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Elimination of both CD4⁺ and CD8⁺ T cells but not B cells eliminates inflammation and prolongs the survival of TGFβ1-deficient mice^{*}

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Abstract

Transforming growth factor β1 (TGFβ1) is a potent negative immunoregulatory molecule. We have previously shown that the autoimmune-mediated weaning-age lethality of *Tgfb1*^{-/-} mice is reversed upon genetic combination with *Scid* or *Rag* null alleles. Here, we show that elimination of T but not B cells is sufficient for the reversal, but elimination of either CD4⁺ or CD8⁺ cells is not. Although elimination of B cells does not rescue TGFβ1-deficient animals from autoimmunity, B cells are hyperresponsive to LPS in the absence of TGFβ1. TGFβ1 deficiency leads to activation of CD8⁺ T cells as suggested by down-modulation of CD8 even in the absence of CD4⁺ T cells. This study provides evidence that both CD4⁺ and CD8⁺ T cells, but not B cells, have the ability to cause inflammation in the absence of TGFβ1. However, though TGFβ1-deficient B cells are hyperresponsive to stimulation, alone they are not sufficient to cause inflammation.

Keywords

Cell proliferation; Growth factors; Inflammation; Knockout mice; TGFβ1; T-lymphocytes and B-lymphocytes

1. Introduction

TGFβ1 is a polypeptide growth factor that gene ablation studies have shown is involved in lymphocyte homeostasis and self-tolerance [1], several types of cancer [2–7], cardiac hypertrophy [8], and platelet aggregation [9]. In the embryo TGFβ1 plays vital roles in pre-implantation and yolk sac development [10,11]. Often, these functions have genetic background dependencies [3,12].

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The multifocal autoimmune disease of *Tgfb1*^{-/-} mice is eliminated by genetic ablation of either *Rag1* or *Rag2* genes [3,8] or by prevention of activation and extravasation of lymphocytes into non-lymphoid organs [13]. TGFβ1-deficient T cells undergo spontaneous activation due to elevated [Ca²⁺]_i levels resulting in a lowered threshold for activation and increased levels of adhesion and co-stimulatory molecules such as LFA-1 [14,15]. Recent studies by several laboratories suggest that TGFβ1 is also of major importance for the generation and function of regulatory T cells (T_{reg} cells) [1,16].

Earlier studies aimed at rescuing *Tgfb1*^{-/-} mice from multifocal inflammation suggested that elimination of MHC II molecules and consequent CD4⁺ T cells is not sufficient to significantly extend the longevity of these mice, even though inflammation is eliminated in many organs such as heart, salivary gland, masseter muscle, and quadriceps muscle (our unpublished observations). On the other hand, elimination of MHC class I antigen presentation and consequently CD8⁺ T cells, by combining *Tgfb1* and *β2 microglobulin* null alleles, is sufficient to eliminate inflammation and prolong survival of the animals [17,18]. Earlier reports also suggested a role for B cells in the inflammatory responses of *Tgfb1*^{-/-} mice since they develop autoantibodies [19].

In order to determine the major lymphocyte subsets that mediate the inflammatory response in *Tgfb1*^{-/-} mice we have eliminated B cells, CD4⁺, CD8⁺ or all T cells by breeding the *Tgfb1* null allele onto *Igh6*^{-/-}, *Cd4*^{-/-}, *Cd8*^{-/-}, or athymic nude backgrounds, respectively. The present study resolves the confusion regarding the lymphocyte population that causes inflammation. Elimination of B cells or CD4⁺ or CD8⁺ T cells alone is not sufficient to significantly alter longevity or eliminate inflammation, whereas elimination of all T cells is sufficient to do both.

2. Materials and methods

2.1. Mice

Tgfb1^{+/-} mice (129 × CF-1 mixed background) were maintained as breeders to generate *Tgfb1*^{-/-} mice [20] and were also bred with *Cd4*^{-/-}, *Cd8a*^{-/-}, *Igh6*^{-/-} (C57BL/6 background; Jackson Laboratory, Bar Harbor, ME), and BALB/c Nude (*nu/nu*) mice (Taconic Farms, Germantown, NY) to generate double null mice. The mice were housed in a specific pathogen-free barrier mouse facility at the University of Cincinnati College of Medicine. All mice were used at the ages described in the text and figure legends.

