} mice, 8–10 weeks of age, were all maintained on DBA/1J background (n>10 generations). DBA/1J and C57Bl/6J control mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). SKG (Balb/c) mice, 12 weeks of age, were maintained as *Ccr2*^{+/+} or as *Ccr2*^{-/-} (BALB/c) (n>6 generations). Mice were housed and bred under specific pathogen-free environment the University of Texas Health Science Center at San Antonio, TX and the Institutional Animal Care and Use Committee approved all procedures.

CIA immunization

Preparation of native bovine collagen type II (CII) was done as previously described (Quinones *et al.* 2004). For CIA, mice were immunized with intradermal (i.d.) injections of CII (in complete Freund's adjuvant; CFA) at the base of the tail to induce arthritis.

Flt3L administration

A set of *Ccr2*^{+/+} or *Ccr2*^{-/-} (DBA/1J) mice received one round of daily intraperitoneal (i.p.) injections of either recombinant human Flt3L (10 µg) or phosphate buffered saline (PBS) for 10 days, prior to CII immunization. The mice were immunized with CII-CFA after the initial set of Flt3L injections and a booster immunization was given after the second set of Flt3L injections. Mice were sacrificed 30 days after the CII booster immunization.

Zymosan administration, tissues and clinical assessment in SKG mice

SKG mice were all primed with an intraperitoneal (i.p.) injection of zymosan, 2.0 mg/ml. The incidence and severity was measured weekly for 5 weeks post-injection. At the time of sacrifice, the spleen and blood samples were collected. The production of autoantibodies in serum samples was measured by ELISA (CII, rheumatoid factor, and zymosan). Mice were monitored daily for the incidence of RA.

Immunohistochemistry

At necropsy, whole joints (paws and feet) from Flt3L-treated *Ccr2*^{-/-} (DBA/1J) mice were collected, tissues formalin-fixed in, and decalcified (10% EDTA) for paraffin embedding.

Sections 5 μm thick were cut and stained for the macrophage marker ER-HR3. Briefly, sections were deparaffinized, hydrated and washed with PBS, followed by antigen retrieval at high temperature in Tris-HCl pH 9.0. Endogenous peroxidase was inactivated using 3% H_2O_2 in 1% NaN_3 for 30 min and nonspecific binding was blocked using an avidin-biotin blocking kit and 5% BSA. ER-HR3 antibodies were used at a concentration of 1:100. Following 1 hr incubation at RT, sections were washed and incubated with their respective secondary antibodies for an additional 45 min at RT. Next, sections were washed and incubated with ABC-HRP (Vector Labs. Burlingame, CA) and subsequently color reaction was developed with Vector NovaRed (Vector Labs. Burlingame, CA). ABC-HRP and Vector NovaRed were used following the manufacturer's instructions. Finally, sections were counterstained with Gill's Hematoxylin and mounted permanently with mounting media.

Isolation of DCs

DCs were isolated from the spleen in a similar protocol as described by Inaba, K. et al. (Inaba *et al.* 2001). In brief, spleens from WT mice were collected and digested with 400U of Collagenase D in HBSS. After incubation for 15 min, a single cell suspension was obtained; cells were centrifuged, resuspended and digested by an additional 15 min in 100U of collagenase D. Cells were centrifuged and RBCs were lysed. Cell suspensions were counted, resuspended in FACS buffer and stained with the following antibodies: CD11c, and CD8 α . DCs expressing both markers were sorted using a BD FACS Aria (BD Biosciences, San Jose, CA).

Transfer of DCs

CD11c⁺ and CD8 α ⁺ DCs were sorted and adoptively transferred in mice. *Ccr2*^{-/-} (DBA/1J) mice received either PBS or 120,000 CD11c⁺ CD8 α ⁺ DCs (in 200 μl of PBS) intravenously (i.v.) into the tail vein. Injections of DCs were administered 2 times per week for a total of 3 weeks. Mice received a total of 960,000 DCs and were followed clinically for 30 days after the final injection.

Clinical assessment of RA

The severity of RA was measured over the course of disease progression and assigned a clinical score, as previously described (Quinones *et al.* 2004). Briefly, joint swelling was examined and clinically scored based on severity. The severity was determined by assignment of swelling/erythema of hind and fore paws from grade 0 (no swelling or erythema) to grade 4 (severe swelling with joint rigidity or deformity). Clinical score of severity was determined from the average joint involvement of hind and fore paws.

