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DEC205$^+$ Dendritic Cell–Targeted Tolerogenic Vaccination Promotes Immune Tolerance in Experimental Autoimmune Arthritis

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Previous studies in mouse models of autoimmune diabetes and encephalomyelitis have indicated that the selective delivery of self-antigen to the endocytic receptor DEC205 on steady-state dendritic cells (DCs) may represent a suitable approach to induce Ag-specific immune tolerance. In this study, we aimed to examine whether DEC205$^+$ DC targeting of a single immunodominant peptide derived from human cartilage proteoglycan (PG) can promote immune tolerance in PG-induced arthritis (PGIA). Besides disease induction by immunization with whole PG protein with a high degree of antigenic complexity, PGIA substantially differs from previously studied autoimmune models not only in the target tissue of autoimmune destruction but also in the nature of pathogenic immune effector cells. Our results show that DEC205$^+$ DC targeting of the PG peptide 70–84 is sufficient to efficiently protect against PGIA development. Complementary mechanistic studies support a model in which DEC205$^+$ DC targeting leads to insufficient germinal center B cell support by PG-specific follicular helper T cells. Consequently, impaired germinal center formation results in lower Ab titers, severely compromising the development of PGIA. Overall, this study further corroborates the potential of prospective tolerogenic DEC205$^+$ DC vaccination to interfere with autoimmune diseases, such as rheumatoid arthritis. ***The Journal of Immunology*****, 2015, 194: 4804–4813.**

Various immune effector cell types, including CD4$^+$ T cells, have been implicated in the pathogenesis of rheumatoid arthritis (RA) (1). Indeed, the synovial tissue of RA patients is infiltrated with IFN-γ– and IL-17–producing CD4$^+$ T cells, resulting in synovial inflammation and joint cartilage destruction (2). Blockade of T cell costimulation with a therapeutic agent has proven to be an effective treatment, further corroborating a critical role for T cells in RA (3). The cartilage proteoglycan (PG) aggrecan is an Ag that is targeted by self-reactive T cells in the joints of RA patients (4–6). In BALB/c mice, immunization with human PG protein induces a form of arthritis (PG-induced arthritis [PGIA]) that recapitulates many clinical aspects of human RA (7, 8). PGIA is a T cell–dependent and B cell–/Ab-mediated autoimmune disease (9–12).

Previous studies have indicated that the selective delivery of Ag to the endocytic receptor DEC205 represents a powerful method to induce Ag-specific T cell tolerance (13–18). In these studies, Ag was targeted to steady-state dendritic cells (DCs) using recombinant anti-DEC205 Abs fused either to whole protein or to specific T cell epitopes, resulting in Ag-specific T cell tolerance. In contrast, administration of anti-DEC205 Abs that lacked the fusion Ag or had been fused to an irrelevant T cell epitope did not (19, 20), indicating that this approach indeed induced Ag-specific alterations in T cell populations. Moreover, coadministration of recombinant anti-DEC205 fusion Abs with adjuvant induced protective immune responses in experimental settings of cancer (21, 22) and infection (22, 23), further demonstrating that steady-state DCs (i.e., immature DCs with low expression levels of costimulatory molecules) are a prerequisite for the induction of DEC205$^+$ DC-targeted T cell tolerance (20, 24).

Recombinant anti-DEC205 Abs fused to a T cell–relevant self-antigen can prevent autoimmunity via interference with pathological T cell responses, both through dominant and recessive tolerance mechanisms. DEC205$^+$ DC-targeted dominant tolerance can be achieved by the Ag-specific enhancement of Foxp3$^+$ regulatory T cell (Treg) activity (15–18, 24, 25), whereas recessive tolerance mechanisms include deletion and induction of anergy in pathogenic T cells (13, 14, 18, 24). DEC205$^+$ DC targeting has been shown to promote immune tolerance in the spontaneous NOD mouse model of autoimmune diabetes (24, 26) and peptide-induced mouse models of experimental autoimmune encephalomyelitis (EAE) (14, 18, 25). Compared to peptide-induced autoimmune models such as

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Abbreviations used in this article: anti–DEC205-PG, recombinant anti-DEC205 proteoglycan fusion Ab encoding proteoglycan peptide 70–84; DC, dendritic cell; DDA, dimethyldioctadecylammonium bromide; dLN, draining lymph node; EAE, experimental autoimmune encephalomyelitis; GC, germinal center; III/10-PG, recombinant isotype control fusion Ab encoding proteoglycan 70–84; LN, lymph node; MedLN, mediastinal LN; PG, proteoglycan; $PG_{70–84}$, proteoglycan peptide 70–84; PGIA, proteoglycan-induced arthritis; RA, rheumatoid arthritis; Teff, T effector; T_{FH}, T follicular helper; Treg, regulatory T.

EAE, PGIA is fundamentally different because disease is induced with whole protein, namely human cartilage–derived PG. Human PG exhibits a high degree of sequence homology with murine PG and shows substantial complexity with regard to contained T cell epitopes. Indeed, systemic immunization with whole PG protein leads to activation of a variety of PG-specific T cell clones, with the amino acids 70–84 of PG ($PG_{70–84}$) representing an immunodominant epitope (27–29). Induction of EAE critically depends on self-reactive T effector (Teff) cells skewed toward the Th17 lineage (30). In PGIA, Th17 cells contribute to pathology (31), but they may not be essential for the induction of disease (32). Furthermore, although their putative role in EAE has remained unclear (33), B cells play an important dual role in PGIA: they are required as APCs for the induction of severe clinical symptoms (11, 34, 35) and for the production of pathological autoantibodies (9, 11, 36, 37).

The overall aim of the present study was to examine whether DEC205$^+$ DC-targeted tolerogenic vaccination with a single immunodominant peptide of PG is suitable to protect against PGIA. Additionally, the impact of DEC205$^+$ DC-mediated tolerance induction on eliciting arthritogenic T and B cell activity in response to systemic PG protein immunization was studied.

Materials and Methods

Mice

Female BALB/c mice (retired breeders) were purchased from Charles River Laboratories. Congenic CD90.1 BALB/c mice were purchased from The Jackson Laboratory and backcrossed to conventional CD90.2 BALB/c (Charles River Laboratories) mice. TCR-5/4E8 BALB/c mice carry a transgenic TCR reactive to the human PG peptide 70–84 (hereafter referred to as TCR-PG) (38). All mice were kept under standard housing conditions at the Central Animal Laboratory of Utrecht University. Animal experiments were approved by the Animal Ethics Committee from Utrecht University.

