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HLA monomers as a tool to monitor indirect allorecognition

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

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25 **Footnotes**

26 1 Contributions

27 EB designed, preformed the experiments and wrote the manuscript. PPvM participated in designing and performing the experiments.

28 DMvdS performed experiments MHH participated in writing of the paper. IID contributed reagents. DR, FHC and CvK designed

29 experiments, discussed results and helped to write the manuscript.

30 Conflict-of-interest disclosure: The authors declare no conflict of interest.

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4   39   **Abbreviations**
5
6   40   APC – antigen presenting cells
7
8   41   DC – Dendritic cell
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10   42   DTH – delayed-typed hypersensitivity
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13   43   EBV – Epstein bar virus
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15   44   ELISPOT - enzyme linked immunosorbent spot
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17   45   mAb – monoclonal antibody
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20   46   MLR – Mixed leukocyte reaction
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22   47   moDC – monocyte-derived dendritic cells
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24   48   PBMC – peripheral mononuclear blood cells
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27   49   PHA - phytohemagglutinin
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29   50   SAL – single antigen lines
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31   51   TLR – Toll like receptor
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34   52   Treg – Regulatory T-cells
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54   **Abstract**

55   Background: Recognition of donor antigens can occur through two separate pathways:

56   the direct pathway (non-self HLA on donor cells) and the indirect pathway (self-restricted

57   presentation of donor derived peptides on recipient cells). Indirect allorecognition is

58   important in the development of humoral rejection, therefore there is an increasing

59   interest in the monitoring of indirect alloreactive T-cells. We have used an in vitro model

60   to determine the optimal requirements for indirect presentation and assessed the risk for

61   semi-direct presentation in this system.

62

63   Methods: HLA typed monocyte-derived dendritic cells (moDC) were incubated with

64   cellular fragments or necrotic cells and incubated with either indirect or direct

65   alloreactive T-cell clones. T-cell reactivity was measured by proliferation or cytokine

66   secretion. HLA-typed moDC, monocytes or PBMCs were incubated with HLA class I

67   monomers, in combination with either direct/indirect T-cell clones.

68

69   Results: Although both were efficiently taken-up, alloreactivity was limited to the semi-

70   direct pathway, as measured by allo-specific CD4 (indirect) and CD8 T-cell clones

71   (direct). In contrast, HLA-A2 monomers were not only efficiently taken up, but also

72   processed and presented by HLA-typed moDC, monocytes or PBMCs. Activation was

73   shown by a dose dependent induction of IFN-γ production and proliferation by the CD4

74   T-cell clone. Antigen presentation was most efficient when the monomers were cultured

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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105 transplant tolerance (20-22). Recently, natural Treg were shown to inhibit direct but not

106 indirect allorecognition (23) Although both pathways have been known for decades,

107 assays monitoring indirect T-cell alloreactivity have not become routine, and as of yet no

108 clinical assay is available (11).

109 Methods to monitor direct alloreactivity include the mixed leukocyte reaction (MLR), the

110 cytotoxic T-cell precursor assay and more recently, the IFN-γ enzyme linked

111 immunosorbent spot (ELISPOT). The assays give a good indication of the potency of

112 directly reactive T-cells (24-27). Methods to measure indirectly reactive T-cells have

113 been previously reviewed and offer many complications (28). These include the use of

114 donor cell fragments as a source of donor antigens, which are presented in the context of

115 self-HLA class II. In theory this method allows the full repertoire of alloantigen available.

116 In practice, this assay is difficult and not very reproducible. Furthermore, reactivity may

117 be due to the semi-direct pathway (29, 30). Synthetic peptides which correspond with the

118 mismatched donor antigens have also been used (31-33). This method has a higher

119 reproducibility but reactivity may be directed at neo-epitopes that are not available *in*

120 *vivo*. The third method makes use of the *trans vivo* delayed-type hypersensitivity (DTH)

121 model, in which recipient cells and donor cellular fragments are injected into the footpad

122 of naïve mice (34-36). Donor cell alloreactivity leads to a DTH response that can be

123 measured by the footpad swelling. The advantage is that a global alloresponse can be

124 measured (37). However, this approach is very complex and technically too difficult to be

125 used as a diagnostic assay.

126 The aim of this study was to develop a reliable and easy tool to monitor indirect

127 allorecognition in renal transplant patients that could be used in a clinical setting.

