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*J Immunol* 2010; 185:2701-2709; Prepublished online 4 August 2010; doi: 10.4049/jimmunol.1000385

http://www.jimmunol.org/content/185/5/2701

Supplementary Material

http://www.jimmunol.org/content/suppl/2010/08/03/jimmunol.1000385.DC1

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Mice Producing Less Reactive Oxygen Species Are Relatively Resistant to Collagen Glycopeptide Vaccination against Arthritis

Tsvetelina Batsalova, Balik Dzhambazov, Dorota Klaczkowska, and Rikard Holmdahl

The bottleneck for the induction of collagen-induced arthritis in mice is the recognition of immunodominant type II collagen (CII) peptide (CII259–273) bound to the MHC class II molecule Aβ. We have shown previously that the posttranslationally glycosylated lysine at position 264 in this epitope is of great importance for T cell recognition and tolerance induction to CII as well as for arthritis development. The Ncf1 gene, controlling oxidative burst, has been shown to play an important role for immune tolerance to CII. To investigate the effect of oxidation on the efficiency of immune-specific vaccination with MHC class II/glycosylated–CII peptide complexes, we used Ncf1 mutated mice. We demonstrate that normal reactive oxygen species (ROS) levels contribute to the establishment of tolerance and arthritis protection, because only mice with a functional oxidative burst were completely protected from arthritis after administration of the glycosylated CII259–273 peptide in complex with MHC class II. Transfer of T cells from vaccinated mice with functional Ncf1 protein resulted in strong suppression of clinical signs of arthritis in B10.Q mice, whereas the Ncf1 mutated mice as recipients had a weaker suppressive effect, suggesting that ROS modified the secondary rather than the primary immune response. A milder but still significant effect was also observed in ROS deficient mice. During the primary vaccination response, regulatory T cells, upregulation of negative costimulatory molecules, and increased production of anti-inflammatory versus proinflammatory cytokines in both Ncf1 mutated and wild type B10.Q mice was observed, which could explain the vaccination effect independent of ROS.

The Journal of Immunology, 2010, 185: 2701–2709.

Rheumatoid arthritis (RA) is a chronic autoimmune disease with unknown etiology that affects ∼0.5% of the population worldwide. It is characterized by inflammation of peripheral joints and bone destruction due to synovial tissue infiltration with immune cells, secretion of proinflammatory cytokines, and matrix metalloproteinases (1). A specific prognostic marker for RA development is the presence of Abs against citrullinated proteins (ACPAs) and rheumatoid factors (RFs; anti-IgG Fc) (2). It has been shown that ACPAs together with certain MHC class II (MHC II) and PTPN22 alleles are strong predictive factors arguing for a role of adaptive immunity in the disease pathogenesis (3). The MHC II region, coding a shared amino acid motif in the peptide-binding pocket of the β-chain of HLA-DR4 and DR1 haplotypes (the shared epitope hypothesis) (4, 5) is the strongest genetic factor. This epitope includes amino acid residues 67, 70, 71, 74, which are similar in all RA-associated HLA-DR alleles and have crucial importance in determining the pocket structure as well as the T cell repertoire and its ability to recognize self peptides (4).

T cells are believed to play a central role in RA pathogenesis. Important arguments for this are the MHC II association, the presence of T cell–dependent ACPA and RF IgG in the serum, and T cell–derived cytokines in the synovial fluid and serum of patients with RA. Moreover, T lymphocytes is a large fraction of the cellular infiltrate, could be ∼40%, in the inflamed synovium (6). It has been shown that the specificity of this milieu could be directed toward different autoantigens—ubiquitously expressed molecules, such as glucose-6-phospho-isomerase, heterogeneous nuclear ribonucleoprotein-A2, the stress protein BiP (7–9), citrullinated filaggrin and vimentin (10), or joint-specific proteins (collagen II, aggrecan, human cartilage gp39) (11–14). Despite decades of intensive research, a single disease-causative autoantigen has not been identified yet. One of these candidate autoantigens, collagen type II (CII), is the major protein in hyaline cartilage and is of particular interest because immunization with CII induces collagen induced arthritis (CIA), the most commonly used animal model for RA.

