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CD27 expression and function during ontogeny of the hematopoietic system

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Abstract
The observation that tumor necrosis factor receptor (TNFR) superfamily member CD27 is expressed on adult hematopoietic stem and progenitor cells (HSPCs) strongly suggests that direct cell-cell contact between immune cells and HSPCs plays a role in regulating hematopoiesis and raised the question whether CD27-signaling could also be involved in the development of the hematopoietic system during embryogenesis. We here report that CD27 is expressed on the majority of definitive hematopoietic stem and progenitor cells in mice from E11.5 onwards. While absence of CD27 on adult HSPCs results in enhanced myeloid-colony forming potential, hematopoiesis is unaffected in CD27/- fetuses when compared to wildtype controls. By overexpressing CD70, the unique ligand of CD27, we previously showed that constitutive triggering of CD27 on HSPCs has pronounced effects on hematopoiesis in adult life. In contrast, in this study we found that CD27-triggering on fetal liver HSPCs via CD70 transgenic (CD70TG) B-cells does not affect hematopoiesis during embryogenesis. Furthermore, our findings reveal that in CD70TG mothers, which have a chronically activated immune system, neither CD70-expressing B cells, nor activated T cells and the pro-inflammatory cytokine interferon-γ (IFN-γ) affect the ontogeny of the hematopoietic system of the fetus. In contrast to the expression of CD27 on fetal HSPCs from the mouse, CD27 expression is lacking on human fetal HSPCs. Taken together, these data show that the fetus is well protected against the activated immune system of the mother and that CD27-expression on fetal HSPCs does not play a pivotal role during embryogenesis.
Introduction
In the mouse embryo the first definitive hematopoietic stem cells (HSCs) arise from haemogenic endothelium in the dorsal aorta at embryonic day 10.5 (E10.5)\textsuperscript{1-4}. After emergence, HSCs are thought to enter the circulation and colonize the fetal liver (FL) from E11 onwards. Thereafter, HSCs eventually colonize other secondary hematopoietic niches like the bone marrow from where the hematopoietic system is further developed\textsuperscript{5}. Via a delicate balance between self-renewal and differentiation, HSCs are capable of generating and maintaining the blood and immune system throughout the entire life-span of an organism\textsuperscript{6,7}. Although being dormant most of the time, the generation and maintenance of HSCs during periods of cellular stress imposes a dynamic and tight regulation of these cells\textsuperscript{8}. An important role in regulation of HSC function lies within the regulatory microenvironment in which these cells reside, the HSC niche\textsuperscript{9}. Within the bone marrow (BM), where most of the adult HSCs reside, two niches have been described. One HSC niche was found near the border of bone marrow and endosteum\textsuperscript{10,11}, the other is located in the perivascular region of blood vessels\textsuperscript{12,13}. During embryogenesis, FL-HSCs are found within and adjacent to sinusoidal networks\textsuperscript{14}. Dependent on cell-cell interaction and signals received within the niche, the fate of HSCs can be altered. HSCs then proliferate and differentiate into more committed progenitor at the expense of maintaining their quiescence and self-renewal capacity. The more differentiated progenitors cells will subsequently give rise to the fully differentiated blood cells of all lineages of the hematopoietic system\textsuperscript{15}. While HSC emergence and differentiation to mature blood cells in the fetus is well described, the actual signals and cell-cell interactions affecting this process still remain to be explored in detail.

