Supplemental Material and Methods

Supplemental methods

Detection of CD6 splice variants

For the analysis of CD6 splice variants, the following primer combinations were used: 5´dD3_2: 5´-GGATCAGTGTGCGATGACA-3´annealing to nucleotides 5'770 - 3'788 and 3´CD6_D: 5´-GAGGAGGTCCAGCAAAGC-3 annealing to nucleotides 5'1760 - 3'1777 (NCBI NM_006725.3) with PCR conditions of initial denaturation 94°C 120 sec, followed by 40 cycles at 94°C for 30 sec, 58°C for 30 sec, 72°C for 60 sec and a final 5 min 72°C synthesis phase.

Phenotyping of NK cell subpopulations during expansion with IL-2 and IL-15

NK cell subsets were isolated using the isolation kits (Miltenyi, Bergisch Gladbach, Germany) for $CD16^+$ NK cells according to the manufacturer's instructions. Isolated NK cells were stained immediately or after 2, 4, 6, and 8 days culture with IL-2 (500 U/ml) or IL-15 (5 ng/ml) using the combinations of CD16-FITC, CD6-PE, CD3-PerCP and CD56-APC. Sorted NK cells (subsets A-C) were cultured for 5d in the presence of IL-2 (500 U/ml), IL-15 (20 ng/ml) or medium and then stained for the expression of CD6 and CD56.

Proliferation assay of sorted NK cells

To assess the proliferative capacity of $CD6^-CD56^{dim}$ and $CD6^+CD56^{dim}$ NK cells, isolated NK cells were labelled with CellTrace Violet (Invitrogen) and incubated for 6 days with IL-2 (500 U/ml), IL-15 (20 ng/ml) or medium. CellTrace Violet dilution was analysed in conjunction with CD6 expression after gating on $CD56^{dim}$ NK cells.

Supplemental Figure legends

Suppl. Figure 1. Different CD6-specific mabs give rise to identical CD6-staining patterns for T and NK cells and CD6 splice variants are detectable in activated NK cells. (A) Indirect staining of PBMC with mab MT-606, MEM-98 and BL-CD6 resulted in an identical distribution of CD6 on the three NK cell subsets A, B and C as defined in Fig. 1A in which directly PE-labeled CD6 mab (M-T605) was used. As expected, virtually all T cells highly express CD6 and the staining patterns with these three different mab are identical to the CD6-PE staining (data not shown). One of four representative experiments is shown. (B) Using two newly defined primer sets binding to exon 4 (SRCR2 domain) and exon 10 (intracytoplasmic domain), full length mRNA (1000bp) was only weakly detectable in fresh NK cells (lane 1 after the marker M). Following 3d culture of NK cells with IL-2 (100 U/ml) (lane 2), the full length amplicon was replaced by several shorter variants bearing deletions of exon 8/9 and /or the ΔD3 domain. Even shorter amplicons arise and are currently investigated for their exact composition. Interestingly, this splice variant pattern is almost identical to an allo-specific $CD8^+$ T cell clone that was used as T cell reference (lane 3).

Suppl. Figure 2. CD6 expression on sorted NK cells (A) or CD16-positively isolated NK cells (B) after cytokine stimulation. (A) NK cells were sorted according to their expression density of CD56 and CD6 (subset A-C) and cultured for 5d with IL-2 (500 U/ml), IL-15 (20 ng/ml) or medium. NK cell subsets A-C were analysed for their CD6 expression whereby expression remains stable on $CD6^+$ NK cells and is not induced on $CD6^-$ NK cells in this setting. One representative experiment of three is shown. (B) In contrast to FACS sorting in (A), the CD16-positive isolation strategy by Miltenyi was used to highly enrich $CD16^+CD56^{dim}$ NK cells that were subsequently cultured with IL-2 (100 U/ml) or IL-15 (20 ng/ml). Expression of CD16, CD56 in combination with CD6 was determined at d2, 4, 6, and

8. Remarkably, no development of $CD56^{dim}$ NK cells into $CD16^{-}CD56^{bright}$ NK cells was observed. However, a new $CD16^{low}/CD56^{low}$ NK cell population appeared that also displayed decreasing CD6 densities compared to the stable $CD16^{+}CD6^{+}$ NK cell subset. A graph of the raw data is plotted on the left in which the $CD6^{+}CD56^{dim}$ NK (A) and the $CD6^{-}CD56^{dim}$ population (B) are subdivided into $CD16^{+}$ and $CD16^{-}$ subsets and calculated according to all NK cells (sum of the four subsets = 100%).

