Suppression of a broad spectrum of liver autoimmune pathologies by single peptide-MHC-based nanomedicines

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Peptide-major histocompatibility complex class II (pMHCII)-based nanomedicines displaying tissue-specific autoantigenic epitopes can blunt specific autoimmune conditions by re-programming cognate antigen-experienced CD4+ T-cells into disease-suppressing T-regulatory type 1 (TR1) cells. Here, we show that single pMHCII-based nanomedicines displaying epitopes from mitochondrial, endoplasmic reticulum or cytoplasmic antigens associated with primary biliary cholangitis (PBC) or autoimmune hepatitis (AIH) can broadly blunt PBC, AIH and Primary Sclerosing Cholangitis in various murine models in an organ- rather than disease-specific manner, without suppressing general or local immunity against infection or metastatic tumors. Therapeutic activity is associated with cognate TR1 cell formation and expansion, TR1 cell recruitment to the liver and draining lymph nodes, local B-regulatory cell formation and profound suppression of the pro-inflammatory capacity of liver and liver-proximal myeloid dendritic cells and Kupffer cells. Thus, autoreactivity against liver-enriched autoantigens in liver autoimmunity is not disease-specific and can be harnessed to treat various liver autoimmune diseases broadly.

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Nanoparticles (NPs) coated with mono-specific type 1 diabetes (T1D), experimental autoimmune encephalomyelitis (EAE) or collagen arthritis (CIA)-relevant peptide-major histocompatibility complex (MHC) class II (pMHCII) molecules can restore normoglycemia in diabetic animals or motor function in paralyzed mice, and resolve joint swelling and destruction in arthritic mice. pMHCII-NPs directly trigger sustained ligation of cognate antigen receptors on autoantigen-experienced FoxP3+CD25+ T-cells, promoting their differentiation into T-regulatory-type-1 (TR1)-like cell progeny in a phagocyte-independent manner, followed by systemic expansion. Consequently, these compounds cannot trigger TR1-like cell formation or expansion in mice that are either disease-free or do not express the cognate autoantigen. These in vivo-expanded TR1-like cells then broadly suppress the polyclonal T-cell responses underlying T1D, EAE, and CIA development in a disease-specific manner, by suppressing local autoantigen presentation and antigen-presenting cell (APC) activation in a cognate antigen-dependent but non-antigen-specific manner (i.e. by recognizing cognate pMHCII molecules on costimulation-competent, autoantigen-loaded APCs).

In autoimmune disorders like T1D, multiple sclerosis (MS) or rheumatoid arthritis (RA), disease results from recruitment of T-lymphocytes and B-lymphocytes recognizing a diverse repertoire of organ-specific autoantigens. In other organ-specific autoimmune disorders, such as liver autoimmune diseases—primary biliary cholangitis (PBC), primary sclerosing cholangitis (PSC) or autoimmune hepatitis (AIH)—the autoimmune response focuses on liver-enriched, non-organ-specific antigens, such as the mitochondrial pyruvate dehydrogenase complex-E2 component (PDC-E2) in PBC; or nuclear, cytoplasmic, or Golgi-enriched proteins, such as F-actin, formimidoyltransferase cyclodeaminase (FTCD), or cytochrome P450 (CYPD2D6) in AIH; or tropomyosin isoform 5 (tTMS5) in PSC, among several others.

Although AIH, PBC, and PSC are considered as distinct diseases, there is a group of patients presenting with features of both cholestatic liver disease and AIH. Furthermore, PBC is frequently associated with extra-hepatic autoimmune conditions. The existence of these overlap syndromes suggests that activation of T-cells targeting such liver-enriched autoantigens may contribute to various liver autoimmune conditions. In that case, pMHCII-based nanomedicines displaying epitopes from antigens relevant to one disease (e.g. from PDC-E2 in PBC) might be able to trigger the formation and expansion of epitope-specific TR1 cells capable of blunting both the corresponding liver autoimmune disease (e.g. PBC) and other liver autoimmune diseases.

We sought to test this hypothesis by asking if pMHCII-based nanomedicines displaying epitopes from various PBC-relevant or AIH-relevant antigens could blunt liver autoimmunity broadly. We find that pMHCII-based nanomedicines displaying epitopes from various liver-autoimmune disease-relevant antigens can blunt not only the relevant liver autoimmune disease (i.e. PDC-based nanomedicines blunt PBC) but also their irrelevant counterparts (i.e. PSC and AIH in addition to PBC). Remarkably, they do so without impairing the ability of the host to mount antibody responses against exogenous antigens, to clear viral or bacterial infections or to kill metastatic allogeneic tumors. Thus, hepatocyte and cholangiocyte autoimmune insults can readily trigger the stimulation of peripheral T-cells recognizing liver-prevalent self-antigens, and such T-cell responses can be harnessed by pMHCII-based nanomedicines to treat liver autoimmunity broadly.

**Results**

**TR1 cell formation and expansion by PBC-relevant pMHCII-NPs.** NOD.3c34 mice, which carry anti-diabetogenic regions from C57BL/6 chromosomes 3 and 4, spontaneously develop a form of autoimmune biliary disease that resembles human PBC. Like >90% of PBC patients, these mice develop autoreactive T-cell and B-cell responses against the dihydrolipoic acid transferase (E2) and dihydrolipoyl dehydrogenase-binding protein (E3BP) components of the PDC complex, leading to biliary epithelial cell destruction, cholestasis, small bile duct proliferation, and liver failure.

We searched for peptides in murine PDC-E2 capable of binding to the NOD/NOD.3c34 class II molecule I Ag7 in silico. I Ag7-based pMHCs displaying two such epitopes (PDC-E2166/181 and PDC-E282/96) or a negative control peptide (the T1D-relevant BDC2.5 mimotope) were purified from culture supernatants of transgenic CHO cells and coated onto functionalized iron-oxide NPs or used to produce pMHC tetramers.

pMHC tetramer staining showed that the peripheral blood of untreated NOD.3c34 (but not NOD) mice harbor both PDC-E2166/181-reactive and PDC-E282/96-reactive but not BDC2.5mimotective CD4+ T-cells, particularly as mice age (Fig. 1a).

Treatment of 15-week-old NOD.3c34 mice with PDC-E2166/181/IAg7-NP (twice a week i.v.) triggered the expansion of the PDC-E2166/181/IAg7 (but not PDC-E282/96/IAg7) tetramer+ T-cell pool in peripheral blood (Fig. 1b), spleen, liver, portal/celiac (liver-draining) lymph nodes, and bone marrow, as compared to control NP-treated NOD.3c34 littersmates (having PBC) or untreated NOD mice (not having PBC) (Fig. 1c, d). In fact, this expansion was associated with significant reductions in the frequencies of endogenous PDC-E282/96/IAg7 tetramer+ cells (Fig. 1d). Treatment with T1D-relevant (but PBC-irrelevant) BDC2.5/IA Ag7-NPs did not trigger cognate T-cell expansion (Fig. 1b–e), confirming that pMHC-based nanomedicines exclusively operate on autoantigen-experienced T-cells (BDC2.5-like CD4+ T-cells are not expected to undergo activation by their cognate beta cell autoantigen in the absence of diabetogenic autoimmunity).

As was the case for the TR1-like CD4+ T-cells induced by T1D-relevant pMHC class II-NPs in NOD mice, the PDC-E2166/181/IAg7 tetramer+ T-cells that expanded in response to PDC-E2166/181/IAg7-NP treatment were CD25+/FoxP3+ and expressed the TR1 markers lymphocyte-activation gene-3 (LAG-3), CD49b (integrin a2 or very-late antigen-2), LAP (transforming growth factor beta latency-associated peptide), program cell death protein-1 (PD1), and inducible T-cell costimulator (ICOS) (Fig. 1f and Supplementary Fig. 1a–c). Although most of the tetramer+ cells expressed LAG-3, expression of CD49b was less penetrant, particularly in the spleen (Supplementary Fig. 1b). This phenotype is similar to that described for the TR1-like CD4+ T-cells arising in NOD mice in response to T1D-relevant pMHC class II-NPs. Increased levels of these markers on tetramer+ CD4+ T-cells isolated from target-organ draining LNs vs. spleen (Supplementary Fig. 1b) is consistent with the positive effects of antigen-induced activation of these cells on TR1 marker expression.

