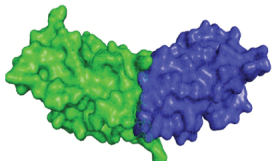

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Plasmacytoid Dendritic Cells Regulate Breach of Self-Tolerance in Autoimmune Arthritis[1]

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Achieving remission in rheumatoid arthritis (RA) remains elusive despite current biological therapeutics. Consequently, interest has increased in strategies to re-establish immune tolerance to provide long-term disease suppression. Although dendritic cells (DC) are prime candidates in initiating autoreactive T cell responses, and their presence within the synovial environment suggests a role in generation and maintenance of autoreactive, synovial T cell responses, their functional importance remains unclear. We investigated the contribution made by plasmacytoid DCs (pDCs) in the spontaneous breach of tolerance to arthritis-related self proteins, including rheumatoid factor, citrullinated peptide, and type II collagen observed in a novel arthritis model. Selective pDC depletion in vivo enhanced the severity of articular pathology and enhanced T and B cell autoimmune responses against type II collagen. pDC may offer a net anti-inflammatory function in the context of articular breach of tolerance. Such data will be vital in informing DC modulatory/therapeutic approaches. *The Journal of Immunology,* **2009, 182: 963–968.**

Rheumatoid arthritis (RA)[4] is a chronic inflammatory disorder characterized by a prearticular phase in which autoimmunity is manifest as the presence of serum autoantibodies, followed by transition to clinical presentation associated with synovial inflammation and related destruction of cartilage and bone. Genome-wide genetic analyses identify risk association with, for example, HLADR4, *PTPN22*, *CTLA4*, and *STAT4* providing robust evidence for a role for adaptive immune mechanisms in RA pathogenesis (1). Recently, clinical benefits have accrued from use of agents, such as abatacept and rituximab, targeting components typically implicated in adaptive immune response (2–4). In parallel, however, convincing data implicate innate immune mechanisms in disease including the identification of a plethora of proinflammatory cytokines, pathogen-associated molecular patterns, and damage-associated molecular patterns expressed in synovium (5) together with the presence of activated neutrophils, NK cells, mast cells, and macrophages (6). Therapeutic strategies targeting innate cytokines such as TNF have had significant clinical impact (1, 6). Nevertheless, many patients remain refractory to current biological interventions and, for those that do respond, true remission associated with re-establishment of immunologic tolerance is rare (1, 6). Dendritic cells (DC) lie at the juxtaposition of innate and adaptive immune function (7, 8). We are exploring the hypothesis that a plausible approach to achieving clinical remission may be by manipulation of DC subsets in disease tissues to capitalize on their potent, inherent immune regulatory properties.

Much phenotypic evidence depicts synovial DCs as cells of varied maturation status, capable of Ag presentation and cytokine production (9, 10). Recently, attention has focused upon the relative contribution of distinct DC subsets to inflammatory processes, namely myeloid (mDC) and plasmacytoid (pDC) (11–13). Thus far, mDC appear to exhibit a primarily proinflammatory effector phenotype, whereas rather less is known of pDC function (11–13). Few data are available, however, concerning the net functions in vivo of such subsets in arthritis induction and particularly in regulating breach of tolerance to arthritis-related autoantigens. We previously observed that exogenously activated and Ag-pulsed mDC induce collagen-specific T cell responses in vivo and can thereby induce inflammatory arthritis (14). To our knowledge, the role of pDC in vivo in arthritis induction has not been explored. pDC have been demonstrated to suppress T cell responses in tumor draining lymph nodes (LNs) (15) and following Ag challenge in experimental asthma models (16), together predicting a net regulatory role for pDC. Moreover, such models explore primarily responses to exogenous Ag. The collagen induced arthritis (CIA) mode in DBA/1 mice is a standard tool for experimental studies in arthritis but lends itself relatively poorly to resolving the role of DC subsets in breach of tolerance to self. Autoimmunity in the CIA model is exogenously engineered by immunization with autoantigen (collagen type II (CII)) and immunostimulators (CFA). Recently, our laboratory has observed that mice receiving Th1 polarized, OVA-specific TCR transgenic (Tg) T cells develop arthritis that is associated with subsequent T and B cell responses to the self-protein, CII, abundantly expressed in the joint (17). The "endogenous" nature of the autoimmune response (that is distinct from the inciting Ag) in this model renders it ideal for investigating breach of immune tolerance in RA. We have used this novel

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[4] Abbreviations used in this paper: RA, rheumatoid arthritis; CCP, cyclic citrullinated peptide; CIA, collagen induced arthritis; CII, collagen type II; DC, dendritic cell; pDC, plasmacytoid DC; mDC, myeloid DC; Treg, regulatory T cell; Tg, transgenic; LN, lymph node; HAO, heat aggregated ovalbumin.

technology to address the fundamental question concerning the contribution of pDCs to the breach of tolerance and induction of arthritis in vivo.

