Modulating T cell homeostasis via TNF and TNFR superfamily members: characterization and function of effector & regulatory T cells
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Chapter 6

Function of CD27 in helper T cell differentiation.

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Abstract

Differentiation of naïve CD4\(^+\) T cells to functional effector T-helper (T\(_H\)) cells is driven by both costimulatory molecules and cytokines. Although polarizing cytokines can induce the differentiation into a particular T\(_H\)-subset, certain costimulatory molecules also seem to affect this polarization process. We have previously found that CD70-transgenic (CD70TG) mice develop large numbers of IFN-\(\gamma\)-producing CD4\(^+\) T cells and we therefore questioned whether CD27 triggering provides an instructive signal for T\(_H\)1 differentiation or rather supports T\(_H\) cell formation in general. Although CD70TG mice on a T\(_H\)1-prone C57Bl/6J background develop more T\(_H\)1 cells, we found that this phenotype is lost when CD70TG mice are backcrossed on a T\(_H\)2-prone Balb/c background, but is not replaced with more T\(_H\)2 cells. Furthermore, CD70-overexpression is not sufficient to drive T\(_H\)17 cell formation, nor does it affect the generation of FoxP3\(^+\) regulatory T cells. Using an \textit{in vitro} setting, we found that CD27-triggering does not provide instructive signals for a specific T\(_H\) cell subset, but rather supports the formation of IFN-\(\gamma\)-producing as well as IL-13-producing CD4\(^+\) T cells depending on the cytokine milieu and genetic background, while inhibiting T\(_H\)17 formation. Induction of allergic airway inflammation in CD70TG Balb/c mice further illustrates that CD27 plays a supportive role in T\(_H\)1 differentiation, without modulating the classical T\(_H\)2 response. This supportive role of CD27 in T\(_H\) cell polarization could not be attributed to a specific modulation of transcription factor expression levels. In summary, this study indicates that CD27 signaling supports T\(_H\)1 differentiation, permits T\(_H\)2 formation, but inhibits T\(_H\)17 formation.
Introduction

Recognition of a MHC-peptide complex via the TCR is the first signal required for effector T cell formation, as it initiates T cell activation and clonal expansion. Subsequent to MHC-peptide binding, T cells depend on a second signal for their survival and proliferation, which is provided in the form of costimulatory molecules. For the final differentiation and polarization to effector cells, activated T cells require a third signal, which is provided by polarizing cytokines (reviewed in (1)). Thus, naïve T cells rely on a triad of signals for their activation and differentiation into an effector population. The large number of molecules that have been implicated in this process either instruct, support or permit the formation of a specific effector T cell population.

Within the CD4 T cell population, a large variety of helper T (TH) cell subsets has been identified, such as TH1, TH2, TH17, TH3, TR1 and TRegs, which have been attributed a specific function in the immune system. Classically, these TH subsets can be distinguished by their cytokine production and/or transcription factor expression. As such, TH1 cells are characterized by the ability to produce high levels of IFN-γ and TNF-α, thereby supporting cell-mediated immunity (reviewed in (2)). On the other hand, humoral immunity is linked to TH2 formation and increased secretion of IL-4, IL-5, IL-6, IL-10 and IL-13 (reviewed in (2). Protective anti-bacterial immunity as well as development of autoimmunity is generally linked to an increase in IL-17 producing TH17 cells (3;4). Next to these effector T cell subsets, two inducible regulatory T cell subsets can be identified by their production of IL-10 and TGF-β, which are respectively referred to as TR1 and TH3 cells (5-7). Finally, naturally occurring regulatory T cells (TRegs) are not characterized by their cytokine production, but are generally distinguished by their expression of the transcription factor FoxP3 (8;9). The function of these TReg subsets is to regulate inflammatory responses and to prevent the induction of autoimmunity.
The differentiation of naïve CD4+ T cells towards these different helper T cell lineages is classically driven by polarizing cytokines, which affect the expression and/or function of instructive transcription factors. TH1 polarization occurs subsequent to the production of IL-12 by antigen presenting cells (APCs), which results in the upregulation of the transcription factor T_Bet in T cells (10). In contrast, TH2 formation is enhanced following IL-4 signalling and through the upregulation of the transcription factor GATA-3(11). Commitment of a T cell to the TH17 lineage is induced by the transcription factor RORγt. In mice, this transcription factor is upregulated by the combination of TGF-β and IL-6, whereas in humans the combination of TGF-β and IL-1-β is necessary (4;12-15). Regulatory T cells are induced by increased levels of TGF-β and retinoic acid and result in the upregulation of the transcription factor FoxP3 (16-19).