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7

129   **Results**

130   **Evidence for semi-direct rather than indirect allorecognition when donor cell**

131   **fragments are used as exogenous antigen source**

132   To set-up and validate a model for indirect allopresentation, a previously characterized

133   CD4 T-cell clone (referred to as 4.44) recognizing HLA-A2 peptide restricted by HLA-

134   DR1was used (38). Incubation of 4.44 with its native ligand in the form of Epstein Bar-

135   Virus (EBV) transformed B-cell line (EBV-LCL) expressing HLA-DR1 and HLA-A2 led

136   to IFN-γ secretion (Figure 1A). Clone 4.44 did not react with HLA-DR1+/HLA-A2-

137   EBV-LCLs, but IFN-γ secretion could be induced when cells were pulsed with HLA-A2

138   peptides containing the relevant epitope (Figure 1B).

139   A CD8 T-cell clone (referred to as 1E2) that recognizes HLA-A2 (39), was used as a

140   readout to exclude semi-direct alloreactivity. Both T cell clones were cultured with single

141   antigen lines (SAL) expressing only HLA-A2 (SAL-A2). T-cell reactivity as measured by

142   IFN-γ was limited to the 1E2 T-cells, confirming specificity of both clones (Figure 1C).

143   To investigate the process of indirect alloreactivity, monocyte-derived dendritic cells

144   (moDC) from HLA-typed donors (HLA-DR1+/HLA-A2-) were used as APCs and were

145   loaded with peptides or fragmented SAL-A2 cells as antigen source. After 48h clone 4.44

146   was added, but apart from the positive control no indirect alloreactivity could be

147   measured in any of the conditions (Figure 1D). In contrast, under similar conditions of

148   loading fragments, when clone 1E2 was used low but significant production of IFN-γ

149   could be detected in 3 out of 5 experiments (Figure 1E). This indicates that the use of cell

150   fragments might result in semi-direct allorecognition when co-cultured with moDC. No

8

151   reactivity was observed when fragments and 1E2 were cultured alone. 1E2 activation was

152   confirmed in all conditions where viable SAL-A2 were used.

153

154   **Processing of necrotic cells leads to semi-direct allorecognition**

155   Professional APCs have specialized mechanisms for the uptake of apoptotic or necrotic

156   cells. Therefore, we investigated whether dead cells could be a more efficient way of

157   loading alloantigen, without the risk of semi-direct allorecognition. Viable, or necrotic

158   SAL-A2 cells were used as previously described (40). HLA-A2 expression on SAL-A2

159   was confirmed on viable and necrotic (Nec) SAL-A2 (Figure 2A). Moreover, SAL-A2

160   were stimulated with IFN-γ to increase HLA-A2 expression on these cells (Figure 2B).

161   Uptake of dead cells, but not viable cells, was confirmed by flow cytometry as well as

162   confocal microscopy (Figure 2C-D). Importantly, after 24h incubation a near complete

163   removal of dead material was observed. This was an active process that did not occur at

164   4°C.

165   Indirect allorecognition studies were performed using HLA-A2-/HLA-DR1+ typed

166   moDC. Whereas strong IFN-γ production was achieved with exogenous peptide loading

167   none of the conditions investigated resulted in detectable activation of the 4.44 clone

168   (Figure 2E). Activation of moDC using different toll-like receptor (TLR) ligands did not

169   result in detectable indirect presentation (data not shown). Comparable to the fragments,

170   a low albeit significant response was found for semi-direct allorecognition by 1E2 when

171   necrotic SAL-A2 cells were used (Figure 2F).

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173 **HLA monomers can be used for antigen loading resulting in indirect allo-**

174 **presentation**

175 As an alternative source of alloantigens we investigated the potential use of HLA-A2

176 monomers. Incubation of HLA-DR1+/HLA-A2- moDC with HLA-A2 led to potent

177 activation of clone 4.44 comparable to exogenously loading of peptides (Figure 3A).

178 This response was dose-dependent and significant IFN-γ production was observed with

179 concentrations starting at 1μg/ml HLA-A2 and kept increasing until 125μg/ml (Figure

180 3B).

181 MoDCs were incubated with 25μg/ml HLA-A2 for various time points and after

182 extensive washing 4.44 was added for an additional 24h. The highest reactivity was

183 observed when moDCs were incubated for at least 4h with the antigen (Figure 3C). Next,

184 moDCs were incubated with HLA-A2 for 4h before extensive washing. 4.44 T-cells were

185 then added at 0h, 24h, or 48h and incubated for an additional 24h (Figure 3D). The

186 highest reactivity was observed when 4.44 were added immediately (0h), while a steady

187 decrease was observed after 24 and 48h, indicating a steady turnover of HLA-peptide

188 complexes.