2.2. Splenocyte proliferation and phenotype analysis

Single cell suspensions were prepared, enumerated, and assayed for their mitogenic response using a [³H]thymidine incorporation assay after 2 days of in vitro culture as described [15]. Phenotype analysis of splenocytes was determined by flow cytometry with the appropriate fluorochrome-conjugated antibodies (BD Pharmingen, San Diego, CA) as described [15].

2.3. Inflammation score

Animals were euthanized following institutional guidelines, and tissues were fixed in 10% neutral buffered formalin. Tissues were dehydrated through a gradient of alcohol and xylene, embedded in paraffin, and 5 μm sections were cut and H&E stained. An inflammation score was assigned to each tissue depending on the severity of the inflammatory cell infiltrate: 0 (no inflammation), 0.5 (very mild), 1.0 (mild), 2 (moderate), 3 (severe), and 4 (very severe). Data for the most commonly affected organs are shown in the figures.

Statistical analysis was done using Student's *t* test for determining the significance between wild-type and mutant mice.

3. Results

3.1. Elimination of B cells does not prevent inflammation in TGFβ1-deficient mice

The multifocal inflammatory disorder in *Tgfb1*^{-/-} mice is the hallmark of their weaning-age lethality (median survival of 21 days) since the severity of inflammation generally correlates with decreased survival. *Tgfb1*^{-/-} *Igh6*^{-/-} mice were monitored daily and euthanized when they displayed a wasting syndrome which typically results in death within 2–5 days after they start losing weight. The inflammation score was determined from histological analysis of heart, liver, lungs, pancreas, salivary gland, and stomach, the organs most severely affected in *Tgfb1*^{-/-} mice. *Tgfb1*^{-/-} *Igh6*^{-/-} mice exhibit inflammatory lesions similar to those of *Tgfb1*^{-/-} *Igh6*^{+/-} mice (Fig. 1). However, the life span of double null mice is extended to a median survival of 35 days as compared to 21 days for *Tgfb1*^{-/-} mice, which includes four mice that lived for 7–9 weeks (Fig. 2). While elimination of either CD4⁺ or CD8⁺ T cells does not increase the longevity of *Tgfb1*^{-/-} mice, elimination of all T cells by combination with BALB/c nude mice eliminates inflammation and increases longevity beyond 2 months (Fig. 2). *Igh6*^{-/-}, *Cd4*^{-/-}, *Cd8*^{-/-} or *nu/nu* mice when combined with *Tgfb1*^{+/-} mice maintain their health up to a year.

3.2. Decreased T-cell numbers in the spleens of double null mice

Analysis of spleen and thymus suggests that B-cell-deficient TGFβ1-mutant mice (*Tgfb1*^{-/-} *Igh6*^{-/-}) exhibit thymic atrophy and severe depletion of cells from both thymus and spleen similar to that which is seen in *Tgfb1*^{-/-} and *Tgfb1*^{-/-} *Igh6*^{+/-} or *Igh6*^{+/-} mice. However, in mice that were euthanized before the rate of weight gain in TGFβ1-deficient animals became less than the rate of weight gain in control animals, cellularity in thymus and spleen was near normal (Figs. 3A–D). Variation from mouse to mouse is probably due to differences in age (see Fig. 2), body weight (data not shown), and health (as determined by differential rates of weight gain or loss) at the time of euthanasia. Flow cytometry analysis of d17 mouse splenocytes reveals a severe reduction of both CD4⁺ and CD8⁺ T cells in the *Tgfb1*^{-/-} *Igh6*^{-/-} mice (Fig. 3). This observation is similar to that obtained from *Tgfb1*^{-/-} mice. Consistently, CD3 and CD8 expression is downregulated in the *Tgfb1*^{-/-} *Igh6*^{-/-} T cells. They also exhibit an anergic response to receptor-mediated mitogenic stimulation but respond well to PMA/ionomycin stimulation [15].