Although polarizing cytokines clearly fulfill a key function in TH cell formation, costimulatory molecules may also play an important role in T cell differentiation and polarization. Several lines of evidence suggest that engagement of the TNFR superfamily member CD27 by its ligand CD70 enhances TH1 cell development. Whereas CD70 is only transiently expressed on APCs and lymphocytes during immune activation (20-24), we have previously shown that constitutive expression of CD70 on B cells induces a strong increase in the numbers of IFN-γ producing CD4+ and CD8+ T cells, thereby enhancing T cell mediated immunity (25-27). CD27 ligation on human T cells not only drives proliferation of CD4+ T cells, but also TH1 polarization via upregulation of IL-12Rβ2 and T_Bet (28). Moreover, human TNFα-induced CD70+ DCs can evoke TH1, but also TH17 responses, although it was not shown whether these responses are indeed dependent on CD27-engagement (29). Finally, CD27 ligation in mice can induce a TH1-type gene expression profile in CD4+ T cells (30), and can under certain conditions promote TH1 cell formation independently of IL-12 (31).
This would suggest that CD27, as a typical “signal 2”, can directly induce T_H1 cell differentiation without the need for the classical “signal 3”.

Based on these observations, we questioned whether triggering through CD27 provides instructive signals for T_H1 differentiation, or that it rather supports the formation of T_H1 cells. As the genetic background of mice has been associated with a predisposition to T_H cell polarization and disease development (32-35), we decided to approach our question by backcrossing CD70TG mice from a T_H1-prone C57Bl/6J to a T_H2-prone Balb/c background. Our data indicate that the strong T_H1 skewing observed in CD70TG mice is highly dependent on the genetic background, as it does not induce T_H1, nor T_H2 skewing on a Balb/c background. Importantly, CD27 ligation during the induction of allergic airway inflammation (AAI), a typical T_H2 response, enhanced the generation of T_H1 cells without affecting the formation of T_H2 cells. Together with in vitro polarization studies, our data indicate that CD27 does not instruct, but rather supports the formation of T_H1 cells, both in vitro and in vivo.

Results

Strain specific enhancement of T_H1 polarization via CD27 ligation under homeostatic conditions

