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Differentiation and functional regulation of human fetal NK cells

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The human fetal immune system is naturally exposed to maternal allogeneic cells, maternal antibodies, and pathogens. As such, it is faced with a considerable challenge with respect to the balance between immune reactivity and tolerance. Here, we show that fetal natural killer (NK) cells differentiate early in utero and are highly responsive to cytokines and antibody-mediated stimulation but respond poorly to HLA class I–negative target cells. Strikingly, expression of killer-cell immunoglobulin-like receptors (KIRs) did not educate fetal NK cells but rendered them hyporesponsive to target cells lacking HLA class I. In addition, fetal NK cells were highly susceptible to TGF-β–mediated suppression, and blocking of TGF-β signaling enhanced fetal NK cell responses to target cells. Our data demonstrate that KIR-mediated hyporesponsiveness and TGF-β–mediated suppression are major factors determining human fetal NK cell hyporesponsiveness to HLA class I–negative target cells and provide a potential mechanism for fetal-maternal tolerance in utero. Finally, our results provide a basis for understanding the role of fetal NK cells in pregnancy complications in which NK cells could be involved, for example, during in utero infections and anti-RhD–induced fetal anemia.

Introduction

Natural killer (NK) cells are innate lymphocytes involved in the immune response against tumors, viruses, and allogeneic cells (1). They are also important for successful placentation in humans and regulate placental development and fetal growth (2). NK cells are perhaps best known for their ability to directly kill target cells (3) but are also potent producers of cytokines (4, 5) and are involved in tuning adaptive immune responses (6–8).

Despite reports on functional human fetal immune responses (9, 10), the fetal immune system is commonly viewed as immature and unresponsive (11, 12). Newborn mice have only low numbers of αβ T cells (13), and most NK cells are not fully mature (14). The lack of more differentiated NK cells at birth in mice was recently shown to be strongly influenced by TGF-β, since mice deficient in TGF-β receptor signaling had elevated numbers of differentiated mature NK cells at birth (15). In contrast to those in the mouse, human αβ T cells can be detected in the fetus as early as gestational week 12 (16). Human NK cells have been detected in fetal liver as early as gestational week 6 and in fetal spleen at gestational week 15 (17). Although fetal liver NK cells have been reported to kill target cells, both by natural and redirected antibody-dependent cellular cytotoxicity (ADCC), they are hyporesponsive compared with adult NK cells (17). Together, previous data thus indicate that human NK cells develop early in utero but are functionally immature compared with adult NK cells.

Although the fetal-maternal interface in the placenta has previously been regarded as a strong barrier, it is today well established that small numbers of cells can pass in both directions (18, 19), in addition to antibodies, proteins, nutrients, and microbes (20). Transfer of maternal antibodies could be beneficial for antiviral ADCC responses by fetal NK cells but can also cause anemia in fetuses of RhD-immunized mothers. Analyzing how antibody-

mediated responses by fetal NK cells are regulated is therefore important for understanding the role of NK cells under these conditions. In addition, the transfer of maternal cells could trigger potentially devastating alloreactive immune responses by fetal T cells and NK cells. The possibility of fetal antimaternal immune responses would thus require mechanisms to ensure fetal-maternal immune tolerance in the developing fetus. We have recently shown that human fetal T cells are highly reactive to stimulation with allogeneic cells but are uniquely prone to develop into regulatory T cells upon stimulation (19), thus providing a mechanism for fetal-maternal T cell tolerance in utero. However, it remains unknown whether there are mechanisms operating to ensure fetal-maternal NK cell tolerance.

NK cell self tolerance and function in adults (21) and neonates (22) is largely controlled via inhibitory receptors binding to HLA class I molecules. The inhibitory receptors expressed by human NK cells include CD94/NKG2A (hereafter referred to as NKG2A) and killer-cell immunoglobulin-like receptors (KIRs) (1). NKG2A binds to HLA-E, a ubiquitously expressed nonclassical HLA class I molecule with very limited polymorphism. Most of the inhibitory KIRs have been reported to bind to distinct groups of HLA class I molecules, where, for example, KIR2DL1 binds to HLA-C2, KIR2DL3 binds to HLA-C1, and KIR3DL1 binds to HLA-Bw4. KIRs are expressed in a stochastic and variegated manner, resulting in a diverse repertoire of KIR-expressing NK cells (23). NKG2A and inhibitory KIRs serve two functions with regard to self tolerance. First, self-HLA class I–specific inhibitory receptors turn off NK cell effector responses during interactions with normal autologous cells, expressing the ligand(s) for the inhibitory receptors (3). Second, in a process referred to as licensing or education, interactions between inhibitory NKG2A and KIRs and their respective HLA class I ligands tune NK cell responses (24). Consequently, NK cells that lack expression of inhibitory receptors for self HLA class I are noneducated and as such are hyporesponsive to HLA class I–
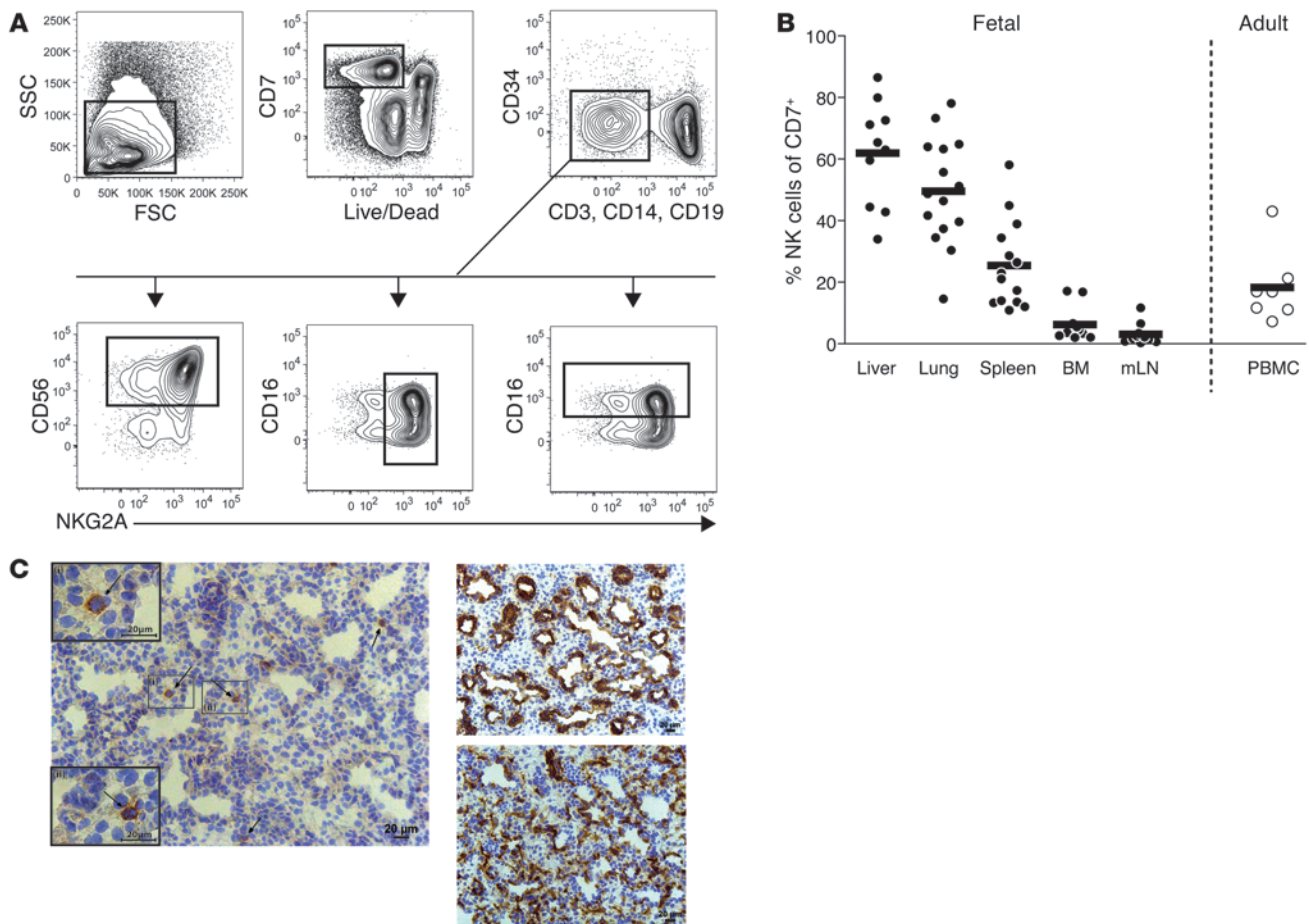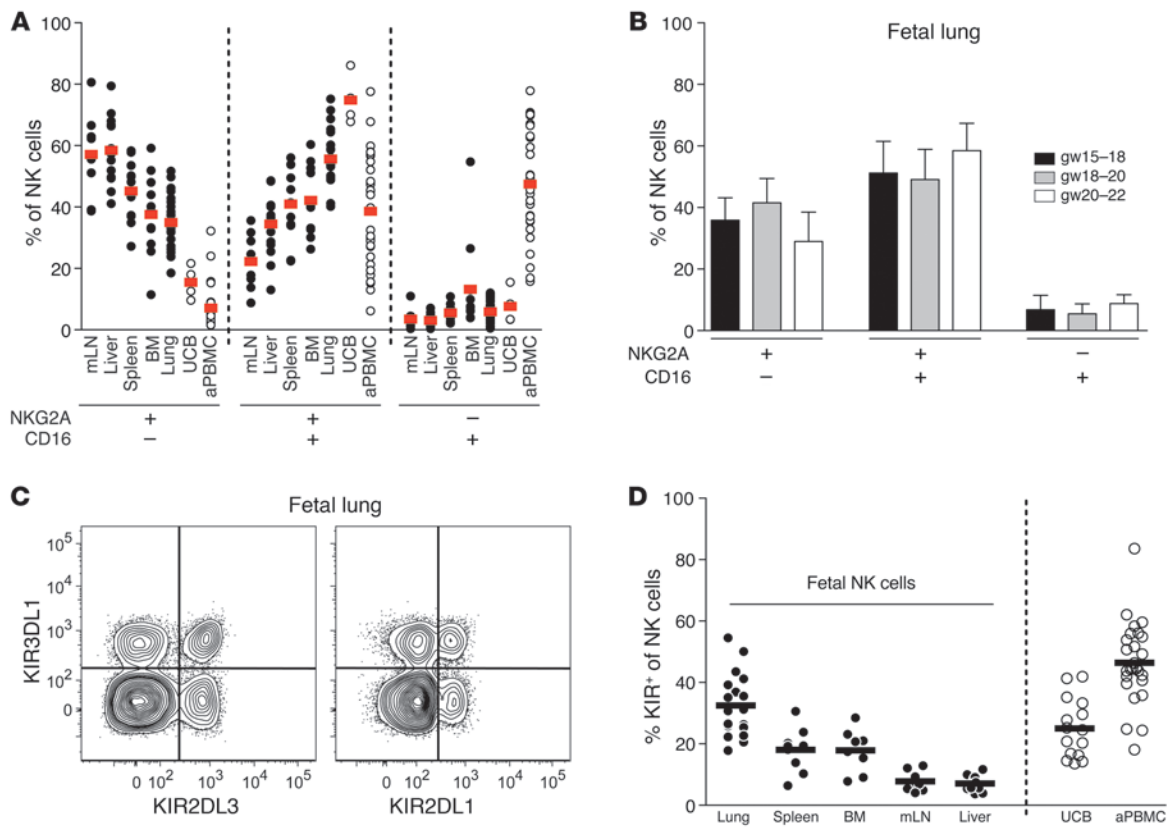
Figure 1
NK cells are present in human fetal tissues. (**A**) Representative staining and gating strategy for identification of NK cells in fetal lung. NK cells were defined using the Boolean algorithm ("CD56$^+$ or NKG2A$^+$ or CD16^{+}") after gating on live CD3$^-$CD14$^-$CD19$^-$CD34$^-$CD7$^+$ cells. For detailed information on the identification of fetal NK cells, see Supplemental Figure 1. (**B**) Frequency of NK cells, as percentages of CD7$^+$ cells in fetal liver ($n = 10$), fetal lung ($n = 15$), fetal spleen ($n = 14$), fetal bone marrow ($n = 11$), fetal mLNs ($n = 11$), and adult PBMCs ($n = 7$). Horizontal bars indicate the mean percentage, and dots indicate the number of samples tested. (**C**) Staining of NKp46$^+$ cells in fetal lung. Arrows indicate NKp46$^+$ cells. Two NKp46$^+$ cells are shown in the top and bottom left corners. The staining is representative of sections from 4 individual fetal lungs at gestational week 18–22. Representative staining of EpCAM expression (epithelial marker, top right) and CD31 expression (endothelial marker, bottom right) in fetal lung sections. Scale bar: 20 μm.