3.3. TGFβ1-deficient B cells from older mice are hyperresponsive to mitogenic stimulation

Four double mutants (*Tgfb1*^{-/-} *Igh6*^{-/-}) and one *Tgfb1*^{-/-} *Igh6*^{+/-} mouse lived much longer (7–9 weeks) than the median age of survival, and the LPS-stimulated mitogenic response of splenic *Tgfb1*^{-/-} *Igh6*^{+/-} B cells is much higher than in control *Tgfb1*^{+/-} *Igh6*^{+/-} cells. As expected, negative control splenocytes from both double mutant *Tgfb1*^{-/-} *Igh6*^{-/-} and single mutant *Igh6*^{-/-} mice do not respond to LPS stimulation due to the lack of mature B cells (Fig. 4A). However, splenocytes from pre-weanling, functional B-cell-containing *Tgfb1*^{-/-} mice (d12–d19) do not exhibit LPS hyperresponsiveness (Fig. 4B). The reason for splenic B-cell hyperresponsiveness in the older *Tgfb1*^{-/-} mouse is unknown, but could have been due to B cells having been exposed to inflammatory cytokines for a prolonged period of time resulting in hypersensitivity to stimulation, or because of a response to self-antigen released from inflammation-induced cell damage.

3.4. Elimination of all T cells prevents inflammation and extends survival of *Tgfb1*^{-/-} mice

Since all *Tgfb1*^{-/-} *Igh6*^{-/-} mice had severe inflammation followed by death, albeit delayed by about 2 weeks, we hypothesized that elimination of T cells alone would prevent inflammation. As expected, combination of *Tgfb1* null mice with athymic nude mice resulted in a 3- to 4-month increase in longevity and the near elimination of inflammatory lesions (Fig. 5). Mild to

moderate inflammation is observed only in 2–3 organs in *Tgfb1*^{-/-} nude mice; most of the organs are free of lesions. Except for lung, liver, and stomach any given tissue is affected only in 3–4 out of 27 *Tgfb1*^{-/-} nude mice. Liver and stomach are affected in only 6 of 27 *Tgfb1*^{-/-} nude mice. It must be mentioned here that inflammation observed in the lungs of *Tgfb1*^{-/-} nude mice is not necessarily the cause of death as we have seen such inflammation in *Rag1* or *Rag2* KO *Tgfb1*^{-/-} mice, which also live for about 3–6 months. Hence, TGFβ1-deficient B cells in the absence of activated T cells are not a major contributor to the multifocal inflammatory disease that kills *Tgfb1*^{-/-} mice. To determine possible roles of TGFβ1 in B cells in the absence of an inflammatory environment, we analyzed splenic B-cell mitogenic responses in *Tgfb1*^{-/-} mice. The data presented in Fig. 6 show that *Tgfb1*^{-/-} B cells are hyperresponsive to mitogenic stimulation (Fig. 6A). This hyperresponsiveness can be inhibited by addition of TGFβ1 (Fig. 6B), which in turn can be reversed by addition of anti-TGFβ1 antibody (Fig. 6C). The data show that hyperresponsiveness of splenic B cells is due to the absence of TGFβ1 rather than any secondary effects of inflammation. However, the total cellularity of spleens of *Tgfb1*^{-/-} nude mice is not significantly different from that of *Tgfb1*^{+/+} control nude mice (Fig. 7). Since nude mice are athymic, they do not have any thymic-derived T cells in the peripheral lymphoid organs such as spleen. It is interesting to note that although there are very few CD3⁺ T cells in the spleens of nude mice, some splenocytes of *Tgfb1*^{-/-} nude mice respond to anti-CD3 stimulation much better than their littermate controls (Fig. 8). Flow cytometric analysis of splenocytes from *Tgfb1*^{-/-} nude and control nude (*Tgfb1*^{+/+} nude) mice reveals that the former indeed have increased numbers of CD3⁺ T cells (Fig. 9), though significantly less than wild-type (*Tgfb1*^{+/+}) mice.

3.5. Elimination of either CD4 or CD8 T cells alone is not sufficient to prevent inflammation in *Tgfb1*^{-/-} mice

The above data confirm that elimination of all T cells prevents inflammation in *Tgfb1*^{-/-} mice. To further identify whether elimination of either CD4⁺ or CD8⁺ T cells is sufficient to prevent inflammation we have combined the *Tgfb1* knockout allele with *Cd4* or *Cd8* null mutations. All of the double knockout mice exhibit severe inflammation and die within 4 weeks after birth, with only 1 of 8 organs (pancreas) having slightly more inflammation in *Tgfb1*^{-/-} *Cd4*^{-/-} mice (Fig. 10). This is consistent with previous data showing that both types of T cells undergo activation in *Tgfb1*^{-/-} mice [15]. Analysis of CD8 expression on *Tgfb1*^{-/-} *Cd4*^{-/-} splenocytes suggests that CD8⁺ T cells are activated as their surface CD8 expression is downregulated (data not shown [15]).

