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## **HLA monomers as a tool to monitor indirect allorecognition**

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### **Citation**

Breman, E., Miert, P. P. van, Steen, D. M. van der, Heemskerk, M. H., Doxiadis, I. I., Roelen, D., ... Kooten, C. van. (2014). HLA monomers as a tool to monitor indirect allorecognition. *Transplantation*. doi:10.1097/TP.000000000000113

Version: Not Applicable (or Unknown)

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**Note:** To cite this publication please use the final published version (if applicable).

## HLA monomers as a tool to monitor indirect allorecognition

Journal:	<i>Transplantation</i>
Manuscript ID:	TPE-2013-1318.R1
Manuscript Type:	Article- Basic & Experimental
Date Submitted by the Author:	n/a
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Classifications:	09.1 Cellular < 09 Immunological Methods, 04.03.2 histocompatibility antigens - major < 04.03 Histocompatibility antigens, genes, including tissue specific antigens < 04 Histocompatibility (Clinical and Experimental), 09 Immunological Methods
Keywords:	HLA, Indirect allorecognition, immune monitoring, allopeptide

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Manuscripts

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29 12 Keywords: Indirect allorecognition, Immune monitoring, HLA, allopeptide,  
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31  
32 13

33  
34 14 Text word count abstract: 208  
35

36 15 Text word count paper: 2914  
37

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39 16

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41 17 List of figures 4 Color Figures 1  
42

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3 **25 Footnotes**  
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5 **26 1 Contributions**  
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7  
8 **27** EB designed, performed the experiments and wrote the manuscript. PPvM participated in designing and performing the experiments.  
9

10 **28** DMvdS performed experiments MHH participated in writing of the paper. IID contributed reagents. DR, FHC and CvK designed  
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12 **29** experiments, discussed results and helped to write the manuscript.  
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15 **30** Conflict-of-interest disclosure: The authors declare no conflict of interest.  
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39 **Abbreviations**

40 APC – antigen presenting cells

41 DC – Dendritic cell

42 DTH – delayed-typed hypersensitivity

43 EBV – Epstein bar virus

44 ELISPOT - enzyme linked immunosorbent spot

45 mAb – monoclonal antibody

46 MLR – Mixed leukocyte reaction

47 moDC – monocyte-derived dendritic cells

48 PBMC – peripheral mononuclear blood cells

49 PHA - phytohemagglutinin

50 SAL – single antigen lines

51 TLR – Toll like receptor

52 Treg – Regulatory T-cells

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3 **54 Abstract**  
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5 55 Background: Recognition of donor antigens can occur through two separate pathways:  
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8 56 the direct pathway (non-self HLA on donor cells) and the indirect pathway (self-restricted  
9  
10 57 presentation of donor derived peptides on recipient cells). Indirect allorecognition is  
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12 58 important in the development of humoral rejection, therefore there is an increasing  
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14 59 interest in the monitoring of indirect alloreactive T-cells. We have used an in vitro model  
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16 60 to determine the optimal requirements for indirect presentation and assessed the risk for  
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18 61 semi-direct presentation in this system.  
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22 62  
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24 63 Methods: HLA typed monocyte-derived dendritic cells (moDC) were incubated with  
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26 64 cellular fragments or necrotic cells and incubated with either indirect or direct  
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28 65 alloreactive T-cell clones. T-cell reactivity was measured by proliferation or cytokine  
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30 66 secretion. HLA-typed moDC, monocytes or PBMCs were incubated with HLA class I  
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32 67 monomers, in combination with either direct/indirect T-cell clones.  
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38 69 Results: Although both were efficiently taken-up, alloreactivity was limited to the semi-  
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40 70 direct pathway, as measured by allo-specific CD4 (indirect) and CD8 T-cell clones  
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42 71 (direct). In contrast, HLA-A2 monomers were not only efficiently taken up, but also  
43  
44 72 processed and presented by HLA-typed moDC, monocytes or PBMCs. Activation was  
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46 73 shown by a dose dependent induction of IFN- $\gamma$  production and proliferation by the CD4  
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48 74 T-cell clone. Antigen presentation was most efficient when the monomers were cultured  
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50 75 for longer time periods (24-48h) in the presence of the T-cells. Using this method, no  
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76 reactivity was observed by the CD8 T-cell clone, confirming no semi-direct  
77 alloreactivity.

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79 Conclusion: we have developed a system that could be used to monitor indirect  
80 alloreactive T-cells.

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## 82 **Introduction**

83 Transplantation has become the standard therapy in end stage renal failure, leading to an  
84 increase in quality of life and survival (1). Despite immunosuppressive regimes that are  
85 very effective in reducing acute rejection, chronic rejection remains a major cause of  
86 allograft loss (2).

87 T-cells are a major driving force in mediating allograft rejection (3). Recipient CD4+ T-  
88 cells can recognize the allograft either through recognition of donor HLA-peptide  
89 complexes on donor antigen presenting cells (APC, direct), or by recognizing self-  
90 restricted donor HLA-derived peptides on recipient APCs (indirect). A third pathway  
91 (semi-direct) has been postulated in which recipient T-cells recognize intact donor HLA-  
92 peptide complexes on recipient APCs (4, 5).

93 T-cells with direct alloreactivity have been shown to play a dominant role in acute  
94 rejection, primarily in the early phase after transplantation, when APCs from donor origin  
95 are still available (6-8). T-cells with indirect alloreactivity are correlated with chronic  
96 rejection in humans (7, 9, 10). The importance of indirect allorecognition in allograft  
97 rejection is multiple and was recently reviewed (11). Briefly, it can provide help and thus  
98 induce alloreactive cytotoxic CD8 T-cells (12, 13). Perhaps more importantly indirect  
99 alloreactive CD4 T-cells are the only cells that can provide help to alloreactive B-cells as  
100 shown in animal models (13-15). These cells have also been implicated in the regulation  
101 of tolerance (16). Regulatory T-cells (Treg) from transplant recipients were shown to  
102 have indirect alloreactivity (17, 18). Furthermore, they were capable of suppressing both  
103 the direct and indirect pathways of allorecognition (19). Animal studies have shown that  
104 Treg with indirect allospecificity can abrogate alloantibody formation and mediate

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3 105 transplant tolerance (20-22). Recently, natural Treg were shown to inhibit direct but not  
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5 106 indirect allorecognition (23) Although both pathways have been known for decades,  
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8 107 assays monitoring indirect T-cell alloreactivity have not become routine, and as of yet no  
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10 108 clinical assay is available (11).

