# HLA alloreactivity by human viral specific memory T-cells

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Chapter 5

# Vaccine induced allo-HLA reactive memory T-cells in a kidney transplantation candidate

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ABSTRACT

*Background:* Allo-HLA reactivity by naturally acquired viral specific memory T-cells is common. However, the effect of successful vaccination on the alloreactive memory T-cell repertoire is unclear. We hypothesized that vaccination could specifically induce allo-HLA reactive memory T-cells.
*Methods:* A varicella zoster virus (VZV) IE62 specific CD8 memory T-cell clone was single cell sorted from a VZV seronegative renal transplant candidate, following response to live attenuated varicella vaccination. To analyze the allo-HLA reactivity, the VZV IE62 specific T-cell clone was tested against HLA typed target cells and target cells transfected with HLA molecules, in both cytokine production and cytotoxicity assays.
*Results:* The varicella vaccine induced VZV IE62 specific T-cell clone specifically produced IFNγ when stimulated with HLA-B*55:01 expressing Epstein-Barr virus (EBV) transformed B-cells and HLA-B*55:01 transfected K562 cells (SALs) only. The clone also demonstrated specific cytolytic effector function against HLA-B*55:01 SALs and PHA Blasts. Cytotoxicity assays using proximal tubular epithelial cell (PTEC) and human umbilical cord endothelial cell (HUVEC) targets confirmed the kidney tissue specificity of the allo-HLA-B*55:01 reactivity, and the relevance of the crossreactivity to clinical kidney transplantation. The results also suggest that molecular mimicry, and not bystander proliferation, is the mechanism underlying vaccine induced alloreactivity.
*Conclusions:* Varicella vaccination generated a *de novo* alloreactive kidney cell specific cytolytic effector memory T-cell in a patient awaiting renal transplantation. Vaccination induced alloreactivity may have important clinical implications, especially for vaccine timing and recipient monitoring.

## INTRODUCTION

Allo-HLA reactivity by naturally acquired viral specific memory T-cells is far more common than anticipated, and the allo-HLA reactivity and virus specificity are mediated via the same T-cell receptor (TCR) (1). 45% of virus specific CD4 and CD8 memory T-cell clones have been shown to be cross-reactive against allo-HLA molecules. Allo-HLA crossreactivity has been shown for Epstein-Barr virus (EBV), Cytomegalovirus (CMV), Influenza virus and Varicella-Zoster Virus (VZV) specific T-cells (1). Alloreactive memory T-cells are a major barrier to successful transplantation because they resist immunosuppression and can rapidly engage effector functions (2-10).

Vaccine preventable infections remain a major source of morbidity and mortality in transplant recipients (11-12). Guidelines for recommended vaccinations to be given before and after transplantation are provided by many centres (11) and whenever possible the full complement of vaccines should be administered prior to transplantation (12). Adaptive immunity and the development of pathogen specific memory T-cells underlie successful vaccination. However the effect of vaccination on the allo-HLA reactive T-cell pool is largely unknown.

Danziger-Isakov and colleagues recently demonstrated that influenza vaccination can have a significant impact on the potency of the alloreactive T-cell repertoire, as determined by IFNγ production in a mixed lymphocyte culture (13). However this study did not determine the origin of the alloreactive memory T-cells or if the alloreactivity was elicited by non-specific reactivation, bystander proliferation or molecular mimicry.

VZV seronegative transplant recipients who contract varicella may suffer lethal consequences (14). Therefore, guidelines for vaccination of solid organ transplant candidates recommend that live attenuated varicella-zoster virus (VZV) vaccination be administered prior to transplantation, amongst others (11). Consequently at the Erasmus Medical Centre all kidney transplantation candidates with negative varicella serology are given the recommended live attenuated varicella vaccine (15). We hypothesized that this varicella vaccination could specifically induce allo-HLA reactive memory T-cells in patients awaiting renal transplantation.


We therefore sorted a VZV specific T-cell clone from a seronegative renal transplant candidate with a demonstrable VZV specific T-cell response following varicella vaccination, using VZV specific peptide/tetramer complex staining. Successful vaccination generated *de novo* HLA-specific alloreactive memory T-cells.

