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Therapy targeting antigen-specific T cells by a peptide-based tolerizing vaccine against autoimmune arthritis

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A longstanding goal has been to find an antigen-specific preventive therapy, i.e., a vaccine, for autoimmune diseases. It has been difficult to find safe ways to steer the targeting of natural regulatory antigen. Here, we show that the administration of exogenous mouse major histocompatibility complex class II protein bounding a unique galactosylated collagen type II (COL2) peptide (A^q–galCOL2) directly interacts with the antigen-specific TCR through a positively charged tag. This leads to expanding a VISTA-positive nonconventional regulatory T cells, resulting in a potent dominant suppressive effect and protection against arthritis in mice. The therapeutic effect is dominant and tissue specific as the suppression can be transferred with regulatory T cells, which downregulate various autoimmune arthritis models including antibody-induced arthritis. Thus, the tolerogenic approach described here may be a promising dominant antigen-specific therapy for rheumatoid arthritis, and in principle, for autoimmune diseases in general.

rheumatoid arthritis | immune tolerance | regulatory T cells | collagen type II | T cell receptor

Significance

Current treatments of autoimmune diseases, like rheumatoid arthritis, is directed to inflammatory consequences of the disease process, with limitations regarding effectiveness and side effects. We have shown in vivo a highly effective tolerogenic vaccine, which consists of a complex between an antigenic glycopeptide from COL2 and a relevant MHCII molecule. The vaccine operates through binding directly to the T cell receptor on the T cell surface, leading to differentiation of the T cell into a distinct regulatory phenotype mediating a dominant tissue-specific tolerance.

Autoimmune diseases affect a large part (5 to 10%) of the population but still lack preventive and curative treatments. Rheumatoid arthritis (RA) is a common, severe, chronic disease characterized by autoimmune inflammation of peripheral joints, leading to progressive cartilage and bone destruction. Existing therapeutics predominantly target common later stages of effector pathways of inflammation, thereby increasing the risk of side effects such as infections. To improve early treatment, the antigen-specific autoimmunity regulating the onset of joint inflammation must be addressed. Before and around clinical onset, the autoimmune response expands to include citrullinated proteins (1). In addition, an immune response to joint proteins, including the major joint protein, type II collagen (COL2) and to citrullinated COL2, appears (2–4). COL2 is of particular interest, as it is the major protein component in cartilage as well as in central lymphoid organs, the thymus, and bone marrow (5–7). Indeed, immunization of COL2 with potent adjuvant induces the development of autoimmune arthritis in rodents (collagen-induced arthritis [CIA]) (8).

Experimental arthritis is dependent on major histocompatibility complex class II region (MHCII) interaction (9). In the mouse, CIA is associated with the MHCII allele A^q (10) and dependent on T cell recognition of the galactosylated 259-273 COL2 epitope ($galCOL2_{259\text{-}273}$) (11, 12). Interestingly, T cells specific for the $galCOL2_{259\text{-}273}$peptide occur in RA patients carrying the DRB1*0401 allele (13–16).

With the aim of developing a therapeutic vaccine, we have found that a recombinant A^q molecule binding the $galCOL2_{259\text{-}273}$ peptide (a protein complex hereafter named A^q–galCol2) could suppress the development of CIA (17, 18). Complexes of A^q containing the COL2 peptide without galactosylation at position 264 had no effect. The reason for this remarkably selective regulatory effect is likely related to the fact that galactosylated COL2 is expressed only in cartilage (19), whereas nonglycosylated COL2 is expressed also in the thymus (5, 7). Thus, the T cell response to nonglycosylated COL2 is regulated by central tolerance mechanisms, whereas the T cell response to the galactosylated antigen is regulated by peripheral tolerance mechanisms.

Several different attempts have been made to develop tolerogenic strategies, but so far with limited clinical success. These approaches include whole-antigen tolerance (20), utilizing altered or unaltered peptide ligands (21–23), forming soluble peptide–MHC complexes, and utilizing various antigen-loaded particles (23). Furthermore, transferring Tregs (24) or tolerogenic dendritic cells (25) has also been studied as a potential approach toward promoting immune tolerance and reducing autoimmune disorders.

An efficient approach is by treatment with recombinant MHCII molecules bound to peptides, also called recombinant T cell receptor ligands (RTLs), which have demonstrated efficacy in several models of autoimmune diseases, such as uveitis, multiple sclerosis, and

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Competing interest statement: R.H., H.B., B.X., V.U., N.S. and S.W. are inventors.

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rheumatoid arthritis (21, 22, 26). The mode of action/s is however unclear. It has been demonstrated that such treatment primary target regulatory dendritic cells delivering the peptide for presentation (27–29), or coupled to particles, directly activating T cells (30). The variable results are likely dependent on the used carrier, peptide or the structure of the peptide–MHCII complex.

We previously reported that the galCOL2$_{259\text{-}273}$ peptide in complex with the soluble A^q MHCII molecules induced self-tolerance in an antigen-specific manner (18). We have now found that the MHCII–peptide complex operates via direct interaction with the cognate T cell receptors, which are facilitated by the positively charged His-tag in the recombinant MHCII molecule, in combination with the galactosylated COL2 peptide. This interaction leads to the activation of distinct nonconventional regulatory T cells.

Results

A^q–galCOL2 Complexes Ameliorate Autoimmune Arthritis.

Consistent with the previous data (18), subcutaneous (s.c.) administration of A^q–galCOL2 complexes using osmotic pumps significantly reduced the severity and incidence of CIA as well as considerably delayed the onset of the arthritis (Fig. 1*A*). We used osmotic pumps instead of i.v. injections to prolong the availability of A^q–galCOL2, based on in vitro and in vivo stability experiments (*SI Appendix*, Fig. S1).

The arthritis scoring data were confirmed by paw histology (Fig. 1 *B* and *C*). Anti-COL2 IgG antibody levels were reduced in the A^q–galCol2-treated group as compared with the control group treated with A^q–CLIP (Fig. 1*D*).

A^q–galCOL2 Complexes Target Antigen-Specific T Cells.

Since Th1-mediated immunity is involved in RA (31, 32) and in animal models for RA (33, 34), we investigated the potential of A^q–galCOL2 to downregulate COL2-specific Th1 cell–mediated immune responses, using a delayed-type hypersensitivity (DTH) test. COL2 challenge markedly increased the thickness of ears in COL2-sensitized mice, and which was attenuated by A^q–galCOL2 administration (Fig. 1*E*), indicating a functional inactivation of COL2-specific Th1 cells.

Next, we tested if T cells from mice treated with A^q–galCOL2 could transfer the suppressive effect. T cells derived from immunized mice injected with A^q–galCOL2 was found to protect