Measurement of T_{reg}

Spleens were harvested from *Ccr2*^{-/-} (DBA/1J) treated with DCs and homogenized to obtain a single-cell suspension. Red blood cells were lysed, splenocytes were counted, resuspended in FACS buffer and surface stained for CD4 and CD25. After fixation/permeabilization cells were stained for FoxP3 and analyzed by FACS to quantify the number of T_{reg}.

Quantification of active TGF β by T cells

Spleens from *Ccr2*^{-/-} (DBA/1J) mice receiving either DCs or PBS were collected. Splenocytes were stimulated in culture with CD3 and CD28 or control (PBS) in culture for 72 hours and the levels of active murine TGF β 1 were quantified using the ELISA kit from R&D (Minneapolis, MN) according to the manufacturer's directions.

Statistical analyses

Data are presented as mean $\pm$ SEM. Groups were analyzed using Stata (StataCorp, College Station, TX). According to the distribution of the data (normal or non-normal), comparisons were done using parametric or non-parametric methods of analysis. Statistical significance was accepted at $p \leq 0.05$.

Results

Reduced level of CD8 α^+ DCs in *Ccr2*^{-/-} (DBA/J) mice

Consistent with our previous results in the C57Bl/6J, *Ccr2*^{-/-} mice in the DBA/1J background were significantly deficient ($p < 0.05$) in CD11c⁺ CD8 α^+ DCs, at baseline compared to controls (Fig. 1). Based on these observations, we determined that the quantitative defect of baseline DCs could also translate into a defect in CIA leading to the arthritic pathogenesis in absence of *Ccr2*. Taken together, the DCs deficiency along with our previous finding that these cells are hematopoietic-derived (Sato *et al.* 2000) suggested to us that DCs are the link between *Ccr2* and tolerance to autoimmune arthritis.

Flt3L treatment increased the susceptibility of CIA in *Ccr2*^{-/-} mice

To investigate the effect of increasing the proportion of DCs on the arthritic phenotype, we administered repeated injections of Flt3L in *Ccr2*^{-/-} mice thereby testing the hypothesis that expansion of DCs in these mice would ameliorate CIA (Fig. 2A). As previously published, the onset of early severe arthritis in the *Ccr2*^{-/-} mice occurred within 4–6 weeks of the first immunization, whereas the control mice remained asymptomatic (Fig. 2B–C). Mice treated with Flt3L displayed heightened and sustained incidence (*Ccr2*^{-/-} Flt3L-treated: vs *Ccr2*^{-/-} PBS-treated, day 53), similar to controls (*Ccr2*^{+/+} Flt3L-treated vs WT PBS-treated, day 53). Unexpectedly, the severity was higher in animals receiving FLT3L (*Ccr2*^{-/-} Flt3L-treated vs *Ccr2*^{-/-} PBS-treated, and *Ccr2*^{+/+} Flt3L-treated vs *Ccr2*^{+/+} PBS-treated) (Fig. 2B–C), contrary to its beneficial effects in autoimmune diabetes (O’Keeffe *et al.* 2005). It was anticipated that Flt3L-mediated enhancement of DCs would improve the phenotype, however this was not the case. In fact, the incidence of arthritis in Flt3L-treated control mice developed to a similar extent as the PBS-treated *Ccr2*^{-/-} mice (Fig. 2B). Moreover, treatment with Flt3L increased the susceptibility to arthritis in both the absence and presence of *Ccr2*, however a statistical significance was not reached at any of the weeks examined or in any combination of the groups ($p = 1.000$, Fisher’s exact test). An examination of the mean arthritis severity (clinical score) showed that Flt3L treatment in either *Ccr2*^{+/+} and *Ccr2*^{-/-} mice did not improve. Similar to our previously published results in C57Bl/6J mice, *CCR2* deficiency alone significantly exacerbated arthritis in PBS-treated mice ($p = 0.000$, Kruskal-Wallis test at days 21, 30, 41, and 53) (Fig. 2C). Interestingly, Flt3L treatment in both *Ccr2*^{+/+} and *Ccr2*^{-/-} mice significantly worsened disease in mice *Ccr2*^{-/-} compared to *Ccr2*^{+/+} mice ($p = 0.003$, Kruskal-Wallis test at days 30, 41, and 53). Examination of specific autoantibodies for CII corroborated our findings (data not shown). The severity of disease after Flt3L treatment in the same mice was further appreciated by histology showing joint inflammation and cell infiltrate in the synovium after Flt3L treatment (Supplementary Fig. 1). Histopathological analysis of the inflamed joints was documented in sections of Flt3L-treated *Ccr2*^{+/+} and *Ccr2*^{-/-} mice. The observed joint inflammation was associated with macrophage recruitment (Supplementary Fig. 1). It is noteworthy to mention that Flt3L treatment increased the absolute number of both the classic (CD11c⁺CD11b⁺CD8 α^-) and tolerogenic (CD11c⁺CD11b⁺CD8 α^+) subsets of DCs (data not shown). Collectively, Flt3L-mediated increase of total DCs were not sufficient to reduce the arthritis susceptibility, and in fact treatment with the cytokine Flt3L augmented the risk of autoimmune arthritis.