RESULTS

*Confirmation of monoclonality and TCR repertoire analyses of the vaccine induced VZV IE62 specific CD8 T-cell clone*

Post-vaccine VZV seroconversion was confirmed (Figure 1a). VZV IE62 specific T-cells were not detectable in the blood prior to vaccination. HLA-A2/ALW tetramer positive CD8 T-cells were detectable in the blood only following live attenuated varicella vaccination (Figure 1b), and were single cell sorted based on tetramer staining. VZV IE62 specific memory T-cell clones were confirmed to bind viral peptide/HLA-A2 tetramer complexes (Figure 1c). RT-PCR and sequencing were performed to confirm monoclonality and determine the TCR usage of the sorted VZV IE62 specific CD8 memory T-cell clones. All clones isolated expressed an identical Va2s1 Vb14s1 TCR, and therefore only a single clone for testing was generated. Sequence analysis of the TCR CDR3 region is shown in table 1.

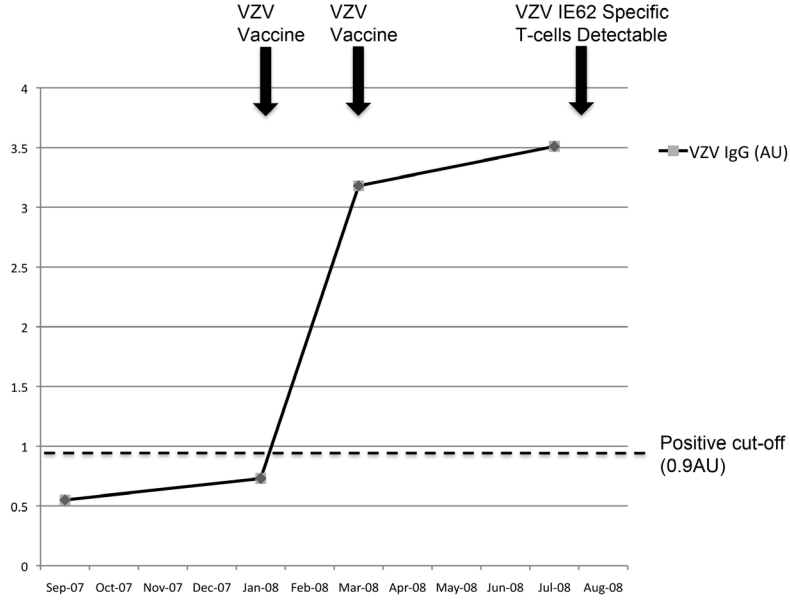


**Figure 1a.**

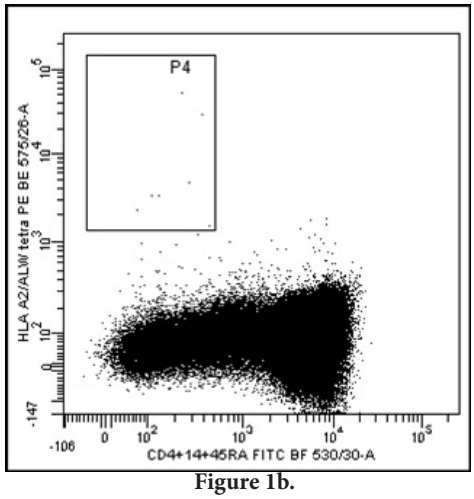


Figure 1b.

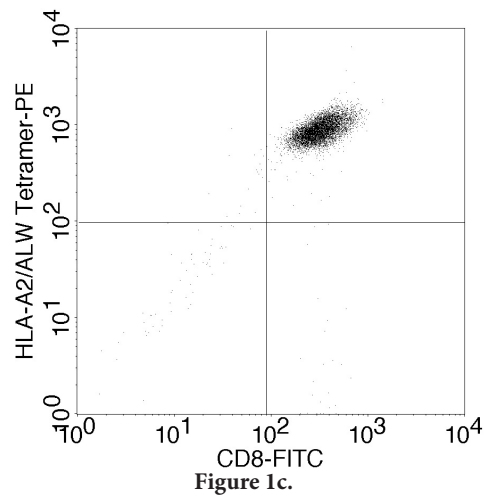


Figure 1c.

