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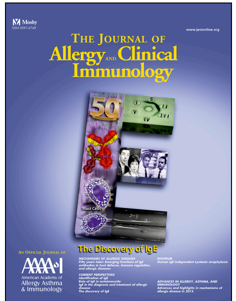

β-lactam hypersensitivity involves expansion of circulating and skin-resident Th22 cells

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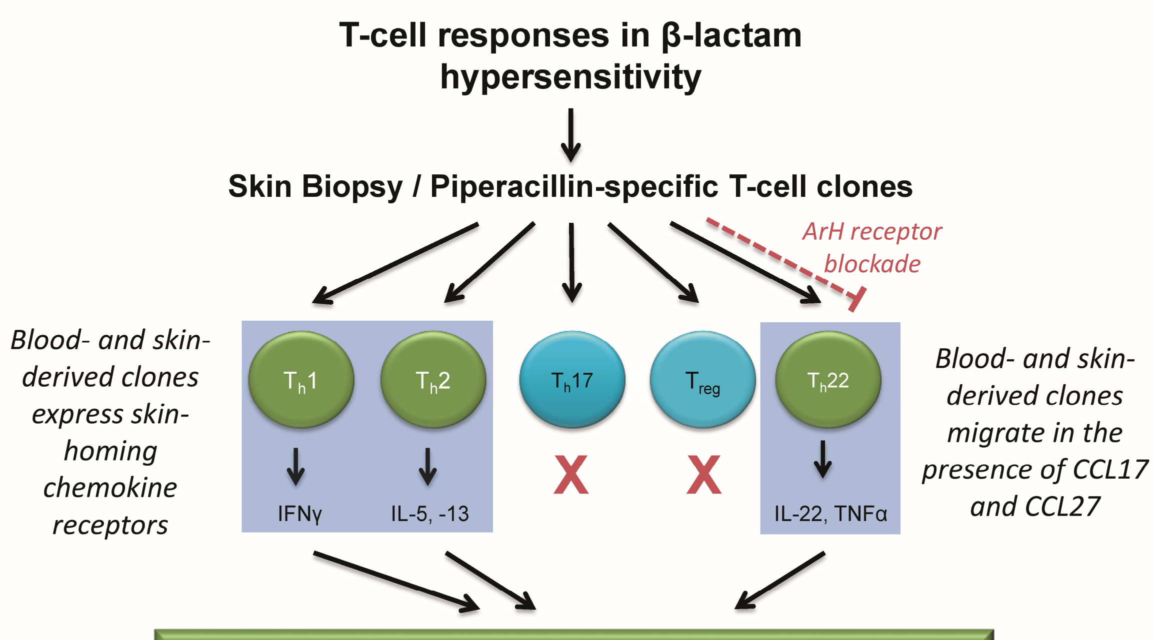
T-cell responses in β-lactam hypersensitivity
Skin Biopsy / Piperacillin-specific T-cell clones
ArH receptor blockade
Blood- and skin-derived clones express skin-homing chemokine receptors
Th1
Th2
Th17
Treg
Th22
X
X
IFNγ
IL-5, -13
IL-22, TNFα
Blood- and skin-derived clones migrate in the presence of CCL17 and CCL27
Drug-specific clones from inflamed skin display cytolytic activity, secreting perforin, granzyme B and Fas L when activated

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1

2 **Title: β-lactam hypersensitivity involves expansion of circulating and skin-**

3 **resident Th22 cells**

4

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9

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24 **Abstract**

25 **Background:** β-lactam hypersensitivity has been classified according to the phenotype and
26 function of drug-specific T-cells; however, new T-cell subsets have not been considered.
27 **Objective:** The objective of this study was use piperacillin as a model of β-lactam
28 hypersensitivity to study the nature of the drug-specific T-cell response induced in the blood
29 and skin of hypersensitive patients and healthy volunteers.

30 **Methods:** Drug-specific T-cells were cloned from blood and inflamed skin and cellular
31 phenotype and function was explored. Naïve T cells from healthy volunteers were primed to
32 piperacillin, cloned and subjected to the similar analyses.

33 **Results:** PBMC and T-cell clones (n=570, 84% CD4+) from blood of piperacillin
34 hypersensitive patients proliferated and secreted Th1/2 cytokines alongside IL-22 following
35 drug stimulation. IL-17A secretion was not detected. Drug-specific clones from inflamed skin
36 (n=96, 83% CD4+) secreted a similar profile of cytokines, but displayed greater cytolytic
37 activity, secreting perforin, granzyme B and Fas L when activated. Blood- and skin-derived
38 clones expressed high levels of skin-homing chemokine receptors and migrated in the
39 presence of the ligands CCL17 and CCL27. Piperacillin-primed naïve T-cells from healthy
40 volunteers also secreted IFN-γ, IL-13, IL-22 and cytolytic molecules. Aryl hydrocarbon
41 (ArH) receptor blockade prevented differentiation of the naïve T-cells into antigen-specific
42 Il-22 secreting cells.