Materials and Methods

Animals

DO11.10 BALB/c TCR Tg mice (18) were inhouse bred. Six- to 8-wk-old female BALB/c mice (Harlan Breeders) were used as recipients. Mice were housed in the University of Strathclyde and procedures were performed according to the U.K. Home Office regulations.

Adoptive transfer of OVA-specific Th1 cells and arthritis model

Arthritis was induced in BALB/c recipients by i.v. transfer of 2×10^6 in vitro, Th1 polarized, KJ1.26$^+$ T cells, followed 1 day later by restimulation in vivo with OVA/CFA and 10 days later heat aggregated ovalbumin (HAO), as described by Maffia et al. (17). Mice were then monitored for development of arthritis by measuring paw thickness with a dial caliper (Kroeplin) or by histological assessment at day 7 following HAO challenge (17). Disease scoring, based on cell infiltration (0 – no cells; 1 - <50 cells; 2 - 50 to 200 cells; 3 > 200 cells), proliferation of the synovial membrane (0 – no proliferation; 2 – more than 3 layers; and 3 - severe), and joint erosion (0 – no erosion; 1 - very mild; 2 - marginal; and 3 - complete) was performed on three joints for each of seven mice per group. An average paw score for each parameter was then calculated across all three joints. The total score for each paw was then calculated by adding the individual parameter scores.

Depletion of pDC in vivo

pDCs were depleted in vivo by i.p. injection of 200 μg 120G8 (Rat IgG1k) Ab (provided by Schering-Plough Research Institute) 1 day before, on the day of, and 2 days after s.c injection of HAO. Control mice received matched injection of either 200 μl PBS or 200 μg of the irrelevant isotype control Ab rat (IgG1k) anti-dog CD8 (a gift from Mark Frewin, Sir William Dunn School of Pathology, Oxford, U.K.). pDC depletion was confirmed by FACS analysis of mPDCA-1 (Miltenyi Biotec) expression on homogenized LN and spleen suspension (Fig. 1, *A–C*). Analysis of B220 (RA3-6B2; BD Pharmingen) and CD11c (HL3; BD Pharmingen) expression confirmed that conventional mDC and B cell populations were not affected by treatment with 120G8, whereas B220-positive CD11c low cells were (Fig. 1, *D–F*). This would be consistent with the previously reported ability of 120G8 treatment to specifically deplete pDCs (19).

Flow cytometry

Single-cell suspensions were prepared from axillary, inguinal, cervical, and mesenteric LN from DO11.10 mice by crushing under Nitex mesh (Cadisch Precision Meshes). For CD4 and Foxp3 analysis, aliquots of these cells were stained with FITC-conjugated CD4 (L3T4; BD Pharmingen) incubated in Cytofix/Cytoperm (BD Pharmingen) and stained with PE-labeled anti-Foxp3 (eBioscience) for the detection of T regulatory cells (Treg). Analysis by flow cytometry was performed using CellQuest software (BD Biosciences).

Restimulation of draining LN cells in vitro

Popliteal LN cells were restimulated with either medium alone, 1 mg/ml OVA, or 50 μg/ml collagen II (CII) (Sigma-Aldrich). Supernatants were harvested at 72 h for analysis of cytokine production, and proliferation was analyzed at 96 h by 3H incorporation as described previously (17).

In vitro activation of T cells by DC populations

A total of 5×10^4 CFSE-labeled DO11.10 TCR Tg T cells were cultured with 5×10^4 mDCs and 5×10^4 pDCs in 96-well round-bottom tissue culture plates for 72 h. The ability to present Ag was limited to one or both DC subset by preincubating either population overnight with 1 mg/ml OVA. DC populations were then washed before coculture removing residual Ag. Flow cytometric analysis was used to determine CFSE dilution by CD4$^+$ cells. mDCs were generated from the bone marrow of BALB/c mice by culture in GM-CSF-conditioned medium (20). pDCs were isolated by magnetic bead separation (pDC isolation kit II; Miltenyi Biotec) from Flt3L-treated BALB/c mice injected i.p. once daily with 10 μg purified human Flt3L (kindly provided by Amgen) for 10 days (21).

