Use of B-cell bound HLA-A2 class I monomers to generate high avidity, allo-
restricted CTL against the leukemia-associated protein Wilms tumor antigen 1

Running title: HLA-A2 monomers stimulate WT1-specific CTL

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Abstract

Recent studies have detected WT1-specific CTL in AML and CML patients and demonstrated that most of these CTL were low avidity. Although HLA-mismatched donors can mount high avidity CTL against HLA-A2-presented peptides of WT1, a dominant anti-allo immune response usually obscures detection of peptide-specific CTL. Here we explored the feasibility of using recombinant HLA-A2 monomers containing single peptide epitopes as immunogens to generate peptide-specific CTL from allogeneic donors. We demonstrate that the coating of HLA-A2-negative B lymphocytes with A2/peptide monomers provides a strong stimulus for autologous peptide-specific CTL. After 3-5 rounds of stimulation a population of CD8+ T cells binding A2/peptide tetramers is easily detectable by FACS analysis. Furthermore, sorted A2/WT1 tetramer-positive CTL display strong cytotoxic activity against leukemia cells expressing WT1 endogenously but not against WT1-negative human tumor cells. Thus, HLA/peptide monomers may be useful to isolate peptide-specific donor lymphocytes for treatment of leukemia patients after HLA-mismatched transplantation.
**Introduction**

WT1 is expressed at high levels in CML, AML and ALL and can serve as marker for disease burden \(^1\)\(^-\)\(^4\). Since WT1 is expressed at low levels in some normal tissues including CD34+ hematopoietic progenitor/stem cells \(^5\)\(^-\)\(^7\), it is likely that tolerance mechanisms may render high avidity CTL unresponsive. Although two recent reports provided evidence for WT1-specific CTL in leukemia patients, in one of these studies it was demonstrated that low avidity CTL were expanded in CML patients \(^8\)\(^,\)\(^9\). In the past, we have shown that the T-cell repertoire of HLA mismatched donors can be used to isolate high avidity CTL against HLA-A2-presented peptide epitopes of WT1 \(^10\)\(^,\)\(^11\). However, in our experience the isolation of such peptide-specific CTL is unsuccessful in many donors, since allogeneic stimulator cells often provoke dominant CTL responses against allogeneic epitopes unrelated to the A2-presented WT1 epitope.

In this report we have explored the possibility of avoiding allogeneic stimulator cells and use instead recombinant HLA-A2/peptide monomers as immunogens. Single chain streptavidin-labelled antibodies that specifically bind to CD20 molecules were used to provide a binding site for biotinylated HLA-monomers on the surface of B-lymphocytes. This allowed us to introduce allogeneic HLA-A2/peptides complexes as the sole antigen into PBMC cultures of HLA-A2-negative donors and to exploit autologous B-lymphocytes as antigen-presenting cells (APC).
Materials and methods

Antibodies: The recombinant scFvSA fusion protein has been previously described 12. This protein, referred to here as anti-CD20SA, consists of the heavy and light chain variable regions of the anti-CD20 antibody B9E9 joint to the streptavidin sequence of Streptomyces avidinii.

Cells: PBMC of four HLA-A2-negative healthy donors, MG, LH, PS, BQ were used after obtaining informed consent. T2 is a TAP-deficient human HLA-A2-positive cell line 13. BV173 and ZR571 are HLA-A2-negative human leukemia and breast cancer lines. CIR and C1R-A2 are HLA-A2-negative and A2-positive EBV-transformed human cell lines. All cells were maintained in RPMI + 10% FCS. In this report HLA-A2-positive cells and monomers/tetramers are of A*0201 subtype.

Peptides: The following HLA-A2-binding peptides were used: the WT1-derived peptide RMFPNAPYL (referred to as pWT126) 10, the Melan-A-derived peptide ELAGIGILTV (pMelA) 14, influenza virus matrix peptide GIILGFVFTL (pFlu) 15, the HIV-Gag peptide SLYNTVATL (pHIV) 16, the telomerase peptide ILAKFHWL (pTel) 17, and the HPV16 E7 peptide YMLDLQPETT (pHPV) 18. In addition the HLA-A1-binding tyrosinase peptide KSDICTDEY (pTyr) 19 and HLA-A3-binding bcr-abl peptide ATGFKQSSK (pBcr) 20 were used.

HLA-A2/peptide complex monomers and tetramers. Recombinant biotinylated HLA-A2 class I monomers and A2 or A3 or B7 fluorescent tetramers containing the described above were obtained from ProImmune Ltd, (Oxford UK).