negative target cells, compared with educated NK cells expressing self-specific inhibitory receptors. Together, education and functional inhibition via NKG2A and KIRs provide a mechanism to ensure efficient recognition of target cells lacking expression of self HLA class I, e.g., allogeneic cells, while maintaining self tolerance. According to this model of NK cell tolerance, fetal KIR-expressing NK cells educated by paternally inherited HLA class I could be reactive against allogeneic maternal cells, unless alternative mechanisms regulate fetal NK cell tolerance.

In addition to regulation of NK cell tolerance via KIRs and NKG2A, cytokines in the environment shape the reactivity of NK cells. For example, adult NK cells can respond directly by producing IFN-γ after stimulation with IL-12, IL-15, and IL-18 (4, 5). Priming of adult NK cells with IL-12 and IL-15 also partially breaks the hyporesponsiveness of noneducated NK cells, allowing them to respond readily to HLA class I–negative target cells (25). Moreover, cytokines such as TGF-β have been demonstrated to suppress NK cell responses to target cells as well as to cytokines

in vitro (26, 27). Expression of TGF-β is increased in fetal lymph nodes compared with that in adult lymph nodes (19), and we have previously shown that TGF-β is involved in the induction of peripheral fetal regulatory T cells (19). However, it is currently not known what effects these cytokines have on fetal NK cells.

Here we have analyzed the differentiation and function of fetal NK cells, allowing us to directly test how fetal NK cells are regulated by inhibitory HLA class I–binding receptors and cytokines, including IL-12, IL-18, and TGF-β. Our data revealed a fundamental difference between fetal and adult NK cells with regards to KIR-mediated education, in that fetal NK cells that expressed KIRs were hyporesponsive. In addition, fetal NK cells were highly susceptible to TGF-β–mediated suppression compared with adult NK cells. Together, the KIR-mediated hyporesponsiveness and TGF-β–mediated suppression provide a mechanism for the decreased reactivity against target cells displayed by fetal NK cells and have implications for the functional regulation of fetal NK cells as well as for fetal-maternal tolerance.

Figure 2
Fetal NK cell differentiation and KIR acquisition starts early in the developing fetus and varies among organs. (**A**) Frequency of NKG2A+CD16−, NKG2A+CD16+, and NKG2A−CD16+ subsets of total NK cells in fetal organs (black circles), full-term umbilical cord blood (UCB; white circles), and adult PBMCs (aPBMC; white circles) (fetal mLN, *n* = 8; liver, *n* = 13; spleen, *n* = 11; bone marrow, *n* = 11; lung, *n* = 24; umbilical cord blood, *n* = 4; adult PBMCs, *n* = 31). (**B**) Frequency of NKG2A+CD16−, NKG2A+CD16+, and NKG2A−CD16+ subsets of NK cells in fetal lung stratified over gestational weeks (gw) 15–18 (black, *n* = 9), 18–20 (gray, *n* = 9), and 20–22 (white, *n* = 6). Error bars represent SD, and bars represent mean. (**C**) KIR2DL1, KIR2DL3, and KIR3DL1 expression on fetal lung NK cells. (**D**) Frequency of NK cells expressing KIR2DL1, KIR2DL3, and/ or KIR3DL1 (defined by the Boolean gate "KIR2DL1+ and/or KIR2DL3+ and/or KIR3DL1+") (lung, *n* = 21; spleen, *n* = 10; bone marrow, *n* = 8; mLN, *n* = 8; liver, *n* = 12; umbilical cord blood, *n* = 14; adult PBMCs, *n* = 26). Horizontal bars indicate the mean percentage, and dots indicate the number of samples tested.

Results

*NK cell differentiation and KIR acquisition start early in fetal development.* NK cells, defined as CD45+CD3−CD56+ cells, have been reported to exist in the human fetal liver as early as gestational week 6 as well as in fetal spleen from gestational week 15 (17). Here we used a more stringent definition of NK cells (CD7+CD3−CD14− CD19−CD34− cells expressing CD56, NKG2A, or CD16) to identify NK cells (Figure 1A and Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/ JCI68989DS1). CD7 is expressed by B cells, T cells, and NK cells (28) and as such is a useful marker of lymphocytes. To verify that staining with CD7 did not exclude a large population of CD7− CD161+ immature stage III pre-NK cells (29), we analyzed the expression of CD7 on CD161+CD3−CD14−CD19−CD45+ lympho-cytes. Virtually all of the CD161+ cells expressed CD7 in bone mar-row, liver, lung, lymph nodes, and spleen (Supplemental Figure 1D), demonstrating that gating on CD7 did not exclude a signifi-cant number of immature NK cells. NK cells were most frequent in fetal liver and lung, in which they made up 50%–60% of the CD7+ cells on average. Relatively high frequencies of NK cells were also

detected in fetal spleen, whereas the frequency was low in fetal bone marrow and mesenteric lymph nodes (mLNs) (Figure 1B).

Although our flow cytometry data demonstrated that NK cells were the dominant lymphocyte population in fetal lung and liver, they did not reveal where the NK cells were located in these tissues. NKp46 is expressed by virtually all fetal NK cells, but by very few other cells (Supplemental Figure 1B), and is thus specific for fetal NK cells in the tissues analyzed. The fetal lungs analyzed in this study (gestational week 15–22) correspond to the canalicular stage of lung development, where clearly visible terminal buds and acinar tubules are lined with EpCAM+ epithelial cells (30). NKp46+ cells were scattered throughout the mesenchyme of the lung, between the terminal buds and acinar tubules, and were only rarely intraep-ithelial in their location (Figure 1C). Also in fetal liver, NKp46+ cells were scattered and had no apparent association with endothelial (CD31+) or epithelial (EpCAM+) structures (data not shown).