In addition, the tetramer+ CD4+, but not the tetramer–CD4+ cells from these mice (FACS-sorted) produced IL-10 (but not IFNy, IL-2, IL-4, IL-9, or IL-17) specifically in response to DCs pulsed with their cognate but not non-cognate peptides ex vivo (PDC-E2166/181 vs. BDC2.5mi, respectively; Fig. 1g). Similar results were obtained in mice treated with PDC-E282/96/IAg7-NPs, displaying a second PDC-E2 derived I Ag7-binding peptide (Fig. 1b–d and Supplementary Fig. 1a–c). These TR1-like CD4+ T-cells were not “exhausted” CD4+ T-cells, because unlike CD4+PD1+LAG-3 +KLRG1+ (killer-cell lectin-like receptor 1) T-cells isolated from old untreated NOD.3c34 mice, PDC-E2166/181/IAg7 tetramer+CD4+LAG-3+CD49b+ T-cell isolated from PDC-E2166/181/IAg7-NP-treated animals proliferated in response to stimulation with anti-CD3/anti-CD28 mAb-coated beads ex vivo, without...
undergoing activation-induced cell death (AICD) (Supplementary Fig. 1d). Thus, as it was the case for T1D, CIA, and EAE-relevant pMHC class II-NTs, PBMC-relevant pMHC class II-NTs induce the formation and expansion of TR1-like CD4+ T-cells in vivo.

**TR1 cell-driven reversal of established PBC.** NOD.c3c4 mice display biliary epithelial proliferation, mononuclear cell infiltration of the biliary tree, massive bile duct involvement, and enlargement of the common bile duct (CBD) by 6–8 weeks (Fig. 2b–d). By ~15 weeks, they display increased total bile acid production (Fig. 2c), accompanied by elevated levels of serum alanine aminotransferase (ALT) (Fig. 2a), massive infiltration of the biliary epithelium by both CD4+ and CD8+ T-cells (Fig. 2f), high titers of anti-nuclear autoantibodies (ANAs) (Fig. 2e, right, also absent in NOD mice) and a nearly three-fold increase in liver weight by ~38 weeks of age (Fig. 2d, bottom).

Treatment of 15-week-old NOD.c3c4 mice with PDC-E2166-181/IAβ7-NTs and PDC-E2165-96/IAβ7-NTs, but not BDC2.5/IAβ7-NTs (displaying a pancreatic beta cell-specific epitope), resulted in significant reductions in all examined biochemical, immunological, macroscopic, and microscopic readouts of PBC (Fig. 3a–f). Similar results were obtained when treatment was initiated at the peak of disease (24 weeks) (Fig. 3g–i and Supplementary Fig. 1e). As was the case for the TR1 cells arising in response to T1D and EAE-specific pMHC class II-NTs 4, mAb-based in vivo blockade of IL-10 and TGFβ in pMHC-NT-treated NOD.c3c4 mice showed that the therapeutic effects of PDC-E2166-181/IAβ7-NTs required these two TR1 cell-derived cytokines (Fig. 4a–c).

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**Fig. 1** Mitochondrial autoantigen-based pMHCII-NPs expand cognate TR1 cells. **a** Percentages of tetramer+ CD4+ cells in blood of NOB vs. NOD.c3c4 mice vs. age (4 and 5 mice/strain, respectively). **b** Percentage of tetramer+ CD4+ cells in blood of pMHCII-NT-treated NOD.c3c4 vs. untreated or control NP-treated NOD or NOD.c3c4 mice. Data correspond to: 2–5 untreated NOD mice (1 experiment); 2–5 untreated and 15 Cys-NP-treated NOD.c3c4 mice and 18 PDC66/181/IAβ7-NT-treated NOD.c3c4 mice (5 experiments); three untreated and five PDC82/96/IAβ7-NT-treated NOD.c3c4 mice (2 experiments); and 3–5 untreated and 3–5 BDC2.5mi/IAβ7-NT-treated NOD.c3c4 mice (2 experiments). **c** Percentages of tetramer+CD4+ cells in various organs from the mice in (c) at the end of therapy. **d** Percentages of tetramer+CD4+ cells in lymphoid organs and liver of NOD.c3c4 mice treated for 9 weeks with PDC82/96/IAβ7-NTs or PDC66/181/IAβ7-NTs. Data correspond to four untreated and three PDC66/181/IAβ7-NT-treated, and six untreated and eight PDC82/96/IAβ7-NT-treated NOD.c3c4 mice. **f** Percentages of tetramer+CD4+ cells in liver and various lymphoid organs in the NOD.c3c4 mice treated with BDC2.5mi/IAβ7-NTs from panel. **f** Expression of TR1 markers by splenic tetramer+CD4+ cells upon stimulation with peptide-pulsed DCs. Data correspond to four PDC66/181/IAβ7-NT-treated NOD.c3c4 mice from three experiments. Data are represented as mean ± SEM. *P* values were calculated via two-way ANOVA (a and b) or Mann–Whitney U (c–e).
To ascertain if the TR1-like CD4+ T-cells arising in these mice in response to pMHC class II-NP treatment were, at least in part, responsible for therapeutic activity, we transferred purified splenic CD4+ cells from PDC-E2166–181/IAβ7-NP-treated or untreated mice into NOD.scid.c3c4 hosts reconstituted with splenocytes from diseased (untreated) NOD.c3c4 donors. As shown in Fig. 4d–g, CD4+ cells from PDC-E2166–181/IAβ7-NP-treated mice had significant disease-suppressive activity as compared to CD4+ T-cells from untreated mice, and treatment of the hosts with PDC-E2166–181/IAβ7-NPs enhanced this effect. The specificity of this in vivo disease-suppressive effect was tested by repeating these adoptive transfer experiments but using FACS-sorted PDC-E2166–181/IAβ7 tetramer+ CD4+ T-cells from PDC-E2166–181/IAβ7-NP-treated NOD.c3c4 donors or control BD2.5mi/IAβ7 tetramer+ CD4+ T-cells from BDC2.5mi/IAβ7-NP-treated NOD mice; only the former, but not the latter, were able to suppress disease progression in the hosts as compared to hosts only receiving effector T-cells (Fig. 4h). Thus, these PDC-E2 epitope-based nanomedicines trigger the formation and expansion of cognate TR1-like cells, which then go on to suppress the progression of PBC.

**Suppression of local and proximal APCs.** We have previously shown that T1D-relevant antigen-specific TR1-like CD4+ T-cells selectively suppress the proinflammatory and antigen-presenting capacity of pancreatic lymph node-associated APCs by recognizing cognate pMHC class II complexes on autoantigen-loaded APCs draining the pancreas (the source of autoantigenic
To investigate whether this is also the case for liver autoimmune disease-relevant TR1-like CD4+ T-cells, we compared the cytokine/chemokine profiles of CD11b+ cells purified from the portal/celiac (liver-draining) and mesenteric (non-draining, control) lymph nodes (PCLN and MLN, respectively) of PDC-E2166/IAg7-NP-treated vs. control NP-treated animals (Supplementary Fig. 2a). LPS-challenged CD11b+ cells of the PCLN of control NP-treated animals secreted significantly higher levels of a broad range of pro-inflammatory cytokines and chemokines (n = 14/30) than their MLN-associated counterparts, consistent with an increased pro-inflammatory capacity of liver-draining CD11b+ cells in diseased mice (Fig. 5a and Supplementary Fig. 2b). Conversely, the PCLN CD11b+ cells of PDC-E2166/IAg7-NP-treated mice secreted significantly lower levels of these 14 pro-inflammatory mediators than both their mesenteric counterparts and the CD11b+ cells isolated from the
Fig. 3 Mitochondrial autoantigen-based pMHCII-NPs blunt PBC in NOD.c3c4 mice. a Changes in serum TBA and ALT levels (n = 15, four experiments; n = 13, three experiments; and n = 8, two experiments (left to right)). b Representative liver micrographs (×4 and ×10) of liver sections from Cys-NP-treated vs. PDC166-181/IAβ7-NP-treated mice (top) and average scores (bottom) (n = 5, two experiments; n = 20, five experiments; n = 17, four experiments; and n = 6, two experiments (left to right)). Scale bars: 100 μm. c and d Representative common bile duct (CBD) images (c, top), average CBD scores/diameters (c, bottom), representative livers (d, top) and average liver scores/weight (d, bottom) (n = 5, two experiments; n = 30, seven experiments; n = 19, four experiments; and n = 8, two experiments (left to right)). e Representative images of treated NOD.c3c4 mice. f Anti-PDC-E2 positive antibody activity levels (PAA, see "Methods" for details) and ANA titers (top), and representative images of Hep2 cells stained with diluted (1:160) sera (bottom) (n = 16, five experiments; n = 13, three experiments; n = 8, two experiments, respectively -left-; n = 7, 8 and 5, 1-experiments, respectively -right-). Scale bars: 100 μm. g Percentages of tetramer+ cells in 38-44 week-old mice treated from 24 weeks (n = 6 and 7, respectively, two experiments). h and i Macroscopic (h) and microscopic scores (i) for the mice in g (n = 31 (h) or 25 (i); n = 19 (h) or 7 (i); and n = 8 (h and i) (left to right)). Data correspond to mean ± SEM. P values were calculated via Mann-Whitney U.

