Restriction of the CD4⁺ T-cell receptor repertoire prevents immune pathology in TGF- β 1 knockout mice

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Mice with a targeted deletion in TGF- β 1 spontaneously develop CD4⁺ T-cell-dependent multifocal inflammatory disease and autoimmune pathology. T cells from TGF- β 1^{-/-} mice are strongly activated, but the mechanisms that lead to T-cell activation and organ pathology are not well understood. Recent work shows that TGF- β 1 raises the threshold for signaling through the TCR, suppressing the response of T cells to mitogenic stimuli. This suggests the possibility that CD4⁺ T cells in TGF- β 1^{-/-} mice become aberrantly activated and cause damage in response to physiologic inputs that ordinarily are not sufficient for cell activation, such as homeostatic MHC–TCR interactions, cytokines, or adhesion molecules. This model predicts that pathology is largely antigen-independent, and that CD4⁺ T cells, regardless of antigen specificity, will become activated in TGF- β 1^{-/-} mice, with subsequent organ pathology. To test this model, we crossed BALB/c-TGF- β 1^{-/-} mice with the DO11.10 TCR transgenic mouse. To obviate the possible development of nonclonotypic TCRs, we also bred in a deficiency in RAG-1. Cohorts of highly inbred BALB/c background TGF- β 1^{-/-} mice with an increasingly restricted CD4⁺ T-cell repertoire (TGF- β 1^{-/-} mice; DO11.10-TGF- β 1^{-/-} mice; DO11.10-RAG-1^{-/-}-TGF- β 1^{-/-} mice) were then analyzed for inflammatory organ pathology and T-cell activation. The data show that progressively restricting the CD4⁺ T-cell repertoire improved survival, ameliorated target organ pathology, and reduced T-cell activation to control levels. Therefore, these results find no support for the involvement of atypical T-cell activation pathways in disease in TGF- β 1^{-/-} mice. Rather, T-cell activation and pathology in TGF- β 1^{-/-} mice appear to be functions of typical TCR activation pathways. This supports the hypothesis that immune pathology in TGF- β 1^{-/-} mice is self-antigen triggered.

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TGF- β 1 is a critical regulator of tolerance and autoimmunity. Mice with a targeted deletion in TGF- β 1 spontaneously develop multifocal inflammatory disease and autoimmune pathology. The lungs and heart are affected in TGF- β 1^{-/-} mice, with variable and strain-dependent involvement of other organs.^{1,2} On the BALB/c background, hepatic pathology in the form of necroinflammatory liver disease is a particularly prominent feature.³ Regardless of genetic background, TGF- β 1^{-/-} mice succumb to progressive wasting and failure to thrive, typically dying before 3–4 weeks of age. The direct participation of the adaptive immune

system is important, as these pathologies are absent in TGF- β 1^{-/-} mice rendered B- and T-cell deficient, such as SCID-TGF- β 1^{-/-} mice⁴ or RAG-1^{-/-}-TGF- β 1^{-/-} mice.⁵ The pathologic role of CD4⁺ T cells has been unequivocally established, as specific depletion of the CD4⁺ T-cell subset using a monoclonal antibody prevents disease development.⁵ In addition, MHC Class II^{-/-}-TGF- β 1^{-/-} double knockout mice (in which CD4⁺ T cells are not generated because of a failure in positive selection) do not develop autoimmune or inflammatory disease.⁶

TGF- β 1 is an important inhibitor of CD4⁺ T cells, inhibiting cell activation at several key mechanistic control points.^{7–9} Importantly, TGF- β 1 inhibits proximal TCR signaling¹⁰ in part by inhibiting the Tec kinase ITK, Ca²⁺ flux, and the nuclear translocation of the NFAT transcription factor.¹¹ TGF- β 1 raises the threshold for TCR signaling and the T-cell response to mitogenic stimuli.¹² As evidence for this, thymocytes from TGF- β 1^{-/-} mice are hyperresponsive to

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TCR-mediated as well as TCR-independent mitogenic stimuli due to a lowered threshold for Ca²⁺-dependent activation.¹³ This suggests the possibility that CD4⁺ T cells in TGF- β 1^{-/-} mice become aberrantly activated through triggers that ordinarily would not be sufficient to lead to activation. The survival of naïve T cells in the periphery is believed to require continual TCR signaling through recognition of self-MHC ligands,^{14,15} although this has been controversial.¹⁶ Perhaps, in the complete absence of TGF- β 1, a lowered TCR activation threshold permits self-MHC-TCR interactions to become sufficiently vigorous as to induce aberrant T-cell activation and autoimmune pathology. Another possibility is that non-MHC signals such as those delivered by cytokines^{17,18} or adhesion molecules (eg LFA-3/CD2^{19,20}) can, in a TGF- β 1-deficient environment, activate T cells *in vivo*. We therefore considered the possibility that disease pathology in TGF- β 1^{-/-} mice results from aberrant activation of CD4⁺ T cells through pathways other than those delivered by specific cognate activating antigen. Nearly all CD4⁺ T cells from TGF- β 1^{-/-} mice possess an activated/memory phenotype,³ consistent with this mode of T-cell activation. Furthermore, in analyses of TCR repertoire usage in BALB/c-TGF- β 1^{-/-} mice, expansion of CD4⁺ T cells occurs approximately equally in T cells from all TCR V β expressing subsets (Richard T Robinson and James D Gorham, unpublished data).