Research based on CIA has been proved useful for studies of pathologic mechanisms relevant to RA development and in validating novel therapies, such as anti–TNF-α treatment (15). Importantly, an MHC II (Abeta) gene has been positioned that is a major factor for development of CIA in mice, and the most susceptible allele is Aβ, which has a peptide–binding pocket similar to the shared epitope in humans (16–18). Autoreactive T cells in both RA and CIA are directed toward the same immunodominant CII peptide 259–273 (14, 19, 20), which binds both Aβ and shared epitope human MHC II molecules. The lysine residue at positions 264 and 270 in this epitope could be posttranslationally modified by hydroxylation and subsequent glycosylation with monosaccharides or disaccharides. It has been demonstrated that glycosylation of the lysine side chains at position 264 is of particular importance for CIA development (21–23). In addition, we have previously shown that CIA in Aβ mice could be blocked by therapeutic administration of soluble MHC II molecules in complex with the galactosylated CII peptide 259–273 (24). Furthermore, we have recently demonstrated the important role of reactive oxygen species (ROS) in...
regulating autoimmune reactions and tolerance induction in animal models of autoimmunity (25–28). This finding came from the positional cloning of the \textit{Ncf1} gene (coding p47^phox subunit of the NADPH oxidase complex) and the identification of \textit{Ncf1} polymorphism in the DA rat strain, resulting in increased susceptibility to arthritis because of low production of ROS. The discovery was later confirmed in mice, where a mutation in the same gene leads to significantly reduced levels of ROS, highly increased autoimmunity, impaired tolerance to CIA, and more severe arthritis (27, 28).

In this study, we investigated the importance of ROS for the effectiveness of our vaccination therapy. We show that CIA in B10. Q mice with reduced ROS production cannot be ameliorated by MHC-II/CII–peptide treatment, which implicates a new important aspect in vaccine development.

Materials and Methods

\textbf{Mice}

\textit{Ncf1} mutated (\textit{Ncf1}^{+/+}) mice on the B10.Q background were used and described previously (28). A point mutation in exon 8 (29) of the \textit{Ncf1} gene in a B6 strain was introduced to the B10.Q/rhd strain by backcrossing for 13 generations, resulting in a genetically clean strain as checked with 10 kilo-base single nucleotide polymorphism markers. B10.Q/rhd mice with a functional \textit{Ncf1} gene were used as a control in all experiments. B10.Q mice were originally provided by J. Klein (Tübingen, Germany), but were kept in our animal house for >10 y. The animals were age- (8–12 wk old) and sex-matched; they were bred and kept at the animal facility of Medical Immunology Research (Lund University, Karolinska Institute) under temperature-, light- and food-controlled conditions. The Lund-Malmö or Stockholm ethical committee approved all animal experiments. All experiments were blinded, and the mice of different experimental groups were mixed in cages to avoid cage-dependent effects.

\textbf{Ags}

Rat collagen type II (CII) was prepared from Swarm chondrosarcoma via limited pepsin digestion as described previously (30). The protein was dissolved in 0.1 M acetic acid and stored at 4°C. The following CII259–273 peptides were used: K264 (unmodified rat CII259–273 with lysine at position 264); GIAFGFKGQVFKGKPGE and GalOK264, denoted also as Gal264 (CII259–273 with β-galactopyranosyl-5-hydroxy-L-lysine at position 264). The synthesis and purification of these peptides were described previously (31, 32). The MOG79–96 peptide (mouse myelin oligodendrocyte glycoprotein; GKVTLRQNVQRFSDEGGY) was produced by Schäfer-N (Copenhagen, Denmark). All peptides were dissolved in PBS and kept at 4°C prior to use. C1 (CII359–369; ARG1LGRGPDA) and U1 (CII494–504; LVGPRGERGF) peptides were synthesized by Schäfer-N, dissolved in 0.1 M acetic acid and stored at 4°C. Con A was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in PBS, sterile filtered, and kept at –20°C until use.

\textbf{Purification of soluble Aβ molecules and preparation of Aβ–peptide complexes}

The design of the constructs for Aβ40- and Aβ42-chains, their cotransfection in SL2 Drosophila cells (CRL-1963; American Type Culture Collection, Manassas, VA), and establishing a stably transfected cell line were described previously (24). For large-scale expression of soluble Aβ, the transfected cells were expanded in serum-free Insect Express medium (PAA Laboratories, West Grove, PA) and 100 μl Tris buffer. The positive protein fractions were concentrated, dialyzed against PBS, and purified further with Superdex 200 gel filtration column (Amersham Biosciences). Finally, the fractions with correct protein mass were concentrated and stored at 4°C until use.