![Figure 3](https://example.com/figure3.png)

Fig. 4 Therapeutic activity of PBC-relevant pMHCII-NPs can be suppressed by IL-10 and TGFβ blockade. a Percentages of tetramer+CD4+ T cells in blood and lymphoid organs of NOD.c3c4.scid hosts reconstituted with whole splenocytes from untreated NOD.c3c4 donors a day after transfusion with splenic CD4+ T-cells from untreated or PDC166-181/IAβ7-NP-treated NOD.c3c4 mice. The hosts were either left untreated or were treated with PDC166-181/IAβ7-NPs. b Representative FACS staining histograms (left) and average mean fluorescence intensity values for TR1 markers on tetramer+CD4+ vs. tetramer−CD4+ T-cells (right) of the hosts treated with PDC166-181/IAβ7-NPs. c and d Macroscopic scores and liver weights (c) and microscopic scores (d) of the mice studied in a. Data in a-d correspond to means ± SEM, were compared with Mann-Whitney U, and correspond to n = 9 (blue in c) or 7 (blue in d), 5 (red in a, c, and d), and 5 mice (green in a, c, and d)/treatment group, respectively. e Percentages of tetramer+ CD4+ T-cells in mice treated with pMHCII-NPs and rat-IgG (control) or blocking rat mAbs against mouse IL-10 or TGFβ. f-g Macroscopic (f) and microscopic (g) scores of the mice studied in a. Data in e-g correspond to n = 5, 3-4 and 4 mouse/treatment group, respectively. h Macroscopic scores, liver weights, and microscopic scores of NOD.c3c4.scid hosts reconstituted with whole splenocytes from untreated NOD.c3c4 donors a day after transfusion of the hosts with: FACS-sorted splenic/PLCN-derived tetramer+CD4+ T-cells from PDC166-181/IAβ7-NP-treated NOD.c3c4 mice; purified PCLN B-cells from PDC166-181/IAβ7-NP-treated NOD.c3c4 mice; FACS-sorted splenic/PLCN-derived tetramer−CD4+ T-cells from BDC2.5mi/IAβ7-NP-treated NOD; or purified PLN B-cells from BDC2.5mi/IAβ7-NP-treated NOD mice. Data in h correspond to n = 5/group (red, blue and yellow) or n = 4/group (orange and cyanine). Data in a-h correspond to mean ± SEM. P values were calculated via Mann-Whitney U.

![Figure 4](https://example.com/figure4.png)

PCLNs of control-NP-treated animals. This indicated that, in NOD.c3c4 mice, treatment with PDC-E2166-181/IAβ7-NPs selectively downregulates the pro-inflammatory capacity of CD11b+ cells draining the liver (Fig. 5a and Supplementary Fig. 2b), presumably because they are loaded with PDC-E2 antigenic material shed from the injured liver and thus display the TR1 cells’ cognate pMHC class II complexes on their surface. pMHC-NP treatment also decreased the secretion of CCL4, IFNy, and IL-10 by PCLN-associated CD11b+ cells, but this reduction was also seen in MLN-associated CD11b+ cells (Supplementary Fig. 2b). Interestingly, Kupffer cells isolated from PDC-E2166-181/IAβ7-NP-treated mice (Supplementary Fig. 2a) also secreted significantly lower levels of 8 of these 30 mediators (Fig. 5b and Supplementary Fig. 2c). Thus, systemic expansion of PDC-E2-specific TR1 CD4+ cells in NOD.c3c4 mice is associated with dramatic inhibition of the pro-inflammatory properties of local
Fig. 5 Liver-specific regulatory network formation. a Cytokine profile of LPS-challenged CD11b+ cells from PCLNs and MLNs. Cytokine profile of Kupffer cells (only statistically significant analytes shown in a and b) (n = 3/group). c Absolute numbers of B-cells (top) and tetramer+ T-cells (bottom) in PCLNs, ILNs, and liver. Data for B-cells correspond to n = 15 and 19 (PCLN), n = 11 and 11 (ILN), and n = 12 and 16, respectively (liver), from 3-5 experiments/organ. Data for tetramer+ cells correspond to n = 14 and 19 (PCLN), n = 11 and 11 (ILN), and n = 15 and 16, respectively (liver), from 3-5 experiments/organ. d Correlation between numbers of B-cells and tetramer+ cells in the PCLNs, liver, and ILNs of PDC166/IAg7-NP-treated mice (n = 19, 16 and 11; 4, 5 and 3 experiments, respectively). e IL-10 secretion by LPS-challenged B-cells from PCLNs, MLNs, and liver (n = 4 and 3 mice/treatment type, respectively). f Representative FACS plots showing conversion of eGFP+ B cells from NOD.IIIO-eGFP donors into eGFP−/CD5+ Bregs in PDC-E2166/IAg7-NP-treated hosts. g Percentages of B cell-to-Breg cell conversion in PDC-E2166/IAg7-NP vs. Cys-NP-treated NOD.c3c4 hosts, in liver, spleen, PCLNs, and ILNs (n = 3 and 4 mice/treatment type, respectively). h Percentages of B cell-to-Breg cell conversion in PDC-E2166/IAg7-NP-treated NOD.c3c4 hosts, in spleen, PCLNs, and MLNs, as a function of the peptide displayed on the B-cell surface (non-cognate: BDC2.5mi, n = 4/organ; cognate: PDC166-181, n = 3/organ). Data correspond to mean ± SEM. P values were calculated via multiple t-test analysis (a and b), Mann–Whitney U (c, e, g and h) or Pearson’s correlation test (d)

and proximal PDC-E2 autoantigen-loaded APC types, largely sparing APCs elsewhere.

Liver B-regulatory (Breg) cell recruitment and formation. The liver and the PCLNs, but not non-liver-draining inguinal LNs (ILNs) of PDC-E2166-181/IAg7-NP-treated mice harbored significantly higher numbers of PDC-E2166-181/IAg7-tetramer+ cells and B-cells than those from control-NP-treated animals (Fig. 5c). In addition, the B-cell and tetramer+ T-cell numbers in liver, albeit not PCLNs, were correlated (Fig. 5d). Furthermore, the liver and PCLN, but not the MLN B-cells of PDC-E2166-181/IAg7-NP-treated mice produced IL-10 in response to LPS, whereas neither the liver nor the PCLN B-cells of control NP-treated animals produced IL-10 (Fig. 5e). Collectively, these observations suggested that, in the liver-draining lymph nodes, PDC-E2166-181/IAg7-NP-induced TR1-like cells promoted Breg cell formation. These B-cells had disease-specific immunoregulatory activity in vivo, because transfer of purified PCLN B-cells from PDC-E2166-181/IAg7-NP-treated NOD.c3c4 mice suppressed the transfer of disease into NOD.scid.c3c4 hosts reconstituted with splenocytes from diseased NOD.c3c4 donors. In
contrast, the pancreatic lymph node-associated B-cells from BDC2.5mi/IA\(^{\beta}\)NP-treated NOD mice, which can protect NOD. scid mice from diabetes transfer\(^7\), had no effect on the ability of splenocytes from sick NOD.c3c4 mice to transfer PBC into NOD. scid.c3c4 hosts, suggesting that these B cell-mediated immunoregulatory effects are antigen-specific (Fig. 4h).