43 **Conclusion:** Together our results reveal that circulating and skin resident antigen-specific IL-
44 22 secreting T-cells are detectable in patients with β-lactam hypersensitivity. Furthermore,
45 differentiation of naïve T-cells into antigen-specific Th22 cells is dependent on ArH receptor
46 signalling.

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48 **Key messages:** (1) β-lactam-specific, IL-22 secreting CD4+ and CD8+ T-cells reside in
49 blood and inflamed skin of hypersensitive patients: (2) β-lactam primed naïve T-cells from
50 healthy volunteers secrete the same cytokines and cytolytic molecules: (3) Differentiation of
51 naïve T-cells into antigen-specific Th22 cells is dependent on ArH receptor signalling.

52

53 **Capsule summary:** New T-cell subsets have not been considered in the context of drug
54 hypersensitivity. Herein, we show that antigen-specific circulating and skin resident CD4+
55 and CD8+ T-cells secrete IL-22 and cytolytic molecules following drug treatment.

56

57 **Keywords:** Human, T-cells, drug hypersensitivity.

58

59 **Abbreviations: S**timulation index, SI; peripheral blood mononuclear cells, PBMC.

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60 **Introduction**

61 To understand the cellular pathophysiology of different forms of hypersensitivity reaction,
62 antigen-specific T-cells have been cloned from the peripheral blood of hypersensitive patients
63 and characterized in terms of cellular phenotype and function.[1-4] Data deriving from these
64 studies indicated that delayed-type hypersensitivity reactions could be categorized according
65 to the phenotype of drug-specific T-cells, the cytokines they secrete and pathways of
66 immune-mediated killing of target cells.[5] Essentially, reactions were divided into 4 subsets.
67 In the last decade, our knowledge of the mechanistic basis of drug hypersensitivity has
68 increased exponentially. For example, we now know that (1) herpes virus reactivation is
69 implicated in certain reactions,[6,7] (2) HLA allele expression is an important determinant for
70 susceptibility[8,9] and (3) specific forms of drug-induced kidney and liver injury should fall
71 under the definition of drug hypersensitivity.[10-13] Despite this, our knowledge of the T-cells
72 that instigate and/or regulate drug hypersensitivity reactions has not progressed significantly
73 in the last decade.

74

75 The cytokine milieu that naïve T-cells are exposed to at the time of antigen exposure and
76 during differentiation is known to determine the nature of the antigen-specific response and
77 the types of secretory molecules that can affect tissue cells. In recent years, memory CD4+
78 and CD8+ T-cells have been shown to secrete distinct cytokine signatures: Th1 (IFN-γ), Th2
79 (IL-4, IL-5, IL-13), Th17 (IL-17, IL-22) and Th22 (IL-22). However, the picture is
80 complicated somewhat since the cytokines IL-17 and IL-22 can also be secreted by Th1/Th2
81 T-cells.[14,15] IL-22 is thought to be of particular importance in allergic skin disease since
82 receptors that the cytokine binds to are expressed on epithelial cells including keratinocytes.[16]
83 Moreover, Th17 and Th22 secreting T-cell subsets tend to express chemokine receptors that
84 promote skin migration (e.g., CCR4, CCR10).[17] Since human skin is protected by discrete

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85 populations of resident and recirculating T-cells[18] we have isolated and cloned drug-specific
86 T-cells from blood and skin of hypersensitive patients and characterized their cellular
87 phenotype and function following antigen recall, to determine whether IL-17 and/or IL-22
88 participate in drug hypersensitivity reactions. Previous studies with human T-cells isolated
89 from the memory T-cell pool have shown a mixed cytokine pattern, with small shifts in
90 balance toward a dominant cytokine. Thus, our patient studies were conducted in parallel to
91 T-cell priming experiments utilizing PBMC from healthy volunteers and a recently described
92 dendritic cell T-cell co-culture system.[19] This assay is simple to manipulate, which allowed
93 us to assess the influence of aryl hydrocarbon (ArH) receptor signalling on the nature of the
94 primary drug-specific response.