\textbf{Vaccination protocol}

Intravenous injections were given through the tail vein. Mice received 100 μg protein complex (Aβ40/GalOK264 or Aβ42/K264) in 200 μl PBS on days 20 and 34 postimmunization. The control groups were injected with either PBS or 100 μg Aβ42/MOG in the same volume (200 μl) and time.

\textbf{CII immunization and CIA evaluation}

To induce CIA, all mice were injected in the skin at the base of the tail with 100 μl emulsate, prepared by mixing 1:1 CFA (Difco/Beckton Dickinson, Sparks, MD) and 100 μg CII. After 35 d, the animals received a 50-μl booster injection of 50-μg CII emulsified 1:1 with immunofluorescence assay (Difco/Beckton Dickinson). Clinical signs of arthritis were evaluated using a 60 points system, giving a maximum score of 15 points per paw. The mice were examined two to three times per week for at least 70 d, and blood was collected at days 35 and 70 postimmunization.

\textbf{Anti-CII Ab measurement}

Serum was collected from all blood samples after centrifugation at 6500 rpm for 20 min, diluted 10 times in PBS, and stored at –20°C. The levels of anti-CII Abs were measured by quantitative ELISA. For this aim, 96-well plates (Costar plates, Corning, Lowell, MA) were coated overnight with 50 μl per well of rat CII (10 μg/ml solution in phosphate buffer, pH 9). The plates were then washed three times with PBS, containing 0.1% Tween 20, and nonspecific binding to the plastic was blocked using 100 μl per well 2% PBS solution of nonfat milk for 1 h at room temperature. After washing, samples were added to the plate in serial dilutions ranging from 1:100 to 1:10^6 and incubated for 2 h at room temperature. To detect specific anti-CII Abs 50 μl per well solution of 1 μg/ml goat anti-mouse IgG (H+L chain) HRP-conjugated Ab (Jackson ImmunoResearch Laboratories, West Grove, PA) was added for 1 h at room temperature. The plates were washed five times and developed using the ABTS system (Roche Diagnostic Systems, Somerville, NJ) and absorbance was measured at the 405 nm wavelength with Wallac Victor 1420 multilabel counter (PerkinElmer, Wellesley, MA). Pooled polyclonal serum from DBA1 mice with known anti-CII Abs concentration was added in serial dilutions to all plates as a positive control.

\textbf{CII epitope-specific ELISA was done using Costar plates coated for 2 h at room temperature with either 4 μg/ml C1 peptide or U1 peptide. The blocking and sample additions were performed as already described; 50 μl per well rat anti-mouse IgG κ biotinylated Ab (1 μg/ml concentration, clone 187.1 from our Ab collection) was added to the plates after washing. The presence of anti-CII epitope specific Abs was revealed via the dissociation-enhanced lanthanide fluorescent immunoassay system using Eu^3+-labeled streptavidin (Wallac, Turku, Finland), and time-resolved fluorescence was measured with Wallac Victor 1420 ELISA reader (PerkinElmer). Monoclonal C1- or U1-specific Abs in serial dilutions were included to each plate as a positive control.

\textbf{Cytokine ELISA and T cell assays}

To determine the T cell response, mice were sacrificed 25 and 50 d after immunization, corresponding to day 3 after the first vaccination injection and day 16 after the second vaccination injection, respectively. Lymphe node and spleen cells (1 × 10^6 cells/well) were collected and restimulated in vitro with 5 μg/ml ConA in DMEM (Life Technologies, Rockville, MD) supplemented with 10% heat-inactivated FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin. After 96 h, cell-free supernatant was collected and stored at –20°C until use.

\textbf{IL-10 and IL-17 cytokine levels were measured by sandwich ELISA. Ninety-six–well Costar plates were coated for 2 h at room temperature with 50 μl per well anti-mouse IL-10 or IL-17 mAbs in a concentration of 2 μg/ml and 1 μg/ml, respectively. After blocking and washing, 50 μl supernatant from ConA-stimulated lymph node and spleen cells were added to the plates in duplicates or triplicates. The samples were incubated at room temperature for 2 h. After washing three times with PBS 0.1% Tween 20, anti-mouse biotinylated IL-10 or IL-17 Abs (1 μg/ml) were used for detection of the respective cytokines. The plates were developed with Eu^3+-labeled streptavidin and fluorescence was measured as denoted in the previous section. Recombinant mouse IL-10 and supernatant from ConA-stimulated splenocytes with known IL-10 concentration were added to each plate as a positive control.}