Although first described to be solely expressed on lymphoid cells\textsuperscript{16-19}, high expression of the tumor necrosis factor (TNF) receptor superfamily member CD27 was also found on adult hematopoietic stem and progenitor cells (HSPCs) in adult mouse BM\textsuperscript{20,21}. CD27 acts as a co-stimulatory molecule during immune responses and induces activation, expansion and differentiation of lymphocyte populations\textsuperscript{22-25}. More recently it is shown that CD27 co-stimulation also lowers the threshold of T cell receptor triggering. This mechanism enables the immune system to generate T-cell responses against low-affinity antigens, thereby generating a broader response to pathogens\textsuperscript{26}. In the adult mouse, expression of the unique ligand of CD27, the type II TNF-related transmembrane glycoprotein CD70, is restricted and is only transiently expressed on T cells, B cells and dendritic cells upon immune activation\textsuperscript{19,27-29}. The observation that CD27 is expressed on adult HSPCs therefore suggested that the activated immune system could affect hematopoiesis. CD27 triggering on hematopoietic stem and progenitor cells (HSPCs) by overexpression of CD70 on B cells indeed inhibits HSPC colony forming potential in vitro and especially B cell differentiation in the BM is abrogated in vivo in adult mice\textsuperscript{21}. In addition, CD27-triggering enhances self-renewal of adult HSCs, thereby inducing accelerated aging of the HSC compartment (De Bruin, unpublished data). Moreover, enhanced CD70 expression also has secondary hematopoietic effects in adult mice due to enhanced interferon-γ (IFN-γ) production by an extended effector T cell pool\textsuperscript{23}. Enhanced production of IFN-γ due to CD27-triggering further depletes B cells\textsuperscript{23}, hampers erythropoiesis\textsuperscript{30} and the formation of eosinophilic and neutrophilic granulocytes\textsuperscript{31,32}, while myelopoiesis is enhanced\textsuperscript{32}.

Given the fact that the absence of CD27 and CD70-mediated triggering of CD27 on adult mouse HSPCs have profound effects on hematopoiesis\textsuperscript{21}, and other immune-system related
singals, like interleukin-1\textsuperscript{33} and tumor growth factor (TGF)-β\textsuperscript{34}, have been found to affect early hematopoiesis, we sought to determine whether CD27 is already expressed on fetal HSCs and whether its expression has a function in shaping the developing hematopoietic system. We here describe the emergence of CD27-expressing HSPCs within the aorta-gonad-mesonephros-region (AGM) and FL of mice as early as E11.5 of gestation. At E12.5 the majority of HSPCs within the FL express high levels of CD27. Absence of CD27 on fetal HSPCs had no effect on hematopoiesis \textit{in vivo}, nor are CD27\textsuperscript{-/-} HSPCs impaired in their functional capacity \textit{in vitro}. Furthermore, by using CD70 transgenic (CD70TG) mice we show that CD27-triggering on HSPCs during ontogeny does not affect hematopoiesis \textit{in vivo} or affects the hematopoietic output \textit{in vitro}.

In humans maternal immune activation during pregnancy can have serious consequences for the conceptus. Not only can it affect development and cause per-term birth in diseases like inflammatory bowel disease\textsuperscript{35-37}, diseases like rubella, hepatitis and cytomegalovirus can cause malformations of the fetus and induce abortion\textsuperscript{38}. By using CD70TG mice as a model for sterile immune activation, we show that the chronic immune activation that is present in CD70TG mothers has no impact on hematopoiesis in the fetus. In contrast to mouse, where CD27-expression on HSPCs is eminent, we found that HSCs from human FL, fetal BM and cord blood do not express CD27.

Taken together, we conclude that the presence, absence or triggering of CD27 on HSPCs does not influence the development of the hematopoietic system in the fetus, but rather has a role in affecting hematopoietic output during immune activation after birth. In addition, we show that the fetus is well protected from maternal immune activation.

**Materials and Methods**

**Mice**

WT, CD27\textsuperscript{-/-}\textsuperscript{22} and CD70TG mice\textsuperscript{23} on a C57BL/6 background were used for experiments. Mice were bred and had access to water and food ad libitum in specific pathogen free conditions in the animal facility of the Academic Medical Center (University of Amsterdam, Amsterdam). All animal experiments were approved by the Experimental Animal Committee of the Academic Medical Center in Amsterdam, The Netherlands, according to institutional and national guidelines.