11 109 Methods to monitor direct alloreactivity include the mixed leukocyte reaction (MLR), the  
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13 110 cytotoxic T-cell precursor assay and more recently, the IFN- $\gamma$  enzyme linked  
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15 111 immunosorbent spot (ELISPOT). The assays give a good indication of the potency of  
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17 112 directly reactive T-cells (24-27). Methods to measure indirectly reactive T-cells have  
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19 113 been previously reviewed and offer many complications (28). These include the use of  
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21 114 donor cell fragments as a source of donor antigens, which are presented in the context of  
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23 115 self-HLA class II. In theory this method allows the full repertoire of alloantigen available.  
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25 116 In practice, this assay is difficult and not very reproducible. Furthermore, reactivity may  
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27 117 be due to the semi-direct pathway (29, 30). Synthetic peptides which correspond with the  
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29 118 mismatched donor antigens have also been used (31-33). This method has a higher  
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31 119 reproducibility but reactivity may be directed at neo-epitopes that are not available *in*  
32  
33 120 *vivo*. The third method makes use of the *trans vivo* delayed-type hypersensitivity (DTH)  
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35 121 model, in which recipient cells and donor cellular fragments are injected into the footpad  
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37 122 of naïve mice (34-36). Donor cell alloreactivity leads to a DTH response that can be  
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39 123 measured by the footpad swelling. The advantage is that a global alloresponse can be  
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41 124 measured (37). However, this approach is very complex and technically too difficult to be  
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43 125 used as a diagnostic assay.

44 126 The aim of this study was to develop a reliable and easy tool to monitor indirect  
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46 127 allorecognition in renal transplant patients that could be used in a clinical setting.  
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3 129 **Results**  
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5 130 **Evidence for semi-direct rather than indirect allorecognition when donor cell**  
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8 131 **fragments are used as exogenous antigen source**  
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10 132 To set-up and validate a model for indirect allopresentation, a previously characterized  
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12 133 CD4 T-cell clone (referred to as 4.44) recognizing HLA-A2 peptide restricted by HLA-  
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15 134 DR1 was used (38). Incubation of 4.44 with its native ligand in the form of Epstein Bar-  
16  
17 135 Virus (EBV) transformed B-cell line (EBV-LCL) expressing HLA-DR1 and HLA-A2 led  
18  
19 136 to IFN- $\gamma$  secretion (Figure 1A). Clone 4.44 did not react with HLA-DR1+/HLA-A2-  
20  
21 137 EBV-LCLs, but IFN- $\gamma$  secretion could be induced when cells were pulsed with HLA-A2  
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23 138 peptides containing the relevant epitope (Figure 1B).  
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27 139 A CD8 T-cell clone (referred to as 1E2) that recognizes HLA-A2 (39), was used as a  
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29 140 readout to exclude semi-direct alloreactivity. Both T cell clones were cultured with single  
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31 141 antigen lines (SAL) expressing only HLA-A2 (SAL-A2). T-cell reactivity as measured by  
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33 142 IFN- $\gamma$  was limited to the 1E2 T-cells, confirming specificity of both clones (Figure 1C).  
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36 143 To investigate the process of indirect alloreactivity, monocyte-derived dendritic cells  
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38 144 (moDC) from HLA-typed donors (HLA-DR1+/HLA-A2-) were used as APCs and were  
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40 145 loaded with peptides or fragmented SAL-A2 cells as antigen source. After 48h clone 4.44  
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42 146 was added, but apart from the positive control no indirect alloreactivity could be  
43  
44 147 measured in any of the conditions (Figure 1D). In contrast, under similar conditions of  
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46 148 loading fragments, when clone 1E2 was used low but significant production of IFN- $\gamma$   
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48 149 could be detected in 3 out of 5 experiments (Figure 1E). This indicates that the use of cell  
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50 150 fragments might result in semi-direct allorecognition when co-cultured with moDC. No  
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3 151 reactivity was observed when fragments and 1E2 were cultured alone. 1E2 activation was  
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5 152 confirmed in all conditions where viable SAL-A2 were used.  
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10 154 **Processing of necrotic cells leads to semi-direct allorecognition**

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12 155 Professional APCs have specialized mechanisms for the uptake of apoptotic or necrotic  
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14 156 cells. Therefore, we investigated whether dead cells could be a more efficient way of  
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16 157 loading alloantigen, without the risk of semi-direct allorecognition. Viable, or necrotic  
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18 158 SAL-A2 cells were used as previously described (40). HLA-A2 expression on SAL-A2  
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20 159 was confirmed on viable and necrotic (Nec) SAL-A2 (Figure 2A). Moreover, SAL-A2  
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22 160 were stimulated with IFN- $\gamma$  to increase HLA-A2 expression on these cells (Figure 2B).  
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24 161 Uptake of dead cells, but not viable cells, was confirmed by flow cytometry as well as  
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26 162 confocal microscopy (Figure 2C-D). Importantly, after 24h incubation a near complete  
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28 163 removal of dead material was observed. This was an active process that did not occur at  
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30 164 4°C.  
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35 165 Indirect allorecognition studies were performed using HLA-A2-/HLA-DR1+ typed  
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37 166 moDC. Whereas strong IFN- $\gamma$  production was achieved with exogenous peptide loading  
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39 167 none of the conditions investigated resulted in detectable activation of the 4.44 clone  
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41 168 (Figure 2E). Activation of moDC using different toll-like receptor (TLR) ligands did not  
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43 169 result in detectable indirect presentation (data not shown). Comparable to the fragments,  
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45 170 a low albeit significant response was found for semi-direct allorecognition by 1E2 when  
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47 171 necrotic SAL-A2 cells were used (Figure 2F).  
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3 173 **HLA monomers can be used for antigen loading resulting in indirect allo-**  
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6 174 **presentation**

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8 175 As an alternative source of alloantigens we investigated the potential use of HLA-A2  
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10 176 monomers. Incubation of HLA-DR1+/HLA-A2- moDC with HLA-A2 led to potent  
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12 177 activation of clone 4.44 comparable to exogenously loading of peptides (Figure 3A).

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15 178 This response was dose-dependent and significant IFN- $\gamma$  production was observed with  
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17 179 concentrations starting at 1 $\mu$ g/ml HLA-A2 and kept increasing until 125 $\mu$ g/ml (Figure  
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19 180 3B).