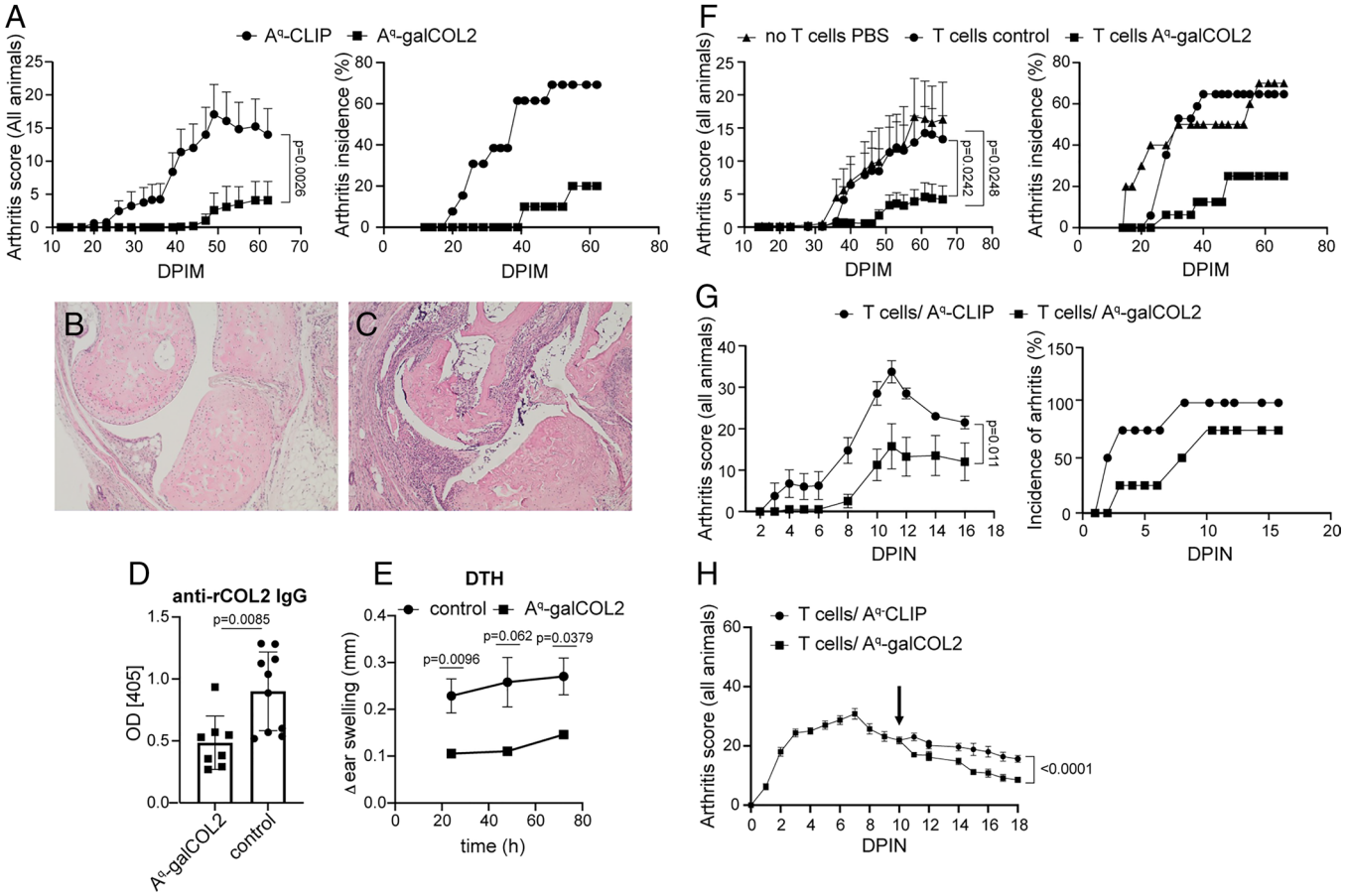

Fig. 1. Tolerogenic collagen peptide-based vaccine attenuates autoimmune pathologies (CIA, DTH, CAIA) in a T cell–dependent manner. (*A*) CIA in vaccinated mice as assessed by mean clinical score of arthritis severity (*Left*) and incidence of arthritis (percentage of affected mice [*Right*]) on different days postimmunization (DPIM). QB mice were immunized with 100 μg COL2 on day 0 and boosted on day 35 with 50 μg COL2. On day 7, mice were vaccinated s.c. with A^q–peptide complexes using osmotic pumps (n = 10 for A^q–galCOL2, n = 13 for A^q–CLIP). (*B*) Histological examination of an ankle joint from A^q–galCOL2-treated QB mice. (*C*) Histological examination of an ankle joint from A^q–CLIP-treated mice demonstrating cell infiltration and cartilage/bone destruction. (*D*) Anti-COL2 IgG serum levels assessed at day 35 during CIA by ELISA. (*E*) DTH reactions (ear swelling) following treatment with A^q–galCOL2 complexes. (*F*) Mean clinical score of arthritis severity (*Left*) and incidence (*Right*) following transfer of T cells from A^q–galCOL2-treated and A^q–CLIP-treated QB mice (n = 5 per group). (*G*) CAIA following transfer of CD4+T cells from A^q–galCOL2-treated and A^q–CLIP-treated BQ mice (n = 5 per group). Mice were immunized with COL2 on day 5 and vaccinated either with 100 μg A^q–galCOL2 or A^q–CLIP using osmotic pumps. On day 15, purified CD4+ T cells were transferred to *BQ.Cia9i* recipients, which were injected with the arthritogenic CAIA cocktail (M2139 and ACC1 anti-COL2 antibodies) on the same day as the T cell transfer. (*H*) CAIA following transfer of CD4$^+$T cells from A^q–galCOL2-treated and A^q–CLIP-treated BQ mice (n = 5 per group). Mice were immunized with COL2 and on day 5 vaccinated either with 100 μg A^q–galCOL2 or A^q–CLIP using osmotic pumps. On day 15, purified CD4+ T cells were transferred to BQ.Fcgr2b$^{-/-}$ recipients, which were injected with the arthritogenic CAIA cocktail (M2139, L10D9 and 15A11 anti-COL2 antibodies) 10 d earlier than the T cell transfer. Results are expressed as mean ± SEM. DPIN, days postinjection.

recipient mice from development of CIA. (Fig. 1*F*). Furthermore, T cells from COL2-immunized mice injected with A^q–galCOL2, was also found to protect recipient mice with CAIA (cartilage antibody-induced arthritis) (Fig. 1*G*). Thus, A^q–galCOL2 treatment activates regulatory cells that can actively suppress joint inflammation mediated by either T cells or only antibodies.

In addition, we investigated if T cells from mice vaccinated with A^q–galCOL2 could suppress established arthritis. For this, we transferred T cells from COL2 immunized mice injected with A^q–galCOL2 into mice with already established arthritis due to a previous induction of CAIA in Fcgr2b$^{-/-}$ mice. We found that A^q–galCOL2-treated T cells have a therapeutic effect on established arthritis (Fig. 1*H*).

Treatment with A^q–galCOL2 Expands Antigen-Specific CD4+ Regulatory T Cells. To characterize the tolerogenic T, we used mice with a transgenic (tg) αβTCR (*HCQ3tg*) (9) and a single β chain *tg* TCR Vb12 mice (35), both specific for the glycosylated form of the COL2259-273 peptide in the context of A^q (7, 36).

First, we found that administration of A^q–galCOL2 to *HCQ3tg* mice led to reduction in the number of galCOL2-specific T cells (Fig. 2*A*). The galCOL2-specific T cells from the *HCQ3tg* mice treated with A^q–galCOL2 had increased expression of cell markers associated with a regulatory phenotype (CD73, folate receptor4 [FR4], programmed death-1 [PD-1], and forkhead box P3 [FoxP3]) (Fig. 2*B*), similar to those observed in *HCQ3tg.MMCtg* mice. (*SI Appendix*, Fig. S2*A*). *MMCtg* mice express the immunodominant T cell epitope of rat/human COL2 (i.e., with 266E). Consequently, *MMCtg* mice display T cell tolerance when immunized with rat COL2 and have a reduced susceptibility to arthritis compared with nontolerized wild-type (wt) mice (7, 37). Prior investigations have postulated that persistent antigen signaling may prompt the emergence of CD73^{hi}FR4^{hi} anergic cells, as illustrated in Fig. 2*B*. These cells possess the capacity to differentiate into either FoxP3-expressing Tregs or IL-10 producing CD49$^+$ LAG3$^+$ Tr1 cells, depending on the specific physiological context (38–40).

To better define the phenotype and to investigate a potential dominant suppressive effect of A^q–galCOL2-treated COL2-specific T cells on activated polyclonal T cells, we utilized HCQ3*tg* recombination activating gene 1 (RAG1)-deficient mice, possessing only HCQ3-specific T cells and a lack of FoxP3-positive T cells (*SI Appendix*, Fig. S2*B*). Since galCOL2-specific T cells from RAG-1-deficient mice retained suppressive activity, the conventional FoxP3-positive Treg cells are dispensable for the effect.

Four days postvaccination, galCOL2-specific T cells from the spleens of *HCQ3tg.RAG1*$^{-/-}$ mice were enriched and cocultured with purified carboxyfluorescein N-succinimidyl ester (CFSE)-labeled CD4+ T cells from wt *BQ* mice, activated with anti-CD3 and anti-CD28 antibodies. As shown in Fig. 2*C*, tolerogenic galCOL2-specific T cells generated in vivo by administration of A^q–galCOL2 efficiently suppressed activation and proliferation of noncognate T cells.

Treatment with A^q–galCOL2 led to expansion of Tr1 cells in COL2-specific T cells from *HCQ3.RAG*$^{-/-}$ mice stimulated in vitro with COL2 (*SI Appendix*, Fig. S2*C*). Similarly, when A^q–galCOL2 complexes were administered to *HCQ3tg.MMCtg* mice, we observed induction of a Tr1-regulatory phenotype (dual expression of LAG3 and CD49b) on galCOL2 T cells (Fig. 2*D*).