To investigate this further, we ascertained the ability of PDC-E2\(^{122-135}/\)DRB4*0101-NPs, PDC-E2\(^{249-263}/\)DRB4*0101-NPs, and PDC-E2\(^{629-641}/\)DRB1*0801-NPs to expand cognate TR1-like T-cells in NOD.scid/Il2rg\(^-/-\) hosts, suggesting that these B cell-mediated immunoregulatory effects are antigen-specific (Fig. 4h).

Continued versus intermittent treatment. We next examined if the size of the cognate TR1 cell pool arising in blood in response to therapy could be used to gauge the need for re-treatment. As the liver is a large organ, we suspected that the blood-residence time of the PDC-E2-specific TR1 cells in diseased NOD. c3c4 mice would be significantly shorter than in diabetic NOD mice, where cells can persist in the circulation for months after treatment withdrawal\(^7\). The blood tetramer\(^+\) T-cell content from most mice declined to ~50% of the original values within 4–6 weeks after treatment withdrawal. Re-treatment rapidly restored these values (Fig. 6a, b). Intermittent therapy given up to 50 weeks of age did not compromise the pharmacodynamic or therapeutic effects of pMHCII-NPs (Fig. 6c–g), as compared to mice treated continuously, supporting the safety of these compounds, even when administered for prolonged periods of time.

pMHCII-NPs versus the standard of care. Ursodeoxycholic acid (UDCA, a hydrophilic bile acid) is the standard of care for PBC. Although effective in ~50% of patients when given early, it is ineffective at advanced stages of PBC\(^1\).

Intake of UDCA-supplemented chow by 6-week-old NOD.c3c4 mice for 14 weeks had a small therapeutic effect on the progression of PBC, as manifested by reductions in liver scores and liver weight, bile duct proliferation and mononuclear cell infiltration, albeit not serum ALT or TBA levels, CBD scores, CBD diameter, or bile duct involvement (Fig. 7a–d). However, when UDCA was given at 24 weeks, it had none of these effects, except for a very significant reduction in CBD diameter, possibly because of its anti-cholestatic effects (Fig. 7e–g). In contrast, PDC-E2\(^{166-181}/\)IA\(^{\beta}\)NP had substantial therapeutic effects in both 6-week-old and 24-week-old animals (Fig. 7a–h).

Therapeutic effects in another PBC model. The NOD.c3c4 model does not fully recapitulate the immunopathology of human PBC, characterized by female prevalence, progression to liver fibrosis, and absence of liver cyst formation. B6 mice carrying a deletion of the IFN\(\gamma\) 3’-untranslated region adenylate uridylicate-rich element (ARE) (ARE-Del\(^{1-17}\)) have a dysregulated Ifng locus, and develop a form of PBC that, like the human disease, primarily affects females and is associated with up-regulation of TBA, production of anti-PDC-E2 autoantibodies, portal duct and lobular liver inflammation, bile duct damage and fibrosis\(^14\). Treatment of female (NODxB6.1FNy ARE-Del\(^{1-17}\)) F1 mice with PDC-E2\(^{166-181}/\)IA\(^{\beta}\).NPs triggered TR1 cell formation/expansion and suppressed the upregulation of TBA and ALT levels, liver inflammation and fibrosis, as compared to mice treated with control NPs (Supplementary Fig. 3a–d). Similar results were obtained in B6.1FNy ARE-Del\(^{1-17}\) mice treated with NPs displaying an IA\(^{\beta}\)-binding PDC-E2-derived epitope (PDC-E2\(^{629-641}/\)IA\(^{\beta}\)NP) (Supplementary Fig. 3e, f), indicating that the therapeutic activity of these compounds is not a peculiarity of the NOD genetic background or its unique MHC class II molecule.

Humanized mice with PBC. DRB4*0101 and DRB1*0801 have been associated with PBC in some studies\(^1\). HLA-DRB-typing of 154 PBC patients from our cohort indicated that 61.7% carried DRB4*01 and 14% DRB1*0801.

Several T-cell epitopes from PDC-E2 binding to two of these HLA-DRB types have been described\(^12,16\). We compared the ability of PDC-E2\(^{122-135}/\)DRB4*0101-NPs, PDC-E2\(^{249-263}/\)DRB4*0101-NPs, and PDC-E2\(^{629-641}/\)DRB1*0801-NPs to expand cognate TR1-like CD4+ T-cells in NOD.scid/Il2rg\(^-/-\) hosts reconstituted with PBMCs from 11 DRB4*0101+ and 5 DRB1*0801+ PBC patients (PBL-NSG mice, Supplementary Fig. 4 and Supplementary Table 1). Supplementary Fig. 4a–c show that the hosts were engrafted with hCD45+ cells containing hCD4+ cells and hCD19+ cells, but no mCD4– cells, and that their mCD45+ cells lacked mCD4+ or hCD4+ cells, as expected. We saw expansion of tetramer+CD49b+/LAC-3+CD4+ T-cells in the spleen and/or liver and LNs from 5/6 PBL-NSG mice treated with PDC-E2\(^{122-135}/\)DRB4*0101-NPs, 4/6 PBL-NSG mice treated with PDC-E2\(^{249-263}/\)DRB4*0101-NPs, and 2/5 PBL-NSG mice treated with PDC-E2\(^{629-643}/\)DRB1*0801-NPs (Supplementary Table 1). Treated responsive mice had significantly higher percentages and absolute numbers of tetramer+ cells in spleen, liver, lymph nodes, and/or bone marrow (Supplementary Fig. 4d, e) than untreated or unresponsive mice, and these cells expressed the TR1 markers CD49b and LAG-3 (Supplementary Fig. 4f).

Disease versus organ specificity. Given that PDC-E2 is an autoantigen expressed in virtually all cell types, our results begged the question of whether PBC-relevant nanomedicines (i.e. PDC-E2\(^{166-181}/\)IA\(^{\beta}\)NP) are disease-specific or not.

PSC is a chronic cholestatic disease characterized by inflammation of intra-hepatic and extra-hepatic bile ducts leading to a fibroobliterative cholangitis with periductal fibrosis around medium and large bile ducts and degenerative changes of the biliary epithelium, in the absence of anti-mitochondrial autoantibodies\(^17\). Aβbc4 knockout mice (lacking the multirdrug resistance protein 3) develop a form of cholangitis similar to human PSC that is caused by impaired biliary phospholipid secretion\(^17\).

AIH is characterized by a portal mononuclear cell infiltration of the liver parenchyma that is associated with presence of ANAs and/or smooth muscle (AIH type 1) or anti-liver kidney microsomal or anti-liver cytosol type 1 autoantibodies, which target the microsomal cytochrome CYP2D6 or FTCD, respectively (AIH Type 2)\(^18\). Recently, it has been shown that infection of NOD mice with a replication-defective adenovirus encoding human FTCD (Ad-hFTCD) triggers a form of chronic AIH that resembles AIH type 2\(^19\).

We reasoned that the large bile duct and parenchymal liver damage that underlie PSC and AIH, respectively, might trigger the release of PDC-E2, CYP2D22 (the mouse CYP2D6 ortholog, herein referred to CYPD) and FTCD, and the priming of autoreactive CD4+ T-cells capable of responding to the corresponding pMHC-NPs. To investigate this, we tested the ability of PDC-E2\(^{166-181}/\)IA\(^{\beta}\)NP-NPs (PBC-relevant) and
CYPD398–412/IA^b^7^-NPs (AIH-relevant) to expand cognate TR1-like CD4^+^ T-cells and ameliorate PSC in NOD.Abc^b^/^- mice. Remarkably, both PDC-E2166–181/IA^b^7^-NPs expanded cognate TR1 CD4^+^ T-cells (Fig. 8a and Supplementary Fig 5) and reduced liver necroinflammation and fibrosis (Fig. 8b), as well as serum ALT and TBA levels (Fig. 8c), as compared to controls. Likewise, PDC-E2166–181/IA^b^7^-NPs (PBC-relevant) and both mFTCD58–72/IA^b^7^-NPs and CYPD398–412/IA^b^7^-NPs (AIH-relevant) triggered cognate TR1 cell expansion (Fig. 8d and Supplementary Fig. 6a, b) and significant reductions in ALT levels, hepatocyte necrosis, liver inflammation, and liver fibrosis in Ad-hFTCD-infected NOD mice (Fig. 8e, f).

