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## Tim-1 Regulates Th2 Responses in an Airway Hypersensitivity Model

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### Summary

T cell immunoglobulin mucin-1 (Tim-1) is a transmembrane protein postulated to be a key regulator of Th2-type immune responses. This hypothesis is based in part upon genetic studies associating Tim-1 polymorphisms in mice with a bias toward airway hyperresponsiveness and the development of Th2-type CD4<sup>+</sup> T cells. Tim-1 is expressed by Th2 CD4<sup>+</sup> T cells on which it has been proposed to function as a co-stimulatory molecule. Tim-1 is also expressed by B cells, macrophages, and dendritic cells, but its role in responses by these cell types has not been firmly established. We generated Tim-1 deficient mice to determine the role of Tim-1 in a murine model of allergic airway disease that depends on the development and function of Th2 effector cells and results in the generation of AHR. We found antigen-driven recruitment of inflammatory cells into airways is increased in Tim-1 deficient mice relative to wild-type mice. In addition, we observed increased antigen-specific cytokine production by splenocytes from antigen-sensitized Tim-1 deficient mice relative to those from controls. These data support the conclusion that Tim-1 functions in pathways that suppress recruitment of inflammatory cells into the airways and the generation or activity of CD4<sup>+</sup> T cells.

### Keywords

Tim-1; asthma; Th2

### Introduction

The incidence and prevalence of atopic diseases are increasing in developed nations. One of these atopic diseases, allergic asthma, is a multifactorial disease with a strong but complex genetic component. Asthma is believed responsible for 250,000 annual deaths worldwide [1] and annual economic costs in the United States are estimated at \$14.7 billion in direct costs

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with an additional \$5 billion in indirect costs such as loss of productivity [2]. Improving our understanding of the functions of genes that contribute to the susceptibility to and severity of asthma are of great consequence to both the treatment and prevention of disease.

Tim-1 was identified as a putative asthma susceptibility gene through fine-mapping of congenic mice with differential susceptibility to development of experimental allergic airway disease [3]. Specific polymorphisms in Tim-1 were associated with the predisposition to the development of Th2 CD4<sup>+</sup> T cell effector responses and airway hyperresponsiveness (AHR) [3]. Tim-1 is a type I transmembrane glycoprotein with an immunoglobulin-like domain at the N-terminus, a glycosylated mucin domain, a single transmembrane domain and a cytoplasmic domain. Tim-1 is expressed by activated CD4<sup>+</sup> T cells. In addition, Tim-1 surface expression is inducible on murine B cells [4] and Tim-1 mRNA has been shown to be constitutively expressed in dendritic cells [5]. Genetic polymorphisms in the gene encoding TIM-1 (HUGO designation *HAVCR1*) have been associated in some populations with susceptibility to asthma, atopy, and elevated IgE [6]. The numerous polymorphisms found and conserved in *HAVCR1* suggest that there may be a survival advantage to these polymorphisms [7].

Given the association of Tim-1 polymorphisms with allergic disease in humans as well as mouse models, a number of studies have sought to elucidate the precise role of Tim-1 in these processes. Treatment of mice with monoclonal antibodies to Tim-1 ameliorates experimental allergic airway disease in mice [8, 9] and in a humanized mouse model of asthma [5]. However, it has also been shown treatment of mice with anti-Tim1 monoclonal antibodies results in T cell proliferation and CD4<sup>+</sup> T cell cytokine production [9–13]. Thus Tim-1 has been proposed to have both activating and inhibitory effects in immune responses (reviewed in [14]).

In this study we generated mice deficient in Tim-1 and evaluated their immune responses to stimulation *in vitro* and *in vivo*. In contrast to the profound anomalies suggested by the antibody studies noted above, we found Tim-1 deficient mice had modest but significant increases in their production of the Th2 cytokines IL-5 and IL-13 as well as the Th17 cytokine IL-17A. *In vivo* allergic airway disease revealed enhanced inflammatory responses in the absence of Tim-1, suggesting its primary role is to dampen, rather than promote, Th2-type immune responses.

## Results

### Immune system development in Tim-1 deficient mice

Tim-1 deficient mice were generated by replacing exons 4 and 5 of *Havcr1*, the gene encoding Tim-1, with a cassette encoding LacZ (Figure 1A and 1B). To allow evaluation of the specific role of Tim-1 and its polymorphisms in development of allergic diseases, we backcrossed the Tim-1 deficient mice to strains of mice with differential susceptibility to allergic airway diseases and Th2 responses, BALB/c and C57BL/6 mice. In concordance with a previous report [4] Tim-1 was detected on the surface of wild type (WT) splenic B cells activated with anti-IgM, but was not detected on Tim-1 deficient B cells (Figure 1C). Splenic B cells also upregulated surface Tim-1 when splenocytes were stimulated with PMA/ionomycin or concanavalin A for 48 hours *in vitro* (data not shown). As expected, *Tim-1* mRNA was not detected in Tim-1 deficient mice (Figure 1D). Expression of *Tim-2*, *Tim-3*, *Tim-4*, and *ITK* was similar in wildtype and Tim-1 deficient mice on both BALB/c and C57BL/6 backgrounds (Figure 1D and 1E), indicating that the Tapr locus was not disrupted by interruption of the *Havcr-1* gene.

Splenocytes from Tim-1 deficient mice of the C57BL/6 and BALB/c backgrounds did not display significant differences in total numbers or percentages of T cells, B cells, NK cells, and macrophages compared to WT (Figure 2 and Supplemental Figure 1). Thymic cellularity and the distribution of cells with respect to developmental stage were also normal in Tim-1 deficient mice (**Supplemental** Figure 2). Bone marrow cellularity and B cell development also appeared normal (data not shown). Serum from naïve WT and Tim-1 deficient mice contained similar levels of total IgG1 and IgG2c or IgG2a (Figure 2 and Supplemental Figure 1). These data indicate that Tim-1 is not required for immune system development or homeostasis.

### Tim-1 influences lung inflammation in experimental allergic airway disease

To determine the role of Tim-1 in the generation of experimental allergic airway disease, WT and Tim-1 deficient mice on the BALB/c background were sensitized by immunization with chicken ovalbumin (OVA) adsorbed to alum and then challenged with aerosolized OVA. Production of OVA-specific IgG1 and IgE in response to immunization were elevated to a similar extent in Tim-1 deficient and WT mice (**Supplemental** Figure 3A). Following aerosolized antigen challenge Tim-1 deficient mice developed a modest but statistically significant increase in eosinophils and macrophages within the airways compared to WT mice (Figure 3A). Levels of IL-13 were significantly elevated in BAL from Tim-1 deficient mice (Figure 3B). Histologic analysis of lung sections revealed similar levels of mucus production and goblet cell hyperplasia in WT and Tim-1 deficient mice (data not shown).