We therefore sought to determine whether autoimmune pathology in TGF- β 1^{-/-} mice requires the presence of a physiologic broad CD4⁺ T-cell TCR repertoire, or, alternatively, whether pathology develops as long as there are CD4⁺ T cells present, regardless of antigen specificity. This is particularly interesting with respect to the pathogenesis of autoimmune liver disease and the significant liver damage observed in BALB/c-TGF- β 1^{-/-} mice, as the liver is subject to injury through 'bystander' pathways that appear not to depend on the presence of a liver-specific antigen.^{21,22} Thus, we crossed BALB/c-TGF- β 1^{-/-} mice with the DO11.10 mouse, a BALB/c background TCR transgenic mouse with CD4⁺ T-cell specificity for a peptide (OVA₃₂₃₋₃₃₉) from chicken ovalbumin. Nearly all T cells from this mouse express the transgenic TCR V α and V β genes as CD4⁺, and recognize OVA presented by the class II MHC I-A^d complex. The rearrangement of endogenous TCR V α gene segments is not completely suppressed in TCR transgenic mice, however, permitting the generation of T cells expressing a second nonclonotypic TCR V α chain and having dual antigen specificities.^{23,24} To obviate this possibility, we also bred in a RAG-1 deletion, rendering TCR transgenic DO11.10-TGF- β 1^{-/-} mice incapable of generating additional TCR specificities. Thus, we generated cohorts of highly inbred BALB/c background TGF- β 1^{-/-} mice with an increasingly restricted CD4⁺ T-cell repertoire (TGF- β 1^{-/-} mice; DO11.10-TGF- β 1^{-/-} mice; DO11.10-RAG-1^{-/-}-TGF-

β 1^{-/-} mice) and analyzed these for survival, target organ pathology, and T-cell activation.

Materials and methods

Mice

Mice were bred at the Dartmouth Medical School American Association for Accreditation of Laboratory Animal Care-accredited animal care facility and were treated humanely, according to National Institutes of Health guidelines. The derivation of inbred BALB/c-TGF- β 1^{+/-} mice has been described.^{3,25} Extensively back-crossed BALB/c-DO11.10 mice and BALB/c-RAG-1^{-/-} mice were obtained from the Jackson Laboratory and bred with BALB/c-TGF- β 1^{+/-} mice. Litters were screened by PCR of DNA from tail snips for *TGFB1* and *RAG1* genotypes as we have described⁵ and for the DO11.10 TCR α transgene using the primers 5'-GAGCAGCTTCCTTCCATCCTGAGAG-3' and 5'-TGGCTCTACAGTGAGTTTGGTGCCA-3'.

Survival Curves

The day of birth was designated as day 0, and while some mice were found to have died without leading indications, most were euthanized when signs indicated suffering or the mice were moribund, including reduced movement throughout the cage, reduced feeding, and hunched posture. Statistical comparisons between survival curves are by the log-rank test, and were calculated using GraphPad Prism v. 4.

Isolation of Intrahepatic and Splenic Lymphocytes

TGF- β 1^{-/-} or littermate control TGF- β 1^{+/-} mice were euthanized by overexposure to CO₂. Livers and spleens were removed, weighed and homogenized in ice-cold serum-free RPMI 164 with a Powergen Tissue Homogenizer (Fisher) at its lowest speed. The homogenized liver was then washed with serum free RPMI and resuspended in 5 ml of 100 U/ml collagenase IV (Sigma Aldrich, St Louis, MO, USA) and 10 U/ml DNase I (Roche, Indianapolis, IN, USA) in serum-free RPMI. The organ/digestion solution mixture was incubated at 37°C for 45 min with inverting every 2 min. After washing with cold 2% FCS/RPMI, spleen or liver cells were resuspended in room temperature 2% FCS/RPMI and collected over a Ficoll (Sigma Histopaque 1.119) step-gradient. Cells were counted using Guava ViaCount and stained for flow cytometric analysis.

Serum Transaminase Analysis

Directly following euthanasia by overexposure to CO₂, blood was collected into heparinized plasma

separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA) from the right atrium of the heart. Plasma was diluted four-fold in sterile PBS and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) concentrations were determined spectrophotometrically using a Roche-Hitachi 917 Automatic Analyzer.

Histological Analysis

Livers, lungs and hearts were dissected out and placed in buffered formalin. Following paraffin embedding, sectioning and staining with H&E, samples were coded and scored on a 0–3 scale in a blinded fashion, using sections of organs from wild-type BALB/c mice as a baseline reference (score 0). For each organ, numerical scores were assigned as follows: Liver: '0' baseline wild-type BALB/c histology; '1' rare portal balloon degeneration and/or rare portal and parenchymal infiltrates but no necrosis; '2' more frequent portal infiltrates and rare interface hepatitis; '3' extensive portal inflammation and bridging necrosis. Lung: '0' baseline wild-type BALB/c histology; '1' rare perivascular and peribronchiolar infiltrates; '2' more frequent perivascular and peribronchiolar infiltrates; '3' extensive cellularity surrounding the vasculature and bronchioles. Heart: '0' baseline wild-type BALB/c histology; '1' focal ventricular subendocardial infiltrates; '2' mild transmural ventricular infiltrates; '3' moderate to extensive diffuse infiltrates in both atria and ventricles.