Reduced CIA after administration of DCs in *Ccr2*^{-/-} mice

The consequence of adoptive transfer with control (*Ccr2*^{+/+}) DCs to CII-immunized *Ccr2*^{-/-} mice was evaluated to test the hypothesis that DCs are required for reducing the arthritis susceptibility in *Ccr2*^{-/-} mice (DBA/1J). To this end, mice received two i.v. injections per week consisting of 120,000 DCs over the course of three weeks. We found that three weeks of direct supplementation with DCs did not improved the arthritic incidence (Fig. 3A). On the other hand, there was a significant reduction in the severity of CIA in *Ccr2*^{-/-} mice (Fig. 3B). The levels of autoantibodies for IgG1 and RF were comparable in *Ccr2*-deficient mice, however IgG2a titers for CII antibodies were significantly reduced in D mice treated with DCs, compared to controls ($p < 0.05$) (Fig. 3C–E) and these levels correlate with the decrease in CIA. These data are indicative of a tolerogenic role for DCs and they act in part via CCR2, during the early development and progression of CIA.

Splenic T_{reg} produced Increased TGFβ-1 expression in *Ccr2*^{-/-} mice treated with DCs

Since peripheral tolerance involves the suppression of immunity by CD4⁺CD25⁺ FoxP3⁺ T_{reg} cells, we examined the numbers of T_{reg} in CII-immunized *Ccr2*^{-/-} mice receiving DCs. The percentages of Treg were not significantly different in CIA *Ccr2*^{-/-} mice compared to PBS-treated controls (Fig. 3F). Given that T_{reg} control effector T cells by suppressing other lymphocytes through cell-cell contacts and by the release of TGFβ-1 (Miossec 2008; Wing *et al.* 2010), we also examined the production of active TGFβ-1. Looking for intrinsic T cell differences, we stimulated CII-immunized T cells from *Ccr2*^{-/-} mice with anti-CD3/CD28 and determined that TGFβ-1 (26.5±0.02 pg/ml) was significantly increased in mice treated with DCs compared controls (22.5±0.78 pg/ml) (Fig. 3G). The significant increase in TGFβ-1 production was not accompanied by an increase in T_{reg}, suggesting that the protective effect of DCs occurred through the augmented immunosuppressive effects of T_{reg}-induced TGFβ-1.