\textbf{T cell transfer}

Two donor groups of B10.Q mice were vaccinated i.v. (day 0) with 200 μg of either Aβ40/GalOK264 or Aβ42/MOG in a total volume of 100 μl PBS without Ca^2+ and Mg^2+ (Life Technologies). The animals were sacrificed 5 d later; spleens were harvested and pooled in two groups according to the type
The organs were passed through a 40-μm cell strainer (BD Discovery, Labware, Sparks, MD), and erythrocytes were lysed using 0.84% NH₄Cl. The cells were washed again with PBS without Ca²⁺ and Mg²⁺, and T lymphocytes were isolated via negative selection. Rat anti-mouse MHC II Abs (M5/114; BD Pharmingen) were added to the cell suspension and incubated for 20 min. The unbound Abs were then washed out and anti-rat IgG Dynabeads (Dynal Biotech, Oslo, Norway) were incubated with the cells according to the manufacturers recommendations. After magnetic sorting, the cells were washed and resuspended (5 × 10⁶ cells/ml) in PBS without Ca²⁺ and Mg²⁺ (Life Technologies). The purity of the cell suspension was evaluated by flow cytometry and was found to be 95% CD3 expressing cells.

At the same time (day 0), four recipient groups (2 groups of B10.Q and 2 groups of B10.Q Ncf1<sup>−/−</sup> mice) were immunized with CII in CFA and evaluated for arthritis development. On day 10 postimmunization, the animals received an i.v. injection of 200 μl with 1 × 10⁶ T cells: one group of B10.Q and one group of B10.Q Ncf1<sup>−/−</sup> mice got T cells from A<sup>q</sup>/GalOK264-treated animals, the other two groups (B10.Q and B10.Q Ncf1<sup>−/−</sup>) received T cells from A<sup>q</sup>/MOG-treated mice. The mice were bled and evaluated for clinical signs of arthritis.

### Flow cytometry analysis

The following Abs labeled with different fluorochromes or biotin were used for staining of surface epitopes: anti-mouse CD4 (clone L3T4, Southern Biotechnology Associates, Birmingham, AL), anti-mouse CD25 (clone PC61, eBioscience, Hatfield, U.K.), anti-mouse CD3 (clone 145-2C11; BD Pharmingen), anti-mouse CD8 (clone 53-6.7; BD Pharmingen), anti-mouse MHC II (clone 7.16.17; BD Pharmingen), anti-mouse CD-pd 1 (clone RPM1-30, BioLegend, San Diego, CA). Anti-FcRII Ab (clone 2.4G2 from our Ab collection) was used prior to staining with the aim of blocking unspecified binding to Fc receptors. Anti-Foxp3 (NRRF-30; eBioscience) and anti-CTLA-4 (UC10-4f10-11; BD Pharmingen) fluorochrome-conjugated Abs were used for intracellular staining.

Erythrocyte-free blood cells and lymph node cells pooled with erythrocyte-free spleen cells were analyzed by flow cytometry. The cells were washed, resuspended in PBS supplemented with 0.5% BSA (Sigma-Aldrich) and 0.01% NaN₃ (FACS buffer), and stained for various surface epitopes with mAbs for 15 min at room temperature. The samples were then washed with FACS buffer and either analyzed immediately or used for additional intracellular staining. The intracellular staining was performed using eBioscience Foxp3 Staining Buffer Set according to the manufacturer’s instructions. The samples were analyzed on an LSRII flow cytometer (Becton Dickinson) using Diva software (Becton Dickinson).

### Statistics

The statistical significance of the data were calculated by Mann-Whitney U test or Kruskal Wallis test using the StatView program (SAS Institute, Cary, NC). p < 0.05 was considered statistically significant.

### Results

#### Normal ROS levels are of importance for vaccination

To investigate the effect of reduced ROS levels on peptide–MHC II therapy, we induced CIA in B10.Q Ncf1<sup>−/−</sup> mice and then vaccinated them on days 20 and 34 after CII immunization. The mice were divided in three groups: 1) injected with glycosylated CII peptide (GalOK264) in complex with A<sup>q</sup>; 2) treated with unmodified CII peptide (K264)/A<sup>q</sup> complex; and 3) PBS control group. Three more animals, the other two groups (B10.Q and B10.Q Ncf1<sup>−/−</sup>) received T cells from A<sup>q</sup>/MOG-treated mice. The mice like in normal B10.Q strain, but this effect is not pronounced and is extremely mild, indicating that reduced ROS levels have a crucial effect on tolerance development and arthritis susceptibility.