**Preparation of the AGM Region and Fetal Liver**

For the generation of embryos, adult mice were submitted to timed pregnancies in the late afternoon. Appearance of a vaginal plug the next morning was considered E0.5. At time-points between E10.5 and E17.5 pregnant females were sacrificed and embryos were isolated. The AGM-region and FL were then dissected using a dissection microscope. AGM regions were first dissociated by immersion in IMDM supplemented with collagenase (0.12% w/v type I, a kind gift from Prof. Dr. E. Dzierzak) for 45 minutes at 37°C. Subsequently, the AGM cell suspensions and FLs were strained through a 70 μm cell strainer to obtain single cell suspensions. For all washing and additional handling steps, cells were taken up in PBS supplemented with 10% fetal calf serum.
Flow Cytometry and Cell Sorting
Monoclonal antibodies used (all from eBioscience, unless stated otherwise) were CD19-Fluorescein isothiocyanate (FITC; 1D3), B220-Phycoerythrin(PE)-Cy7 (RA3-6B2), CD4-PE (GK1.5), CD8α-allophycocyanin (APC; 53-6.7), GR-1-FITC (RB6-8C5), CD11b-APC-Alexa780 (M1/70), Ter119-PE-Cy5.5 (Ly-76) and CD70-PE (FR70). For identification of HSPCs by flow cytometry, cells were first incubated with a lineage cocktail of biotin-conjugated antibodies directed against CD4, CD8α, B220, Gr1 and Ter119. As CD11b / MAC-1 is described to be expressed on fetal hematopoietic progenitor cells, this marker was not used in our lineage cocktail. Subsequently, cells were washed and incubated with streptavidin-PE, Sca-1-PE-Cy7 (D7), c-Kit-APC-Alexa780 (2B8) and CD27-APC (LG.7F9). All stainings were performed in the presence of anti CD16/32block (2.4G2, a kind gift of Louis Boon, Bioceros, Utrecht, The Netherlands). HSPCs were defined as c-Kit+ Sca-1+ in the AGM-region at all time-points and E11.5 from FL. From E12.5 onwards FL-HSPCs were defined as Lin- C-Kit+ Sca-1+ (LKS). Data acquisition was done using a FACS Calibur or FACS Canto II (BD Biosciences). Cells were sorted using a FACS Aria (BD Biosciences). Data was analyzed using FlowJo Software (Treestar).

Semi Solid Colony Assays
FACS-sorted FL-HSPCs or total FL cells were cultured in complete methylcellulose medium (Methocult m3434, Stem Cell Technologies) at 250 or 100.000 nucleated cells, respectively, in 6-wells plates. For FACS-sorted LKS cells, colonies were typed and scored at day 12. For total FL cells analysis on the number and type and colonies generated was done at day 8.

Human Fetal Liver, Fetal Bone Marrow and Cord Blood Analysis
With informed consent and approval of the Medical Ethical Committee of the Academic Medical Center / University of Amsterdam, 14 to 20 week old human FL and fetal BM samples were obtained from elective abortions. Cord blood was obtained with informed consent and approval of the Medical Ethic Committees of local hospitals. To obtain single cells suspensions from fetal BM and FL, tissues were first mechanically disrupted and subsequently filtered over a mesh. FL, fetal BM and cord blood CD34+ cells were then isolated from single cell suspensions using a Ficoll-Paque Plus (GE-Healthcare) density gradient and subsequent magnetic enrichment using the CD34 Progenitor Cell Isolation Kit (Miltenyi Biotech). Obtained cell fractions from FL and fetal BM were labelled with CD3-FITC (SK7), BDCA2-FITC, CD56-FITC CD10-FITC, CD34-PE-Cy7, CD38-PerCP (HIT2), CD133-PE, CD45-Alexa700 (2D1 and HI30), and CD27-APC (LG.7F9) for FACS analysis. Cord blood samples were labelled with CD34-PE-Cy7 (8G12), CD38-PerCP-Cy5.5 (HIT2), CD45-PacificBlue (T29/33) and CD27-APC (LG.7F9). Data acquisition was done using a LSR II cytometer (BD Biosciences) and analyzed using FlowJo Software (Treestar).