Recombinant fusion Ab production

Eukaryotic expression vectors encoding the mouse IgG1 IgH chain cDNA of cloned anti-DEC205 (NLDC-145) and III/10 isotype control, and their respective Ig-κ L chain cDNA, were provided by the Nussenzweig laboratory at Rockefeller University (13, 14). To improve the specificity of DEC205-mediated targeting, the rat IgG2a C regions of the original NLDC-145 and isotype control Abs had been replaced with mouse IgG1 C regions, which additionally carry point mutations interfering with Fc receptor binding (13). DNA PG fragments (oligonucleotide 1, forward, 5′-CTA-GCG-ACA-TGG-CCA-AGA-AGG-AGA-CAG-TCT-GGA-GGC-TCG-AGG-AGT-TCG-GTA-GGT-TCA-CAA-ACA-GGG-CC-3′, reverse, 5′-TTC-AGT-GGC-CCT-GTT-TGT-GAA-CCT-ACC-GAA-CTC-CTC-GAG-CCT-CCA-GAC-TGT-GTC-CTT-CTT-GGC-CAT-GTC-G-3′; oligonucleotide 2, forward, 5′-ACT-GAA-GGG-CGC-GTG-CGG-GTC-AAC-AGT-GCC-TAT-CAG-GAC-AAG-TAT-TAT-GAC-GGT-AGG-ACA-TGA-TAG-GC-3′, reverse, 5′-GGC-CGC-CTA-TCA-TGT-CCT-ACC-GTC-ATA-ATA-CTT-GTC-CTG-ATA-GGC-ACT-GTT-GAC-CCG-CAC-GCG-CCC-3′) encoding the human $PG_{70–84}$ (ATEGRVRVNSAYQDK) were added in frame to the C terminus of anti-DEC205 (anti–DEC205-PG) and III/10 (III/10-PG) Abs. Recombinant Abs were produced as described before (17, 20).

In vitro and in vivo APC targeting

After preparation of splenic single-cell suspensions using collagenase D (Roche), CD11c$^+$ DCs were FACS purified and incubated with recombinant Abs or free $PG_{70–84}$ peptide (37°C). After 3 h, DCs were washed extensively and cocultured with 1×10^5 FACS-purified CFSE-labeled PG-specific T cells. On day 4, T cell proliferation was analyzed by flow cytometry. To track DEC205$^+$ DC targeting in vivo, allophycocyanin-conjugated anti-DEC205 (clone NLDC-145) or respective isotype control Ab was administered i.p. (5 μg/200 μl PBS) and splenocytes were analyzed by flow cytometry after 21 or 2 h, as indicated.

Treatment, arthritis, and T cell transfer

Cohorts of mice were injected i.p. with anti–DEC205-PG, as indicated (see the figure legends for details on amounts and time points). III/10-PG isotype control Abs and PBS injections were included for comparison, as indicated. PGIA was induced by i.p. injection of 250 μg PG protein and 2 mg dimethyldioctadecylammonium bromide (DDA; Sigma-Aldrich) on days 0 and 21, as described before (39). PG (aggrecan) was purified from human articular cartilage by 4 M guanidinium chloride extraction and depleted of glycosaminoglycan side chains using endo-β-galactosidase (0.22 mU/mg dry weight) and testicular hyaluronidase (5 U/mg dry weight) (39). Onset and severity of arthritis was determined using a visual scoring system based on swelling and redness of paws (39). For adoptive transfers, CD4$^+$TCR-PG$^+$ T cells were isolated from spleens of naive TCR-5/4E8-Tg mice by negative selection with sheep anti-rat IgG Dynabeads (Dynal, Invitrogen) using excess amounts of mAbs to B220 (RA3-6B2), CD11b (M1/70), MHC class II (M5/114), and CD8 (YTS169). Enriched CD4$^+$TCR-PG$^+$ T cells were routinely ~90–95% pure. Conventional CD90.2 or congenic CD90.1 BALB/c recipient mice were injected i.v. with indicated amounts of CFSE (Molecular Probes)-labeled T cells.

Flow cytometry, intracellular cytokine staining, and ELISA

Single-cell suspensions were prepared from spleen and draining lymph nodes (dLNs) of the paws (brachial, axillary, popliteal). For flow cytometry, cells were stained with mAbs to CD5 (53-7, 3), CD90.2 (53-2.1) (eBioscience), CD4 (RM4-5), or CD25 (PC61) (BD Biosciences). Foxp3 (FJK 16S) staining was carried out according to the manufacturer's protocol (eBioscience). Prior to intracellular staining with mAbs to IL-17 (eBio17; eBioscience) and IL-10 (JES5-16; BD Biosciences), T cells were stimulated with PMA (50 ng/ml)/ionomycin (500 ng/ml) for 5 h in the presence of brefeldin A (1 μg/ml; all from Sigma-Aldrich). Samples were analyzed on a FACSCanto II (BD Biosciences). PG-specific Abs in sera were measured by ELISA as described elsewhere (39). PG-specific IgG1 and IgG2a Ab levels were compared with a standard of pooled sera from arthritic mice and expressed in relative units.

Statistical analysis

For statistical analysis, Prism (GraphPad Software) was used. Two-tailed Student *t* tests and one-way ANOVA (two-tailed) with Bonferroni correction were applied, when applicable. Differences were considered significant at $p < 0.05$.

Results

DEC205$^+$ DC targeting of PG-derived peptide Ag via recombinant anti-DEC205 fusion Abs

Under steady-state conditions in vivo, the endocytic DEC205 receptor is highly expressed on migratory DCs (such as Langerhans cells and dermal CD103$^+$ DCs) and on lymphoid-resident DCs with a CD11c$^+$CD8$^+$ surface marker phenotype (25), whereas DEC205 expression is low or negligible on conventional CD11c$^+$ CD8$^-$ DCs, T cells, and B cells (13) (Supplemental Fig. 1A). For DEC205$^+$ DC targeting in the PGIA model, we produced recombinant Abs consisting of the immunodominant amino acids 70–84 of human PG ($PG_{70–84}$) (27, 28) fused either to the IgH chain C terminus of cloned DEC205 (anti–DEC205-PG) or to the III/10 isotype control (III/10-PG) Ab. To confirm that the $PG_{70–84}$ peptide delivered by the anti–DEC205-PG fusion Ab was properly processed and presented, FACS-purified splenic CD11c$^+$ DCs were incubated for 3 h with various concentrations of either anti–DEC205-PG or III/10-PG control Ab (5, 20, 50, 200, and 500 ng) or with equivalent amounts of free synthetic $PG_{70–84}$ peptide (500 ng fusion Ab corresponds to ~10 ng free peptide Ag). After unbound Ab and synthetic peptide Ag were removed by extensive washing, DCs were cocultured with Ag-specific CD4$^+$ T cells expressing a transgenic TCR reactive to $PG_{70–84}$ (TCR-PG) (Supplemental Fig. 1B). Analysis of cocultures at day 4 demonstrated that preincubation of CD11c$^+$ DCs with low amounts of the anti–DEC205-PG Ab (≤20 ng, corresponding to ≤0.4 ng $PG_{70–84}$ peptide) was sufficient to induce vigorous proliferation of TCR-PG$^+$ T cells (Supplemental Fig. 1B). Preincubation with increased levels of the III/10-PG isotype Ab (≥200 ng) or free $PG_{70–84}$ peptide (≥4 ng) controls also induced TCR-PG$^+$ T cell prolifer-

ation (Supplemental Fig. 1B), indicating that DEC205 targeting improved Ag presentation to T cells as compared with III/10-PG isotype Ab or $PG_{70–84}$ peptide controls. In addition to efficient T cell proliferation, cocultures of anti–DEC205-PG-targeted DCs and TCR-PG^+ T cells could be further characterized by the production of high amounts of IFN-γ and low but detectable amounts of other pro- and anti-inflammatory cytokines (IL-4, IL-17a, IL-6, IL-10; data not shown), indicating that cell isolation and culture conditions promote maturation and/or activation of initially immature DCs, resulting in the loss of their tolerogenic potential in vitro. In contrast to $CD11c^+$ DCs (Supplemental Fig. 1B), FACS-purified B cell subpopulations (follicular and marginal zone B cells from spleen, peritoneal B-1a cells), which had been preincubated with anti–DEC205-PG fusion Abs, were largely ineffective in promoting proliferation of cocultured $CD4^+$TCR-PG^+ T cells (data not shown). In complementary in vivo experiments, i.p. injection of otherwise nonmanipulated wild-type mice with fluorochrome-conjugated anti-DEC205 mAbs resulted in efficient targeting of splenic $CD11c^{hi}CD8^+$ DCs but poorly labeled $CD11c^{hi}CD8^-$ DCs, $CD4^+$ T cells, and $B220^+$ B cells from the same anatomical location (Supplemental Fig. 1C, 1D). This observation is consistent with previous studies (25) in that high steady-state expression levels of DEC205 (Supplemental Fig. 1A) correlate with the preferential uptake of the anti-DEC205 Ab in vivo (Supplemental Fig. 1C, 1D).

