Chapter 2.

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Human cytotoxic T lymphocytes specific for a single minor histocompatibility antigen HA-1 are effective against human lymphoblastic leukaemia in NOD/scid mice

Lothar Hambach, MD[1], Bart A Nijmeijer, PhD[2], Zohara Aghai[1], Marianke LJ van Schie[2], Marca HM Wauben[1], PhD, JH Frederik Falkenburg, MD, PhD[2], Els Goulmy, PhD[1]
[1]Department of Immunohaematology and Blood Transfusion, [2]Department of Haematology, Leiden University Medical Centre, Leiden, The Netherlands.

Donor lymphocyte infusions (DLIs) after human leukocyte antigen (HLA)-matched allogeneic stem cell transplantation (SCT) can induce curative graft-versus-leukaemia (GvL) effects [1]. Cytotoxic T lymphocytes (CTLs) recognising minor histocompatibility antigens (mHags) HA-1 and HA-2 on the recipient's haematopoietic cells coincide with leukaemia remissions after DLI [2]. This indicates, that therapeutic boosting of only those CTLs that recognise the haematopoiesis restricted mHags HA-1 and HA-2 (HA-1 and HA-2 CTLs) might increase the GvL effect. The risk of graft-versus-host disease (GvHD) will be low because non-haematopoietic cells will not be recognised [3]. The efficacy of this therapeutic concept is unclear, since a causal relationship between mHag CTL appearance and clinical responses could not yet be made. In vitro assays that show HA-1 and HA-2 CTLs killing leukaemic cells and inhibiting leukaemic progenitor cell outgrowth [3] support the role of mHag CTLs as mediators of the GvL activity. The predictive value of these in vitro assays for the in vivo efficacy of human CTLs is however unknown. Compared to the in vitro situation, different cytotoxic mechanisms might be relevant, the accumulation of toxic soluble factors may be different and the time and chance of CTL – target cell interaction might be lower in vivo. Recently, a non-obese diabetic/severe combined immune deficiency (NOD/scid) human leukaemia model has been used to show the anti-leukaemic efficacy of T cell therapy in vivo [4]. Allo-HLA T cell lines served as model for T cell therapy, but are, due to a high risk of GvHD, clinically not applicable. Here, we used the same NOD/scid mouse leukaemia model to confine the postulated in vivo anti-leukaemic efficacy of CTLs recognising only a single HLA ligand, the mHag HA-1.

The human leukaemia cells used in this model were derived from the HLA-A2 and HA-1 ($HA\text{-}1^{A2}$) positive patient 'OF' suffering from chronic myeloid leukaemia in lymphoid blast crisis. The latter leukaemia cells were chosen because of their very aggressive progression and minimal growth variation in NOD/scid mice [4]. The CTL lines and clones for adoptive cellular transfer were generated as described [5]. In short, peripheral blood mononuclear cells (PBMCs) of healthy blood donors were stimulated with HA-1 (VLHDDLLEA) or CMV (NLVPMVATV) peptide pulsed dendritic cells and restimulated weekly. The HA-1 CTL line contained 6 % $HA\text{-}1^{A2}$ tetramer positive cells, the CMV CTL line generated with PBMCs from the same donor showed 14 % CMV^{A2} tetramer staining cells (Figure 1a,c). The HA-1 and CMV CTL clones showed bright staining with the relevant tetramers (Figure 1b,d). The HA-1 CTL line and clone displayed mHag specific cytotoxicity against OF leukaemia cells (Figure 1e, f). The CMV CTL line and clone lysed only CMV peptide loaded target cells (Figure 1g,h).

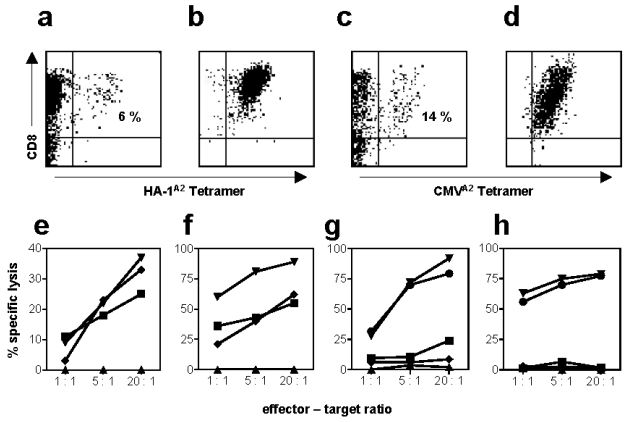

Figure 1. Tetramer staining and specific lysis of in vitro generated CTLs. (a - d) HA-1^{A2} and CMV^{A2} tetramer (x-axis) and CD8 staining (y-axis) of the HA-1 and CMV CTL lines and clones. HA-1 CTL line (e) and clone (f) show mHag HA-1 specific cytotoxicity against HA-1 natural expressing or HA-1 peptide loaded target cells. CMV CTL line (g) and clone (h) show CMV peptide specific lysis of CMV peptide loaded target cells; x-axis, effector-target ratio; y-axis, percent specific lysis. Target cells: squares: Epstein-Barr virus-/lymphoid cell line (EBV-LCL) positive for HA-1; triangles: EBV-LCL negative for HA-1; reverse triangles: EBV-LCL pulsed with the relevant peptide, HA-1 or CMV; diamonds: leukaemia OF positive for HA-1; circles: leukaemia OF loaded with CMV-peptide.