Statistical analysis.
Results are expressed as mean ± SD. Statistical analysis of groups was performed using Graphpad Prism 5, using either a non-paired 2-tailed Student t-test when comparing two groups or a one-way ANOVA with Bonferroni correction when more then 2 groups. P<0.05 was considered statistically significant.
Figure 1. CD27-expressing HSPCs in mouse embryonic tissues.
CD27 expression analysis on definitive HSPCs from AGM and fetal liver. (a) Representative dot plots and histograms from flow cytometric analysis of CD27 expression on HSPCs from E12.5 fetal liver. The % of positive cells is based on stainings on HSPCs from CD27-/- fetal livers. Data is representative for 2 independently performed experiments containing at least 5 mice per group. (b) Flow cytometric analysis of CD27 expression on HSPCs from E10.5-E12.5 AGM regions and E11.5-E12.5 fetal livers. E10.5 and E11.5 data are representative for 1 experiment containing at least 4 mice per group, E12.5 data are representative for 2 experiments containing at least 5 mice per group. (c) Colony forming capacity of sorted CD27+ and CD27- WT fetal liver HSPCs (Lin- C-kit+ Sca-1+) at E12.5 from 2 different experiments. Data is expressed as bar graphs +/- SD and represents counts in duplo for each sample.
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Results

**CD27-expressing HSPCs in mouse embryonic tissues.**
While CD27-expressing HSPCs can be found in adult mouse BM, it is unknown when CD27 expression is initiated during development and whether it is functionally involved on emerging HSPCs in early life. Therefore, CD27 expression was analyzed by FACS on HSPCs from E10.5-E12.5 mouse AGM-regions and FLs (Fig. 1a-b). No CD27-expression was found on HSPCs from the AGM-region at E10.5, using CD27-/- embryos as negative controls. However, profound expression of CD27 was found on HSPCs in both the AGM-region and FL from E11.5 onwards (Fig. 1a-b). Although the majority of FL-HSPCs at E12.5 express CD27, there is a small population where CD27-expression is absent (Fig. 1a-b). To examine functional differences between CD27+ and CD27- HSPCs these subsets were sorted and cultured in

![Figure 1.](image1)

**Figure 2. Absence of CD27 on fetal HSPC does not impair HSC function during ontogeny.**
Analysis of the hematopoietic precursor compartment of WT and CD27-/- fetal livers at E12.5. (a) Absolute number of cells in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD from mice analysed in two independent experiments, with each group containing 4 mice. * indicates a significant difference (P<0.05) between using a 2-tailed Student t-test. (b) Absolute number of LKS cells in fetal liver at E12.5 as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD from mice analysed in two independent experiments, with each group containing 4 mice. (c) Colony forming capacity of sorted WT and CD27-/- sorted fetal liver HSPCs (LKS-cells) from 2 different experiments. Data is expressed as bar graphs +/- SD and represents counts in duplo for each sample.
semi-solid colony forming assays. The presence or absence of CD27 on HSPCs had no effect on the functional capacity of these cells as both CD27⁺ and CD27⁻ HSPCs gave rise to the same amount and type of colonies in two independently performed experiments (Fig. 1c). CD27 is thus expressed on fetal HSPCs from E11.5 onwards, but does not discriminate between functionally different subsets of HSPCs in the embryo.

**Absence of CD27 on fetal HSPC does not impair HSC function during ontogeny.**

Next we checked whether the absence of CD27 affected the development of the hematopoietic system. CD27⁻/⁻ mice are viable and show no apparent phenotype (data not shown). We observed that in the E12.5 wildtype embryos total FL cell numbers are slightly higher than in FLs of CD27⁺ embryos (Fig. 2a). However, both the percentage and absolute number of LKS cells of E12.5 CD27⁻/⁻ FLs was comparable to wildtype FL (Fig. 2b). In addition, E12.5 CD27⁻/⁻ FL LKS cells gave rise to the same amount and types of hematopoietic colonies when compared to WT FL LKS cells, suggesting that E12.5 CD27⁻/⁻ FL cells are similarly competent as WT FL cells and indicating that CD27 is not required for hematopoiesis during ontogeny (Fig. 2c).