This ability of ubiquitous autoantigen-based pMHC-nanomedicines to blunt liver autoimmunity in an organ-specific rather than disease-specific manner also occurred in NOD.c3c4 mice treated with CYPD398–412/IA^b^7^-NPs (Fig. 9a–c). In fact, the latter were as efficient as PDC-E2166–181/IA^b^7^-NPs at expanding cognate TR1 cells (Fig. 9a) and blunting PBC in 15-week-old mice (Fig. 9b, c).

Collectively, these observations suggest that hepatocyte (AIH) and bile duct epithelial (PBC and PSC) damage in liver
autoimmunity results in the delivery of significant amounts of liver-prevalent autoantigens, including PDC-E2, CYPD2D6, and FTCD to local and proximal APCs. In turn, this enables autoreactive CD4+ T-cell priming (a sine qua non requirement for pMHC-NP-induced TR1 cell formation\(^1\)), cognate TR1 cell generation by pMHC-NPs, Breg cell formation, and suppression of the pro-inflammatory capacity of local and proximal autoantigen-loaded APCs.

**Normal immunity is spared.** We next investigated if persistent expansion of PDC-E2\(_{166-181}/\text{IA}^\beta\)-specific TR1 cells results in suppression of normal immunity against infection and cancer.

Cohorts of NOD.c3c4 mice received doses of PDC-E2\(_{166-181}/\text{IA}^\beta\)-NPs or control NPs twice a week for 9 weeks. At the end of therapy, the mice were given an i.v. injection of recombinant vaccinia virus. The viral titers in the ovaries of females 14 days after infection were similar in both cohorts of mice and substantially lower than those found at the peak of infection, indicating that pMHCI1-NP therapy did not impair cellular immunity against the virus-infected cells (Fig. 10a).

To probe this further, we infected PDC-E2\(_{166-181}/\text{IA}^\beta\)-NP-treated or untreated NOD.c3c4 mice with a laboratory strain of influenza (HKx31 –H3N2–) i.p. to induce heterologous (shared) immunity against a subsequent lethal infection with an...
H1N1 strain of Influenza (PR8) given via the intranasal route. As shown in Fig. 10b, c, pMHC-II-NP treated NOD.c3c4 mice mounted protective immunity against the PR8 infection, as documented by decreased viral load in lung tissue and clinical signs of active infection, despite systemic presence of cognate TR1-like CD4+ T-cells (Supplementary Fig. 7a).

Similar results were obtained in mice infected with the intracellular pathogen *Listeria Monocytogenes* (LM). LM-infected PDC-E2166-181/IAβ7-NP-treated and untreated NOD.c3c4 mice were equally efficient at clearing the bacteria from both the spleen and liver, consistent with unimpaired immunity against this intracellular pathogen (Fig. 10d and Supplementary Fig. 7b). This outcome was also true when the mice were infected with LM shortly before initiation of pMHC-NP treatment. As shown in Supplementary Fig. 7c–f, diseased NOD.c3c4 mice infected with LM immediately before initiation of treatment and treated for 5 consecutive weeks had, at the end of follow up, significantly reduced disease scores, as well as numbers of LM colony forming units (cfu) in both the liver and spleen. Importantly, this reduction in spleen and liver LM cfu was similar in pMHC-NPs-treated vs. untreated mice. Thus, treatment suppressed liver inflammation without impairing the ability of the host to clear the pathogen, presumably because the mechanisms involved in clearance of this intracellular pathogen are not impaired by TR1 cell-driven immunoregulation.

PDC-E2166-181/IAβ7-NP-treated and untreated NOD.c3c4 mice also produced similar titers of anti-dinitrophenyl (DNP) antibodies upon immunization with the hapten-carrier conjugate DNP-keyhole limpet hemocyanin (KLH) (Fig. 10e), indicating that these compounds do not impair humoral immunity against foreign antigens.

Lastly, systemic expansion and liver accumulation of PBC-suppressing PDC-E2-specific TR1-like CD4+ T-cells (Fig. 10f–n) did not impair the ability of pMHC-NP-treated NOD.c3c4 mice to mount immune responses against allogeneic colon carcinoma (CT26) and melanoma (B16/F10) liver metastases arising upon intra-splenic injection, as compared to untreated NOD.c3c4 mice or syngeneic hosts (Balb/c and C57BL/6J, respectively) (Fig. 10i–q).

Thus, despite targeting a systemically expressed antigen, PDC-E2166-181/IAβ7-specific TR1 cells do not impair cellular or humoral immunity against local or systemic foreign antigens. This is potentially so because bacterial/viral antigenic load in local APCs transiently overwhelms the APCs’ ability to present PDC-E2 epitopes to cognate TR1-like CD4+ T-cells, hence the manifestation of their immunoregulatory properties.
Fig. 9 AIH-relevant pMHCII-NPs are as efficient as PBC-relevant pMHCII-NPs to blunt PBC in NOD.c3c4 mice. a Percentage of tetramer+ cells in untreated/Cys-NP-treated (n = 7), or CYPD398-IAF7-NP-treated mice (n = 5). b and c Average microscopic (b) or macroscopic (c) liver scores for the mice treated with PDC166-IAF7-NPs or PDC82-IAF7-NPs (n = 23, five experiments for b and n = 27, six experiments for c) vs. CYPD398-IAF7-NPs (n = 5, one experiment), as compared to untreated/Cys-NP-treated mice (n = 20, six experiments for b and n = 30, seven experiments for c). Data correspond to the mean ± SEM. P values were calculated via Mann–Whitney U.

Discussion
We have used mice undergoing PBC, PSC, or AIH, as well as NSG mice humanized with PBMCs from PBC patients to investigate whether hepatocyte and/or cholangiocyte destruction in autoimmunity results in the stimulation of autoreactive T-cells capable of responding to disease-relevant and irrelevant pMHCII-based nanomedicines.

We found that nanomedicines displaying various liver-prevalent antigenic peptides triggered TR1-like cell formation and expansion in mice undergoing various liver autoimmune diseases, as well as in NSG mice humanized with PBMCs from PBC patients. As a result, these nanomedicines effectively blunted PBC, PSC, and AIH in various genetic backgrounds by suppressing liver inflammation, even when initiated at the peak of disease severity. In the liver, disease suppression involved TR1 cell-driven local Breg cell formation, required both IL10 and TGFβ, could be transferred by both tetramer+CD4+ T-cells and PCLN-associated B-cells from treated donors, and was associated with profound suppression of the pro-inflammatory capacity of both liver and liver-proximal myeloid DCs as well as Kupffer cells. In contrast, a nanomedicine displaying a pancreatic beta cell-specific epitope was unable to trigger cognate TR1 cell responses in NOD.c3c4 mice undergoing liver autoimmunity in the absence of pancreatic autoimmunity, consistent with the sine qua non requirement for autoantigen-experience in T-cell responsiveness to pMHCII-NPs.

Importantly, suppression of liver inflammation by these nanomedicines did not compromise immunity against viruses (vaccinia, influenza), intracellular bacteria (Listeria), or metastatic (liver) allogeneic tumors. The TR1-like CD4+ T-cells that are triggered by pMHC-based nanomedicines can only effect regulation when they engage cognate pMHC class II of professional APCs that are loaded with endogenous autoantigen. Such APCs must capture autoantigen shed from the damaged liver cells and therefore are only present in significant numbers in the target organ or in draining lymphoid tissue. As a result, it is not surprising that pMHC-based nanomedicines do not impair immunity against systemic infections or against vaccines, as the liver-distal APCs that orchestrate these immune responses are not loaded with liver-derived autoantigens. For intra-hepatic or liver-proximal immunity, such as against a LM infection, the infected liver APCs may be overwhelmed with LM-derived antigens, decreasing their ability to elicit TR1 cell function and suppression (by dilution of cognate pMHCs at the expense of pathogen-derived pMHC complexes below the threshold required for TR1 cell activation). These cells may therefore be spared from suppression. Given the short half-lives of myeloid-derived APCs (days), replacement of these APCs by uninfected ones might be sufficient to support continued TR1-mediated immunoregulation. Alternatively, the mechanisms involved in clearance of this intracellular pathogen and allogeneic liver tumor metastases may not be impaired by TR1 cell-driven immunoregulation.

