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MALT1-Deficient Mice Develop Atopic-Like Dermatitis Upon Aging

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MALT1 plays an important role in innate and adaptive immune signaling by acting as a scaffold protein that mediates NF-κB signaling. In addition, MALT1 is a cysteine protease that further fine tunes proinflammatory signaling by cleaving specific substrates. Deregulated MALT1 activity has been associated with immunodeficiency, autoimmunity, and cancer in mice and humans. Genetically engineered mice expressing catalytically inactive MALT1, still exerting its scaffold function, were previously shown to spontaneously develop autoimmunity due to a decrease in Tregs associated with increased effector T cell activation. In contrast, complete absence of MALT1 does not lead to autoimmunity, which has been explained by the impaired effector T cell activation due to the absence of MALT1-mediated signaling. However, here we report that MALT1-deficient mice develop atopic-like dermatitis upon aging, which is preceded by Th2 skewing, an increase in serum IgE, and a decrease in Treg frequency and surface expression of the Treg functionality marker CTLA-4.

Keywords: atopic dermatitis, skin inflammation, MALT1, lymphocytes, Tregs, Th2, aging

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INTRODUCTION

MALT1 (Mucosa-associated lymphoid tissue lymphoma translocation protein 1) is an intracellular signaling protein that plays an important role in several cell types, including lymphoid and myeloid cells as well as non-hematopoietic cells (1). MALT1 is best known for its role as a scaffold protein in T cell receptor (TCR)- and B cell receptor (BCR)-induced nuclear factor-κB (NF-κB) signaling, leading to the activation and proliferation of T and B cells, respectively (2, 3). Moreover, MALT1-mediated NF-κB signaling plays a key role in the proliferation of certain B cell lymphomas, such as MALT1 lymphoma and activated B cell-like diffuse large B cell lymphoma (ABC-DLBCL) (4–13). TCR or BCR stimulation, as well as oncogenic mutations in specific signaling proteins, leads to the formation of a so-called CBM signaling complex, consisting of CARD11 (also known as CARMA1), BCL10 and MALT1 (8, 14–18). In this complex, MALT1 acts as an adaptor to recruit the E3 ubiquitin ligase TRAF6, whose activity facilitates the recruitment and activation of downstream NF-κB signaling proteins (19–21). The importance of the CBM complex is illustrated by the impaired TCR-induced NF-κB activation in T cells isolated from *Card11*-, *Bcl10*-, and *Malt1*-knock-out (KO) mice, respectively (2, 3, 22, 23).

In addition to its scaffold function, MALT1 also acts as a cysteine protease. TCR stimulation leads to the MALT1-mediated cleavage of several substrates including BCL10, the deubiquitinases A20 and CYLD, the NF-κB family member RelB, the RNA-binding and RNA-destabilizing proteins Roquin-1/2, Regnase-1, and N4BP1, the E3 ubiquitin ligase HOIL1, and MALT1 itself (24–34).

Although the specific biological role of cleavage of each of these substrates is still largely unclear, MALT1 proteolytic activity contributes to the fine-tuning of TCR-induced gene expression, lymphocyte activation and proliferation, and regulatory T cell (Treg) development and function. Consequently, inhibition of MALT1 proteolytic activity has been proposed as an interesting therapeutic approach for autoimmune diseases and certain cancers, which is further supported by promising results with MALT1 protease inhibitors in preclinical mouse models (12, 13, 35–37). Of note, MALT1 mutation in humans, causing the absence or very low expression of MALT1, leads to combined immunodeficiency (CID), which is characterized by several bacterial, fungal, and viral infections, indicating that targeting MALT1 activity may not be without risk (38–43). Moreover, it was recently shown that *Malt1* protease-dead (PD) knock-in mice expressing a catalytically inactive MALT1 mutant spontaneously develop multi-organ inflammation due to defects in T cell homeostasis (44–49). This was rather unexpected since inflammation was never described for mice that are completely deficient in MALT1. However, in the present paper we show that *Malt1*-KO mice develop atopic-like dermatitis upon aging.

RESULTS

Malt1-KO Mice Spontaneously Develop Skin Lesions, Accompanied by Elevated Serum Cytokine Levels

Malt1-KO mice were housed under SPF conditions and monitored for macroscopic clinical signs on a regular basis. Interestingly, the mice were found to develop skin lesions upon aging, with an average disease onset of 161 days (**Figures 1A,B**). The *Malt1*-KO mice suffer from erosive lesions in the neck and face region, with the epidermis showing acanthosis, hyperkeratosis, and parakeratotic scaling, as well as $CD3^+$ T cell infiltration (**Figure 1C**). Similar lesions were observed in another independent *Malt1*-KO LacZ reporter mouse line (**Figure 1A**), indicating that the observed phenotype is strain-independent. Next to full body *Malt1*-KO mice, also T cell-specific ($Malt1^{FL/FL}CD4\text{-}Cre^{Tg/+}$) and keratinocyte-specific ($Malt1^{FL/FL}K5\text{-}Cre^{Tg/+}$) *Malt1-KO* mice were monitored for skin lesions over time, but these mice did not develop any skin lesions (**Figure 1B**), indicating that absence of MALT1 in T cells or keratinocytes, as such, is not sufficient to induce skin inflammation. *Malt1*-KO mice that develop skin inflammation were found to have increased serum levels of the pro-inflammatory cytokines IL-2, IL-4, IL-6, IL-17, IFN-γ, and TNF (**Figure 1D**). To assess if increased serum cytokine levels reflect a more general inflammation in MALT1-deficient mice, we analyzed H&E stained sections of lung, liver, stomach, colon, small intestine, lacrimal glands and salivary glands. However, no differences were observed between *Malt1*-KO and WT mice for all these tissues (**Figure 2A**). In addition, we checked blood glucose levels in young (±20 weeks) and older mice (7–8 months) to determine possible pancreatic inflammation, but also here MALT1 deficiency had no effect (**Figure 2B**). Together, our data demonstrate that MALT1 deficiency in mice specifically results in an inflammatory skin phenotype upon aging.