189 ~~As m~~Monocytes are the major ~~a more readily available source of~~ APCs within the

190 PBMCs, we investigated their capacity to present alloantigens via the indirect pathway.

191 Similarly to moDC, monocytes showed a strong capacity to present HLA-A2 and activate

192 4.44 T-cells, comparable to the exogenous peptide (Figure 3E-F). This response was

193 dose-dependent and significant levels of IFN-γ were observed at concentrations of

194 1μg/ml HLA-A2, reaching an optimum at 25μg/ml (Figure 3F).

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10

196   **PBMCs can present monomer derived peptides to indirect allorecognizing T-cells**

197   **clones without evidence for semi-direct allorecognition**

198   Since the afore mentioned experiments were performed with purified populations, we

199   next addressed the question whether the use of total PBMC would affect the sensitivity or

200   specificity of the above described model. HLA-DR1+/HLA-A2- PBMCs were pulsed

201   with HLA-A2 peptides or incubated with HLA-A2 monomers in the presence/absence of

202   4.44. Again using either exogenous peptide or HLA-A2 monomers, a strong and dose-

203   dependent response of the T-cell clone could be detected (Figure 4A-B). In the absence of

204   the T-cell clone no IFN-γ production could be detected indicating that the addition of

205   HLA-A2 monomer does not activate CD8 T-cells with direct allospecificity (Figure 4A).

206   The absence of semi-direct allorecognition could be further confirmed with the 1E2

207   clone, which was completely unresponsive when incubated with the HLA-typed PBMCs

208   and increasing concentrations of HLA-A2, as depicted by IFN-γ production as well

209   proliferation (Figure 4C-D). This is in stark contrast to clone 4.44 which was highly

210   positive in both conditions.

211   To investigate the sensitivity of this model, various PBMCs concentrations were tested

212   and incubated for 24 or 48h with a set amount of HL-A2 and 4.44 (Figure 4E). 48h and

213    $1x10^5$ PBMCs yielded overall a better T-cell response. When titrating the T cell clone, as

214   little as 8-40 T-cells were sufficient to induce detectable levels of IFN-γ when $1x10^5$

215   PBMCs were incubated with 25μg/ml HLA-A2 for 48h (Figure 4F).

216   Finally, we could confirm that antigen presentation was completely HLA-DR dependent.

217   The cytokine production and proliferation was completely and dose-dependently

11

218   prevented when cultures with 4.44 were incubated with a blocking antibody against

219   HLA-DR (Figure 4G-H).

220

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

13

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

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

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

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

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

249   limited to low levels of IFN-γ and is most likely due to the limited HLA class I molecules

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

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

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

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

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

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

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

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

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

259   by the T-cell clone.

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

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

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

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

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

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

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

267   reactivity to peptides derived from autologous HLA molecules. Peptides used for the

268   detection of indirect allorecognition include peptides derived from the β1 domain of HLA

269   class II (32, 51-53) or the α1 domain of HLA class I (33, 54). In one study, where

270   peptides from a complete HLA-A2 molecule were used, reactivity was seen in the

271   hypervariable region as well as from the α3 subunit and the trans-membrane domain (52).

272   However, as no peptides from self-HLA were taken as a control it is unclear what the

273   reactivity means, and whether these peptides would be formed when naturally processed.

274   Use of longer peptides could be a good alternative, as these would require processing and

275   are able to induce T-cell reactivity in other model systems of antigen presentation (53,

276   54).

277   HLA monomers offer solutions to many of the problems depicted earlier. However, there

278   are two distinct disadvantages in using monomers. The one is the high price involved in

279   making highly pure monomers and secondly the relatively high concentration needed to

280   induce a reproducible T-cell response.

281   The quest to induce long-term graft tolerance and/or survival starts with an accurate

282   depiction of the recipient's immune-response against the donor allograft. Although many

283   studies are aimed at monitoring and measuring operational tolerance and organ rejection

284   (55, 56), no reliable test exists other than the actual clinical outcome. Measuring indirect

285   allorecognition could give a broad view into how the recipient immune-system is shaping

286   its response to the allograft and be helpful in tailoring an individual immune-suppression

287   regime to prolong graft survival.

288

289 **Materials and methods**

290 **Cell culture and reagents**

291 Peripheral blood mononuclear cells (PBMC), monocytes and monocytes-derived

292 dendritic cells (moDC) were cultured in RPMI-1640 (PAA, Austria) supplemented with

293 10% FCS (Bodinco, the Netherlands), 5,000 U/ml penicillin and 5 mg/ml streptomycin

294 (Invitrogen, USA).