Preadministration of anti–DEC205-PG Ab ameliorates clinical symptoms in PGIA

We performed a series of in vivo experiments to assess the tolerogenic potential of $DEC205^+$ DC targeting in the PGIA model (Fig. 1A–D). Previous studies in TCR non–transgenic mouse models of autoimmune diabetes (17) and encephalomyelitis (18) established that the induction of immune tolerance by $DEC205^+$ DC-targeted self-antigen critically depends on the administration of anti-DEC205 fusion Abs under subimmunogenic conditions (i.e., low Ag dose, limited T cell costimulation, steady-state DCs) (24, 40). Therefore, our studies on $DEC205^+$ DC targeting in PGIA focused on single low-dose administration of the anti–DEC205-PG Ab prior to the induction of PGIA by immunization with whole PG protein and DDA, a potent adjuvant that exerts a strong stimulatory effect via activation of innate immune cells, including DCs (39). To this end, cohorts of adult mice were i.p. injected with various amounts (50 ng, 1 μg, 5 μg) of anti–DEC205-PG or III/10-PG fusion Abs, either on day –10 (Fig. 1A, 1C) or on day -20 (Fig. 1B, 1D) before induction of PGIA. Cohorts of mice that had been pretreated with PBS were included for comparison (Fig. 1A–D).

In contrast to the III/10-PG isotype control Ab and PBS, pretreatment of otherwise nonmanipulated mice with a single dose of anti–DEC205-PG Ab substantially ameliorated PGIA (Fig. 1A–C), as judged by delayed onset and overall reduced severity of disease symptoms. This holds true for a range of anti–DEC205-PG Ab doses (50 ng, 1 μg, 5 μg), although we observed some variability in efficacy of anti–DEC205-PG-mediated autoimmune protection between individual experiments (for comparison, see "αDEC205-PG 1 μg" in Fig. 1A–C). Possible reasons underlying such experimental variability may include differences between independent cohorts of mice, for example, in the activation status of DCs and/or the number of arthritogenic, PG-reactive cells among the overall pool of peripheral $CD4^+$ T cells. To directly assess the impact of PG-reactive $CD4^+$ T cell numbers on $DEC205^+$ DC-targeted PGIA protection, we injected cohorts of recipient mice with 2×10^6 $CD4^+$TCR-PG^+ T cells prior to tolerogenic anti–DEC205-PG Ab administration and subsequent PGIA induction by PG/DDA immunization (Fig. 1D). The results show that increased numbers of PG-reactive $CD4^+$ T cells can indeed abrogate the protective effect of 50 ng anti–DEC205-PG Ab observed in Fig. 1B, whereas 1 μg anti–DEC205-PG Ab was still found to efficiently interfere with the establishment of high clinical PGIA scores (Fig. 1D).

Overall, preadministration of 1 μg III/10-PG isotype control Ab had only a minor, if any, effect on the development of PGIA (Fig. 1C, 1D). Preadministration of 5 μg III/10-PG Ab resulted in some amelioration of the clinical PGIA score, albeit to a much lesser extent as compared with the same amount of anti–DEC205-PG Ab (Fig. 1A). Preadministration of equivalent amounts of free $PG_{70–84}$ peptide Ag did not appreciably interfere with PGIA induction (data not shown).

In previous studies, different mechanisms underlying $DEC205^+$ DC-targeted induction of T cell tolerance have been proposed, including conversion of initially $CD4^+Foxp3^-$ T cells into $Foxp3^+$ Treg cells (15), as well as deletion and induction of an anergic phenotype in residual T cells, which was accompanied by increased expression levels of CD5 on anergic T cells (13) and abrogated secretion of effector cytokines (18). In our studies, the analysis of mice that had been protected from PGIA by $DEC205^+$ DC targeting (Fig. 1B, day 58 after immunization with PG/DDA) revealed no alterations in the proportion of $Foxp3^+$ Treg cells (Fig. 1E) and expression levels of CD5 (Fig. 1F) among $CD4^+$ T cells in dLNs of the paws, as compared with mice with active PGIA. Nevertheless, amelioration of PGIA by $DEC205^+$ DC targeting correlated with reduced numbers of IL-17^+ and IFN-γ^+ cells among dLN-derived $CD4^+$ T cells (Fig. 1G), whereas no changes were observed in effector cytokine production by $CD4^+$ T cells from spleen (data not shown). Furthermore, the analysis of humoral immune responses revealed that anti–DEC205-PG-protected mice exhibit reduced serum levels of PG-specific IgG2a Abs (Fig. 1H), which is in agreement with the notion that PG-specific IgG2a autoantibody titers correlate with onset and severity of clinical PGIA symptoms (41, 42).

Tracking PG-reactive $CD4^+$ T cells during anti–DEC205-PG-mediated DC targeting in vivo

After adoptive transfer into otherwise nonmanipulated recipient mice, CFSE-labeled $CD4^+$TCR-PG^+ T cells accumulate in peripheral lymphoid tissues of the host but fail to undergo cell division (Fig. 2A), indicating that the cognate self-antigen is not presented by lymphoid tissue-residing APCs under steady-state conditions. However, and in contrast to the III/10-PG isotype control Ab, administration of minute amounts of anti–DEC205-PG Ab (50 ng) induced robust proliferation of adoptively transferred $CD4^+$TCR-PG^+ T cells, with $43 \pm 11.3\%$ divided cells at day 4 (Fig. 2A, 2B). The administration of increased amounts of anti–DEC205-PG Ab (1 μg) only marginally increased the proportion of proliferating $CD4^+$TCR-PG^+ T cells ($50 \pm 5.5\%$ divided cells, Fig. 2B). Notably, the proportion of divided TCR-PG^+ T cells in anti–DEC205-PG Ab-treated mice markedly dropped by day 14 (50 ng, $23 \pm 2.9\%$, 1 μg, $28 \pm 5.7\%$; Fig. 2E), which is consistent with a scenario of initial proliferative expansion and subsequent deletion of divided TCR-PG^+ T cells (15). Furthermore, compared with III/10-PG Ab-treated mice, anti–DEC205-PG-mediated DC targeting initially (day 4) resulted in higher proportions of proliferating $CD4^+$TCR-PG^+ T cells expressing Foxp3 (Fig. 2C) and IL-10 (Fig. 2D), whereas no differences in Foxp3 expression could be observed at day 14 after adoptiver transfer (Fig. 2F). Overall, these data suggest that anti–DEC205-PG-mediated DC targeting promotes the deletion of PG-specific