Ensuing T cell activation, the genetic background predisposes polarization to a specific helper T cell subset (32;38). To determine whether CD27 ligation differentially affects helper T cell polarization depending on the genetic background, we backcrossed the CD70TG C57Bl/6J mice on a Balb/c background and studied the in vivo effects of CD70-driven costimulation. By direct stimulation of WT and CD70TG splenocytes with PMA/ionomycin and brefeldin A, we confirmed that CD70TG C57Bl/6J mice have increased percentages of IFN-γ production by CD4⁺ T cells and decreased production of the T_H2 cytokines IL-5 and IL-13 by CD4⁺ T
Figure 1. **T\(h\) profile of CD70 TG mice on different genetic backgrounds.** Wild type (wt) and CD70 TG C57Bl/6J and Balb/c mice were analyzed for their intrinsic cytokine production capacity directly ex vivo. A representative staining on a (A) C57Bl/6J and (C) Balb/c background for IFN-\(\gamma\), IL-4, IL-5, IL-13 and IL-17 production by CD\(4^+\) T cells in WT and CD70 TG mice after stimulation with PMA/ionomycin is shown. The percentage of cytokine production by WT or CD70 TG derived CD\(4^+\) T cells on a (B) C57Bl/6J (average of 4 mice ± SD) or (D) Balb/c (average of 3 mice ± SD). background. Asterisks denote significant differences (* \(p<0.05\); ** \(p<0.005\)).
cells (Fig. 1A-B). No significant changes were identified for the cytokines IL-4 and IL-17 (Fig. 1A-B). This indicates that CD27 ligation enhances T_{H1} lineage commitment on a C57Bl/6J background (25). In contrast, we found that CD70TG Balb/c mice showed no significant difference in the production of the T_{H1} cytokine IFN-γ by CD4^{+} T cells (Fig. 1C-D). Importantly, constitutive CD27 triggering in Balb/c mice did not enhance T_{H2} differentiation either (Fig. 1C-D). These data indicate that CD27 triggering enhances T_{H1} differentiation in a strain-dependent manner.

**CD27 ligation does not affect the regulatory T cell compartment**

Since CD4^{+} T_{Regs} also express CD27 (39;40), we assessed whether enhanced CD27 triggering by CD70 could affect the formation and/or activation of this distinct T cell subset. This is particularly important because T_{Regs} might also influence T_{H} cell formation. CD4^{+} T_{Regs} are characterized by their expression of the transcription factor FoxP3, high levels of CD25 and can be distinguished into two subsets based on CD103 and CD62L expression (8;9;41). CD70TG mice showed no significant difference in the percentage and absolute numbers of CD4^{+} T_{Regs} for both the C57Bl/6J (Fig. 2A-B) and Balb/c (Fig. 2E-F) mouse strain. The expression of CD27 on T_{Regs} was significantly downregulated in CD70TG mice, indicative of an interaction with CD70 (Fig. 2C/G). However, this did not correlate with an increase in the activation state of these cells, as T_{Regs} in CD70TG C57Bl/6J mice showed a small increase in CD103 expression, but no difference was observed for CD62L and CD25 expression (Fig. 2C-D). In addition, T_{Regs} in CD70TG Balb/c mice did not differ in CD25, CD103 and CD62L expression (Fig. 2G-H). These data indicate that although CD27 is expressed on CD4^{+} T_{Regs}, enhanced ligation through CD70 does not affect the number nor the activation status of these cells.
Figure 2. CD70 driven costimulation does not affect regulatory T cell development.

The regulatory T cell compartment was analysed for WT and CD70 TG C57Bl6J (A-D) and Balb/c (E-H) mice. (A,E) Representative staining for FoxP3 within the splenic CD4⁺ T cell population. (B, F) The percentages and absolute numbers of splenic derived regulatory T cells in mice. (C, G) Representation and (D, H) average expression of the percentage of CD4⁺FoxP3⁺ T cells that express CD27, CD25, CD103 and CD62L, based on gate in respective histograms. Asterisks denote significant differences (* p<0.05; ** p<0.005).
Strain specific enhancement of IFN-γ producing cells via CD27 ligation under non-polarizing conditions

To determine if CD27 ligation provides an instructive, supportive or permissive signal for T_{H1} cell polarization, we performed T cell stimulation assays under specific polarizing conditions using naïve WT T cells and providing CD27 triggering by the addition of either WT or CD70TG B cells. These experiments revealed that CD27 ligation enhanced the formation of IFN-γ producing T cells under non-polarizing (T_{H0}) conditions for C57Bl/6J derived cells (Fig. 3A), but only marginally affected IFN-γ production for Balb/c derived cells (Fig 3B). CD27 ligation did not enhance nor inhibit the specific formation of cytokine producing cells under T_{H1} and T_{H2} polarizing conditions (Fig 3A).

