Function of CD27 in 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-γ-producing CD4+ T cells and we therefore questioned whether CD27 triggering provides an instructive signal for T_H1 differentiation or rather supports T_H cell formation in general. Although CD70TG mice on a T_H1-prone C57Bl/6J background develop more T_H1 cells, we found that this phenotype is lost when CD70TG mice are fully backcrossed on a T_H2-prone Balb/c background, but is not replaced with more T_H2 cells. Furthermore, CD70-overexpression is not sufficient to drive T_H17 cell formation, nor does it affect the generation of FoxP3+ regulatory T cells. Using an in vitro setting, we found that CD27-triggering does not provide instructive signals for a specific T_H cell subset, but, depending on the cytokine milieu and genetic background, supports T_H1 cell formation, while it inhibits the formation of T_H17 but not T_H2 cells. Induction of allergic airway inflammation in CD70TG Balb/c mice further illustrates that CD27 plays a supportive role in T_H1 differentiation in vivo, without modulating the classical T_H2 response. This supportive role of CD27 in T_H cell polarization could not be attributed to a specific change of transcription factor expression levels. In summary, this study indicates that CD27 signaling does influence T_H cell differentiation, but that it is highly dependent on the conditions and genetic background.
Function of CD27 in T helper cell differentiation

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. 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, Th1 and Threg, 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. 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. Protective anti-bacterial immunity as well as development of autoimmunity is generally linked to an increase in IL-17 producing Th17 cells. 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 Th1 and Th3 cells. Finally, naturally occurring regulatory T cells (Threg) are not characterized by their cytokine production, but are generally distinguished by their expression of the transcription factor FoxP3. The function of these Threg 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 TBet in T cells. In contrast, Th2 formation is enhanced following IL-4 signalling and through the upregulation of the transcription factor GATA-3. 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-1β is necessary. Regulatory T cells are induced by increased levels of TGF-β and retinoic acid and result in the upregulation of the transcription factor FoxP3. 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, 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.

In addition, microarray analysis of activated effector-type CD4+ T cells from WT vs CD27-deficient mice revealed that CD27-triggering can induce a Th1-like gene expression profile. In human T cells, CD27 ligation drives proliferation of CD4+ T cells, but also Th1 polarization via upregulation of IL-12Rβ2 and TBet. Moreover, human TNF-α-induced CD70+ DCs can
evoke T\(_h\)1, but also T\(_h\)17 responses, although it was not shown whether these responses are indeed dependent on CD27-engagement\(^{31}\). Finally, CD27 ligation in mice can under certain conditions promote T\(_h\)1 cell formation independently of IL-12\(^{32}\). Overall, these data suggest that CD27, as a typical "signal 2", can directly induce T\(_h\)1 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\(_h\)1 differentiation, or that it rather supports the formation of T\(_h\)1 cells. As the genetic background of mice has been associated with a predisposition to T\(_h\)1 cell polarization and disease development\(^{33-36}\), we decided to approach our question by backcrossing CD70TG mice from a T\(_h\)1-prone C57Bl/6J to a T\(_h\)2-prone Balb/c background. Our data indicate that the strong T\(_h\)1 skewing observed in CD70TG mice is highly dependent on the genetic background, as it does not induce T\(_h\)1, nor T\(_h\)2 skewing on a Balb/c background. Importantly, CD27 ligation during the induction of allergic airway inflammation (AAI), a typical T\(_h\)2 response, enhanced the generation of T\(_h\)1 cells without affecting the formation of T\(_h\)2 cells. Together with in vitro polarization studies, our data indicate that CD27 does not instruct, but rather supports the formation of T\(_h\)1 cells, both in vitro and in vivo.

**Materials and Methods**

**Mice**

CD70TG mice were generated on a C57Bl/6J background, maintained heterozygously and bred in the animal department of the Academic Medical Center (Amsterdam, The Netherlands) under specific-pathogen-free conditions\(^{26}\). To generate CD70TG Balb/c mice, mice were backcrossed 10x with wild type (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: allophycocynanin-conjugated 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 allophycocynanin-conjugated anti-CD8 (clone Ly-2); PE- or allophycocynan-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 10^6 cells/well in a 96-well round-bottom plate and stimulated for 6 hours with 1 ng/ml PMA and 1 mM ionomycin, of which the last 4 hours was in the presence of 1 ng /ml Brefeldin A (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).