MALT1 Deficiency Results in Defective Treg Development and CTLA-4 Expression via a T Cell Intrinsic Mechanism in Both Young and old Mice

Malt1-KO mice are known to have a defect in Treg development (44, 46, 50, 51), which could be responsible for the skin inflammation in aging MALT1-deficient mice. However, it has been reported by Brüstle et al. that whereas young *Malt1*-KO mice have severely reduced numbers of Tregs in blood and thymus, 1 year old *Malt1*-KO mice have normal Treg numbers in blood, which was suggested to reflect the generation of inducible Tregs (iTregs) in aging mice (51). Of note, this study did not mention the development of skin lesions in aged mice. We therefore analyzed the number of Tregs ($Foxp3^+CD25^+CD4^+$ T cells) in young and aged (±7 months old) *Malt1*-KO mice. In agreement with the above mentioned previous studies, Treg numbers were reduced in thymus, lymph nodes (LN), and spleen of lesion-free young mice. However, in contrast to the study by Brüstle et al., we found that the number of Tregs were equally reduced in thymus, LN and spleen of aged *Malt1*-KO mice that developed skin lesions (**Figures 3A,B** and gating strategy in **Supplementary Figures 1**, **2**). The reason for this discrepancy is still unclear, but different findings may reflect specific differences in mouse housing conditions. Similar to the full *Malt1*-KO mice, also T cell specific *Malt1*-KO mice had a reduced Treg frequency in their thymus, spleen, and LN (**Figure 3C**), indicating a T cell intrinsic role of MALT1 in Treg development. We next investigated whether the remaining MALT1-deficient Tregs are functional. CTLA-4 expression on Tregs is known to compete with CD28 on T cells for binding to CD80 and CD86, as well as to reduce the surface expression of CD80 and CD86 on antigen presenting cells, resulting in reduced T cell proliferation and cytokine production (52, 53). We therefore assessed CTLA-4 surface expression on splenocytes from WT and *Malt1*-KO mice that were stimulated *in vitro* for 4 h with phorbol myristic acid/Ionomycine (PMA/IO). As shown in **Figure 3D**, a reduced frequency of Tregs that express surface CTLA-4 could be observed in *Malt1*-KO mice compared to WT mice, suggesting that the remaining MALT1-deficient Tregs are functionally impaired (gating strategy in **Supplementary Figure 2**).

Activation and CTLA-4 Surface Expression of $CD4^+$ T Cells Is Altered in *Malt1*-KO Mice

Since we observed increased $CD3^+$ T cell infiltration in the diseased skin of *Malt1*-KO mice, we further investigated whether the proliferation and activation of $CD4^+$ T cells is affected in MALT1-deficient mice. For this purpose, we purified $CD4^+$ T cells and labeled them with CFSE to measure their proliferation after 72 h stimulation with anti-CD3 and anti-CD28. This showed that MALT1-deficient $CD4^+$ T cells can proliferate, albeit to a lesser extent than WT $CD4^+$ T cells (**Figure 4A**), which is similar to what has previously been described (45). To assess