Having identified NK cells in different fetal organs, we next asked whether fetal NK cells were predominantly immature or whether we could find evidence of NK cell differentiation. Based on cur-rent models for adult NK cell differentiation (25, 31), we examined
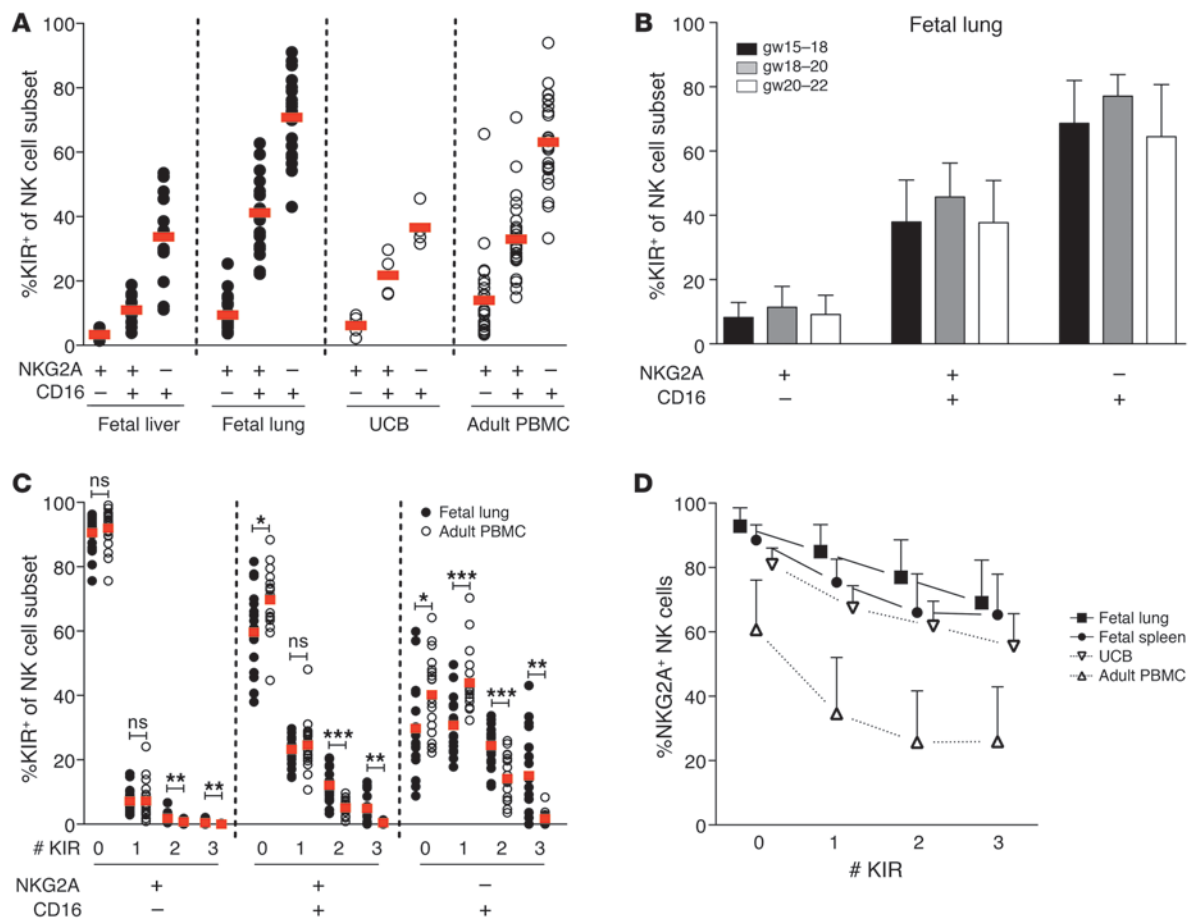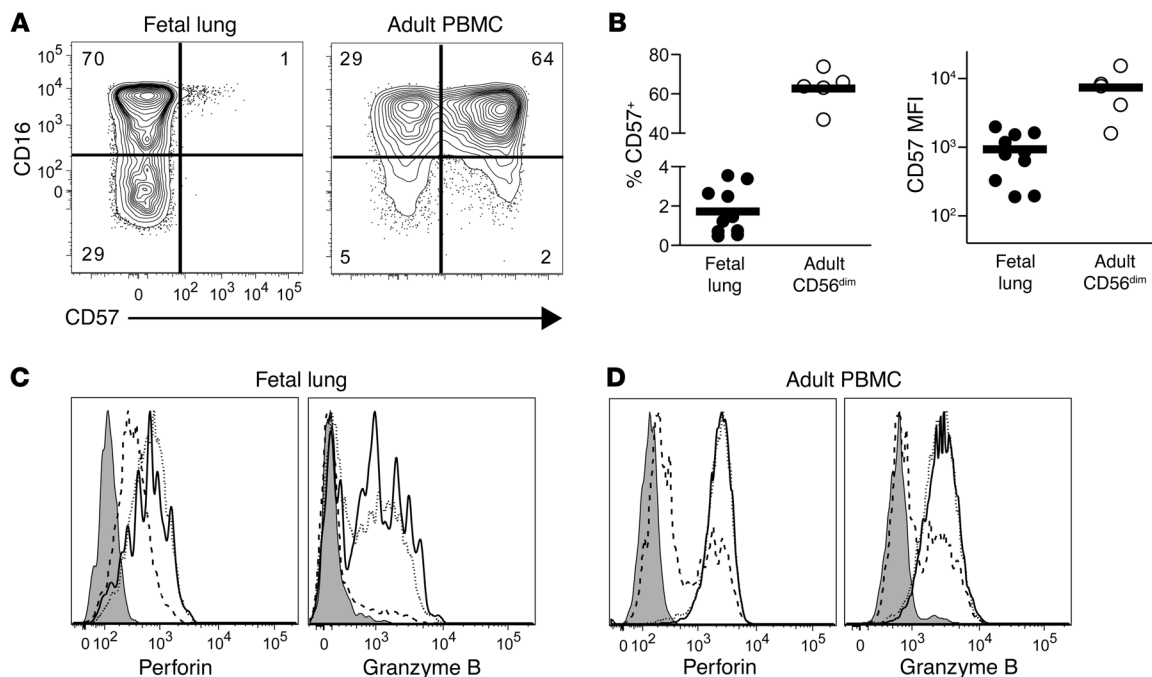
Figure 3

KIR expression on fetal NK cells increases with differentiation, and coexpression of several KIRs is more common on differentiated fetal lung NK cells than on adult PBNK cells. (**A**) Frequency of NK cells expressing KIR2DL1, KIR2DL3, and/or KIR3DL1 (defined by the Boolean gate "KIR2DL1+ and/or KIR2DL3+ and/or KIR3DL1+") within each subset (liver, n = 12; lung, n = 21; full-term umbilical cord blood, n = 4; adult PBMCs, n = 26). (**B**) Frequency of fetal lung NK cells expressing KIR2DL1, KIR2DL3, and/or KIR3DL1, stratified over gestational weeks 15–18 (black, n = 4), 18–20 (gray, n = 8), and 20–22 (white, n = 6). (**C**) Frequency of NK cells coexpressing 0, 1, 2, or 3 KIRs (KIR2DL1, KIR2DL3, and/or KIR3DL1) in NKG2A+CD16–, NKG2A+CD16+, and NKG2A–CD16+ subsets of NK cells from fetal lung (black circles, n = 11) and adult PBMCs (white circles, n = 11). *P < 0.05; **P < 0.01; ***P < 0.001, 2-tailed Mann-Whitney test performed. ns, not significant. (**D**) Frequency of NKG2A+ NK cells in subsets with 0, 1, 2, or 3 KIR (KIR2DL1, KIR2DL3, and/or KIR3DL1) in fetal lung (n = 19), spleen (n = 9), umbilical cord blood (n = 11), and adult PBMCs (n = 25). Error bars represent SD, and bars represent mean. Horizontal bars indicate the mean percentage, and dots indicate the number of samples tested.

3 subsets of NK cells: NKG2A+CD16– (corresponding to CD56bright), NKG2A+CD16+, and NKG2A–CD16+ (corresponding to CD56dim NK cells with an increasingly differentiated phenotype). All 3 subsets were present in all fetal organs studied, but the distribution varied substantially among organs, with most differentiated NK cells in fetal lung and the least differentiated NK cells in lymph nodes and liver (Figure 2A). The expression of CD56 was in general high on fetal NK cells, and we could not readily discriminate discrete CD56bright from CD56dim NK cell populations in fetal tissues. Nevertheless, in line with adult NK cell differentiation, the mean fluorescence intensity of CD56 decreased with increasing differentiation of fetal NK cells (Supplemental Figure 2). However, fetal NK cells were less differentiated compared with adult peripheral blood NK (PBNK) cells, and approximately 90% of the fetal NK cells expressed NKG2A, irrespective of organ (Figure 2A). Interestingly, there was no change in the distribution of the NKG2A+CD16–,

NKG2A+CD16+, and NKG2A–CD16+ subsets during gestational weeks 15–22 (Figure 2B and data not shown), suggesting that NK cell differentiation in the human fetus starts before gestational week 15 and is stable from gestational week 15–22.

Similar to NKG2A, KIR expression is also associated with differentiation of CD56dim NK cells in adults (25). To our knowledge, only one previous report has studied KIR expression on fetal NK cells, showing that they are expressed by a small subset of fetal liver NK cells at gestational week 14 (32). However, it is currently not known whether KIRs are expressed on NK cells in other fetal organs or whether KIR expression is associated with fetal NK cell differentiation, as is the case for adult PBNK cells. All the fetal organs studied here contained NK cells that expressed KIR2DL1, KIR2DL3, and KIR3DL1, the 3 major inhibitory KIRs, but the frequency varied considerably among the organs studied (Figure 2, C and D). Fetal lung contained the highest frequency of KIR+ NK cells (≈30%),

Figure 4
The frequency of CD57+ NK cells in fetal lung is low. (**A**) Representative staining of CD16 and CD57 on NK cells from fetal lung and adult PBNK. (**B**) Frequency and mean fluorescence intensity of CD57 expression on NK cells from fetal lung (black circles) and adult PBNK (white circles). Bars represent mean. Representative stainings of perforin and granzyme B expression in NKG2A+CD16− (long dashed lines), NKG2A+CD16+ (dotted line), and NKG2A−CD16+ (solid line) subsets of NK cells in (**C**) fetal lung and (**D**) adult PBMC. The non-NK cell Boolean gate was used as a reference (filled gray). The stainings are representative of 3 independent stainings in 3 individual donors.

followed by spleen and bone marrow (≈20%). In contrast, expression of KIRs on NK cells from fetal mLNs and liver was low (<10%) (Figure 2D). Similar to adult PBNK cells (25), KIR expression was associated with differentiation and was increasingly more common on fetal NKG2A+CD16+ and NKG2A−CD16+ NK cells, compared with that on NKG2A+CD16− NK cells (Figure 3A). Surprisingly, the frequency of KIR+ cells on NKG2A+CD16+ and NKG2A−CD16+ fetal lung NK cells was at least as high as that on adult PBNK cells, with an average of ≈70% of NKG2A−CD16+ fetal lung NK cells expressing KIR (Figure 3A). In contrast, the frequency of KIR-expressing NK cells in fetal liver and full-term umbilical cord blood was lower than in fetal lung and adult PBNK cells, irrespective of which subset was analyzed (Figure 3A). The frequency of KIR-expressing fetal NK cells did not increase between gestational weeks 15 and 22 (Figure 3B and data not shown), in line with the stable distribution of NKG2A+CD16−, NKG2A+CD16+, and NKG2A−CD16+ fetal NK cell subsets during this period of time (Figure 2B).