Figure 3. Genetic differences on T cell polarization following CD70 driven T cell costimulation in vitro. T cell polarization assays were performed using WT derived naïve CD4^{+} T cells and WT or CD70 TG derived B cells. T cells were cultured under T_{H0} (non-polarizing), T_{H1} or T_{H17} polarizing conditions for a period of 3 days or under T_{H2} polarizing conditions for 7 days. (A) C57Bl/6J or (B) Balb/c polarized cells were studied for their capacity to produce T_{H1}, T_{H2} or T_{H17} associated cytokines upon PMA-ionomycin stimulation after the specified polarization.
Moreover, CD70 driven costimulation did not affect T cell polarization under T\textsubscript{H1} conditions in experiments using cells obtained from Balb/c mice (Fig 3B). CD27 ligation enhanced the formation of IL-13 producing cells under T\textsubscript{H2} polarizing conditions (Fig 3B), but the induction of IL-13 was less efficient for Balb/c than C57Bl/6J cells. Interestingly, CD27 triggering did inhibit the formation of IL-17 producing cells on both genetic backgrounds (Fig 3A&B). These data indicate that CD27 ligation \textit{in vitro} enhances IFN-\gamma production under non-polarizing conditions, does not have a major effect on T\textsubscript{H1} or T\textsubscript{H2} induction under polarizing conditions, but does inhibit T\textsubscript{H17} induction.

\textbf{CD70 driven costimulation does not inhibit T\textsubscript{H2} polarization during allergic airway inflammation}

As these experiments indicate that CD27 triggering does not induce an instructive signal for T\textsubscript{H1} development, but does affect T\textsubscript{H2} differentiation to some extent, we further examined the impact of CD27 triggering on T\textsubscript{H2} differentiation \textit{in vivo}. Therefore, we used the allergic airway inflammation (AAI) model, which induces T\textsubscript{H2} cells that can amplify allergic inflammation via the production of cytokines, chemokines and enhancing IgE production (reviewed in (42)). Although the pathogenesis of AAI also includes the recruitment of other T cell subsets into the lung, T\textsubscript{H2} cells play an essential role in the inflammatory response (reviewed in (43)). In addition, blockade of the T\textsubscript{H2} cytokines IL-4 and IL-13 by antibodies or neutralizing fusion proteins, respectively, resulted in reversing and/or preventing allergen-induced airway hyperresponsiveness during sensitization and challenge phases (44-47). We found that both WT and CD70TG Balb/c mice had a significant infiltration of CD4\textsuperscript{+} T cells in the lung and thus allowed us to investigate the effect of CD27 ligation on T\textsubscript{H} cell polarization (Fig. 4A). WT mice showed increased numbers of IL-4, IL-13 and IFN-\gamma producing CD4\textsuperscript{+} T cells in the lung of OVA challenged mice compared to PBS-treated mice (Fig. 4B).
Importantly, CD70TG mice showed normal numbers of IL-4 and IL-13 producing CD4⁺ T cells, but a significant increase of IFN-γ producing CD4⁺ T cells compared to challenged WT mice (Fig. 4B). This indicates that even in a TH2 inflammation model, CD27 stimulation in vivo enhances TH1 cell formation while permitting TH2 cell polarization.

Figure 4. CD70 TG mice show parallel TH1 and TH2 polarization during allergic asthma model.

Allergic airway inflammation (AAI) was induced in WT and CD70 TG Balb/c mice to specifically promote TH2 polarization. (A) Absolute total number of infiltrated pulmonary CD4⁺ T cells on day 32 of AAI induction. (B) The absolute number of pulmonary CD4⁺ T cells which produce IL-4, IL-13 and IFN-γ in WT (average of 8 mice ± SD) and CD70 TG Balb/c (average of 7 mice ± SD) mice. Asterisks denote significant differences (* p<0.05; ** p<0.005).