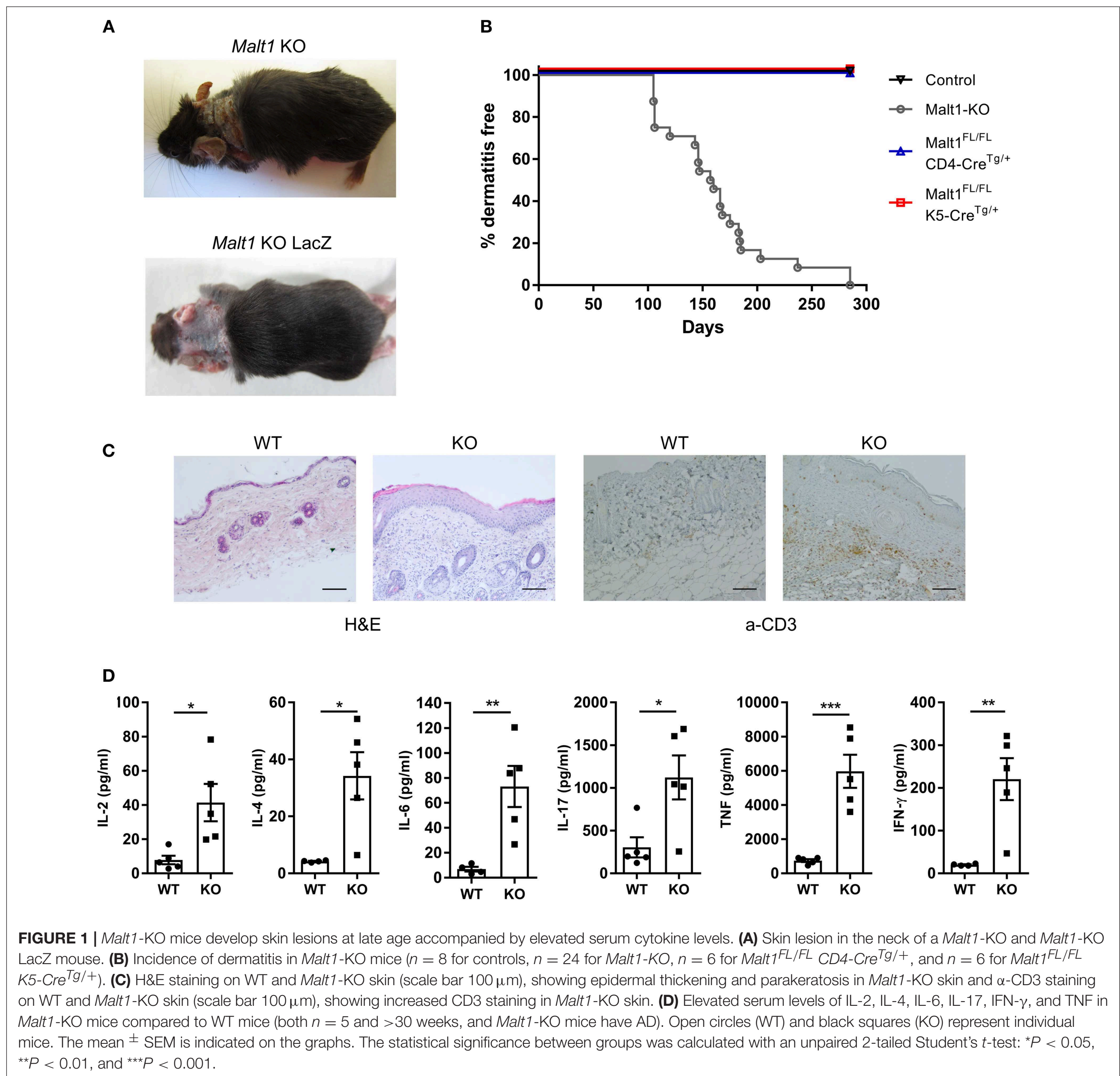

FIGURE 1 | *Malt1*-KO mice develop skin lesions at late age accompanied by elevated serum cytokine levels. **(A)** Skin lesion in the neck of a *Malt1*-KO and *Malt1*-KO LacZ mouse. **(B)** Incidence of dermatitis in *Malt1*-KO mice ($n = 8$ for controls, $n = 24$ for *Malt1-KO*, $n = 6$ for *Malt1*$^{FL/FL}$ *CD4-Cre*$^{Tg/+}$, and $n = 6$ for *Malt1*$^{FL/FL}$ *K5-Cre*$^{Tg/+}$). **(C)** H&E staining on WT and *Malt1*-KO skin (scale bar 100 μm), showing epidermal thickening and parakeratosis in *Malt1*-KO skin and α-CD3 staining on WT and *Malt1*-KO skin (scale bar 100 μm), showing increased CD3 staining in *Malt1*-KO skin. **(D)** Elevated serum levels of IL-2, IL-4, IL-6, IL-17, IFN-γ, and TNF in *Malt1*-KO mice compared to WT mice (both $n = 5$ and >30 weeks, and *Malt1*-KO mice have AD). Open circles (WT) and black squares (KO) represent individual mice. The mean ± SEM is indicated on the graphs. The statistical significance between groups was calculated with an unpaired 2-tailed Student's *t*-test: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

the activation of CD4$^+$ MALT1-deficient T cells, we stimulated splenocytes for 4 h with PMA/IO and determined the frequency of CD44$^+$CD4$^+$ T cells, so-called effector CD4$^+$ T cells. Notably, *Malt1*-KO mice had a reduced frequency of effector CD4$^+$ T cells (**Figure 4B**), which is consistent with previous findings (2).

Since not only Tregs but also effector CD4$^+$ T cells can use surface CTLA-4 to suppress proliferation of effector CD4$^+$ T cells (54–56), we assessed the expression of CTLA-4 on the surface of effector CD4$^+$ T cells from *Malt1*-KO and WT mice. In contrast to WT mice, *Malt1*-KO mice showed a strong reduction in the frequency of effector CD4$^+$ T cells that express surface CTLA-4 (**Figure 4C** and gating strategy in **Supplementary Figure 2**). In addition, the expression of CTLA-4 on the remaining MALT1-deficient surface CTLA-4$^+$ effector CD4$^+$ T cells was also reduced, as determined by the surface CTLA-4 mean fluorescent intensity (**Figure 4D**). These data clearly show that besides being important for CTLA-4 expression on the surface of Tregs, MALT1 is similarly important for the expression of CTLA-4 on the surface of effector CD4$^+$ T cells. Together, these data show that although MALT1 deficiency leads to reduced activation and proliferation of stimulated CD4$^+$ T cells, it also lowers the immune suppressive functions of both Tregs and effector CD4$^+$ T cells, which could contribute to disease development.

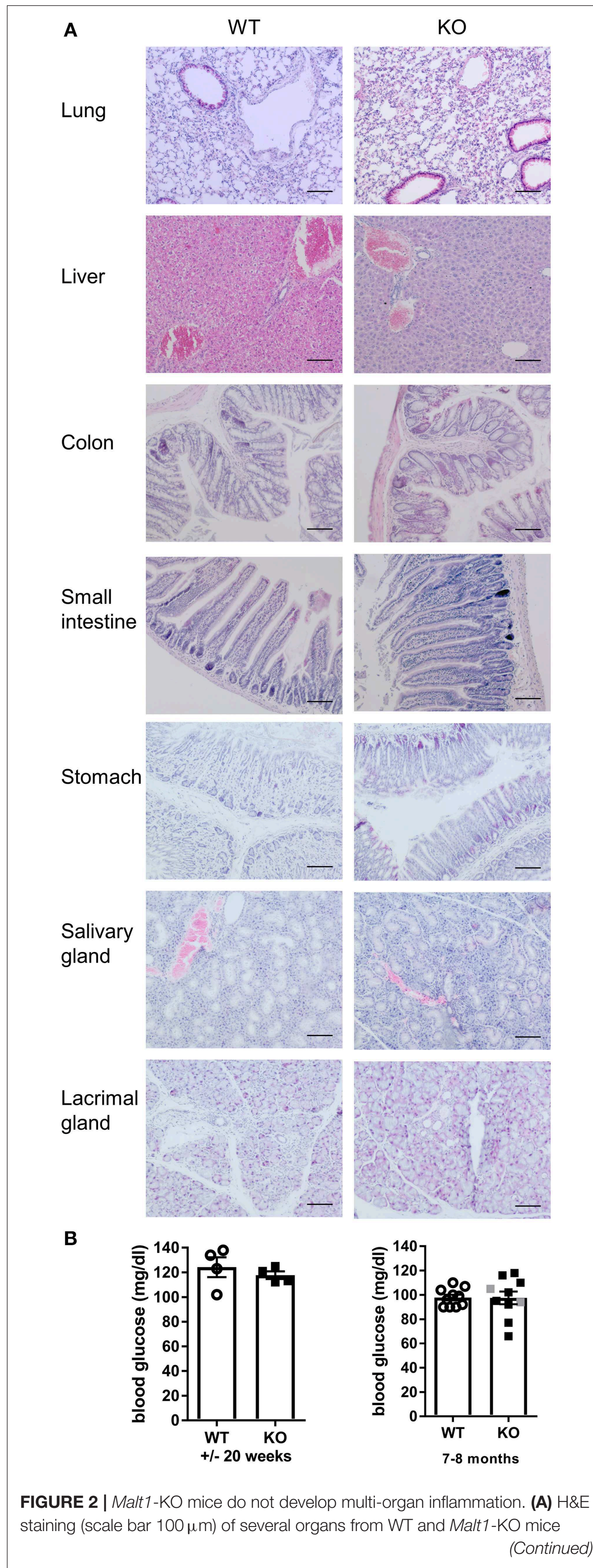

FIGURE 2 | *Malt1*-KO mice do not develop multi-organ inflammation. **(A)** H&E staining (scale bar 100 μm) of several organs from WT and *Malt1*-KO mice *(Continued)*