We recently showed that expression of multiple KIRs on single adult CD56dim NK cells is associated with differentiation (25). Similar to adult NK cells, expression of multiple KIRs on single fetal lung NK cells was increasingly more frequent on NKG2A+CD16+ and NKG2A−CD16+ NK cells, compared with that on NKG2A+CD16− NK cells (Figure 3C). Interestingly, the simultaneous expression of ≥2 KIRs was more frequent on NKG2A+CD16+ and NKG2A−CD16+ fetal lung NK cells compared with that on adult NKG2A+CD16+ and NKG2A−CD16+ PNBK cells, respectively (Figure 3C). The acquisition of multiple KIRs was also associated with a lower frequency of NKG2A expression, although not as pronounced as that observed in adult PBNK cells (Figure 3D), indicat-

ing that downregulation of NKG2A occurs at a later stage of NK cell differentiation, subsequent to acquisition of KIRs.

Terminal differentiation of adult NK cells is also associated with expression of CD57 (25, 33). A small percentage of fetal lung NK cells expressed low levels of CD57, compared with the abundant and high expression found on PBNK cells (Figure 4, A and B). Although the frequency of CD57-expressing NK cells was higher in NKG2A−CD16+ fetal NK cells compared with that in NKG2A+CD16+ and NKG2A+CD16− fetal NK cells, the frequency was still much lower compared with that in adult NKG2A−CD16+ NK cells (data not shown). This indicated that the observed differentiation of fetal NK cells, as evidenced by the loss of NKG2A and acquisition of multiple KIRs, was in itself not sufficient to drive any substantial upregulation of CD57. Finally, we also analyzed the intracellular expression of perforin and granzyme B in fetal NK cells. A majority of the fetal NK cells in the lung expressed both perforin and granzyme B (Figure 4C), albeit at a lower level than that observed in adult PBNK cells (Figure 4D). The expression of perforin and granzyme B was higher in the more differentiated NKG2A+CD16+ and NKG2A−CD16+ fetal NK cells, compared with that in NKG2A+CD16− NK cells, indicating that the differentiation was also accompanied with functional maturation (Figure 4C).

In summary, our analysis revealed that several fetal organs contain a high frequency of KIR-expressing, differentiated NK cells, which suggests that they may be functional and that NKG2A and KIRs may be involved in the regulation of fetal NK cell responses.

Fetal NK cells display decreased responses to HLA class I–negative target cells but are highly responsive to cytokine stimulation. It was reported previously that NK cells in fetal liver are able to kill HLA class I–neg-
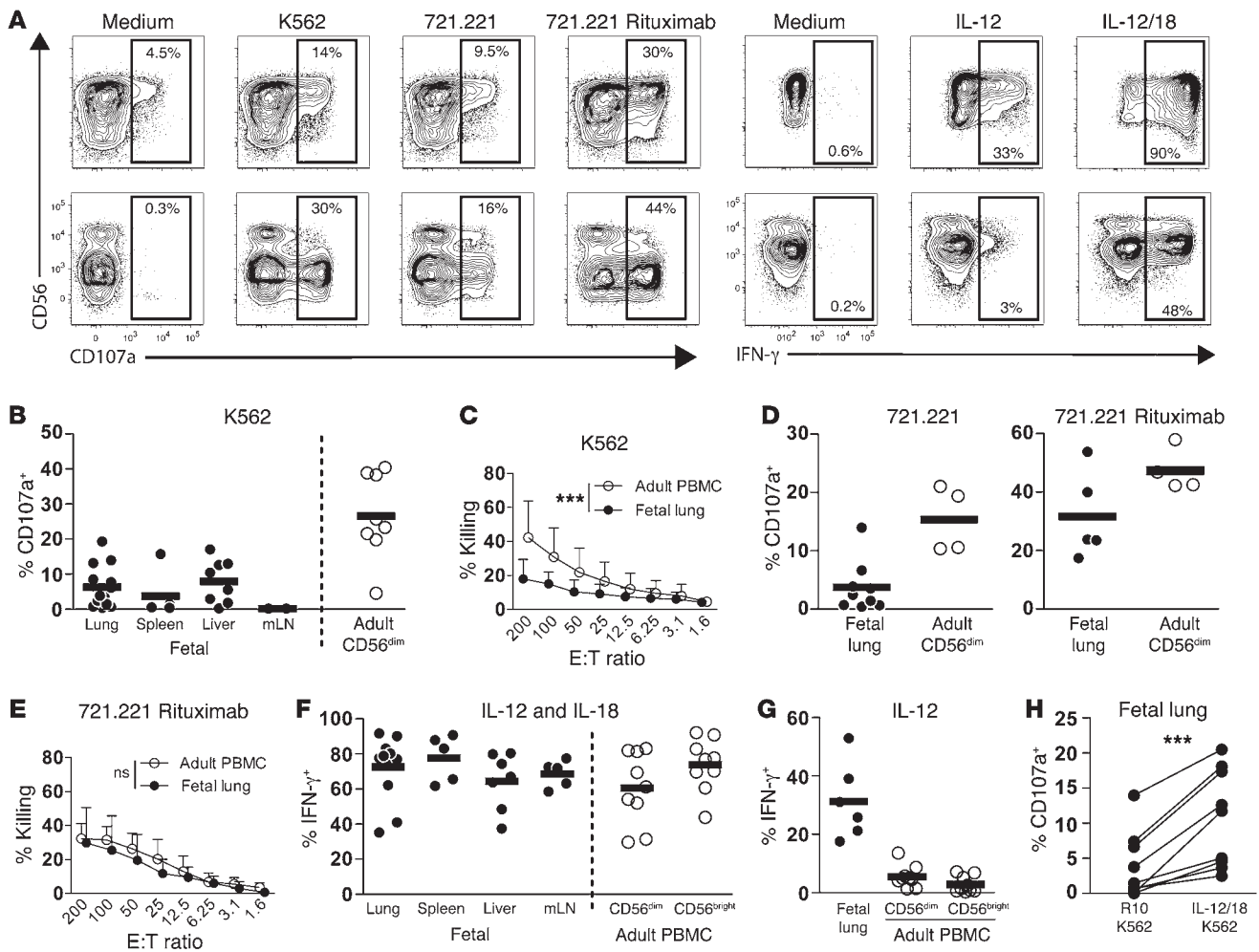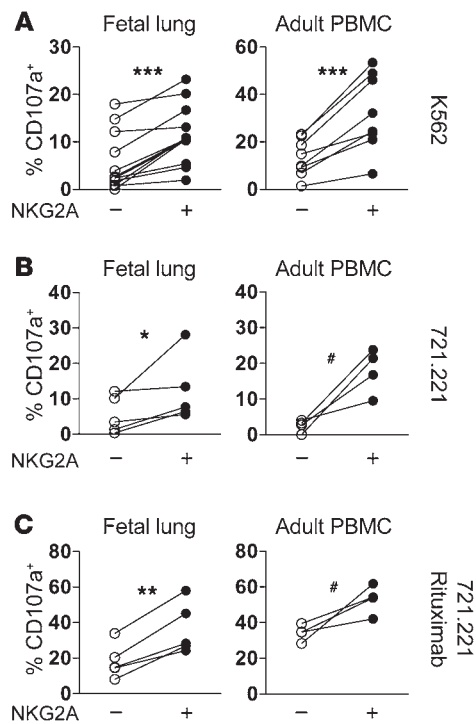
Figure 5
Fetal NK cells degranulate in response to target cells and are highly responsive to cytokine stimulation. (**A**) Representative plots of degranulation (measured as CD107a cell surface expression) and IFN-γ production by fetal lung NK cells (top row) and adult PBNK cells (bottom row), following stimulation with target cells (left) or cytokines (right). (**B**) NK cell degranulation (fetal lung, $n = 14$; fetal spleen, $n = 5$; fetal liver, $n = 8$; fetal mLN, $n = 2$; adult PBMCs, $n = 7$) and (**C**) cytotoxicity in response to K562 cells (fetal lung, $n = 7$; adult PBMCs, $n = 9$). ***$P < 0.001$. (**D**) NK cell degranulation in response to 721.221 cells and rituximab-coated 721.221 cells and (**E**) mean cytotoxicity against rituximab-coated 721.221 cells (fetal lung, $n = 6$; adult PBMCs, $n = 10$). (**F**) Frequency of IFN-γ–positive NK cells after stimulation with IL-12 and IL-18 or (**G**) IL-12 alone. (**H**) Frequency of NK cells degranulating after overnight priming with IL-12 and IL-18, followed by coculture with K562 cells. Bars represent mean, and error bars represent SD. Horizontal bars indicate the mean percentage, and dots indicate the number of samples tested. ***$P < 0.001$, paired t test. The frequency of responding (CD107a+ or IFN-γ+) cells cultured in the control (R10 medium alone) was subtracted in **B**, **D**, **F**, and **G**. The degranulation response from control wells stimulated with medium alone (left) or IL-12/18 (right), without K562 coculture, was subtracted in **H**. (**C** and **E**) Linear regression analysis was performed, testing whether slopes were significantly different.

ative cells and anti-CD16–coated P815 cells, but not as efficiently as adult PBNK cells (17). To date, it is neither known whether fetal NK cells in other organs are functional nor whether fetal NK cells respond to cytokine stimulation. Therefore, we tested fetal and adult NK cell responses in parallel with several different stimuli, including HLA class I–negative cells, antibody-coated cells, and cytokines. For practical reasons, we chose to compare fetal tissue NK cells with adult blood NK cells, as it was logistically very hard to coordinate simultaneous collection of adult and fetal tissues on the same day. However, a separate comparison of matched adult blood and adult lung NK cells revealed that they had a similar degree of differentiation, as measured by expression of CD16, NKG2A, and CD57 (data not shown). A much larger fraction of adult PBNK cells

compared with fetal NK cells expressed high levels of CD57 (Figure 4, A and B), a molecule associated with late NK cell differentiation and altered NK cell function (25, 33). In order to compare adult and fetal NK cells with a similar degree of differentiation, we therefore analyzed CD57− NK cells in all subsequent functional analysis.