**Figure 1. Varicella vaccine induced VZV IE62 specific CD8 memory T-cells.**
Generation of the T-cell clone was performed by single cell sorting based on HLA-A*0201/ALWALPHAA specific tetramer staining. (a) A VZV seronegative renal transplantation candidate was given varicella vaccination, with seroconversion confirmed after 6 weeks. A booster vaccination was then given. Vaccine was administered immediately after collection of serum samples. Following vaccination VZV IE62 specific T-cells became detectable in the peripheral blood, and were then single cell sorted based on HLA-A2/ALW tetramer staining. VZV IE62 specific T-cells were not detectable in the blood prior to vaccination. AU=Arbitrary Units. Seropositivity cut-off value=0.9AU (b): HLA-A2/ALW specific T-cells were sorted 5 months following varicella vaccination of a VZV seronegative transplantation candidate. P4=Sort gate. Total events=200000. Sorted events=16. (c) T-cell clone is >99% HLA-A2/ALW tetramer binding and clonality was confirmed with TCR PCR. All sorted clones possessed an identical Va2s1 Vb14s1 TCR. HLA typing of renal transplantation candidate from whom VZV IE62 specific clone was sorted - HLA-A*02:01,-; B*13,40; Cw3,Cw6; DRB1*07,-.


*Allo-HLA crossreactivity of the vaccine induced VZV IE62 specific T-cell clone*
To screen for the ability of the varicella vaccine induced T-cell clone to exert alloreactivity, the clone was tested against a panel of EBV-LCLs selected to cover almost all frequently occurring HLA class I and II molecules. The T-cell clone produced IFNγ when stimulated with EBV-LCLs 5 and 19 only (Figure 2). These two EBV LCLs shared expression of the HLA-B*55:01 molecule, which was not expressed on any other tested EBV LCL. The T-cell clone recognized HLA-A*02:01 expressing EBV LCLs only when exogenously loaded with the ALW peptide (positive control), and did not produce IFNγ when cultured in IL-2 containing medium alone (negative control). Therefore the screening results were highly suggestive that varicella vaccination had induced a de novo HLA-B*55:01 alloreactive memory T-cell.

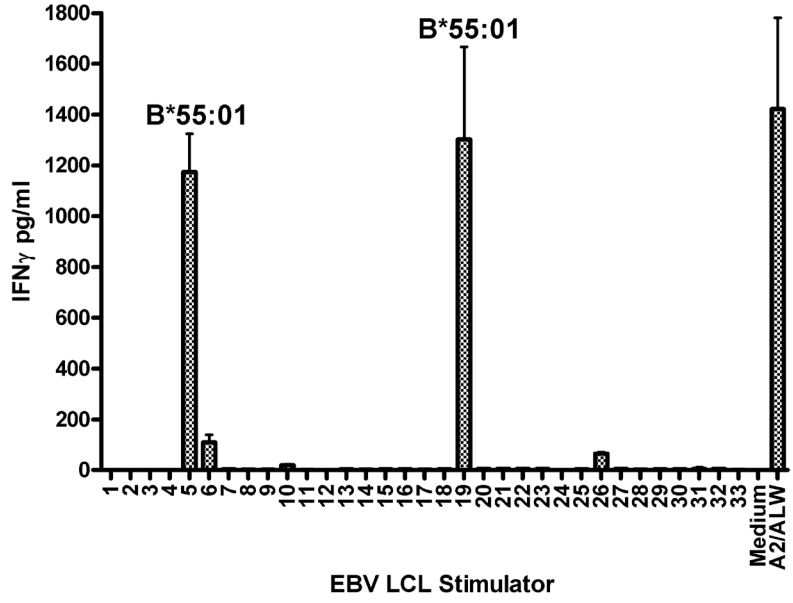


**Figure 2. Allo-HLA-B*55:01 crossreactivity by the varicella vaccine induced VZV IE62 specific T-cell clone.** The VZV IE62 specific T-cell clone was stimulated with a panel of EBV LCLs, selected to cover all common HLA class I and II molecules, in a 24 hour IFNγ ELISA. The T-cell clone produced IFNγ when stimulated with EBV-LCLs 5 and 19 only. These two EBV LCLs shared expression of the HLA-B*55:01 molecule, which was not expressed on any other tested EBV LCL. The T-cell clone recognized a HLA-A*02:01 EBV LCL only when loaded with the ALW peptide (Positive control– A2/ALW). Responder=10000 cells, Stimulator=50000 cells. Experiments performed in duplicate, mean values shown with SD. The screening results strongly suggest that varicella vaccination had induced a de novo HLA-B*55:01 alloreactive memory T-cell.