Flow Cytometric Analysis

All antibodies used for flow cytometric analysis were purchased from BD Pharmingen (San Diego, CA, USA) or CalTag Laboratories (Burlingame, CA, USA). Ficoll-purified mononuclear cells were washed with FACS buffer (2% FCS in PBS) and stained with antibodies that recognize CD3 ϵ (clone 17A2), CD4 (clone RM4-5), the DO11.10 TCR (clone KJ1-26) and CD62L (clone MEL-14), washed, fixed in 1% methanol-free formaldehyde in FACS buffer, and analyzed using a FACSCanto cytometer (BD Biosciences, San Jose, CA, USA). For all surface markers, positive staining was established using appropriate isotype controls.

Results

Restricting the TCR Repertoire Prolongs Survival of TGF- β 1^{-/-} Mice

TCR transgenic DO11.10-TGF- β 1^{-/-} mice lived significantly longer than their nontransgenic TGF- β 1^{-/-} counterparts (Figure 1). While nontransgenic TGF- β 1^{-/-} mice had a median survival of 15.25 days, DO11.10-TGF- β 1^{-/-} mice had a median survival of 35.0 days, and statistical analysis of the two curves

Figure 1 DO11.10-TGF- β 1^{-/-} mice have increased survival. The survival rates of TGF- β 1^{-/-} mice that are either nontransgenic, DO11.10- or DO11.10-RAG-1^{-/-} are shown. The median survivals for nontransgenic ($n=8$), DO11.10- ($n=20$), and DO11.10-RAG-1^{-/-} ($n=12$) TGF- β 1^{-/-} mice were 15.25, 35.0 and 60.75 days, respectively. The difference in survival curves for the nontransgenic TGF- β 1^{-/-} mice and DO11.10-TGF- β 1^{-/-} mice is statistically significant ($P<0.0001$; indicated by asterisk). The difference in survival curves for the DO11.10-TGF- β 1^{-/-} mice and the DO11.10-RAG-1^{-/-}TGF- β 1^{-/-} mice is statistically not significant (NS). 'RAG-1^{+/+}' is used to indicate that data for RAG-1^{+/+} mice and for RAG-1^{+/-} mice are combined.

showed them to be strongly significantly different ($P<0.0001$; log-rank analysis). Thus, restricting the CD4⁺ T-cell repertoire using the DO11.10 TCR transgenes significantly prolongs survival of TGF- β 1^{-/-} mice. DO11.10-TGF- β 1^{-/-} mice on a RAG-1^{-/-} background survived even longer (median survival of 60.75 days) than did RAG-1-intact DO11.10-TGF- β 1^{-/-} mice, but this difference did not reach statistical significance ($P=0.10$). In our previous study,⁵ RAG-1^{-/-}TGF- β 1^{-/-} mice had a median survival of 111.0 days and the survival curves of DO11.10-RAG-1^{-/-}TGF- β 1^{-/-} mice here and RAG-1^{-/-}TGF- β 1^{-/-} mice from that study are calculated to be not statistically significantly different ($P=0.07$). However, in the previous study, most RAG-1^{-/-}TGF- β 1^{-/-} mice were euthanized prior to the onset of morbidity, so the conclusion that the survival of these two groups is not different must remain tentative.

Restricting the TCR Repertoire Ameliorates Organ Pathology in TGF- β 1^{-/-} Mice

A prominent feature of the TGF- β 1^{-/-} phenotype on the BALB/c background is a significant degree of necroinflammatory liver disease, reflected in elevated circulating concentrations of the hepatocyte enzymes AST and ALT, indicative of the presence of hepatocellular damage. We have previously demonstrated that hepatic pathology is dependent on CD4⁺ T cells.⁵ To test whether DO11.10-TGF- β 1^{-/-} mice and DO11.10-RAG-1^{-/-}TGF- β 1^{-/-} mice develop hepatocellular damage, we measured plasma AST and ALT in mice of each genotype (Figure 2).

As expected, TGF- $\beta 1^{-/-}$ mice exhibited high plasma AST activity and high plasma ALT activity by 11–12 days of age. In samples from DO11.10-TGF- $\beta 1^{-/-}$ mice, AST and ALT were lower than in TGF- $\beta 1^{-/-}$ samples (Figure 2a; $P < 0.0001$ for both AST and

ALT), indicating that the presence of the DO11.10 TCR transgenes largely protects TGF- $\beta 1^{-/-}$ mice from hepatocellular damage. Notably, AST and ALT were somewhat higher on average in DO11.10-TGF- $\beta 1^{-/-}$ mice than in littermate control DO11.10-TGF- $\beta 1^{+/+}$ mice, and this was statistically significant for ALT ($P = 0.02$), but not for AST. Inspection of the data shows that elevated AST and ALT were observed for some but not all DO11.10-TGF- $\beta 1^{-/-}$ samples. Stratification of the data (Figure 2b) shows that AST and ALT increased with age, with statistically significant regression lines. Notably however, for the older DO11.10-TGF- $\beta 1^{-/-}$ mice, elevations in AST and ALT were much more moderate than those typically observed for TGF- $\beta 1^{-/-}$ mice.