CD70 driven transcription factor regulation

To investigate how CD27 ligation affects CD4⁺ T cell differentiation on a molecular level, we examined the impact of CD70-driven costimulation on transcription factor expression, as changes on this level would be expected if CD27 ligation would provide instructive signals
for Th cell formation, the transcription factor T\textsubscript{Bet} is the main transcription factor associated with Th\textsubscript{1} polarization. T\textsubscript{Bet} induces the expression of IL-12Rβ2, thereby allowing cells to differentiate to Th\textsubscript{1} cells following IL-12 signaling (10;48;49). We found that T\textsubscript{Bet} mRNA is induced under Th\textsubscript{1} polarizing conditions compared to naïve T cells, maintained under Th\textsubscript{1} polarizing conditions, but downregulated under Th\textsubscript{2} or Th\textsubscript{17} conditions. Importantly, CD27 ligation did not affect mRNA expression of T\textsubscript{Bet} under these conditions (Fig. 5A). GATA3 is the central transcription factor responsible for Th\textsubscript{2} polarization (11), and is essential for the cytokine profile associated with Th\textsubscript{2} polarized cells (11;50;51). We found that GATA3 transcript levels remained similar to levels found in naïve T cells under Th\textsubscript{0} conditions, were downregulated under Th\textsubscript{1} and Th\textsubscript{17} conditions, and were upregulated under Th\textsubscript{2} conditions. However, CD27 triggering did not affect the GATA3 transcription expression levels under any conditions (Fig. 5B). The transcription factor RORγt, which is important for Th\textsubscript{17} lineage commitment (15), was only found under Th\textsubscript{17} conditions and was not regulated by CD27 stimulation (Fig. 5C). The transcription factors TWIST and FOG have been implicated in a negative feedback loop for Th\textsubscript{1} and Th\textsubscript{2} polarization, respectively (52-54). We found that both factors were downmodulated with respect to naïve T cells under all polarizing conditions and that CD27 ligation did not affect the respective transcription factor expression levels (Fig. 5D-E). Finally, we investigated the transcription factor Blimp-1. Expression of Blimp-1 has been associated with increased Th\textsubscript{2} polarization through active repression of Th\textsubscript{1} associated genes, such as T\textsubscript{Bet} and IFN-γ (55). We found that Blimp-1 expression remained similar to naïve T cells in cells cultured under Th\textsubscript{1} polarizing conditions and was downregulated under all other conditions. However, enhanced signalling through CD27 did not influence Blimp-1 expression in any culture conditions (Fig. 5F).
Thus, we conclude that CD27 triggering does not influence the expression of instructive transcription factors, which is in line with the notion that CD27 supports, but does not instruct the formation of IFN-γ producing CD4+ T cell following T cell activation.

**Figure 5. Enhanced CD70 driven IFN-γ production is not transcriptionally regulated.**

Naïve CD4+ T cells were stimulated under specific T H0, T H1, T H2 and T H17 polarizing conditions in the presence of WT or CD70 TG C57Bl/6J derived B cells. Transcript levels encoding (A) TBet, (B) GATA3, (C) RORyγt, (D) TWIST, (E) FOG and (F) Blimp were analysed by qPCR, normalized with HPRT for each condition and are depicted as the number of copies of the respective transcript per HPRT transcript.
Discussion

In the present study, we show that although CD70-overexpression strongly promotes the formation of IFN-γ producing CD4⁺ T cells, costimulation through CD27 does not induce differentiation of TH1 cells per se. Instead, CD27 seems to support TH1 cell formation, but this is dependent on the culture conditions and genetic background of the mice. The latter has been associated with a predisposition towards TH1 or TH2 polarization, i.e. C57Bl/6J mice are more prone towards TH1 cell development, whereas Balb/c mice are more TH2 prone. This variance in polarization can be attributed to a difference in a dominant genetic locus between the different genetic backgrounds, but could also be related to differences in chromatin remodeling subsequent to receptor signaling (56). In this respect, it has been suggested that Balb/c mice are less capable of sustaining responsiveness to IL-12 compared to C57Bl/6J mice, thereby decreasing their TH1 polarizing capacity (57;58). Although it is unknown which genetic differences are responsible for the distinct phenotypes of C57Bl/6J and Balb/c CD70TG mice, it could be that these differences are associated with the enhanced availability of the TH1 locus in C57Bl/6J mice. As there is some IFN-γ production during AAI even in Balb/c mice, this would suggest that the TH1 locus is accessible during these conditions and that this might be the reason why CD27 ligation is capable of enhancing the formation of IFN-γ producing CD4⁺ T cells (Fig. 4).