**Table 1. Sequence analysis of the VZV TCR CDR3 region**

| | AV2S1 | N | Ja40 | BV14S1 | NDN | Jb2.7 |
|---|---|---|---|---|---|---|
| VZV | C A V | F | T S G T Y K Y I F | C A S S S | D R G | Y E Q Y F |
| | tgt gcc gtg | tt | t acc tca gga acc tac aaa tac atc ttt | tgt gcc agc agt t | cc gac cgg ggt | tac gag cag tac ttc |

TCR sequences were analysed using IMGT/V-QUEST version 3.2.16 (Brochet et al. Nucl. Acids Res. (2008) 36 (suppl 2): w503-508). The junction analysis of the TCRα -and TCRβ chains are shown left to right. The one-letter amino acid code is shown above the first nucleotide of the codon. Variable gene segments are depicted according to Arden nomenclature (Arden et al. Immunogenet 1995).

*Confirmation of allo-HLA-B*55:01 crossreactivity by the vaccine induced VZV IE62 specific T-cell clone*

To confirm the allo-HLA crossreactivity of the VZV IE62 specific T-cell clone against the allogeneic HLA-B*55:01 molecule, the T-cell clone was tested for IFNγ production using HLA transfected K562 cells (SALs) as stimulators. Strong IFNγ production was only elicited by the HLA-B*55:01 transfected SAL (Figure 3) (***p<0.0001). IFNγ production was also elicited by HLA-A*02:01 SAL loaded with ALW peptide (positive control). No IFNγ production was elicited by culture with medium alone, non transfected K562 cells or HLA-A*0201 SALs (negative controls).

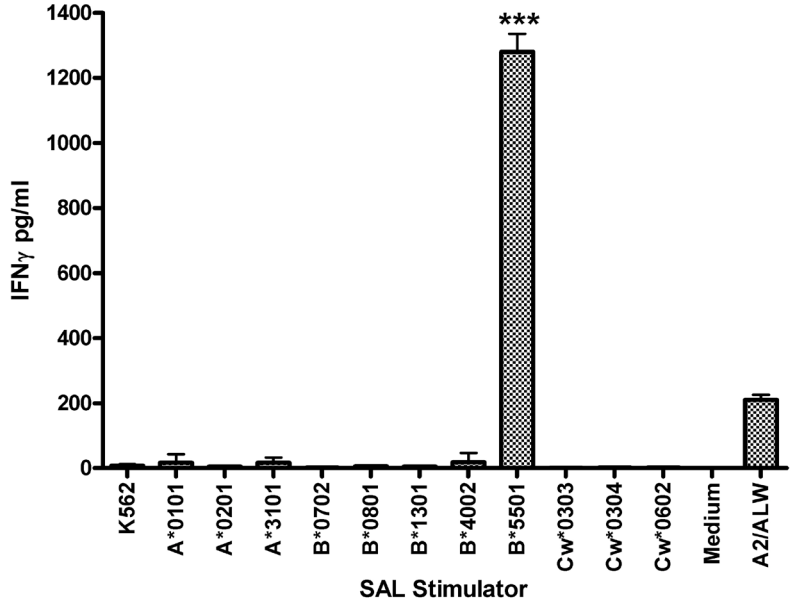


**Figure 3. Confirmation of allo-HLA-B*55:01 reactivity by the varicella vaccine induced VZV IE62 specific T-cell clone.** The VZV IE62 specific T-cell clone recognized only K562 cells transfected with allogeneic HLA-B*55:01, in a 24 hour IFNγ ELISA (***p<0.0001; comparison to K562 cell). SAL A2 was recognized only when exogenously loaded with ALW peptide (Positive control – A2/ALW). Non-transfected K562 cells, SAL A*02:01 (HLA restriction of clone), HLA matched and allo-HLA transfected SALs (other than HLA-B*55:01) were not recognized. Responder=10000 cells, Stimulator=50000 cells. Experiments performed in duplicate, mean values shown with SD.