95

96

97 Piperacillin hypersensitivity was selected as the study model for several reasons. Firstly,
98 piperacillin is a β-lactam antibiotic and antibiotic hypersensitivity is the most common form
99 of drug-induced allergic disease. Secondly, piperacillin forms drug antigens directly in
100 patients and *in vitro* through the selective covalent modification of lysine residues on proteins
101 such as human serum albumin.[20] Thirdly, piperacillin is a commonly prescribed drug and the
102 frequency of hypersensitivity has been estimated to be as high as 35% in patients with cystic
103 fibrosis.[21] Finally, PBMC and blood-derived T-cell clones have been shown to be activated *in*
104 *vitro* in the presence of piperacillin and piperacillin albumin conjugates.[22]

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108 **Methods**

109 **Human subjects and cell isolation/separation**

110 Venous blood (20-60ml) was collected from 4 piperacillin hypersensitive patients with cystic
111 fibrosis and 4 control subjects who had been exposed to at least one course of piperacillin
112 with no recorded adverse event. PBMC were isolated and used for the lymphocyte
113 transformation test, generation of EBV-transformed B-cells and T-cell cloning. Punch biopsy
114 samples (3mm) were obtained from 2 of the hypersensitive patients (patients 1 and 2)
115 following a positive 24h intradermal skin test. The intravenous preparation of Tazocin
116 (piperacillin-tazobactam) was used for intradermal testing at a concentration of 2mg/ml. A
117 volume of 0.02ml was injected using a 0.5mm X 16mm insulin needle, this created an
118 injection wheal of around 3mm. Between 24 to 48 hours an infiltrated erythema over 5mm
119 was seen and a punch biopsy performed. Skin was broken up using a scalpel and cultured in
120 medium containing IL-2 (100IU/ml) for 5 days. T-cells migrating into culture medium were
121 collected, passed through a 50µm strainer and used for T-cell cloning.

122

123 PBMC were also isolated from 120ml of blood from 4 healthy volunteers to study the
124 priming of naïve T-cells with piperacillin. Monocytes and naïve T-cell populations were
125 separated using magnetic microbeads (Miltenyi Biotech; Bisley, UK). CD14+ cells were
126 positively selected from total PBMC. For isolation of naïve T-cells, pan negative T-cell
127 separation was performed using an anti-T-cell antibody cocktail. CD3+ cells were then
128 subject to positive selection to remove the unwanted CD25+ T_{reg} and memory cells
129 (CD45RO+). The naïve T-cells prior to priming were consistently greater than 98% pure. The
130 purity of the CD14+ monocytes varied depending on the level of neutrophil contamination in
131 the PBMC preparation but was generally greater than 80%. Monocyte-derived dendritic cells
132 were 100% positive for CD40, CD58, CD86 and Class I and Class II expression.

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133

134 Approval for the study was obtained from the Liverpool and Leeds local research ethics
135 committees and informed written consent was received from participants prior to inclusion in
136 the study.

137

138 **Medium for T-cell culture and cloning**

139 Culture medium consisted of RPMI-1640 supplemented with pooled heat-inactivated human
140 AB serum (10%, v/v), HEPES (25mM), L-glutamine (2mM), transferrin (25µg/mL),
141 streptomycin (100µg/mL), and penicillin (100U/mL). IL-2 (100IU/ml) was added to establish
142 drug-specific T-cell lines and clones.

143

144 **Lymphocyte transformation test and PBMC ELIspot**

145 PBMC (1.5x10^5 cell/well) from hypersensitive patients and tolerant controls were incubated
146 with piperacillin (0.5-4mM) or tetanus toxoid (5µg/mL, as a positive control) in culture
147 medium for 5 days. [^{3}H]thymidine was added for the final 16h of the experiment. IFN-γ, IL-
148 13, IL-17 and IL-22 secreting PBMC were visualized using ELIspot (MabTech, Nacka
149 Strand, Sweden) by culturing PBMC (5x10^5 cell/well) in culture medium with piperacillin
150 (0.5-2mM) for 48h.

151

152 **Priming of naïve T-cells from healthy volunteers**

153 CD14+ monocytes were cultured for 8 days in culture medium containing GM-CSF and IL-4
154 (800U/ml) to generate dendritic cells. On day 7, TNF-α (25ng/ml) and LPS (1µg/ml) were
155 added to induce maturation. Mature dendritic cells (0.8x10^5/well; total volume 2ml) were
156 cultured with CD3+CD25-CD45RO- naive T cells (2.5x10^6/well) and piperacillin (2mM) or
157 nitroso sulfamethoxazole (50µM; as a positive control) in a 24-well plate for 7 days. The

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158 experiment was repeated following inclusion of: (1) TGFβ (5ng/ml), IL1-β (10ng/ml) and IL-
159 23 (20ng/ml) to polarize the induction protocol towards a Th17 phenotype or TNFα
160 (50ng/ml) and IL-6 (20ng/ml) to polarize towards a Th22 phenotype and (2) ArH receptor
161 agonist (VAF347, 50nM) or ArH receptor antagonist (CH-223191, 100nM; Millipore,
162 Watford, UK).