Collectively, these results have several important implications for our understanding of both normal immunity and treatment of autoimmunity. First, they demonstrate that tissue destruction in specific autoimmune diseases has the potential to trigger the stimulation or possibly outright activation of autoreactive T-cells recognizing many, perhaps all, of their antigenic components, suggesting that the antigenic repertoires in autoimmune diseases may be much more extensive than currently thought. We note
that the pMHCs tested herein were designed in silico, using online MHC-binding algorithms, raising the possibility that any peptide capable of binding to self-MHC molecules, from a whole host of proteins expressed by hepatocytes and/or cholangiocytes, might be recognized by, and be able to activate peripheral T-cells. Second, our observations imply that penetrance of central and peripheral T-cell tolerance to highly expressed antigens is remarkably incomplete, even in disease-resistant genetic backgrounds. From an evolutionary standpoint, such pervasive auto-reactivity may have been sustained because it functions as a source of regulatory cells capable of extinguishing pathology. Third, from a translational standpoint, this study has identified disease-modifying compounds for several complex liver autoimmune diseases that share common immunopathological pathways and represent unmet clinical needs20,21. Finally, these observations suggest that a few pMHCII-based nanomedicines displaying ubiquitous epitopes and HLA class II molecules encoded in oligomorphic HLA loci might be sufficient to treat various liver autoimmune diseases without impairing normal immunity.

**Methods**

*Mice.* NOD/LtJ, BALB/c, C57BL/6, NOD.scid.Illg2-known (NSG), NOD.c34 and FVB/N.Abcb4−/− (Abcb4 or ATP-binding cassette transporter, sub-family B, member 4) mice were purchased from the Jackson Laboratory (Bar Harbor, ME). IFNs ARE-Del−/− B6 mice were obtained from H. Young (NIH, Bethesda, MD). NOD.c34 scid mice were generated by backcrossing (NOD.c34 x NOD.scid) F1 mice with NOD.c34 mice for five generations, followed by intercrossing of mice heterozygous for the scid mutation and homozygous for the B6 chromosome 3 and 4.
intervals from NOD.scid mice. NOD.Apbc4−/− mice were obtained by backcrossing the mutant Apbc4 allele from FVB/N-Apbc4−/− mice onto the NOD/LtJ background for six generations. These studies were approved by the Institutional Animal Care Committee of the Medical University of South Carolina.

Human subjects. HLA-DRB*0401+ PBC patients were recruited under informed consent approved by the Institutional Review Board at Hospital Clinic (see Supplementary Table 1 for demographic and other patient details). All the work with human participants complied with all the relevant ethical regulations and was approved by the Hospital Clinic human ethics review board.

Cell lines, pathogens, and tumors. CHO-S, B-SC-1, MDCK, 293T, R16/10, and CT26 cell lines were purchased from the ATCC (Manassas, VA). The H3N2 HKx31 and H1N1 PR8 influenza strains were from P. Thomas (St. Jude Children’s Research Hospital, Memphis, TN). LM was obtained from DMX Corporation (Philadelphia, PA).

Antibodies and reagents. FITC, PE, APC, PerCP, Alexa Fluor 647, BV605, or biotin-conjugated mAbs against mouse CD4 (RM4-5; C57BL/6 background) for fluorescence-activated cell sorting (FACS) were purchased from eBioscience (San Diego, CA). Anti-latent-fat-stained avidin incubation (15 min at RT), blood leukocytes, and single cell suspensions were stained with anti-CD49b (AK7, Pierce Biosciences, San Jose, CA), PerCP-conjugated polyclonal goat anti-LAG-3 IgG (Novus Biologicals, Littleton, CO), anti-CD25 (PC61), and anti-CD279 (PD1; J43), anti-CD19 (5/100; used as a co-staining with pMHC tetramer (5μM) transport channel) for 30 min at 4 °C. After washing, cells were fixed (1% paraformaldehyde in PBS) and analyzed with FACScan, FACSaria, or BD LSRII cytometers. For phenotypic analyses, the cells were incubated with anti-FcR Abs, scFv, or biotin-conjugated anti-CD49b (AK7, Pierce Biosciences). The mixture was cooled to room temperature and mixed with 30 μl mouse biotinylated streptavidin for 30 min at 4 °C. After washing, cells were stained with anti-CD49b (AK7, Pierce Biosciences) and biotin-conjugated anti-CD49b (AK7, Pierce Biosciences). The reaction was stirred for 1 h and heated to 260 °C with reflux for 2 h. The mixture was centrifuged at 2000g for 30 min. The NPs were washed twice using magnetic MACS columns (Miltenyi Biotec, Auburn, CA) and stored in water at room temperature or 4 °C. The concentration of iron was determined spectrophotometrically at 410 nm in 2 N hydrochloric acid (HCl). Free cysteines (controls) or mAbs, carrying a free carboxylterminal Cys, were conjugated to the maleimide-functionalized PMA in 50 μM phosphate buffer, pH 6.0, containing 10% v/v ethanol and 50% v/v glycerol in PBS. The mAbs conjugated NPs were separated from free mAbs using magnetic columns, sterilized by filtration through 0.2 μm filters and stored in water or PBS at 4 °C. Quality control was done using transmission electron microscopy, dynamic light scattering, and native and denaturing gel electrophoresis. pMHC content was measured using Bradford assay (Thermo Fisher Scientific) and SDS-PAGE.

Purification of exhausted CD4+ and TR1-like CD4+ T-cells. Exhausted CD4+ T-cells (CD4+PD1+KLH13+LAG3+) were FACS-sorted from the spleens of 6–8-week-old NOD.scid mice (n = 4). PDC-E21661–181/IAg7−/−NP-induced TR1-like CD4+ T-cells (CD4+PDC-E21661660/IAg7−/−tetramer+LAG3+LAG3+CD4+CD25+) were FACS-sorted from the spleens of 22–30-week-old NOD.scid mice that had received 14 or 29 doses of PDC-E21661660/IAg7−/−NPs, starting at 15 weeks of age (n = 1 and 3, respectively). The sorted cells were stimulated with anti-CD3/CD28 mAb-coated beads for 36 h, to measure the extent of AICD (as measured using the viability staining die 7-AAD), or for 6 days, to compare their proliferative response (as measured by BrdU incorporation using the FITC BrdU Flow kit from BD Biosciences).

Generation of FTCD-expressing adenovirus. A replication-deficient adenovirus expressing human formiminotransferase cyclodeaminase (Ad-hFTCD) (a target antigen in AHI Type 2) was generated by cloning the hFTCD DNA sequence directly into Adeno-X Adenoviral System 3 (CMV) using In-Fusion® HD cloning technology and Stellar Competent cells (Clontech, Mountain View, CA). Cloned Ad-FTCD was amplified in Ad-293 T cells and purified using Adeno-X Maxi Purification Kit (Clontech). The viral titer was measured using the End-point Dilution Assay or Adeno-X Rapid Titer Kit (Clontech).

UDCA treatment. Cohorts of 5–6 or 24-week-old male and/or female NOD.scid mice were untreated, fed a diet supplemented with 0.5% UDCA (BOC Sciences, Upton, NY; TestDiet, Richmond, IN), or treated with pMHCII-NP for 14 or 9 weeks, respectively, and sacrificed for pMHCII tetramer staining, pBcr scoring and biochemical testing.

pMHCII-NP therapy for PBC in various genetic backgrounds. Cohorts of 15-week-old male and/or female NOD.scid mice with established PBC were treated with 20 μg pMHCII-NP or Cy5-labeled NPs containing 10 nM of each peptide for 8 weeks unless indicated otherwise. Liver disease scoring involved macroscopic evaluation of cyst content (0–5 for all experiments, except Fig. 6f, where cyst content was scored from 0–8), liver weight, and CBD diameter (0–4), as well as microscopic evaluation of bile duct involvement (0–4), bile duct proliferation (0–4), and mononuclear cell infiltration (0–4). In other experiments, treatment was initiated at the peak of disease (24 weeks of age) and given twice a week for 14 weeks. Intermittent treatment involved treating mice twice a week from 15 to 24 weeks of age, then withdrawing treatment until the percentages of tetramer+ cells dropped to ~50% of the levels seen at treatment withdrawal (measurements in peripheral blood were done once every 2 weeks), re-treating mice twice a week until the percentages of tetramer+ cells reached original values, and repeating this cycle until 50 weeks of age.