*Cytotoxicity of the vaccine induced VZV IE62 specific T-cell clone*

To confirm that the VZV IE62 specific CD8 T-cell clone was also cytolytic against allogeneic cells expressing the HLA-B*55:01 molecule, we performed a cytotoxicity assay using SALs and PHA blasts as target cells (Figures 4a and 4b). The VZV IE62 specific T-cell clone specifically lysed HLA-B*55:01 expressing SALs and PHA blasts in proportion to the E/T ratio (Figure 4a and 4b) (**p=0.0007 and **p=0.0002 respectively), whereas HLA-A*02:01 expressing PHA blasts and SALs were not lysed unless exogenously loaded with the ALW peptide. Cytotoxicity was low, as compared to the HLA-A2$^+$ viral peptide loaded positive controls after 4 hours, but increased after overnight cytotoxicity assay (Figure 4b) (***p<0.0001).

*Tissue Specificity of the vaccine induced VZV IE62 specific T-cell clone*

To investigate tissue (kidney) specificity of the crossreactive alloresponse by the VZV IE62 specific memory T-cells, we used PTECs and HUVECs as a model system for human kidney transplantation. The VZV IE62 specific T-cell clone demonstrated specific cytolytic effector function against a HLA-B*55:01 expressing PTEC, in a 4 hour cytotoxicity assay (Figure 5a). This confirms that the vaccine induced memory T-cells in this kidney transplantation candidate can recognize normal kidney tissue cell types present in a transplanted kidney. However a HLA-B*55:01 expressing HUVEC was not targeted by the VZV IE62 specific T-cell clone, in a 4 hour cytotoxicity assay, even with IFNγ pre-stimulation to increase the amount of HLA-B*55:01 expression (Figure 5b).

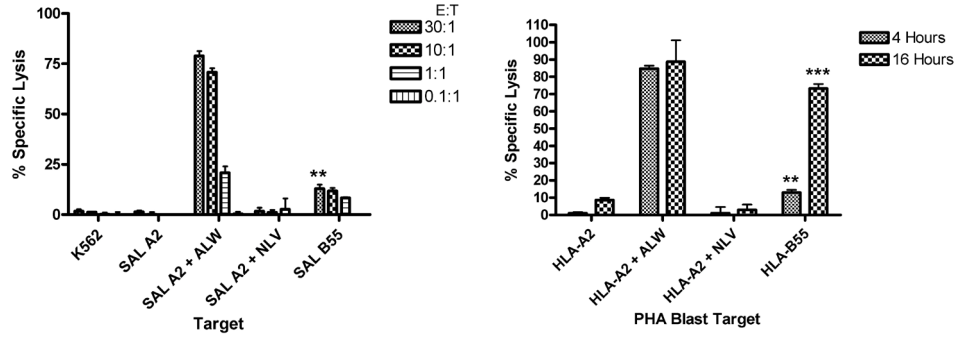


**Figure 4. Varicella vaccine induced VZV IE62 specific memory T-cell clone is cytolytic against allogeneic HLA-B*55:01 expressing target cells.** (A) Cytotoxicity assay using effector T-cell clone demonstrates cytotoxic effector function against HLA-B*55:01 SAL (**p=0.0007; comparison to K562 cell ratio 30:1), in proportion to effector:target ratio. Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD. (B) VZV IE62 allogeneic cell cytotoxicity capacity increases with time. Cytotoxicity assay using effector T-cell clone demonstrates cytotoxic effector function against HLA-B*55:01 expressing PHA blasts (** p=0.0002; comparison to HLA-A2 PHA blast at 4 hours), in a 4 hour cytotoxicity assay. Cytotoxicity capacity of the VZV IE62 specific T-cell clone against HLA-B*55:01 PHA blast increased with longer co-culture time (***p<0.0001; comparison between HLA-B*55:01 PHA blast target cell at 4 and 16 hours). E:T ratio=30:1, Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD.