4. Discussion

Tgfb1^{-/-} mice develop severe inflammation in several organs, the most severely affected being heart, liver, lung, pancreas, salivary gland, and stomach. The inflammation is mainly due to extravasation of activated lymphocytes into non-lymphoid organs as this is prevented by anti-LFA-1 treatment or by combination with immunodeficient backgrounds [13]. In order to determine the possible genotype of lymphocytes that are capable of causing inflammation in *Tgfb1*^{-/-} mice we have combined the *Tgfb1* null mutation with that of B-cell- (*Igh6*^{-/-}) and T-cell- (*nu/nu*) deficient backgrounds. Consistent with our recent findings that TGFβ1-deficient T cells are spontaneously activated because of reduced threshold levels of activation, *Tgfb1*^{-/-} mice on a nude background live longer and do not develop significant levels of inflammation even after 2 months of age. Consistently, *Tgfb1*^{-/-} *Igh6*^{-/-} mice do develop severe inflammation in almost all the organs that are usually affected in *Tgfb1*^{-/-} mice, though there is a delay in the onset of inflammation of about 2 weeks [21], suggesting that B cells contribute to the acceleration of inflammation, either through secretion of cytokines that can decide the fate of T cells that differentiate into either Th1 or Th2 cells, or by acting as APC [22]. Unexpectedly, four outlier *Tgfb1*^{-/-} *Igh6*^{-/-} mice and one *Tgfb1*^{-/-} *Igh6*^{+/-} mouse lived

2–8 weeks longer, but they still succumbed to inflammation. These data suggest that TGFβ1 is essential for T-cell but not B-cell tolerance. In the absence of TGFβ1, both B and T cells extravasate into non-lymphoid organs and cause inflammation that is independent of any infection since the inflammatory response still occurs in germ-free *Tgfb1*^{-/-} mice [21]. The autoantibodies previously seen in *Tgfb1*^{-/-} mice [19] may be secondary to T-cell activation since elimination of B cells does not prevent inflammation. The delay in development of inflammation in B-cell-deficient *Tgfb1*^{-/-} mice could be due to decreased self-antigen presentation to T cells since B cells can act as APC. The reason for the survival of nude *Tgfb1*^{-/-} mice beyond 3 months of age without any significant inflammation is consistent with the fact that most *Tgfb1*^{-/-} *Rag1*^{-/-} and *Tgfb1*^{-/-} *Rag2*^{-/-} mice live past 3 months of age without significant inflammation [6,8]. It is not known why those immunodeficient TGFβ1-deficient mice that do not die from colon cancer still die from 3 to 6 months of age, but we have no evidence that it is immune system related. A wasting syndrome may be the primary cause of death of immunodeficient *Tgfb1*^{-/-} mice that live for 3–6 months in the absence of inflammation.

Although we have observed T-cell anergy in *Tgfb1*^{-/-} mice that live only about 3 weeks [15], we do not find any difference in the mitogenic response of splenic B cells in these mice. However, B cells in older *Tgfb1*^{-/-} mice (splenocytes from nude *Tgfb1*^{-/-} mice and the one *Tgfb1*^{-/-} *Igh6*^{+/-} mouse that lived longer) were hyperresponsive to mitogenic stimulation, and the hyperresponsiveness was due to the absence of TGFβ1 since the addition of TGFβ1 along with LPS inhibited the hyperresponsiveness. This could be due to accumulation of hyperresponsive B cells in sufficient numbers in the aged *Tgfb1*^{-/-} mice due to a lack of TGFβ1. A recent in vivo study by Sharon Wahl and her co-workers suggested that the inflammatory response in 2- to 3-week-old *Tgfb1*^{-/-} mice upregulates *Tlr4* expression which is responsible for increased LPS sensitivity. However, it is not clear whether the upregulated *Tlr4* expression in non-lymphoid organs is due to the presence of inflammatory leukocytes, cytokines or both [23]. It has been shown that B cells produce TGFβ1 upon activation by LPS and these B cells retain TGFβ1 on their surface and induce T-cell tolerance [24]. Since we do not find any hyperresponsiveness of splenocytes from 2- to 3-week-old *Tgfb1*^{-/-} mice which have moderate to severe inflammation, the hyperresponsiveness observed in splenocytes from aged *Tgfb1*^{-/-} nude mice which do not develop any significant inflammatory lesions could be primarily due to absence of TGFβ1 and not *Tlr4* upregulation. It is interesting to note that autoantibody production and immune complex deposition in autoimmune diseases such as lupus nephritis may depend on active T-cell–B-cell collaboration, and elimination of T-cell help renders B cells ineffective in disease manifestation [25]. In some animal models autoimmune gastritis results in loss of parietal cells due to the presence of parietal cell autoantibodies [26]. We have observed parietal cell loss in the stomachs of *Tgfb1*^{-/-} mice (unpublished observation) which could be due to autoantibody production by B cells in the presence of activated T cells [19]. However, none of the *Tgfb1*^{-/-} nude, *Tgfb1*^{-/-} *Igh6*^{-/-} or *Tgfb1*^{-/-} *Rag2*^{-/-} mice exhibited parietal cell loss in their stomachs, suggesting that *Tgfb1*^{-/-} B cells require activated T cell help for autoantibody production.