In vivo cytokine blocking experiments, mAbs against HRPN (rIgG1), IL-10 (JJ5–2A3), or TGF-β (1D11) (BioXcell, West Lebanon, NH) were given i.p. twice a week at 500 μg per dose for 2 weeks, followed by 200 μg per dose for 7 additional weeks. Mice were randomized into cytokine-neutralizing mAb-treatment (anti-IL-10 or anti-TGFβ) or HRPN-ratIgG1 groups.
In experiments involving (NOD x B6.1FVB-ARE-De−/−) F1 and B6.1FVB-ARE-De−/− mice, 10-week-old male and female mice were treated for 5–6 weeks. Histopathologic severity in the liver was scored by examining portal inflammation, lobular inflammation, and granuloma formation from 0 to 4, and bile duct damage from 0 to 2. The extent of portal inflammation and bile duct damage were scored from 0 to 4 based on the ratio between affected vs. unaffected area. The extent of lobular inflammation and granuloma formation were scored from 0 to 4 based on number of lesions per specimen. Lesion scores were obtained by adding the scores for both severity and lesion number. The severity of fibrosis was scored on a 0–6 scale as follows: 0, no fibrosis; 1, fibrous expansion in very few portal areas with or without small fibrous septa; 2, fibrous expansion in most portal areas with small fibrous septa; 3, partially expanded pseudepithelial areas with very few portal-to-portal bridging; 4, fibrous expansion in all portal areas with marked bridging (portal-to-portal and portal-to-central); 5, marked bridging with very few nodules (incomplete cirrhosis); and 6, complete cirrhosis. Studies using NOD mice involved treating cohorts of 10-week-old pre-diabetic female NOD/Ltj mice with 20 µg of PMHCII-NPs or Cys-NPs i.v. twice weekly for 5 weeks.

**pMHCI-II therapy for PSC in NOD.Abc4−/− mice.** Cohorts of 5–6-week-old male and/or female NOD.Abc4−/− mice with established PSC were treated with 20 µg of PMHCII-NPs or Cys-NPs i.v. twice weekly for 5–6 weeks. Histopathologic lesions were graded using the Ishak scoring system, which evaluates both fibrosis (0–6), as well as necroinflammatory sequelae of biliary cholangitis, including interface hepatitis (0–4), confluent necrosis (0–6), lobular inflammation (0–4), and portal inflammation (0–4).

**pMHCI-II therapy for AIH in NOD mice.** We induced AIH by infecting 5–6-week-old female NOD/Ltj mice with an adenosine encoding human FTCD (Ad-hFTCD, 1010 plaque forming units (PFU) i.v.), as previously described. Four weeks later, cohorts of mice with established AIH were treated with 20 µg of PMHCII-NP s or Cys-NPs (i.v.) twice weekly for 5–6 weeks. Histopathological scoring was done using the Ishak scale as above.

**pMHCI-II therapy in human PBMC-reconstituted NSG hosts.** PBMCs from HLA-DRB4*0101+ PBC patients (recruited under informed consent approved by the Institutional Review Board at Hospital Clinic) were depleted of CD8+ T-cells (to reduce the magnitude of GVHD in the hosts) using anti-CD8 mAb-coated magnetic beads (Miltenyi Biotech, Auburn, CA) and injected i.v. (2 × 107) into 8–10-week-old NSG hosts. Mice were treated with 30–40 µg PMHCII-NPs starting on day 5 after PBMC transfusion, twice a week for 5 consecutive weeks, or left untreated. Therapy-induced expansion of cognate CD4+ T-cells was measured in liver, peripheral LNs, and spleen (Supplementary Table 1). The gender, age, anti-DNP antibody titer, and cytokine content were measured using an anti-DNP Ig ELISA Kit (Alpha Diagnostic International).

**Cytokine secretion assays.** Splenic and portal/celiac lymph node (pLN) cell suspensions were obtained from all cohorts treated from pMHCI-II-NP-treated mice and cells depleting CD19– B-cells (EasySep™ Mouse CD19 Positive Selection Kit, Stem Cell Technologies, Vancouver, BC) and CD8+ T-cells (CD8 Magnetic Particles, BD Biosciences). Cells were stained with pMHCI tetramers and sorted by flow cytometry. The sorted cells (2–5 × 106) were challenged with bone marrow-derived DCs (2 × 106) pulsed with 2 µg/ml peptide. Forty-eight hours later, supernatants were harvested for measurement of cytokine content by luminex cytokine assay.

To ascertain whether pMHCI-II-NP therapy promoted the recruitment/formation of IL-10-secreting B-cells, mesenteric LNs, pLN, and liver cell suspensions were enriched for B-cells using a CD19 enrichment kit (Stem Cell Technologies). The cells (2–3 × 107 in 200 µl) were stimulated in duplicate with LPS (1 µg/ml, Sigma) for 24 h in RPMI-1640 media containing 10% FCS. The levels of IL-10 in the supernatants were measured using Luminex®, 40 cells and liver were cut into several pieces, weighted, and homogenized in PBS containing 0.35% Triton X-100. Serial dilutions of the lysates were then plated onto BHI-agar containing 5 µg/ml erythromycin, incubated for 24–48 h at 37 °C and the number of colonies counted. In other experiments, mice were infected with LM immediately before the initiation of pMHCI-NP therapy and sacrificed on day 3 to confirm presence of LM cfu in both liver and spleen, or on day 25, after termination of treatment (treatments given in 2 doses/week for 5 weeks).

Cellular immunity to liver metastatic tumors was ascertained upon intra-splenic injection of B16/F10 melanoma and CT26 colon carcinoma tumors into syngeneic (C57BL/6j or Balb/c, respectively) or allogeneic hosts (pMHCI-NP-treated or untreated NOD.c3c4 mice). A small incision was made in the abdomen, under isoflurane anesthesia, to partially expose the spleen. Tumors (0.2 ± 105 and 0.1 ± 106 for B16/F10 and CT26, respectively, in 100 µl of PBS) were injected slowly for 1 min into the exposed spleen. Ten minutes later, the spleen was removed and the peritoneal and skin layers sutured. pMHCI-II-NP therapy was resumed within 5–7 days after surgery and continued until the end of follow-up. Mice were monitored for up to 19–21 days and euthanized for tetramer staining, PBMC sorting, and tumor burden measurement. For 16B16/F10 injected mice, tumor burden was assessed by measuring liver weight and counting the number of metastases, easily distinguishable from liver parenchyma. In CT26-injected animals, tumor burden was scored histologically by measuring the hepatic area (HAP) occupied by metastatic tumors.

To evaluate humoral immunity, pMHCI-II-NP-treated and untreated mice were immunized i.p. with 100 µg of DNP-KLH (Alpha Diagnostic International, San Antonio, TX) in CFA and boosted again 3 weeks later as previously described. Mice were sacrificed 10 days later, to measure serum anti-DNP antibody titers using an anti-DNP Ig ELISA Kit (Alpha Diagnostic International).
In vivo Breg induction assay. Splenic B-cells from NOD.IL10tm1Flv (Tiger) mice were enriched using an EasySep Mouse B-cell Isolation Kit (Stem Cell Technologies) and pulsed with BDC2.5m or PDC-E2181 peptides (10 μg ml⁻¹) for 2 h at 37 °C as previously described1. The peptide-pulsed B-cells were washed twice with PBS, labeled with PKH26 (Sigma) and transfused (3 × 10⁷) into pMHC-NP-treated or untreated mice. The hosts were killed 7 days later and their spleens, MLNs, PCLNs, and liver mononuclear cells were labeled with anti-B220-APC and biotinylated anti-CD1d or anti-CD5 mAbs followed by Streptavidin-PerCP. PKH26⁺ B-cells were analyzed for presence of eGFP⁺/CD1d181 and eGFP⁺/CD5⁺ cells by flow cytometry.