FIGURE 2 | (*Malt1*-KO mice had skin lesions and the WT and KO mice were between 30 and 40 weeks old). **(B)** Blood glucose levels in mice of ±20 weeks (left, WT: $n = 5$ and KO: $n = 4$) and mice of 7–8 months (right, WT: $n = 10$ and KO: $n = 8$). Open circles (WT), gray squares (KO), and black squares (KO + AD = KO mice with atopic dermatitis) represent individual mice. The mean ± SEM is indicated on the graphs. The statistical significance between groups was calculated with an unpaired 2-tailed Student's *t*-test: no significant difference was found.

MALT1 Deficiency Causes Th2 Skewing Accompanied by Increased Serum IgE Levels

We next investigated whether the impaired Treg development in *Malt1*-KO mice has an impact on the T-helper (Th) cell populations. To this end, we stimulated splenocytes with PMA, IO, and Brefeldin A for 4 h, and determined the percentage of Th2 (IL-4 producing $CD44^+CD4^+$ T cells) (**Figure 5A**) and Th1 cells (IFN-γ producing $CD44^+CD4^+$ T cells) (**Figure 5B**), respectively (gating strategy in **Supplementary Figure 3**). MALT1-deficient mice (±20 weeks, skin lesion free) repeatedly showed largely similar levels of IFN-γ-producing Th1 cells, while the IL-4 producing Th2 cells were significantly increased. Since IL-4, secreted by Th2 cells, is known to induce B cell Ig isotype switching from IgM to IgE (57), we determined the serum IgE levels from *Malt1*-KO and WT mice of several ages. In agreement with the increased Th2 frequency, IgE levels were clearly elevated in *Malt1*-KO mice at any time point tested and preceded lesion onset (**Figure 5C**). Furthermore, in the ear skin of *Malt1*-KO mice with lesions, we found elevated mRNA levels of *Tslp* and *Il22* (**Figure 5D**), which are both known to promote Th2 responses (58, 59). Collectively, these data indicate that MALT1 deficiency leads to Th2 skewing and IgE production, which might contribute to skin lesion development.

DISCUSSION

We report that aging *Malt1*-KO mice suffer from atopic-like dermatitis accompanied by elevated serum cytokine levels and preceded by Th2 skewing and elevated serum IgE levels. No inflammation could be observed at other sites of the body. In contrast to *Malt1*-KO mice, skin lesions were never reported in mice fully deficient in one of the other components of the CBM complex, BCL10 and CARD11, or in the upstream activator PKCθ, even though BCL10 and PKCθ deficient mice were followed up until 6 months of age (22, 60). Notably, despite being part of the same signaling pathway, MALT1, BCL10, and PKC-θ deficiency have also been reported to differentially affect TCR-induced proliferation, IL-2 production, and NF-κB activation in T cells, with MALT1-deficient T cells showing a milder impairment than BCL10- and PKCθ-deficient T cells, suggesting they may have divergent functions and act in additional signaling pathways (61).

We further report that atopic-like dermatitis in aging *Malt1*-KO mice is associated with a decrease in the number and function of Tregs in the thymus and periphery. We propose that the