Whereas CD70 clearly stimulates TH1 cell development, it has also been shown that TNFα-induced CD70⁺ DCs can induce Th17 responses in humans (29). Our data clearly indicate that CD27 triggering in murine T cells inhibit formation of TH17 cells in vitro, but it is not yet clear what the underlying mechanism of this inhibition is. Our transcription factor analysis would argue against a direct effect of CD27 ligation on the expression of RORγt in this setting (Fig. 5C). Instead, it is more likely that the CD27-mediated increase in IFNγ production affects TH17 cell formation, as it has been well documented that loss of IFNγ secretion
promotes T\(_{H17}\) formation, whereas IFN\(_{\gamma}\) negatively can directly inhibit formation of the T\(_{H17}\) lineage (reviewed in (59)). Nevertheless, our data raise the question if CD70 overexpression, which enhances anti-viral immunity (27), would actually inhibit anti-bacterial responses? We have not yet been able to addressed this question, but we have previously shown that absence of CD27 at least does not affect the outcome of an infection with Mycobacterium tuberculosis (60). Whether the number of IL-17 producing cells was affected in these CD27-deficient mice during this infection was not investigated in this study.

As T cell immunity can be seen as a balance between activation and regulation, we postulated that CD27 signalling could influence T\(_{Reg}\) numbers and/or their activation state. Importantly, other TNFR superfamily members have been shown to influence T\(_{Reg}\) numbers and/or function. GITR is capable of promoting T\(_{Reg}\) proliferation without impairing its regulatory function (Unpublished observation and (61)), whereas OX40 inhibits the induction of regulatory T cells from effector T cells (62). We found that T\(_{Reg}\) express CD27, and that transgenic overexpression of CD70 resulted in a significant reduction of membrane bound CD27, indicative for an interaction with CD70. However, both the activation state and absolute numbers of regulatory T cells were not affected in CD70TG mice on both the C57Bl/6J and Balb/c background. Thus, these data suggest that regulatory T cells are not accountable for the differences observed in helper T cell formation between C57Bl/6J and Balb/c mice.

Classically, the generation of IFN-\(\gamma\) producing T\(_{H1}\) polarized cells occurs following the upregulation and activation of the transcription factor T\(_{BET}\). T\(_{BET}\) activation results in an upregulation of IL-12R\(\beta2\), and the subsequent formation of active IL-12R. In addition, T\(_{BET}\) plays an important role in chromatin remodeling (63), thus allowing transcription of T\(_{H1}\) dependent genes. Steinman \textit{et al.} (31) showed that CD27 ligation could induce formation of IFN-\(\gamma\) producing CD4\(^+\) T cells in an IL-12 independent manner, suggesting that CD27
signalling can promote helper T cell polarization independently of the classical polarization via the cytokine environment. These observations are congruent with our results showing that CD27 ligation enhances formation of IFN-γ producing CD4⁺ T cells under non-polarizing conditions in vitro (Fig. 3). However, we also show that the T_H1 polarizing effects of CD27 are not due to changes of T_Bet expression levels (Fig. 5), though the effects of CD27 ligation on the function of T_Bet are not known. A possible mechanism for the observed effects could be the specific modulation of proliferation and/or survival via CD70 driven costimulation. CD27 has been described to enhance expansion of TCR stimulated CD8⁺ T cells in an IL-2 independent manner without affecting differentiation and cytokine production (64). In addition, it has also been shown that CD27 ligation can promote expression of the anti-apoptotic protein Bcl-Xl in human CD4⁺ T cells (28). Therefore, it remains possible that CD27 triggering stimulates proliferation and/or survival of T_H1 polarized cells, thereby inducing the accumulation of IFN-γ producing T cells. In line with this, ligation of OX40, another member of the TNFR superfamily, can also induce proliferation and survival of CD4 T cells and thereby enhance the pool of T_H1 (65;66). However, the difference with CD27 is that this costimulatory effect of OX40 is not specific for T_H1 cells, as it can also enhance the pool of T_H2 cells, depending on the model (67-69).