Increased CIA severity and incidence in *Ccr2*^{-/-} mice are T cell-independent

In view of the fact that CIA is regarded as a T- and B cell-dependent process, involving both the innate and acquired immune systems, we sought to determine the contribution of T cell-dependent mechanisms on CIA in *Ccr2*^{-/-} mice. To this end, we genetically inactivated CCR2 in SKG mice to test the hypothesis that CCR2 acts independent of T cell selection. Zymosan-treated SKG x *Ccr2*^{-/-} mice showed a trend towards reduced spleen DCs compared to control animals, however, the difference did not reach significance (Fig. 4A). The incidence and severity (Fig. 4B–C) of autoimmune arthritis between SKG x *Ccr2*^{-/-} mice and control mice were quite similar across a five-week period (Fig. 4B–C). Given that SKG mice alone (Sakaguchi *et al.* 2003) and CII-immunized *Ccr2*^{-/-} mice (Quinones *et al.* 2004) are independently capable of producing high autoantibody, we measured the serological autoimmune response in SKG x *Ccr2*^{-/-} mice. Circulating antibodies were initially high in SKG x *Ccr2*^{-/-} mice for specific CII (IgG1: 1.04±0.61 vs 0.13±0.04 optical density), RF (IgM: 1.27±0.06 vs 0.13±0.04 optical density), and zymosan antibodies (IgG1: 1.04±0.61 vs 0.13±0.04 and IgG2a 0.97±0.02 vs 0.06±0.22 optical density), nevertheless these levels tapered off across serial dilutions similar to controls (Fig. 4D–G). Based on the finding that the inflammatory arthritis persisted in the same way as controls, we determined that the T cells defect driving arthritis in SKG mice did not exacerbate the already heightened arthritis seen in CCR2 deficient mice.

Discussion

We previously demonstrated that *Ccr2*^{-/-} mice became highly susceptible to autoimmune arthritis in response to CII-immunization (Quinones *et al.* 2004). In this study we explored the mechanism behind severe CIA in *Ccr2*^{-/-} mice by cross-comparing two models of

experimental autoimmune arthritis (arthritis-prone DBA/1J and SKG mice). We first showed that *Ccr2*^{-/-} (DBA/1J) mice exhibited a significant defect in DCs. These results were in accord with our previous study in non-infected *Ccr2*^{-/-} (C57Bl/6J) mice, where lower numbers of DCs were displayed in unchallenged as well challenged conditions to *Leishmania major* (Sato *et al.* 2000) suggesting that the DCs defect transcended across different strains of mice. Previously, we showed that *Ccr2*^{-/-} mice had an enhanced susceptibility to experimental arthritis and that disease onset occurred much earlier and more robust than in control mice (Quinones *et al.* 2004). Here we show that repeated injections of Flt3L to CII-immunized *Ccr2*^{-/-} mice did not reverse the severe arthritic phenotype of *Ccr2*^{-/-} mice, rather Flt3L treatment augmented CIA in both *Ccr2*^{-/-} mice and control mice. Contrary to a previous study suggesting that Flt3L could be used to treat autoimmune diseases (O’Keeffe *et al.* 2005), our results showed that Flt3L treatment increased the susceptibility and incidence of CIA in *Ccr2*^{-/-} and control mice. The outcome seemed to have been the result of a non-specific increase of both DCs and non-DCs by Flt3L; indeed, *in vivo* administration of rhFlt3L was previously shown to increase the total numbers of DCs including CD4⁻CD8⁻ and CD4⁻CD8⁺ subsets (O’Keeffe *et al.* 2002). Furthermore, caution should be exercised when using Flt3L as a therapeutic treatment of autoimmune arthritis. Our results prompted us to investigate specifically the ability of DCs to regulate the severe arthritic phenotype.

DCs induce tolerance and hold the potential of being therapeutic in autoimmunity. Since numerous publications have described tolerogenic DCs as targets of immune therapy and specifically as a treatment of RA (Khan *et al.* 2009; Lebre *et al.* 2009; Morelli *et al.* 2007), in this study we focused on the specific role of DCs in the pathogenesis of arthritis. Results presented here assigned DCs as inhibitors of CIA in *Ccr2*^{-/-} (DBA/1J) mice. In line with these results, DCs arising from TNF α or IL-10-treated BM cells ameliorated CIA in DBA/1J mice compared to LPS stimulated controls (Healy *et al.* 2008; van Duivenvoorde *et al.* 2007). Unique to our study was treatment with a purified population of CD11c⁺CD11b⁻CD8 α ⁺ DCs. The DCs were sorted by flow cytometry and administered after the initial CII-immunization twice per week for a period of three weeks. Early treatment with pure DCs significantly reduced CIA severity and the reduced autoantibody production confirming our hypothesis that DCs are key regulators of immune responses in autoimmune arthritis. Further, our results highlighted the protective effect of DCs mediated via *Ccr2*.