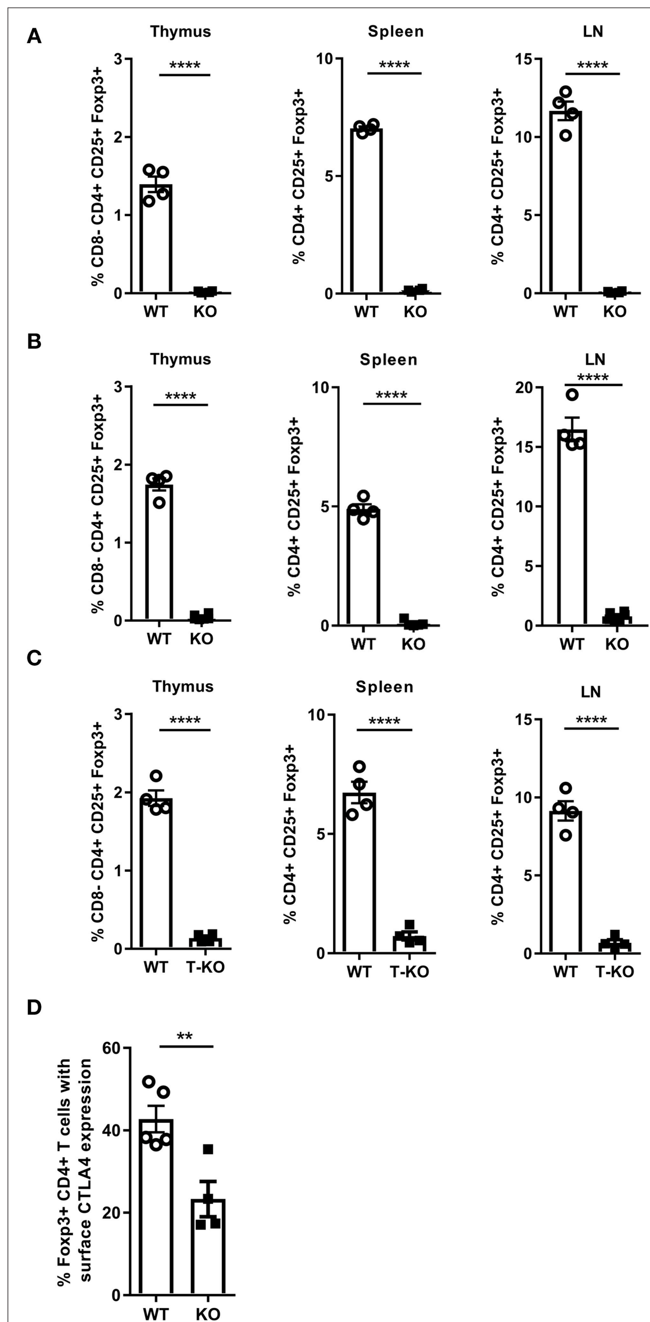

FIGURE 3 | MALT1 plays a T cell-intrinsic role in Treg development. **(A)** Percentage of Tregs (Foxp3$^+$CD25$^+$CD4$^+$ T cells) in the thymus, spleen and lymph nodes of *Malt1*-WT and *Malt1*-KO mice without skin lesions (WT: $n = 4$ and KO: $n = 4$, age 10–12 weeks). **(B)** Percentage of Tregs (Foxp3$^+$CD25$^+$ CD4$^+$ T cells) in the thymus, spleen and lymph nodes of older WT and *Malt1*-KO mice with skin lesions (WT: $n = 4$ and KO: $n = 6$, age 6.5–8.5 months). **(C)** Percentage of Tregs (Foxp3$^+$CD25$^+$CD4$^+$ T cells) in the thymus, spleen and lymph nodes of WT (*Malt1$^{FL/FL}$CD4-Cre$^{+/+}$*, $n = 4$), and T-KO (*Malt1$^{FL/FL}$CD4-Cre$^{Tg/+}$*, $n = 4$) mice. **(D)** Percentage of splenic Tregs (Foxp3$^+$CD4$^+$ T cells) expressing CTLA-4 on their surface after stimulation for 4 h with PMA and IO in WT ($n = 5$) and *Malt1*-KO ($n = 5$) mice. The mean ± SEM is indicated on the graphs. The statistical significance between groups was calculated with an unpaired 2-tailed Student's *t*-test: $^{**}P < 0.01$ and $^{****}P < 0.0001$.

reduction in immune suppressive Tregs leads to a disruption of normal immune homeostasis and contributes to the activation of effector T cells and allergic skin inflammation. In this context, we could measure more Th2 cells producing IL-4, which is known to play multiple roles in promoting atopic-like dermatitis (62). A severe Treg reduction is also seen in patients suffering from immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome (OMIM #304790), caused by mutations in the *FOXP3* gene (63) as well as mice with a mutation in the *Foxp3* gene, so called scurfy mice (64). The scurfy mice and the IPEX patients illustrate variable autoimmune disorders. IPEX patients can suffer from type 1 diabetes mellitus and thyroid disease, increased IgE levels, asthma and food allergies, while dermatitis and increased IgE levels are also present in the scurfy mice (63, 65–67). A lack of functional Tregs is a common feature in *Malt1*-KO mice, scurfy mice and IPEX patients (44, 46, 50, 51, 68–70). Moreover, a scurfy-like phenotype was described for mice (*Malt1$^{FL/FL}$Foxp3-cre$^{Tg/+}$*) with a specific deletion of *Malt1* in Tregs (36, 49). However, while *Malt1*-KO mice as well as MALT1 CID patients display impaired T cell activation (3, 22, 38–47), this is not the case for IPEX patients, scurfy mice and mice only lacking *Malt1* in Tregs, where there is a failure to control T cell activation due to the absence of Tregs or the reduced functionality of Tregs leading to lymphoproliferation and autoimmunity, resulting in death (36, 49, 63, 65–67, 71).

CTLA-4 is a known functionality marker on Tregs and is required for their inhibitory function (52, 53). We show that the remaining Tregs in *Malt1*-KO mice are also functionally impaired as demonstrated by a reduced CTLA-4 expression. The reduced Treg frequency and functionality we observed in *Malt1*-KO mice is associated with an increased Th2 frequency. Th2 cells were previously shown to expand disproportionally upon depletion of Tregs, which tightly control the Th2 population via induction of apoptosis of Th2, but not Th1 cells (72). Moreover, Tian et al. showed that addition of recombinant CTLA-4-Ig to Treg depleted mice induces Th2 apoptosis and thus reduces the Th2 expansion, illustrating the tight control by Tregs on the Th2 population (72). In addition to Tregs, also effector CD4$^+$ T cells make use of surface CTLA-4 to inhibit effector CD4$^+$ T cells, albeit with much lower efficiency than Tregs (54–56). Notably, we show that CTLA-4 expression is also reduced on effector CD4$^+$ T cells in *Malt1*-KO mice, which may also contribute to disease pathogenesis. Interestingly, CTLA-4 mRNA is post-transcriptionally regulated by the endonuclease Regnase-1 and the RNA-binding proteins Roquin-1 and-2, which were shown to be inactivated by MALT1-mediated cleavage, leading to stabilization of CTLA-4 and many other mRNA molecules (28, 29). Most likely, reduced CTLA4 expression in MALT1-deficient Tregs and effector CD4$^+$ T cells reflects the absence of Regnase-1 and Roquin cleavage, leading to CTLA-4 mRNA degradation and reduced CTLA-4 protein expression.

Mice that are completely deficient in MALT1 as well as mice expressing a catalytically inactive (protease-dead) mutant MALT1 have a reduced number of Tregs, but only *Malt1*-PD mice develop severe autoimmune symptoms (44–51). Impaired TCR-mediated effector T cell activation, normally mediated by the MALT1 scaffold function, has been proposed to prevent