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22 181 MoDCs were incubated with 25 $\mu$ g/ml HLA-A2 for various time points and after  
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24 182 extensive washing 4.44 was added for an additional 24h. The highest reactivity was  
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26 183 observed when moDCs were incubated for at least 4h with the antigen (Figure 3C). Next,  
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28 184 moDCs were incubated with HLA-A2 for 4h before extensive washing. 4.44 T-cells were  
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30 185 then added at 0h, 24h, or 48h and incubated for an additional 24h (Figure 3D). The  
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32 186 highest reactivity was observed when 4.44 were added immediately (0h), while a steady  
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34 187 decrease was observed after 24 and 48h, indicating a steady turnover of HLA-peptide  
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36 188 complexes.

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41 189 ~~As m~~Monocytes are the major a more readily available source of APCs within the  
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43 190 PBMCs, we investigated their capacity to present alloantigens via the indirect pathway.  
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46 191 Similarly to moDC, monocytes showed a strong capacity to present HLA-A2 and activate  
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48 192 4.44 T-cells, comparable to the exogenous peptide (Figure 3E-F). This response was  
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50 193 dose-dependent and significant levels of IFN- $\gamma$  were observed at concentrations of  
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52 194 1 $\mu$ g/ml HLA-A2, reaching an optimum at 25 $\mu$ g/ml (Figure 3F).

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3 196 **PBMCs can present monomer derived peptides to indirect allorecognizing T-cells**  
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5 197 **clones without evidence for semi-direct allorecognition**  
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8 198 Since the afore mentioned experiments were performed with purified populations, we  
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10 199 next addressed the question whether the use of total PBMC would affect the sensitivity or  
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12 200 specificity of the above described model. HLA-DR1+/HLA-A2- PBMCs were pulsed  
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14 201 with HLA-A2 peptides or incubated with HLA-A2 monomers in the presence/absence of  
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16 202 4.44. Again using either exogenous peptide or HLA-A2 monomers, a strong and dose-  
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18 203 dependent response of the T-cell clone could be detected (Figure 4A-B). In the absence of  
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20 204 the T-cell clone no IFN- $\gamma$  production could be detected indicating that the addition of  
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22 205 HLA-A2 monomer does not activate CD8 T-cells with direct allospecificity (Figure 4A).  
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24 206 The absence of semi-direct allorecognition could be further confirmed with the 1E2  
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26 207 clone, which was completely unresponsive when incubated with the HLA-typed PBMCs  
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28 208 and increasing concentrations of HLA-A2, as depicted by IFN- $\gamma$  production as well  
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30 209 proliferation (Figure 4C-D). This is in stark contrast to clone 4.44 which was highly  
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32 210 positive in both conditions.  
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38 211 To investigate the sensitivity of this model, various PBMCs concentrations were tested  
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40 212 and incubated for 24 or 48h with a set amount of HL-A2 and 4.44 (Figure 4E). 48h and  
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42 213  $1 \times 10^5$  PBMCs yielded overall a better T-cell response. When titrating the T cell clone, as  
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44 214 little as 8-40 T-cells were sufficient to induce detectable levels of IFN- $\gamma$  when  $1 \times 10^5$   
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46 215 PBMCs were incubated with 25 $\mu$ g/ml HLA-A2 for 48h (Figure 4F).  
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50 216 Finally, we could confirm that antigen presentation was completely HLA-DR dependent.  
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52 217 The cytokine production and proliferation was completely and dose-dependently  
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3 218 prevented when cultures with 4.44 were incubated with a blocking antibody against  
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6 219 HLA-DR (Figure 4G-H).  
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221 **Discussion**

222 Indirect allorecognition is considered one of the important factors in mediating chronic  
223 allograft rejection and influencing long term graft outcome (7, 41-43). However, at the  
224 moment, no reliable test is available to monitor indirect allorecognition in organ  
225 transplant recipients. A major hurdle is the antigen loading, that requires a high  
226 sensitivity to detect low frequency indirect T-cells, and a high specificity to exclude T-  
227 cells with direct specificity. In the current study, we have successfully developed a novel  
228 *in vitro* method to measure indirect allorecognition. By using T-cell clones that  
229 specifically recognize HLA-A2 through the indirect (HLA-A2 peptides restricted by  
230 HLA-DR1) or direct (HLA-A2) pathway of allorecognition, we could demonstrate that  
231 semi-direct allorecognition is not an issue in our model system, where HLA-A2  
232 monomers were used as a source of alloantigen.

233 In experiments using HLA-A2 expressing cells as donor antigen source (either as cell  
234 fragments or as necrotic cell) reactivity was limited to the semi-direct pathway of  
235 alloreactivity. This raises some questions on the conclusions drawn from previous studies  
236 that have routinely used cellular fragments to measure indirect alloreactivity (7, 10, 44).  
237 Those studies did not include controls to distinguish between semi-direct and indirect  
238 allorecognition and it is therefore possible that the semi-direct pathway has a contribution  
239 to the allorecognition measured in those studies.

240 The semi-direct pathway involves the incorporation of alloantigen intact on the cell  
241 surface and has been shown to occur with DCs (45) monocytes and T-cells (46, 47). The  
242 process of membrane transfer is a subject of many studies and the mechanisms involved  
243 are now slowly unraveled (48). Interestingly, cross-dressed DCs (DCs that express an

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3 244 acquired HLA-peptide complex) have been shown to prime CD8 T-cells in mice (45, 49).

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5 245 This suggests that semi-direct allorecognition could potentially lead to T-cell priming,

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8 246 which is an important factor in interpreting model systems that use cell fragments or

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10 247 apoptotic and necrotic cells to monitor indirect allorecognition. Reactivity against HLA

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12 248 class I molecules was observed in 3 out of 5 experiments performed. The reactivity was

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15 249 limited to low levels of IFN- $\gamma$  and is most likely due to the limited HLA class I molecules

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18 250 transferred. The conditions in most experiments involving membrane transfer are short

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20 251 term (2h), it is possible that longer time periods (as utilized in our experiments) reduce

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22 252 the efficiency, and thereby the reactivity. However, we observed no indirect alloreactivity

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25 253 in any of the conditions utilizing cellular fragments, or dead cells. Although the material

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27 254 is taken-up and processed there is relative little HLA class I molecules when compared

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29 255 with all other proteins available, that will also be processed and presented and therefore

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31 256 will generate large amounts of competing peptides that will not be recognized by the T

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33 257 cell clone. Furthermore, the T-cell clone only recognizes one epitope in the context of

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35 258 HLA class II, while the APC presents multiple epitopes which might not be recognized

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38 259 by the T-cell clone.

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41 260 Another strategy to monitor indirect presentation has been the use of synthetic peptides.