NK cells from fetal lung, spleen, and liver degranulated in response to stimulation with HLA class I–negative K562 cells, as measured by CD107a upregulation, but to a lower degree than adult PBNK cells (Figure 5, A and B). Adult PBNK cells responded at a similar level as that of matched adult lung NK cells (Supplemental Figure 3A), verifying that the use of adult PBNK cells in the assays is representative for adult lung NK cells also. When lung and liver NK cells from the same fetus could be directly compared,

Figure 6
NKG2A educates fetal NK cells. Degranulation by NKG2A−CD57−KIR− and NKG2A+CD57−KIR− fetal lung NK cells and NKG2A−CD57−KIR−CD56dim and NKG2A+CD57−KIR−CD56dim adult PBNK cells after stimulation with (**A**) K562 cells, (**B**) 721.221 cells, or (**C**) rituximab-coated 721.221 cells. The degranulation in cultures with medium alone was subtracted from the response to the indicated stimulation. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, paired t test; #$P < 0.05$, Wilcoxon matched-pairs signed-rank test.

fetal NK cells from the liver responded better than those from the lung (data not shown). Fetal lung NK cells also killed K562 cells (Figure 5C), indicating that the observed degranulation correlated with killing of target cells. However, fetal NK cells killed K562 cells to a lower extent than adult PBNK cells, in line with the reduced degranulation by fetal NK cells after stimulation with K562 cells. In addition to K562 cells, fetal NK cells also degranulated in response to HLA class I–negative 721.221 cells (Figure 5D). To test to what extent fetal NK cells could mediate ADCC, we stimulated them with 721.221 cells coated with rituximab (a therapeutic humanized antibody against CD20). Fetal lung NK cells responded strongly to rituximab-coated 721.221 cells, both with respect to degranulation (Figure 5D) and cytotoxicity (Figure 5E), indicating that stimulation via CD16 at least partially overrides the fetal NK cell hyporesponsiveness observed against HLA class I–negative target cells.

In contrast to the decreased responsiveness to HLA class I–negative target cells, fetal NK cells responded strongly to stimulation with IL-12 and IL-18. A majority (≈70%–80%) of the NK cells from all fetal organs tested (lung, spleen, liver, and mLN) produced IFN-γ after stimulation with IL-12 and IL-18 (Figure 5, A and F). The frequency of IFN-γ–producing fetal NK cells was at least as high as that in adult CD56dim and CD56bright PBNK cells (Figure 5F). In addition, fetal lung NK cells also responded strongly to

stimulation with IL-12 alone (Figure 5G), whereas adult PBNK cells responded poorly, suggesting that fetal NK cells have a lower threshold of activation with respect to cytokine stimulation. In addition to direct activation of NK cells, stimulation with IL-12 and IL-18 can prime target cell responses by adult NK cells. Similarly, overnight culture with IL-12 and IL-18 significantly increased the functional responsiveness to K562 cells by fetal lung NK cells (Figure 5H). Taken together, our results demonstrate that fetal NK cells are highly responsive to cytokine stimulation but are hyporesponsive to HLA class I–negative target cells.

NKG2A educates fetal NK cells. The finding that fetal NK cells had a decreased response to HLA class I–negative target cells indicated that there are differences in the regulation of fetal and adult NK cell function. Education via NKG2A and KIRs is critical for optimal NK cell responses to HLA class I–negative or allogeneic cells in adults (24), but it is currently unknown whether these receptors educate fetal NK cells. We have previously shown that fetal tissue lymphocytes express normal levels of HLA class I (19), and we also verified the expression of HLA class I molecules on fetal cells in this study (Supplemental Figure 4), demonstrating that ligands for both NKG2A and KIRs are expressed in the fetal tissues. NKG2A has a strong educational effect on NK cells in adults, and NKG2A+ NK cells in adults respond well to stimulation with HLA class I–negative target cells (23, 34). In addition, NKG2A confers an educational signal to NK cells during the early phases of the reconstitution of the immune system after hematopoietic stem cell transplantation (35). Given the high frequency of NKG2A+ fetal NK cells (Figure 2A), we asked whether NKG2A conferred an educational signal in the developing fetus. To compare NK cell education via NKG2A in fetal and adult NK cells, we analyzed degranulation after stimulation, and gated on CD57−KIR− NK cells, thus normalizing for the effects of differentiation coupled to expression of CD57 (25, 33) and KIR-mediated education (21). NKG2A+CD57−KIR− fetal lung NK cells (as well as fetal liver and spleen NK cells; data not shown) responded better than NKG2A−CD57−KIR− NK cells after stimulation with K562 cells (Figure 6A) as well as after stimulation with 721.221 cells (Figure 6B) and rituximab-coated 721.221 cells (Figure 6C). Confirming previous studies, adult NKG2A+CD57−KIR− PBNK cells also responded better than NKG2A−CD57−KIR− PBNK cells after stimulation with K562 cells (Figure 6A), 721.221 cells (Figure 6B), or rituximab-coated 721.221 cells (Figure 6C). Similarly, adult lung NKG2A+ NK cells responded better than adult lung NKG2A− NK cells to stimulation with K562 cells (Supplemental Figure 3B). Thus, NKG2A educates fetal and adult NK cells, thereby rendering them reactive to target cells lacking HLA-E, while potentially remaining tolerant to HLA-E–expressing cells.

KIR expression on fetal NK cells results in hyporesponsiveness. In addition to the education via NKG2A, adult NK cells are educated via the interaction between KIR and self-HLA class I molecules (24). KIR-mediated education and functional inhibition are important for the regulation of NK cell allogeneic responses, in which target cells lack ligand(s) for inhibitory KIRs. As such, education of fetal NK cells via paternally inherited HLA class I could potentially result in the generation of fetal NK cells reactive toward allogeneic maternal cells. We have previously used HLA class I–negative target cells as a model to reveal potential alloreactive NK cell responses (36). The use of K562 and 721.221 cells facilitates the comparison among different donors and provides a more sensitive tool compared with primary allogeneic target cells, which normally induce
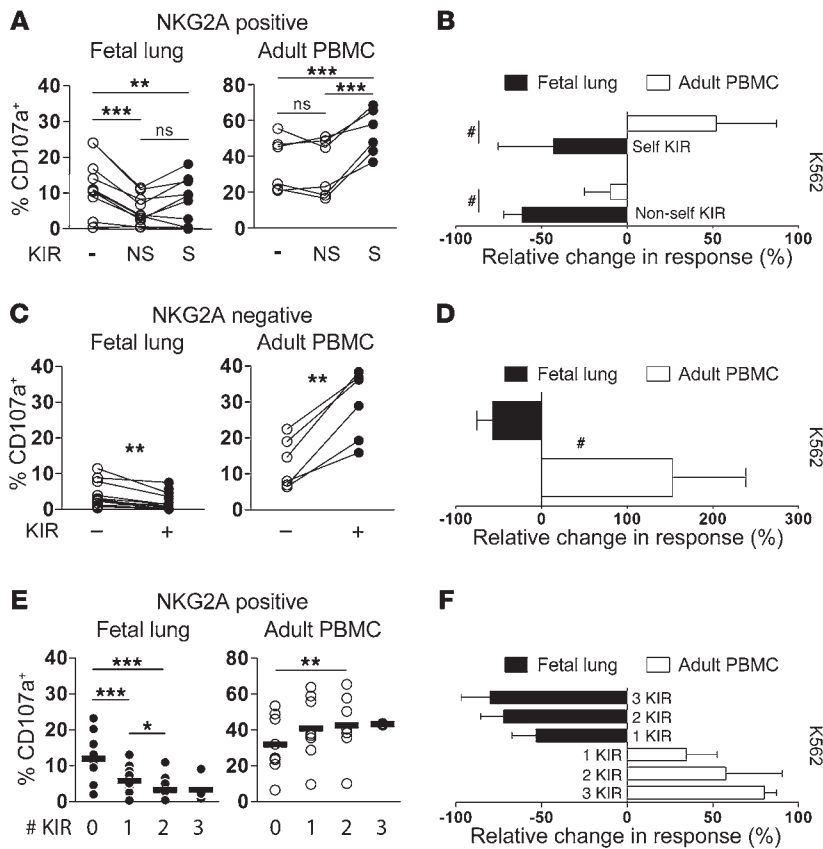
Figure 7
KIR-expressing fetal NK cells are hyporesponsive to stimulation with K562 cells. (**A**) Degranulation by NKG2A+CD57– fetal lung NK cells and NKG2A+CD57–CD56dim adult PBNK cells expressing no KIR, only self KIR (S), or only non-self KIR (NS) (defined using a Boolean function of KIR2DL1, KIR2DL3, and KIR3DL1 expression and KIR-HLA ligand genotype) after stimulation with K562 cells. (**B**) Relative change in response to K562 cells compared with NKG2A+CD57– NK cells lacking KIR expression. (**C**) Degranulation by NKG2A–CD57– fetal lung NK cells and NKG2A–CD57–CD56dim adult PBNK cells expressing no KIR or any combination of KIR (as in **A**) after coculture with K562 cells. (**D**) Relative change in response to K562 cells compared with NKG2A–CD57– NK cells lacking KIR expression. (**E**) Degranulation by NKG2A+CD57– fetal lung NK cells and NKG2A+CD57–CD56dim adult PBNK cells expressing 0, 1, 2, or 3 KIRs (KIR2DL1, KIR2DL3, and/or KIR3DL1) after stimulation with K562 cells. (**F**) Relative change in degranulation compared with NK cells lacking expression of KIRs. Bars represent mean, and error bars represent SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, 1-way ANOVA analysis of repeated measurements with Bonferroni's post-test; #$P < 0.001$, unpaired t test. Horizontal bars indicate the mean percentage, and dots indicate the number of samples tested.