To evaluate the in vivo anti-leukaemic efficacy of HA-1 CTLs, NOD/scid mice were inoculated with 10^7 OF leukaemia cells. 3 days later, when the mice were still in a status of minimal disease, $3x10^7$ HA-1 CTLs or as control CMV CTLs or PBS were administered intravenously. The mice received interleukin 2 (IL-2) intraperitoneally daily and leukaemia and T cells were monitored in peripheral blood samples weekly as described earlier [4]. Mice receiving the control CMV CTLs or PBS showed no significant difference in leukaemia progression (Figure 2a,b), hereby excluding non-specific anti-leukaemic effects by the procedure of human CTL infusion. Mice injected with the HA-1 CTL clone showed a significantly delayed leukaemia development ($p<0.001$, regression analysis using a generalised linear model) by more than 30 (Figure 2a) or 50 (Figure 2b) days. This demonstrates for the first time, that CTLs specific for a single mHag HA-1 can induce strong anti-leukaemic effects under in vivo conditions. The long delay of leukaemia outgrowth indicates significant eradication of leukaemia repopulating cells by the HA-1 CTL clone. The latter strong effect is the result of the *in vivo* interaction between the CTLs and the leukaemia cells. This is crucially different from the report of Bonnet et al. [6], wherein reduced engraftment of leukaemic precursor cells in NOD/scid mice was observed only after *in vitro* coincubation of leukaemic cells with mHag CTLs. We used a leukaemia subtype (i.e. blast crisis of chronic myeloid leukaemia) as disease model that is clinically mostly unresponsive to unselected DLIs [1]. The high in vivo potency of HA-1 CTLs against this leukaemia suggested, that enrichment of adoptively transferred lymphocytes for HA-1 CTLs might overcome the limitations of unselected DLI. However, infusion of the HA-1 CTL line was not effective (Figure 2b). The HA-1 CTL line contained 6% HA-1 CTLs. Thereby, the number of infused HA-1 CTLs was 20-fold lower than with the HA-1 CTL clone (Figure 1a). This indicates, that the ratio between HA-1 CTLs and leukaemia cells in vivo is relevant for their effective interaction. This hypothesis is supported by the fact that transfer of the HA-1 CTL clone during overt leukaemia was also not effective (Figure 2c).

Since the HA-1 CTL clone did not entirely prevent the development of progressively growing tumours, immune escape of leukaemia may have occurred. However, leukaemic cells isolated from mice treated with the HA-1 CTL clone were equally sensitive to in vitro killing by HA-1 CTLs as leukaemic cells that had not been passaged in NOD/scid mice (Figure 2f). Thus, therapy-induced loss of the HA-1 target

antigen is unlikely. Tracking of adoptively transferred CTLs on day 3 after CTL administration showed broad and specificity-independent distribution of these cells in lung, liver, spleen and bone marrow of the mice (Figure 3a,b). The involvement of spleen and bone marrow in leukaemic engraftment [4] supports an interaction of the HA-1 CTLs with the leukaemia cells in our model. Thus, the lacking of complete leukaemia eradication may not be caused by the inability of the CTLs to interact with the leukaemia cells in the various organs.