**Triggering of CD27 by CD70 does not impair HSC function during ontogeny.**

Expression of CD27 on HSPCs during ontogeny suggests that triggering of CD27 by CD70 affects HSPC function. To test this hypothesis we made use of CD70 transgenic (CD70TG) mice, which express CD70 under control of the B-cell specific human CD19 promotor. At E17.5, when a substantial amount of CD19⁺ cells can be found in the FL, we observed in CD70TG embryos a clear population of B220⁺ CD19⁺ B cells that expressed high levels of CD70 (Fig. 3a). Total FL B cell numbers were comparable between CD70TG and control mice (Fig. 3b), this in contrast to adult CD70TG mice, where B cell differentiation is impaired and B cells are gradually depleted. Compared to WT embryos, expression of CD27 is downregulated on LKS cells in CD70TG embryos, indicating that CD27 on HSPCs has been triggered (Fig. 3c). This is in line with earlier results that show downregulation of CD27 expression upon interaction with CD70. Surprisingly, triggering of CD27 induced a modest, but significant, increase in the number of LKS cells compared to WT (Fig. 3d), which was also seen in CD27⁻/⁻ embryos. The presence of a higher number of LKS cells however had no consequences for hematopoietic lineage differentiation as the absolute cell number of FLs was comparable between groups (Fig. 3e). In addition, the functional hematopoietic differentiation capacity of CD27-triggered LKS cells was unaltered as both the number and types of colonies formed were comparable to WT and CD27⁻/⁻ embryos (Fig. 3f). These data thus show that CD27 indeed can be triggered on HSPCs during ontogeny, but it does not have clear consequences for the number of HSPCs and hematopoietic cells in vivo or the functional capacity of these cells in vitro.

**Chronic immune activation in the mother does not affect ontogeny of her progeny.**

Although the maternal and fetal blood supply are separated by the placenta, it is unclear whether immune activation in the mother, or activated immune cells from the mother that cross the placenta, affect the unborn fetus. As described earlier, CD27-triggering itself, and the consequential high number of effector T cells and elevated IFN-γ levels in CD70TG mice have profound effects on hematopoiesis in adult mice. This chronic state of immune
Figure 3. Triggering of CD27 by CD70 does not impair HSPC function during ontogeny.

Analysis of the effects of constitutive CD27-triggering on E17.5 fetal liver HSPCs. (a) Representative histogram from CD70 expression on B-cells using flow cytometric analysis. Data are representative for at least 3 mice per group. (b) Absolute B cell numbers in fetal liver. Data are expressed as bar graph +/- SD. Each group contains at least 3 mice per group. (c) Representative histogram from CD27 expression on E17.5 HSPCs (LKS-cells) using flow cytometric analysis. Data are representative for at least 3 mice per group. (d) Absolute number of HSPCs (LKS-cells) in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD. Each group contains at least 3 mice. * indicates a significant difference (P<0.05) between WT and the analyzed groups using a one-way ANOVA with Bonferroni correction. (e) Absolute number of cells in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD. Each group contains at least 3 mice. (f) Colony forming capacity of sorted fetal liver HSPCs from WT, CD27 +/- and CD70TG embryos. Data is expressed as bar graphs +/- SD and represents counts in duplo for three samples per group.
Figure 4. Chronic immune activation in the mother does not affect ontogeny of her progeny.