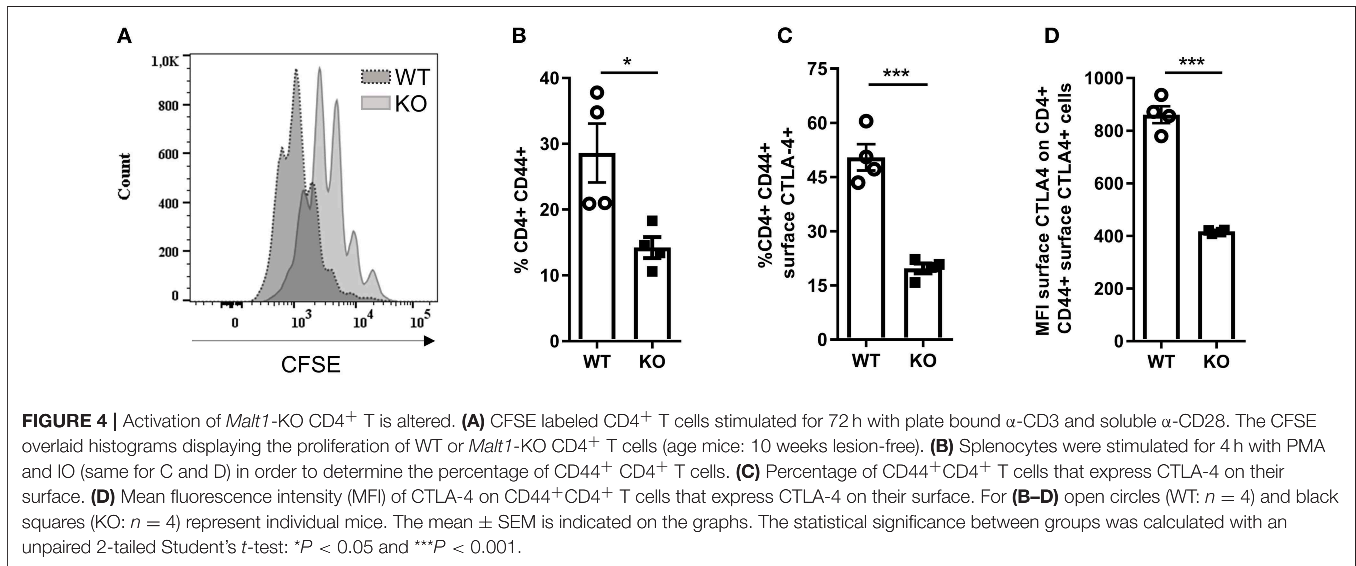

FIGURE 4 | Activation of *Malt1*-KO CD4$^+$ T is altered. **(A)** CFSE labeled CD4$^+$ T cells stimulated for 72 h with plate bound α-CD3 and soluble α-CD28. The CFSE overlaid histograms displaying the proliferation of WT or *Malt1*-KO CD4$^+$ T cells (age mice: 10 weeks lesion-free). **(B)** Splenocytes were stimulated for 4 h with PMA and IO (same for C and D) in order to determine the percentage of CD44$^+$ CD4$^+$ T cells. **(C)** Percentage of CD44$^+$CD4$^+$ T cells that express CTLA-4 on their surface. **(D)** Mean fluorescence intensity (MFI) of CTLA-4 on CD44$^+$CD4$^+$ T cells that express CTLA-4 on their surface. For **(B–D)** open circles (WT: $n = 4$) and black squares (KO: $n = 4$) represent individual mice. The mean ± SEM is indicated on the graphs. The statistical significance between groups was calculated with an unpaired 2-tailed Student's *t*-test: *$P < 0.05$ and ***$P < 0.001$.

FIGURE 5 | Th2 skewing and elevated IgE levels in *Malt1*-KO mice. **(A)** Splenocytes of 20 weeks old mice without skin lesions were stimulated for 4 h with PMA and IO (same for B), in order to determine the percentage of CD44$^+$CD4$^+$ T cells expressing IL-4 (WT and KO: $n = 8$). **(B)** Percentage of CD44$^+$ CD4$^+$ T cells expressing IFN-γ (WT and KO: $n = 8$). **(C)** IgE levels in serum collected from WT and *Malt1*-KO mice of ±15, ±20, and >30 weeks (15 weeks WT and KO: $n = 4$, at 20 weeks WT: $n = 8$ and KO: $n = 6$ and at 30 weeks WT: $n = 6$ and KO: $n = 9$). Open circles (WT), black squares (KO), and gray squares (KO + AD) represent individual mice. **(D)** mRNA expression levels of *Malt1*, *Tslp*, and *IL22* (relative to reference genes *Hprt1* and *Ubc*) in ears of *Malt1*-WT mice ($n = 7$), *Malt1*-KO mice ($n = 11$), and *Malt1*-KO + AD mice ($n = 3$) suffering from dermatitis. The mean ± SEM is indicated on the graphs. For **(A–C)** all results were obtained by flow cytometry the statistical significance between groups was calculated with an unpaired 2-tailed Student's *t*-test. For **(D)** the statistical significance between groups was calculated with a one-way ANOVA with a Tukey's multiple comparison test. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

spontaneous inflammation in full *Malt1*-KO mice (1). Our present finding that *Malt1*-KO mice spontaneously develop skin inflammation upon aging, implicates a role for MALT1-independent antigen or cytokine receptor signaling leading to low or intermediate T cell activation. The reduced frequency of functional Tregs in combination with a lowered effector T cell activation causes a gradual and selective expansion of Th2 cells, culminating in allergic skin inflammation without autoimmunity or generalized inflammation.

Skin inflammation in atopic dermatitis is assumed to arise due to a misdirected immune response against harmless antigens on the one hand, and to skin barrier defects on the other hand

(73). We propose that scratching may cause local skin barrier defects, which in combination with the Treg deficiency and the Th2 skewing favors the specific development of skin lesions in *Malt1*-KO mice. This is further supported by a report showing that tape stripping in combination with Treg depletion results in skin thickening, increased IL-4 and IL-13 mRNA levels in the skin, and elevated serum IgE levels (74). Of note, whereas an increase in IL-4 was already detectable in *Malt*1-KO mice before the development of skin lesions, elevated levels of *Tslp* and *Il22* mRNA, which are known to promote expression of Th2 cytokines, such as IL-4, could only be detected in lesional ear skin. A possible explanation might be that increased *Tslp* and *Il22* levels only occur upon skin barrier disruption in skin lesions, which is in agreement with an observed increase in TSLP expression upon tape stripping (75, 76).