To systematically characterize the regulatory phenotype of galCOL2-specific CD4$^+$T cells, we analyzed the proteomic changes (Fig. 2*E*). GalCOL2-specific CD4$^+$ T cells from *HCQ.3tg* mice, immunized with COL2 and treated with the A^q–galCOL2 ex vivo, were FACS sorted with galCOL2/A^q tetramers and analyzed by liquid chromatography with tandem mass spectrometry (LC-MS/MS). The results confirmed the anergic phenotype in galCOL2-specific CD4+ T cells from A^q–galCOL2-vaccinated mice (reflected by CD73, FR4 overexpression, and hyporesponsiveness to TCR stimulation). It also showed overexpression of diacylglycerol kinase α (DGKα), an enzyme critical for anergy induction via depletion of diacylglycerol (41), and a decreased expression of Ras-related GTP- binding protein C (Rragc), a small G-protein that mediates a crucial step in the activation of the mechanistic target of rapamycin (mTOR) signaling cascade, driving T cell activation and differentiation (42–44). We found enhanced expression of a surface molecule, V-type immunoglobulin domain-containing suppressor of T cell activation (VISTA), that may contribute to the tolerogenic potency of A^q–galCOL2-treated antigen-specific CD4$^+$T cells (45, 46). VISTA is a potent negative regulator of T cell responses that actively imposes quiescence on naïve T and myeloid cells and inhibits T cell activation and cytokine production. Moreover, VISTA is required for inducible Treg generation and stability, as genetic deficiency of VISTA on the T cells reduces inducible Treg differentiation (47). The overexpression of VISTA was confirmed by immunophenotyping of galCOL2-MHCII-tetramer enriched CD4$^+$ T cells recovered from BQ mice immunized with COL2 and treated with A^q–galCOL2 (Fig. 2*F*). Of importance, the phenotypic analysis of the tetramer-enriched galCOL2-specific CD4+ T cells from wt *BQ* mice immunized with COL2 and vaccinated with A^q–galCOL2 revealed an identical regulatory T cell phenotype to the one found in *HCQ.3tg* mice (Fig. 2*B*).

Treatment with VISTA neutralizing antibodies led to a reduction in the suppressive potency of tolerized galCOL2-specific HCQ3*tg* T cells in ex vivo suppression assays. In contrast, blocking antibodies against CD73 or PD1 had minimal or no effect, respectively (Fig. 2*G*). VISTA neutralizing antibodies also had a minimal impact on the responder T cell activity (Fig. 2*H*). To further investigate the role of VISTA in the suppressive effect of galCOL-2-specific HCQ3*tg* T cells, we pretreated with anti-VISTA antibodies, leading to a decreased suppressive effect, confirming a critical role of VISTA (Fig. 2*H*). Finally, to confirm the antigen specificity of the observed tolerogenic effect, we investigated the T cell response against ovalbumin (OVA), after vaccination. *SI Appendix*, Fig. S2*D* indicates that there were no significant variations in the T cell response to OVA after vaccination with A^q–galCOL2. Overall, our results indicate that treatment with A^q–galCOL2 leads to the generation of a population of distinct antigen-specific nonclassical regulatory T cells.

Direct Interaction of A^q–galCol2 Vaccine with the Specific TCR. The MHCII–COL2 complexes are designed to directly target TCR. However, an alternative possibility is that the COL2–MHCII complex could also be processed by antigen presenting cells (APCs). We tested these two possibilities using DR4 restricted APCs, showing that processing and peptide presentation were much more efficient with COL2259-273 peptide than with COL2259-273 peptide–MHCII complexes to stimulate COL2-specific DR4-restricted T cells (Fig. 3 *A* and *B* and *SI Appendix*, Figs. S4 and S6). IL-2 responses elicited under in vitro conditions of direct T cell contact with DR4–COL2 complexes plateau at stimulus concentrations between 5 and 10 μg/mL corresponding to the level of IL-2 release induced by 1 μg/mL anti-CD3 IgG (*SI Appendix*, Fig. S6). Accordingly, this direct mode of DR4-COL2-induced T cell stimulation proved to be much more efficient compared to the endocytosis-dependent indirect pathway of T cell activation that required 50-100 times higher protein concentrations of DR4–COL2 complexes (up to 500 μg/mL) delivered to APCs to cause low levels of T cell-mediated IL-2

