**Project name: Alloreactive NK cell therapy in breast cancer**

Our project is based on the consistent observations that mouse breast cancer can be cured by alloreactive donor "natural killer cells" (NK cells). Cure is NK cell dose dependant. This holds also true for human breast cancer implanted in immune deficient mice after treatment with human NK cells.

It is our hypothesis that high doses of alloreactive NK cells will also cure patients. The limitation for immediately starting a clinical trial last year is that these high numbers of alloreactive NK cells could not be achieved with any method known at that time. We had preliminary data that huge NK cell expansion can be achieved with human leukocyte cultures, but these NK cell products contain also a cell subset that is harmful to the patient, i.e. the T lymphocytes.
Our objective was to produce NK cell products devoid of T lymphocytes using commercially available GMP grade T cell depletion devices at different time points during the expansion. This was the best strategy at the time, despite being laborious and expensive, and this proposed in the A Sister's Hope grant application.

Early Spring 2012, while working on our NK cell expansion protocol, we got into contact with a German company, Zellwerk GmBH, situated in the Berlin area. This company appeared to be able to expand NK cells on huge scale using an original bioreactor. The starting material is only 100 ml peripheral blood. They manage to get expansion rates of over 1000 fold (see attached flyer). Furthermore, when expanded NK cells (passage 1) are subjected to renewed expansion, they immediately start to expand further without a lag phase (passage 2). It seems even possible to expand the NK cells in a 3$^{rd}$ passage.

Collaboration started late Summer. At first, the viability of Zellwerk's NK cell products appeared too low for our purpose. Because the expansion cultures take 3-4 weeks, it took us several months before we, after slightly changing the expansion protocol, managed to increase viability to >90%.
Fortunately, the NK cell product does not contain any T lymphocytes. The T cell depletion is performed before starting the expansion process, and in contrast to the method we used in the past, this does not preclude successful NK cell expansions.

Using the renewed expansion protocol for better viability, we thus far were able to test batches of expanded NK cells from three different German blood donors. The cytotoxic capacity of the expanded NK cells was tested using different tumor cell lines as targets. The K562 cell line, i.e. an universal target for all human NK cells because of the lack of inhibitory NK cell receptor ligand expression, was killed with great efficacy by all three batches of expanded NK cells (Figure 1). More importantly, the breast cancer cell line MCF-7 was also killed as good as using freshly isolated NK cells. Kill is less than for K562, as in case of K562 all NK cells of a given donor get activated because of the absence of ligands for inhibitory NK cell receptors, while for MCF7 this is only the subset of NK cells that is not inhibited by the inhibitory ligands present on MCF7.

NK cell alloreactivity requires that (subsets of) NK cells are there that bear inhibitory receptors for which the patient's tumor cells does not have ligands. In that case NK cells are not inhibited and kill the tumor cells. We thus had to demonstrate that after NK cell expansion NK cell alloreactivity had remained, and this was doubtlessly indeed the case (Figure 2).

In conclusion, in this year 2012 we succeeded in achieving our objective "to expand alloreactive NK cells with high cytotoxic potential and without any T cells by a GMP approved method". This is one important step closer to clinical application of human NK cells in patients with breast cancer.

Next steps are:

1. The establishment of a donor bank that allows us to expand NK cells for every patient depending on their genomic KIR ligand (3 different donor batches required)
2. Testing the efficacy of expanded NK cells in mice with implanted human breast cancer
3. Starting clinical studies in patients with metastasized breast cancer