Suppl. Figure 3. Flow cytometric analysis of CD107a following mab-stimulation. CD107a surface expression on NK cells stimulated with plate-bound mab against various activating NK receptors is shown as bar graph in Fig. 4D. In these dot plots, pre-activated PBMC were incubated for 4h with the specific plate-bound mabs against CD16 (3G8) or NKp46 (Bab284), CD6 and CD107a expression was analysed on NK cells (gated on $CD56^{+}CD3^{-}$) in correlation to CD56 density ($CD56^{bright/dim}$). The CD107a-positive NK cells in each subset are indicated as percentages of all NK cells.

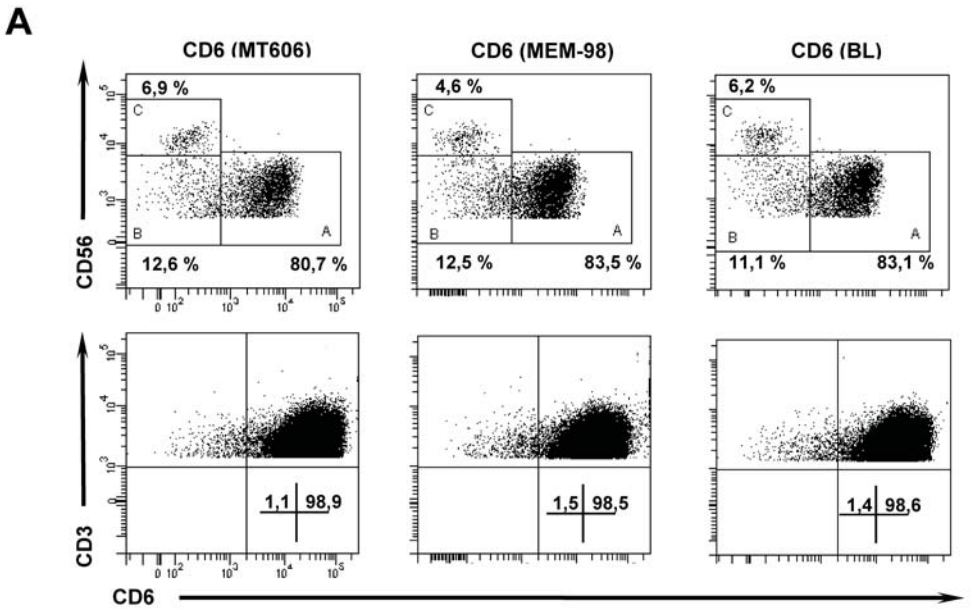
Suppl. Figure 4. Secretion of and intracellular cytokine staining (ICS) for IFN-γ, TNF-α and CCL5 (RANTES) following mab-stimulation of NK cells. (A) Negatively isolated NK cells ($1x10^5$/ 200µl) were stimulated by plate-bound mab against CD6 (MT-606, MEM-98 and BL-CD6) or isotype control MOPC21 with IL-2 (200 U/ml) for 24 h and IFN-γ, TNF-α, CCL5 (RANTES) were quantified using the multiplex technology. (B) Fresh PBMC ($2x10^5$) were activated by plate-bound mab for 6h in medium alone or supplemented with IL-2 (500 U/ml) or IFN (50 U/ml) or PMA/ionomycin followed by ICS for CXCL10 (IP-10). Staining with CD16/ CD6/ CD3/ CD56 allowed the distribution of $CXCL10^{+}$ NK cells according to our subsets A-C. Less than 1% of NK cells were positive for CXCL10 (IP-10) even after PMA/ionomycin activation, but a substantial increase was observed in the presence of IFN with isotype mab that was not further elevated by CD6-triggering. This observation indicates

that CXCL10 (IP-10) is only produced in NK cells following IFN signalling analogous to T cells.