In vitro immunisation protocol. PBMCs were incubated with anti-CD20SA (10µg/ml) diluted in PBS for 1 hour at room temperature, washed and incubated with the biotinylated A2/peptide monomers (0.5µg/ml in PBS) for 30 minutes at room temperature, washed and plated at 3x10^6 cells/well in 24-well plates in RPMI with 10% human AB serum. IL-7 (R+D Systems, Minneapolis, MN) was added on day 1 at 10ng/ml and IL-2 (Chiron, Harefield, UK) was added at 10U/ml on day 4 (18). Over a 5-
week period cells were re-stimulated weekly with fresh PBMCs prepared as above, mixed with responder cells at a 1:1 ratio and plated at $3 \times 10^6$/well in 24-well plates.

**FACS staining/sorting.** Approximately $10^6$ PBMCs were incubated with tetramer-PE for 30 minutes at $37^\circ$C, followed by anti-CD8-FITC/APC for 20 minutes at $4^\circ$C, followed by FACS-Calibur analysis. Sorting was done with a FACS-Vantage. Sorted tetramer-positive Cells were expanded in in 24 well plates using per well $2 \times 10^5$ sorted cells, $2 \times 10^6$ irradiated A2-negative PBMC as feeders, $2 \times 10^4$ CD3/CD28 beads/ml (Dynal Oslo Norway) and IL-2 (1000u/ml).

**Chromium release assay.** CTL assays were performed as described previously and the specific lysis was calculated as:

$$\% \text{ lysis} = \frac{\text{experimental cpm} - \text{spontaneous cpm}}{\text{maximum cpm} - \text{spontaneous cpm}} \times 100$$
Results and Discussion

It has been shown previously that recombinant HLA-A2 monomer/peptide complexes can be used to stimulate peptide-specific, self-HLA-restricted CTL. In this study we tested if monomers can generate HLA-A2-restricted CTL specific for leukemia-associated peptides in HLA-A2-negative donors. This is relevant for the development of peptide-specific immunotherapy options for leukemia patients undergoing HLA-mismatched stem cell transplantation.

We focused on two HLA-A2-presented CTL epitopes, a WT1-derived peptide pWT126 and a MelanA-derived peptide pMelA. WT1 is a target for immunotherapy of leukemia while Melan-A can function as target in melanoma. In the self-restricted setting CTL against WT1 are rare whereas CTL against Melan-A are frequent. Using anti-CD20SA antibodies B-lymphocytes of HLA-A2-negative donors were decorated with A2/peptide monomers and used as APC for autologous T-lymphocytes. After 4 rounds of stimulation with pMelA monomers FACS analysis revealed that nearly 16% of CD8+ cells stained with pMelA tetramers but not with a panel of control tetramers (figure 1A-E), and cytotoxicity assays revealed that the CTL displayed pMelA-specific killing activity (figure 1F). Similarly, FACS analysis of cultures stimulated 5 times with pWT126 monomers showed up to 9.5% of CD8+ cells staining specifically with A2/pWT126 tetramers (figure 1G-K) and cytotoxicity assays revealed pWT126-specific killing activity (figure 1L). In total 3 HLA-A2-negative donors were stimulated with pWT126 monomers resulting in the generation of pWT126-specific CTL, as determined by tetramer staining, in all donors after 4 or 5 stimulations (range 0.7-9.5% tetramer+ CD8+ cells). Four donors were stimulated with pMelA monomers and all developed specific CTL after 3 to 5 stimulations (range 5.5-23% tetramer+ CD8+ cells). In addition 2 donors were stimulated with pFlu-monomers and one donor with pHIV monomers resulting in the generation of CTL specific for these epitopes in all cases (data not shown). Although this indicates that monomers can reliably prime and expand peptide-specific CTL, a side-by-side comparison would be required to compare the efficiency of CTL induction by T2 cells and monomers.

Stimulation with allogeneic monomers may also expand high avidity CTL that bind the allogeneic monomer independent of the presented peptide. We found that such high
avidity, peptide-independent, alloreactive CTL have a distinct tetramer staining profile. For example, figure 2A-H shows the analysis of a bulk culture stimulated with A2/pWT126 monomers. In this culture CD8+ cells bind a panel of A2 tetramers in a peptide non-specific fashion, but not HLA-A1 and A3 tetramers. The individual A2 tetramers appeared to bind to the same CD8+ population, since the percentage of positive cells did not increase when all tetramers were combined (figure 2F). Although we cannot rule out tetramer-binding to the inhibitory receptor immunoglobuline-like-transcript-2 (ILT2), the lack of binding of HLA-A1/A3 tetramers suggest that this is unlikely. Together, the data suggest that tetramer analysis can serve to identify cultures containing high avidity pan-A2-alloreactive CTL, which would be expected to pose a high risk of GvHD if used for DLI in post-transplant patients. In this study we found that allogeneic monomers stimulated more often peptide-specific CTL (10 cases) than pan-A2-alloreactive CTL (1 case).