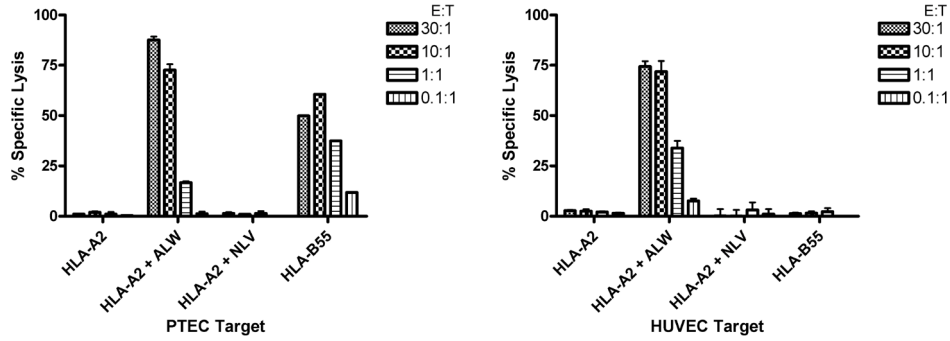


**Figure 5. Tissue specificity of the allo-HLA reactivity by the VZV IE62 specific T-cell clone.**
(A) The VZV IE62 specific T-cell clone demonstrates specific cytolytic effector function against a HLA-B*55:01 expressing PTEC, in a 4 hour cytotoxicity assay. Thus confirming that the vaccine induced memory T-cells can recognize normal renal tissue cell types present in a transplanted kidney. Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD. Assay with HLA-B*55:01 PTEC target performed in single due to low target cell numbers. (B) A HLA-B*55:01 expressing HUVEC was not targeted by the VZV IE62 specific T-cell clone, in a 4 hour cytotoxicity assay, even following IFNγ pre-stimulation to increase the HLA-B*55:01 expression. Targets=5000 cells. Experiment performed in triplicate, mean values shown with SD.


## DISCUSSION

Transplant candidates are at increased risk of infectious complications and every effort should be made to assure that they complete the full complement of recommended vaccinations prior to transplantation. We addressed whether successful vaccination may also induce alloreactive memory T-cells. In this study we show that live attenuated varicella vaccine can induce *de novo* human cytolytic allo-HLA reactive memory T-cells, in a kidney transplantation candidate. It also suggests that successful vaccination induces alloimmunity against specific allo-HLA molecules via peptide dependent molecular mimicry.

The VZV IE62 specific memory T-cells specifically recognized the allogeneic HLA-B*55:01 molecule. This allorecognition resulted in both cytokine production *and* cytotoxicity. We also demonstrated that this VZV specific T-cell from a kidney transplantation candidate can exert allo-HLA reactivity against normal kidney cell types present in transplantation tissue, thus demonstrating the clinical relevance of the vaccine induced response to kidney transplantation.

Lower cytotoxicity against allo-HLA-B*55:01 expressing PHA blasts and SALs in a four hour cytotoxicity assay, as compared to the positive controls (ALW peptide loaded HLA-A2$^+$ target

cells), is not unexpected. The HLA-A2 expressing PHA blasts and SALs were exogenously loaded with excess amount of viral peptide, while the cross-reactive alloresponses are dependent on presentation of endogenous self-peptide. Furthermore the lower percentage of specific lysis against PHA blasts, increased to levels comparable to the positive controls after an overnight cytotoxicity assay. Alloreactive memory T-cells are able to persist and may be capable of causing long-term damage, especially at times when immunosuppression is tapered (3).

The lack of recognition of HLA-B*55:01$^+$ HUVECs supports the conclusion that alloreactivity is tissue specific, and that the vaccine induced alloreactivity is HLA and endogenous peptide dependent. These results support molecular mimicry, and not bystander proliferation, as the mechanism for the vaccine induced alloreactivity. Similarly a HLA-DR3 restricted human tetanus toxoid-specific T-cell clone was previously found to give HLA-DR4 specific alloreactivity (16).

The presently described VZV IE62 specific T-cell expresses a Vβ14 TCR and recognizes the allogeneic HLA-B*55:01 molecule. Another T-cell clone with the same specificity and the same Vβ usage sorted from an individual with naturally acquired VZV infection was previously also reported to give allogeneic HLA-B*55:01 crossreactivity (1). VZV IE62 specific T-cell clones have also been reported to give allogeneic HLA-A*02:05 and HLA-B*57:01 reactivity, however these T-cell clones did not express Vβ14 TCRs (1).

The crossreactive potential of antigen specific T-cells is difficult to predict. However data presented here supports the notion that memory T-cells with the same antigen specificity and the same Vβ usage will exert alloreactivity against the same allo-HLA molecule.