**T:\_ 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 T\_0, T\_1 or T\_17 polarizing conditions, or 7 days under T\_2 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 αCD3 (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 T\_1 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 T\_2 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 T\_17 polarization, 3 ng/ml TGF-β (R&D Systems) and 20 ng/ml IL-6 (Peprotech) was included. At the end of the culture period, cells were washed and 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. 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.
The primers used to detect mRNA transcripts are as follows:

\[
\begin{align*}
\text{mTBet:} & \quad 5'-\text{CAACAACCCCTTTGCCAAAG}-3' \quad (\text{forward}) \\
& \quad 5'-\text{TCCCCCAAGCAGTTGACAGT}-3' \quad (\text{reverse}); \\
\text{mGATA3:} & \quad 5'-\text{AGAACCGGCCCTTATCAA}-3' \quad (\text{forward}) \\
& \quad 5'-\text{AGTTCGCGCAGGATGTCC}-3' \quad (\text{reverse}); \\
\text{mRORγt:} & \quad 5'-\text{TGTCCTGGGCTACCCTACTG}-3' \quad (\text{forward}) \\
& \quad 5'-\text{GTGCAGGAGTAGGCCACATT}-3' \quad (\text{reverse}); \\
\text{mTWIST:} & \quad 5'-\text{CGCACGCAGTCGCTGAACG}-3' \quad (\text{forward}) \\
& \quad 5'-\text{GACGCGGACATGGACCAGG}-3' \quad (\text{reverse}); \\
\text{mFOG:} & \quad 5'-\text{TCCCCTGAGAGAGAAGAACCG}-3' \quad (\text{forward}) \\
& \quad 5'-\text{GCAGCATCCTAGCCAGCA}-3' \quad (\text{reverse}); \\
\text{mHPRT:} & \quad 5'-\text{TGAAGAGCTACTGTAATGATCAGTCAAC}-3' \quad (\text{forward}) \\
& \quad 5'-\text{AGCAAGCTTGGCAACCTAACCA}-3' \quad (\text{reverse}).
\end{align*}
\]

**Statistical analysis**

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

**Results**

**Transgenic CD70 expression on B cells on a C57Bl/6J and Balb/c background**

Ensuing T cell activation, the genetic background predisposes polarization to a specific helper T cell subset\(^{33,39}\). 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 to study the *in vivo* effects of CD70-driven costimulation. Flow cytometric analysis confirmed high CD70 expression on transgenic B cells in both backgrounds, though expression was slightly lower in Balb/c mice (Fig. 1a). CD70 overexpression induced a significantly downregulation of CD27 in all T cells from CD70TG mice in both strains (Fig. 1b), which is indicative of a productive interaction between CD27 and CD70. The functional consequence of this enhanced CD27-mediated costimulation is an increase in effector-memory T cell (CD44\(^{hi}\) CD62L\(^{lo}\)) formation in CD4 and CD8 T cells\(^{26}\), though this was more pronounced in C57Bl/6J mice than in BALB/c mice (Fig. 1c-d). Although the phenotype of CD70TG mice seemed less severe on a BALB/c background, the degree of B cell depletion\(^{26}\) was comparable to C57Bl/6 mice (Fig. 1e). Thus, enhanced CD27-driven costimulation induces strong effector T cell formation and a concomitant loss of B cells, both on a C57Bl/6J and Balb/c background.

**Strain specific enhancement of T$_{H1}$ polarization via CD27 ligation under homeostatic conditions**

To investigate the impact of CD27-triggering on T$_{H1}$-cell polarization, we analyzed the cytokine profile of CD4 T cells by direct stimulation of WT and CD70TG splenocytes with PMA/ionomycin and brefeldin A. Based on these experiments, we conclude that CD70TG C57Bl/6J mice have increased percentages and absolute numbers of IFN-γ production by
CD4⁺ T cells and decreased production of the TH2 cytokines IL-5 and IL-13 by CD4⁺ T cells (Fig. 2a-b). No significant changes were identified for IL-17 and a small increase in the absolute, but not relative number of IL-4 producing T cells (Fig. 2a-b). In contrast with C57Bl/6 mice, we found no significant difference in IFN-γ-producing CD4⁺ T cells in CD70TG mice on a Balb/c background (Fig. 2c-d). Importantly, constitutive CD27 triggering in Balb/c mice did not enhance TH2 differentiation either, but even reduced the levels of IL-5 and IL-13 producing CD4⁺ T cells (Fig. 2c-d). These data demonstrate that in the steady state situation CD27 triggering reduces TH2 differentiation and enhances TH1 differentiation, but the latter only on a TH1-prone background.