Maastricht, December 26, 2012

Michel van Gelder

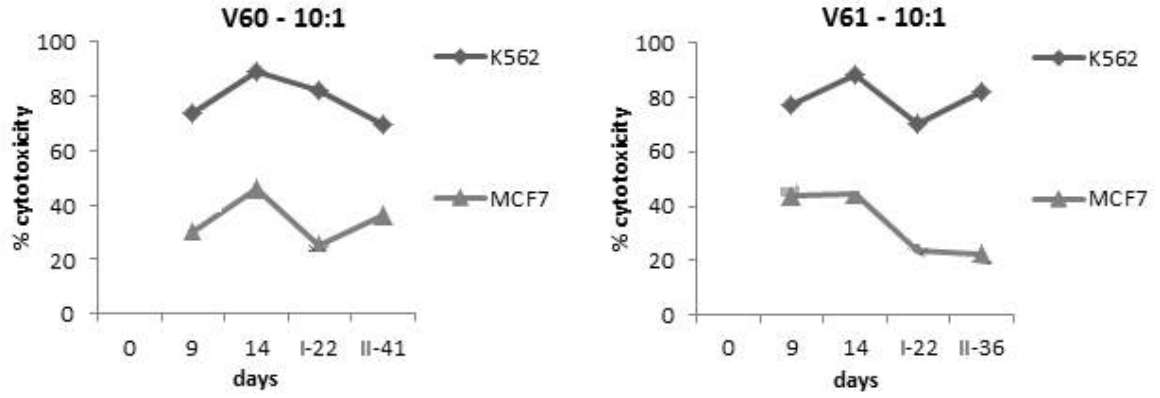


**Figure 1. Cytotoxic capacity of expanded NK cells from 2 donors at different time points during expansion.** Expanded NK cells from different time points (as indicated on the x-axis, . I and II means second and third procedure respectively) were incubated with K562 or MCF7 tumor cells in an effector to target ratio of 10:1. Kill of fluorescently labeled tumor target cells was measured after overnight incubation by 7AAD staining on the FACS. K562 has no ligands for inhibitory receptors, what allows all NK cells to get activated and kill the tumor cells. MCF7 bears 2 ligands for inhibitory NK cell receptors resulting in the activation of less NK cells compared to K562 and hence lower cytotoxicity of this target cell line. **Conclusion: the kill capacity of expanded NK cells against the two tumor cell lines remain within the normal references for freshly isolated NK cells.**

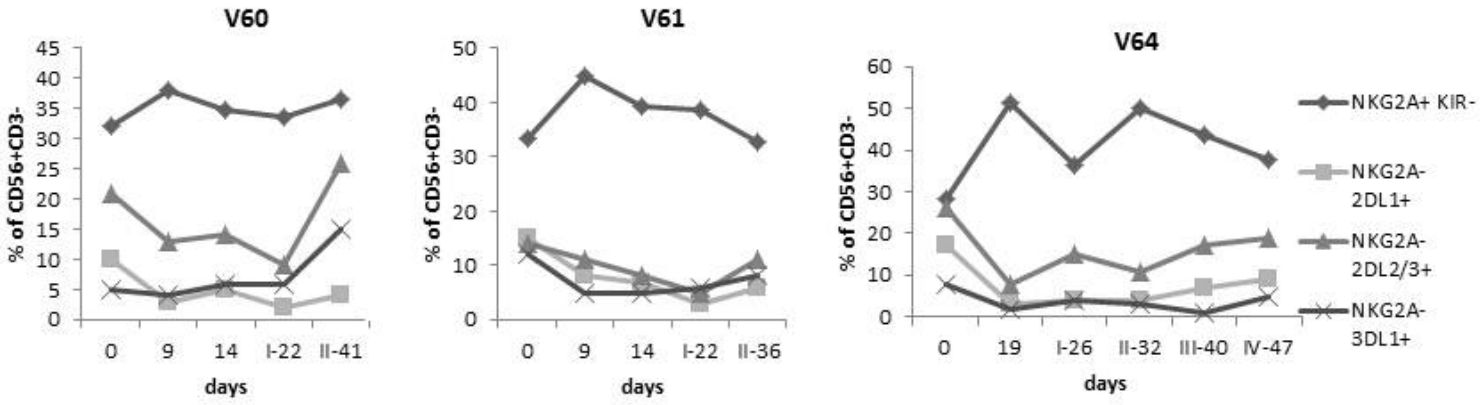


**Figure 2. Inhibitory KIR expression of expanded NK cells from 3 different donors at different time points during expansion.** Expanded NK cells from different time points (as indicated on the x-axis, . I, II, III and IV means second, third, fourth and fifth expansion procedure respectively) were analyzed for KIR expression by FACS using antibodies for the appropriate KIRs. NKG2A is the KIR for a ligand that is usually expressed on every cell and therefor does not represent the alloreactive NK cell subsets. On the other hand, NKG2A negative NK cells (indicated as NKG2A-) expressing either 2DL1, 2DL2/3 or 3DL1 represent alloreactive NK cells as these are inhibited only by their corresponding ligands that are not all expressed on cells of patients selected for their genomic KIR ligand signature. **Conclusion: the percentages of the different alloreactive NK cell subsets remain within the normal intra-individual limits during NK cell expansion.**