295 Single antigen lines (SAL) were created by transfecting K562 cells with a plasmid

296 construct containing a HLA-A2 heavy chain gene (SAL-A2) (57). SAL-A2 were

297 maintained in IMDM supplemented with 10% FCS, pen/strep and G-418 (Roche,

298 Germany) at a concentration of 250 μg/ml for the selection of stably transfected SALs.

299 HLA-A*0201 derived peptides of the region 99-122 of HLA-A2 were synthesized. HLA-

300 A*0201 heavy chains were produced in *E.coli.* Monomer refolding around the

301 melanoma-associated pmel 17 peptide (YLEPGVTA) in the presence of β2-

302 microglobulin was achieved as previously described (58). Monomers were purified by gel

303 filtration HPLC and tested routinely.

304 T-cells clones 4.44 (CD4+) and 1E2 (CD8+), recognizing HLA-A2 (aa98-120) restricted

305 by HLA-DR1 and HLA-A2 respectively, have been previously described (38, 59). They

306 were maintained in IMDM medium (PAA, Austria) with 5% FCS (Bodinco, the

307 Netherlands), 5% normal human serum (Sanquin, the Netherlands), 100 IU/ml

308 recombinant IL-2 (Chiron, Novartis, USA) 5,000 U/ml penicillin, 5 mg/ml streptomycin,

309 and 2 mM L-glutamine (Gibco, invitrogen, USA). Expansion was achieved by

310 stimulation with phytohemagglutinin (PHA, 0.8 μg/ml, Murex Biotec Limited, Dartford,

311 UK) and PBMCs in a ratio of 1:5. Cells were harvested after two weeks and either frozen

312   or used in experiments after a resting period of 2-3 days. T-cell specificity was routinely

313   tested.

314   Stable Epstein-Barr virus (EBV)-transformed B-cell lines (EBV-LCL) were generated

315   from an HLA-DR1+/HLA-A2- donor using standard procedures (60). HLA-A2+ EBV-

316   LCLs were generated by transducing a retroviral vector encoding for HLA-A*0201 into

317   the donor (HLA-A2-) EBV-LCLs (61).

318

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

320   moDCs were generated from buffy coats as previously described (62). Briefly PBMCs

321   were isolated from buffy coats (Sanquin, the Netherlands) of healthy (HLA-A2$^-$,HLA-

322   DR1$^+$) individuals using Ficoll/amidotrizoaat (pharmacy, LUMC, the Netherlands)

323   density gradient, followed by CD14 microbeads magnetic cell sorting (Miltenyi Biotec,

324   The Netherlands) according to manufactures protocol. Monocytes were cultured in 6 well

325   plates (Costar, USA) in RPMI-1640 supplemented with 10 ng/ml IL-4 and 5 ng/ml GM-

326   CSF (Gibco, Invitrogen, USA). Cytokines were refreshed every 2-3 days and cells were

327   allowed to differentiate for at least 6 days before harvesting.

328

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

330   moDCs were co-cultured with necrotic, apoptotic or fragmented SAL-A2 cells. Necrosis

331   and apoptosis was induced as previously described (40). Briefly necrosis was induced by

332   heating cells to 56°C for one hour and confirmed by light microscopy and annexin-V/PI

333   staining. Cell fragments were generated by 3 rounds of freeze-thawing and confirmed by

334   light microscopy. $5x10^5$ moDCs were co-cultured at a 1:1 ratio with SAL-A2 cells for a

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335   period of at least 24h in a 96 round well plate. 5×10^3 4.44 ~~cells~~ (CD4 indirect) or 1E2

336   (CD8 direct/semi-direct) cells were added for an additional 48h incubation. Supernatants

337   were then harvested and IFN-γ production was measured.

338

Phagocytosis assay

340   Phagocytosis was quantified by flow cytometry or fluorescence microscopy as previously

341   described (40). Briefly moDCs were labeled with PKH26 (Sigma-Aldrich) or stained

342   with HLA-DR mAb. Necrotic, apoptotic or fragmented SAL-A2 cells were stained with

343   CFSE prior to induction of cell death and then co-cultured with moDCs at a ratio of 1:1

344   (5×10^4 cells). Analysis was conducted at 2 or 24h post co-culture. Fluorescence was

345   assessed with FACSCalibur or LSR-II or with a Leica SP5 confocal scanning laser

346   microscope and the analysis preformed with ImageJ imaging software.