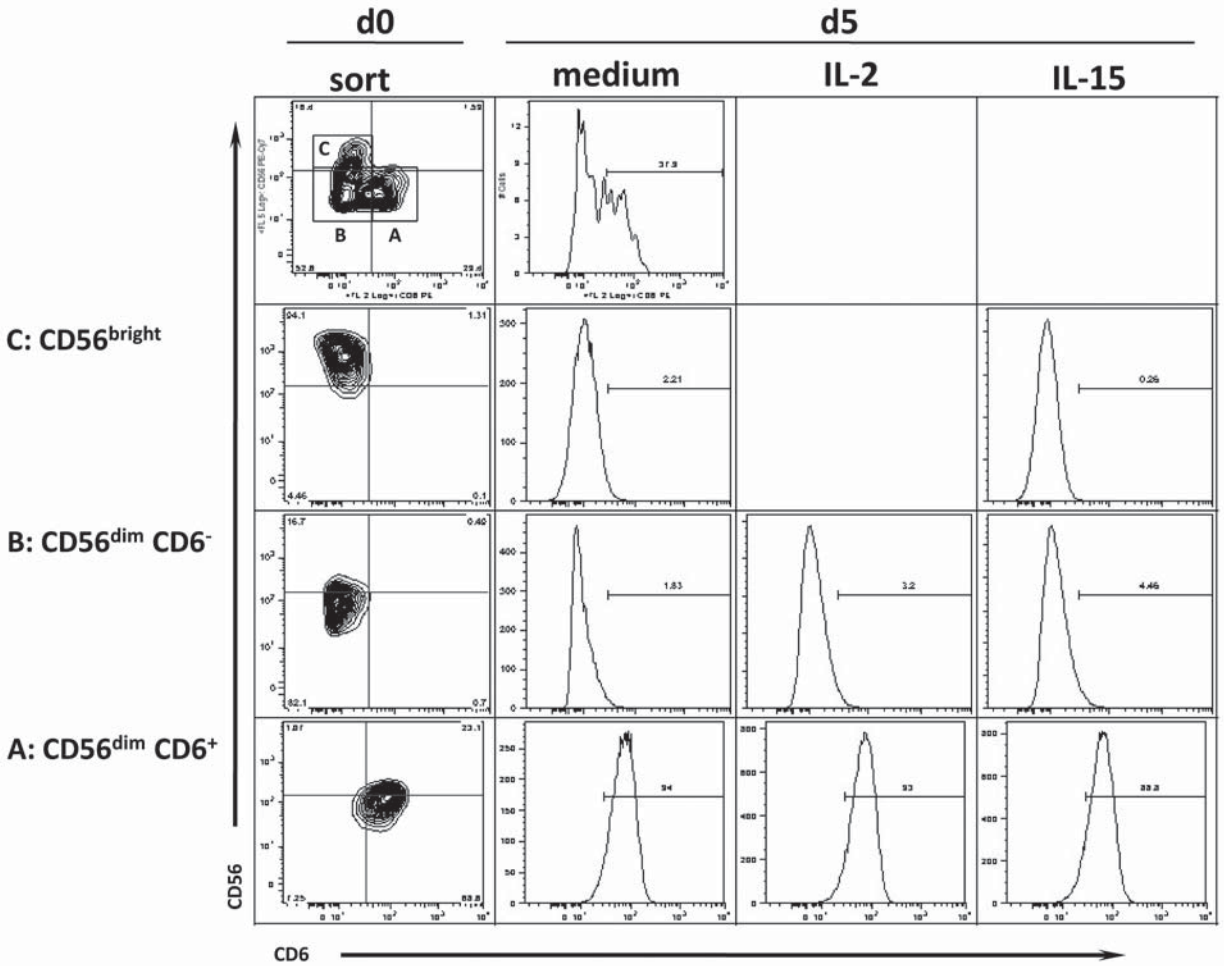
Suppl. Figure 5. KIR expression on NK subsets of healthy donor PBMC defined by their CD16 expression. Peripheral NK cells from healthy donors (n = 16) were analyzed with respect to their CD16 and KIR expression. Each data point indicates one donor and the mean values are depicted as black bars (± SEM). KIR expression is almost exclusively found on $CD16^+$ NK cells, independent of the KIR gene analyzed. As almost all $CD16^+$ NK cells belong to the $CD56^{dim}$ NK subset, this result is in accordance with published data. $CD56^{dim}CD16^+$ NK cells predominantly express CD6 (Fig. 1C), thus, KIR are mainly expressed by this NK cell subpopulation which correlates with the results shown in Fig. 2E.