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43 261 *In vivo* APCs present donor derived peptides to autologous T-cells restricted by their own

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45 262 HLA class II molecules. Each HLA class II molecule has its own repertoire of peptides

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47 263 that can be presented. Prediction of HLA class II epitopes, is complex and it is unlikely

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49 264 that a set of peptides can be used to predict individual alloreactivity (50). Current tests

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52 265 that use synthetic peptides have the risk of creating neo-epitopes, epitopes that are not

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55 266 created in the natural antigen processing manner (31-33, 51). This may even lead to

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3 267 reactivity to peptides derived from autologous HLA molecules. Peptides used for the  
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5 268 detection of indirect allorecognition include peptides derived from the  $\beta$ 1 domain of HLA  
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8 269 class II (32, 51-53) or the  $\alpha$ 1 domain of HLA class I (33, 54). In one study, where  
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10 270 peptides from a complete HLA-A2 molecule were used, reactivity was seen in the  
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12 271 hypervariable region as well as from the  $\alpha$ 3 subunit and the trans-membrane domain (52).  
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15 272 However, as no peptides from self-HLA were taken as a control it is unclear what the  
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17 273 reactivity means, and whether these peptides would be formed when naturally processed.  
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20 274 Use of longer peptides could be a good alternative, as these would require processing and  
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22 275 are able to induce T-cell reactivity in other model systems of antigen presentation (53,  
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24 276 54).  
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27 277 HLA monomers offer solutions to many of the problems depicted earlier. However, there  
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29 278 are two distinct disadvantages in using monomers. The one is the high price involved in  
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31 279 making highly pure monomers and secondly the relatively high concentration needed to  
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33 280 induce a reproducible T-cell response.  
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35  
36 281 The quest to induce long-term graft tolerance and/or survival starts with an accurate  
37  
38 282 depiction of the recipient's immune-response against the donor allograft. Although many  
39  
40 283 studies are aimed at monitoring and measuring operational tolerance and organ rejection  
41  
42 284 (55, 56), no reliable test exists other than the actual clinical outcome. Measuring indirect  
43  
44 285 allorecognition could give a broad view into how the recipient immune-system is shaping  
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46 286 its response to the allograft and be helpful in tailoring an individual immune-suppression  
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48 287 regime to prolong graft survival.  
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3 289 **Materials and methods**  
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6 290 **Cell culture and reagents**  
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8 291 Peripheral blood mononuclear cells (PBMC), monocytes and monocytes-derived  
9  
10 292 dendritic cells (moDC) were cultured in RPMI-1640 (PAA, Austria) supplemented with  
11  
12 293 10% FCS (Bodinco, the Netherlands), 5,000 U/ml penicillin and 5 mg/ml streptomycin  
13  
14 294 (Invitrogen, USA).  
15

16  
17 295 Single antigen lines (SAL) were created by transfecting K562 cells with a plasmid  
18  
19 296 construct containing a HLA-A2 heavy chain gene (SAL-A2) (57). SAL-A2 were  
20  
21 297 maintained in IMDM supplemented with 10% FCS, pen/strep and G-418 (Roche,  
22  
23 298 Germany) at a concentration of 250 µg/ml for the selection of stably transfected SALs.  
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26  
27 299 HLA-A\*0201 derived peptides of the region 99-122 of HLA-A2 were synthesized. HLA-  
28  
29 300 A\*0201 heavy chains were produced in *E.coli*. Monomer refolding around the  
30  
31 301 melanoma-associated pmel 17 peptide (YLEPGVTA) in the presence of β2-  
32  
33 302 microglobulin was achieved as previously described (58). Monomers were purified by gel  
34  
35 303 filtration HPLC and tested routinely.  
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38  
39 304 T-cells clones 4.44 (CD4+) and 1E2 (CD8+), recognizing HLA-A2 (aa98-120) restricted  
40  
41 305 by HLA-DR1 and HLA-A2 respectively, have been previously described (38, 59). They  
42  
43 306 were maintained in IMDM medium (PAA, Austria) with 5% FCS (Bodinco, the  
44  
45 307 Netherlands), 5% normal human serum (Sanquin, the Netherlands), 100 IU/ml  
46  
47 308 recombinant IL-2 (Chiron, Novartis, USA) 5,000 U/ml penicillin, 5 mg/ml streptomycin,  
48  
49 309 and 2 mM L-glutamine (Gibco, invitrogen, USA). Expansion was achieved by  
50  
51 310 stimulation with phytohemagglutinin (PHA, 0.8 µg/ml, Murex Biotec Limited, Dartford,  
52  
53 311 UK) and PBMCs in a ratio of 1:5. Cells were harvested after two weeks and either frozen  
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3 312 or used in experiments after a resting period of 2-3 days. T-cell specificity was routinely  
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5 313 tested.

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7  
8 314 Stable Epstein-Barr virus (EBV)-transformed B-cell lines (EBV-LCL) were generated  
9  
10 315 from an HLA-DR1+/HLA-A2- donor using standard procedures (60). HLA-A2+ EBV-  
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12 316 LCLs were generated by transducing a retroviral vector encoding for HLA-A\*0201 into  
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14  
15 317 the donor (HLA-A2-) EBV-LCLs (61).

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### 19 319 **Generation of monocytes-derived dendritic cells**

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21  
22 320 moDCs were generated from buffy coats as previously described (62). Briefly PBMCs  
23  
24 321 were isolated from buffy coats (Sanquin, the Netherlands) of healthy (HLA-A2<sup>-</sup>, HLA-  
25  
26 322 DR1<sup>+</sup>) individuals using Ficoll/amidotrizoat (pharmacy, LUMC, the Netherlands)  
27  
28  
29 323 density gradient, followed by CD14 microbeads magnetic cell sorting (Miltenyi Biotec,  
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31  
32 324 The Netherlands) according to manufactures protocol. Monocytes were cultured in 6 well  
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34 325 plates (Costar, USA) in RPMI-1640 supplemented with 10 ng/ml IL-4 and 5 ng/ml GM-  
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36 326 CSF (Gibco, Invitrogen, USA). Cytokines were refreshed every 2-3 days and cells were  
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39 327 allowed to differentiate for at least 6 days before harvesting.