### MHC II/GalOK264 vaccination reduces anti-CII Ab levels

Anti-CII Ab levels in all treated animals were measured on days 35 and 70 after CII immunization. Concordantly with arthritis data in B10.Q mice, we observed significantly decreased serum concentration of anti-CII IgG (Fig. 1C, 1D), confirming that glycosylated CII peptide–MHC II treatment affects Ag-specific T cells and they fail to stimulate pathogenic anti-CII Ab secreting plasma cells. Interestingly, the A<sup>q</sup>/GalOK264-treated Ncf1<sup>−/−</sup> mice also exhibited significantly lower levels of anti-CII IgG (Fig. 1E, 1F). This result was surprising for us considering the lack of strong arthritis suppression in this strain. The data illustrate that the CII peptide vaccination operates in the same way in Ncf1<sup>−/−</sup> and normal littermates suppressing both T cell and B cell anti-CII responses. However, the suppression of CIA is not efficient in the absence of normal ROS levels in mice with mutated Ncf1 gene.

Possibly the fine specificity of the anti-CII Ab response might differ between the B10.Q Ncf1<sup>−/−</sup> and B10.Q mice, and we attempted to find this difference by analyzing the response to certain CII epitopes. It has been shown that Abs directed to C1 and U1 epitopes from CII are arthritogenic and correlate with chronic arthritis (33). The response against C1 and U1 epitopes is immunodominant in both CIA and RA (34). In our vaccination experiment, as expected, the levels of anti-C1 and anti-U1 Abs were reduced in the A<sup>q</sup>/GalOK264-treated groups (Fig. 2), confirming again the specific effect of A<sup>q</sup>/GalOK264 vaccination. However, a trend was observed that the suppression of the response to these specific epitopes was less pronounced in the B10.Q Ncf1<sup>−/−</sup> mice.

### A<sup>q</sup>/GalOK264 treatment leads to expansion of Tregs and elevated expression of PD-1 on CD4<sup>+</sup> T cells

Next we analyzed the effect of vaccination on different T cell subsets, hoping for a clue to the role of ROS production. B10.Q and B10.Q Ncf1<sup>−/−</sup> mice were immunized with CII as described in the previous section. The animals from each strain were divided in two groups and vaccinated on days 20 and 34 postimmunization with either A<sup>q</sup>/GalOK264 or A<sup>q</sup>/MOG79–96. The A<sup>q</sup>/MOG79–96 complex suppresses experimental autoimmune encephalomyelitis (B. Dzhambazov, unpublished data), showing that it is functional in vivo, and thus an optimal negative control. Two different time points after giving the CII treatment were chosen to evaluate possible changes in various T cell subgroups. Five days after the first vaccination (day 25 postimmunization), and 16 d after the second vaccination (day 50 postimmunization) blood was collected from each group and analyzed by flow cytometry. These time points were chosen with the aim of studying the dynamics of the T cell populations during and after arthritis onset. Erythrocyte-free cell suspensions were stained for the following T cell markers: CD4, CD8, CD25, Foxp3, PD-1 and CTLA-4. Figs. 3 and 4 indicate the differences observed among the CD4<sup>+</sup> T cell population. No differences were found in the CD8<sup>+</sup> T cell subset (Supplemental Figs. 3, 6). Five days after A<sup>q</sup>/GalOK264 treatment, we detected an increase in the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells, which confirms the importance of CTLA-4 as a specific marker for regulatory T cells. The majority of CD4<sup>+</sup> CTLa-4<sup>+</sup> T cells were also Foxp3<sup>+</sup> (Fig. 3), which confirms the importance of CTLA-4 as a specific marker for regulatory T cells.
populations (Fig. 3), indicating that CD4+PD-1+ cells constitute a distinct subset of anergic T lymphocytes. The data led us to hypothesize that regulatory T cells act in concert with anergic CD4+ T cells in suppressing autoimmune reactions and arthritis development. At day 50 postimmunization, the differences in CD4+CD25+ Foxp3+ and CD4+PD-1+ populations were not prominent (Fig. 4, Supplemental Fig. 4), demonstrating that these regulatory T cell subsets decrease with time after vaccination and after exerting their function. However, at this time point (day 50) no difference was noted between B10.Q Ncf1<sup>p</sup>/p and B10.Q mice.

We could not identify a distinct mechanism for the lack of full arthritis amelioration in the B10.Q Ncf1<sup>p</sup>/p strain. A possible explanation for this fact is that the numbers of regulatory T cells, PD-1+ cells, and CTLA-4+ T cells are lower in Ncf1<sup>p</sup>/p mice compared with the normal B10.Q Aq/GalOK264-treated mice (Supplemental Figs. 2, 5).