347

Indirect/direct allorecognition assay using HLA class I monomers

349   To monitor the pathways of~~indirect~~ allorecognition moDC or monocytes (DR1$^+$/A2$^-$)

350   were plated (3×10^4) in round 96-well plates (Costar, USA) and incubated with different

351   concentrations of HLA-A2 monomers (HLA-A2) or peptides. T-cells were added to the

352   culture ~~(5×10^3, 4.44 or 1E2)~~ and incubated at different time points. A CD4 T-cell clone

353   (5×10^3, 4.44) was used as a readout for indirect allorecognition and a CD8 T-cell clone

354   (5×10^3, 1E2) for direct/semi-direct allorecognition. Part of the supernatant was harvested

355   and cells were pulsed with 0.5μCi ^{3}H (Life science products Inc. USA) and incubated for

356   24h. Similarly (1×10^5) PBMCs were co-cultured with (5×10^3) T-cells (4.44 or 1E2) with

357   different concentrations of HLA-A2. Co-cultures were incubated for 48h, supernatants

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358   collected and cells pulsed with ^{3}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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362 **Acknowledgements**

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553   **Figure legends:**

554   **Figure 1. T-cell clones recognize cellular fragments through semi-direct**

555   **allorecognition but not through indirect allorecognition**. A) $5x10^3$ CD4 T-cells (4.44)

556   were cultured with their native ligand HLA-A2+/HLA-DR1+ EBV-LCLs. IFN-γ

557   secretion was measured in the supernatants after 24h. B) HLA-A2-/HLA-DR1+ EBV-

558   LCLs were pulsed with different HLA-A2 derived peptides and co-cultured with 4.44 T-

559   cells. After 24h IFN-γ was measured in the supernatants. C) An HLA-A2 recognizing

560   CD8 T-cell clone (1E2) and the 4.44 were co-cultured with K562 or HLA-A2 transfected

561   K562 cells (SAL-A2) for 48h. Supernatants were then collected and IFN-γ measured. D)

562   HLA-Typed moDC (HLA-DR1+/HLA-A2-) were cultured with or without fragments of

563   SAL-A2 cells (1:1 ratio at $5x10^4$ cells) for 48h after which $5x10^3$ 4.44 T-cells were added

564   with or without HLA-A2 peptides. E) moDCs were also cultured with 1E2 cells (as

565   described in D). All experiments were repeated three times, graphs represent the mean

566   +SD of a single experiment conducted in triplicate. Dashed lines represent the detection

567   limit of the ELISA used.

568

569   **Figure 2. T-cell clones recognize necrotic cells through semi-direct allorecognition**

570   **but not through indirect allorecognition.** A-B) SAL-A2 cells were stained with HLA-

571   A2-PE labeled antibodies or isotype control. Cells were either viable or necrotic. IFN-γ

572   stimulated SAL-A2 cells were stimulated with 100ng/ml IFN-γ for 24h prior to HLA-A2

573   labeling. C-D) moDCs were labeled with PKH26 and SAL-A2 cells were labeled with

574   CFSE. Necrosis was then induced and as a control viable cells were used. 1x10^4 SAL-

575   A2 cells were incubated in a 1:1 ratio with moDCs for 2 or 24h. Uptake of necrotic cells

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576   by moDCs was quantified by flow cytometry double positive populations in the dotplots

577   represent phagocytosis by moDCs which was also confirmed by confocal microscopy. E)

578   Typed moDC (HLA-DR1+/HLA-A2-) were cultured with living/necrotic or IFN-γ

579   stimulated necrotic SAL-A2 cells (1:1 ratio at $5x10^4$ cells) for 48h after which $5x10^3$ 4.44

580   T-cells were added with or without HLA-A2 peptides. F) moDCs were also cultured with

581   1E2 cells (as described in E). All experiments were repeated three times, graphs represent

582   mean + SD of a single experiment conducted in triplicate. Confocal imaging was repeated

583   twice. Dashed bar depicts the detection limit.

584

585   **Figure 3. moDCs and monocytes present monomers to T-cells clones in a time and**

586   **dose dependent manner.** A-B) HLA-DR1+/HLA-A2- moDCs were incubated with

587   HLA-A2 derived peptides(peptide pulsed) or HLA-A2 monomers(HLA-A2) in co-

588   cultures with or without the 4.44 T-cells for 24h. Supernatants were collected and IFN-γ

589   measured. HLA-A2- moDCs presented HLA-A2 monomer derived peptides in a dose

590   dependent manner to the T-cells. C) HLA-Typed moDCs were incubated with HLA-A2

591   monomers for 1,2,4 and 6h, then washed thoroughly before the 4.44 T-cells were added

592   for an additional 24h incubation. After a 24h incubation IFN-γ was measured in the

593   supernatants D) HLA-Typed moDCs were incubated with HLA-A2 monomers for 4h and

594   then thoroughly washed. T-cells were then added immediately (0h), 24h or after 48h.