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### 42 43 329 **Indirect allorecognition assay using cells as source of HLA class I antigens**

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46 330 moDCs were co-cultured with necrotic, apoptotic or fragmented SAL-A2 cells. Necrosis  
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48 331 and apoptosis was induced as previously described (40). Briefly necrosis was induced by  
49  
50 332 heating cells to 56°C for one hour and confirmed by light microscopy and annexin-V/PI  
51  
52 333 staining. Cell fragments were generated by 3 rounds of freeze-thawing and confirmed by  
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55 334 light microscopy.  $5 \times 10^5$  moDCs were co-cultured at a 1:1 ratio with SAL-A2 cells for a

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3 335 | period of at least 24h in a 96 round well plate.  $5 \times 10^3$  4.44-cells (CD4 indirect) or  $1 \times 10^2$   
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5 336 | (CD8 direct/semi-direct) cells were added for an additional 48h incubation. Supernatants  
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8 337 | were then harvested and IFN- $\gamma$  production was measured.  
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### 11 12 339 | **Phagocytosis assay**

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15 340 | Phagocytosis was quantified by flow cytometry or fluorescence microscopy as previously  
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17 341 | described (40). Briefly moDCs were labeled with PKH26 (Sigma-Aldrich) or stained  
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19 342 | with HLA-DR mAb. Necrotic, apoptotic or fragmented SAL-A2 cells were stained with  
20  
21 343 | CFSE prior to induction of cell death and then co-cultured with moDCs at a ratio of 1:1  
22  
23 344 | ( $5 \times 10^4$  cells). Analysis was conducted at 2 or 24h post co-culture. Fluorescence was  
24  
25 345 | assessed with FACSCalibur or LSR-II or with a Leica SP5 confocal scanning laser  
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27 346 | microscope and the analysis performed with ImageJ imaging software.  
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### 33 34 348 | **Indirect/direct allorecognition assay using HLA class I monomers**

35  
36 349 | To monitor the pathways of indirect allorecognition moDC or monocytes ( $DR1^+/A2^-$ )  
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38 350 | were plated ( $3 \times 10^4$ ) in round 96-well plates (Costar, USA) and incubated with different  
39  
40 351 | concentrations of HLA-A2 monomers (HLA-A2) or peptides. T-cells were added to the  
41  
42 352 | culture ( ~~$5 \times 10^3$ , 4.44 or  $1 \times 10^2$~~ ) and incubated at different time points. A CD4 T-cell clone  
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44 353 | ( $5 \times 10^3$ , 4.44) was used as a readout for indirect allorecognition and a CD8 T-cell clone  
45  
46 354 | ( $5 \times 10^3$ ,  $1 \times 10^2$ ) for direct/semi-direct allorecognition. Part of the supernatant was harvested  
47  
48 355 | and cells were pulsed with  $0.5 \mu\text{Ci } ^3\text{H}$  (Life science products Inc. USA) and incubated for  
49  
50 356 | 24h. Similarly ( $1 \times 10^5$ ) PBMCs were co-cultured with ( $5 \times 10^3$ ) T-cells (4.44 or  $1 \times 10^2$ ) with  
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52 357 | different concentrations of HLA-A2. Co-cultures were incubated for 48h, supernatants  
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358 collected and cells pulsed with <sup>3</sup>H for another 24h when proliferation was measured.  
359 Inhibition of indirect allorecognition was achieved by titration of mouse anti-human  
360 HLA-DR (B8.11.2, IgG2b) antibodies to the co-cultures.  
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3 362 **Acknowledgements**  
4

5 363 The authors would like to acknowledge Dr. Hetty de Boer from the department of  
6  
7  
8 364 Nephrology of the Leiden University Medical Centre, for providing help in confocal  
9  
10 365 imaging. The research conducted in this manuscript was financed with a grant (project  
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12 366 code C09.2304) from the Dutch Kidney Foundation.  
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3 553 **Figure legends:**  
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5 554 **Figure 1. T-cell clones recognize cellular fragments through semi-direct**  
6 **allorecognition but not through indirect allorecognition.** A)  $5 \times 10^3$  CD4 T-cells (4.44)  
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8 were cultured with their native ligand HLA-A2+/HLA-DR1+ EBV-LCLs. IFN- $\gamma$   
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10 secretion was measured in the supernatants after 24h. B) HLA-A2-/HLA-DR1+ EBV-  
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12 LCLs were pulsed with different HLA-A2 derived peptides and co-cultured with 4.44 T-  
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14 cells. After 24h IFN- $\gamma$  was measured in the supernatants. C) An HLA-A2 recognizing  
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16 CD8 T-cell clone (1E2) and the 4.44 were co-cultured with K562 or HLA-A2 transfected  
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18 K562 cells (SAL-A2) for 48h. Supernatants were then collected and IFN- $\gamma$  measured. D)  
19  
20 HLA-Typed moDC (HLA-DR1+/HLA-A2-) were cultured with or without fragments of  
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22 SAL-A2 cells (1:1 ratio at  $5 \times 10^4$  cells) for 48h after which  $5 \times 10^3$  4.44 T-cells were added  
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24 with or without HLA-A2 peptides. E) moDCs were also cultured with 1E2 cells (as  
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26 described in D). All experiments were repeated three times, graphs represent the mean  
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28 +SD of a single experiment conducted in triplicate. Dashed lines represent the detection  
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30 limit of the ELISA used.  
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41 569 **Figure 2. T-cell clones recognize necrotic cells through semi-direct allorecognition**  
42 **but not through indirect allorecognition.** A-B) SAL-A2 cells were stained with HLA-  
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44 A2-PE labeled antibodies or isotype control. Cells were either viable or necrotic. IFN- $\gamma$   
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46 stimulated SAL-A2 cells were stimulated with 100ng/ml IFN- $\gamma$  for 24h prior to HLA-A2  
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48 labeling. C-D) moDCs were labeled with PKH26 and SAL-A2 cells were labeled with  
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50 CFSE. Necrosis was then induced and as a control viable cells were used.  $1 \times 10^4$  SAL-  
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52 A2 cells were incubated in a 1:1 ratio with moDCs for 2 or 24h. Uptake of necrotic cells  
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3 576 by moDCs was quantified by flow cytometry double positive populations in the dotplots  
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6 577 represent phagocytosis by moDCs which was also confirmed by confocal microscopy. E)  
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8 578 Typed moDC (HLA-DR1+/HLA-A2-) were cultured with living/necrotic or IFN- $\gamma$   
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10 579 stimulated necrotic SAL-A2 cells (1:1 ratio at  $5 \times 10^4$  cells) for 48h after which  $5 \times 10^3$  4.44  
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12 580 T-cells were added with or without HLA-A2 peptides. F) moDCs were also cultured with  
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15 581 1E2 cells (as described in E). All experiments were repeated three times, graphs represent  
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17 582 mean + SD of a single experiment conducted in triplicate. Confocal imaging was repeated  
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20 583 twice. Dashed bar depicts the detection limit.  
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24 585 **Figure 3. moDCs and monocytes present monomers to T-cells clones in a time and**  
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27 586 **dose dependent manner.** A-B) HLA-DR1+/HLA-A2- moDCs were incubated with  
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29 587 HLA-A2 derived peptides(peptide pulsed) or HLA-A2 monomers(HLA-A2) in co-  
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31 588 cultures with or without the 4.44 T-cells for 24h. Supernatants were collected and IFN- $\gamma$   
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33 589 measured. HLA-A2- moDCs presented HLA-A2 monomer derived peptides in a dose  
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35 590 dependent manner to the T-cells. C) HLA-Typed moDCs were incubated with HLA-A2  
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37 591 monomers for 1,2,4 and 6h, then washed thoroughly before the 4.44 T-cells were added  
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39 592 for an additional 24h incubation. After a 24h incubation IFN- $\gamma$  was measured in the  
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41 593 supernatants D) HLA-Typed moDCs were incubated with HLA-A2 monomers for 4h and  
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43 594 then thoroughly washed. T-cells were then added immediately (0h), 24h or after 48h.  
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45 595 Supernatants were harvested after 24h. E-F) HLA-DR1+/HLA-A2- monocytes were  
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47 596 pulsed with an HLA-A2 derived peptide or different concentrations of monomers (HLA-  
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49 597 A2) and co-cultured in the presence or absence of 4.44 T-cells. After 24h incubation  
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51 598 IFN- $\gamma$  was measured in the supernatants. All experiments were conducted in triplicate,  
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3 599 results indicate the mean + SD of one experiment in triplicate. Dashed bars indicate the  
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5 600 detection limit of the ELISA used. ND – Not detectable.