VZV infects about 95% of the population (15,17-18). The immediate early (IE62) protein is required for the initiation of VZV replication, and VZV IE62 specific T-cells are correlated with immunity after VZV infection (19-20). VZV IE62 specific T-cells were found in 12/19 (63%) of stem cell transplantation patients with VZV reactivation, and 3/18 (17%) serologically positive healthy donors, indicating that this HLA-A2 restricted epitope is commonly used in HLA-A2 positive individuals (21). Here we show that live attenuated varicella vaccination also induces memory T-cells with identical specificity. Therefore HLA-B*55:01 mismatching may be an unacceptable mismatch in HLA-A2$^+$ transplantation recipients, but further database studies are warranted.

Live attenuated vaccines are very potent at eliciting protective T-cell immunity because they possess many antigenic targets, provide natural co-stimulation and can usually replicate to a limited extent. Recently activated effector memory T-cells may have differential or even no requirements for co-stimulatory signals, as compared to resting peripheral blood memory T-cells (22-23), and the CD8 memory T-cell state of readiness is antigen dependent and actively maintained but reversible (24-25). Therefore live attenuated vaccination may enable quantitatively and qualitatively stronger allo-HLA reactivity from the newly generated crossreactive memory T-cells. Similarly alloreactive memory T-cells in an already seropositive transplant candidate or recipient could be activated by booster vaccination, thereby enabling alloreactive

effector function in the absence of co-stimulation.

Alloreactive memory T-cells are central mediators of the immune-mediated injury to the allograft and therefore results presented here may have important clinical implications. We suggest vaccination could be given at least three months prior to expected transplantation to avoid the peak of specific cellular alloreactivity, as also suggested by others (13). Closer monitoring could be applicable to transplanted patients following recent vaccination. Live attenuated vaccination may be particularly potent at activating alloreactive memory T-cells. Further clinical studies on the effects of vaccination on the alloreactive T-cell repertoire are required.

Finally, Danziger-Isakov and colleagues demonstrated an effect of Influenza vaccination on cellular alloreactivity in humans (13), and favoured the conclusion that the observed increase in T-cell alloreactivity may be due to non-specific reactivation of a variety of T-cells clones. However their work lacked mechanistic studies to help understand the immunological process behind the reported observations. Furthermore they did not study HLA specificities of the cellular alloreactivity. Nonetheless, the absence of self responses in those transplant recipients who also showed evidence of anti-influenza reactivity suggests that specific allo-HLA reactivity is likely. Our previous work (1,26) combined with results presented here strongly suggests that molecular mimicry underlies the effect of vaccination on cellular alloreactivity.

Transplant candidates are at increased risk of infectious complications mandating a full complement of recommended vaccinations prior to transplantation. We provide evidence that live attenuated vaccination is associated with the generation of de novo HLA specific alloreactive memory T-cells, likely via molecular mimicry. Vaccination induced alloreactivity may have important clinical implications, especially for vaccine timing and recipient monitoring. Future work should determine if induction of HLA specific alloreactivity is a common characteristic of human vaccination.

## MATERIALS AND METHODS

*Renal transplantation candidate*

HLA-A*02:01$^{+}$, varicella seronegative renal transplantation candidates were considered for participation in this study. The patient from whom the VZV specific T-cell clone was sorted is a 52 year old male with end stage renal disease due to diabetes mellitus type 2, receiving haemodialysis therapy, with HLA typing HLA-A*02:01,-; B*13,40; Cw3,Cw6; DRB1*07,-. The candidate had never previously received an organ transplant. History taking confirmed no previous varicella infection or vaccination. Two separate serum samples taken before varicella vaccination were both VZV seronegative (Figure 1a). A commercially available ELISA kit was used (Vidas Varicella Zoster IgG, BioMerieux, Marcy l'Etoile, France). The patient was vaccinated with a live attenuated varicella vaccine (Varilix, GlaxoSmithKline) on two occasions, separated by six weeks.