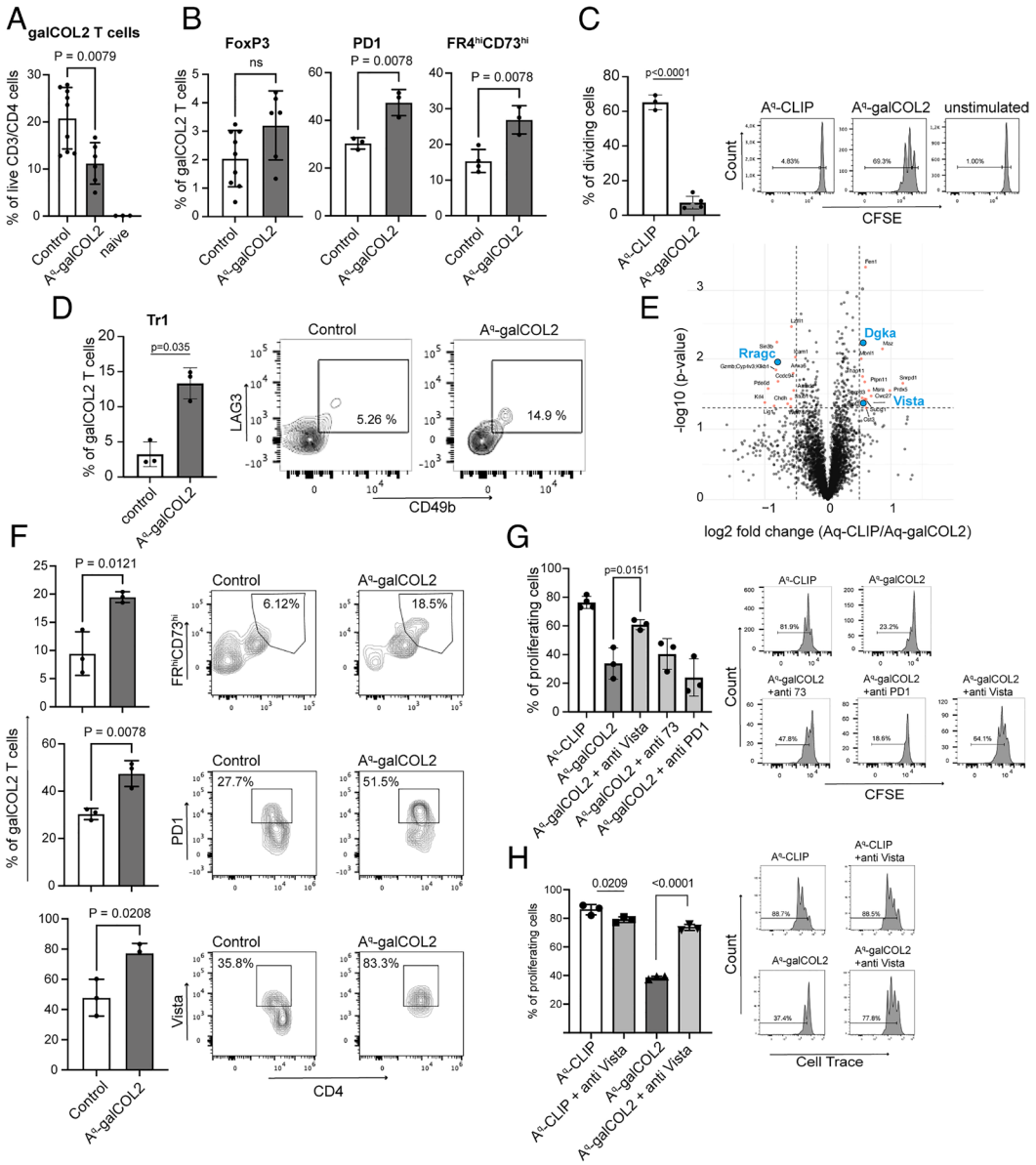

Fig. 2. Identification of regulatory phenotype of antigen-specific CD4+ T cells associated with the therapeutic effects of the A^q–galCOL2 vaccine. (*A*) Treatment with A^q–galCOL2 complexes reduces frequencies of galCOL2-specific T cells. HCQ3*tg* mice were immunized with COL2/CFA, 5 DPIM osmotic pumps filled with A^q–peptide complexes were implanted (2001D, 100 μg). Twelve DPIM T cells in dLN were analyzed with galCOL2 peptide/A^q tetramer staining using flow cytometry. (*B*) Vaccination with the A^q–galCOL2 complexes induces tolerogenic phenotype in galCOL2-specific CD4+ T cells. Upregulation of Foxp3, CD73, FR4, and PD1 was assessed by either galCOL2 peptide/A^q tetramer staining and immunophenotyping or a congenic marker CD45.1. (*C*) *BQ.HCQ3tg.Rag1*$^{-/-}$ T cells from the vaccinated mice suppress anti-CD3/CD28-induced polyclonal T cell proliferation. GalCOL2-specific CD4+ T cells from the vaccinated *BQ.HCQ3tg.Rag1*$^{-/-}$ mice (suppressor cells) were cocultured with 1×10^5 purified CD45.2 expressing CFSE-labeled CD4+ T cells from *BQ* mice (responder cells) in vitro at a ratio of 1:3. FACS analysis was performed 3 d after stimulation with anti-CD3/CD28 Abs. Quantification (*Left*) and representative gating (*Right*) are shown. (*D*) Induction of T regulatory phenotype by A^q–galCOL2 complexes in *BQ.HCQ3tg.MMCtg* mice. *BQ.HCQ3tg.MMCtg* mice were immunized with COL2/CFA, at 5 DPIM mice were vaccinated with A^q–peptide complexes. Day 12 postimmunization, the expression of CD49b and LAG3 (markers for Tr1 phenotype) within the splenic gal-COL2/A^q tetramer+ CD4+ T cells were analyzed by flow cytometry (gated on live CD3$^+$CD4+ galCOL2/A^q tetramer+ cells). (*E*) Volcano plot comparing the proteomic profile of the galCOL2-specific T cells treated with A^q–CLIP with the galCOL2-specific T cells treated with A^q– galCOL2 complex. *BQ.HCQ.3tg* mice were immunized with COL2/CFA. 5 DPIM osmotic pumps (2001D) filled either with 100 μg of A^q–galCOL2 complex (n = 3) or control A^q–CLIP complex (n = 4) were implanted. On 10 DPIM iLN cells were collected, Ag-specific CD4+ T cells were labeled with galCOL2/Aq tetramers and sorted by BD FACS Aria flow FACS sorter. The sorted cells were subjected to LC-MS/MS. Proteins involved in T cell tolerance and T cell effector functions that were significantly and substantially up- or down-regulated are highlighted in blue. Rrgac, Ras-related GTP-binding protein C, Dgka, Diacylglycerol kinase alpha; VistaV-domain immunoglobulin suppressor of T cell activation. (*F*) Vaccination with A^q–galCOL2 leads to elevated expression of VISTA, CD73, FR4, and PD1 on gal-COL2-specific T cells from wt mice. BQ wt mice were immunized with COL2/CFA, at 5 DPIM osmotic pumps filled with A^q–peptide complexes were implanted (n = 3 per group). 10 DPIM splenocytes and dLN cells were recovered, stained with the PE-labeled galCOL2/A^q tetramer and enriched using anti-PE magnetic beads. The phenotypic analysis of the enriched galCOL2-specific CD4+ T cells were performed by flow cytometry (gated on live CD4+ galCOL2/A^q tetramer+ cells). (*G*) VISTA is required for the suppressive effect of tolerogenic galCOL2-specific cells. Osmotic pumps filled with A^q–peptide complexes were implanted s.c. (n = 3 per group) to *BQ.HCQ3tg* mice. Day 6 after implantation splenocytes were recovered, CD4+T cells were purified and co-cultured with purified CFSE-labeled CD4+ T cells from *BQ* mice in the presence or in the absence of the blocking antibodies targeting VISTA, CD73, or PD1. FACS analysis was performed 3 d after stimulation with anti-CD3/CD28 Abs. Quantification (*Left*) and representative gating (*Right*) are shown (*H*) Osmotic pumps filled with A^q–peptide complexes were implanted s.c. (n = 3 per group) to BQ.HCQ3*tg*.Rag1$^{-/-}$ mice. Day 5 after implantation splenocytes were recovered, CD4$^+$T cells were purified and cocultured with purified violet cell trace-labeled CD4$^+$ T cells from BQ mice. CD4$^+$T cells from A^q–CLIP vaccinated mice were cocultured in the presence or in the absence of the blocking antibody targeting VISTA. Meanwhile CD4$^+$T cells from A^q–galCOL2 treated mice were incubated for 1 h at 4 °C with 100 μg/mL anti-VISTA antibody or isotype control. Afterward, cells were washed and added to the naïve BQ T cells. FACS analysis was performed 3 d after stimulation with anti-CD3/CD28 Abs. Quantification (*Left*) and representative gating (*Right*) are shown. Each dot represents individual mice. Results are expressed as mean ± SD.