Invasive measures of airway resistance (Rn) were modestly but significantly elevated in Tim-1 deficient mice at baseline and low doses of methacholine, but this increase in airway resistance was not statistically significant at elevated doses of methacholine (Figure 3C). Lung elastance, an inverse measure of tissue elastic recoil, was significantly elevated in Tim-1 deficient mice at baseline and with increasing doses of methacholine (Figure 3C). Loss of elastic recoil is associated with increased risk for near-fatal asthma [15]. Using stains that reacted with mast cell granules, we detected fewer numbers of mast cells in lungs of Tim-1 deficient mice following asthma challenge (**Supplemental** Figure 3B). However, numbers of peritoneal mast cell detected in WT and Tim-1 deficient mice both prior to and after immunization with alum and ova, were similar (data not shown). This suggests that the difference in detected mast cells seen in the allergic airway model does not represent either an intrinsic defect in mast cell development or a global defect in mast cell recruitment.

Compared to those from wild-type mice, splenocytes from Tim-1 deficient mice sensitized to OVA secreted significantly increased amounts of IL-4, IL-5, IL-13, IL-10, and IL-17A when rechallenged with antigen *in vitro* (Figure 3D). Levels of IFN $\gamma$  produced by splenocytes from WT and Tim-1 deficient mice were not significantly different. Increased cytokine production did not appear to be secondary to a difference in total T cells within the spleen, as the numbers of CD4<sup>+</sup> T cells (and proportions of naïve and effector/memory cells) were similar in WT and Tim-1 deficient mice, both in naïve and immunized mice (data not shown).

To determine if the background of the mice plays a role in the effect of Tim-1 in allergic disease we also performed sensitization and airway challenge with OVA using WT and Tim-1 deficient mice of the C57BL/6 background. Bronchoalveolar lavage cellularity tended to be increased in Tim-1 deficient mice, particularly lymphocytes and eosinophils (**Supplemental** figure 4A), but did not reach statistical significance. Similar to Tim-1 deficient mice on the BALB/c background, levels of IL-13 were significantly elevated in BAL from Tim-1 deficient mice on the C57BL/6 background (**Supplemental** Figure 4B). Although we could not detect IL-4 in splenocyte antigen recall experiments, the production of IL-5 and IL-13 were significantly elevated in Tim-1 deficient splenocytes, again similarly

to that found for mice on the BALB/c background (**Supplemental Figure 4C**). While a trend for elevated IL-17A was also observed, it did not achieve statistical significance. Thus, it appears that in the absence of Tim-1 expression enhanced Th2 cytokine production and airway inflammation occur regardless of the background strain of the mice.

### **Tim-1-deficient T cell polarization *in vitro* does not reveal enhanced cytokine levels**

As Tim-1 is expressed by both antigen presenting cells and CD4<sup>+</sup> T cells, we determined whether increased cytokine production by Tim-1 deficient splenocytes was due to an intrinsic CD4<sup>+</sup> T cell effect of Tim-1. Tim-1 deficient and WT T cells were stimulated *in vitro* under Th1, Th2, and Th17 polarizing conditions and cytokine production assessed by ELISA following restimulation. Cytokine production by Tim-1 deficient CD4<sup>+</sup> T cells from mice of either the BALB/c or the C57BL/6 background was not significantly different from that produced by WT cells (Figure 4A and data not shown). In addition, polyclonal T cell proliferation in response to stimulation with anti-CD3 was not altered in Tim-1 deficient CD4<sup>+</sup> T cells at any concentration tested, either in the presence or absence of anti-CD28 stimulation (Figure 4B).

### **Tim-1 deficient mice develop enhanced responsiveness to immunization with alum and Ovalbumin**

Tim-1 deficiency resulted in modest but consistently enhanced immunologic responses *in vivo*. This enhancement could be due to an increase in response to primary sensitization or to an increased recall response to aerosolized OVA during the challenge phase. To elucidate whether the effect of Tim-1 deficiency seen *in vivo* was occurring during antigen sensitization, we immunized WT and Tim-1 deficient mice on the BALB/c background with OVA adsorbed to alum, then restimulated splenocytes *in vitro*. Similar to the results obtained following sensitization and aerosol challenge *in vivo*, Tim-1 deficient mice on the BALB/c background produced increased levels of IL-5, IL-13, and IL-17A during splenocyte restimulation *in vitro* (Figure 5). Tim-1 deficient mice on the C57BL/6 background demonstrate enhanced IL-5 and IL-13 production during *in vitro* restimulation with antigen, and similar increases in IFN $\gamma$  to WT (**Supplemental Figure 5**). They also developed similar levels of OVA-specific serum IgG1. IL-4, IL-10 and IL-17A cytokine levels were below the level of detection in C57BL/6 splenocytes. Thus, it appears that the deficiency of Tim-1 drives enhanced Th2 cytokine production during allergen sensitization.

### **Increased cytokine production in Tim-1 deficient mice is not T cell-intrinsic**

*In vivo*, but not *in vitro*, stimulated CD4<sup>+</sup> T cells lacking Tim-1 have enhanced cytokine production. To more closely mimic the conditions for *in vivo* stimulation we crossed DO11.10 mice carrying the transgenic TCR for OVA peptide to the Tim-1 deficient mice of the BALB/c background and stimulated these transgenic T cells with WT antigen-presenting cells and OVA peptide in the presence of polarizing cytokines. Polarization of WT or Tim-1 deficient DO11.10 CD4<sup>+</sup> T cells *in vitro* did not reveal any differences in Th1, Th2, or Th17 polarization (data not shown) or proliferation (Figure 4C), suggesting the lack of Tim-1 in CD4<sup>+</sup> T cells is not sufficient to drive the enhanced cytokine production seen in the Tim-1 deficient mouse. To determine the role of Tim-1 on antigen presenting cells we utilized OT-II mice that carry a transgenic TCR for OVA peptide on the C57BL/6 background. Polarization of Tim-1 sufficient OT-II transgenic T cells with Tim-1 deficient T cell-depleted splenic APCs resulted in similar levels of IL-4 in Th2 polarizing conditions and IFN $\gamma$  in Th1 polarizing conditions (data not shown). However, polarization of WT OT-II transgenic T cells with Tim-1 deficient APCs under Th17 conditions resulted in enhanced IL-17A production relative to that obtained when Tim-1 sufficient APCs were used (Figure 6A). These data suggest that Tim-1 expression on antigen-presenting cells could alter T cell

polarization and that Tim-1 may play a role in the development of not only Th2 and allergic responses but also Th17 responses. Addition of a blocking antibody to Tim-1 resulted in a further increase in IL-17A production in cultures with both Tim-1 deficient and Tim-1 sufficient APCs (Figure 6A), suggesting that Tim-1 expression by CD4+ T cells negatively regulates cytokine production in this culture system.