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10 602 **Figure 4. PBMCs can present HLA-A2 monomer derived peptides via HLA-DR1**

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12 603 **with no measurable semi-direct alloreactivity.** A-B)  $1 \times 10^5$  Typed HLA-DR1+/HLA-

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15 604 A2- PBMCs were incubated with different concentrations of HLA-A2 monomers or

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17 605 pulsed with an HLA-A2 peptide and co-cultured with or without  $5 \times 10^3$  4.44 T-cells. 48h

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20 606 after incubation supernatants were collected and IFN- $\gamma$  measured. C-D) PBMCs were

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22 607 incubated with different concentrations of HLA-A2 monomers and co-cultured with

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25 608 either the indirect recognizing clone 4.44 (grey circles) or with the direct recognizing

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27 609 clone 1E2 (white circles) for 48h. IFN- $\gamma$  and proliferation were analyzed as previously

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29 610 described. E) Different PBMC numbers were incubated with 25 $\mu$ g/ml HLA-A2 in the

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32 611 presence of  $5 \times 10^3$  4.44 for 24 or 48h. Supernatants were then harvested and IFN- $\gamma$

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35 612 measured. F) To test the minimal amount of T-cells needed to detect a response 4.44 were

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37 613 titrated and 25 $\mu$ g/ml HLA-A2 monomer was added in combination with  $1 \times 10^5$  HLA-

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39 614 typed PBMCs for 48h. Supernatants were then collected and IFN- $\gamma$  measured G-H)

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42 615 PBMCs were co-cultured with 4.44 T-cells in the presence of 25 $\mu$ g/ml HLA-A2 for 24h

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45 616 with or without anti-HLA-DR monoclonal antibodies (mAb). The reciprocal dilution of

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47 617 the HLA-DR mAb is set on the x-axis. All experiments were conducted at least 3 times

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49 618 shown is the mean + SD of one experiment in triplicate. Dashed line represents the

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51 619 detection limit of the ELISA used. ND – Not detectable.

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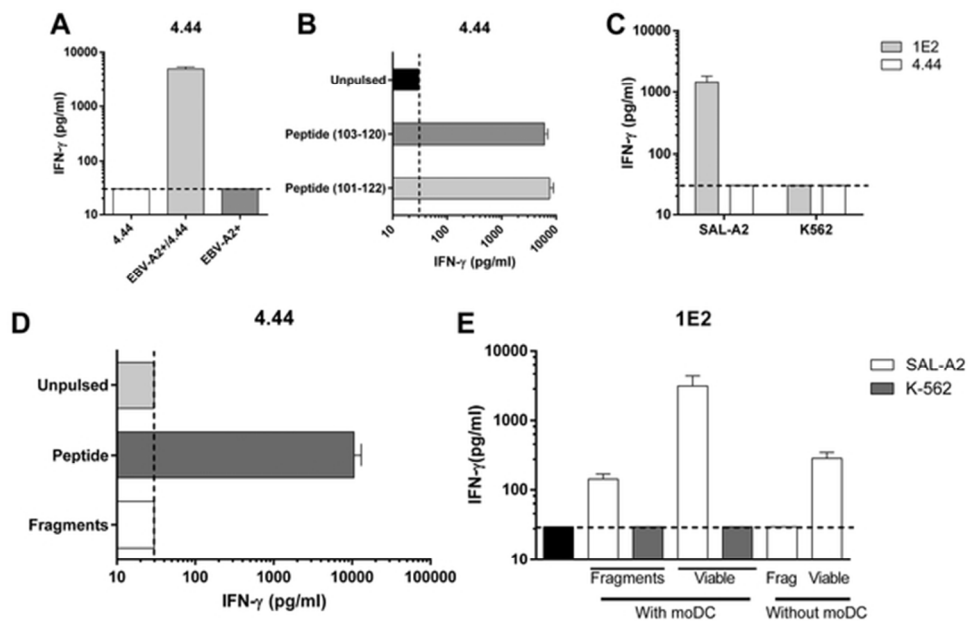


Figure 1. T-cell clones recognize cellular fragments through semi-direct allorecognition but not through indirect allorecognition. A)  $5 \times 10^3$  CD4 T-cells (4.44) were cultured with their native ligand HLA-A2+/HLA-DR1+ EBV-LCLs. IFN- $\gamma$  secretion was measured in the supernatants after 24h. B) HLA-A2-/HLA-DR1+ EBV-LCLs were pulsed with different HLA-A2 derived peptides and co-cultured with 4.44 T-cells. After 24h IFN- $\gamma$  was measured in the supernatants. C) An HLA-A2 recognizing CD8 T-cell clone (1E2) and the 4.44 were co-cultured with K562 or HLA-A2 transfected K562 cells (SAL-A2) for 48h. Supernatants were then collected and IFN- $\gamma$  measured. D) HLA-Typed moDC (HLA-DR1+/HLA-A2-) were cultured with or without fragments of SAL-A2 cells (1:1 ratio at  $5 \times 10^4$  cells) for 48h after which  $5 \times 10^3$  4.44 T-cells were added with or without HLA-A2 peptides. E) moDCs were also cultured with 1E2 cells (as described in D). All experiments were repeated three times, graphs represent the mean +SD of a single experiment conducted in triplicate.