Effect of Aq/GalOK264 treatment on IL-10 and IL-17 production
To evaluate anti-inflammatory and proinflammatory cytokine levels after peptide–MHC II administration, we measured the production of IL-10 and IL-17 in the supernatant of ConA-stimulated in vitro lymph node and spleen cells from treated mice 5 d after vaccination. As shown in Fig. 5, the treatment with Aq/GalOK264 resulted in significantly increased production of IL-10, compared with the control group treated with Aq/K264. IL-17 levels also were dramatically decreased, indicating an important shift in cytokine production. This difference suggests that Aq/GalOK264 vaccination mediates a suppressive effect on Th17 T cells and also, as shown in the previous section, switches both anergy in CII-specific cells and regulatory T cell expansion, leading to production of the anti-inflammatory cytokine IL-10.

However, the same effects on cytokine levels by the vaccination was evident also in B10.Q Ncf1<sup>p</sup>/p mice. Thus, although the changed cytokine pattern can contribute to an explanation for the vaccination effect, it cannot explain the relative resistance to vaccination in the Ncf1 mutated mice (i.e., in mice lacking ROS capacity).

T cells from vaccinated B10.Q mice completely suppress arthritis development in B10.Q mice, but only partially in B10.Q Ncf1<sup>p</sup>/p mice
We have shown previously that the A<sup>q</sup>/GalOK264 vaccination effect is mediated by T cells (24). Transfer of purified T cells from A<sup>q</sup>/GalOK264 treated B10.Q mice can completely suppress CIA. To further extend our studies in B10.Q Ncf1<sup>p</sup>/p mice, we performed a transfer of T cells from vaccinated B10.Q mice to CII
immunized B10.Q Ncf1<sup>−/−</sup> mice, using B10.Q mice as positive controls. The donors were vaccinated with either A<sup>9</sup>/GalOK264 or A<sup>9</sup>/MOG as negative controls. Interestingly, T cells from A<sup>9</sup>/GalOK264-vaccinated mice with a functional Ncf1 gene were able to suppress arthritis severity, but not incidence in B10.Q Ncf1<sup>−/−</sup> mice, whereas transfer to B10.Q mice led to complete blockage

**FIGURE 2.** Anti-CII epitopes specific response in B10.Q and B10.Q Ncf1<sup>−/−</sup> mice after vaccination. A, Anti-C1 epitope response in B10.Q mice. B, Anti-C1 epitope Ab titers in B10.Q Ncf1<sup>−/−</sup> mice. C, Anti-U1 epitope Abs in B10.Q mice. D, Anti-U1 Ab response in B10.Q Ncf1<sup>−/−</sup> mice. Sample sera were collected at days 35 and 70 postimmunization and anti-CII epitope Abs were measured by ELISA. The treatment groups consisted of seven to nine mice. The data are shown as mean ± SE. *p < 0.05.

**FIGURE 3.** Induction of regulatory T cells and PD-1 expression in CD4<sup>+</sup> T cells 5 d after A<sup>9</sup>/GalOK264 vaccination (day 25 postimmunization). A, Expression of PD-1, CTLA-4, CD25, and Foxp3 on CD4<sup>+</sup> cells in A<sup>9</sup>/GalOK264- and A<sup>9</sup>/MOG79–96-treated B10.Q mice. B, Expression of PD-1, CTLA-4, CD25, and Foxp3 on CD4<sup>+</sup> cells in A<sup>9</sup>/GalOK264- and A<sup>9</sup>/MOG79–96-treated B10.Q Ncf1<sup>−/−</sup> mice. Erythrocyte-free blood cells from A<sup>9</sup>/GalOK264- and A<sup>9</sup>/MOG79–96-treated B10.Q (n = 9) and B10.Q Ncf1<sup>−/−</sup> (n = 10) mice were stained for the following surface and intracellular markers: CD4, CD8, CD25, PD-1, CTLA-4, Foxp3. A standard gate for lymphocytes was set to exclude dead cells and aggregates. Statistics (%)) were calculated regarding the staining of the negative control. The bars represent mean values ± SE.
of arthritis (Fig. 6). Thus, this finding again confirms the strong vaccination effect on arthritis in normal mice, but not in Ncf1 mutated mice. Importantly, it also shows that this difference is related more to secondary stimulation or effector mechanisms in the donor mice rather than priming stimulation in the recipient.