We further investigated the role of Tim-1 on CD4+ T cells by performing adoptive transfer experiments and stimulating the cells *in vivo*. Adoptive transfer of Tim-1 sufficient or deficient transgenic CD4+ T cells into WT or Tim-1 deficient mice demonstrated that deficiency of Tim-1 on both CD4+ T cells and non-CD4+ cells is required for the statistically significant enhanced production of IL-5; this enhancement is lost when only the host or the transferred CD4+ T cells lacks Tim-1 (Figure 6B). Interestingly, we did not observe differences in IL-17A production between groups. We did not observe differences in numbers of DO11.10 cells recovered from recipient groups, or differences in upregulation of CD44 and downregulation of CD62L on the transferred cells (data not shown). Based on these experiments it appears that expression of Tim-1 on both CD4+ T cells and non-CD4+ cells regulates antigen-specific cytokine production.

In order to determine which cell type within the T cell depleted splenocyte population is responsible for the increased cytokine production *in vitro*, we performed co-culture experiments (Figure 6C). Wildtype OT-II CD4+ T cells have no significant difference in IL-17A release when stimulated by wildtype or Tim-1 deficient bone marrow derived dendritic cells. However, stimulation of WT OT-II CD4+ T cells by Tim-1 deficient B cells or B cell depleted splenocytes resulted in significantly more IL-17A release upon restimulation than from WT OT-II CD4+ T cells initially activated by wildtype B cells. These data identify the B cell as a critical Tim-1 expressing cell responsible for downregulating IL-17A release, although the results for the B cell depleted splenocytes suggest other splenic antigen presenting cells have similar effects.

## Discussion

Studies of congenic mice and *in vivo* experiments with antibodies to Tim-1 predicted that Tim-1 deficient mice would develop reduced Th2 responses, reduced IgE, and reduced airway hyperreactivity in an asthma model. In particular, the initial identification of Tim-1 as a potential gene of interest in the development of atopic disease followed the fine mapping of increased IL-4 production and airway hyperreactivity to the *Tapr* locus containing the Tim-1 family of molecules. Polymorphisms in Tim-1 were associated with Th2 differentiation and airway hyperresponsiveness. CD4+ T cells with the *Tapr* locus from Th2-susceptible mice produced more IL-4 than CD4+ T cells with the *Tapr* locus from Th2-resistant mice regardless of the *Tapr* locus of the dendritic cells used to activate the T cells. This suggested Tim-1 acted in T cells to drive Th2 responses in a CD4+ T cell-intrinsic manner [3]. These studies in mice suggesting a critical role for Tim-1 in development of atopic disease and airway hyperresponsiveness were strongly supported by subsequent human studies. Independent studies performed in separate human populations have repeatedly linked susceptibility to atopic diseases to TIM-1 alleles. These studies have suggested an essential role for TIM-1 in driving the development of allergic disease.

Contrary to these predictions, we found that Tim-1 deficient mice produce IgE and develop airway hyperresponsiveness to a similar extent as WT mice, and develop slightly increased airway inflammation. We found that IL-4, IL-5, IL-10, IL-13, and IL-17A were all produced to greater extent by Tim-1 deficient rather than WT cells during *in vitro* antigen recall with splenocytes from asthma-challenged mice. Although small differences in airway resistance were detected between Tim-1 deficient and sufficient mice, a much more striking increase in

lung elastance was detected in the lungs of Tim-1 deficient mice. It is currently unclear whether this is due to increased fibrosis or increased air trapping within alveoli.

In addition, our studies suggest a role for Tim-1 on the antigen presenting cell and in particular the B cell, rather than the T cell as has been previously reported [3]. There are a number of possible explanations for this discrepancy: the earlier studies did not systematically examine the role of antigen presenting cells nor did they examine the importance of the individual members of the *Tapr* locus in the T cell response. It may be that other members of the locus drive a T cell response that overshadows the more subtle antigen presenting cell driven response described in our study. Another perhaps more likely possibility is the interruption of the Tim-1 gene may remove both positive and negative control of the T cell as suggested by antibody studies [5, 8–12], exposing a role for Tim-1 on the antigen presenting cell in modulating the T cell response. Tim-1 engagement on B cells by antibodies has been suggested to regulate B cell function and induction of tolerance [16, 17].

As alluded to above, our findings of enhanced inflammation in Tim-1 deficient mice are discordant with some of the findings for mice treated with anti-Tim-1 antibodies. While the reasons for this are unclear, it may relate to the multiple domains and multiple proposed ligands of Tim-1. A number of distinct ligands have been postulated to bind Tim-1: phosphatidylserine via a specific pocket within the ligand-binding domain [22, 23], Tim-4 (possibly via bridging apoptotic bodies) [24–26], LMIR5/CD300b within a cleft of the ligand-binding domain distinct from the phosphatidylserine-binding pocket [27], and possibly via homotypic interactions to other Tim-1 molecules [23, 28]. It is likely that antibody treatment will interrupt Tim-1 binding to some but not all of these many ligands, depending on the individual ligand and the domain of Tim-1 to which it binds. An example of the divergent functions of individual ligands and binding domains of Tim-1 can be shown by the binding of CD300b to its discrete binding domain in Tim-1 resulting in a proinflammatory signal and neutrophil recruitment, while the independent binding to Tim-1 of phosphatidylserine from apoptotic bodies may be predicted to negatively modulate inflammatory responses. Accordingly, removing these two opposing stimuli may result in loss of both pro and anti inflammatory signals and the phenotype revealed in our studies.

This idea is supported by the detailed analysis of antibody binding and epitope characterization performed in some of the earlier studies (reviewed in [14]), wherein either inflammatory or anti-inflammatory roles were defined for Tim-1 depending upon whether the mucin, stalk or IgV domains were implicated. These studies carefully characterized anti-Tim-1 antibodies' binding specificity to the separate IgV, mucin and stalk domains of Tim-1 and showed that antibody binding to the IgV or stalk domains resulted in decreased inflammatory responses while treatment with antibodies with binding to the mucin and stalk domains was associated with increased inflammatory responses [9]. Additional studies focused on the mucin and IgV domains of Tim-1 suggest requirements for both domains in ligand binding with mucin domain modulating IgV ligand binding [23, 24, 28]. This need for both domains is also supported by a separate study of hepatitis A virus binding requirements [29]. Together with our study this could suggest Tim-1 can be activating when bound at some domains and inhibitory when bound at others; thus it is not unexpected the result of a complete deficiency is different from that seen with specific, individual epitope/ligand blockade.

Initial descriptions of Tim-1 indicated that it is upregulated on Th2 cells following multiple rounds of *in vitro* polarization [10] and that it is detectable on CD4+ T cells found in lung-draining lymph nodes following airway challenge with antigen [30]. Although we detected Tim-1 mRNA in Th2 cells polarized *in vitro*, which is consistent with other publications [3,

31], we could not detect surface Tim-1 on Th2 cells, and we also could not detect it on lung-draining lymph nodes from mice challenged with antigen via the airways. This is consistent with other reports in which surface expression of Tim-1 was not detected on Th2 cells [4, 5].