Dashed lines represent the detection limit of the ELISA used.

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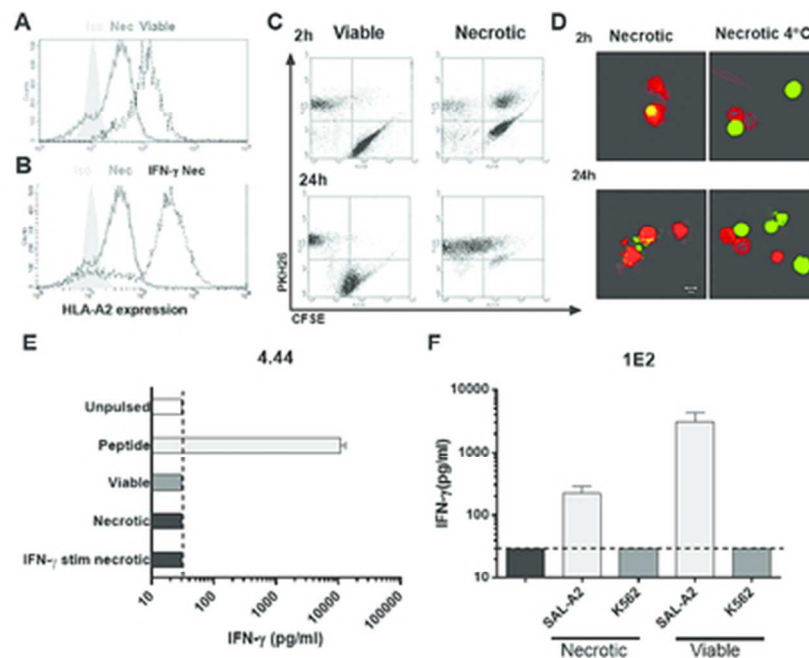


Figure 2. T-cell clones recognize necrotic cells through semi-direct allorecognition but not through indirect allorecognition. A-B) SAL-A2 cells were stained with HLA-A2-PE labeled antibodies or isotype control. Cells were either viable or necrotic. IFN- $\gamma$  stimulated SAL-A2 cells were stimulated with 100ng/ml IFN- $\gamma$  for 24h prior to HLA-A2 labeling. C-D) moDCs were labeled with PKH26 and SAL-A2 cells were labeled with CFSE. Necrosis was then induced and as a control viable cells were used.  $1 \times 10^4$  SAL-A2 cells were incubated in a 1:1 ratio with moDCs for 2 or 24h. Uptake of necrotic cells by moDCs was quantified by flow cytometry double positive populations in the dotplots represent phagocytosis by moDCs which was also confirmed by confocal microscopy. E) Typed moDC (HLA-DR1+/HLA-A2-) were cultured with living/necrotic or IFN- $\gamma$  stimulated necrotic SAL-A2 cells (1:1 ratio at  $5 \times 10^4$  cells) for 48h after which  $5 \times 10^3$  4.44 T-cells were added with or without HLA-A2 peptides. F) moDCs were also cultured with 1E2 cells (as described in E). All experiments were repeated three times, graphs represent mean + SD of a single experiment conducted in triplicate. Confocal imaging was repeated twice. Dashed bar depicts the detection limit. 17x14mm (600 x 600 DPI)