CD27 ligation does not affect the regulatory T cell compartment
Since regulatory T cells also express CD27⁴¹, we assessed whether enhanced CD27 triggering by CD70 could affect the formation and/or activation of this distinct T cell subset.

Figure 1. Impact of CD70-overexpression in C57Bl/6J and Balb/c mice.
CD70 expression on B220⁺ B cells (a) and CD27 expression on CD3⁺ T cells (b) in WT and CD70TG spleens from C57Bl/6J or Balb/c mice. Control in b represents background staining on T cells without the CD27 antibody. Quantification of TEM cells (CD44⁺CD62L⁻) in CD4 (c) or CD8 (d) T cells in WT and CD70TG spleens from C57Bl/6J or Balb/c mice. (e) Quantification of the relative (left) and absolute (right) number of B220⁺ B cells in WT and CD70TG spleens from C57Bl/6J or Balb/c mice.
Figure 2. T<sub>p</sub> profile of CD70TG mice on different genetic backgrounds.

WT and CD70TG 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-γ, IL-4, IL-5, IL-13 and IL-17 production by CD4<sup>+</sup> T cells in WT and CD70TG mice after stimulation with PMA/ionomycin is shown. The percentage of cytokine producing cells by WT or CD70TG derived CD4<sup>+</sup> 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).
This is particularly important because T_{Reg} might also influence T_H cell formation. Regulatory T cells 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^{9,10,42}. CD70TG mice showed no significant differences in the percentage and absolute numbers of CD4^+ regulatory T cells on a C57Bl/6J background (Fig. 3a-b), though numbers were reduced in Balb/c mice (Fig. 3e-f). The expression of CD27 on regulatory T cells was significantly downregulated in CD70TG mice (Fig. 3c,g). However, this did not correlate with an increase in the activation state of these regulatory T cells. CD70TG C57Bl/6J mice showed a small increase in CD103 expression, but no difference was observed for CD62L and CD25 expression (Fig. 3c-d). In addition, CD70TG Balb/c mice did not show relevant changes in expression of CD25, CD103 and CD62L (Fig. 3g-h). These data indicate that although CD27 is expressed on T_{Reg}, enhanced ligation through CD70 does not affect the activation status of these cells. Overall, the observed changes in T_{Reg} homeostasis in CD70TG mice on a C57Bl/6 vs. Balb/c background do not explain the respective changes in effector CD4^+ T cell profile.

Strain-specific enhancement of IFN-γ producing cells via CD27 ligation under non-polarizing conditions
The cytokine profile of WT and CD70TG splenocytes indicated that CD27-costimulation has a stimulating effect on the formation of T_{H1} cells, at least in C57Bl/6 mice, and an inhibitive effect on T_{H2} cells on both backgrounds (Fig. 2). However, since the conditions and timing under which these T cells were initially activated is unknown, these data do not allow us to conclude whether CD27 triggering provides instructive or supportive signals for T_H cell polarization. Therefore 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. Based on two independent experiments we conclude that that CD27 ligation enhanced the formation of IFN-γ producing T cells under non-polarizing (T_H0) conditions for C57Bl/6J derived cells, but not for Balb/c derived cells (Fig. 4). IL-4 production was not affected under these conditions. Importantly, CD27 ligation did neither enhance nor inhibit the formation of IFN-γ or IL-4 producing cells under T_H1 and T_H2 polarizing conditions in both genetic backgrounds (Fig. 4). Interestingly, CD27 triggering did reproducibly inhibit the formation of IL-17 producing T cells derived from C57Bl/6 mice, but this was not consistently found with Balb/c-derived T cells (Fig. 4). These data indicate that CD27 ligation provides supportive signals for IFN-γ production under non-polarizing conditions, but repressive signals for IL-17 production; however, this could only be concluded for C57Bl/6-derived cells. Because of this strain-specific effect and because CD27-triggering did not affect T cell differentiation under T_H1 or T_H2 conditions, we conclude that CD27-mediated costimulation does not provide instructive signals for T_H cell polarization.

CD70-driven costimulation does not inhibit T_H2 polarization during allergic airway inflammation
Although CD27-costimulation in vitro does not instruct T_H cell development, this does not explain why CD70TG mice had significantly fewer T_H2 cells (Fig. 2). To test whether CD27 triggering inhibits T_H2 formation in vivo, we exposed WT and CD70TG BALB/c mice to the allergic airway inflammation (AAI) model. This model induces T_H2 cells that amplify allergic inflammation via the production of cytokines, chemokines and enhancing IgE production^{43}. 
Figure 3. Impact of CD70-driven costimulation on regulatory T cells.
The regulatory T cell compartment was analysed for WT and CD70TG 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) quantification of CD4+FoxP3+ T cells that express CD27, CD25, CD103 and CD62L, based on the gate in the respective histograms. Asterisks denote significant differences (* p<0.05; ** p<0.005).
Figure 4. Impact of CD70 triggering on T cell polarization in vitro.