*Generation of VZV IE62 specific CD8 memory T-cell clone*

We used the newly validated ALWALPHAA/HLA-A*02:01 restricted epitope from the immediate early 62 (IE62) protein of varicella zoster virus to detect de-novo virus specific memory T-cells generated following varicella vaccination (21). VZV IE62 specific T cells were isolated from the peripheral blood as previously described (1,26). Briefly, PBMCs were harvested and labeled with HLA-A2/ALW tetrameric complexes for 30 minutes at 4 $^{0}$C in RPMI without phenol red, supplemented with 2% FCS, washed three times and single cell sorted at 4 $^{0}$C using the FACS vantageTM (Becton Dickinson). Tetramer positive CD8 T-cells were non-specifically stimulated every 2 weeks with feeder cell mixture containing irradiated allogeneic PBMCs (3500 Rad), 800ng/ml phytohaemagglutinin (PHA), 100 IU/ml IL-2 in IMDM medium supplemented with glutamine, human serum (5%) and fetal calf serum (5%).

*Confirmation of T-cell clonality*

TCRα and TCRβ rearrangements were analyzed on the VZV IE62 specific T-cell clone.
Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany). Oligo dT primed first-strand cDNA was synthesized from 1 µg RNA template using Superscript III reverse transcriptase (Invitrogen corporation, Carlsbad, CA, USA). First RT-PCR was performed to determine the TCR AV and BV usage, using primers that cover the complete TCR repertoire. Sequencing templates were obtained performing high fidelity PCR using Pfx50 DNA Polymerase (Invitrogen Corporation, Carlsbad, CA, USA). Each reaction contained forward primers targeting the Va2S1 or Vb14S1 variable region and reverse primers specific for the alpha and beta chain constant region. Amplicons spanning the variable, CDR3 and joining regions were purified using ExoSAP-IT (GE Healthcare, Buckinghamshire, UK) according to manufacturer's protocol. Thermo sequenase primer cycle sequencing (GE healthcare) reactions were performed using a CY5 labeled M13 sequencing primer (Sigma-Aldrich, St. Louis, MO, USA) according to manufacturer's protocol. Sequencing reactions were run on an ALFexpress DNA sequencer (GE Healthcare), and analyzed with sequence analyser 2.10 software (GE Healthcare).

*Cytokine assays*
Screening for allo-HLA crossreactivity of the VZV IE62 specific T-cell clone was done using a panel of EBV transformed B-cells (EBV LCLs) selected to cover almost all frequently occurring HLA class I and II molecules, using IFNγ production as readout. IFNγ production was also used to confirm the allo-HLA crossreactivity by the VZV IE62 specific T-cell clone, using single HLA transfected K562 cells (SALs) as stimulator (27). 10000 T-cells were co-cultured with 50000 irradiated stimulator cells in a final volume of 150µL IMDM culture medium supplemented with 10% fetal calf serum and 100IU/mL IL-2. After 18 hours of incubation, supernatants were harvested and IFNγ production was measured using standard enzyme-linked immunosorbent assay (ELISA, U-Cytech, Netherlands).

*Cytotoxicity assays*
The VZV IE62 specific T-cell clone was evaluated for cytotoxicity by incubating 5000 target cells with serial dilutions of the T-cell clone for 4 hours in a standard $^{51}$Cr-release cytotoxicity assay. Target cells used in the standard cytotoxicity assays include PHA Blasts, single HLA transfected K562 cells (SALs) (26-27), proximal tubular epithelial cells (PTECs) (28-31) and human umbilical vein endothelial cells (HUVECs) (32-34). Cytotoxicity assays involving PTEC and HUVEC target cells were performed before and after IFNγ treatment, 500 units/ml for 24 hours. PHA blasts were also incubated with the T-cell clone in an overnight cytotoxicity assay (16 hours), however, SALs were not suitable targets in an overnight assay due to $^{51}$Cr leakage. Target cells were incubated with $^{51}$Cr for 60 minutes. Supernatants were harvested for gamma counting: *percent specific lysis= (experimental release-spontaneous release)/(Max release-spontaneous release) x 100%.* Results are expressed as the mean of triplicate samples.


*Statistics*
Values for specific lysis are presented as the mean of triplicate wells, with standard deviation (SD). Values for IFNγ production are presented as the mean of duplicate wells, with standard deviation. Comparative analyses are non-parametric (unpaired) t-tests, $p<0.05$ is considered to be significant. Statistics are derived using Graph Pad Prism 4 for Windows (Version 4.02, 2004).

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