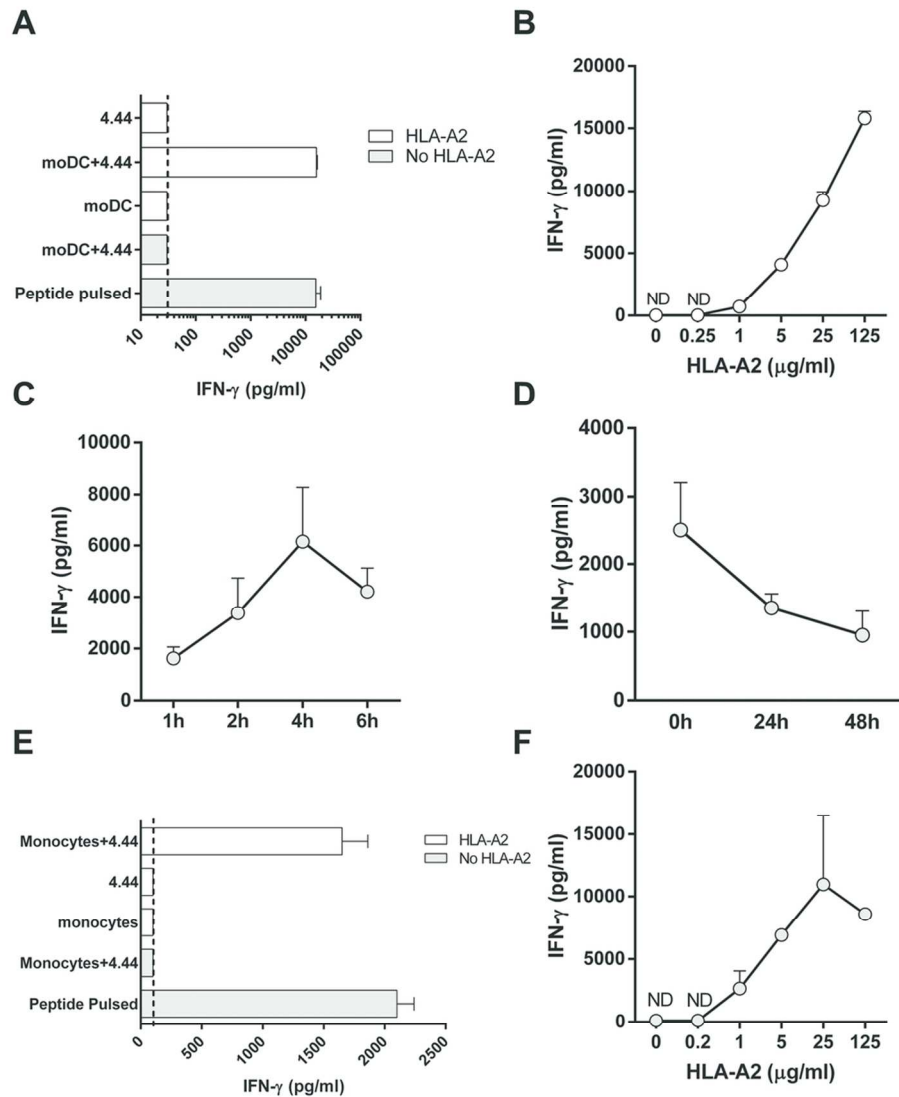


Figure 3. moDCs and monocytes present monomers to T-cells clones in a time and dose dependent manner. A-B) HLA-DR1+/HLA-A2- moDCs were incubated with HLA-A2 derived peptides(peptide pulsed) or HLA-A2 monomers(HLA-A2) in co-cultures with or without the 4.44 T-cells for 24h. Supernatants were collected and IFN-γ measured. HLA-A2- moDCs presented HLA-A2 monomer derived peptides in a dose dependent manner to the T-cells. C) HLA-Typed moDCs were incubated with HLA-A2 monomers for 1,2,4 and 6h, then washed thoroughly before the 4.44 T-cells were added for an additional 24h incubation. After a 24h incubation IFN-γ was measured in the supernatants D) HLA-Typed moDCs were incubated with HLA-A2 monomers for 4h and then thoroughly washed. T-cells were then added immediately (0h), 24h or after 48h. Supernatants were harvested after 24h. E-F) HLA-DR1+/HLA-A2- monocytes were pulsed with an HLA-A2 derived peptide or different concentrations of monomers (HLA-A2) and co-cultured in the presence or absence of 4.44 T-cells. After 24h incubation IFN-γ was measured in the supernatants. All experiments were conducted in triplicate, results indicate the mean + SD of one experiment in triplicate. Dashed bars indicate the detection limit of the ELISA used. ND – Not detectable.

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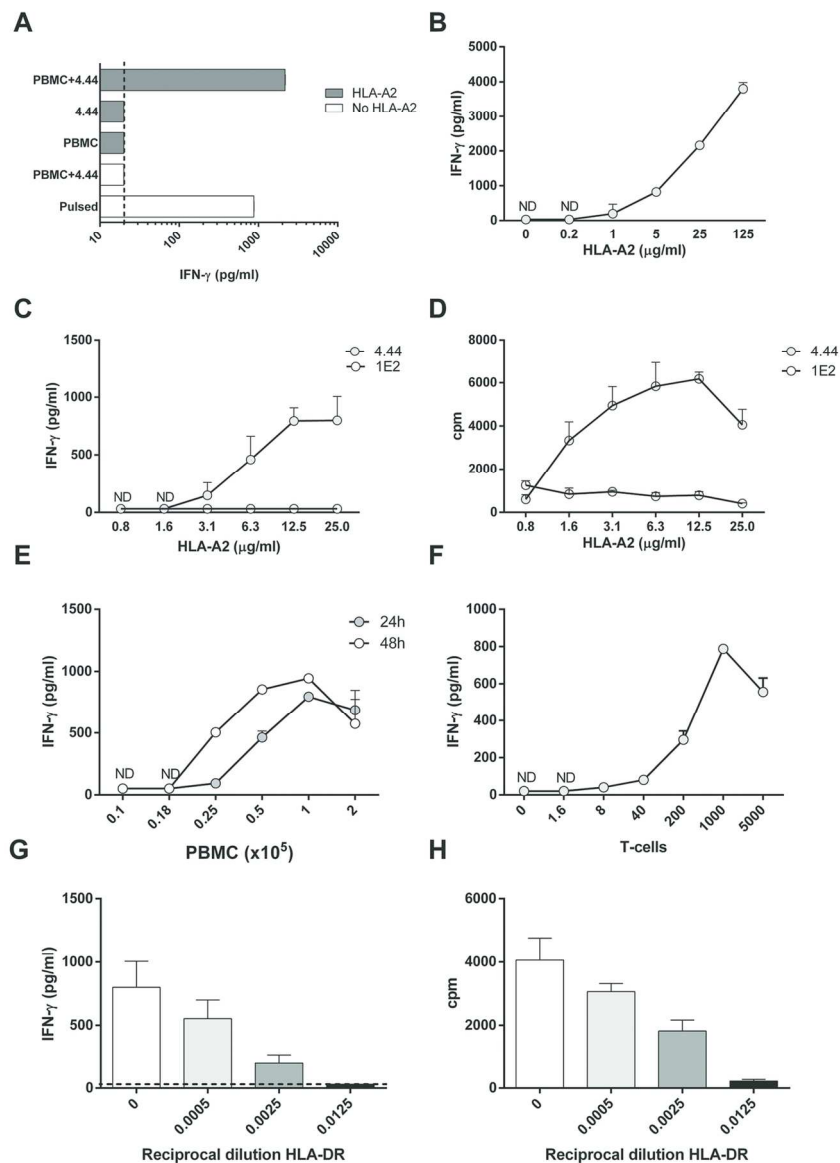


Figure 4. PBMCs can present HLA-A2 monomer derived peptides via HLA-DR1 with no measurable semi-direct alloreactivity. A-B)  $1 \times 10^5$  Typed HLA-DR1+/HLA-A2- PBMCs were incubated with different concentrations of HLA-A2 monomers or pulsed with an HLA-A2 peptide and co-cultured with or without  $5 \times 10^3$  4.44 T-cells. 48h after incubation supernatants were collected and IFN- $\gamma$  measured. C-D) PBMCs were incubated with different concentrations of HLA-A2 monomers and co-cultured with either the indirect recognizing clone 4.44 (grey circles) or with the direct recognizing clone 1E2 (white circles) for 48h. IFN- $\gamma$  and proliferation were analyzed as previously described. E) Different PBMC numbers were incubated with  $25 \mu$ g/ml HLA-A2 in the presence of  $5 \times 10^3$  4.44 for 24 or 48h. Supernatants were then harvested and IFN- $\gamma$  measured. F) To test the minimal amount of T-cells needed to detect a response 4.44 were titrated and  $25 \mu$ g/ml HLA-A2 monomer was added in combination with  $1 \times 10^5$  HLA-typed PBMCs for 48h. Supernatants were then collected and IFN- $\gamma$  measured G-H) PBMCs were co-cultured with 4.44 T-cells in the presence of  $25 \mu$ g/ml HLA-A2 for 24h with or without anti-HLA-DR monoclonal antibodies (mAb). The reciprocal dilution of the HLA-DR mAb is set on the x-axis. All experiments were conducted at least 3 times shown is the mean

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