T cell polarization assays were performed using WT derived naïve CD4+ T cells and WT or CD70TG derived B cells. T cells were cultured under TH0 (non-polarizing), TH1 or TH17 polarizing conditions for a period of 3 days or under TH2 polarizing conditions for 7 days. C57Bl/6J or Balb/c polarized cells were studied for their capacity to produce TH1, TH2 or TH17 associated cytokines upon PMA-ionomycin stimulation after the specified polarization. This experiment was performed twice with similar outcome.

Although the pathogenesis of AAI also includes the recruitment of other T cell subsets into the lung, TH2 cells play an essential role in the inflammatory response. In addition, blockade of the TH2 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. We found that both WT and CD70TG Balb/c mice had a significant infiltration of CD4+ T cells in the lung, which allowed us to investigate...
the effect of CD27 ligation on T<sub>H</sub> cell polarization in vivo (Fig. 5a). WT mice showed increased numbers of IL-4, IL-13 and IFN-γ producing CD4<sup>+</sup> T cells in the lung of OVA challenged mice compared to PBS-treated mice (Fig. 5b). Importantly, CD70TG mice showed normal numbers of IL-4 and IL-13 producing CD4<sup>+</sup> T cells and rather unexpectedly even showed a significant increase of IFN-γ producing CD4<sup>+</sup> T cells compared to challenged WT mice (Fig. 5b). Thus, we conclude that CD27-mediated costimulation does not inhibit T<sub>H</sub> subspecies formation, neither in vitro nor in vivo, though it can under certain circumstances promote T<sub>H1</sub> differentiation.

**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 T<sub>H</sub><sub>1</sub> cell formation. The transcription factor T<sub>Bet</sub> is the main transcription factor associated with T<sub>H</sub><sub>1</sub> polarization. T<sub>Bet</sub> induces the expression of IL-12Rβ2, thereby allowing cells to differentiate to T<sub>H</sub><sub>1</sub> cells following IL-12 signalling<sup>11,49,50</sup>. We found that T<sub>Bet</sub> mRNA is induced under T<sub>H</sub><sub>0</sub> polarizing conditions compared to naïve T cells, maintained under T<sub>H</sub><sub>1</sub> polarizing conditions, but downregulated under T<sub>H</sub><sub>2</sub> or T<sub>H</sub>17 conditions. Importantly, CD27 ligation did not affect mRNA expression of T<sub>Bet</sub> under these conditions (Fig. 6a). GATA3 is the central transcription factor responsible for T<sub>H</sub>2 polarization<sup>12</sup>, and is essential for the cytokine profile associated with T<sub>H</sub>2 polarized cells<sup>12,51,52</sup>. We found that GATA3 transcript levels remained similar to levels found in naïve T cells under T<sub>H</sub>0 conditions, were downregulated under T<sub>H</sub>1 and T<sub>H</sub>17 conditions, and were upregulated under T<sub>H</sub>2 conditions. However, CD27 triggering did not affect the GATA3 transcription expression levels under any conditions (Fig. 6b). The transcription factor RORγt, which is important for T<sub>H</sub>17 lineage commitment<sup>16</sup>, was only found under T<sub>H</sub>17 conditions and was not regulated by CD27 stimulation (Fig. 6c). The transcription factors TWIST and FOG have been implicated in a negative feedbackloop for...
T\textsubscript{H}1 and T\textsubscript{H}2 polarization, respectively\textsuperscript{53-55}. 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. 6d-e). 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\textsuperscript{+} T cell following T cell activation.

**Figure 6. Enhanced CD70-driven IFN-γ production is not transcriptionally regulated.**

Naïve CD4\textsuperscript{+} T cells were stimulated under specific TH0, TH1, TH2 and TH17 polarizing conditions in the presence of WT or CD70TG C57Bl/6J derived B cells. Transcript levels encoding (a) TBet, (b) GATA3, (c) RORγt, (d) TWIST, and (e) FOG were analysed by qPCR, normalized with HPRT for each condition and depicted as the number of copies per HPRT transcript. This experiment was performed twice with similar outcome.
Chapter 2