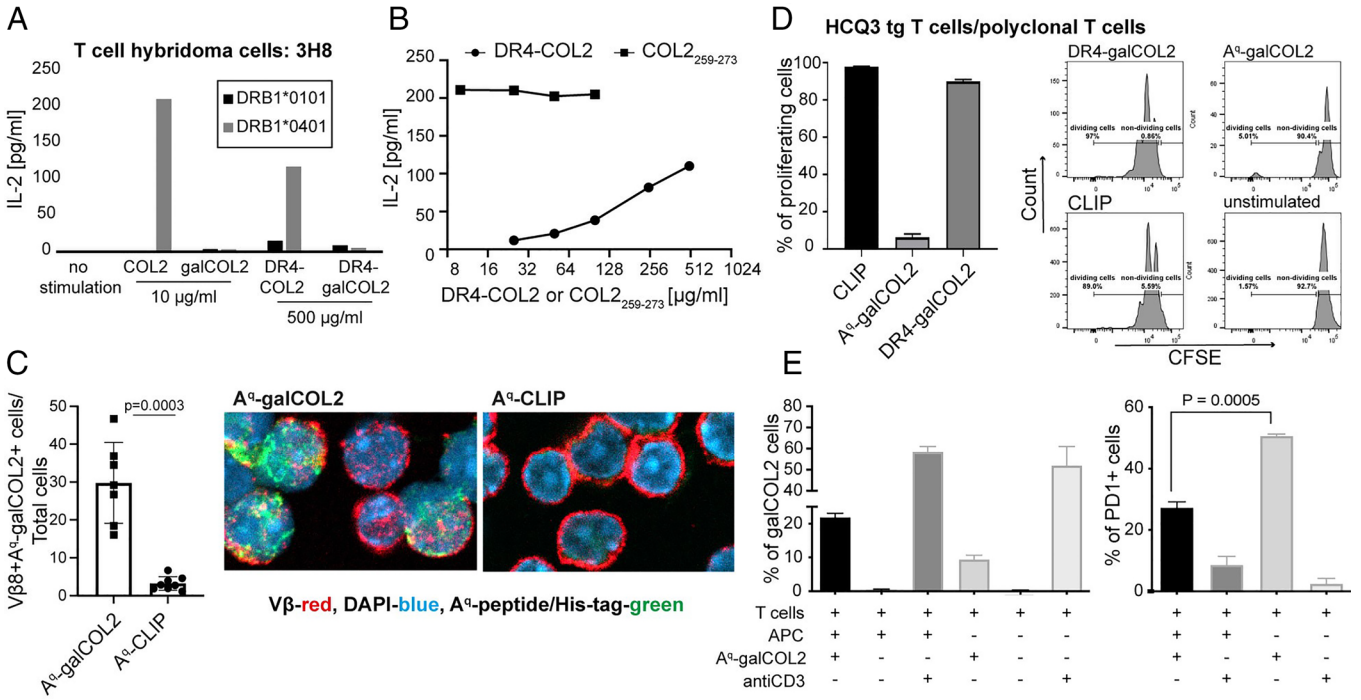

Fig. 3. A^q–galCOL2 vaccine operates through the direct interaction with the specific T cells/TCR. (*A*) A single experiment shows an inefficient presentation of COL2 peptides from the MHCII (DR4)-COL2 peptide complex upon processing by APC. APCs (10^6/mL) derived from CD3-depleted PBMC of HLA-DR-genotyped healthy donors (homozygous DRB1*0401 vs. homozygous DRB1*0101) were antigen-pulsed with either the synthetic COL2259-273 peptide or DR4-COL2259-273 complexes, subsequently PFA-fixed and finally cocultured with hybridoma 3H8 T cells (10^6/mL in a 24 well plate). Secretion of IL-2 was assessed by ELISA. (*B*) Titration curves for IL-2 production using APCs from a DRB1*0401 allele carrier and the COL2259-273 peptide or DR4-COL2259-273 complexes in the concentration range indicated under identical experimental conditions obtained from a single experiment. (*C*) Colocalization of TCR complexes as detected by anti-Vβ8 TCR antibodies (*Left*) and visualization of a direct interaction of the A^q–galCOL2 complexes with galCOL2-specific T cells by immunofluorescence microscopy (*Middle* and *Right*). *BQ.HCQ3tg.Rag1$^{-/-}$CD45.1* mice were immunized with COL2/CFA. 8 DPIM dLN cells were collected, and stained for DNA (DAPI, in blue), A^q–galCOL2 complexes bound to anti-His tag Abs (secondary antibody labeled with AF488, in green), and Vβ8 T cell receptor (secondary antibody labeled with APC, in red). A^q–CLIP complexes bound to anti-his Ab served as a negative control (100× magnification). (*D*) GalCOL2 peptide-based vaccine complexes lacking a relevant MHCII molecules do not suppress polyclonal T cell proliferation. *BQ.HCQ3tg.Rag1$^{-/-}$CD45.1* mice were s.c. administered either with A^q–galCOL2 (can directly stimulate *HCQ3tg* T cells, since they are A^q-restricted) or DR4-galCOL2 (cannot directly stimulate *HCQ3tg* T cells and is mainly acting as a galCOL2 peptide transporter) using 2001D osmotic pumps (100 µg/mouse). A^q–CLIP served as a negative control. At day 4 postimplantation, CD45.1 expressing galCOL2-specific *HCQ3tg* T cells were collected and cocultured with CD45.2 expressing purified and CFSE-labeled CD4+ T cells from BQ mice (1×10^5 cells/well) in the presence of anti-CD3/CD28 antibodies (Ag-specific T cells/polyclonal T cells ratio 1:3). Dilution of CFSE was analyzed 3 d later by flow cytometry. (*E*) Direct stimulation of the antigen-specific T cells by A^q–galCOL2 more efficiently up-regulate tolerance marker, PD1. CD45.2-expressing purified CD4+ T cells from *HCQ3tg* mice were cocultured with or without CD45.1-expressing splenocytes/APC in the presence of 25 µg/mL of A^q–galCOL2. After 4 d of the cell culture, galCOL2-specific T cells were flow cytometrically analyzed by galCOL2/A^q tetramer staining and immunophenotyping and gated on galCOL2/A^q tetramer$^+$CD45.2$^+$CD3$^+$CD4+ live cells. Spleens from four to five animals were pooled and analyzed in triplicates. Results are expressed as mean ± SD.

release (*SI Appendix*, Fig. S5). Thus, prior uptake of the DR4–COL2–peptide complexes by APCs is unlikely to be the main cause of T cell activation upon in vivo application.

Next, we investigated whether the MHCII–COL2 complexes could bind directly to COL2-specific TCR on T cells. T cells from *HCQ3tg* mice were incubated with A^q–galCOL2 or A^q–CLIP and detected with anti-penta-His, whereas TCRs were detected with anti-Vβ8 TCR antibodies. The A^q–galCOL2 complexes clearly bound to the surface of the galCOL2-specific T cells and colocalize with TCR (Fig. 3*C*).

To address the functional effect of the direct ligation of TCR by A^q–galCOL2, we used *HCQ3tg.RAG1$^{-/-}$* mice, which were treated either with A^q–galCOL2 or DR4-galCOL2. Only galCOL2-specific T cells, generated in vivo by administration of the A^q–galCOL2 complex, efficiently suppressed activation and proliferation of polyclonal T cells. In contrast, galCOL2-specific T cells from the same mouse strain but vaccinated with DR4-COL2, which cannot directly interact with the A^q restricted HCQ3 TCR, had only very weak or no suppressive effects (Fig. 3*D*).

To further test the functional effects of MHCII-COL2/TCR binding, we incubated A^q–galCOL2 with purified HCQ3*tg*CD4$^+$T cells in the presence or absence of APC (Fig. 3*E*). A stronger expansion of galCOL2/A^q tetramer-specific T cells was observed in the presence of APC, but the tolerogenic phenotype, reflected by PD1 expression, was more pronounced in T cells incubated in the absence of APC. Similarly, a direct effect of the human analogue DR4-galCOL2 on pure CD4$^+$T cells from *DRB1*0401*-positive donors produced IL-2 without additional costimulation (*SI Appendix*, Fig. S7).

Taken together, these data indicate that the MHCII–COL2 complexes generate tolerogenic antigen-specific T cells by directly interacting with the TCR, without a need for APCs.

Positively Charged Polycationic Tags Potentiate the Binding of A^q–galCOL2 to the TCR. To explain why MHCII-COL2 could directly bind to the relevant TCR, we investigated the different components of the recombinant protein. Different parts of the DR4-galCOL2 proteins were systematically removed and the modified protein tested in T cell hybridoma assays (*SI Appendix*, Fig. S8*A*). Only constructs with a polyhistidine tag (His-tag), or an equivalently charged tag, at the C-terminal end of the polypeptide comprising the DRB1*0401 or A^q β-chain were able to stimulate the cells. In a search for potential targets of the positively charged tag we selected charged glycosaminoglycans (GAGs), which are detectable in considerable concentrations on cell surfaces and surrounding matrix. We coated microtiter wells with concentrated chondroitin sulfate (CS), hyaluronan, or heparan sulfate solutions

and performed in vitro stimulation experiments of T hybridoma cells by adding the DR4–COL2 peptide complexes to the fluid phase. As a control, experiments were performed in microtiter plates with blocked surfaces in the absence of the extracellular matrix (ECM) components. The stimulating recombinant DR4–COL2 complexes used only differed by the presence or absence of the - His-tag. We also included a mutated recombinant variant of the DR4–COL2 complex in which the 6His-tag was replaced by a triplet of negatively charged amino acid residues (DED).

We found that only MHCII-COL2 containing the His-tag could induce a considerable IL-2 response under these conditions (Fig. 4*A*). This T cell activating capacity is critically dependent on the presence of a CS-coated surface of the microtiter well. Accordingly, our studies show that the 6His-tag in the DR4–COL2 complex has a functional role in providing an improved spatial orientation of the peptide-binding groove for eliciting TCR activation via an impact on the interaction with CS. In addition, subsequent studies showed that the interaction of the His-tagged DR4-COL2 with CS bound to a solid phase enhances its capacity to stimulate an IL-10 response by COL2-specific T cells (*SI Appendix*, Fig. S8*B*). The remarkable selectivity of the agonistic interaction of His-tagged DR4-COL2 with CS but not with other GAGs, e.g., hyaluronate, for T cell activation prompted us to investigate the binding affinities of the respective interactions by surface plasmon resonance (SPR) investigations. An equilibrium dissociation constant (KD) of 4.86 μM indicated a moderate binding affinity of the His-tagged DR4-COL2 for CS that was slightly better than that of DR4-COL2 without the His-tag (KD = 10.93 μM). In accordance with the functional T cell activation studies, the measurements did not reveal any binding of the His-tagged DR4–COL2 complex to hyaluronan (Fig. 4*B*).

Next, we analyzed the importance of the His-tag for the therapeutic vaccine effect. The His-tagged complexes (A^q–galCOL2) resulted in a more efficient protection against ear swelling in a DTH model (Fig. 4*C*).

To test the importance of the His tag in a CIA experiment, we had four different treatment groups: A^q–peptide complexes with and without His-tag, a histone 2A N-terminal peptide (H2AN-tag), which possesses a positive charge comparable to His-tag but is a natural sequence from the conserved protein, and the A^q–CLIP construct (which has a 6His-tag) as a control (Fig. 4*D* and *SI Appendix*, Fig. S9*C*). The A^q–galCOL2 complex without His-tag showed a similar effect as the negative control (A^q–CLIP). A^q–galCOL2 containing the histone 2A N-terminal sequence, with the highest pI value, displayed the most pronounced suppressive effect on arthritis. The results clearly demonstrate that the 6His-tag, as well as other positively charged sequences, crucially contribute to the therapeutic effect of the vaccine.