These data demonstrate that T cells but not B cells are the primary effectors in the inflammatory response that is observed in *Tgfb1*^{-/-} mice, although B cells accelerate disease progression. Earlier studies indicated that β2 microglobulin-deficient (MHC class I-deficient) mice live 2 months longer than *Tgfb1*^{-/-} mice, whereas MHC class II-deficient *Tgfb1*^{-/-} mice live about 1 week longer due to elimination of CD8⁺ and CD4⁺ T cells, respectively [17,18]. Accordingly, we would expect that elimination of CD8⁺ T cells but not CD4⁺ T cells would rescue *Tgfb1*^{-/-} mice from the inflammatory disease and early lethality, but this was not the case. Elimination of either CD4⁺ or CD8⁺ T cells alone was not sufficient to decrease significantly the inflammatory load, and survival time was increased by only a week in either case. Though the reason for the discrepancy in the longevity of β2 microglobulin-deficient *Tgfb1*^{-/-} mice

(3–4 months) and *Cd8^{-/-} Tgfb1^{-/-}* mice (1 month) is unclear, it suggests unknown mechanisms of antigen cross-presentation between these two types of T cells in autoimmune-prone mice and a flexibility in the TCR repertoire of autoimmune T-cell responses [27–29]. The other possibility is that β 2 microglobulin, in addition to being associated with MHC I molecules, is associated with several other molecules such as CD1d and FcRN [30]. The absence of β 2 microglobulin might cause a severe deficiency in self-antigen presentation and also eliminate several other cell types in addition to CD8⁺ T cells [31–33]. It is important to note here that in *Ctla4^{-/-}* mice elimination of CD4⁺ but not CD8⁺ T cells is sufficient to rescue the mice from their autoimmune disease [34]. Consequently, even though both *Tgfb1^{-/-}* and *Ctla4^{-/-}* mice develop an overtly similar autoimmune disease, TGF β 1 seems to play an important role in tolerance of both CD4⁺ and CD8⁺ cells, whereas CTLA4 regulates tolerance primarily in CD4⁺ cells. It has also been reported that MHC II is upregulated in non-lymphoid organs in *Tgfb1^{-/-}* mice, a phenotype which is eliminated in the absence of inflammation on *Scid* or *β 2m^{-/-}* backgrounds [13,18]. Our data suggest that unlike normal T-cell responses which are MHC-restricted, autoimmune T-cell responses may have relaxed restriction.

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Fig. 1.

B-cell elimination does not eliminate inflammation in *Tgfb1*^{-/-} mice. Inflammation in the vital organs of *Tgfb1*^{-/-} *Igh6*^{+/-} (B-cell-sufficient) (A) and *Tgfb1*^{-/-} *Igh6*^{-/-} (B-cell-deficient) mice (B). Inflammation score: 1 for mild, 2 for moderate, 3 for severe, and 4 for very severe. *n* = 4 for (A) and *n* = 8 for (B). Age of the mice is 2–9 weeks. Heart (*p* = 0.09), liver (*p* = 0.20), lung (*p* = 0.13), pancreas (*p* = 0.40), salivary gland (*p* = 0.35), and stomach (*p* = 0.50).

Fig. 2.