595   Supernatants were harvested after 24h. E-F) HLA-DR1+/HLA-A2- monocytes were

596   pulsed with an HLA-A2 derived peptide or different concentrations of monomers (HLA-

597   A2) and co-cultured in the presence or absence of 4.44 T-cells. After 24h incubation

598   IFN-γ was measured in the supernatants. All experiments were conducted in triplicate,

599   results indicate the mean + SD of one experiment in triplicate. Dashed bars indicate the

600   detection limit of the ELISA used. ND – Not detectable.

601

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

603   **with no measurable semi-direct alloreactivity.** A-B) $1x10^5$ Typed HLA-DR1+/HLA-

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

605   pulsed with an HLA-A2 peptide and co-cultured with or without $5x10^3$ 4.44 T-cells. 48h

606   after incubation supernatants were collected and IFN-γ measured. C-D) PBMCs were

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

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

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

610   described. E) Different PBMC numbers were incubated with 25μg/ml HLA-A2 in the

611   presence of $5x10^3$ 4.44 for 24 or 48h. Supernatants were then harvested and IFN-γ

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

613   titrated and 25μg/ml HLA-A2 monomer was added in combination with $1x10^5$ HLA-

614   typed PBMCs for 48h. Supernatants were then collected and IFN-γ measured G-H)

615   PBMCs were co-cultured with 4.44 T-cells in the presence of 25μg/ml HLA-A2 for 24h

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

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

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

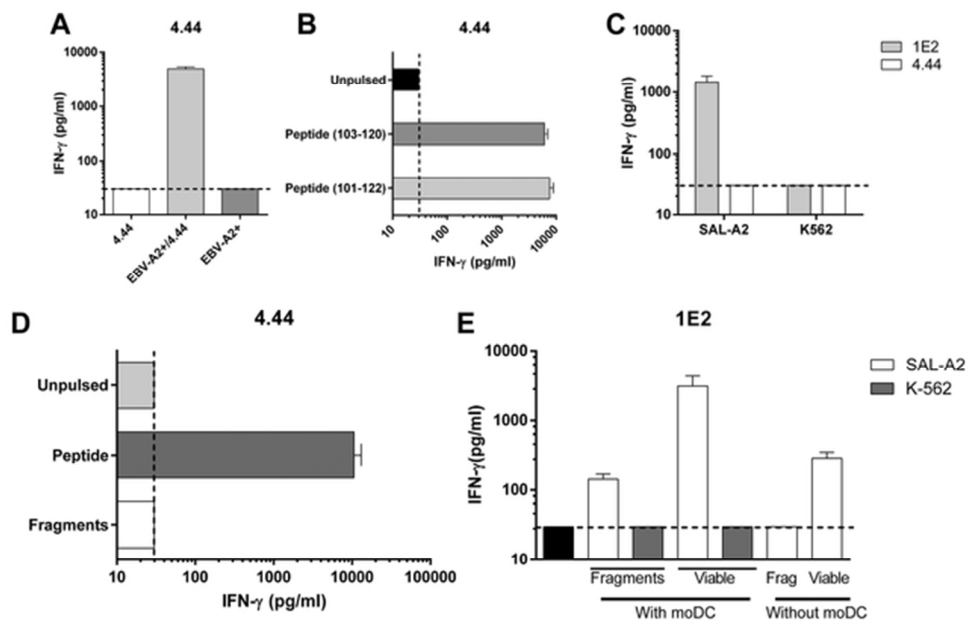619   detection limit of the ELISA used. ND – Not detectable.