To understand the impact of tagged vs untagged A^q–galCOL2 complexes in antigen-specific T cells, we performed a proteomic analysis of galCOL2-specific CD4+ T cells from HCQ3*tg* mice immunized with COL2.

Upon treatment with tagged A^q–galCOL2 (tolerogenic potency) vs un-tagged A^q–galCOL2 (no tolerogenic potency) complexes, the expression levels of most detected proteins altered in opposite directions (Fig. 4*E*),

Taken together, the results demonstrate that positively charged tags/sequences are required for induction of tolerogenic properties in antigen-specific T cells, and, consequently, for protection against autoimmune arthritis.

Based on the above results, we propose that interactions with negatively charged proteoglycans in the membrane both on the same (cis) or neighboring (trans) T cells or extracellular matrices likely increase the avidity of MHCII-galCOL2 and the specific TCR (Fig. 4*F*). The His-tag might also contribute to a favorable orientation of the MHCII–COL2 complex for interaction with the TCR, which is viewed as an anisotropic mechanoreceptor with signaling properties sensitive to steric impact (48).

Vaccination with A^q–galCOL2 Attenuates Autoimmunity and Arthritis in Models Not Directly Involving COL2. Next, we investigated if the A^q–galCOL2 vaccine can regulate T cells specific for another arthritogenic antigen. We immunized A^q-expressing mice with the GPI325-339 peptide derived from glucose-6-phosphate isomerase (GPI), a ubiquitous protein that induces experimental arthritis and is recognized by T cells which do not cross-react with COL2 (49, 50). Treatment with A^q–galCOL2 attenuated arthritis development (Fig. 5*A*) and reduced hGPI325-339 peptide-specific T cell responses (Fig. 5*B*). Next, galCOL2-specific T cells were tracked at different time points after GPI325-339 immunization. We found that in naïve BQ mice, only low levels of galCOL2-specific T cells were detectable, but the numbers increased a few days after immunization, just before the onset of arthritis (Fig. 5*C*).

To explore if the numbers of COL2-specific T cells was associated with a possible vaccination effect for protection against arthritis, we administered the A^q–galCOL2 either 4 d before or 3 d after the immunization. We found that the vaccination effect was associated with the occurrence of galCOL2-specific T cells (Fig. 5*D*).

To investigate if the A^q–galCOL2 vaccine modulate galCOL2-specific T cells during arthritis development, *BQ* mice were immunized with the GPI325-339 peptide and injected day 3 with A^q–galCOL2. Day 10 postimmunization, the galCOL2-specific T cells from dLN were tracked using galCOL2/A^q tetramers. We found that vaccination with A^q–galCOL2 reduced the expansion of galCOL2-specific T cells (Fig. 5*E*).

Phenotyping of the galCOL2-specific CD4+ T cells after vaccination with A^q–galCOL2 complexes in hGPI325-339 peptide-induced arthritis revealed a similar phenotype to that observed in CIA (Fig. 5*F*).

In conclusion, these data demonstrate that A^q–galCOL2 displays a dominant suppressive effect on autoimmune arthritis not dependent on the specificity of the pathogenic autoreactivity.

Discussion

We describe an effective tolerogenic vaccine, with a prospect to use for treatment of RA, which consists of a complex between an antigenic glycopeptide from COL2 and a relevant MHCII molecule (17, 18). The unique effect of the vaccine is based on that it operates through binding directly to the TCR on the T cell surface, leading to differentiation of the T cell into a distinct regulatory phenotype mediating a dominant tissue-specific tolerance. Thus, this type of TCR-directed vaccine is likely applicable in all autoimmune diseases.

T cells against COL2, specific for relevant COL2 peptides, are activated in most RA patients (14, 15). Lymphocytes reactive with COL2 are of significant interest as COL2 is not only a major tissue-specific protein expressed in joint cartilage but is also expressed in bone marrow and thymus (6, 7). In thymus, COL2 is expressed in medullary thymic epithelial cells, but only in its nonglycosylated forms, whereas the glycosylated forms dominate in joint cartilage (19). Therefore, a key function of T cells to COL2 might be to maintain peripheral tolerance and thereby contribute to a dominant protection against joint inflammation. Immunization of mice, using strong adjuvants, can break this tolerance and induce arthritis, but in the normal state COL2 T cell reactivity might be predominantly of regulatory importance in both mice