Discussion

Treatment with a CII glycopeptide bound to a relevant MHC II molecule induces a profound therapeutic vaccination against CIA. However, our results clearly demonstrate that this strong vaccination effect against arthritis requires a functional ROS response. The ROS-mediated effect on the vaccination operates on T cells subsequent to the vaccination and is not related to the priming of T cells, leading to a skewed cytokine response or the induction of regulatory T cells.

Posttranslational modifications of the CII protein play a critical role for development of autoimmune arthritis (14, 21, 22, 35). It is now clear that the O-linked galactose on hydroxy-lysine at position 264 is recognized by T cells and plays a crucial role for CIA development and tolerance induction. Our group has shown that therapeutic vaccination with the GalOK264 peptide in complex with the murine MHC II molecule Aq could completely ameliorate CIA (24). This effect is present even when CIA is dependent on other MHC II molecules, which indicates an important dominant way of action. The vaccinating effect is possible to transfer with T cells, and it indicates a dominant tolerance mechanism. Our laboratory has shown previously that limited levels of ROS result in breakage of tolerance threshold (27), and the question arose as to whether the vaccination effect is dependent on a functional ROS production. Although it was clear from the experiments that a functional ROS response provided a situation in which the vaccination effect was much more efficient, it was still possible to vaccinate in mice lacking a ROS response. In both B10.Q and B10.Q Ncf1−/− mice there was a remarkable suppression of the anti-CII response and T cell production of inflammatory cytokines, whereas the production of IL-10 and a remarkable expansion of regulatory T cells could be seen after the vaccination. It has been demonstrated that regulatory T cells are potent inducers of immunologic tolerance and could actively mediate an autoimmunity suppressive effect (36–38). In addition, diminished function of regulatory T cells is involved in the pathogenesis of RA (39), confirming their importance in down-modulating autoimmune responses in arthritis. In our experiments, we observed markedly increased numbers of CD4+CD25+Foxp3+ cells in the A9/GalOK264-treated group compared with the mice administered with A9/MOG complexes. These findings suggest that MHC II/glyco-CII peptide vaccination could induce expansion of in mice. However, our results clearly demonstrate that this strong vaccination effect against arthritis requires a functional ROS response. The ROS-mediated effect on the vaccination operates on T cells subsequent to the vaccination and is not related to the priming of T cells, leading to a skewed cytokine response or the induction of regulatory T cells.

Posttranslational modifications of the CII protein play a critical role for development of autoimmune arthritis (14, 21, 22, 35). It is now clear that the O-linked galactose on hydroxy-lysine at position 264 is recognized by T cells and plays a crucial role for CIA development and tolerance induction. Our group has shown that therapeutic vaccination with the GalOK264 peptide in complex with the murine MHC II molecule Aq could completely ameliorate CIA (24). This effect is present even when CIA is dependent on other MHC II molecules, which indicates an important dominant way of action. The vaccinating effect is possible to transfer with T cells, and it indicates a dominant tolerance mechanism. Our laboratory has shown previously that limited levels of ROS result in breakage of tolerance threshold (27), and the question arose as to whether the vaccination effect is dependent on a functional ROS production. Although it was clear from the experiments that a functional ROS response provided a situation in which the vaccination effect was much more efficient, it was still possible to vaccinate in mice lacking a ROS response. In both B10.Q and B10.Q Ncf1−/− mice there was a remarkable suppression of the anti-CII response and T cell production of inflammatory cytokines, whereas the production of IL-10 and a remarkable expansion of regulatory T cells could be seen after the vaccination. It has been demonstrated that regulatory T cells are potent inducers of immunologic tolerance and could actively mediate an autoimmunity suppressive effect (36–38). In addition, diminished function of regulatory T cells is involved in the pathogenesis of RA (39), confirming their importance in down-modulating autoimmune responses in arthritis. In our experiments, we observed markedly increased numbers of CD4+CD25+Foxp3+ cells in the A9/GalOK264-treated group compared with the mice administered with A9/MOG complexes. These findings suggest that MHC II/glyco-CII peptide vaccination could induce expansion of
regulatory T cell population, which actively suppresses the autoantigen-specific immune responses. As shown by a recent in vitro study, it is possible to induce de novo generation of Ag-specific CD4+CD25+ regulatory T cells from human CD4+CD25– after stimulation with MHC II–peptide complex (40). Conceivably, a similar process takes place after administration of Aq/GalOK264 complexes.