Our findings suggest a more modest role for Tim-1 in the development of allergic diseases than has been previously predicted. However, this conclusion must be considered in the context of the human data; TIM-1 has been linked to susceptibility to atopic diseases in multiple independent studies in the United States, Asia and Australia (reviewed in [6]). The extrapolation of our finding to humans is complicated further by the presence of Tim-2 in mice and its absence in humans. As murine Tim-1 and Tim-2 share 66% sequence homology, human Tim-1 is similarly homologous to both murine Tim-1 (41%) and Tim-2 (36%). It may be that Tim-2 in mice serves an overlapping role to that of Tim-1; the functional homology and redundancy of Tim-1 and Tim-2 are an area of ongoing investigation.

In conclusion, Tim-1 deficiency appears to mediate an effect that is not consistent with phenotypes seen from treating mice with antibodies to Tim-1. Like Tim-4 deficient mice, it appears that Tim-1 deficiency in mice does not lead to overt morbidity and mortality, but a subtle deviation of the immune response. It may have been predicted, given the original linkage of Tim-1 with Th2 predisposition or resistance in mouse strains, that deletion of Tim-1 in these strains would result in conversion to Th2 resistance or predisposition, respectively. While our studies confirm a role for Tim-1 in these diseases, it is still unclear by what mechanism Tim-1 may serve a pivotal role in the development of atopic disease.

## Materials and Methods

### Mice

Tim-1 deficient mice were generated in collaboration with Regeneron. 129 ES cells were electroporated with targeting constructs and selected for targeted alleles. Chimeric mice were backcrossed for 10 generations onto C57BL/6 (Jackson) or BALB/c (Charles River) backgrounds. The following primers were used to genotype mice: sense 5'-GTTTGCTGCCTTATTTGTGTCCTGG-3'; antisense WT 5'-CAGACATCAACTCTACAAGGTCCAAGAC-3'; antisense KO 5'-GTCTGTCCTAGCTTCCTCACTG-3'. DO11.10 and OT-II TCR transgenic mice were purchased from Jackson. OT-II mice were maintained by intercrossing and mice homozygous for the TCR transgenes were used for experiments. DO11.10 transgenic mice were crossed to Tim-1 deficient mice or wild type mice purchased from Charles River (BALB/c) and genotyped according to Jackson labs protocols online. Mice heterozygous for the TCR transgene were used for experiments. Mice were housed in barrier facilities and used in experiments at 8–14 weeks of age. All experiments were performed in accordance with NIH guidelines using protocols approved by the University of Iowa Institutional Animal Care and Use Committee.

### Reagents

Antibodies for flow cytometry, ELISA, *in vitro* polarization and proliferation were obtained from e-bioscience and BD Pharmingen. AffiniPure F(ab')<sub>2</sub> fragment goat anti-mouse IgM  $\mu$ -chain specific antibody was purchased from Jackson ImmunoResearch. PMA and ionomycin were purchased from CalBiochem. Imject alum and Imject ovalbumin (OVA) were purchased from Pierce. Recombinant murine IL-4, IL-6, and IL-12 were purchased from BD Pharmingen. Recombinant human TGF $\beta$  was purchased from Peprotech. Recombinant murine IL-2 was purchased from ebioscience. Dynal mouse CD4 negative selection kits and Dynal Thy1.2 beads were purchased from Invitrogen. CD4 (L3T4), CD43,

and CD62L microbeads were purchased from Miltenyi Biotech. OVA<sub>323-339</sub> peptide was purchased from GenScript. Functional-grade anti-Tim-1 (clone RMT1-10) rat monoclonal antibody has been previously characterized and was kindly provided by Hideo Yagita (Juntendo University, Tokyo, Japan) [11]. Primary murine cells were cultured in RPMI 1640/10% w/v FCS/1000 U/mL penicillin/1000 µg/mL streptomycin/20 mM L-glutamine/10 mM sodium pyruvate/5% w/v non-essential amino acids/50 µM β-mercaptoethanol. Bone marrow dendritic cells were cultured in DMEM/10% w/v FCS/1000 U/mL penicillin/1000µg/mL streptomycin.

### Flow cytometry

Cells were suspended in PBS containing 5% FBS and 0.09% sodium azide and preincubated with anti-CD16/32. Cells were incubated with fluorochrome- or biotin-conjugated antibodies for 30 min on ice and washed. When necessary, cells were resuspended in buffer containing streptavidin- or avidin-conjugated fluorophore, incubated for 30 min on ice, and washed. Flow cytometric analysis was performed using an LSR II (BD) and data were analyzed using FlowJo (Tree Star, Inc.). The following antibodies were obtained from e-bioscience, Biolegend, Caltag, and BD Pharmingen. FITC conjugates B220 (RA3-6B2), CD4 (RA3-6B2), CD11b/Mac-1 (M1/70), CD21/35 (7G6), CD122 (TM-β1); PE conjugates B220 (RA3-6B2), Gr-1/Ly6G (RB6-8C5), Tim-1 (RMT1-4), Tim-4 (RMT4-54), CD3e (145-2C11), CD4 (RA3-6B2), CD11b/Mac-1 (M1/70), CD23 (B3B4), rat IgG2b (eB149/10H5); APC conjugates B220 (RA3-6B2), NK1.1 (PK136), CD4 (RA3-6B2), CD8α (53-6.7), streptavidin; APC-Cy7 conjugates CD4 (RA3-6B2); PerCP conjugates B220 (RA3-6B2), CD4 (RA3-6B2) and streptavidin; PE-Cy7 conjugates B220 (RA3-6B2), IgM (II/41), CD4 (RM4-5), CD11c (N418); biotin conjugates CD43 (S7), CD49b (DX5).

### Analysis of *Tapr* locus gene expression by real-time PCR and FACS

Expression analysis of *Tim-1*, *Tim-2*, *Tim-3*, and *Itk* in wildtype and Tim-1 deficient cells was assessed by quantitative real-time PCR as previously described [32]. Briefly, naïve CD4<sup>+</sup>CD62L<sup>hi</sup> T cells were cultured as previously described in Th1, Th2, or Th17 polarizing conditions for 5 days.  $1.5 \times 10^5$  cells were restimulated with platebound anti-CD3 (10 µg/mL) for 6–8 hours. RNA was isolated with Trizol reagent (Invitrogen) and transcribed to cDNA using SuperScript III cDNA synthesis kit (Invitrogen). Quantitative real-time PCR was performed with POWER SYBR Green Master Mix and the PRISM 7700 Detection system (Applied Biosystems), and analysis was performed using the deltaCT method with normalization of target cDNA levels to Hprt cDNA levels. Sequences of primers were obtained from the following references: Tim-1, Tim-2: [33], Itk: [34], HPRT [32]. Tim-4 expression on peritoneal lavage cells of naïve wildtype and Tim-1 deficient mice was assessed by flow cytometry.