ELISA and multiplex bead cytokine analysis

Anti-OVA, anti-collagen, and anti-IgG2a Abs were detected by ELISA as previously described (17). Anti-cyclic citrullinated peptides (CCP) Ab levels were detected using DIASTAT anti-CCP kit (Axis-Shield). Multiplex bead cytokine analysis of IL-5 and IFN-γ in supernatant was conducted according to the manufacturer's instructions (Biosource).

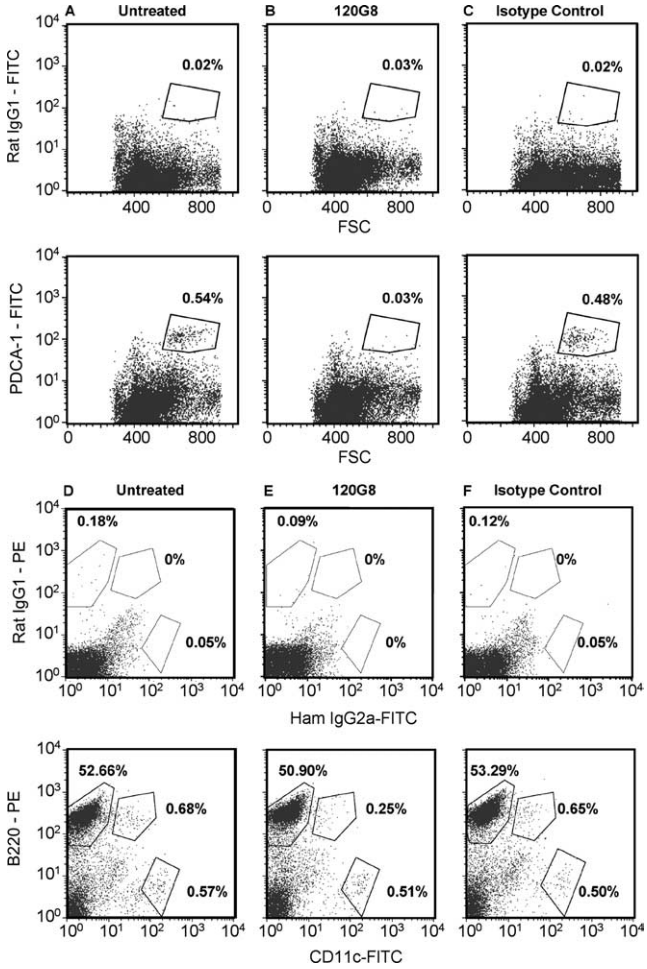

FIGURE 1. 120G8 depletes pDC in spleen and LN. The pDC and mDC in spleen and LN harvested on day 5 from BALB/c mice given (*A*) 200 μl PBS, (*B*) 200 μg 120G8 pDC-depleting Ab, or (*C*) 200 μg rat anti-dog CD8 IgG2a-irrelevant isotype control i.p. per day for 4 days was determined by staining with the pDC-specific mPDCA-1 or isotype control. Analysis of B220 and CD11c populations (*D–F*) revealed that B cell and mDC populations were not affected by treatment with 120G8. FACS analysis of LN samples are shown.

Statistics

Statistical analysis was done using Minitab software (Minitab), and results were compared using one-way ANOVA or single Student's *t* test. A *p* value of < 0.05 was considered significant.

Results

In vivo depletion of pDCs exacerbates immunopathology of experimental arthritis

We previously demonstrated that OVA-specific T cell transfer followed by peri-articular injection of HAO results in development of unilateral peripheral arthritis associated with spontaneous generation of responses to articular autoantigens such as CII (17). Unlike the CIA model, this experimental approach provides the opportunity to examine the contribution of pDC to breach of tolerance observed in arthritis. The role of pDC was analyzed through depletion with the mAb 120G8 (19) that induced satisfactory depletion of pDC in spleen and LN, as confirmed by FACS analysis (Fig. 1) as previously reported (19). Depletion of pDC at the time