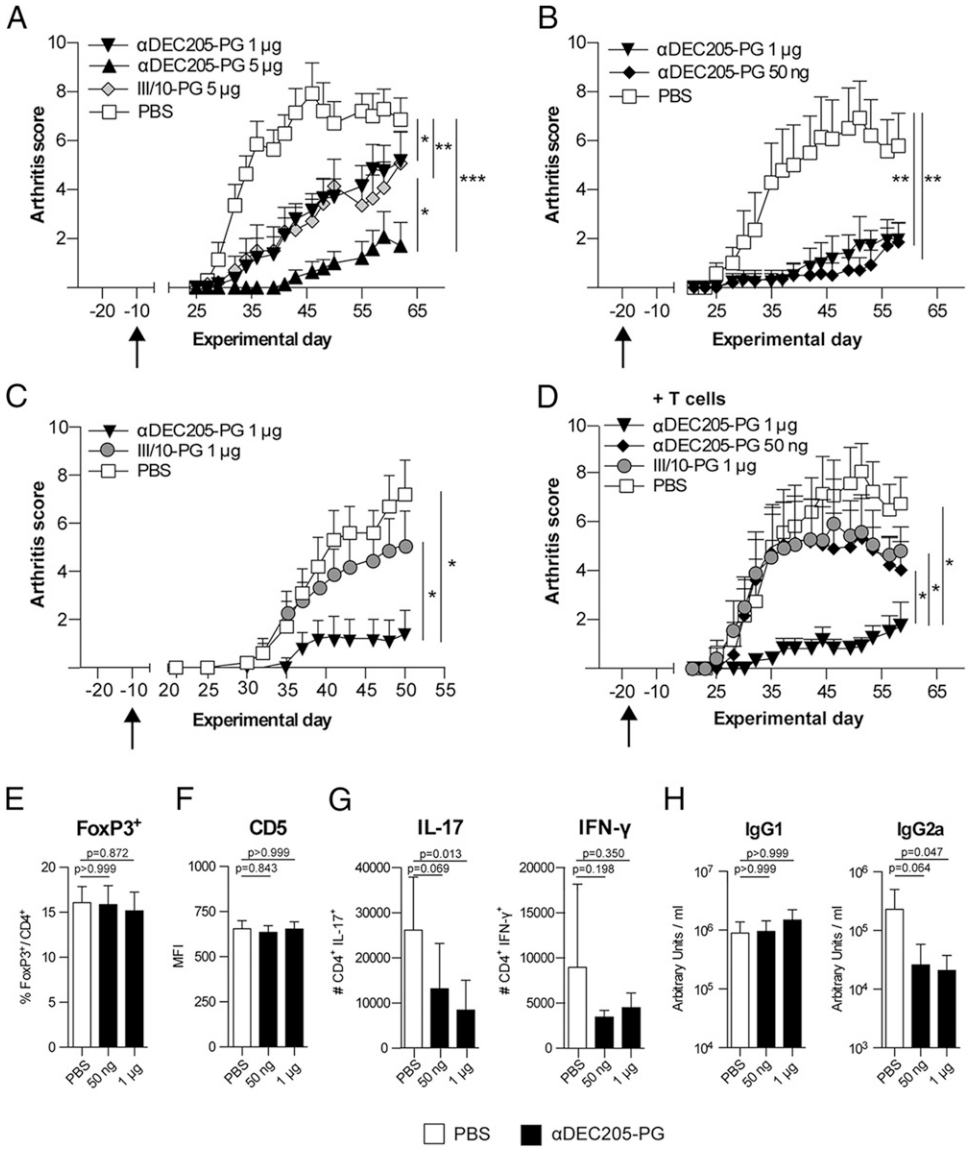

FIGURE 1. Preadministration of recombinant anti–DEC205-PG Abs delays disease onset and ameliorates clinical symptoms of PGIA. (**A**–**D**) Effect of DEC205$^+$ DC targeting on disease course. Cohorts of mice were injected with PBS or indicated amounts (50 ng, 1 μg, 5 μg) of recombinant fusion Abs (anti–DEC205-PG or III/10-PG) on either day −10 (A and C) or −20 (B and D) before induction of PGIA by administration of whole PG protein and DDA. Mice in (D) additionally received 2×10^6 PG-reactive CD4$^+$CD25$^-$ T cells 1 d prior to recombinant Ab or PBS injection, as indicated. Shown are mean arthritis scores ± SEM of five to seven mice per group. The area under the curve was used as measure of disease severity. $*p < 0.05$, $**p < 0.01$, $*** p < 0.001$, statistically significant differences between recombinant Ab and PBS-injected control groups (one-way ANOVA) or the "αDEC205-PG 5 μg" and the "III/10-PG 5 μg" group (Student *t* test). (**E**–**H**) Cohorts of mice as in (B) were analyzed on day 58 after PGIA induction. Expression of (E) Foxp3, (F) CD5, as well as (G) IL-17 and IFN-γ among CD4$^+$ T cells from paw-dLNs. Shown are mean values ± SD of six mice per group. (H) PG-specific IgG1 and IgG2a Abs in serum, as revealed by ELISA.

Teff cells, which coincides with a transient increase in PG-reactive Foxp3$^+$ Treg cells.

Tracking PG-reactive CD4$^+$ T cells after anti–DEC205-PG-mediated DC targeting and PG/DDA immunization in vivo

To further dissect the tolerogenic effect of DEC205$^+$ DC targeting on PG-reactive CD4$^+$ T cells in PGIA, we next tracked proliferation and accumulation of adoptively transferred populations of CD4$^+$TCR-PG$^+$CD90.2$^+$ T cells in congenic CD90.1 recipient mice after recombinant fusion Ab administration and subsequent immunization with PG/DDA (Fig. 3A). In control mice that had been pretreated with PBS, the congenically marked CD4$^+$TCR-PG$^+$ T cells were readily detectable in spleen (Fig. 3B, 3E) and LNs (data not shown) of recipient mice and exhibited robust proliferation, as revealed by the complete loss of the CFSE dye in the vast majority of cells (Fig. 3C, 3D). In contrast, pretreatment with 1 μg anti–DEC205-PG Ab, and to a lesser extent with 1 μg III/10 PG Ab, resulted in a marked reduction of CD4$^+$ TCR-PG$^+$ T cell accumulation (Fig. 3B, 3E) and CFSE dilution (Fig. 3C, 3D). These data indicate that deletion and induction of an anergic phenotype may at least partly contribute to the autoimmune protection by anti–DEC205-PG-mediated DC targeting. Additionally, we observed that the small population of residual PG-specific CD4$^+$CD90.2$^+$ T cells, which was maintained after anti–DEC205-PG Ab or III/10 PG Ab treatment and PG/DDA immunization (Fig. 3B, 3E), exhibited increased proportions of Foxp3$^+$ T cells (Fig. 3F). This observation raises the possibility that the observed reduction in TCR-PG$^+$ T cell proliferation after recombinant fusion Ab treatment and PG/DDA immunization