### T cell purification and in vitro polarization

CD4<sup>+</sup>CD62L<sup>hi</sup> T cells were isolated from 8–12 week mice by sequential purification with CD4<sup>+</sup> T cell negative selection (Dyna) and CD62L<sup>hi</sup> positive selection (Miltenyi). Cells were cultured in unbiased (Th0), Th1, Th2, and Th17 conditions for 5 days. CD4<sup>+</sup>CD62L<sup>hi</sup> cells were polarized as previously described [34] at  $5 \times 10^5$  cells/well coated with plate-bound anti-CD3 (10 µg/mL) and anti-CD28 (10 µg/mL). T cell polarizing conditions were as follows. Th1: anti-IL-4 (2 µg/mL), IL-12 (4 ng/mL), IL-2 (20 U/mL); Th2: anti-IFNγ (2 µg/mL), IL-4 (1000 U/mL), IL-2 (20 U/mL); Th17: anti-IL-4 (2 µg/mL), anti-IFNγ (2µg/mL), IL-6 (10 ng/mL), TGFβ (5 ng/mL); Th0: 20 U/mL IL-2. OT-II CD4<sup>+</sup> CD62L<sup>hi</sup> T cells were isolated as previously described and cultured in Th17 polarizing conditions with  $1 \times 10^6$  Thy1.2-depleted irradiated splenocytes (2500 rads), purified B cells, B cell-depleted splenocytes, or bone marrow-derived dendritic cells, 2 µM OVA<sub>323-339</sub> peptide, and 10 µg/mL anti-Tim-1 (RMT1-10) as indicated.  $5 \times 10^5$  cells/well were restimulated with



platebound anti-CD3 (10  $\mu\text{g}/\text{mL}$ ) for 48 hours (ELISA). Resting follicular B cells were prepared from splenocytes by negative selection with Miltenyi CD43 beads, with the remaining positively selected cells constituting the B cell-depleted fraction. Bone marrow dendritic cells were cultured at  $0.5\text{--}1.5 \times 10^6$  cells/mL with 10 ng/mL recombinant murine GM-CSF (R&D systems) in DMEM/10% w/v FCS/1000 U/mL penicillin/1000  $\mu\text{g}/\text{mL}$  streptomycin/50  $\mu\text{M}$   $\beta$ -mercaptoethanol. Half of the media and GM-CSF was replaced every other day for 6–8 days. Loosely adherent cells were harvested and used for T cell stimulation.

### T cell proliferation in vitro

$2 \times 10^5$  CD4+CD62L<sup>hi</sup> T cells were activated for 48 hours as previously described [34] on antibody-coated plates, and pulsed with 1  $\mu\text{Ci}$  of tritiated thymidine 12 hours before harvest.  $1 \times 10^5$  OT-II or DO11.10 CD4+CD62L<sup>hi</sup> T cells/well were cultured with  $2 \times 10^5$  irradiated Thy1.2-depleted splenocytes/well and OVA<sub>323-339</sub> peptide for 48 hours, and pulsed with 1  $\mu\text{Ci}$  tritiated thymidine 8 hours before harvest.

### Antibody and cytokine ELISAs

Detection of serum isotypes from unimmunized mice was performed following manufacturer's instructions. Concentrations of antigen-specific IgG1 were determined by coating plates with OVA (Pierce) or pan-IgG (to generate a standard curve). Purified IgG1 was used to generate a standard curve. Biotinylated anti-IgG1 was used for detection. Concentrations of antigen-specific IgE were determined using clone EM95.3 [35] (purified from hybridoma supernatant) as a capture antibody. Purified IgE (BD Pharmingen) was used to generate a standard curve. Total IgE was determined using biotinylated anti-IgE (BD Pharmingen) for detection. Ovalbumin specific IgE was determined using biotinylated ovalbumin (prepared using an EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce)) for detection. Unless otherwise stated, all ELISA reagents were purchased from Southern Biotech.

Cytokine ELISAs for IL-4, IL-5, IL-13, IL-10, IL-17A, and IFN $\gamma$  were performed with Ready-Set-Go kits (ebioscience) or paired ELISA antibodies (ebioscience).

### Immunization protocols

Mice (8–12 weeks) were immunized i.p. with 100  $\mu\text{g}$  OVA (Pierce) adsorbed to alum (Imject alum, Pierce) on days 1, 8, and 15, then euthanized on day 25. Serum was collected by cardiac puncture and spleens were mechanically disrupted by passing through 100  $\mu\text{m}$  nylon screens. Following red blood cell lysis (PharmLyse, BD Pharmingen)  $2 \times 10^6$  cells were plated in 24 well plates. Supernatants were harvested 96 hours later for analysis by ELISA. Mice were immunized s.c. at the tail base with 50  $\mu\text{g}$  OVA emulsified in CFA (Pierce) and serum and spleens were harvested 10 days later.  $3 \times 10^6$  splenocytes were cultured with OVA in 24 well plates for 72 hours. Supernatants were harvested for ELISA. For adoptive transfer,  $1 \times 10^6$  DO11.10 CD4+ T cells from transgenic WT or Tim-1 KO mice were transferred to recipients i.v., and 48 hours later mice were immunized once i.p. with 100  $\mu\text{g}$  OVA adsorbed to alum. 10 days after immunization, splenocytes from each group of recipients were pooled and CD4+ T cells were isolated by MACS positive selection.  $1 \times 10^6$  transgenic T cells were plated in 24 well plates with  $2 \times 10^6$  irradiated BALB/c splenocytes and 0–10  $\mu\text{M}$  OVA<sub>323-339</sub> peptide in a total of 1 mL medium. Supernatants were harvested after 72 hours. Transgenic T cells were detected with anti-DO11.10 TCR (KJ1-26).

## Asthma model and assessment of airway hyperreactivity

Mice (8–12 weeks) were immunized i.p. with 10 µg OVA (Sigma) adsorbed to alum (Sigma) on days 1 and 8, then exposed to aerosolized 10% OVA for 40 minutes on days 15 and 16. Whole-body plethysmography was performed on day 17, and airway resistance was assessed invasively (Flexivent) on day 18. Mice were euthanized, and spleens, mediastinal lymph node, and serum were collected. Splenocytes were stimulated *in vitro* as above. Mediastinal lymph node cells were cultured at  $1.5 \times 10^6$  cells/well in 48 well plates for 4 days. BAL was performed with 1 mL PBS a total of three times, and cells were stained with Wright-Geimsa and analyzed with light microscopy. Lungs were fixed in 10% buffered formalin (Fisher), placed under vacuum pressure, embedded in paraffin, and stained with H&E, ABPY, PAS, and sirius red. Slides were evaluated by a veterinary pathologist. Inflammation and mucus secretion were scored for severity, and mast cells and eosinophil numbers were enumerated.

## Statistics

Statistical analysis was performed using GraphPad Prism version 4.0. Details of statistical tests applied to each experiment are noted in the figure legends.