Persistent severe dermatitis (7/9) and increased serum IgE levels (4/8) have been described in patients with loss of function mutations in MALT1 (38, 40–43). Similarly, dermatitis has been reported in genome-wide association studies for *CARD11* (77) and is also one of the clinical features found in most patients with loss of function mutations in *CARD11* (78–80). Also mice that have hypomorphic mutations in *Card11* display dermatitis, reduced Tregs, and increased IgE and Th2 levels (81–83). The proposed mechanism for disease development is the tight relationship between Tregs and Th2 cell levels (72, 82). However, our T cell-specific *Malt1*-KO mice did not develop skin lesions, suggesting that absence of MALT1 in T cells only is not sufficient to drive skin inflammation in aging mice. CARD11 is a member of the CARD-CC protein family, which also contains CARD9, CARD10 (also known as CARMA3), and CARD14 (also known as CARMA2) (84), which can all form distinct CBM complexes in a cell-type specific manner. Recently, Peled at al. reported that two loss-of-function mutations in *CARD14* are associated with atopic dermatitis (85). CARD14 is mainly expressed in keratinocytes and activates MALT1 signaling in keratinocytes (86, 87), which led us hypothesize that MALT1 deficiency in keratinocytes was driving atopic-like dermatitis. However, we also did not observe any skin lesions in keratinocyte-specific *Malt1*-KO mice. Possibly, combined deficiency in T cells and keratinocytes is needed to induce the atopic skin phenotype in aged mice. Alternatively, we cannot exclude a role for other cell types as well.

In general, the here established relationship between impaired MALT1-dependent TCR signaling, partial Treg deficiency, and dysregulated accumulation of Th2 cells, may provide a mechanistic basis to explain the allergic responses in patients carrying *MALT1* and *CARD11* mutations, and invites future studies investigating associations between atopy and genetic variations in other components of the TCR-MALT1 signaling pathway.

MATERIALS AND METHODS

Mice

Malt1-KO mice (backcrossed for more than 10 generations into C57BL/6 background) were a kind gift from Dr. T. Mak (Toronto, Canada). Another *Malt1* allele from EUCOMM ($Malt1^{tm1a(EUCOMM)Hmgu/+}$) was derived from ES cells and subsequently back-crossed to germline-expressing *Flpe*-deleter mice (88) to generate a conditional *Malt1*-deficient allele ($Malt1^{FL/+}$). The ES cells were also backcrossed to a germline-expressing *Cre*-deleter mouse (89) to obtain an alternative full deficient allele with a LacZ reporter ($Malt1^{IRES-LacZ/+}$). To generate a T-cell specific knock-out, $Malt1^{FL/+}$ was further crossed to $CD4\text{-}Cre^{Tg/+}$ mice (90) and $K5\text{-}Cre^{Tg/+}$ (91) and offspring was inter-crossed to select for Flpe-deleter-negative T cell-specific ($Malt1^{FL/FL}CD4\text{-}Cre^{Tg/+}$) and skin-specific ($Malt1^{FL/FL}$ $K5\text{-}Cre^{Tg/+}$) MALT1-deficient mice. *CD4-Cre* is always kept heterozygote by selecting one parent $CD4\text{-}Cre^{Tg/+}$ and the other parent as *Cre*-negative. The specificity of *CD4-Cre* was confirmed via western blot (**Supplementary Figure 4**) using rabbit monoclonal anti-MALT1 (SC-28246, Santa Cruz) and anti-Cre (6905-3, Merck Millipore). The *K5-Cre* is always kept heterozygote by selecting a male $K5\text{-}Cre^{Tg/+}$ and a female as *Cre*-negative, to avoid female germline transmission (91). Mice were housed in individually ventilated cages in a specific pathogen-free (SPF) facility. Mice were supplied with water and food *ad libitum* and experiments were performed in compliance with the guidelines of the University of Ghent Ethics Committee for the use of laboratory animals (EC2011-024 and 2013-066). Mice were monitored regularly for signs of dermatitis, consisting of hair loss in the facial, ear and neck region, together with redness, skin thickening, and scratching.

Genotyping

For *Malt1*-KO-mice we used the primers MALT1-F (GTGCTC TTGTAA TTTTCTGTGCTC), MALT1 WT-R (GGGTACATC ATGGCCTGAACAGTTG), and MALT1 KO-R (GGGTGGGAT TAGATAAATGCCTGCTC), resulting in 172 bp (WT) and 272 bp (KO) PCR products. The genotypings were made using GoTaq Green Hot Start (Promega) master mix, with a typical PCR program: 5 min 94°C denaturation, 35–40 cycles [45 s 94°C|30 s 60°C|45 s 72°C] and 10 min 72°C final elongation.

For the $Malt1^{tm1a(EUCOMM)Hmgu/+}$ derived mice we monitored the *Malt1* Flox-allele or KO allele with the primers MALTcKO-F (GTTTCTCAGGTCTTTAGTTCATGTC), CoMLT-3-R (TAT ACTCTACATCTCCATGGT), MALTcKO-R (TTGTTTTGC AGATCTCTGCC), and MLT-LacZ-F (TCGCTACCATTACCA GTTGGT) resulting in 280 bp (WT), 400 bp (FL), 345 (KO), or 514 bp (KO-LacZ) PCR products. *Flp* was detected with the primers Flp-F (TTAGTTCAGCAGCACATGATG) and Flp-R (GGAGGATTTGATATTCACCTG), resulting in a 370 bp PCR fragment. K5-Cre was detected with the primers Cre-F (TGC CACGACCAAGTGACAGCAATG) and Cre-R (AGAGAC GGAAATCCATCGCTCG) producing a 374 bp PCR fragment. CD4-Cre was detected with primers CD4-Cre-R (TCAAGG CCAGACTAGGCTGCCTAT) and CD4-Cre-F2 (TCTCTGTGG CTGGCAGTTTCTCCA) producing a 300 bp PCR fragment. The genotypings were made using the GoTaq Green Hot Start (Promega) master mix, with a typical PCR program: 5 min 95°C denaturation, 35–40 cycles [30 s 95°C|30 s 55–60°C|60 s 72°C] and 10 min 72°C final elongation.

Histology and Immunohistochemistry

Skin, lung, liver, colon, small intestine, stomach, lacrimal gland, and salivary gland samples were fixed with 4% paraformaldehyde and imbedded in paraffin. Sections (5 µm) were stained with hematoxylin and eosin. Skin sections were also stained with anti-CD3 (clone CD3-12; Serotec). Images were acquired with a BX51 discussion microscope (Olympus) with PixeLink camera under 100× magnification.