very weak in vitro responses, even by freshly isolated adult NK cells (our own unpublished observations). Similar to NKG2A, education via self-HLA–specific inhibitory KIRs is evident in the early phases of immune reconstitution after hematopoietic stem cell transplantation in adults (35), indicating that education via KIRs could also be functional during the development of fetal NK cells. To investigate whether this was the case, we compared the degranulation of NKG2A+CD57–KIR– NK cells with NKG2A+CD57– NK cells expressing either only self-HLA class I–binding KIRs (self KIR) or only non-self-HLA class I–binding KIRs (non-self KIR) in response to K562 cells. Surprisingly, fetal lung NKG2A+CD57– NK cells expressing either only self or non-self KIRs responded significantly less than did fetal NKG2A+CD57– NK cells lacking KIR expression (Figure 7, A and B). Similarly, fetal lung NKG2A–CD57– NK cells expressing KIRs responded less well to stimulation than did fetal NKG2A–CD57– NK cells lacking expression of KIRs (Figure 7, C and D). Due to the low frequency of NKG2A–CD57– fetal NK cells, further division into self- and non-self KIR+ NK cells in this subset was unfortunately not possible.

In contrast to the response patterns of fetal NK cells, adult NKG2A+CD57– PBNK cells expressing self KIRs responded better to stimulation with K562 cells than did NKG2A+CD57– PBNK cells lacking KIRs or expressing non-self KIRs (Figure 7, A and B), confirming that self KIRs educate adult NK cells. Similarly, adult NKG2A–CD57– PBNK cells expressing KIRs responded better to stimulation with K562 cells compared with NKG2A–CD57– PBNK cells lacking KIRs (Figure 7, C and D). To address whether the observed KIR-mediated hyporesponsiveness was a general feature of fetal NK cells rather than a lung-specific feature, we compared

degranulation by adult NK cells from matched lung tissue and peripheral blood. Interestingly, adult lung NK cells degranulated to a similar level as adult PBNK cells from the same donor and were educated by both NKG2A and KIRs (Supplemental Figure 3, B–D). Finally, NKG2A+KIR+ fetal liver NK cells were hyporesponsive compared with NKG2A+KIR– fetal liver NK cells (Supplemental Figure 3E), whereas NK cells from adult liver have previously been demonstrated to be educated via KIRs (37). Together, this indicates that the KIR-mediated hyporesponsiveness is indeed a feature unique to fetal NK cells.

As we had observed that a large proportion of the fetal lung NK cells expressed multiple KIRs, we next asked whether the decreased responsiveness was dependent on the number of KIRs expressed. Strikingly, expression of 1 single KIR was sufficient to induce hyporesponsiveness in fetal NK cells, and coexpression of 2 or 3 KIRs further dampened the response (Figure 7, E and F). In contrast, expression of 1 or more KIRs on adult PBNK cells resulted in an increased responsiveness (Figure 7, E and F). To test whether the observed hyporesponsiveness of fetal NKG2A+CD57–KIR+ NK cells also extended to other stimuli, we analyzed NK cell responses to 721.221 cells alone as well as to 721.221 cells coated with rituximab. In all settings, KIR+ fetal NK cells responded significantly less well than did KIR– fetal NK cells (Figure 8).

We have previously reported that overnight priming of adult PBNK cells with cytokines is sufficient to break the hyporesponsiveness of noneducated adult NK cells to stimulation with K562 cells (25). To test whether cytokine priming was sufficient to break the hyporesponsiveness of fetal KIR+ NK cells, we cultured them overnight in IL-12 and IL-18 and subsequently measured
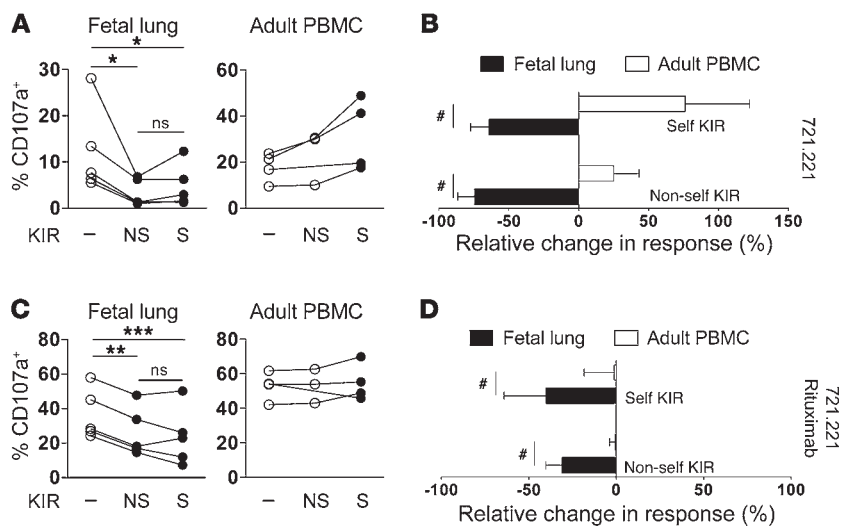
Figure 8
KIR-expressing fetal NK cells are hyporesponsive to 721.221 cells and rituximab-coated 721.221 cells. (**A**) Degranulation by NKG2A⁺CD57⁻ fetal lung NK cells and NKG2A⁺CD57⁻CD56ᵈⁱᵐ adult PBNK cells expressing no KIR, only self KIR, or only non-self KIR (defined using Boolean function of KIR2DL1, KIR2DL3, and KIR3DL1 expression and KIR-HLA ligand genotype) after stimulation with 721.221 cells. (**B**) Relative change in degranulation compared with NK cells lacking expression of KIRs after stimulation with 721.221 cells. (**C**) Degranulation against rituximab-coated 721.221 cells by fetal and adult NK cells as in **A**. (**D**) Relative change in degranulation compared with NK cells lacking expression of KIRs after stimulation with rituximab-coated 721.221 cells. The degranulation in unstimulated cultures was subtracted from the response to the specific stimulations. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, 1-way ANOVA analysis of repeated measurements with Bonferroni's post-test; #$P < 0.001$, unpaired t test. Error bars represent SD.

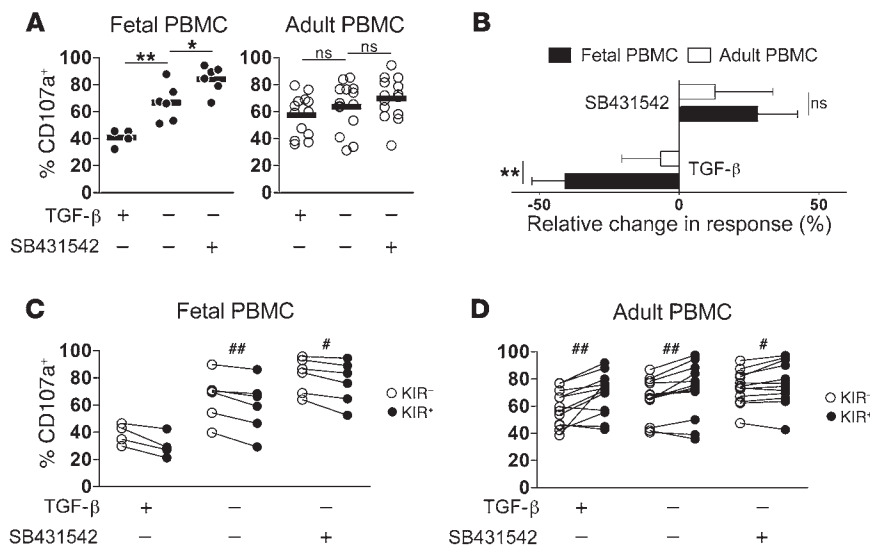addition of recombinant TGF-β strongly dampened fetal NK cell responses to rituximab-coated 721.221 cells (Figure 9, A and B). In contrast, adult PBNK cells were only marginally affected by the addition of TGF-β or the TGF-β receptor kinase signaling inhibitor (Figure 9, A and B), indicating that fetal NK cells are more susceptible to TGF-β–mediated suppression than adult NK cells.