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Figure 1. T-cell clones recognize cellular fragments through semi-direct allorecognition but not through indirect allorecognition. A) 5x103 CD4 T-cells (4.44) were cultured with their native ligand HLA-A2+/HLA-DR1+ EBV-LCLs. IFN-γ 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-γ 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-γ measured. D) HLA-Typed moDC (HLA-DR1+/HLA-A2-) were cultured with or without fragments of SAL-A2 cells (1:1 ratio at 5x104 cells) for 48h after which 5x103 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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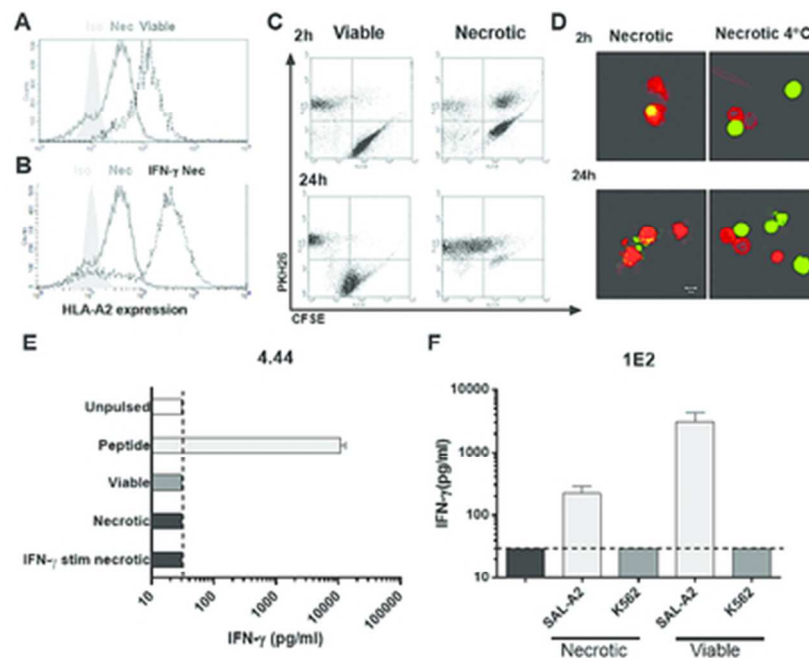49
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Figure 2. T-cell clones recognize necrotic cells through semi-direct allorecognition but not through indirect allorecognition. A-B) SAL-A2 cells were stained with HLA-A2-PE labeled antibodies or isotype control. Cells were either viable or necrotic. IFN-γ stimulated SAL-A2 cells were stimulated with 100ng/ml IFN-γ 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. 1x10^4 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-γ stimulated necrotic SAL-A2 cells (1:1 ratio at 5x104 cells) for 48h after which 5x103 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.
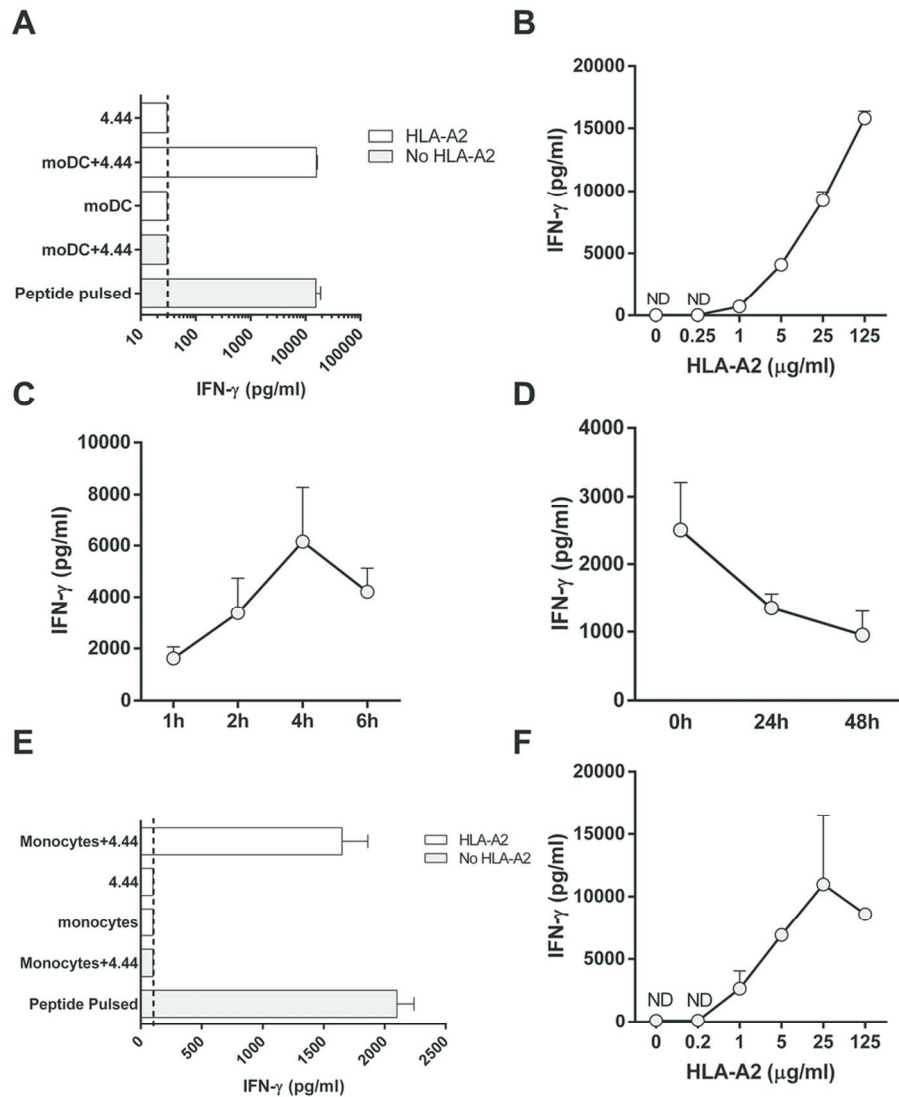17x14mm (600 x 600 DPI)