Recent evidence has accumulated that DCs stimulate the expansion of T_{reg} that suppress the autoimmune response (Li *et al.* 2008; Swee *et al.* 2009; Taylor *et al.* 2008; Yamazaki *et al.* 2009). It is well recognized that self-tolerance involves suppression by CD4⁺CD25⁺FoxP3⁺ T_{reg} cells. Here, we found the early percentage of splenic CD4⁺CD25⁺FoxP3⁺ T_{reg} between mice treated with DCs and controls was almost identical, yet we previously reported an increase in the percentage and total numbers of activated CD4⁺CD44⁺ T cells from the draining lymph nodes in CII-immunized *Ccr2*^{-/-} mice (Quinones *et al.* 2004). It is noteworthy to mention that our study spanned three weeks where differences were undetectable. It is possible that at later time points, over the course of the disease, significant differences in T_{reg} numbers may become apparent. Interestingly, *Ccr2* is expressed on a subset of T cells and have been shown positive for markers of T_{reg} (CD25⁺CD105⁺). These cells were identified at later stages of CIA and were capable of suppressing T and B cell proliferation (Bruhl *et al.* 2004; Quinones *et al.* 2005). Nevertheless, DCs have been shown to expand Tregs (Akbari *et al.* 2001; Bhattacharya *et al.* 2011; Huang *et al.* 2010; Wang *et al.* 2008; Yamazaki *et al.* 2008) and the presence of TGF β -1 is central to this process (Wang *et al.* 2008). Taking our results a step further, we investigated the levels of TGF β -1, a key immunosuppressive mediator of T_{reg}, and found a significant increase of TGF β -1 in mice treated with DCs. It is possible that TGF β -1 was sufficiently augmented to reverse the

effects of CIA in *Ccr2*^{-/-} mice. One mechanism by which that could be possible is via the induction of FoxP3⁺ cells and their direct influence over the effector T cell immune responses. It was recently described that TGFβ-1-induced FoxP3 sustains further the expression of T_{reg} while repressing IL-23R expression, thereby suppressing Th17 differentiation and expression of IL-17 (Zhou *et al.* 2008). In line with these results, the increased TGFβ-1 was accompanied by the production of less CII autoantibodies in *Ccr2*^{-/-} mice treated with DCs.

Genetic inactivation of *Ccr2* in zymosan-treated SKG (Balb/c) mice with spontaneous induction of arthritis produced a minor increase in the incidence of arthritis and in the production of RF autoantibodies. However, no other parameters examined were significantly different from controls. We had anticipated that if T cells were involved, the arthritic incidence and severity in SKG x *Ccr2*^{-/-} mice would have been exaggerated compared to that seen in SKG mice, alone. Since loss of *Ccr2* did not significantly alter the SKG phenotype, we conclude that *Ccr2* is not acting through a T cell-mediated process. Moreover, since the T cell self-reactivity in SKG mice was shown to be dependent on APC-induction of IL-6 and on the generation of Th17 cells (McInnes *et al.* 2007) we can say that the *Ccr2* arthritic phenotype is Th17-independent, and produced exclusive of T cell receptor function. Therefore, it is more likely that enhanced phenotype of *Ccr2*^{-/-} mice is not linked to a T cell defect but rather to reduced DCs.

In conclusion, *Ccr2*^{-/-} mice in the arthritis-susceptible DBA1/J strain possess a quantitative defect of DCs. The Flt3L-induced expansion of non-specific DCs was not sufficient to overcome the enhanced CIA. By contrast, CIA was ameliorated through the specific increase of DCs. Furthermore, the *Ccr2*-dependent protective effect of DCs in CIA was mediated via the increased expression of TGFβ-1 and its immunosuppressive effects. These results substantiate a role for DCs in the pathogenesis of autoimmune arthritis. We propose that DCs deficiency contribute to the severe arthritic phenotype of *Ccr2*^{-/-} mice.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

DC	dendritic cells
RA	rheumatoid arthritis
CAIA	collagen antibody induced arthritis
CIA	collagen induced arthritis
BM	bone marrow
T_{reg}	regulatory T cells
Ccr2	CC-chemokine receptor-2
Flt3L	fms-like tyrosine kinase 3 ligand
APC	antigen presenting cell

CII	type II collagen
CFA	complete Freund's adjuvant

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Figure 1.