## Acknowledgments

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## List of Abbreviations

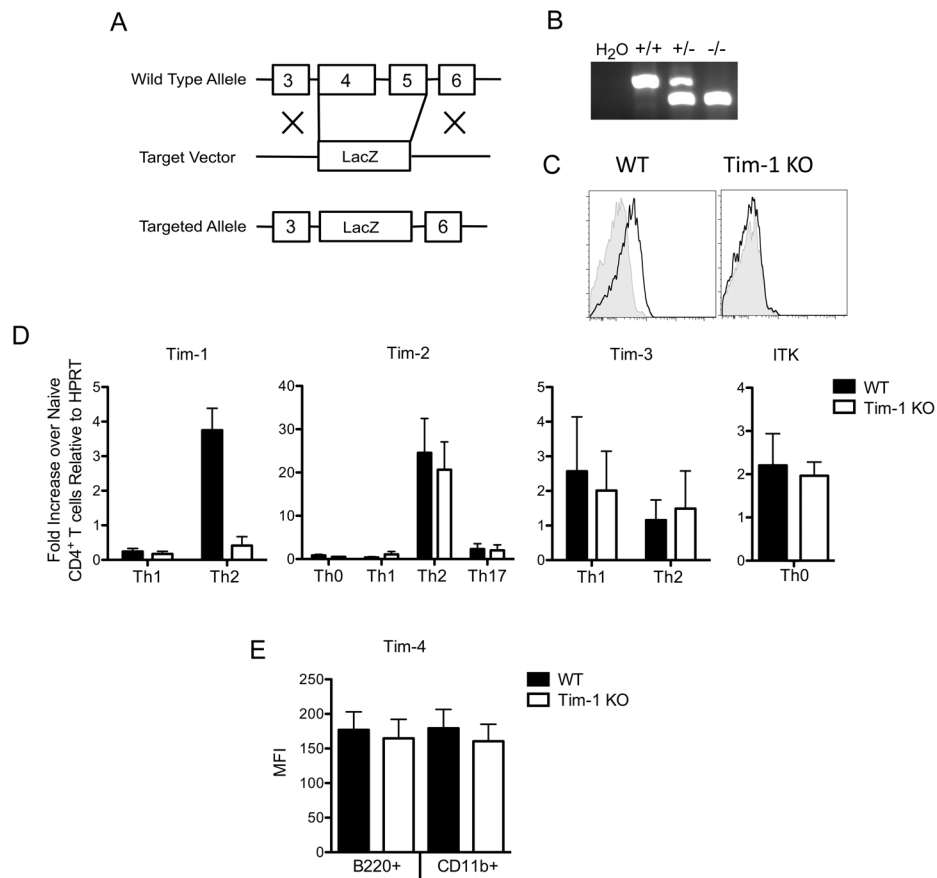
<b>AHR</b>	airway hyperresponsiveness
<b>OVA</b>	ovalbumin
<b>BAL</b>	bronchoalveolar lavage

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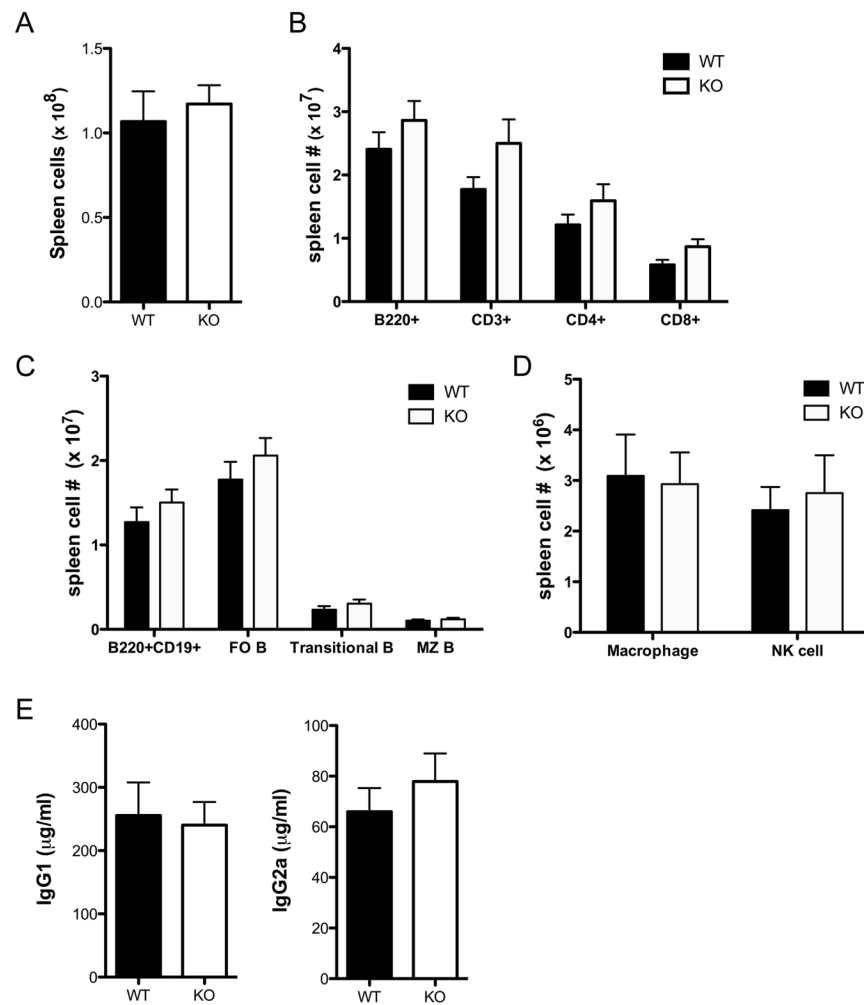
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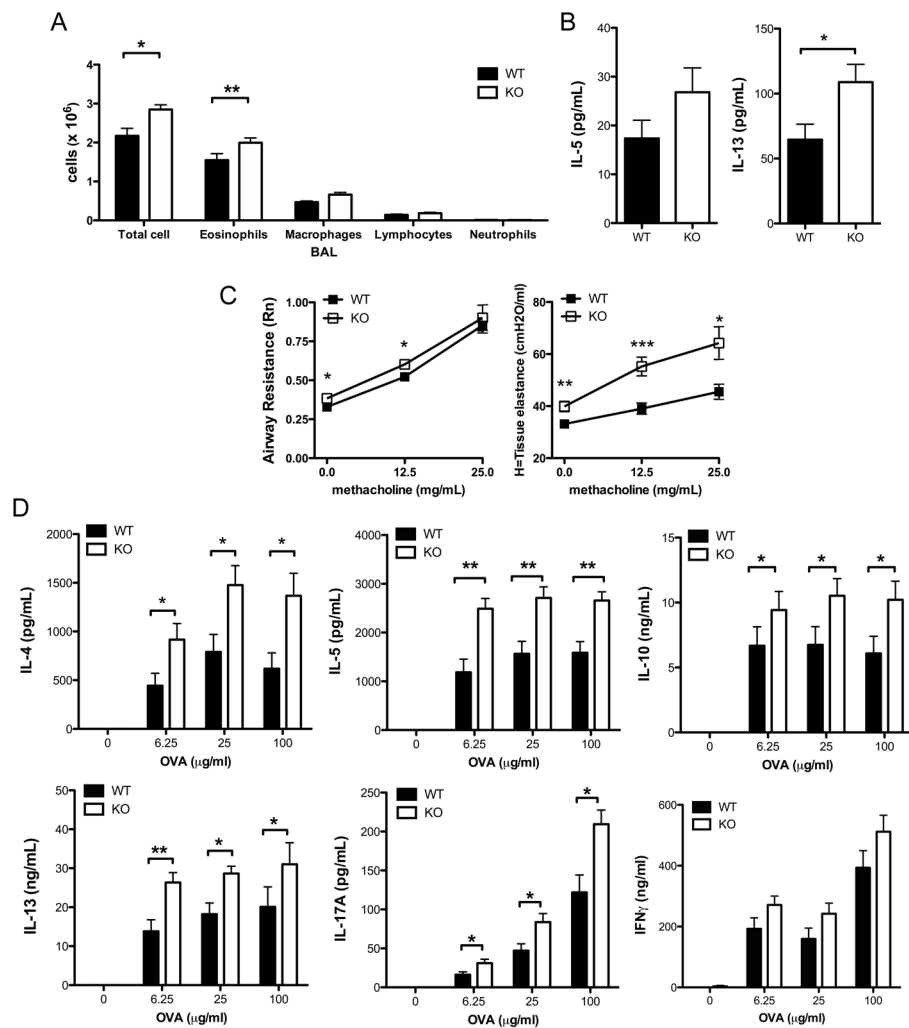
**Figure 1. Generation of Tim-1 deficient mice**