Suppl. Figure 6. $CD6^+CD56^{dim}$ NK cells have lower proliferative capacity than CD6-$CD56^{dim}$ NK cells. Isolated NK cells were labelled with CellTrace Violet and incubated with IL-2, IL-15 or medium for 6 days. The dilution of CellTrace Violet, as indicator of cell division, was assessed on $CD56^{dim}$ NK cells in conjunction with CD6 expression. As expected from the phenotypic characterization, $CD6^+$ NK cells displayed the lowest proliferative capacity under these cytokine-driven conditions. One representative example of three is shown.

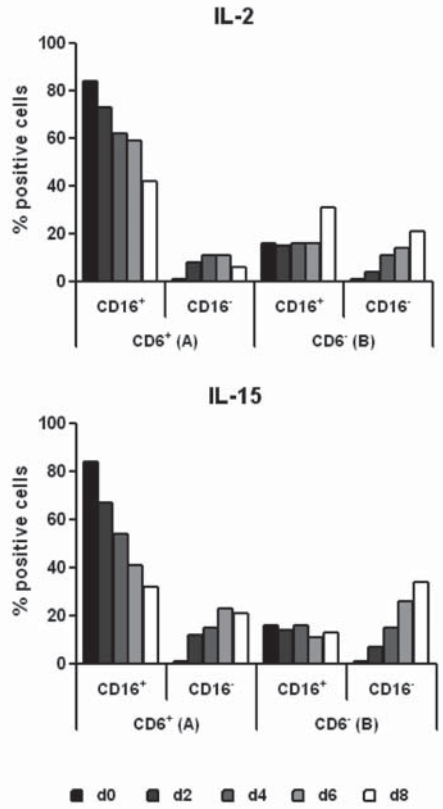
Supplemental Figure 1