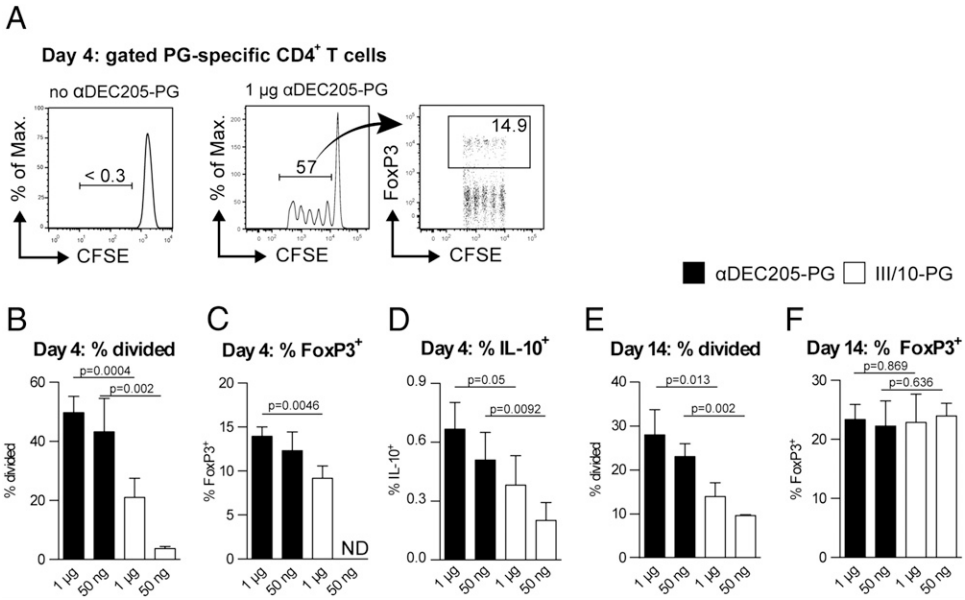

FIGURE 2. Tracking PG-reactive CD4$^+$ T cells during anti–DEC205-PG-mediated DC targeting in vivo. CD4$^+$TCR-PG$^+$CD90.2$^+$ T cells were labeled with CFSE and injected i.v. into conventional CD90.2 (**A**–**D**) or congenic CD90.1 (**E** and **F**) recipient mice. The next day, recipient mice were treated i.p. with 50 ng or 1 μg anti–DEC205-PG Ab. Recipient mice were analyzed by flow cytometry at day 4 (A–D) and day 14 (E and F) after adoptive transfer. (A) Representative flow cytometry of CFSE dilution among gated CD4$^+$TCR-PG$^+$ T cells from spleen of recipient mice that were either left untreated (*left*) or had been injected with 1 μg anti–DEC205-PG Ab (*right*). Numbers in histograms and dot plot indicate the percentage of cells in the respective gate or quadrant. The line with arrowhead indicates the gating scheme. Bar graphs show the mean percentage ± SD of (B and E) divided cells, as well as (C and F) Foxp3 and (D) IL-10 expression among CD4$^+$TCR-PG$^+$ T cells at indicated time points (ND, not detected). Data are from a single experiment with three to five mice per group representative of two experiments performed. Cohorts of mice that received the same anti–DEC205-PG Ab dose were compared using a Student *t* test.

(Fig. 3D) might be due to increased suppressor activity of PG-specific Foxp3$^+$ Treg cells.

Preadministration of the anti–DEC205-PG fusion Ab compromises germinal center formation via abrogation of T follicular helper cell differentiation

Mice that had been protected from severe PGIA symptoms by anti–DEC205-PG preadministration exhibited reduced serum levels of PG-specific IgG2a Abs (Fig. 1H), raising the possibility that tolerogenic DEC025$^+$ DC vaccination may impinge on humoral immunity by abrogating CD4$^+$ T cell help for the generation of germinal center (GC) B cells. To experimentally address this possibility, we first analyzed the kinetics of GC formation in peritoneal cavity–draining, mediastinal LNs (MedLNs) (43) after recombinant fusion Ab administration and subsequent PG/DDA immunization (Fig. 4A). At all time points analyzed, the proportion of CD19$^+$ B cells with a GL-7$^+$FAS$^+$ GC B cell phenotype was found to be reduced in MedLNs of anti–DEC205-PG-pretreated mice, as compared with mice that had been injected with the III/10-PG isotype control Ab (Fig. 4A). Notably, secondary immunization with PG/DDA failed to overcome the impaired formation of GCs in anti–DEC205-PG-pretreated mice (Fig. 4A).

Next, we sought to assess the impact of DEC205$^+$ DC targeting on the generation of PG-specific CD4$^+$ T follicular helper (T_{FH}) cells, which play a crucial role in the development of GCs and Ag-specific B cell immunity (44, 45). For this, recipient mice were injected with congenically marked CD4$^+$TCR-PG$^+$ T cells prior to recombinant fusion Ab administration and immunization with PG/DDA. Analysis was performed at day 10 after PG/DDA immunization, that is, during the early phase of GC formation and prior to clinical manifestation of PGIA. At this early time point after PG/DDA immunization, anti–DEC205-PG-pretreated mice already exhibited moderately reduced numbers of splenic GL-7$^+$FAS$^+$ GC B cells (Fig. 4B) and decreased serum titers of PG-specific Abs (Fig. 4E), as compared with the indicated control groups. In these experiments, pretreatment with the anti–DEC205-PG Ab resulted in substantially reduced numbers of PG-reactive CD4$^+$CD90.2$^+$ T cells with a PD-1$^+$CXCR5$^+$ T_{FH} phenotype (Fig. 4C, 4D).

The observed reduction in GC formation after tolerogenic DEC205$^+$ DC targeting (Fig. 4B–D) could be a consequence of dominant tolerance mechanisms (e.g., increased Foxp3$^+$ Treg cell activity, Fig. 3F) or of deletional tolerance (Fig. 3E) that limits the availability of PG-specific CD4$^+$ T cells giving rise to GC-supporting T_{FH} cells. To address this issue, we adoptively transferred congenically marked, PG-specific populations of either CD4$^+$CD25$^+$Foxp3$^+$ Treg cells or CD4$^+$CD25$^-$Foxp3$^-$ Teff cells (Fig. 5A) into recipient mice, which were subsequently injected with the anti–DEC205-PG Ab followed by PG/DDA immunization to induce GC formation. Compared to cohorts of PBS-injected control mice, pretreatment with the anti–DEC205-PG Ab reduced GC development in both recipients of Treg and Teff cells, as judged by the proportion of CD19$^+$ B cells with a GL-7$^+$FAS$^+$ GC B cell phenotype (Fig. 5B). However, in anti–DEC205-PG Ab- and PBS-treated Treg cell recipient mice, the reduction in GC B cells did not correlate with percentages and numbers of PG-specific Foxp3$^+$ Treg cells (Fig. 5D). This observation strongly indicates that PG-specific Foxp3$^+$ Treg cells are unlikely to be responsible for the reduction in GC development, suggesting that the deletion of PG-specific Teff cells (derived from both the recipient and the small Teff cell population within transferred enriched Treg cells) impinges on GC development. Consistently, the reduced availability of PG-specific CD4$^+$CD90.2$^+$ T cells in anti–DEC205-PG-treated Teff cell recipients (Fig. 5C) correlated with reduced populations of PG-specific CD4$^+$PD-1$^+$CXCR5$^+$ T_{FH}