**A.** Schematic of knockout allele. **B.** Genotyping PCR of Tim-1 wild-type, heterozygous, and knockout alleles. **C.** FACS staining of splenocytes from BALB/c wild-type or Tim-1 deficient mice activated for 72 hours with 10  $\mu$ g/mL anti-IgM. CD19<sup>+</sup> gated cells were stained with anti-Tim1 (black line) or isotype control (shaded histogram). **D.** Quantitative PCR analysis of *Tim-1*, *Tim-2*, *Tim-3* and *Itk* in Tim-1 KO and WT mouse CD4<sup>+</sup> T cells. Results are representative of three (*Tim-1*, *Tim-2*, *Tim-3*) or two (*Itk*) independent experiments. **E.** Surface expression of Tim-4 on WT and Tim-1 KO peritoneal cells via FACS analysis. Results are representative of two independent experiments. Statistical analysis was prepared with unpaired two-tailed t tests. Error bars represent standard error of mean. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001.



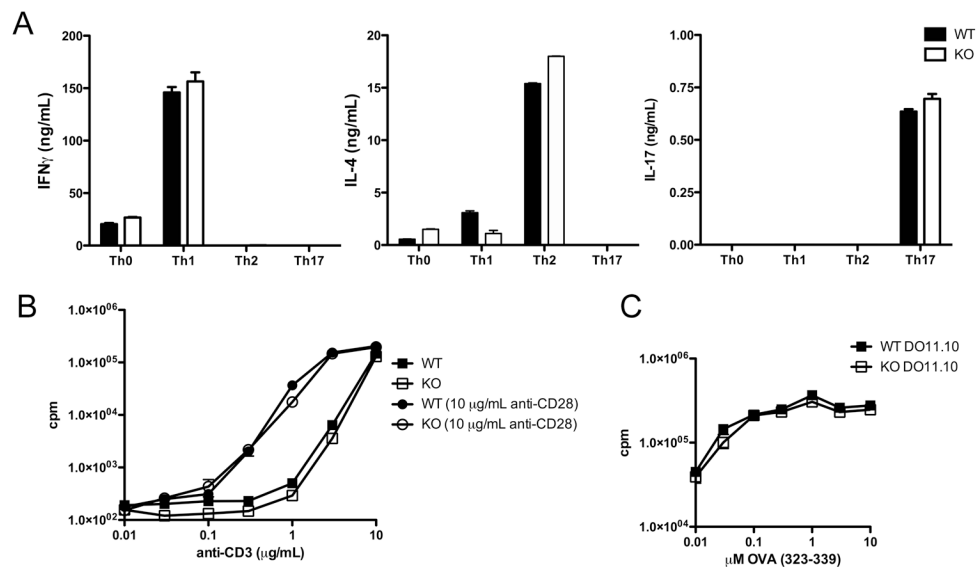
**Figure 2. Distribution of immune cells in BALB/c Tim-1 deficient mice**

**A.** Numbers of splenic cells in wild-type and Tim-1 deficient mice. **B.** Numbers of B220<sup>+</sup>, CD3<sup>+</sup>, CD4<sup>+</sup>B220<sup>-</sup> and CD8<sup>+</sup>B220<sup>-</sup> splenocytes in wild-type and Tim-1-deficient mice. **C.** Numbers of B220<sup>+</sup> splenocyte B cell subsets in wild-type and Tim-1 deficient mice **D.** Numbers of splenic macrophages (CD3<sup>-</sup>B220<sup>-</sup>CD11b<sup>+</sup>GR1<sup>-</sup>) and NK cells (CD3<sup>-</sup>B220<sup>-</sup>DX5<sup>+</sup>CD122<sup>+</sup>). n=9 WT and 9 Tim-1 KO mice, age 9–13 weeks (**A–D**). **E.** ELISA for total serum IgG1 and IgG2a. n=6 WT and 6 Tim-1 KO mice, age 9–13 weeks (**E**). Statistical analysis was prepared with unpaired two-tailed t tests. Error bars represent standard error of mean.



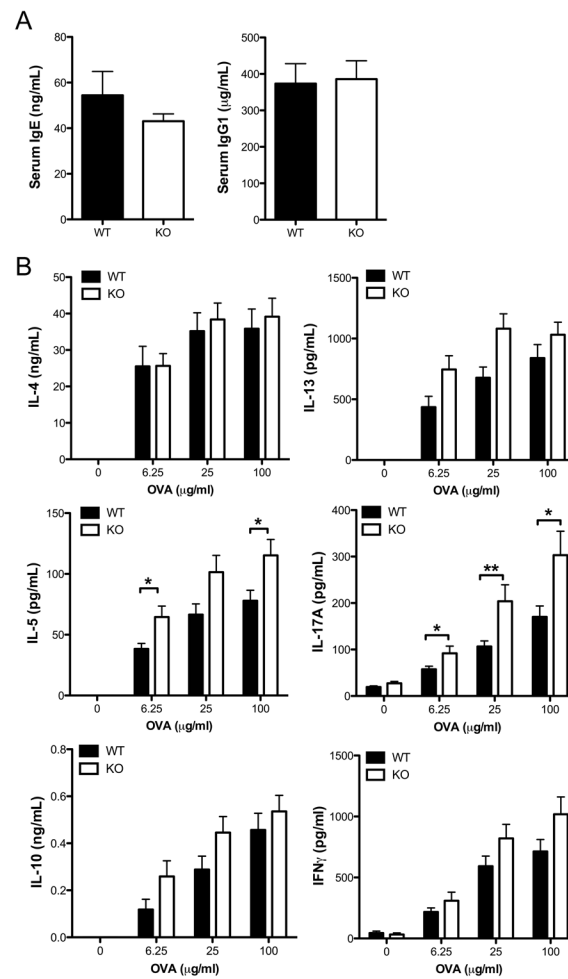
**Figure 3. Enhanced airway inflammation and cytokine production from BALB/c Tim-1 deficient mice challenged in asthma model**