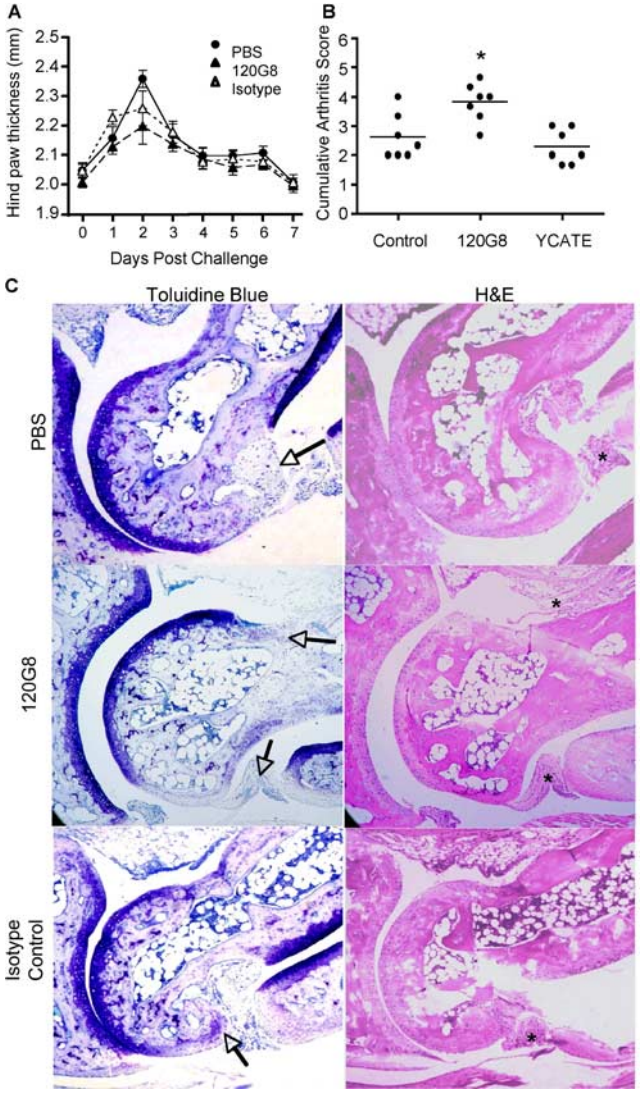

FIGURE 2. Depletion of pDCs enhances synovial hyperplasia, cartilage degradation and pannus invasion in experimental arthritis induced by breaking self-tolerance to joint specific Ags. *A*, The left-hind paw thickness in mm of PBS (●), pDC-depleted (▲), and isotype control (△) mice was measured using dial calipers just before challenge with HAO and every day thereafter for 7 days (days 0–7), $n = 10$. *B*, At day 7 after peri-articular OVA challenge to the left hind paw, mice were sacrificed and left-hind limbs were processed for histological analysis. Cartilage integrity was determined by toluidine blue staining and cellular infiltrate assessed by H&E. Three joints of each ankle were scored on infiltrate, synovial proliferation, and cartilage erosion, and the total score calculated as described in *Materials and Methods*. ∗, $p < 0.05$ compared with isotype control. *C*, Representative sections of control (PBS), pDC-depleted (120G8), and isotype control mice are shown. ↑ indicate lightened areas consistent with cartilage erosion. ∗ indicate areas of synovial hyperplasia and inflammatory cell infiltration. pDC-depleted mice demonstrated more extensive cartilage erosion, inflammatory cell infiltration, and synovial hyperplasia as compared with PBS and isotype control groups. $n = 7$ mice per group.