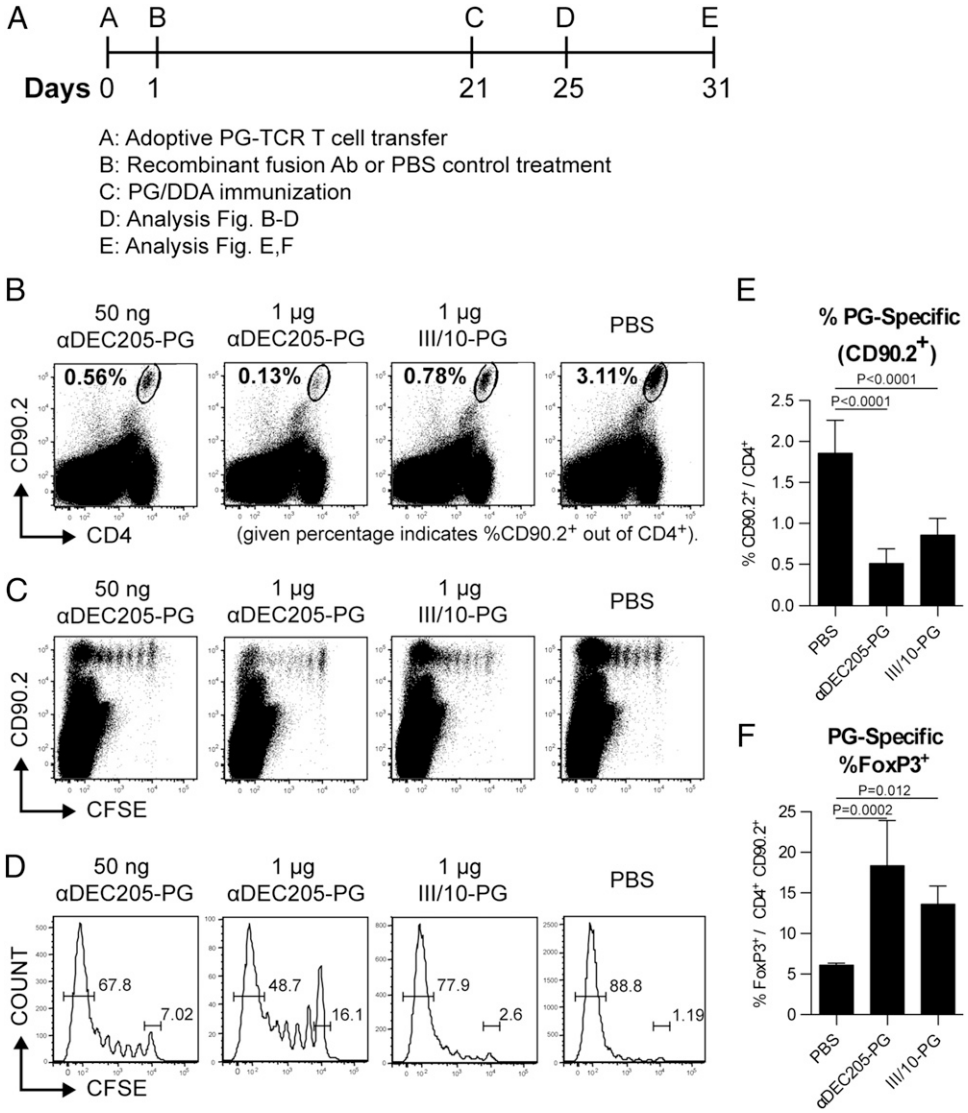

FIGURE 3. Tracking PG-reactive $CD4^+$ T cells after anti–DEC205-PG-mediated DC targeting and PG/DDA immunization in vivo. (**A**) CFSE-labeled $CD4^+TCR\text{-}PG^+CD90.2^+$ T cells were i.v. injected into congenic CD90.1 hosts. The next day, recipient mice were i.p. injected either with indicated amounts of recombinant fusion Abs (anti–DEC205-PG or III/10-PG) or with PBS. At day 21 after adoptive TCR-PG^+ T cell transfer, recipient mice were immunized with PG/DDA, as indicated. The spleens of recipient mice were collected at day 25 (**B**–**D**) or day 31 (**E** and **F**) and analyzed by flow cytometry. (B–D) Representative flow cytometry (B) of $CD4^+CD90.2^+$ T cells among total splenocytes, as well as CFSE dilution among (C) $CD90.2^+$ T cells and (D) gated $CD4^+CD90.2^+$ T cells at day 25 after adoptive TCR-PG^+ T cell transfer. Data are from a single experiment (two to three mice per group) representative of three experiments performed. Numbers in dot plots in (B) and histograms in (D) indicate the percentage of cells within the respective gate. (E and F) After adoptive transfer of $CD4^+TCR\text{-}PG^+CD90.2^+$ T cells, recipient mice were i.p. injected with 200 ng recombinant fusion Ab (anti–DEC205-PG or III/10-PG) or PBS, followed by PG/DDA immunization, as indicated in (A). Composite percentage (E) of adoptively transferred $CD4^+CD90.2^+$ T cells and (F) proportion of $Foxp3^+$ cells among gated $CD4^+CD90.2^+$ T cells at day 31 after adoptive TCR-PG^+ T cell transfer are shown. Data are shown as mean percentage ± SD of six mice per group from a single experiment representative of two experiments performed (one-way ANOVA).

cells (Fig. 5E) and $CD19^+GL\text{-}7^+FAS^+$GC B cells (Fig. 5B) in an experimental system with overall very low numbers of PG-specific $Foxp3^+$ Treg cells (Fig. 5D).

Discussion

Recombinant anti-DEC205 Abs fused to disease-relevant autoantigens have been developed and used successfully for tolerogenic protection in autoimmune disease models, including autoimmune diabetes (24, 26) and EAE (14, 18, 25). In the present study, we show that administration of a single dose of recombinant anti-DEC205 Ab fused to the cartilage-derived immunodominant $PG_{70–84}$ efficiently ameliorates the severity of PG-induced arthritis. PGIA is an experimental arthritis model that substantially differs from previously employed autoimmune models because disease is induced by immunization with a complex whole protein and different immune effector cells are involved in autoimmune destruction.

Our findings further demonstrate that anti–DEC205-PG treatment alters the PG-specific $CD4^+$ T cell population in a complex way: PG-specific $CD4^+$ T cells proliferate extensively early (i.e., 4 d) after anti–DEC205-PG administration and the percentage of $Foxp3^+$ Treg cells is enhanced at this time point. However, the proportion of proliferated PG-specific T cells has dropped distinctly by day 14. This is in agreement with earlier reports where low-dose $DEC205^+$ DC targeting in steady-state mice initially promoted the proliferative expansion of Ag-specific $CD4^+$ T cells, followed by cell death of most cells that had been activated by