Comparison of the hematopoietic compartment of E12.5 embryos from a WT and CD70TG mother. (a) Representative dot plots from cytometric analysis of Sca-1 expression on HSPCs from fetal livers from embryos isolated from a WT or CD70TG mother and bone marrow from a WT or CD70TG mother. (b) Representative histograms from flow cytometric analysis of CD27 expression on fetal HSPCs from a WT or CD70TG mother. (c) Absolute HSPC cell number in fetal liver. Data are expressed as bar graph +/- SD. Each group contains at least 5 mice per group. (d) Colony forming capacity of sorted fetal liver HSPCs from embryos from a WT and CD70TG mother. Data is expressed as bar graphs +/- SD and represents counts in duplo for three samples per group. (e) Absolute number of cells in fetal liver as determined with flow cytometric analysis. Data are expressed as bar graph +/- SD. Each group contains at least 5 mice per group. (f) Representative dot plots from cytometric analysis of hematopoietic lineages present in fetal livers from embryos isolated from a WT or CD70TG mother.
activation of the mother could potentially also affect the development of the hematopoietic system of the fetus. To investigate this, we analysed the hematopoietic compartment in fetuses of CD70TG mothers at E14.5, when the adaptive immune system is undeveloped. As IFN-γ is known to induce Sca-1 expression on HSPCs\textsuperscript{21,30-32}, Sca-1 expression was analysed on Lin\(^{-}\) c-Kit\(^{+}\) cells of E14.5 FLs. In contrast to CD70TG mothers themselves, where Sca-1 was highly expressed on BM-HSPCs, embryos carried by these mice did not display upregulation of Sca-1 (Fig. 4a), strongly suggesting that IFN-γ, produced by the mother, does not pass the placenta. Although still being controversial, it has been proposed that both maternal and fetal cells in general, and lymphocytes in particular, might cross the placenta and induce tolerance\textsuperscript{41-45}. Therefore, we next checked whether B cells or activated T cells from a CD70TG mother could cross the placenta and alter hematopoiesis in the fetus. In contrast to the mother itself, where CD27 is downregulated on BM HSPC, we did not observe downregulation of CD27 on FL LKS cells in any of her fetuses (Fig. 4b), indicating that CD70TG B cells do not or only in very small numbers cross the placenta. In addition, we did not find alterations in the absolute number of LKS cells present in the FLs of embryos from CD70TG mothers when compared to their WT littermates (Fig. 4c), nor was their functional capacity affected or was the FL cellularity altered (Fig. 4d-e). Furthermore, we did not observe any differences in lineage development in the FL as the percentages of granulocytic (GR-1\(^{+}\)), monocytic (CD11b\(^{+}\)) and erythroid (Ter119\(^{+}\)) cells are similar between fetuses from WT and CD70TG mothers (Fig. 4f). The absence of B220\(^{+}\) B cells and CD4\(^{+}\) and CD8\(^{+}\) T cells at this time-point (Fig. 5f) further indicate that during immune activation lymphocytes do not detectably cross the placental barrier and do not affect hematopoiesis. Taken together, within this model the placenta thus protects the developing hematopoietic system of the fetus from chronic immune activation within the mother.

**Human fetal HSPCs lack CD27-expression.**

Although CD27 expression was not found on the vast majority of CD34\(^{+}\) cells from BM of adult humans\textsuperscript{20,46}, it was recently reported that CD27 is expressed to a certain extent on normal HSCs in human and that it is particularly high on human leukemic HSCs\textsuperscript{47}. To further investigate whether CD27 is expressed on human fetal HSPCs as in mouse fetal tissues, we also examined human FL, fetal BM and cord blood. Whereas we could find clear expression of CD27 on Lin\(^{-}\) CD34\(^{+}\) cells from FL and fetal BM, CD27 is largely absent from the HSCs (Lin\(^{-}\) CD34\(^{+}\) CD38\(^{-}\)) in these organs (Fig. 5a-b) as well as in cord blood (Fig. 5c). Also the hematopoietic progenitor populations (Lin\(^{-}\) CD34\(^{+}\) CD38\(^{-}\)) of FL, fetal BM and cord blood are mostly negative for CD27 expression. These data demonstrate that, in contrast to mouse, the vast majority of human fetal tissue HSPCs lack the expression of CD27, suggesting that CD27 also does not play a major role in shaping the human hematopoietic system during ontogeny.

**Discussion**

Here we describe the expression of the TNF-receptor superfamily member CD27 on definitive HSPCs in mouse fetuses from E11.5 onwards (Fig. 1a-b), suggesting that CD27 could play a role in development of the hematopoietic system. These data correlate well with a recently described comprehensive transcriptomic analysis of developing HSCs in murine embryos\textsuperscript{48},
in which CD27 mRNA could be first detected in HSCs from the AGM-region at E11.5 and in FL HSCs from E12.5 onwards (see also http://hsc.hms.harvard.edu). In contrast to the adult situation however, neither the absence, nor the triggering of CD27 has an effect on hematopoiesis in the fetus as in both cases the number of LKS-cells and the functional capacity of these cells is comparable to control cells (Fig. 1c, Fig. 2a-c, Fig. 3d/f). The absence of hematopoietic effects in the fetus due to constitutive CD27-triggering could be due to the duration of CD70-binding. As it takes up until E17.5 for a substantial amount of CD70TG B cells to arise, constitutive CD27-triggering on fetal HSPCs is up until birth relatively short, giving little time to develop hematopoietic differences. This in contrast to adult CD70TG mice, where from E17.5 onwards CD27-triggering on HSPC occurs and which in time could induce more pronounced hematopoietic effects. Although we found some significant differences between WT, CD27−/− and CD70TG embryos regarding cell numbers (Fig. 2a, Fig. 3d), these differences might reflect small discrepancies between gestation time within an experiment.