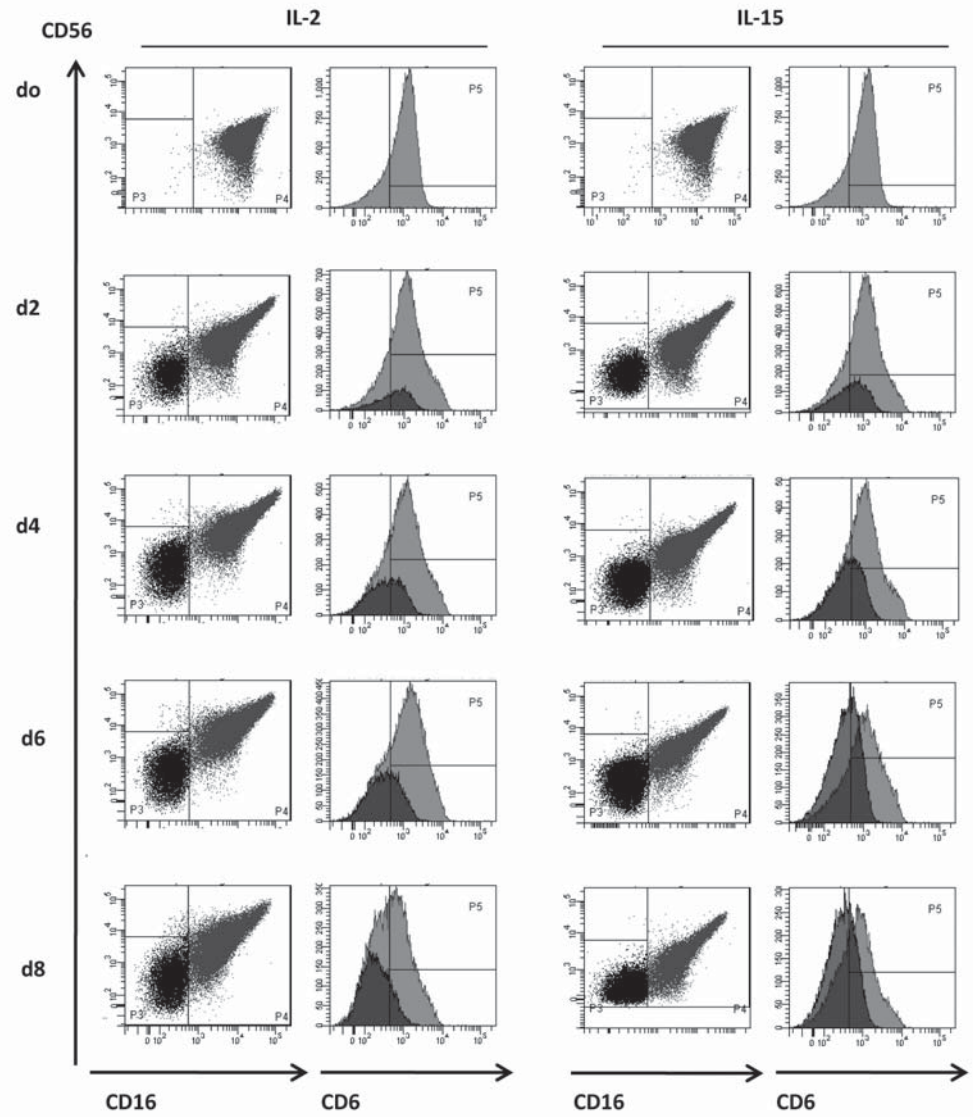


Supplemental Figure 2 A



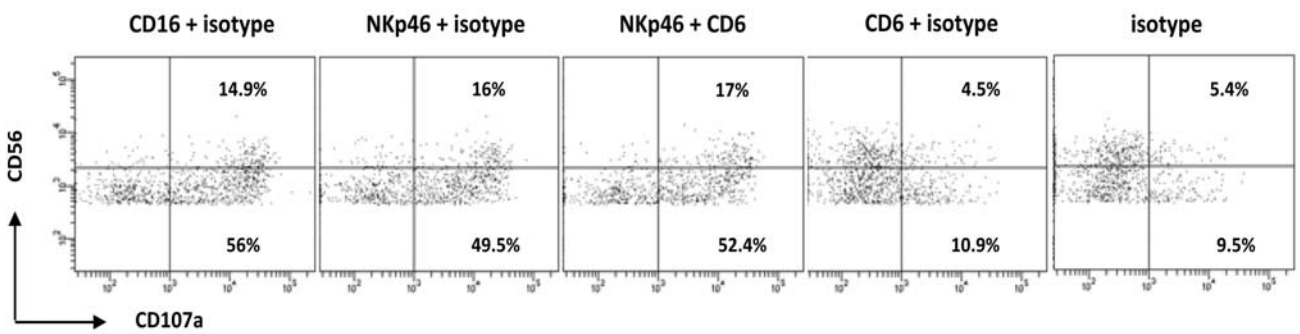
Supplemental Figure 2 B



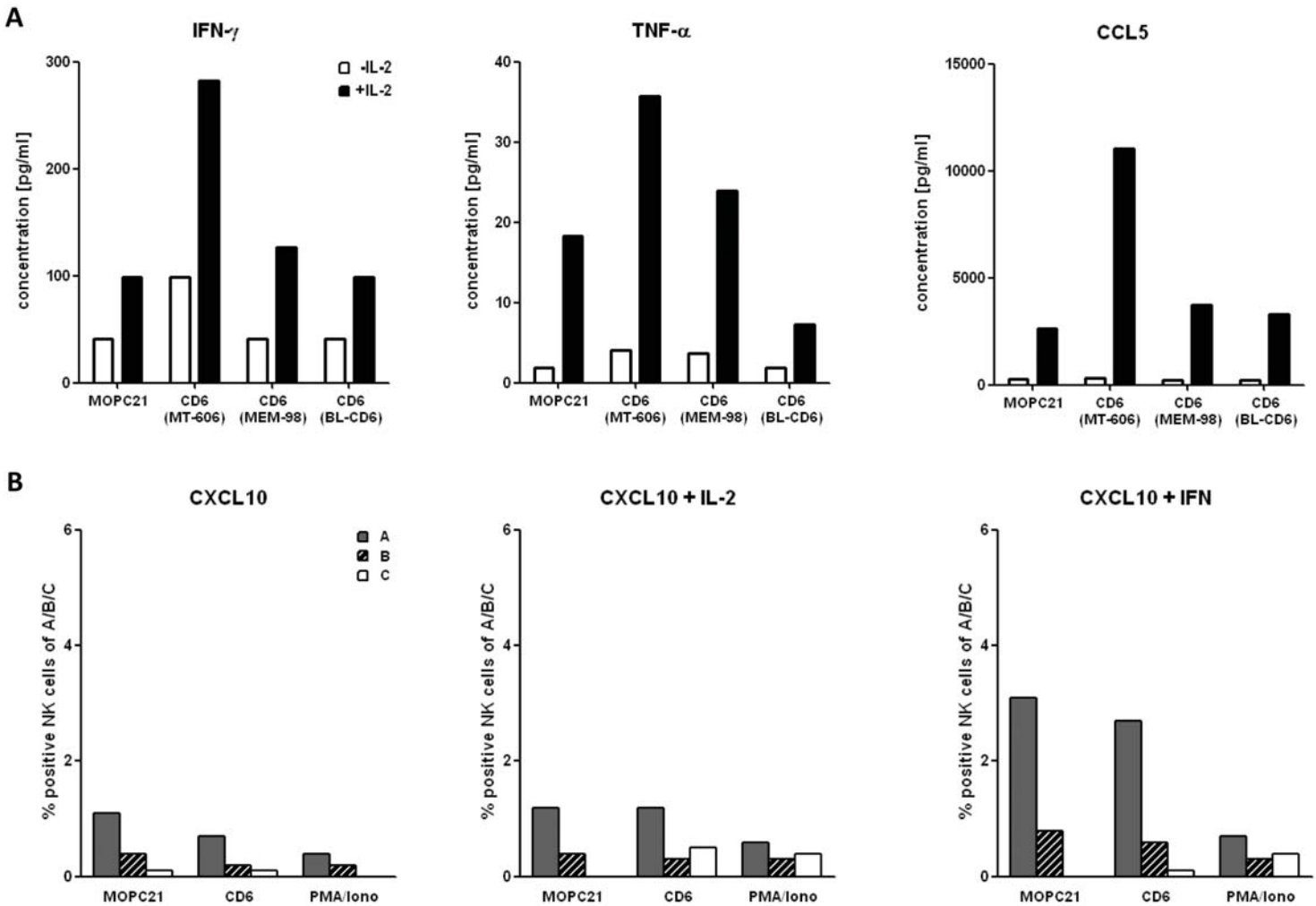


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Supplemental Figure 3



Supplemental Figure 4



Supplemental Figure 5