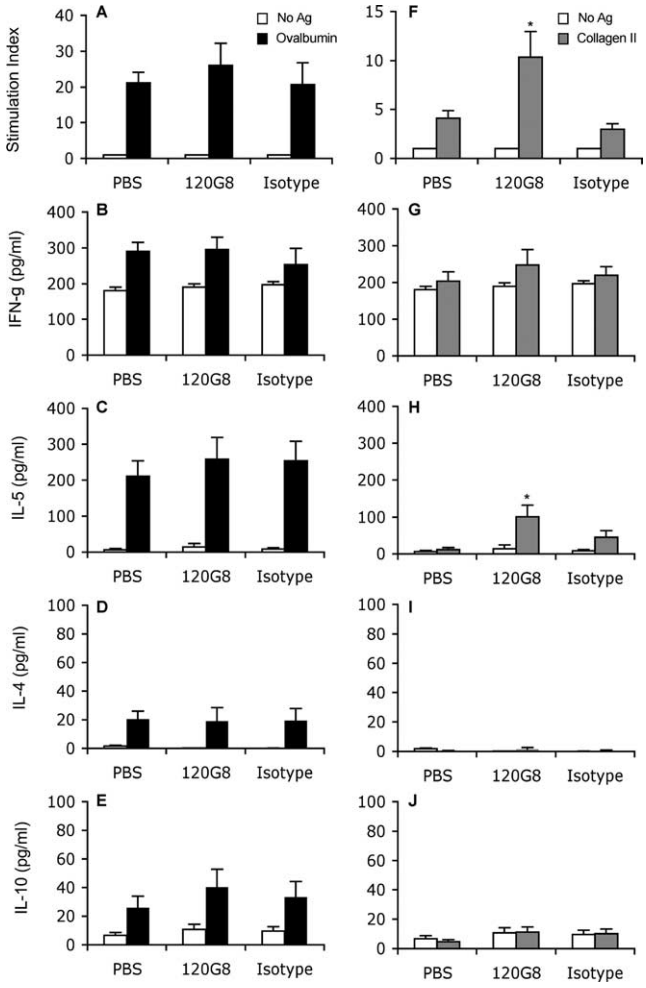

FIGURE 3. Depletion of pDCs increases collagen-specific proliferation ex vivo. *A* and *F*, Draining popliteal LN were collected at day 7 from PBS, pDC-depleted (120G8), and isotype control mice and cultured with medium alone, 50 μg/ml CII, or 1 mg/ml OVA. [^{3}H]Thymidine incorporation was measured at 96 h. Results are presented as the mean stimulation index ± SEM for each group, $n = 10$ per group. ∗ $p < 0.05$. Then, (*B* and *G*) IFN-γ, (*C* and *H*) IL-5, (*D* and *I*) IL-4, and (*E* and *J*) IL-10 levels were measured at 72 h. Data are the mean ± SEM for each group, $n = 10$ per group. ∗, $p < 0.05$ compared with isotype control.

of HAO challenge (day 0) did not produce differences in acute swelling observed between groups up to 1 wk after challenge (Fig. 2*A*). Upon histological examination, however, we observed an increased pathology score (Fig. 2*B*) and a distinct pathology (Fig. 2*C*) following pDC depletion. Mice ($n = 10$) that received i.p. PBS or irrelevant isotype control (rat anti-Dog CD8 IgG1k) exhibited moderate erosion of cartilage (toluidine blue staining) associated with mild inflammatory cell infiltration (H&E staining), commensurate with the relatively early time point in the model when we harvested joints for examination. In contrast, pDC depletion resulted in extensive synovial hyperplasia, cartilage degradation, and pannus invasion, suggesting that immune pathology was accelerated in this group. No footpad swelling was observed in the contralateral paw consistent with the localized nature of this model (data not shown). Together these data suggest that pDC are exerting subtle, net suppressive, effects in this model.

In vivo depletion of pDCs increases autoimmune T cell responses

Our primary objective in this study was to address the role of pDC in the development of autoreactivity. Single-cell suspensions prepared from the draining popliteal LN from each individual mouse were cultured with or without previously optimized concentrations of CII (50 μg/ml) or OVA (1 mg/ml). As expected, given the Th1 polarized OVA-specific cells transferred in the model, all groups exhibited similar levels of proliferation and cytokine production in the presence of OVA (Fig. 3, *A–E*). In contrast, LN cultures from the pDC-deplete group exhibited significantly increased levels of

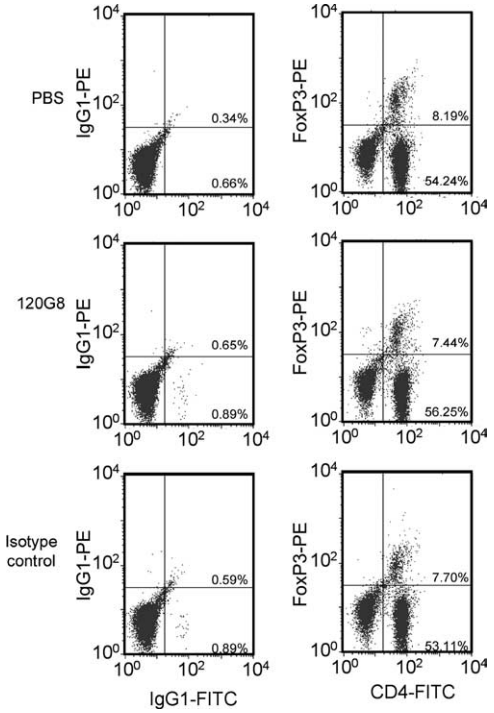

FIGURE 4. Depletion of pDCs does not alter number of Foxp3$^+$ Tregs in the draining LN. The percentage of CD4$^+$/Foxp3$^+$ (Tregs) in the draining LN of mice receiving PBS, pDC-depleting Ab (120G8) or isotype control was measured at day 7, in single-cell suspensions by FACS analysis. The percentage of CD4$^+$/Foxp3$^+$ cells in all groups was comparable. One representative FACS plot from each group is shown.