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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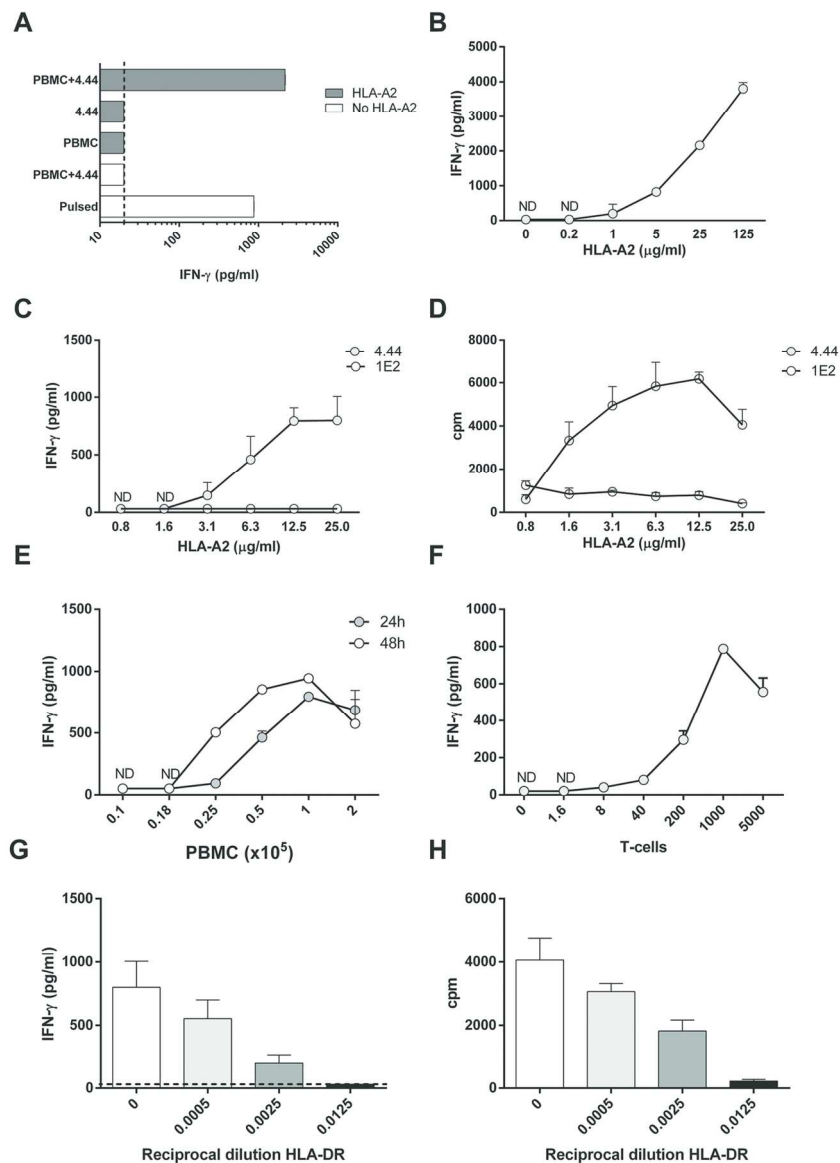41
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Figure 4. PBMCs can present HLA-A2 monomer derived peptides via HLA-DR1 with no measurable semi-direct alloreactivity. A-B) 1x105 Typed HLA-DR1+/HLA-A2- PBMCs were incubated with different concentrations of HLA-A2 monomers or pulsed with an HLA-A2 peptide and co-cultured with or without 5x103 4.44 T-cells. 48h after incubation supernatants were collected and IFN-γ 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-γ and proliferation were analyzed as previously described. E) Different PBMC numbers were incubated with 25µg/ml HLA-A2 in the presence of 5x103 4.44 for 24 or 48h. Supernatants were then harvested and IFN-γ measured. F) To test the minimal amount of T-cells needed to detect a response 4.44 were titrated and 25µg/ml HLA-A2 monomer was added in combination with 1x105 HLA-typed PBMCs for 48h. Supernatants were then collected and IFN-γ measured G-H) PBMCs were co-cultured with 4.44 T-cells in the presence of 25µg/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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+ SD of one experiment in triplicate. Dashed line represents the detection limit of the ELISA used. ND – Not detectable.
58x80mm (600 x 600 DPI)