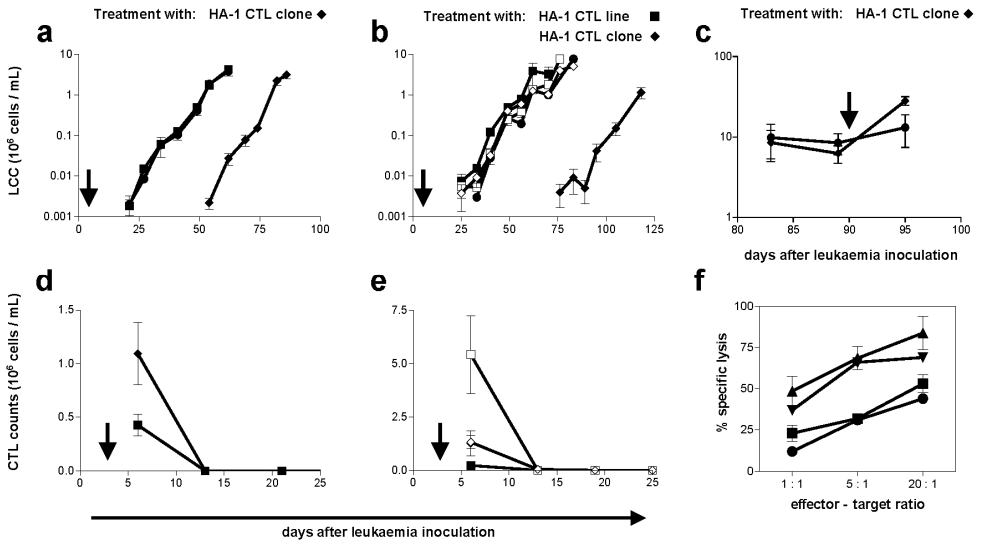

Figure 2. Leukaemic cell counts, T cell counts and in vitro lysis of leukaemic cells after treatment in vivo. (a,b) Leukaemic cell counts after treatment of minimal disease. (a) and (b) describe two sets of experiments**.** On the x-axis is depicted the time after leukaemia inoculation. On the y-axis are depicted the leukaemic cell counts (LCC) +/- SEM in the peripheral blood; the LCC lower than $8x10^6$/ml are shown. In (a) each line represents the mean of the results of 4 (PBS), 5 (HA-1 CTL clone) and 6 (CMV CTL clone) mice +/- SEM. In (b) each line represents the mean of the results of 4 (HA-1 CTL line and clone), 3 (CMV CTL line), 2 (CMV CTL clone) and 1 (PBS) mice +/- SEM. The arrow indicates the time-point of CTL or PBS administration. Treatment: open diamonds: HA-1 CTL line; closed diamonds: HA-1 CTL clone; open squares: CMV CTL line; closed squares: CMV CTL clone; circles: PBS. (c) Leukaemic cell counts after treatment of overt leukaemia. Each line represents the mean of the results of 2 mice +/- SEM. The arrow indicates the time-point of CTL or PBS administration. Treatment: closed squares: HA-1 CTL clone; circles: PBS. (d, e) T cell counts after treatment of minimal disease. On the x-axis are depicted the time after leukaemia inoculation. On the y-axis of (d) and (e) are depicted the mean CD3+ cell counts +/- SEM in the peripheral blood of the mice of the experiments (a) and (b) respectively. The arrow indicates the time-point of CTL administration. (f) In vitro lysis of leukaemic cells after successful treatment in vivo. The in vitro lysis (+/- SEM) of leukaemic cells *before* (circles, reverse triangles) inoculation and of leukaemia cells recovered on day 125 *after* inoculation from mice (n=4) successfully treated with HA-1 CTL clone (squares, triangles) are shown. Effector cells were an HA-1 CTL clone (circles, squares) and an allo HLA-A2 specific CTL clone (triangles, reverse triangles).

On day 10 after HA-1 CTL administration, the CTLs could not be detected anymore in the peripheral blood (Figure 2d,e). Likewise, beyond 10 days after CTL infusion, also the slopes of leukaemia progression curves (Figure 2a,b) did not differ between the successfully treated and the untreated groups. This indicates absence of immunological control during leukaemic progression. IL-2 appeared not sufficient to maintain the administered CTLs in vivo. These results are in accordance with earlier reports in mice [7] and in men [8] showing rapid clearance of mono- or polyclonal CTLs specific for a single epitope. The rapid disappearance of transferred CTLs in our study is not necessarily a problem of the

used NOD/scid mouse model. Allo-HLA specific T cell lines can expand upon antigen recognition in NOD/scid mice [4]. The reason for the better maintenance of the allo-HLA T cell lines might be found in the different composition and way of generation of these T cell lines, i.e. co-transfer of cytokine producing CD4 T helper cells and in vitro stimulation against multiple (unknown) epitopes that are present in vivo. We are currently investigating the putative supportive roles of co-transferred non-tumour reactive CD4 T helper cells in combination with professional antigen presenting cells in mHag specific CTL responses against tumours.

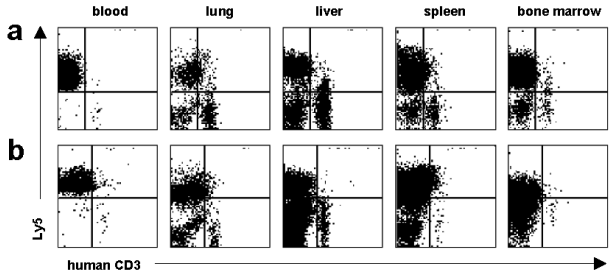

Figure 3. CTL distribution in vivo. Relevant mice were sacrificed 3 days after the treatment with the HA-1 CTL clone (a) and CMV CTL clone (b). Lymphocytes were enriched from peripheral blood and organ suspensions by ficoll gradient. Depicted are human CD3 (x-axis) and mouse Ly-5 (y-axis) staining of peripheral blood and organ suspensions of representative mice.

In conclusion, we provide the first direct evidence for in vivo interaction of human CTLs specific for only one mHag HA-1 leading to human leukaemia eradication. Our results show the crucial role of numbers and maintenance of HA-1 CTLs in the anti-leukaemic effector phase of human mHag specific CTLs in vivo. The overall limited persistence of adoptively transferred CTLs is a crucial starting point for improvements of cellular adoptive immunotherapy. Our findings are important for the rationale to apply mHag HA-1 specific immunotherapy of leukaemia in men either by adoptive cellular transfer and/or by mHag HA-1 peptide vaccination.

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