Finally, we examined if CTL that specifically bind A2/pWT126 tetramers were of sufficient avidity to kill human leukemia cells expressing WT1 endogenously. Cell sorting was used to enrich for CD8+ cells that stained with A2/pWT126 tetramers (figure 2I,J). The enriched CTL killed the A2-positive WT1-expressing BV173 leukemia cells efficiently, but not the A2-positive WT1-negative ZR571 breast cancer cells or C1R-A2 cells unless they were loaded with the pWT126 peptide. This indicates that the tetramer-positive CTL killed human tumor cells in a WT1-specific, HLA-A2-restricted fashion.

In summary, we have presented data showing that allogeneic monomeric HLA class I/peptide complexes presented by autologous B-lymphocytes can stimulate and expand peptide-specific allo restricted CTL. If produced to GMP grade, HLA/peptide monomers may become useful reagents to enrich for peptide-specific donor lymphocytes for the treatment of leukemia patients following HLA-mismatched transplantation.
References:


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Figure legends

Figure 1: Recombinant HLA-A2/peptide monomers can stimulate allo-restricted, peptide-specific CTL. (A-E) PBMC of an HLA-A2-negative donor were stimulated 4 times in vitro with autologous B-lymphocytes coated with A2/pMelA monomers. Responding T lymphocytes were stained with FITC-labelled anti-human CD8 antibodies and PE-labelled A2 tetramers containing the peptide epitopes indicated in each panel. The percentage of tetramer-binding CD8+ T lymphocytes is also indicated in each panel. (F) The killing activity of same cultures stained in A-E was tested in Cr-release assays using the human TAP-deficient, HLA-A2-positive T2 cells coated with the indicated peptides as targets. Filled bars show the killing activity (E:T, 10:1) of cultures that were stimulated with A2/pMelA monomers in the presence of IL-2, IL-7, and open bars show the killing activity of control cultures stimulated in parallel with cytokines only. (G-K) PBMC of an HLA-A2-negative donor were stimulated 5 times in vitro with autologous B-lymphocytes coated with A2/pWT126 monomers and stained with FITC-labelled anti-human CD8 antibodies and PE-labelled A2 tetramers containing the peptide epitopes indicated in each panel. The percentage of tetramer-binding CD8+ T lymphocytes is also indicated in each panel. (L) The killing activity of same cultures stained in G-K was tested against T2 cells coated with the indicated peptides. Filled bars show the killing activity (E:T, 10:1) of cultures that were stimulated with A2/pWT126 monomers in the presence of IL-2, IL-7, and open bars show the killing activity of control cultures stimulated in parallel with cytokines only.

Figure 2: Identification of pan-A2 alloreactive T cells and tetramer sorting of allo-restricted, WT1-specific CTL. (A-H) PBMC of an HLA-A2-negative donor were stimulated 6 times in vitro with autologous B-lymphocytes coated with A2/pWT126 monomers and stained with FITC-labelled anti-human CD8 antibodies and PE-labelled A2 tetramers containing the peptide epitopes indicated in each panel. Panel F shows the FACS profile of a sample stained simultaneously with all 5 A2 tetramers. Panel G and H show the FACS profile after staining with HLA-A1 and HLA-A3 tetramers, respectively. (I-K) FACS profile of a bulk CTL line established from an HLA-A2-negative donor after 7 rounds of stimulation with A2/pWT126 monomers followed by non-specific expansion.
with CD3/CD28 beads and IL-2. Cells were stained with APC-labelled anti-human CD8 antibodies and PE-labelled A2/pWT126 tetramers before (I) and after (J) FACS sorting of CD8+ tetramer+ T cells. The tetramer+ cells did not bind A2-tetramers containing control peptides. (K) Killing activity of sorted CD8+ tetramer+ CTL against A2+ WT1+ leukaemia cells (BV173), A2+ WT1- breast cancer cells (ZR571) and EBV-transformed C1R-A2 cells coated with pWT126 peptides. Uncoated cells and cells coated with the A2-binding pWT235 peptide were used as control.
Figure 1:
Figure 2:
Use of B-cell bound HLA-A2 class I monomers to generate high avidity, allo-restricted CTL against the leukemia-associated protein Wilms tumor antigen 1