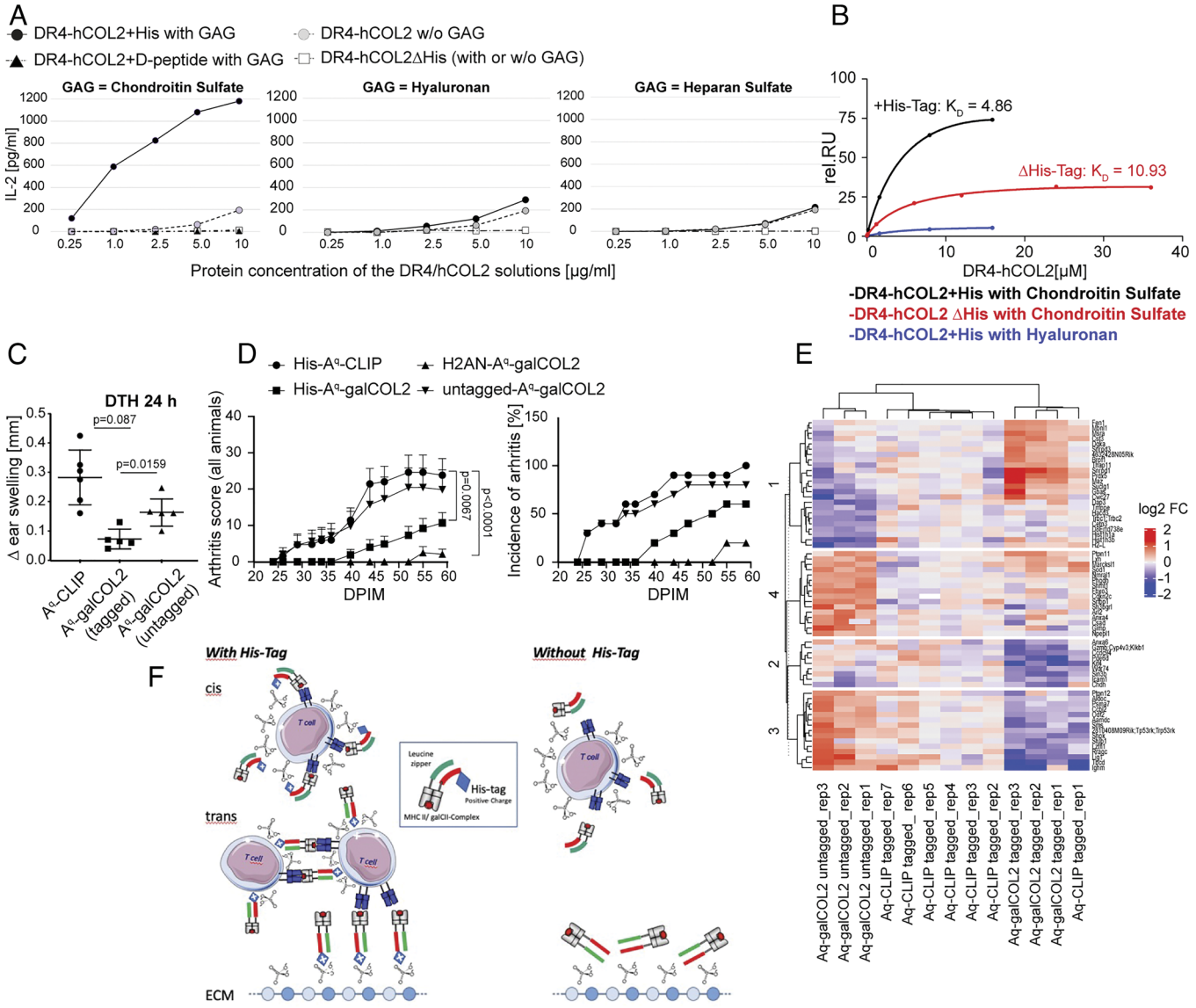

Fig. 4. Functional role of His-tag/other functionally equivalent tags in vitro and in vivo. (*A*) Impact of the His-tag in DR4-COL2 complexes and its interaction with GAG on T cell activation. 1×10^5 antigen-specific T-hybridoma cells were seeded in wells with or without precoating of 10 mg/mL of the respective GAG. The cells were stimulated for 24 h with various DR4-COL2-peptide complexes at different final concentrations between 0.25 and 10 µg/mL. T cell activation was judged via IL-2 secretion. (*B*) SPR measurement of CS binding by DR4/COL2 either with or without His-tag. Biotinylated CS was coupled to a streptavidin-chip and DR4-COL2 complexes with or without His-tag were applied at increasing concentrations. The binding curves are shown with the respective KD values (in µM) determined using the Biacore software. SPR measurements using His-tagged DR4-COL2 complexes, and a chip surface identically coated with biotinylated hyaluronan demonstrate a lack of binding affinity. (*C*) His-tag increases A^q–galCOL2 complex activity in DTH. COL2/CFA-immunized and vaccinated with 100 µg of A^q–galCOL2 (2001D on 4 DPIM). *QB* mice 11 DPIM were intradermally injected with 10 µg of COL2 to the left ear and with solvent to the right ear. Groups: A^q–CLIP (n = 5), A^q–galCOL2 complexes (n = 5). Ear swelling was measured after 24 h. (*D*) Requirement of the His-tag/other positively charged amino acid sequence for vaccination efficiency with the A^q–galCOL2 complexes in CIA. *QB* mice were immunized with COL2/CFA and 2001D pumps filled with 100 µg A^q–peptide complexes were implanted s.c. on 7 DPIM. Treatment groups (10 mice/per group): His-tagged A^q–galCOL2, A^q–galCOL2 containing histone H2A N-terminal sequence (H2AN-tag), His-tagged A^q–CLIP, A^q–galCOL2 complex without any of these tags. Mean clinical score of arthritis severity, including both arthritic and healthy mice (*Left*) and incidence of arthritis (*Right*) are shown. Results are expressed as mean ± SEM. (*E*) Heat map comparing the proteomic profile of the galCOL2-specific T cells treated with untagged vs tagged A^q–galCol2 complexes. *HCQ.3tg* mice were immunized with COL2/CFA. 5 DPIM osmotic pumps (2001D) filled either with 100 µg of tagged Aq–galCOL2 complex (n = 3) or untagged Aq–CLIP complex (n = 3) were implanted. Aq–CLIP used as a negative control (n = 7). On 10 DPIM iLNs cells were collected and antigen-specific CD4+ T cells were labeled with galCOL2/Aq tetramers and sorted by BD FACS Aria flow FACS sorter. Sorted cells were analyzed by LC-MS/MS. Pooled data of two experiments are shown. (*F*) Model illustrating a proposed mechanism of action of positively charged tags/His-tag.

and humans. It is thus of interest that, out of numerous autoantigen-specific T cells detected in RA, T cells reactive with the galactosylated COL2259-273 peptide frequently occur (14, 15). The COL2259-273 peptide binds well to both the mouse A^q molecule, associated with the development of CIA, and the human DR*0401 molecule, associated with RA (10, 13, 15).

It has been known for a long time that induction of antigen-specific tolerance is effective in mouse models, but it has been difficult to translate these protocols to humans. The basis of the experimental models is that the targeted antigen has already been defined to be of importance for the development of disease, and most protocols induce tolerance by providing tolerogenic dendritic cells with this antigen (51). This normally induces a predominantly antigen-specific tolerance that blocks the autoimmune disease driven by the same antigen and requires targeting of tolerogenic dendritic cells, which could increase the risk of adverse activation leading to an aggressive rather than regulatory T cell response.