Figure 5. Absence of CD27 expression on human fetal HSPCs.
CD27 expression analysis on definitive HSPCs from human fetal liver, fetal bone marrow and cord blood. (a) Representative dot plots from flow cytometric analysis of CD27 expression on HSPCs from human fetal liver. Data is representative for 2 pools of 2 fetal livers. (b) Representative dot plots from flow cytometric analysis of CD27 expression on HSPCs from human fetal bone marrow. Data is representative for 4 fetal bone marrow samples. (c) Representative dot plots from flow cytometric analysis of CD27 expression on HSPCs from cord blood. Data is representative for 2 different cord blood samples.
Another explanation for the lack of effects of CD27-triggering could be lying in differences in the downstream signalling upon CD27-triggering between adult and fetal HSCs. Upon binding to CD70, the intracellular tail of CD27 is engaged to TNFR-associated factor 2 (TRAF2) and TRAF5\(^{49,50}\), ultimately activating the canonical and non-canonical nuclear-factor-κB (NFκB) pathways, as well as the c-Jun-N-terminal kinase (JNK) pathway\(^{49-51}\). Activation of these pathways upon CD27 triggering in activated CD4+ T cells leads to the transcription of genes related to induction of Th1 responses and the upregulation of the anti-apoptotic B-cell chronic lymphocytic leukemia / lymphoma 2-like 1 (Bcl-X\(_L\))\(^{32,53}\) in adult mice. In CD8 T cells it mainly induces survival by induction of IL-2, Bcl-X\(_L\) and Pim expression\(^{54,55}\). The effects on gene expression by CD27 triggering on HSPCs is largely unknown, but recent data from our lab show that constitutive CD27-triggering on adult long term repopulating HSCs results in upregulation of genes related to cell cycle progression, DNA replication, DNA repair responses, myeloid differentiation and stress responses, while genes related to regulation of adaptive immune responses, induction of cell death and cell cycle arrest are downregulated, ultimately leading to enhanced HSC self-renewal and accelerated ageing of HSCs (De Bruin; unpublished data). Although CD27 expression on fetal HSPCs is downregulated in CD70TG embryos, which is indicative for CD27-triggering\(^{21,40}\), we show that this has no effect on the number of HSPCs \textit{in vivo} and the functional capacity of HSPCs \textit{in vitro} (Fig. 3d,f). Although we did not specifically analyse cell cycle status, these data suggest that cycling of HSPCs is not induced. In addition we did not find an enhanced myeloid output based on the total cell numbers and functional capacity of fetal HSPCs (Fig 3e-f), as seen in adult CD70TG mice. Although we cannot exclude any effect of CD27-triggering on HSPCs based on gene expression, our data suggests that CD27-signaling on fetal hematopoietic progenitors might be blocked or overruled by other developmental signalling cues. From these data we can thus conclude that although CD27 is expressed on fetal hematopoietic progenitors within the mouse, this co-stimulatory molecule is not involved in shaping the hematopoietic system of the fetus. We therefore state that it isn’t until immune activation is induced after birth that CD27-triggering on HSPCs becomes important in affecting the hematopoietic output.

Whereas CD27-expression is evident on human leukemia stem cells (LSCs) and contributes to disease progression by induction of Wnt target genes\(^{47}\), CD27 expression on healthy human HSPCs is absent or low\(^{20,47,56}\). In addition to these data, we show that we did not find convincing CD27 expression on fetal HSCs from FL, fetal BM and cord blood (Fig. 5). As expression was very low and only found on a minority of HSCs, the impact of CD27-signaling on hematopoiesis therefore would not be of significant influence. Given the fact that in adult mice CD27 stimulation on HSPCs extensively influences the hematopoietic system, it is tempting to explore whether other co-stimulatory proteins on both adult and fetal HSPCs are expressed, and whether their triggering is involved in the shaping of the hematopoietic system during fetal life and subsequent alterations of it during immune activation in adult life. This could give more insight in how the immune system can regulate the hematopoietic system and give feedback at the progenitor level.