**A.** BAL cellularity determined by cytospin. **B.** Cytokine levels in BAL fluid measured by ELISA. **C.** Invasive measure of airway resistance in response to 0, 12.5, or 25 mg/mL methacholine. **D.** Antigen-specific cytokine production by splenocytes restimulated *in vitro* with 0, 6.25, 25, or 100  $\mu$ g/mL OVA for 96 hours. Statistical analyses were performed using unpaired two-tailed t tests (**A**, **B**, **C**) and Mann-Whitney tests (**D**).  $n = 12$  wild-type and 11 Tim-1 KO mice (**A**, **D**).  $n = 16$  wild-type and 15 Tim-1 KO mice (**B**, **C**). Error bars represent standard error of mean. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ .



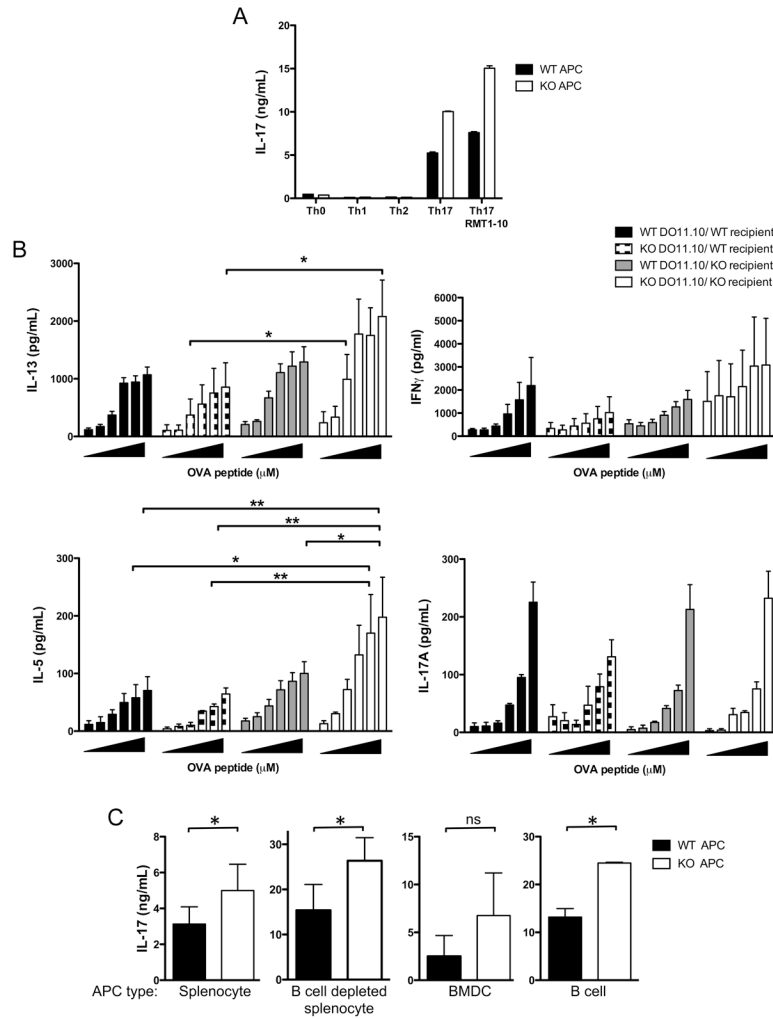
**Figure 4. T cell differentiation and proliferation are unaffected in Tim-1 deficient mice**  
**A.** CD4+CD62L<sup>hi</sup> T cells were cultured in unbiased (Th0) or Th1, Th2, or Th17 polarizing conditions and restimulated. Cytokines were measured by ELISA. **B.** Proliferation of CD4+CD62L<sup>hi</sup> T cells activated with platebound antibodies. **C.** Proliferation of DO11.10 cells sufficient (WT) or deficient (KO) for Tim-1 activated with OVA<sub>323-339</sub> peptide and wild-type irradiated T cell-depleted splenocytes. Experiments were performed at least three times; representative experiment shown. Error bars represent standard deviation of samples measured in triplicate.





**Figure 5. Immunization of BALB/c wild-type and Tim-1 deficient mice with OVA emulsified in alum**

**A.** ELISA of OVA-specific serum IgE and IgG1. **B.** Antigen-specific cytokine production by splenocytes restimulated *in vitro* with 0, 6.25, 25, or 100 µg/mL OVA for 96 hours. Results from 6 wild-type mice and 6 Tim-1 KO mice. Statistical analysis was performed using unpaired two-tailed t tests (**A**) and Mann-Whitney tests (**B**). Error bars represent standard error of the mean. \* $p < 0.05$ , \*\* $p < 0.01$ .



**Figure 6. Adoptive transfer of Tim-1 sufficient and deficient DO11.10 transgenic CD4<sup>+</sup> T cells prior to immunization of BALB/c wildtype and Tim-1 deficient mice with ovalbumin emulsified in alum**

**A.** OT-II CD4<sup>+</sup>CD62L<sup>hi</sup> Tim-1 sufficient T cells polarized *in vitro* with irradiated wild-type or Tim-1 KO T cell-depleted splenocytes produced increased IL-17A. Representative of three independent experiments. Error bars represent standard deviation of samples measured in triplicate. **B.** Tim-1 sufficient or Tim-1 deficient DO11.10 cells were transferred into WT or KO recipient mice, followed by immunization with OVA adsorbed to alum. 10 days post-immunization, purified CD4<sup>+</sup> T cells (pooled from each group of recipient mice) were restimulated *in vitro* with OVA<sub>323-339</sub> peptide and wild-type irradiated splenocytes for 72 hours. Antigen-specific cytokine production by purified CD4<sup>+</sup> T cells was assessed by ELISA. Results of three independent experiments are shown, with 4 recipient mice per group. Statistical analysis was performed using repeated measures 2-way ANOVA. Error bars represent standard error of mean from three independent experiments. **C.** OT-II CD4<sup>+</sup>CD62L<sup>hi</sup> Tim-1 sufficient T cells were polarized *in vitro* with wild-type or Tim-1 KO irradiate splenocytes, B cell-depleted splenocytes, BMDC, or B cells. Results are graphed as mean and SEM of at least three (splenocyte, B cell depleted splenocyte) or two (B cell, BMDC) experiments. Statistical analysis was prepared with unpaired two-tailed t tests. Error bars represent standard error of mean. \* $p < 0.05$ , \*\* $p < 0.01$ .