In conclusion, these data indicate that CD27 signalling specifically enhances the pool of IFN-γ producing CD4⁺ T cells, not by providing instructive polarizing signals, but most likely by the combination of sensitizing these cells for IL-12 mediated signaling and by acting on the proliferation and/or survival of these cells.
Methods

Mice

CD70TG mice were generated on a C57Bl/6J background and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions (25). To generate CD70TG Balb/c mice, mice were backcrossed 10x with WT Balb/c mice (Harlan). WT mice were obtained from CD70TG C57Bl/6J or Balb/c littermates. Mice were used at 6-12 weeks of age, age- and sex-matched within experiments and were handled in accordance with institutional and national guidelines.

Cell staining and flow cytometry

Single-cell suspensions were obtained by mincing the specified organs through 40 µm cell strainers (Becton Dickinson). Erythrocytes were lysed with an ammonium chloride solution and cells were subsequently counted using an automated cell counter (Casy, Schärfe system). Cells (5 x 10^5- 5 x 10^6) were collected in staining buffer (PBS with 0.5% bovine serum albumin (Sigma)) and stained for 30 min at 4°C with antibodies in the presence of anti-CD16-CD32 (FcBlock, clone 2.4G2; kind gift from Dr. Louis Boon, Bioceros, The Netherlands). The following monoclonal antibodies were obtained from Pharmingen: allophycocyanin-conjugated (APC) anti-B220 (clone RA3-6B2); peridinin chlorophyll protein-conjugated (PerCP) anti-CD3ε (clone 145-2C11); Fluorescein isothiocyanate-conjugated (FITC) anti-CD3ε (clone 17A2); PE- or PerCp-conjugated anti-CD4 (clone L3T4); PerCp-, FITC or APC-conjugated anti-CD8 (clone Ly-2); PE- or APC- conjugated anti-CD62L (clone MEL-14). Antibodies used from eBioscience: PE-conjugated anti-FoxP3 (clone NRRF-30); FITC-conjugated anti-CD44 (clone IM7); FITC-conjugated anti-CD27 (clone LG.7F9). Intracellular stainings for FoxP3 were performed using Foxp3 Fixation/Permeabilization Concentrate and Diluent (eBioscience), according to the manufactures protocol. Data were collected on a
FACSCalibur or FACSCanto (Becton Dickinson) and were analyzed with FlowJo software (Treestar, Inc.).

**T cell stimulation assays**

*Direct ex vivo cytokine production.* Splenocytes were plated at 1 x 10⁶ cells/well in a 96-well round-bottom plate and stimulated for 2 hours with 1 ng/ml PMA and 1 μM ionomycin. After 2 hours the protein-secretion inhibitor Brefeldin A was added at 1 μg/ml final concentration (Sigma). Hereafter, cells were stained for CD4 and CD8 followed by fixation and permeabilization. Cells were then incubated for 30 min with fluorescent labelled antibodies against either IFN-γ, IL-17, IL-4, IL-5, IL-13 (eBioscience/BD).