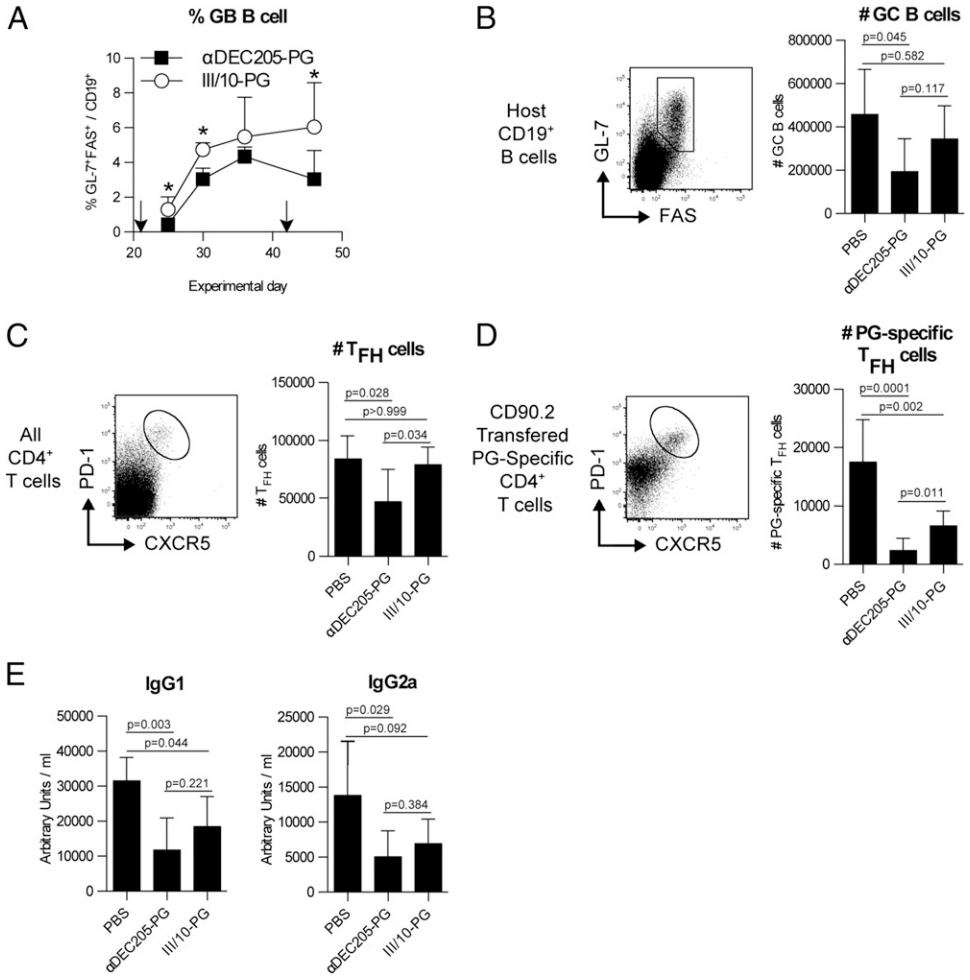

FIGURE 4. Impact of anti–DEC205-PG Ab preadministration on GC formation and T_{FH} cell differentiation. (**A**) Kinetics of GC formation in MedLNs. At days 21 and 42 (indicated by arrowheads) after recombinant Ab administration (1 μg, anti–DEC205-PG or III/10-PG), mice were immunized with PG/DDA. At indicated time points (days 26, 31, 37, and 47), MedLNs were analyzed by flow cytometry for the presence of $CD19^+$ B cells with a $GL\text{-}7^+FAS^+$ GC B cell phenotype. Data are shown as mean percentage ± SD of six mice per group (Student *t* test). (**B**–**E**) PG-specific T_{FH} cell differentiation and IgG Ab titers. $CD4^+TCR\text{-}PG^+CD90.2^+$ T cells were adoptively transferred into congenic CD90.1 hosts. The next day, recipient mice were i.p. injected with either PBS or recombinant fusion Abs (1 μg, anti–DEC205-PG or III/10-PG). At day 21 after adoptive PG-specific T cell transfer, recipient mice were immunized with PG/DDA. At day 31, flow cytometric analysis of splenocytes and serum ELISA was performed. (B) Representative FACS plot of GL-7 and FAS expression among gated $CD19^+$ B cells and absolute numbers of $CD19^+GL\text{-}7^+FAS^+$ GC B cells. (C) Representative FACS plot of PD-1 and CXCR-5 expression among gated $CD4^+$ T cells and absolute numbers of total $CD4^+$ T cells with a $PD\text{-}1^+CXCR\text{-}5^+$ T_{FH} phenotype. (D) Representative FACS plots for the identification of transferred PG-specific $CD4^+CD90.2^+$ T cells and absolute numbers of $CD4^+CD90.2^+PD\text{-}1^+CXCR\text{-}5^+$ T_{FH} cells. (E) PG-specific IgG1 and IgG2a Abs in serum, as revealed by ELISA. (A–E) $n = 6$ mice/group. (B–E) Similar data were obtained from at least two [one for (A)] independent experiments. One-way ANOVA or Student *t* test (anti–DEC205-PG versus III/10-PG) was applied. $^*p < 0.05$.

anti–DEC205-targeted Ag. The few residual T cells that were maintained after the contraction phase were refractory to proliferation upon restimulation in vitro (13, 15, 17). Indeed, immunization with PG in adjuvant 21 d after anti-DEC205-PG treatment resulted in lower numbers of PG-specific $CD4^+$ T cells than there were present in PBS-treated animals. This indicated that anti–DEC205-PG treatment induced deletion of PG-specific $CD4^+$ T cells and/or that PG-specific $CD4^+$ T cells in the PBS-treated animals proliferated more vigorously compared with the $CD4^+$ T cells in the anti–DEC205-PG-treated animals. The latter option would imply that anti–DEC205-PG treatment either anergized (part of the) PG-specific $CD4^+$ T cells and/or that proliferation was suppressed, for example, by Treg cells. Because the increase in $Foxp3^+$ Treg cells after anti–DEC205-PG treatment appears only transient, this is suggestive of a minor role of these cells in protection. However, we noticed that the small population of residual PG-specific $CD4^+$ T cells resulting from anti–DEC205-PG-mediated deletion/anergy induction contained increased proportions of $Foxp3^+$ Treg cells after PG immunization, possibly due to preferential deletion and/or reduced proliferative expansion of PG-specific $CD4^+Foxp3^-$ T cells, leaving the question presently unanswered. Additionally, adoptive transfer of total populations of splenic $CD4^+$ T cells from anti–DEC205-PG-treated donor mice failed to control the induction of PGIA upon immunization of recipient mice with PG protein/DDA (data not shown), further suggesting that $Foxp3^+$ Treg cells and dominant tolerance may play a minor role in DEC205-targeted prevention of PGIA.

Interestingly, treatment of mice with the III/10-PG isotype control also induced PG-specific $CD4^+$ T cell deletion and/or anergy, indicating that $PG_{70–84}$ peptide delivered in subimmunogenic format to resting DCs is sufficient to induce Ag-specific deletion/anergy, although to a lesser extent as compared with DEC205 targeting.