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 T⁺₁ cells per se. Instead, CD27 seems to support T⁺₁ 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 T⁺₁ or T⁺₂ polarization, i.e. C57Bl/6J mice are more prone towards T⁺₁ cell development, whereas Balb/c mice are more T⁺₂ 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 remodelling subsequent to receptor signalling. 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 T⁺₁ polarizing capacity. 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 epigenetic regulation of the Th1 locus plays an important role in this process. It has been reported that CD27-triggering on CD4 T cells downregulates expression of Prmt1, an enzyme that methylates histones. If CD27-costimulation influences the Th1 locus, this could explain why the effects at steady state are more clear in C57Bl/6 mice (Fig. 2), because this locus might be less accessible in BALB/c mice. The fact that there is IFN-γ production during AAI even in BALB/c mice implies that the Th1 locus does become accessible during these conditions, which could be the reason why CD27 ligation is capable of enhancing the formation of IFN-γ producing CD4⁺ T cells also in this model (Fig. 5). It could also be that the CD27-costimulatory pathway acts in concert with transcription factors involved in Th1 formation and is therefore more effective on the C57Bl/6 background. Moreover, it has been shown that CD27 exerts its costimulatory effect by enhancing T cell proliferation and/or survival, though it is not clear if these effects are relevant for our current study, as it is difficult to comprehend why such effects would be dependent on a particular T cell subset and the genetic the background of the mice. In comparison, 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⁺₁, but also of T⁺₂ cells, depending on the model. More molecular studies are required to understand the genetic differences between C57Bl/6J and Balb/c mice regarding Th1 cell formation and how CD27-mediated costimulation influences this process.

Whereas CD70 clearly stimulates T⁺₁ cell development, it has also been shown that TNF-α-induced CD70⁺ DCs can induce Th17 responses in humans. Our data clearly indicate that CD27 triggering in T cells, at least on the C57Bl/6J background, inhibits the formation of T⁺₁ cells in vitro (Fig. 4). It could well be that this is due to the enhanced IFN-γ secretion, since loss of IFN-γ secretion promotes T⁺₁7 formation, whereas IFN-γ negatively affects formation of the T⁺₁7 lineage. This also correlates with the strain-specific effect, since the strong increase on IFN-γ production was more profound on cells from C57Bl/6J than Balb/c mice. Interestingly, it has recently been shown in γδ T cells that CD27-positive cells produce IFN-γ and that IL-17 production is restricted to CD27-negative cells, also indicating that CD27-signaling and IL-17 production are not compatible. IL-17 producing T cells were not significantly altered in CD70TG mice, but numbers were very low and it would therefore be important to induce T⁺₁7 formation in these mice, for instance by bacterial infection, to examine the impact of CD27-triggering on IL-17 production in vivo. We have previously
shown that absence of CD27 does at least not affect the outcome of a bacterial infection with the intracellular pathogen Mycobacterium tuberculosis, but IL-17 production by CD4 T cells was unfortunately 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, whereas OX40 inhibits the induction of regulatory T cells from effector T cells. We found that T.Regs 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 in C57Bl/6J mice, though T.Reg numbers were modestly reduced on the Balb/c background. Nevertheless, these changes do not correlate with the observed changes in CD4 effector T cells, suggesting 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-γ 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-12Rβ2, and the subsequent formation of active IL-12R. In addition, T.Bet plays an important role in chromatin remodeling, thus allowing transcription of T.H1 dependent genes and instructing T.H1 cell formation. Steinman et al. showed that CD27 ligation could enhance formation of IFN-γ 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. Although the underlying mechanism was not investigated in that study, the findings are congruent with the hypothesis that CD27 triggering does not instruct, but rather supports T.H1 cell formation. This supporting effect is substantiated by the finding that CD27-triggering does not affect T.Bet expression levels (Fig. 6). It has been reported that IFN-γ can enhance T.Bet expression, but this feedback system might not be relevant in this system, as T.Bet levels were also comparable between T.H0 and T.H1 cells (Fig 6), despite a much higher number of IFN-γ producing cells under T.H1 compared to T.H0 conditions (Fig. 4).

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.

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Reference List


31. Iwamoto S, Iwai Si, Tsujiyama K et al. TNF-{alpha} Drives Human CD14+ Monocytes to Differentiate into CD70+ Dendritic Cells Evoking Th1 and Th17 Responses. J.Immunol. 2007;179:1449-1457.


64. Ohshima Y, Yang LP, Uchiyama T et al. OX40 costimulation enhances interleukin-4 (IL-4) expression at priming and promotes the differentiation of naive human CD4(+) T cells into high IL-4-producing effectors. Blood 1998;92:3338-3345.