Histology and immunohistochemistry. Livers were fixed in 10% formalin for 2 days, embedded in paraffin, cut into 5 μm sections and stained with H&E or Picrosirius Red. We scored (−0.5 cm²) sections from the four liver lobes from each mouse (right and left, median and caudal) and a minimum of four portal triads per lobe section (16 portal triads/mouse). For immunohistochemistry, liver tissues were embedded in Tissue-Tek OCT, sectioned into 30 μm cryosections and stored on slides at −80 °C. Slides were fixed in chilled aceton, washed with PBS, treated with a 1:10 dilution of 30% H₂O₂ in PBS, washed again, and stained with anti-mouse CD4 (GK1.5) or CD8 (Lyt-2) antibodies (1.5 h, 4 °C). After washing, the slides were stained with a biotinylated goat anti-rat secondary antibody (1:200 dilution), incubated with horseradish peroxidase (HRP)-conjugated streptavidin, followed by 3.3-diaminobenzidine (DAB) substrate. Slides were counterstained with hematoxylin before mounting.

ALT and TBA assays. ALT levels in serum were determined using a kit from Thermo Fisher Scientific following the manufacturer’s protocol. Briefly, serum samples were mixed with pre-warmed (37 °C) In VitroALT (GPT) Liquid Stable Reagent at 1:10 ratio and OD readings were taken for 3 min at 1 min intervals in a nanodrop at a 340 nm wavelength, 37 °C. The slope was calculated by plotting absorbance vs. time using linear regression and multiplied with a factor to obtain ALT levels in serum (U/l) as described in the kit. Serum TBA levels were determined using NOVA Lite Anti-nuclear and anti-mitochondrial autoantibodies

References

1. Clemente-Casares, X. et al. Expanding antigen-specific regulatory networks to treat autoimmunity. Nature 530, 434–440 (2016).
2. Singha, S. et al. Peptide-MHC-based nanomedicines for autoimmunity function as T-cell receptor microclustering devices. Nat. Nanotechnol. 12, 701–710 (2017).
3. Lieberman, S. & DiLorenzo, T. A comprehensive guide to antibody and T-cell responses in type 1 diabetes. Tissue Antigens 62, 359–377 (2003).
4. Walter, U. & Santamaría, P. CD8⁺ T cells in autoimmunity. Curr. Opin. Immunol. 17, 624–631 (2005).
5. Jones, D. E. Pathogenesis of primary biliary cirrhosis. Postgrad. Med. J. 84, 23–33 (2008).
6. Hov, J. R., Boberg, K. M. & Karlsen, T. H. Autoantibodies in primary sclerosing cholangitis. World J. Gastroenterol. 14, 3781–3791 (2008).
7. Zabolotnaya, K., Rigopoulou, E. & Dakekos, G. N. Autoantibodies and autoantigens in autoimmune hepatitis: important tools in clinical practice and to study pathogenesis of the disease. J. Autoimmune Dis. 1, 2 (2004).
8. Floreni, A. et al. Extrahepatic autoimmune conditions associated with primary biliary cirrhosis. Clin. Rev. Allergy Immunol. 48, 192–197 (2015).
9. Irie, J. et al. NOD.c4 congenic mice develop autoimmune biliary disease that serologically and pathogenetically models human primary biliary cirrhosis. J. Exp. Med. 203, 1209–1219 (2006).
10. Robe, A. J., Kirby, J. A., Jones, D. E. & Palmer, J. M. A key role for autoreactive B cells in the breakdown of T-cell tolerance to pyruvate dehydrogenase complex in the mouse. Hepatology 41, 1106–1112 (2005).
11. Kita, H. et al. Quantitative and functional analysis of PDC-E2-specific autoreactive cytotoxic T lymphocytes in primary biliary cirrhosis. J. Clin. Invest. 109, 1231–1240 (2002).
12. Shimoda, S. et al. Identification and precursor frequency analysis of a common T cell epitope motif in mitochondrial autoantigens in primary biliary cirrhosis. J. Clin. Invest. 102, 1831–1840 (1998).
13. Pares, A., Caballeria, L. & Rodes, J. Excellent long-term survival in patients with primary biliary cirrhosis and biochemical response to ursodeoxycholic acid. Gastroenterology 130, 715–720 (2006).
14. Bae, H. R. et al. Chronic expression of interferon-gamma leads to murine autoimmune cholangitis with a female predominance. Hepatology 64, 1189–1210 (2016).
15. Qin, B. et al. Association of human leukocyte antigen class II with susceptibility to primary biliary cirrhosis: a systematic review and meta-analysis. PLoS ONE 8, e79580 (2013).
16. Chow, I. T. et al. Differential binding of pyruvate dehydrogenase complex-E2 epitopes by DRB1*08:01 and DRB1*11:01 is predicted by their structural motifs and correlates with disease risk. J. Immunol. 190, 4516–4524 (2013).
17. Pickert, P. et al. Characterization of animal models for primary sclerosing cholangitis (PSC). J. Hepatol. 60, 1290–1303 (2014).
18. Lonnig, M. S., Ma, Y., Miel–Vergani, G. & Vergani, D. Aetiopathogenesis of autoimmune hepatitis. J. Autoimmun. 34, 7–14 (2010).
19. Hardike-Wolenski, M. et al. Genetic predisposition and environmental danger signals initiate chronic autoimmune hepatitis driven by CD4 + T cells. Hepatology 58, 718–728 (2013).
20. Pares, A. Novel treatment strategies for primary biliary cholangitis. Semin. Liver Dis. 37, 60–72 (2017).
21. Lazaridis, K. N. & LaRusso, N. F. Primary sclerosing cholangitis. N. Engl. J. Med. 375, 2501–2502 (2016).
22. Pickert, P. et al. Effects of ursodeoxycholic and cholic acid feeding on hepatocellular transporter expression in mouse liver. Gastroenterology 121, 170–183 (2001).
23. Yang, G. X. et al. CD8 T cells mediate direct biliary ductule damage in nonobese diabetic autoimmune biliary disease. J. Immunol. 186, 1259–1267 (2011).
24. Ishak, K. et al. Histological grading and staging of chronic hepatitis. J. Hepatol. 22, 696–699 (1995).
25. Pickert, P. et al. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. Gastroenterology 127, 261–274 (2004).
26. de Vries, E. M. et al. Applicability and prognostic value of histologic scoring systems in primary sclerosing cholangitis. J. Hepatol. 63, 1212–1219 (2015).
27. Tate, M. D., Brooks, A. G. & Reading, P. C. The role of neutrophils in the upper and lower respiratory tract during influenza virus infection of mice. Respir. Res. 9, 57 (2008).
28. Otten, M. A. et al. Experimental antibody therapy of liver metastases reveals functional redundancy between Fc gammaRI and Fc gammaRIV. *J. Immunol.* **181**, 6832–6836 (2008).

29. te Velde, E. A. et al. Enhanced antitumour efficacy by combining conventional chemotherapy with angiotatin or endostatin in a liver metastasis model. *Br. J. Surg.* **89**, 1302–1309 (2002).

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Author contributions

C.S.U. produced the pMHCs and executed most of the experiments with contributions from K.S. (Figs. 1c–e; 5a, b; 8a, d; 4a; 10f–q; 6c; Supplementary Fig. 3a), R.H.N. (Figs. 3b, 8b, 8f, 10a–d, 4d, 6g), J.Y. (Fig. 5e–g), and Y.Y. (Figs. 3f and 2e), and contributed to writing the manuscript with P. Santamaria. S.S. was responsible for pMHC-NP and pMHC tetramer production and quality control. Y.Y. produced iron oxide NPs and designed the pMHC constructs for the study. A.P. recruited the PBC patients and coordinated HLA typing. J.B. performed the experiments on Supplementary Fig. 4 and Supplementary Table 1 under the supervision of P. Serra. P. Santamaria designed the study, supervised and coordinated its execution and wrote the manuscript with contributions from C.S.U.

Additional information

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**Competing interests:** P. Santamaria is Scientific Founder of Parvus Therapeutics, Inc. and has a financial interest in the company. The remaining authors declare no competing interests.

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