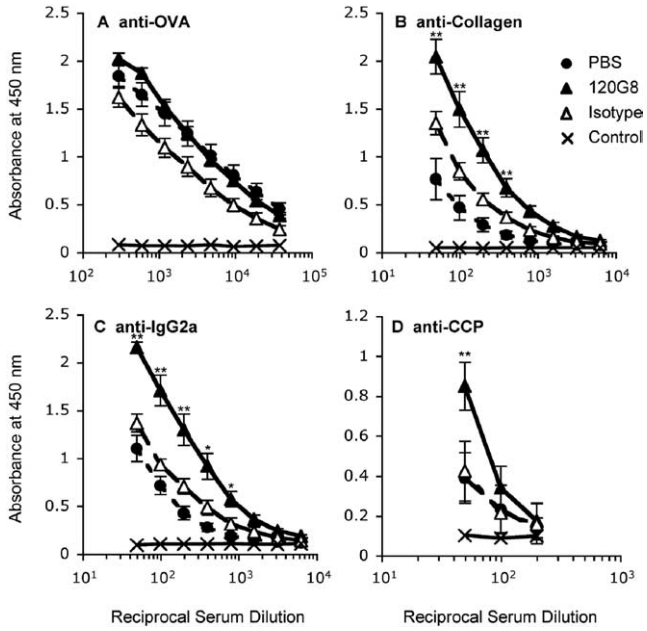

FIGURE 5. Depletion of pDC increases serum anti-OVA Ab, anti-collagen Ab, anti-IgG2a Ab, and anti-CCP Ab. Then, (*A*) serum anti-OVA IgG2a, (*B*) anti-collagen IgG2a, (*C*) anti-IgG2a, and (*D*) anti-CCP Ab levels were measured on day 7 from control (PBS), pDC-depleted (120G8) and isotype control mice by ELISA using doubling serum titrations. Serum levels were measured for each individual animal per group with results presented as mean ± SEM, $n = 10$ mice per group. *, $p < 0.05$, ** < 0.01, 120G8 vs isotype control.

autoantigen (CII)-specific proliferation compared with PBS ($p = 0.028$) and isotype control ($p = 0.011$) groups (Fig. 3*F*), consistent with an enhanced breach of self-tolerance. Previous studies indicate that the course of RA synovitis is determined by the balance between pro-(Th1) and anti-(Th2) inflammatory cytokines (22), while Ag presentation by pDC has been shown to influence the development of these responses (23). Analysis of IFN-γ release indicated that CII-specific Th1 responses were not significantly altered in the pDC-depleted group compared with PBS and isotype controls (Fig. 3*G*). Although, IL-5 release was enhanced compared with isotype control ($p = 0.049$) (Fig. 3*H*), there was no corresponding increase in production of other Th2 cytokines, IL-4 (Fig. 3*I*) and IL-10 (Fig. 3*J*), in response to CII. These data suggest that within the highly Th1-polarized environment induced in the model, depletion of pDCs does not act to alter the Th1/Th2 polarization of the nascent CII response.

Depletion of pDCs does not alter number of Foxp3$^+$ Tregs in the draining LN

The regulation of self-reactive effector responses by specialized populations of Tregs is considered a major mechanism by which tolerance is maintained and autoimmune disease avoided. We, therefore, examined the number of CD4$^+$Foxp3$^+$ Tregs in single-cell suspensions prepared from the draining popliteal LN from each treatment group by FACS analysis. No significant alterations in the number of CD4$^+$Foxp3$^+$ Tregs was observed comparing pDC deplete (7.44%), PBS (8.19%), or isotype control (7.70%) groups (Fig. 4). Moreover, we identified comparable CD4$^+$ expansion in the draining LN of all groups. These data suggest the observed alterations in T cell autoreactivity reflect local articular damage and release of CII in the context of damage.