Elimination of all T cells extends the longevity of $Tgfb1^{-/-}$ mice. $Tgfb1^{-/-}$ mice were generated on $Cd4^{-/-}$, $Cd8^{-/-}$, $Igh6^{-/-}$ or nu/nu backgrounds by combining the $Tgfb1$ null allele with the respective backgrounds.

Fig. 3.

Decreased cellularity of spleen and thymus of $Tgfb1^{-/-} Igh6^{-/-}$ mice. Unaltered splenic cellularity (A and C) but decreased thymic cellularity (B and D) in $Tgfb1^{-/-} Igh6^{-/-}$ (A and B) and $Tgfb1^{-/-} Igh6^{+/+}$ (C and D) mice. Decreased percentage of CD4⁺ T cells (E) and CD8⁺ T cells (F) in d17 double null mouse. Note that CD8 intensity is decreased on double null T cells. The decrease in T-cell subsets was more obvious in a d21 double null mouse which was runted, and there were fewer cells in the spleen.

Fig. 4.

Splenocytes from older *Tgfb1*^{-/-} mice are hyperresponsive to LPS stimulation. Splenocytes from 9-week (A) or d12 (B) mice were cultured in the presence of LPS and their proliferative response was measured by [^3H]thymidine uptake after 2 days of culture. LPS response in (B) represents four experiments, each done in triplicate.

Fig. 5.

Mild to moderate inflammation in *Tgfb1*^{-/-} nude mice. Histological analysis of tissues for inflammatory lesions represented as percent of mice with lesions in the organs. Please note that most of the organs are free of lesions. Except for lung, liver, and stomach any given tissue is affected only in 3–4 *Tgfb1*^{-/-} nude mice out of 27. Liver and stomach are affected in only 6 of 27 *Tgfb1*^{-/-} nude mice.

Fig. 6. Splenocytes are hyperresponsive to LPS stimulation in 2-month-old *Tgfb1*^{-/-} nude mice. Splenocytes were cultured in the presence of LPS (A), LPS + TGFβ1 (B) or LPS + TGFβ1 + anti-TGFβ1 antibody (C) and proliferative response was measured by [³H]thymidine uptake in triplicates after 2 days of culture. LPS response is representative of four experiments and TGFβ1 inhibition is representative of two experiments.

Fig. 7.

Unaltered splenic cellularity in nude $Tgfb1^{-/-}$ mice. Total splenocytes were counted using trypan blue dye exclusion and represented with mean cell number as histogram bar and individual mouse by the closed ($Tgfb1^{+/+}$ nude) or open ($Tgfb1^{-/-}$ nude) circles ($n = 11$; age range 6–11 weeks; median age 9 weeks). $p = 0.24$.

Fig. 8.

Splenocytes are hyperresponsive to anti-CD3 stimulation in *Tgfb1*^{-/-} nude mice. Splenocytes from 2-month-old nude mice were stimulated with anti-CD3 antibody and proliferation was measured. Data represent one of the three experiments in which there was a similar hyperresponsiveness to anti-CD3.

Fig. 9.

Increased numbers of T cells in the spleens of *Tgfb1*^{-/-} nude mice. Percentage of CD4⁺ and CD8⁺ T cells in the spleens of 2-month-old *Tgfb1*^{+/+} (A), *Tgfb1*^{+/+} nude (B), and *Tgfb1*^{-/-} nude (C) mice. (D) CD3 expression on splenocytes of *Tgfb1*^{+/+} hairy (red color), *Tgfb1*^{+/+} nude (blue color), and *Tgfb1*^{-/-} nude (green color) mice. (E) Percent CD3⁺ T cells in *Tgfb1*^{+/+} nude (●) and *Tgfb1*^{-/-} nude (○) mice. Mean %CD3⁺ T cells is represented by histograms.

Fig. 10.

Severity of inflammation in $Tgfb1^{-/-} Cd4^{-/-}$ and $Tgfb1^{-/-} Cd8^{-/-}$ mice is similar. Each symbol represents one mouse and represents the highest level of inflammation score found in each organ. $n = 7$ for $Tgfb1^{-/-} Cd4^{-/-}$ (A) and $n = 12$ for $Tgfb1^{-/-} Cd8^{-/-}$ mice (B). Salivary gland ($p = 0.33$), masseter ($p = 0.40$), diaphragm ($p = 0.15$), heart ($p = 0.07$), lung ($p = 0.17$), liver ($p = 0.19$), pancreas ($p = 0.05$), and stomach ($p = 0.42$).