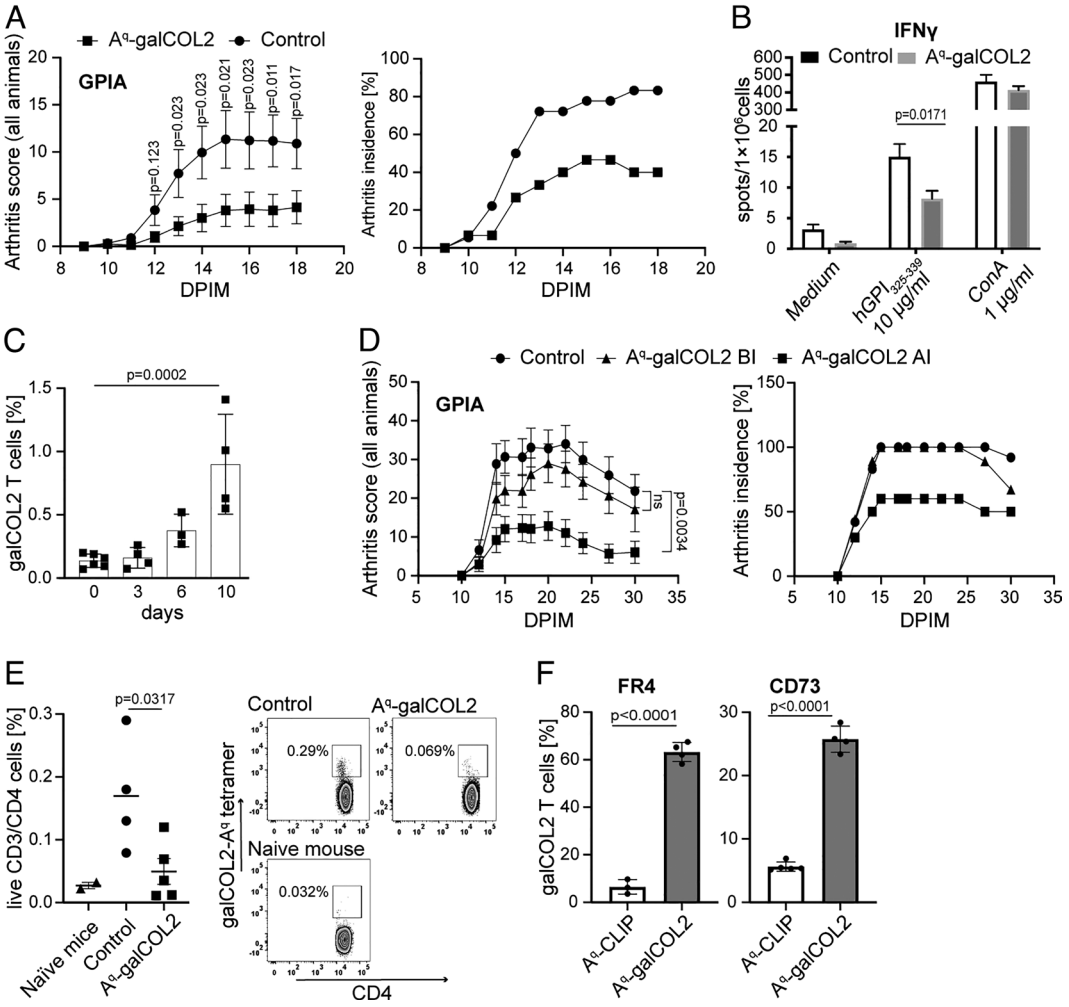

Fig. 5. A^q–galCOL2 vaccination attenuates the tissue-specific autoimmunity/inflammation via modulating galCOL2-specific T cells. (*A*) Comparison of the mean clinical score (*Left*) and incidence (*Right*) of human glucose-6- phosphate isomerase (hGPI)325-339 peptide-induced arthritis (GPIA) in two independent experiments in *BQ* mice. (*B*) Cytokine-secreting cells as determined by ELISPOT. At the end of the arthritis experimentsplenocytes were recovered and re-stimulated in vitro with the hGPI 325-339 peptide for 24 h. ConA was used as a positive control. (*C*) Kinetics of galCOL2-specific CD4$^+$T cell expansion upon immunization with hGPI325-339 peptide. *BQ* mice were immunized with hGPI325-339 peptide/CFA at indicated time points and the galCOL2-specific cells recovered from the dLN were analyzed by galCOL2/A^q tetramer staining. Frequencies of galCOL2/A^q tetramer-positive CD4+ T cells are shown. (*D*) A^q–galCOL2 does not attenuate GPIA mean clinical score (*Left*) or incidence (*Right*) if administered before experimental arthritis induction. A^q–galCOL2 complexes were administered s.c. to *BQ* mice using osmotic pumps either 4 d before GPIA induction (BI) or 3 DPIM (AI). (*E*) Vaccination with A^q–galCOL2 complexes modulates galCOL2-specific T cells in hGPI325-339 peptide-induced arthritis model in *BQ* mice. *BQ* mice were immunized with hGPI325-339 peptide/CFA and on 3 DPIM osmotic pumps filled with A^q–galCOL2 or A^q–CLIP were implanted (2001D). 10 DPIM the galCOL2-specific cells obtained from the dLN were analyzed by galCOL2/A^q tetramer staining. Quantification of galCol2/A^q tetramer-positive CD4$^+$T cell frequencies (*Left*) and representative FACS plots (*Right*) are shown. (*F*) Tolerogenic phenotype of galCOL2-specific CD4+ T cells after vaccination with A^q–galCOL2 complexes in hGPI325-339 peptide-induced arthritis. On day 0, *BQ* mice were immunized with hGPI peptide/CFA. On day 2, *BQ.HCQ3tg.RAG$^{-/-}$.CD45.1* cells (4 × 10^6 cells/mouse) were transferred to the *BQ* recipients. 3 DPIM, mice were treated either with A^q–galCOL2 or with A^q–CLIP (pump 2001D, 100 µg, n = 4 per group). 10 DPIM dLN cells were recovered and analyzed by flow cytometry and gated on CD45.1$^+$CD3$^+$CD4+ live cells. Results are expressed as the mean ± SEM.

The ability of our approach where MHCII–COL2 peptide complexes bind directly to TCR and mediate their effect directly through T cells, without the need of involving dendritic cells, reduces the risk of adverse immune activation. A recent study with nanoparticles linked to relevant peptide–MHCII complexes against autoimmune diseases showed that a protective dominant and tissue-specific tolerance could be induced (30). The COL2259-273 peptide was therapeutic in transgenic DR4-expressing mice in the CIA model, similar to our findings using A^q- expressing mice. However, there are some differences between the studies. First, regarding which peptides were effective. Based on our results, we have concluded that only certain peptides can be regulatory, as they need to allow the specific T cells to pass thymus selection and be activated to Treg cells in the periphery. The discrepancy could possibly be explained by the fact that Clemente–Casares et al. (30) used DR4 transgenic mice, which are known to lack physiologic expression of DR4 in APCs and have altered B cell function (52), which may lead to a disturbed natural tolerance. Another difference is that we could achieve tolerance without linking the complexes to nanoparticles. Our hypothesis is that the 6-His tag in our specific peptide-MHCII construct strengthened the interaction with the TCR, and thus nanoparticles are not needed for the effect.

It is also of critical importance that the encounter with TCR leads to an efficient transformation of the peripheral T cells to gain a distinct regulatory phenotype; the identification and characterization of this phenotype will allow us to follow it in humans. Interestingly, vaccination in a strong natural T cell tolerance mouse model where COL2 is modified to express a COL2259-273 peptide with stronger binding affinity to MHCII (as in humans) leads to the predominance of T cells with expression of LAG3 and CD49b, described as a typical TR1 regulatory cell. Our findings

show that the effector phase of the regulatory COL2-specific T cells triggered by the vaccine is tissue-specific but not antigen-specific. This is certainly a favorable feature for a successful treatment and is expected from a dominant regulatory effect. Importantly, even though the suppressive effect is not antigen-specific, it requires the presence of the target antigen in the tissue, regardless of whether COL2 is injected, as in the DTH reaction or the CIA model, or affects the cartilaginous joints like in the GPI peptide-induced arthritis.

The ability to induce dominant tolerance makes it possible to treat RA patients with a vaccine targeting COL2-specific T cells, even if autoreactivity to COL2 is not directly involved in driving RA in the patients. The induced Tregs operate in the joint where there is an ongoing inflammation that is likely to be driven by other joint autoantigens. Still, as COL2 is present in joints due to normal release from cartilage, regulatory T cells will become activated and have the capacity to downregulate pathogenic T cells recognizing other autoantigens. A major problem with translating tolerance-based therapies into the clinic is the need to revert an already established disease rather than prevent initial disease onset (53). A vaccine given to a patient with an already established disease thus needs to both induce new T cells with regulatory function and also revert pathogenic T cells to a more quiescent state. Our data suggest that administration of the A^q–galCOL2 complexes induces the generation of antigen-specific T cells with a regulatory phenotype in both new onsets and established disease situations. This was shown, both in mice expressing COL2 with a MHCII high-affinity peptide in which natural Tregs could be targeted, and in mice with an established disease where the activated pathogenic T cells are not well tolerized due to the natural low-affinity interaction with MHCII by mouse COL2-derived peptides.

A key feature of the A^q–galCOL2 vaccine is that it directly interacts with the TCR complex of the targeted T cells. Due to the low affinity of such interactions, it could not be predicted that a monovalent antigenic peptide MHCII complex could reach the affinity threshold. However, the vaccine is a recombinant protein and we identified that the molecular explanation was the inserted His-tag, which strengthened the interaction with the TCR on the cell surface. Deletion of the His- tag abolished both binding and the vaccination effect.

In conclusion, we describe a vaccine that directly targets autoreactive, naturally regulatory T cells in mice, mediating a suppressive and tissue-specific effect on inflammation and the development of arthritis in peripheral joints. In mouse models, the vaccine can reverse an established autoimmune process. As the same peptide-specific T cells are targeted in the human context, the vaccine is a good candidate for clinical testing.

Materials and Methods

Mice. All mouse strains used are on the same genetic background (C57Bl10.Q), kept in the identical specific pathogen-free environment in the same animal house. Mouse experiments follows standard protocol, to avoid any genetic, environmental, or experimental bias, and are ethically approved.

In Vivo Functional Models. We used collagen-induced arthritis (CIA) and glucose phosphoisomerase-induced arthritis as T cell-mediated models for RA; and cartilage antibody-induced arthritis (CAIA) as an antibody-mediated model for RA. We used the delayed type of hypersensitivity (DTH) model to test Th1 function in vivo. Joints were analyzed by clinical scoring and histology. Serum antibody responses and cytokine secretion were measured by ELISA. T cell responses were measured by ELISPOT assays, antigen-specific tetramers and cellular phenotyping by flow cytometry. The proteomic changes by vaccination were analyzed with mass-spectrometric analysis on galCOL2-specific $CD4^+$T cells.

Construction and Analysis of Vaccines and Tetramers. The vaccine is a complex with the extracellular domain of MHCII (murine Aq or human DRA/DR*0401) covalently linked with a peptide from COL2, triple helical position 259 to 273; (GIAGFKGEQGPKGEP) or with a Galactose at position 264 (GIAGFK[Gal-Hyl]GEQGPKGEP), or a CLIP peptide (*SI Appendix*, Fig. S9 *A* and *B*). The vaccine was modified including sequencing of different charges (*SI Appendix*, Fig. S9*C*). The mice were treated with the vaccine constructs by an osmotic pump. Tetramers were constructed by adding a biotin site binding to streptavidin and was used for detecting antigen-specific T cells.

Affinity measurements were made with SPR. Interactions with charged polysaccharides were performed in solid phase assays.

Patients and Healthy Donors. All blood sample donors gave prior written consent for study inclusion and were positively genotyped for *DRB1*04:01* carrier status. All patients had well-established disease with a duration of >3 y and were receiving treatment with methotrexate as a disease-modifying antirheumatic drug.

Statistical Analysis. The statistical software package GraphPad Prism was utilized to conduct statistical calculations. The Mann–Whitney *U* test was employed to analyze the antibody levels, in vitro lymphocyte assays, and arthritis severity data. In addition, Student's *t* test was used to determine the frequencies of populations determined by flow cytometry. To evaluate the statistical significance of arthritis severity, the area under the curve for each mouse was calculated and compared.

Additional details of material and methods are to be found in *SI Appendix*.

Patient and Public Involvement. Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Ethics Approval. All blood sample donors gave prior written consent for study inclusion. The current study has been approved by the ethical approval committee of the University Hospital Frankfurt, Germany.

Data, Materials, and Software Availability. All data relevant to the study are included in the article or uploaded as *SI Appendix*. The mass spectrometry proteomics data files have been deposited to ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository with the data identifier PXD034792 (54).

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