In samples from DO11.10-TGF- $\beta 1^{-/-}$ mice on a RAG-1^{-/-} background, AST and ALT were lower than in TGF- $\beta 1^{-/-}$ samples (Figure 2a; $P < 0.0001$ for both AST and ALT). AST and ALT were higher on average in DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice than in littermate control DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{+/+}$ mice, and this was statistically significant both for AST ($P = 0.006$) and for ALT ($P = 0.0006$). Stratification of the data (Figure 2c) shows that AST and ALT did not increase with age for DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice, unlike what is observed for DO11.10-TGF- $\beta 1^{-/-}$ mice on a RAG-1 intact (ie RAG-1^{+/+}) background (Figure 2b). For ALT, there was an apparent decrease with age, but the regression line was not statistically significant. Three DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice had very high ALT levels (> 200 U/l; circled in Figure 2a and c). These same mice had no comparable elevation in AST (eg see Figure 2c), and extensive histologic analyses of multiple liver sections from these three mice revealed fairly unremarkable histology, with no visible hepatocellular damage, and only occasional, mild portal inflammation (data not shown). Thus, for $\sim 10\%$ of DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice, ALT is strongly elevated, but this is apparently dissociated from other obvious signs of liver damage such as elevated AST or histologic abnormalities; the elevation in ALT for this subgroup of DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice remains unexplained at this point. Finally, and as we have previously reported (non-TCR transgenic), RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice did not exhibit elevation in either AST or ALT (Figure 2a).

Figure 2 DO11.10-TGF- $\beta 1^{-/-}$ mice and DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice are protected from hepatocellular damage. **(a)** AST and ALT were measured in plasma collected at day of age 11 or greater from mice of the various genotypes indicated. Each point represents data from one mouse. $n = 10$ –30 mice per group. Statistical significance between groups was tested by Student's t -test. Relevant P -values are shown above horizontal lines. NS: not significant. **(b, c)** AST and ALT values from DO11.10-TGF- $\beta 1^{-/-}$ mice **(b)** and from DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice **(c)** are stratified by age of the mouse. In **(a)** and **(c)**, the ALT values for the three DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice exhibiting ALT > 200 U/l are circled.

Figure 3 DO11.10-TGF- $\beta 1^{-/-}$ mice and DO11.10-RAG-1 $^{-/-}$ -TGF- $\beta 1^{-/-}$ mice are protected from end-organ inflammation. H&E stains of representative sections from (a) liver (b) lung and (c) heart of mice of the indicated genotypes. Arrows indicate inflammatory infiltrates in sections of organs from DO11.10-TGF- $\beta 1^{-/-}$ mice.

We examined the pathology of organ damage histologically. Livers from littermate control TGF- $\beta 1^{+/-}$ mice, regardless of RAG-1 or DO11.10 status, were histologically normal (Figure 3a, and data not shown). The livers from TGF- $\beta 1^{-/-}$ mice showed a periportal infiltrate of mononuclear cells and neutrophils. Inflammation varied in intensity from one portal area to the next but all portal areas were affected. The composition of cells varied, with some portal areas having more neutrophils than others. Interface hepatitis was moderate. Periportal balloon degeneration was diffuse. All TGF- $\beta 1^{-/-}$ livers showed severe and extensive bridging necrosis. Livers from DO11.10-TGF- $\beta 1^{-/-}$ mice showed none of the widespread necrosis and extensive inflammation of TGF- $\beta 1^{-/-}$ livers. Inflammation was seen but varied from mouse to mouse. Some DO11.10-TGF-

$\beta 1^{-/-}$ livers showed no difference from wild type, while other DO11.10-TGF- $\beta 1^{-/-}$ livers showed mild portal inflammation (Figure 3a, arrow) with predominantly mononuclear cells and some neutrophils. Interface hepatitis was rare and mild, and was characterized by hepatocytes ringed by lymphocytes. Livers from DO11.10-TGF- $\beta 1^{-/-}$ mice on a RAG-1 $^{-/-}$ background exhibited no necrosis, and no detectable parenchymal or portal inflammation.

In sections of lung, TGF- $\beta 1^{-/-}$ samples showed extensive perivascular (Figure 3b) and peribronchiolar (not shown) inflammation. The histology of DO11.10-TGF- $\beta 1^{-/-}$ lungs was variable. When observed, inflammation in the lung consisted of rare collection of mononuclear cells around vessels, bronchioles (Figure 3b, arrow), or, rarely, in the parenchyma. In DO11.10-RAG-1 $^{-/-}$ -TGF- $\beta 1^{-/-}$ mice,

perivascular inflammation was rarely seen, similar to sections of littermate DO11.10-RAG-1^{-/-}-TGF- β 1^{+/-} controls.

Sections of myocardium from TGF- β 1^{-/-} mice (Figure 3c) showed an infiltrate of mononuclear cells diffusely in the atria and ventricles. In the ventricles, this was predominantly subendocardial. In DO11.10-TGF- β 1^{-/-} hearts, rare foci of mononuclear cells were present (Figure 3c, arrow), except in one animal in which the heart showed a more extensive infiltrate of mononuclear cells. In DO11.10-RAG-1^{-/-}-TGF- β 1^{-/-} hearts, mononuclear inflammation was absent. Notably, in some DO11.10-RAG-1^{-/-}-TGF- β 1^{-/-} hearts, we observed a focal, predominantly neutrophilic, perivascular infiltrate around a large vessel by the atrium (not shown), which was not observed in littermate DO11.10-RAG-1^{-/-}-TGF- β 1^{+/-} control hearts.