Taken together, these data indicate that pretreatment with the $PG_{70–84}$ peptide, especially by DEC205 targeting, induced T cell deletion and/or anergy, whereas proportions of $Foxp3^+$ Treg cells

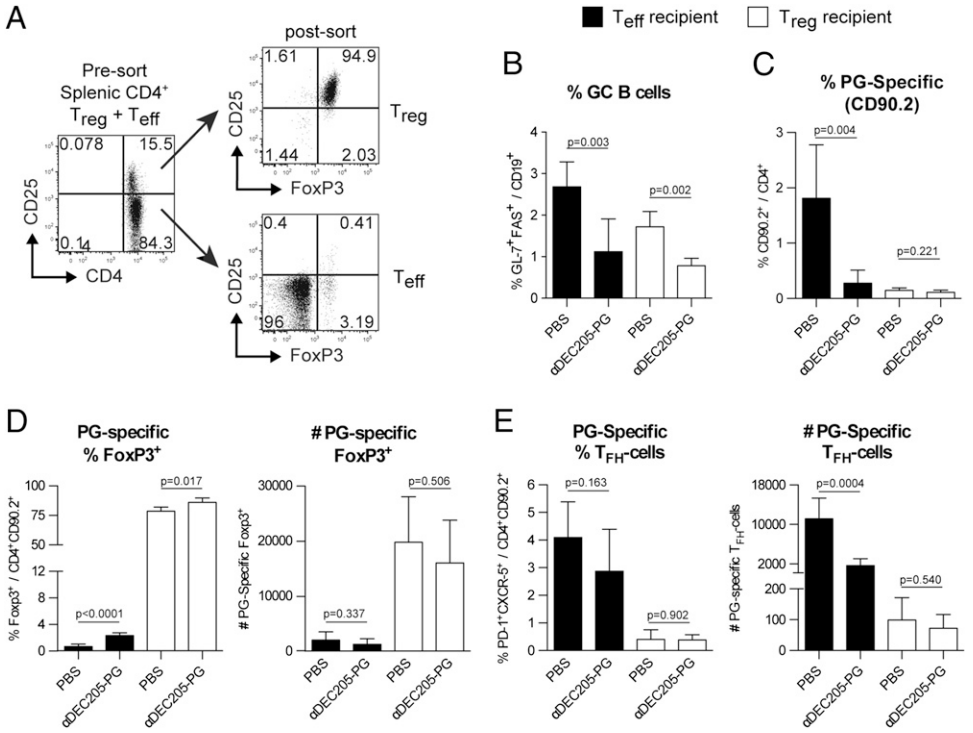

FIGURE 5. Tolerogenic anti–DEC205-PG Ab treatment impinges on GC formation via abrogation of T_{FH} cell activity. FACS-purified populations of conventional $CD4^+CD25^-Foxp3^-$ Teff (1.7×10^6/recipient mouse) and $CD4^+CD25^+Foxp3^+$ Treg (0.3×10^6/recipient mouse) cells from peripheral lymphoid tissues of $CD90.2^+TCR\text{-}PG^+$ donor mice were i.v. injected into congenic CD90.1 hosts. The next day, recipient mice were i.p. injected with either PBS or 1 μg anti–DEC205-PG fusion Ab. At day 21 after adoptive $TCR\text{-}PG^+$ T cell transfer, recipient mice were immunized with PG/DDA. At day 32, splenocytes of recipient mice were analyzed by flow cytometry. (**A**) Dot plots depict presort analysis of CD4 and CD25 expression (*left*), as well as postsort analysis of intracellular Foxp3 expression among FACS-purified $CD4^+CD25^+$ Treg cells (*top right*) and $CD4^+CD25^-$ Teff cells (*bottom right*). Numbers in dot plots indicate the percentage of cells within the respective quadrant. Arrowheads indicate the sorting scheme. (**B**–**E**) Data from recipients of Treg cells and Teff cells are shown as open and filled bars, respectively. (B) Percentage of $CD19^+$ B cells with a $GL\text{-}7^+FAS^+$ GC B cell phenotype. (C) Percentage of adoptively transferred $CD90.2^+$ cells among endogenous $CD4^+$ T cells. (D) Percentage (*left*) and numbers (*right*) of $Foxp3^+$ cells among gated $CD4^+$ $CD90.2^+$ T cells. (E) Percentage (*left*) and numbers (*right*) of adoptively transferred $CD4^+CD90.2^+$ T cells with a $PD\text{-}1^+CXCR\text{-}5^+$ T_{FH} phenotype. Data in (B)–(E) are shown as mean values ± SD of six (Teff) and 5 (Treg) mice per group. Statistical analysis was performed using a Student *t* test.

were increased in the residing cells at the same time. Future studies are warranted to elucidate the relative contributions of recessive and dominant tolerance mechanisms in DEC205-mediated protection from PGIA.

As both anti–DEC205-PG Ab and III/10-PG isotype Ab could induce Ag-specific T cell deletion and/or anergy, but only anti–DEC205-PG was suppressive in PGIA, subsequent studies were warranted to elucidate the exact role of DEC205 targeting in disease suppression. We showed that the anti–DEC205-PG-mediated changes in transferred PG-specific $CD4^+$ T cells compromised the formation of GCs and PG-specific Abs essential for the induction of PGIA, whereas the III/10-PG isotype control was insufficient. Smaller GCs and lower Ab titers were also observed when animals were treated without the transfer of $CD4^+$ T cells (data not shown). Experiments in which PG-specific Treg cells or Teff cells were transferred showed that the effects on GC development were mainly caused by a reduction in potentially pathogenic PG-specific $CD4^+$ T cells as compared with control-treated animals, rather than by increased numbers of PG-specific Treg cells. Nevertheless, these experiments do not absolutely exclude the possibility that Treg cells may contribute to the observed reduction in GC development in anti–DEC205-PG-treated animals.

In summary, our data support a model in which $PG_{70–84}$-specific $CD4^+$ T cells are anergized/deleted and therefore cannot provide the necessary support to GC B cells as differentiated T_{FH} cells. In line with this interpretation, Murad et al. (27) found that immunization with molecularly manipulated PG without the $PG_{70–84}$ T cell epitope significantly diminished the subsequent immune response compared with wild-type Ag, further corroborating that the absence of a single immunodominant T cell specificity has the potential to compromise the entire immune response to a protein Ag. The fact that the human PG used for disease induction is highly homologous to the murine PG may be an important aspect in this, because it presumably limits the number of high-affinity T cell epitopes present in human PG.

Recently, two other C-type lectin receptors were shown to exhibit a tolerance inducing capability as well. Idoyaga et al. (25) recently demonstrated that anti-Langerin Ab fused to EAE-specific peptides could ameliorate clinical symptoms of EAE. This finding is consistent with the notion that DEC205 is also highly expressed on $Langerin^+$ DCs. However, targeting of $DEC205^-$ $DCIR2^+$ DCs with anti-DCIR2 fusion Abs was similarly effective in promoting tolerance in EAE as anti-DEC205 fusion Abs, although the tolerance mechanisms might be different (J.N. Stern and K. Kretschmer, manuscript in preparation). These findings indicate that the DEC205 receptor or even $DEC205^+$ DCs are not unique in their potential to induce tolerance.

Overall, the present study in PGIA further corroborates the clinical potential of prospective tolerogenic $DEC205^+$ DC vaccination with self-antigen. However, successful human translation of these findings in preclinical mouse models of autoimmunity will depend on our detailed knowledge on relevant self-antigens. In fact, a significant number of RA patients respond to PG-derived T cell epitopes (5, 6, 46, 47), but their relative contribution to the

overall progression of disease has remained largely unknown. Additionally, previous studies indicated that DEC205$^+$ DC targeting of self-antigen alone might not be sufficient to interfere with ongoing autoimmune responses (17, 18). In this context, the combination of DEC205-targeted self-antigen with other immunomodulatory strategies (e.g., transient coadministration of immunosuppressive drugs) may represent a particularly promising and safe approach to bring tolerogenic DEC205$^+$ DC targeting in human autoimmunity within reach (24, 48).

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Disclosures

The authors have no financial conflicts of interest.

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