RNA Extraction, cDNA Synthesis, and Quantitative Real-Time PCR

After sacrifice, ears were collected and incubated overnight in RNA later at 4°C before long term storage at −70°C. For RNA extraction, the ears were transferred to TRIzol reagent (Invitrogen) and homogenized using the Precellys 24 (Bertin technologies with CK26 beads). After phenol/chloroform phase separation, RNA was isolated using the Aurum total RNA mini kit (Bio-Rad). cDNA was synthesized using the SensiFAST™ cDNA synthesis kit (Bioline), according to manufacturer's instructions. Quantitative PCR was done with a LightCycler 480 (Roche) using sensiFAST™ SYBR No-ROX kit (Bioline) with a total of 10 ng cDNA and 300 nM of specific primers in a 10 µl reaction. Real-time reactions were done in triplicates. The following specific primers were used (5′-3′): *Hprt1* Fwd AGTGTTGGATACAGGCCA GAC and *Hprt* Rev CGTGATTCAAATCCCTGAAGT; *Ubc* Fwd AGGTCAAACAGGAAGACAGACGTA and *Ubc* Rev TCA CACCCAAGAACAAGCACA; *Malt1* Fwd GGACAAAGTCGC CCTTTTGAT and Rev TCCACAGCGTTACACATCTCA; *Il22* Fwd AGACAGGTTCCAGCCCTACAT and *Il22* Rev TCT TCTGGATGTTCTGGTCGT; *Tslp* Fwd TCTCAGGAGCCT CTTCATCCT and *Tslp* Rev CTCACAGTCCTCGATTTGCT. Analysis was done with qBase software (Biogazelle). Values were normalized to two reference genes, as determined by Genorm analysis.

Blood Glucose Levels

A drop of blood from the tail was applied to a test strip and the glucose level was measured with a Freestyle lite glucose meter (Abbot).

Flow Cytometry

Detection of Tregs

Single cell suspensions of thymus, spleen, and lymph nodes were surfaced stained with Aqua Live/dead fixable stain (Life Technologies) or Fixable Viability Dye eFluor 506 (eBioscience), anti-CD16/CD32 Fc block (clone 2.4G2; BD Biosciences), anti-CD3-V450 (clone 17A2; BD Biosciences) or anti-CD3 eFluor450 (clone 17A2; eBioscience), anti-CD4-FITC (clone GK1.5; BD Biosciences or eBioscience), anti-CD25-PercPcy5.5 (clone PC61; BD Biosciences) for 20 min. Next, cells were permeabilized for 30 min, followed by 30 min of intracellular staining for anti-Foxp3-PE (clone FJK-16s; eBioscience). For the intracellular staining, the Foxp3 buffer set (eBioscience) was used and all incubation steps were done on ice.

CTLA-4 Expression of Tregs and CD44⁺CD4⁺ Effector T Cells

Splenocytes cultured in complete medium (RPMI 1640 medium supplemented with 10% FCS, Sodium Pyruvate, L-glutamine, antibiotics, and 2-Mercaptoethanol) were stimulated with PMA (50 ng/ml) and ionomycin (IO) (1 µg/ml) for 4 h at 37°C. The cells were stained as mentioned above, but anti-CD44-APC-efluor780 (clone IM7; eBioscience) and anti-CTLA-4 PE-eFluor610 (clone UC10-4B9; eBioscience) were also included in the surface staining.

Analysis of Cytokines by Intracellular Cytokine Staining

Splenocytes were cultured in complete medium and stimulated with PMA (50 ng/ml), IO (500 ng/ml) and Brefeldin A (1 µg/ml) for 4–5 h at 37°C. Stimulated cells were washed, surface stained with anti-CD16/CD32, Aqua Live/dead fixable stain or Fixable Viability Dye eFluor 506, anti-CD3-v450 or anti-CD3 eFluor450, anti-CD4-FITC, APC-anti-CD44-APC eFluor780, for 20 min. Next, cells were fixed and permeabilized for 30 min. using the Foxp3 buffer set, followed by intracellular staining with anti-IL4-APC (clone 11B11; eBioscience) and anti-IFNγ-PE-Cy7 (clone XMG1.2; BD Pharmingen) for 30 min.

Proliferation of CD4⁺ T Cells

CD4⁺ T cells isolated with the MACS CD4⁺ T cell isolation kit II were labeled with 2.5 µM CellTrace™ CFSE (Life Technologies) according to the manufacturer's protocol. The cells were cultured in complete medium for 72 h with 5 µg/ml plate bound anti-CD3 (145-2C11; BD Pharmingen) and 1 µg/ml soluble anti-CD28 (37.51; BD Pharmingen) and 50 IU/ml recombinant mIL-2 (PSF, VIB). Cells were surface stained with Aqua Live/dead fixable stain, anti-CD4-FITC and fixed as mentioned above.

All data were obtained with a LSRII flow cytometer (BD Biosciences) and FlowJo Software (Treestar, Inc, Ashland, Ore) was used for data analysis.

Analysis of IgE and Cytokines in Serum

Peripheral blood samples were collected for serum preparation. The level of IgE in serum was determined using the mouse IgE ELISA Ready-SET-Go kit (eBioscience) and the concentration of IgE was calculated using GraphPad Prism 6 (GraphPad Software, Inc). The levels of IL-2 (171-G5003M), IL-4 (171-G5005M), IL-6 (171-G5007M), IL-17 (171-G5013M), IFN-γ (171-G5017M), and TNF (171-G5023M) was determined by Bio-Plex (Biorad) according to the manufacturer's conditions.

Statistical Analysis

Statistical analysis (indicated in the figure legends) was performed with GraphPad Prism 7.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the manuscript/**Supplementary material**.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Ghent Ethics Committee for the use of laboratory animals (EC2011-024 and 2013-066).

AUTHOR CONTRIBUTIONS

AD, DM, JS, and RB designed the experiments. AD performed all the experiments, except for **Figure 1C** (done by DM). EV and GB provided the technical assistance for **Figure 5D** and YD provided the technical assistance for **Figure 3B**. YD and MK assisted with the genotyping of mice. AD, JS, and RB contributed to the scientific discussion and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02330/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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