High-power views of tissues from BALB/c-TGF- β 1^{-/-} mice and from DO11.10-TGF- β 1^{-/-} mice show additional details of the inflammatory lesions observed in these mice. A high-power image of a section of TGF- β 1^{-/-} liver (Figure 4a) shows an interface area with portal side to the left and parenchyma to the right. Mononuclear cells emanate and surround hepatocytes. Degrees of cell death are represented with ballooning of cytoplasm and fading nuclei, and an apoptotic body is seen at the upper right (arrow). The cells appear to be predominantly mature lymphocytes. A section from a DO11.10-TGF- β 1^{-/-} liver (Figure 4b) shows an interface area adjacent to a vessel on the left and parenchyma to the right. Mononuclear cells composed mostly of mature lymphocytes infiltrate between hepatocytes and can be seen clearly surrounding one hepatocyte (arrow). While an

apoptotic body is not seen, ballooning hepatocyte cytoplasm and fading nuclei are present. In a section from TGF- β 1^{-/-} lung (Figure 4c), a dense peribronchiolar infiltrate of mononuclear cells is seen. In a high-power image from a DO11.10-TGF- β 1^{-/-} lung (Figure 4d), a perivascular mononuclear infiltrate obliterates the wall. Necrosis of the vessel wall is not seen. A section of a high-power image from TGF- β 1^{-/-} heart (Figure 4e) shows subendocardial inflammation in a ventricle with the lumen to the right. Mononuclear cells infiltrate between myocytes. A mitotic figure is present (arrow). Myocytes are in various stages of death with nuclei fading and striations lost. A high-power section from a DO11.10-TGF- β 1^{-/-} heart (left ventricular wall) shows myocytes infiltrated by lymphocytes but not extensively (Figure 4f). The myocytes appear viable with dark nuclei and striations.

To extend the histological analyses, liver, lung, and heart sections were analyzed in a blinded fashion and scored on a semiquantitative scale (0 = no pathological changes; 3 = severe pathological changes; see Materials and methods for details). Figure 5 shows the compiled data from a total of 5–7 TGF- β 1^{-/-} mice per group. These results show that restricting the T-cell repertoire through introduction of the DO11.10 TCR transgenes largely but not completely reduced pathological changes in the liver, lung and heart of TGF- β 1^{-/-} mice. Additional introduction of the RAG-1 deletion into DO11.10-TGF- β 1^{-/-} mice further attenuated inflammatory pathological changes. We note that a few DO11.10-RAG-1^{-/-}-TGF- β 1^{-/-} mice demonstrated some pathological changes involving innate immune cells of the myeloid series, including the presence of alveolar macrophages in the lung, and neutrophilic perivas-

Figure 4 High-power images of histopathologic changes in TGF- β 1^{-/-} mice and in DO11.10-TGF- β 1^{-/-} mice. Representative high-power images of sections from liver, lung and heart of TGF- β 1^{-/-} and DO11.10-TGF- β 1^{-/-} mice are shown.

Figure 5 Semiquantitative assessment of histopathologic changes in organs. Each point represents one mouse. Analysis was carried out in a blinded fashion and used a 0–3 point scale for each organ, as detailed in Materials and methods.

cular infiltrates in the atrium of the heart (not shown).

TGF- $\beta 1^{-/-}$ mice uniformly show severe liver pathology as assessed either histologically or by plasma transaminase levels; by contrast, in DO11.10-TGF- $\beta 1^{-/-}$ mice, these measurements are lower, but do exhibit mouse-to-mouse variability. We explored this variability further. To determine whether the elevations in transaminases in DO11.10-TGF- $\beta 1^{-/-}$ mice reflected the degree of liver histopathology, we compared these two metrics of liver disease. The data show no obvious positive correlation between the degree of liver histopathology and either AST (Figure 6a) or ALT (Figure 6b). In addition, the degree of liver histopathology does not correlate with mouse age (Figure 6c); by contrast, plasma transaminase levels in DO11.10-TGF- $\beta 1^{-/-}$ mice do show a small positive correlation with age (see Figure 2b). As observed for liver, the degree of histopathology in either lung (Figure 6d) or heart (Figure 6e) does not correlate with age in DO11.10-TGF- $\beta 1^{-/-}$ mice.

Restricting the TCR Repertoire Prevents Hepatic CD4⁺ T-Cell Lymphocytosis in TGF- $\beta 1^{-/-}$ Mice

BALB/c-TGF- $\beta 1^{-/-}$ livers exhibit a marked CD4⁺ lymphocytosis, with 5- to 10-fold higher numbers of CD4⁺ T cells in the liver compared with littermate controls.^{3,5,25} To determine if hepatic CD4⁺ T-cell accumulation is affected by restricting the TCR repertoire, hepatic CD4⁺ T cells from the various mice were counted. As we previously reported, nontransgenic TGF- $\beta 1^{-/-}$ mice exhibited a marked hepatic CD4⁺ lymphocytosis compared with littermate controls ($P < 0.05$; Figure 7a). For DO11.10 mice, the number of intrahepatic CD4⁺ T cells was not different between DO11.10-TGF- $\beta 1^{-/-}$ mice and littermate DO11.10-TGF- $\beta 1^{+/+}$ controls. This was true for both the RAG-1-intact and RAG-1-deficient