Given their sensitivity to TGF-β, we next investigated the possibility that the observed KIR-mediated hyporesponsiveness was caused by an increased sensitivity of KIR⁺ fetal NK cells to TGF-β, compared with those lacking expression of KIRs. By analyzing NK cells in fetal cord blood, we were at the same time able to investigate whether the observed KIR-mediated hyporesponsiveness was a general feature of fetal NK cells or whether it was unique to fetal NK cells from tissues. Similar to fetal lung NK cells, NKG2A⁺CD57⁻KIR⁺ fetal blood NK cells responded less well to stimulation with rituximab-coated 721.221 cells than did NKG2A⁺CD57⁻KIR⁻ fetal blood NK cells (Figure 9, C and D), corroborating that KIR-mediated hyporesponsiveness is a general feature of fetal NK cells. However, the addition of TGF-β or the TGF-β receptor kinase inhibitor (SB431542) did not reverse the KIR-mediated hyporesponsiveness (Figure 9, C and D), indicating that the differential regulation of fetal versus adult NK cell education via KIRs is independent of TGF-β signaling.

Discussion
Here we demonstrate that human fetal NK cells differentiate early in utero and are highly responsive to cytokine stimulation and antibody-coated target cells but hyporesponsive to HLA class I–negative target cells. Taken together, our data indicate that fetal NK cells can be involved in cytokine- and antibody-mediated NK cell responses in utero, while remaining hyporesponsive to HLA class I–negative or allogeneic cells.

The functional consequence of the early development of the human immune system, as opposed to the relatively late development in the mouse, remains unknown. Given the long gestational period in humans, and the risk of exposure to pathogens this might entail, it is possible that humans have evolved capabilities to respond against infections already in utero. Indeed, recent studies have shown that intrauterine infections are much more common than previously believed and have been estimated to account for a quarter of the 12.6 million preterm births occurring annually (38). In addition, herpes viruses, including CMV, have been detected in fetal tissues after stillbirths (39, 40), and CMV-specific CD8 T cell responses have been reported in cord blood from gestational week 28 (9). From gestational week 10, the fetus starts to inhale amniotic fluid (41) and as such is exposed to the contents of amniotic fluid, including maternal cells, antibodies, cytokines, and pathogens (42). Given the strong responses to cytokines and antibody-coated cells by fetal NK cells, but weak responses to HLA class I–negative cells, it is tempting to speculate that the unusually high frequency of differentiated NK cells in the

responses to K562 cells. Although cytokine priming enhanced the fetal NK cell responses to K562 cells, the KIR⁺ fetal NK cells were still clearly hyporesponsive compared with KIR⁻ fetal NK cells (Supplemental Figure 5B), indicating that the KIR-mediated hyporesponsiveness is maintained even after cytokine priming.

Together, these results suggest that KIR expression does not educate fetal NK cells, but instead confers hyporesponsiveness, and as such provides a potential mechanism for fetal NK cell hyporesponsiveness to HLA class I–negative target cells.

TGF-β efficiently suppresses fetal NK cells. In addition to regulation of NK cell responsiveness via NKG2A and KIRs, other mechanisms may contribute to dampening fetal NK cell responses in vivo. Several studies have shown that human NK cells are sensitive to TGF-β–mediated suppression in vitro (26, 27). It was also recently reported that TGF-β is responsible for the immaturity of neonatal NK cells in mice (15). We have previously demonstrated that TGF-β and TGF-β–related transcripts are expressed at a higher level in the developing human fetus, compared with that in adults (19), supporting the possibility that it could be involved in suppressing fetal NK cell responses in vivo. To investigate whether human fetal NK cells were affected by TGF-β, we cultured fetal cord blood mononuclear cells and adult PBMCs in the presence of either recombinant TGF-β or a TGF-β receptor kinase inhibitor (SB431542) prior to stimulation with rituximab-coated 721.221 cells (Figure 9, A and B). Upon inhibition of TGF-β receptor signaling, fetal cord blood NK cells responded significantly better compared with control cultures (Figure 9, A and B). Conversely,

Figure 9
Fetal NK cells are highly susceptible to TGF-β–mediated suppression. (**A**) Degranulation by NKG2A+CD57− fetal cord blood NK cells and NKG2A+CD57− CD56dim adult PBNK cells after culture with TGF-β or TGF receptor kinase inhibitor (SB431542) for 48 hours and subsequent stimulation with rituximab-coated 721.221 cells for 2 hours. (**B**) Relative change in degranulation in response to rituximab-coated 721.221 cells after treatment with TGF-β or SB431542, compared with medium control. (**C**) Degranulation by NKG2A+CD57− fetal cord blood NK cells and (**D**) NKG2A+CD57−CD56dim adult PBNK cells expressing no KIR (KIR−) or any combination of KIR2DL1, KIR23DL3, or KIR3DL1 (KIR+) after culture with TGF-β or TGF-β receptor kinase inhibitor (SB431542) for 48 hours and subsequent stimulation with rituximab-coated 721.221 cells for 2 hours. Data are summarized from 3 individual experiments. Bars represent mean, and error bars represent SD. *$P < 0.05$, **$P < 0.01$, Mann-Whitney test; #$P < 0.05$, ##$P < 0.01$, paired t test.

fetal lung helps in protecting against fetal infections via production of cytokines and ADCC, while remaining hyporesponsive to allogeneic cells. Moreover, after birth, as the newborn inhales air for the first time, the lungs will be readily exposed to pathogens in the air, and the immediate presence of functional immune cells in the lung might thus serve an important function. The finding that fetal NK cells can mediate strong ADCC responses also opens up the possibility for a role of NK cells as effector cells in, for example fetal anemia, which is dependent on transfer of maternal anti-Rhesus antibodies to the fetus.

An alternative, but not mutually exclusive, possibility is that the fetal immune system regulates development of different tissues via secretion of cytokines. For example, the development of the lung, and in particular production of human pulmonary surfactant proteins, is influenced by both IFN-γ and TGF-β (43), thus potentially providing a role for IFN-γ produced by NK cells in utero, even in the absence of infection. In this regard, it is interesting to note that the threshold for cytokine responsiveness by fetal NK cells was lower compared with that for adult NK cells, as evidenced by the robust production of IFN-γ by fetal lung NK cells in response to IL-12 alone.

It has become increasingly clear that the fetal-maternal interface is much less of a barrier than previously believed. For example, we have previously shown that maternal cells can be detected in fetal lymph nodes from a majority (16 out of 18) of fetuses analyzed (19), and maternal cells, antibodies, and bacteria can be detected in amniotic fluid (20). Maternal cells in the offspring can even be detected into adulthood and have been associated with

increased graft survival in kidney transplantation (44). The transfer of maternal cells to the fetus could potentially induce harmful fetal antimaternal immune responses. Fetal T cells are uniquely prone to develop into regulatory T cells upon stimulation with allogeneic cells, thus providing a mechanism for fetal-maternal T cell tolerance in the developing fetus (19). Conversely, in mice, fetal cells can pass over to the mother and induce maternal regulatory T cell responses to fetal antigens (45, 46). It is conceivable that fetal NK cells have also evolved mechanisms to avoid allorecognition of maternal cells, while maintaining responsiveness to cytokines and pathogens. In the mouse, it was recently suggested that the late development of mouse NK cells, in which expression of Ly49 receptors (the ortholog of human KIRs) is detected only after birth, helps in avoiding the potentially devastating effects of having a mature differentiated pool of NK cells recognizing maternal allogeneic cells in utero (47). In contrast to mouse NK cells, our data reveal that human NK cells develop early, express KIRs, and are likely to be able to mediate functional responses in vivo in the developing human fetus. From the perspective of fetal NK cells, maternal cells can be semiallogeneic with respect to HLA-A, HLA-B, and HLA-C, whereas HLA-E is shared between the mother and the fetus. In adults, recognition of missing self HLA-A, HLA-B, and HLA-C is mediated via educated KIR-expressing NK cells, whereas recognition of missing HLA-E is mediated via NKG2A (24). In the fetus, the KIR-induced hyporesponsiveness in NK cells could potentially prevent the recognition of maternal allogeneic cells by fetal NK cells. Importantly, the KIR-mediated hyporesponsiveness was independent of HLA class I specificity, indicating that it is an intrinsic feature of KIR expression on fetal NK cells and not dependent on interactions with HLA class I expressed by maternal or fetal cells. In addition, HLA-E expression by maternal cells would inhibit fetal NK cells via the interaction with NKG2A. We therefore suggest that the differential education via NKG2A and KIRs provides a mechanism for fetal-maternal tolerance, while maintaining responses to antibody-coated target cells and cytokines.

The mechanism underlying the differential education via KIRs in fetal and adult NK cells remains to be identified. One possibility, given the observed sensitivity of fetal NK cells to TGF-β, was that KIR-expressing fetal NK cells would be more sensitive to this cytokine. However, despite its effects on the overall fetal NK cell response, TGF-β did not affect the observed KIR-mediated hyporesponsiveness, indicating that other mechanisms are responsible. In addition, priming with IL-12 and IL-18 did not break the KIR-mediated hyporesponsiveness by fetal NK cells, whereas it is sufficient to break hyporesponsiveness by noneducated adult NK cells (25). This indicates that KIR-expressing fetal NK cells remain hyporesponsive to allogeneic cells even in conditions in which

cytokines normally priming NK cells are present, e.g., during infections. It is noteworthy that there has been no definite mechanism identified for the education of adult NK cells via inhibitory receptors to date. However, in mice, it is clear that it requires functional immunoreceptor tyrosine-based inhibitory motifs of the MHC class I–binding Ly49 receptors but is independent of SHP-1 signaling (48). In addition, educated mouse NK cells have an altered membrane distribution of activating receptors, which has been proposed to be pivotal for NK cell education (49). We showed recently that adult human KIR-expressing T cells are hyporesponsive compared with T cells lacking expression of KIRs, independent of KIR ligands being present in the host or not (50), akin to what we observed here for fetal KIR-expressing NK cells. Similar to that in fetal NK cells, the T cell responsiveness was decreased with an increasing number of inhibitory KIRs expressed by the same T cell. Thus, it is possible that fetal KIR-expressing NK cells and adult KIR-expressing T cells share a common mechanism to shut down functional responses via inhibitory KIRs in an HLA-independent manner. Alternatively, adult NK cells, but not fetal NK cells and adult T cells, possess pathways that allow educational signals via the inhibitory KIRs.