In the absence of disease DC are reduced in *Ccr2*^{-/-} mice in arthritic-prone DBA/1J background. DC (CD11c⁺, CD11b⁻ CD8α⁺) were quantified from single-cell suspensions from spleens of *Ccr2*^{+/+} (n=9) and *Ccr2*^{-/-} (n=12) mice using fluorescent-labeled antibodies. Data represents mean±SEM.

Figure 2. Flt3L treatment does not prevent arthritis in arthritic-prone mice deficient in *Ccr2* (A–B) Mice were treated with rhFlt3L (10 μ g) i.p. for ten consecutive days, challenged with CII and the regime repeated (n=5/group). (A) Schematic of treatment protocol where day 0 marks collagen type II and complete Freund's adjuvant (CII-CFA) immunization and day 30 denotes the end of Flt3L treatment and time of CII booster administration. (B) Mean arthritis incidence from weekly measurements, post-CII immunization. Mice treated with Flt3L did not improve the incidence over PBS-treated mice rather Flt3L significantly increased the incidence of *Ccr2*^{-/-} mice above *Ccr2*^{+/+} treated with Flt3L (p=1.000 by Fisher's exact test at days 30, 41 and 53). (C) The mean arthritis severity (clinical score) significantly increased in Flt3L-treated *Ccr2*^{-/-} mice (p=0.003 by Kruskal-Wallis test at days 30, 41 and 53) and in *Ccr2*^{+/+} mice (p=0.003). Data presented as mean $\pm$ SEM of 5 mice/group. Statistical significance denoted by an asterisk (*) for p=0.000 for PBS-treated *Ccr2*^{+/+} vs *Ccr2*^{-/-} mice and (**) for p=0.003 Flt3L-treated *Ccr2*^{+/+} vs *Ccr2*^{-/-} mice.

Figure 3. Direct administration of CD8α⁺ DC improved the early arthritis incidence, severity and levels of autoantibodies produced in mice deficient of Ccr2 (DBA/J) mice
 (A–E) Mice received biweekly injections of CD11c⁺ CD8α⁺ DC (120,000 DC/injection) on the same day as collagen administration and during the disease progression. The mice were monitored for clinical follow up for 3 weeks (n=6/group). (A) Maximum joint incidence of arthritis for *Ccr2*^{-/-} not receiving CD8α⁺ DC (38%) was greater when compared to those that received CD8α⁺ DC (6%) mice. (B) Severity of arthritis decreased with CD8α⁺ DC treatment (DC: 0.04±0.04 vs PBS: 0.46±0.18). (D–E) CII and zymosan autoantibodies measurements at 1–3 weeks in serially diluted serum showed a significant reduction in the levels of IgG2a produced in mice receiving DC. (F) Percent gated of CD4⁺CD25⁺FoxP3⁺ Treg from spleen of DC-treated *Ccr2*^{-/-} mice (*p*=ns). (G) Active TGFβ-1 production from anti-CD3/CD28-stimulated serum samples. Data presented as mean±SEM of 6 mice/group and with statistical significance by t test was reached at *p*<0.05 denoted by an asterisk (*).

Figure 4.

CD8α⁺ DC counts, incidence, severity and autoantibodies in SKG x *Ccr2*^{-/-} (DBA/1J) mice. (A–E) Mice were primed with zymosan i.p. and monitored for 5 weeks (n=3). (A) Represents the DC (CD11c⁺CD11b⁻CD8⁺) from *Ccr2*^{+/+} (40x10⁵ ± 10 x10⁵ cells) and *Ccr2*^{-/-} (26x10⁵ ± 5x10⁵ cells) spleens. (B) Maximum joint incidence of arthritis for *Ccr2*^{+/+} (100%) and *Ccr2*^{-/-} (100%) mice. (C) Clinical scores from arthritic paws in *Ccr2*^{+/+} (2.8±0.7) and *Ccr2*^{-/-} (3.3±0.6) mice. (D–G) Elevated production of collagen and zymosan autoantibodies from measurements at 5 weeks post-zymosan in serially diluted serum (n<3). Asterisk (*) indicates statistical significance p<0.05.