groups of mice. Concomitant with the increase in hepatic CD4⁺ T cells, there was a decrease in CD4⁺ T-cell numbers in the spleens of TGF- $\beta 1^{-/-}$ mice, but this did not reach statistical significance ($P = 0.13$; Figure 7b). A similar decrease in splenic T cells was observed in DO11.10-TGF- $\beta 1^{-/-}$ mice and this was statistically significant compared with littermate control DO11.10-TGF- $\beta 1^{+/+}$ mice ($P < 0.05$). In DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{-/-}$ mice, splenic CD4⁺ T-cell numbers were also lower than in littermate control DO11.10-RAG-1^{-/-}-TGF- $\beta 1^{+/+}$ mice ($P = 0.11$). As there was no similar increase in hepatic CD4⁺ T cells in DO11.10-TGF- $\beta 1^{-/-}$ mice, the mechanisms responsible for hepatic CD4⁺ T-cell accumulation and splenic CD4⁺ T cell decrease may be distinct. Thus, limiting the TCR repertoire prevents the accumulation of CD4⁺ T cells in the liver, but has little effect on the diminution of splenic T-cell numbers.

T-Cell Activation in TGF- $\beta 1^{-/-}$ Mice is Dependent upon a Full TCR Repertoire

High expression of CD62L is characteristic of naïve CD4⁺ T cells, and a decrease in CD62L expression reflects prior antigen exposure and cell activation. To examine the effects of restricting the TCR repertoire on the state of T-cell activation in TGF- $\beta 1^{-/-}$ mice, we examined CD62L expression on CD4⁺ T cells from spleens and livers from our various TGF- $\beta 1^{-/-}$ lines. In control TGF- $\beta 1^{+/+}$ splenic CD4⁺ T cells, 87.1% expressed high CD62L (Figure 8a), consistent with a naïve phenotype. By contrast, only 26.8% of splenic CD4⁺ T cells from TGF- $\beta 1^{-/-}$ mice expressed high CD62L (Figure 8b), consistent with a high degree of T-cell activation. In DO11.10-TGF- $\beta 1^{+/+}$ mice, two populations of CD4⁺ T cells were identified. One population stained with the clonotypic antibody KJ1-26, indicating the expression of both the TCR α

Figure 6 DO11.10-RAG-1^{+/}TGF-β1^{-/-} mice: no positive correlation between histopathology and transaminase levels, or between histopathology and age. Data from DO11.10-RAG-1^{+/}TGF-β1^{-/-} mice displayed in Figures 2 and 5 are correlated. The analysis shows no obvious positive correlation between the extent of liver histopathology and levels of either (a) AST or (b) ALT. In addition, no correlation is seen between extent of histopathology in (c) liver (d) lung or (e) heart and the age of the mouse.

and TCRβ transgenes. Gating on this population, 90.5% of cells were CD62L^{hi} (Figure 8c). In the equivalent population in DO11.10-TGF-β1^{-/-} mice, 60.6% of cells were CD62L^{hi} (Figure 8d). A second CD4⁺ population did not stain with KJ1-26, probably reflecting cells that do not express the clonotypic TCR, but use instead an alternative TCRVα chain. Gating on the CD4⁺KJ1-26⁻ population, 68.8 and 45.0% of cells were CD62L^{hi} in DO11.10-TGF-β1^{+/+} mice and DO11.10-TGF-β1^{-/-} mice, respectively (Figure 8c and d). With the addition of the RAG-1 deficiency, and the prevention of endogenous *TCRVA* gene rearrangement and the usage of alternative TCRVα chains, all CD4⁺ T cells stained positive with the KJ1-26 mAb. Whether TGF-β1 was intact (Figure 8e) or deleted (Figure 8f), about 90% of CD4⁺ T cells were CD62L^{hi}, indicating a low degree T-cell activation in these cell populations. A summary of data from three mice in each group (Figure 8g) indicates that introduction of the DO11.10 TCR transgenes reduces T-cell activation in TGF-β1^{-/-} mice, and that further introduction of the RAG-1 deletion eliminates T-cell activation. Inspection of the flow cytometry profiles from

DO11.10-TGF-β1^{-/-} splenic CD4⁺ T cells (Figure 8d) reveals that T-cell activation preferentially occurs in KJ1-26⁻ T cells, which presumably have antigen specificities other than OVA, and may be responsive to autoantigens.

We extended our analysis of CD62L expression to liver CD4⁺ T cells. Forty two percent of control nontransgenic TGF-β1^{+/+} liver T cells expressed high CD62L expression (Figure 9a). Similar expression patterns were observed in hepatic DO11.10-TGF-β1^{+/+} KJ1-26⁺CD4⁺ T cells, and hepatic DO11.10-RAG-1^{-/-}TGF-β1^{+/+} KJ1-26⁺CD4⁺ T cells, with 41.3% (Figure 9c) and 43.6%, (Figure 9e) respectively, expressing high CD62L. Examining the hepatic CD4⁺ T-cell population in TGF-β1^{-/-} mice, we observed that, as in the spleen, most cells were CD62L^{lo} (Figure 9b). In DO11.10-TGF-β1^{-/-} samples, an intermediate frequency (22.0%) of hepatic T cells were CD62L^{hi} (Figure 9d), and in DO11.10-RAG-1^{-/-}TGF-β1^{-/-} T cells, the expression of CD62L expression returned to control frequencies, at 41.8% (Figure 9f). A summary of data from three mice in each group is shown (Figure 9g). Among the KJ1-26⁻population in the DO11.10-TGF-β1^{+/+} mice,

Figure 7 Reduced numbers of intrahepatic CD4⁺ T cells in DO11.10-TGF- β 1^{-/-} mice and DO11.10-RAG-1^{-/-}TGF- β 1^{-/-} mice. The total numbers of CD4⁺ T cells present in the (a) liver and (b) spleen were determined for mice of each genotype shown. The number of CD4⁺ T cells was normalized to organ weight. $n = 3-7$ mice per group. Bars show the mean $\pm$ s.d. * $P < 0.05$.