Depletion of pDC increases serum autoantibody production

We next investigated autoreactivity in the B cell compartment. Serum obtained at day 7 was investigated for the presence of anti-OVA, anti-CII, IgG rheumatoid factor (anti-IgG2a as a surrogate), and anti-CCP Abs. Mice in each treatment group had high levels of serum anti-OVA Abs (Fig. 5*A*) confirming that transferred cells had delivered efficient B cell help. This was not altered by pDC depletion as T cell priming had already been achieved in vitro before cell transfer. In contrast, serum anti-CII, anti-IgG2a, and anti-CCP Ab titers (Fig. 5, *B–D*) were significantly increased in the pDC-deplete group as compared with PBS (CII, $p = 0.003$; IgG2a, $p = 0.001$; CCP, $p = 0.017$) and isotype control groups (CII, $p = 0.025$; IgG2a, $p = 0.017$; $p = 0.003$; CCP, $p = 0.003$). Together these data are consistent with an exacerbated breach of tolerance to self-Ags of particular relevance to inflammatory arthritis induced by specific pDC depletion.

Ag-specific effects of DC subsets on CD4$^+$ T cell cytokine production

The studies above suggested that pDC may act to inhibit activation of autoreactive T cells. To examine this further in a mechanistic manner, we adopted a reductionist in vitro approach. OVA TCR Tg T cells were incubated with equal numbers of pDC and mDC, but Ag presentation was restricted to one subset or other, or both (Fig. 6). Presentation of OVA by mDC in the presence of pDC that lacked Ag resulted in significant Ag-specific T cell proliferation (Fig. 6*A*), Th1 (IFN-γ; Fig. 6*B*), Th2 (IL-5 and IL10; Fig. 6, *C* and *D*), and inflammatory (TNF and IL-6; Fig. 6, *E* and *F*) cytokine production. In contrast, presentation of Ag by pDC in the absence of mDC Ag presentation did not result in significant T cell proliferation or cytokine responses. However, inclusion of Ag-presenting pDC acted to significantly inhibit Ag-presenting mDC induced

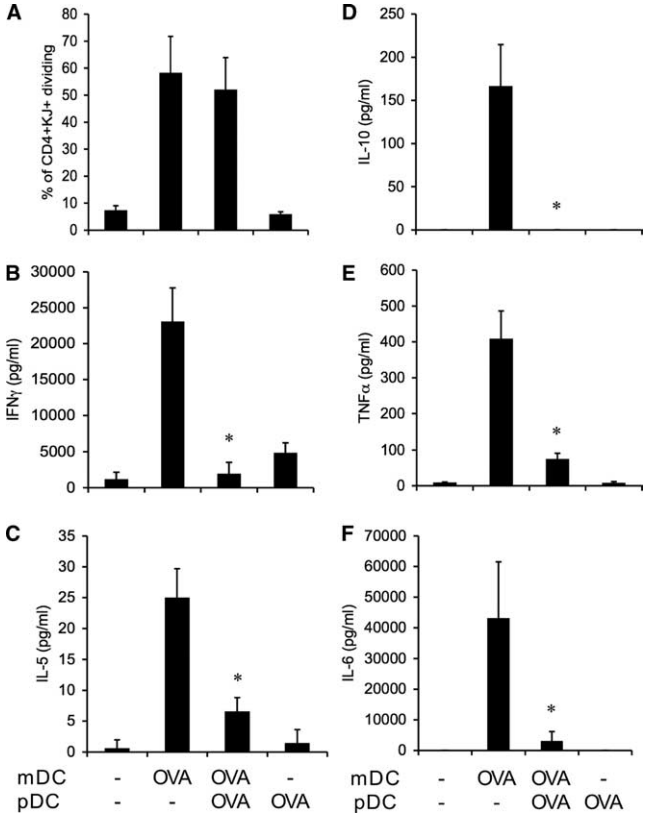

FIGURE 6. pDC inhibition of mDC-primed $CD4^+$ T cell cytokine production. CFSE-labeled DO11.10 $CD4^+$ cells were incubated for 72 h with mixed DC populations where either mDC, pDC, or both APC were pulsed with OVA unpulsed mDC and pDC; OVA mDC and unpulsed pDC; both pDC and mDC pulsed with OVA; unpulsed mDC and OVA pDC. The percentage of dividing DO11.10 T cells was determined by flow cytometric analysis of CFSE dilution in responding $KJ1.26^+$ $CD4^+$ cells (*A*). Levels of IFN-γ were determined by ELISA (*B*). IL-5, IL-10, TNF-α, and IL-6 production was measured by luminex cytometric analysis (*C–F*, respectively). Results are presented as mean ± SEM of six replicates in a representative experiment of three, *, $p < 0.01$, mDC pulsed with Ag vs mDC and pDC pulsed with Ag.

production of the T cell cytokines tested (IFN-γ, $p < 0.001$; IL-5, $p < 0.001$; IL10, $p < 0.001$; TNF, $p < 0.001$; and IL-6, $p = 0.003$) while sparing proliferation. These results suggest that presentation of Ag by pDC can act to block the acquisition of effector function by T cells that are activated by mDC.