Within a mother’s womb, the fetus is considered an allograft. Besides that the placenta allows nutrient and gas exchange from the mothers blood to the fetus and waste exchange from fetus to mother it also serves as a physical immunological barrier that prevents transmission of pathogens from mother to child and avoiding the immune system from the mother attacking the fetus. A variety of immune cells have however been proposed to play
a role in the maternal tolerance to the fetus. Early during pregnancy decidual natural killer cells (dNKs) are present in copious amounts in the decidua, accompanied by macrophages, T cells and DCs\(^57\). To date it is generally accepted that cell-free fetal DNA can be found in the maternal circulation and maternal IgG can cross the placental barrier to induce passive immunisation in the fetus\(^58;59\). However still controversial, maternal lymphocytes and other hematopoietic cells have been described to pass the placenta as well\(^61;64;45;60\). Using CD70TG mice we however did not find any evidence for the transfer of maternal activated lymphocytes or inflammatory cytokines, such as IFN-\(\gamma\), across the placental barrier, as neither CD27-downregulation nor Sca-1 upregulation was found on FL-HSPCs (Fig. 4a-b). Although we cannot exclude that lymphocytes did pass the placental barrier, the numbers would have been very low and certainly did not severely affect the hematopoietic output from fetal HSPCs (Fig. 4c-f). Interestingly, IFN-\(\gamma\) is produced in relatively high levels by dNKs and plays an important role in vasculature remodelling and angiogenesis upon implantation of the placenta\(^61;62\). IFN-\(\gamma\) is nevertheless not required for the implantation of the conceptus and further pregnancy, as IFN-\(\gamma\)\(^-\) mice breed normally and generate normal litters (data not shown). Although IFN-\(\gamma\) might be beneficial for the conceptus, elevated levels of IFN-\(\gamma\) in the placenta have also been linked to pregnancy loss\(^63;64\), as IFN-\(\gamma\) inhibits T regulatory cell (Treg) formation\(^65\). Trophoblasts within the placenta induce systemic proliferation of Tregs and attracts them to the fetal-maternal interface of the placenta by production of chorionic gonadotropin\(^66;67\). Here Tregs contribute to the maternal tolerance to the fetus by suppressing autoimmune responses and allogeneic responses against the fetus. Absence of Tregs in the placenta leads to a failure of gestation\(^66\). Although CD70TG mice have elevated numbers of IFN-\(\gamma\) producing effector T cells\(^23\), we have no indications that the litter number of CD70TG mice is smaller compared to control mice (data not shown) and that Treg function is abrogated in the placenta of CD70TG mice.

With respect to the influence of maternal immune activation on fetal development we did not observe any effects on the conceptus regarding hematopoiesis in mice (Fig. 5). To what extent other parameters are influenced is unknown, but is has been described that immune activation during pregnancy can induce spontaneous abortion, cause pre-term birth, affect birth weight, affect fetal brain development, affect the fetal adaptive immune system and can lead to cognitive impairment and dopaminergic hyperfunction in humans\(^35-37;68-71\). In utero infection with pathogens like rubella, hepatitis, congenital cytomegalovirus (CMV), herpes simplex virus (HSV) and human immunodeficiency virus (HIV) can result in severe congenital disorders, malformations and pregnancy loss, especially during the first trimester of gestation when the fetus is still incapable of mounting immune responses\(^38\). Here we show by using a model of sterile chronic maternal immune activation that excludes vertical transmission of pathogens, that a high degree of chronic maternal immune activation is not sufficient to influence hematopoietic development in the fetus. To what extent fetal hematopoiesis is affected by maternal immunity in general and CD27-triggering on HSPCs in particular in case of pathogen spreading over the placenta largely unknown and requires further research using disease models. Gaining further insight in how maternal immune responses develop while maintaining tolerance to the conceptus could give valuable leads to prevent congenital defects and pregnancy loss due to infection.
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Reference List

1. de Bruijn MF, Speck NA, Peeters MC, Dzierzak E. Definitive hematopoietic stem cells first develop within the major arterial regions of the mouse embryo. EMBO J. 2000;19:2465-2474.