CD62L^{hi} expression was decreased (15.6%) compared with the KJ1-26⁺ population (41.3%) (Figure 9c), and CD62L expression was further reduced (6.9%) in the equivalent cell population in TGF- β 1^{-/-} mice (Figure 9d). Taken together, these results show that progressive restriction of the TCR repertoire progressively prevents T-cell activation in TGF- β 1^{-/-} mice.

Discussion

Mice with a targeted deletion of the gene encoding the regulatory molecule TGF- β 1 develop an unrestrained Th1-mediated immune pathology^{3,5,25} and die within weeks of birth.^{1,2} On the BALB/c background, TGF- β 1^{-/-} mice develop severe necroinflammatory liver disease and is a model for the human disease autoimmune hepatitis (AIH).²⁶ The presence of CD4⁺ T cells is required for inflammatory disease and early death, but whether the immune response is antigen specific has not been

established. Recently, it has been reported that TGF- β 1 significantly lowers the activation threshold for TCR triggering.^{12,13} Indeed, nearly all CD4⁺ T cells isolated from TGF- β 1^{-/-} mice express cell surface markers indicating cellular activation.³ Moreover, expansion in the liver occurs in T cells representing all TCRV β chain subsets (Richard T Robinson and James D Gorham, unpublished data). Together, these data suggest the possibility that in the absence of TGF- β 1, T cells become readily activated and induce pathology *in vivo* through cellular pathways that normally would not result in T-cell activation. Possibilities include self-MHC-TCR interactions and cytokine-receptor interactions that ordinarily act to maintain T-cell survival in the periphery, but do not activate T cells under normal physiologic conditions.^{14,15,17} As the liver is susceptible to T-cell dependent 'bystander' injury (ie not mediated by liver-specific antigens),^{21,22} this hypothesis needed to be considered to further understand this mouse model of autoimmune hepatocellular damage. Our current study finds no support for such modes of T-cell activation and target organ injury in TGF- β 1^{-/-} mice.

To test the hypothesis that the presence of CD4⁺ T cells is sufficient for the development of end organ damage independent of cognate antigen-TCR interaction, we bred TGF- β 1^{-/-} mice with chicken ovalbumin-specific DO11.10 TCR transgenic mice. The presence of the DO11.10 TCR transgenes prolonged the survival of TGF- β 1^{-/-} mice, ameliorated end-organ damage, and caused a reduction in the degree of cellular activation within the CD4⁺ T-cell population. In addition, we bred to homozygosity a null allele for RAG-1 onto DO11.10-TGF- β 1^{-/-} mice. This further improved survival, reduced end-organ damage, and completely eliminated aberrant cellular activation within the CD4⁺ T-cell population. These data indicate that CD4⁺ T-cell activation in TGF- β 1^{-/-} mice does not occur 'non-specifically,' and that the mere presence of CD4⁺ T cells is not sufficient for pathology. Rather the data support a model in which T-cell activation and the associated end-organ damage are driven by 'typical' TCR-activation signals, albeit in a pathologic autoimmune-prone environment (ie the absence of TGF- β 1). That CD4⁺ T-cell numbers in spleens and livers were similar between DO11.10-TGF- β 1^{-/-} and littermate control DO11.10-TGF- β 1^{+/+} mice and between DO11.10-RAG-1^{-/-}TGF- β 1^{-/-} and littermate control DO11.10-RAG-1^{-/-}TGF- β 1^{+/+} mice indicates that the peripheral signals that maintain CD4⁺ T-cell numbers are operative in TCR transgenic TGF- β 1^{-/-} mice; however, these pathways are not sufficient to induce T-cell activation or T-cell dependent end-organ pathology.

Interestingly, in DO11.10-TGF- β 1^{-/-} mice, a moderate degree of inflammation in liver, lung and heart was observed at the histological level. As RAG-1 is intact in these animals, and some rearrangement of the endogenous TCR α locus is therefore permitted,

Figure 8 Reduced activation of splenic CD4⁺ T cells in DO11.10-TGF- β 1^{-/-} mice and DO11.10-RAG-1^{-/-}TGF- β 1^{-/-} mice. (a–f) Splenic mononuclear cells from mice of the indicated genotypes were stained for CD3 or for the DO11.10 TCR (KJ1-26), and co-stained for CD4 and CD62L. CD62L expression was assessed using the gates shown. Representative histograms of CD62L expression are shown for each population of gated cells. Numbers indicate the percentage of gated CD4⁺ cells that are CD62L^{hi}. (g) Combined CD62L^{hi} expression data from three to seven mice per group. Bars show the mean $\pm$ s.d.

it is likely that some CD4⁺ T cells expressing endogenous TCR α genes are generated in DO11.10-TGF- β 1^{-/-} mice. Indeed, we observed in both spleen

and liver the presence of a significant population of CD4⁺ T cells that did not stain with the clonotypic KJ1-26 monoclonal antibody, and therefore do not