Discussion

As mentioned above, the CIA model has proven an excellent means to test interventions to treat arthritis; however, it does not provide a good system to investigate the breach of self-tolerance associated with autoimmune disease, as it involves direct immunization with an autoantigen prepared in adjuvant. Our previous studies established a model of arthritis where OVA-specific T cell transfer and immunization resulted in spontaneous recognition of autoantigens such as collagen (17). In the current study, while control mice developed early histological signs of arthritis including moderate erosion of cartilage and inflammatory cell infiltration of the synovial membrane in joints proximal to OVA challenge as described previously (17), pDC depletion resulted in a distinct pathology involving extensive synovial hyperplasia, cartilage degradation, and pannus invasion. The exacerbation of histopathology in pDC-depleted animals was also apparent in ex vivo immunological analyses. As expected, similarly strong levels of OVA-specific proliferation and release of IFN-γ and IL-5 were observed, irrespective of pDC depletion, demonstrating that activation or regulation of T cell restimulation is unaffected by pDC depletion. In contrast, cultures from the pDC-depletion group exhibited significantly increased levels of autoantigen (CII)-specific proliferation compared with control groups, consistent with an enhanced breach of self-tolerance. Although pDCs have been associated with the Th1/Th2 phenotype of the developing T cell response (21, 23), in the present model of arthritis, we did not see a strong effect of pDC depletion on the polarization of the nascent CII-specific response.

The regulation of self-reactive effector responses by specialized populations of Tregs is considered a major mechanism by which tolerance is maintained and autoimmune disease avoided. In models of allograft tolerance, all alloantigen presenting cells are pDCs (24). Moreover, a number of groups have reported that pDC in the LN are essential for the induction of $CD4^+CD25^+Foxp3^+$ Tregs (24). Both immature and mature mDCs have been associated with the maintenance and proliferation of Tregs (25). We, therefore, examined the number of $CD4^+Foxp3^+$ Tregs in single-cell suspensions prepared from the draining popliteal LN from each treatment group. However, flow cytometric analysis did not identify any significant alterations in the number of $CD4^+Foxp3^+$ Tregs between the pDC-depleted or control groups. Moreover, we identified comparable $CD4^+$ expansion in the draining LN of all groups.

Autoantibodies against Ig (rheumatoid factor) and citrullinated peptides (anti-CCP) are widely used biomarkers in RA and are implicated in disease pathogenesis (26); however, few exogenously induced murine models exhibit such Ab production particularly of anti-CCP specificity (27, 28). In the current study, we examined serum obtained at day 7 for anti-OVA, anti-CII, anti-IgG2a, and anti-CCP by ELISA. Consistent with our ex vivo immunological analyses, all animals, regardless of treatment group, had high levels of serum anti-OVA Abs. In contrast, serum anti-CII, anti-IgG2a, and anti-CCP Abs were significantly increased in the pDC depletion group as compared with control groups. Together these data are consistent with an exacerbated breach of tolerance to self-Ags induced by specific pDC depletion, indicating that pDC may have a central role in regulating autoreactive T cell responses and autoantibody production. To directly analyze whether pDC Ag presentation could affect T cell activation by mDC, we performed in vitro studies examining the effect of combinations of Ag-presenting and non-Ag-presenting pDC or mDC on primary T cell proliferation and effector cytokine production. The results indicated that presentation of Ag by pDC could block the acquisition of effector function by T cells induced by Ag-presenting mDC.

In summary, this study demonstrates for the first time that pDC play a role in suppressing arthritogenic autoimmunity arising via endogenous pathways initiated by an irrelevant nonarticular Ag. Removal of pDCs resulted in increased joint histopathology including cartilage degradation and synovial hyperplasia. Significantly, the spontaneous generation of joint-Ag (collagen)-specific T cell proliferation and elevated serum anti-CII, anti-IgG2a, and anti-CCP Ab levels were elevated in pDC-depleted mice and in vitro, Ag-presenting pDC could act to block effector function in T cells incubated with Ag-presenting mDC. These studies clarify the mechanisms whereby immunological regulation can be breached by bystander responses to infectious agents such as bacteria and viruses that have been implicated in triggering and exacerbating RA (29, 30).

Disclosures

The authors have no financial conflict of interest.

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