Another interesting observation is the elevated number of PD-1–expressing CD4 T cells in the animals treated with MHC-II/glyco–CII peptide complexes. PD-1 (CD279) is a coinhibitory molecule expressed by T cells, B cells, and monocytes (41). To date, there are several reports indicating PD-1 as an essential player in maintaining peripheral tolerance and downmodulating autoimmunity. Blocking of the PD-1 pathway during experimental autoimmune encephalomyelitis or diabetes was shown to exacerbate the disease (42, 43). Moreover, polymorphisms in the human PD-1 gene are associated with development of systemic lupus erythematosus, rheumatoid arthritis, diabetes, and multiple sclerosis (44–47). Hatachi et al. (48) have reported that CD4+PD-1+ T lymphocytes expressing a significant amount of IL-10 accumulate as unique anergic T cells in RA synovial fluid. These findings are consistent with our data, with the only difference being that we observed more systemic rather than local distribution of these cells. Such anergic T cells, as shown by others (49–52), could exert a regulatory role mediated by cytokines, such as IL-10, which influence other T cells, APCs, or both, allowing establishment and maintenance of tolerance.

Based on the detected significant increase of CD4+PD-1+ cells and IL-10 production in the glycopeptide-treated mice, we hypothesize that these anergic cells could act in concert with regulatory T lymphocytes to induce tolerance to collagen a few days after Aq/GalOK264 treatment. It was evident that CD4+CD25+ regulatory T cells exert their action only in the initial process of establishing CII unresponsiveness. We did not detect a pronounced difference in the regulatory T cell subpopulations at the later time point following glycopeptide vaccination. However, there was a tendency for a stronger although not significant distinction in CD4+PD-1+ subgroup. This fact suggests that the induced regulatory subsets are short-lived and that their major role is to prime peripheral tolerance to CII, whereas the PD-1+ cells are possibly long-term players in these events. Interestingly, as demonstrated by the CIA data (Fig. 1A), CII unresponsiveness persists once it is established, and the end result is strong and long-term suppression of autoimmune arthritis development. Importantly, further experiments are needed to clarify this phenomenon and reveal in detail the mechanisms involved.

In addition, we were able to detect significant reduction in IL-17 levels. IL-17 is a proinflammatory cytokine secreted by a novel, recently identified T cell subset—Th17. It has been shown that IL-17 levels are markedly increased in the synovial fluid of patients with RA (53). Moreover, the role of this cytokine in RA pathogenesis is supported by the findings that IL-17 inhibits proteoglycan synthesis and promotes production of other proinflammatory
cytokines like IL-1, TNF-α, IL-6, and IL-8 by synoviocytes, monocytes, and macrophages (54). IL-17-deficient mice have significantly reduced CIA severity and incidence (55). In our study, the reduced IL-17 levels after Aq/GalOK264 treatment support the possibility of active suppression on pathogenic T and B cells.

There are several mechanisms that could provide an explanation for the vaccination effect. In addition, the pronounced effects on the induction of regulatory T cells, induction of phenotypic tolerant T cells, and the cytokine response suggest that the glycosylated CII peptide evoked a broader response than activation of only CII-specific T cells. This finding could provide an explanation for the highly pronounced vaccination effect with glyco CII peptides in CIA compared with the relatively mild vaccination of experimental autoimmune encephalomyelitis using the MHC-II/MOG peptide complex (B. Dzhambazov, personal communication). Importantly however, these mechanisms could not explain why the vaccination was not operating as efficiently in ROS-deficient mice. Importantly, the vaccination effect on arthritis was much stronger when T cells were transferred to normal B10.Q mice than to B10.Q with deficient ROS production. Thus, the effects on the immune response during the induction of vaccination, such as a skewed cytokine response and induction of regulatory T cells, operating in the donor B10.Q are not the critical factors. Instead there are ROS dependent mechanisms in the recipient mice that could involve a secondary activation of the transferred T cells in the recipient mice. A corresponding effect was observed in rats treated with phytol, an inducer of the ROS response (56). In this case treatment of the recipient of arthritogenic T cells could downregulate the development of arthritis. These findings also could have implications for human RA. The glycosylated CII peptide binds to the human DR4/DR1 MHC-II molecules in a manner similar to α1, extending its galactose side chains to the TCR (4, 57, 58). Moreover, T cell specificity directed to this glycosylated CII peptide binds to the human DR4/DR1 MHC-II molecules associated with arthritis in humans and in rheumatoid mice. Proc. Natl. Acad. Sci. USA 96: 9960–9965.