It remains to be established when and how the shift from KIR-induced hyporesponsiveness to KIR-mediated education takes place. We have previously shown that distinct populations of T cells are generated from human fetal hematopoietic stem cells compared with the adult hematopoietic stem cells, indicative of a layered development of human T cells (51). A layered development of mouse B cells was suggested over 20 years ago (52), and it was recently reported that expression of Lin28b is a hallmark for fetal lymphoid progenitors giving rise to innate-like immune cells in mice (53). Similarly, NK cell development might be layered. It is therefore possible that the KIR-induced hyporesponsiveness reflects a feature unique to a first wave of fetal NK cell development, which is gradually substituted by an adult-like NK cell development, in which KIR-mediated education is occurring.

The finding that fetal NK cell differentiation, as measured by expression of NKG2A, CD16, and KIRs, is stable between gestational week 15 and 22 indicates that the output, differentiation, and death of fetal NK cells is already at a steady state during this time period, despite large changes in cellularity and size of the different organs. Given that NK cells can be detected in fetal liver from gestational week 6 (17), it is likely that the first wave of developing NK cells have a more dynamic phase in the first trimester, which is followed by a steady-state phase in the second trimester. The finding that full-term cord blood NK cells, in contrast to fetal NK cells, are educated by self-HLA class I–specific KIRs (22), suggests that the transition from fetal NK cell development to more adult-like NK cell development occurs in the third trimester.

Finally, the KIR-mediated hyporesponsiveness of NK cells described in this study, together with the induction of regulatory T cells in the developing fetus (19), have implications for clinical settings of in utero transplantation, as it is possible that both fetal NK cells and T cells could tolerate allogeneic grafts.

Methods

Human tissues and blood. Tissues and blood samples used were collected after informed consent and with approval from the Karolinska Institutet Ethics review board and the UCSF Committee on Human Research. Human fetal tissues were obtained from elective abortions at the Women's Options Center at San Francisco General Hospital, with approval from the UCSF Committee on Human Research. Collection of fetal blood (gestational weeks 18–36) by ultrasound-guided choriocentesis was part of the therapeutic procedure for fetal anemia and approved by the regional ethical committee in Stockholm, Sweden. Adult lung tissues and matched peripheral blood were obtained from patients at the Karolinska University Hospital undergoing lobectomy due to suspicion of lung cancer. All tissues were derived from healthy areas of the resected lobe, and the regional ethical committee in Stockholm, Sweden, approved collection of material. Adult and fetal PBMCs were isolated by density centrifugation (Ficoll-Hypaque, GE Healthcare). To avoid any potential maternal contamination of the fetal tissues, the fetal organs were dissected from intact fetuses and extensively washed with cold R10 medium (RPMI1640 with 10% FCS, penicillin, streptomycin, and L-glutamine) before further processing. Using this protocol, we have shown previously that the frequency of maternal cells is routinely <0.1% and that maternal contamination is not a factor affecting the analyses of fetal immune cells. Subsequently, fetal liver and fetal and adult lung tissue were cut into pieces. Liver, lung, and mLNs were incubated for 30 minutes at 37°C in serum-free R10 with 0.25 mg/ml collagenase type 2 (Sigma-Aldrich), followed by washing with R10. Spleens were passed through a 70-μm cell strainer to obtain single cell suspensions. Subsequently, mononuclear cells from spleen, lung, liver, and bone marrow were isolated by density centrifugation on a Ficoll gradient.

HLA and KIR genotyping. Genomic DNA was isolated from tissues or blood using the DNeasy Blood & Tissue Kit (QIAGEN). KIR genotyping was performed using PCR-SSP technology with a KIR typing kit (Olerup-SSP). KIR ligands were determined with the KIR HLA Ligand Kit (Olerup-SSP), which detects the HLA-C1, HLA-C2, and Bw4 motifs.

Antibodies and flow cytometry. Antibodies and clones against the following proteins were used: CD3 (S4.1/AKA 7D6, Qdot 655, Invitrogen, or UCHT1, PE-Cy5, Beckman Coulter), CD7 (M-T701, Alexa Fluor 700, BioLegend, or Horizon V450, BD Biosciences), CD14 (TÜK4, Qdot 655, Invitrogen, or M5E2, Horizon V500, BD Biosciences), CD16 (3G8, PerCP, BioLegend), CD19 (SJ25-C1, Qdot 655, Invitrogen, or HIB19, Horizon V500, BD Biosciences), CD34 (581, ECD, Beckman Coulter), CD45 (HI30, Alexa Fluor 700, BioLegend), CD56 (N901, ECD, Beckman Coulter, or NCAM 16.2, Qdot 705, or PE-Cy7, BD Biosciences), CD57 (HCD57, Pacific Blue, BioLegend), CD127 (A7R34, APC-eFluor 780, eBioscience), KIR2DL1 (143211, FITC, R&D Systems), KIR2DL3 (180704, biotin, R&D Systems), KIR3DL1 (DX9, biotin, Alexa Fluor 700, or Brilliant Violet 421, BioLegend), HLA-DR (TÜ36, APC, BD Biosciences), NKG2A (Z199, APC, or Pacific Blue, Beckman Coulter), NKp46 (9E2, Pacific Blue, or biotin, BioLegend), CD107a (H4A3, APC-Cy7, PE, or FITC, BD Biosciences), IFN-γ (β27, PE, BD Biosciences), HLA-Bw4 (biotin, OneLambda), HLA-Bw6 (biotin, OneLambda), HLA class I (W6/32, APC, eBioscience), HLA-E (3D12, PE, BioLegend), Perforin (dG9, FITC, eBioscience), and Granzyme B (GB11, PE, eBioscience). Biotinylated antibodies were detected using streptavidin-Qdot 565, 585, 605, or 705 (Invitrogen). All samples were stained with Live/Dead Aqua (Invitrogen) to discriminate between live and dead cells. Samples were analyzed using a Beckton Dickinson LSRII SORP flow cytometer equipped with 4 lasers (405 nm, 488 nm, 532 nm, and 647 nm). Data was analyzed using FlowJo 9.5.3 (Tree Star).

Functional assays. Prior to use in degranulation assays, isolated mononuclear cells were rested overnight in R10 at 37°C, 5% CO_2. 10^6 mononucleated cells were cocultured with 10^5 target cells (K562 cells or 721.221 cells, with or without 1 μg/ml rituximab) for 2 hours in the presence of anti-CD107a, followed by staining with antibodies. Cytotoxicity assays were performed by labeling K562 cells or 721.221 cells with CellTrace Violet (Invitrogen) and coculturing these with mononuclear cells at indicated effector/target ratios for 4 hours. Cells were subsequently stained with Live/Dead Fixable Dead Cell Stain (Invitrogen). Cytotoxicity was cal-

culated using the following formula: percentage of dead cells in samples cocultured with effector cells − percentage of dead cells in samples with target cells alone, where dead target cells were defined by the Live/Dead Viability Dye (Invitrogen). The effect of rituximab-induced cytotoxicity was calculated using the following formula: percentage dead rituximab-coated 721.221 cells cocultured with effector cells − percentage dead 721.221 cells without rituximab cocultured with effector cells. In experiments in which TGF-β1 (R&D Systems) or TGF-β receptor kinase inhibitor (SB431542, Sigma-Aldrich) were used, mononuclear cells were cultured in R10 for 48 hours with either human TGF-β1 (5 ng/ml) or the TGF-β receptor kinase inhibitor (SB431542) (1 μM) before addition of target cells and anti-CD107a mAb. For analysis of cytokine responsiveness, mononuclear cells were cultured for 12 hours with IL-12 alone (10 ng/ml, PeproTech) or with a combination of IL-12 and IL-18 (10 ng/ml and 100 ng/ml, respectively; PeproTech), followed by addition of monensin and Brefeldin A and an additional 6 hours of culture.

Immunohistochemistry. Fetal lung and liver were imbedded in Tissue-Tek OCT and stored at –80°C until sectioning. 5-μm tissue sections from frozen fetal samples were placed on SuperFrost Ultra Plus slides (Histolab) and stored at –80°C until staining. Before staining, the sections were air dried for 10 minutes and thereafter fixed in 4% paraformaldehyde (Sigma Aldrich) for 20 minutes on ice. Sections were incubated with Bloxall (Vector Laboratories) for 10 minutes and then blocked in Innovex background blocker (Innovex Biosciences) for 20 minutes at room temperature. Samples were incubated with purified mouse anti-human NKp46 (195314, R&D Systems), mouse anti-human EpCAM (Ber-EP4, Dako-Cytomation), or mouse anti-human CD31 (JC/70A, Abcam) overnight at more than 4°C. Isotype-matched antibodies served as negative controls. Subsequently, sections were incubated with ImmPRESS mouse or rabbit secondary antibodies (Vector Laboratories) for 30 minutes at room temperature and specific staining was detected by incubation with ImmPACT DAB (Peroxidase substrate, Vector Laboratories). Tissue sections were then counterstained with Hematoxylin (Histolab) and mounted with Kaisers's glycerol gelatine (Merck Millipore). Positive staining was visualized by light microscopy (Leica DM 4000B).

Statistics. For comparisons of independent groups, the Student's *t* test or the Mann-Whitney *U* test were performed. For comparisons of matched groups, the paired Student's *t* test or Wilcoxon matched test were performed. One-way ANOVA with a Bonferroni post-analysis was used for comparisons of multiple groups. Statistical analyses were performed using Prism software version 5.0 (GraphPad Software Inc.).

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