*Th cell polarization.* Naïve (CD44⁻CD62L⁺) CD4⁺ T cells and B (B220⁺) cells were electronically gated and sorted using a FACSAria cell sorter (Becton Dickinson). The purity of cells sorted using this method was consistently > 96%. Sort purified naïve CD4⁺ T cells from WT mice were then stimulated for 3 days under Th0, Th1 or Th17 polarizing conditions, or 7 days under Th2 polarizing conditions, in the presence of WT or CD70TG derived B cells in a 1:1 ratio. All T cell polarization conditions included plate-bound γ302CD3 (clone 145-2C11, 5 μg/ml), soluble αCD28 (clone PV-1, 1 μg/ml) (both a kind gift from Dr. Louis Boon, Bioceros, The Netherlands) and soluble IL-2 (25 ng/ml) (Invitrogen). For Th1 polarization 10 ng/ml IL-12 (R&D Systems) and 5 μg/ml αIL-4 (clone 11B11, a kind gift from Louis Boon, Bioceros) was added. For Th2 polarization 50 ng/ml IL-4 (R&D Systems), 5 μg/ml αIL-12 (clone c17.8) and 20 μg/ml αIFN-γ (clone XMG 1.2, both mAbs were a kind gift from Louis Boon, Bioceros) was added. For Th17 polarization, 3 ng/ml TGF-β (R&D Systems) and 20 ng/ml IL-6 (Peprotech) was included. Following stimulation, cells were stimulated with 1 μM ionomycin, 1 ng/ml PMA and 1 μg/ml Brefeldin A for 5 hours. Cells were then stained for
CD4 and CD8 followed by fixation and permeabilization and stained for IL-4, IL-5, IFN-γ, IL-17, IL-10 and IL-13 as described above.

Allergic Airway inflammation

Wild type and CD70TG Balb/c mice were sensitized to OVA by i.p injection of 20 µg OVA (Fluka, Switzerland) in a 200 µl alumimun potassium sulfate suspension (Sigma-Aldrich, Germany) on day 0 and 14 (36). Mice were then challenged on day 28, 29 and 30 by i.n. administration of 100 µg OVA in 50 µl of PBS. Control sensitization was performed with aluminum potassium sulfate in PBS and control challenge was performed with PBS alone. All mice were sacrificed on day 32 and serum, spleen and lung was collected.

Quantitative real-time PCR (qPCR) analysis

RNA was isolated from naïve T cells and polarized cells (as described above) using TRIzol (Invitrogen), and cDNA was prepared by reverse transcription of 0.5 µg RNA. The resulting cDNA was subjected to qPCR analysis with the LightCycler System (Roche Diagnostics) in microcappilary tubes with a QuantiTect SYBR Green PCR kit solution (Qiagen). HPRT was used as a reference. Relative changes were calculated by the 2^ΔΔCT method (37). The primers used to detect mRNA transcripts are as follows: mTBet, 5’-CAACAACCCC TTTGCCAAAG-3’ (forward) and 5’-TCCCCCAAGCAGTGACAGT-3’ (reverse); mGATA3, 5’-AGAACCAGCCCTTATCAA-3’ (forward) and 5’-AGTTCGCGC AGGATGTCC-3’ (reverse); mRORγt, 5’-TGCTCCTGG GCTACCCTACTG-3’ (forward) and 5’-GTGCAGGAGTAGGCCACATT-3’ (reverse); mTWIST, 5’-CGCACGCGTGCTGACG-3’ (forward) and 5’-GACGCGGACATGGACG-3’ (reverse); mFOG, 5’-TCCCCTG AGAGAGAAACCG-3’ (forward) and 5’-GGCATTCTTGGGA
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ACTGTGT-3’ (reverse); mHPRT, 5’-TGAAGAGCTACTGTAATGATCAGTCAAC-3’ (forward) and 5’-AGCAAGCTTGCAACCTTAACCA-3’ (reverse).

Statistical analysis

Statistical analysis of the data was performed using the unpaired Student’s $t$-test. Asterisks denote significant differences (* $p<0.05$, ** $p<0.005$).

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Reference List


CD27 Supports Th1 Differentiation