Figure 9 Reduced activation of intrahepatic CD4⁺ T cells in DO11.10-TGF-β1^{-/-} mice and DO11.10-RAG-1^{-/-}-TGF-β1^{-/-} mice. (a–f) Intrahepatic mononuclear cells from mice of the indicated genotypes were stained for CD3 or for the DO11.10 TCR (KJ1-26), and co-stained for CD4 and CD62L. CD62L expression was assessed using the gates shown. Representative histograms of CD62L expression are shown for each population of gated cells. The lower apparent baseline expression of CD62L in hepatic T cells compared with splenic T cells appears to be related to the use of collagenase/DNAse to isolate liver lymphocytes (Tamar J Kitzmiller, unpublished). The numbers indicate the percentage of gated CD4⁺ cells that are CD62L^{hi}. (g) Combined CD62L^{hi} expression data from three to seven mice per group. Bars show the mean + s.d.

express the transgenic TCR $\alpha\beta$ combination. Rather, they presumably express a TCR complex likely composed of the transgenic TCR β subunit paired with an endogenous TCR α subunit, as previously described for DO11.10 mice.²⁴ In addition, KJ1-26⁺ cells from these mice may express a second endogenous TCR α subunit, allowing the expression of two distinct TCR complexes (one clonotypic, the other nonclonotypic) on the same cell, as previously observed for wild-type T cells.^{27,28} Nonclonotypic TCRs should permit promiscuous antigen recognition by these cell populations, the expansion of self-reactive T cells, and the development of some inflammatory changes in the target organs. This inflammation appears to progress slowly, indicating either that the restricted TCR repertoire does not permit the generation of highly pathogenic T-cell clones, or that such clones are kept in check by the concomitant development of effective regulatory T cells. Our current studies do not permit us to distinguish between these two possibilities.

The human disease AIH is associated with the accumulation of CD4⁺ T cells in the liver producing the Th1 cytokines IFN- γ and TNF- α .^{29,30} While AIH is largely assumed to be an antigen-specific disease, the identification of liver specific antigens that may drive the T-cell aspect of the disease has been frustratingly elusive. Support for antigen specificity in the pathology of the immune response in AIH has relied heavily on the presence of high titer autoantibodies, such as antinuclear antibodies or anti-smooth muscle antibodies.³¹ In some subtypes of AIH, specific autoantigens have been identified, such as cytochrome P450 2D6,³² or formiminotransferase cyclodeaminase.³³ However, in the majority of AIH cases, autoantigens recognized by immunoglobulins have not been identified. Moreover, it is generally believed that pathology in AIH is primarily mediated by T cells, with autoantibodies playing only a minor role in pathogenesis.³⁴ Analysis of CDR3 usage in hepatic T cells from AIH patients indicates that the intrahepatic T-cell population is oligoclonal, not polyclonal.^{35–37} This supports an antigen-specific model for the expansion of intrahepatic T cells in AIH, but the identities of the particular antigen(s) that may be involved remain unknown. In the current work, we find evidence in support of the hypothesis that T-cell activation and end-organ damage in TGF- β 1^{-/-} mice are driven by specific antigen(s). These antigens are likely to be 'true' self-antigens, rather than those derived from intestinal flora, as TGF- β 1^{-/-} mice raised under germ-free conditions nevertheless develop the characteristic autoimmune pathology.³⁸ This is in contrast to results from IL-2^{-/-} mice or TCR α ^{-/-} mice in which the development of autoimmune colitis is eliminated when mice are raised in germ-free conditions.^{39,40} Identifying the particular self-antigens driving autoimmune liver disease in BALB/c-TGF- β 1^{-/-} mice should yield insight into the

pathogenesis of autoimmune diseases of the liver and other organs.

Human AIH has not been associated with a specific deficiency in TGF- β 1 production. Indeed, available data support the converse. Serum TGF- β 1 is elevated in AIH patients with active disease and returns to control levels at disease remission.⁴¹ Similarly, TGF- β 1 mRNA and protein expression is elevated in liver biopsies from AIH patients with active disease, but not in biopsies from patients in remission.⁴¹ Interestingly, a recent study shows that AIH patients exhibit both an elevation of TGF- β 1 serum levels as well as a deficiency in expression of a TGF- β 1 receptor subunit in PBMC.⁴² This suggests that the elevation in TGF- β 1 cytokine expression may be accompanied by a reduced ability of immune cells to respond to TGF- β 1, at least during active disease. Animal models show that T lymphocyte responsiveness to TGF- β 1 is a critical factor in the development of immune mediated hepatocellular damage. Mice expressing a transgenic dominant-negative TGF- β 1 receptor specifically in T cells are more susceptible than control mice to experimental AIH induced by injection of liver extracts.⁴³ No data exist on the expression of TGF- β 1, its receptor, or the responsiveness of T cells or other immunocytes to TGF- β 1 at the time of onset of AIH. We speculate that the elevated TGF- β 1 seen in serum and liver during active AIH flares may represent compensatory overexpression of this immunosuppressive cytokine. Given the recognized role of TGF- β 1, through its effects on liver stellate cells, in the development of fibrosis and cirrhosis,⁴⁴ understanding the relationship between TGF- β 1 expression and cellular response, and the onset, progression, and remission of AIH, remains an important research objective.

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