163

164 Primed T-cells ($1x10^5$/well) were re-stimulated using dendritic cells ($4x10^3$/well) and either
165 piperacillin (0.5mM-2mM) or nitroso sulfamethoxazole (12.5-50µM). After 48h, proliferative
166 responses and cytokine release were measured using [^{3}H]thymidine and IFN-γ, IL-13, IL-17
167 or IL-22 ELIspot, respectively.

168

169 **Generation of EBV-transformed B-cells**

170 Epstein-Barr virus transformed B-cell lines were generated from PBMC and used as antigen
171 presenting cells in experiments with T-cell clones.

172

173 **Generation of drug-specific T-cell clones**

174 PBMC ($1\text{-}5x10^6$/ml) from hypersensitive patients were incubated with piperacillin (2mM) in
175 IL-2 containing medium to establish drug-responsive T-cell lines. After 14 days, T-cells were
176 serially diluted (0.3-3 cells/well), and subjected to PHA-driven expansion (5µg/ml).
177 Irradiated allogeneic PBMC ($5x10^4$/well) were added as feeder cells. After 28-42 days, clones
178 expanded to approximately $5x10^5$ cells were tested for piperacillin specificity by culturing the
179 drug (2mM; 200µl total volume) with clones ($5x10^4$ cells/well) and irradiated EBV-
180 transformed B-cells ($1x10^4$ cells/well) for 48h in triplicate cultures per experimental
181 condition. Proliferation was measured by the addition of [^{3}H]thymidine followed by

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182 scintillation counting. Clones with a stimulation index (mean cpm drug-treated wells / mean
183 cpm in control wells) of greater than 2 were expanded and analysed further.
184
185 T-cells from inflamed patient skin and healthy volunteer PBMC after priming were cloned
186 using the same procedure.
187
188 Dose-dependent proliferative responses and the profile of secreted cytokines (IFN-γ, IL-13,
189 granzyme B, Fas L, perforin, IL-17 and IL22) were measured using [^{3}H]thymidine and
190 ELIspot, respectively. The ELIspot reader accurately counts spots up to approximately 400;
191 thus, giving an upper limit to the assay. Not all T-cells in a clonal population are capable of
192 responding. There are several reasons for this: 1, TCR stimulation and co-stimulation must be
193 optimal; 2, the level/form of antigen presented by individual antigen presenting cells may not
194 be optimal; 3, some of the T cells may not interact with antigen presenting cells and 4, the
195 ability of the T-cell to respond depends on the cell being in resting stage or G_0 of the cell
196 cycle. Thus, preliminary experiments were conducted to optimize cell numbers. Eventually,
197 T-cell clones ($5x10^4$) were cultured with irradiated antigen presenting cells ($1x10^4$) and
198 piperacillin (2mM; 200µl) for 48 h to analyse cytokine secretion. Representative ELIspot
199 images showing IL-22 secretion from piperacillin-treated clones are displayed in Figure E1
200 (in the journals online repository). T-cell clones cultured in medium alone have low spot
201 counts ie below the 100 cut-off value which represents “the negative”. The 100 cut-off value
202 was calculated by taking the mean + 2 SD of control wells of 2 key cytokines (IFN-γ and
203 IL22) and granzyme B (70, 96 and 88 sfu, respectively). 95% of control values are expected
204 to lie within this band around the mean. Flow cytometry and ELISA were used to measure
205 expression of the key cytokines IFN-γ and IL-22. Cell phenotyping was performed by flow
206 cytometry. TCR Vβ expression was measured using the IOTest® Beta Mark, TCR Vβ

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207 Repertoire Kit (Beckman Coulter). Antibodies used for flow cytometry staining purchased
208 from BD Biosciences (Oxford, UK) were CD4-APC (clone RPA T4), CD8-PE (clone
209 HIT8a), CCR4-PE (clone 1G1), CLA-FITC (clone HECA-452); from eBIoscience Ltd
210 (Hatfield, UK) were IFNg-Alexa Fluor 488 (clone 4S.B3), IL-13-PE (clone 85BRD), IL-22-
211 eFluor660 (clone 22URTI), isotype controls mouse IgG1-Alexa Fluor 488, mouse IgG1-PE,
212 mouse IgG1-eFluor660 and from R&D Systems (Abingdon, UK) were CCR1-Alex Fluor 488
213 (clone 53504), CCR2-PE (clone 48607), CCR3-FITC (clone 61828), CCR5-FITC (clone
214 CTC5), CCR6-APC (Clone 53103), CCR8 –PE (clone 191704), CCR9-APC (clone 248621),
215 CCR10-PE (clone 314305), CXCR1-FITC (clone 42705), CXCR3-APC (clone 49801),
216 CXCR6-PE (clone 56811) and E cadherin-Alexa Fluor 488 (clone 180224). Approximately
217 $1x10^5$ T cell clones were stained for surface markers using directly conjugated antibodies.
218 The cells were incubated on ice for 20 min and then washed with 1ml 10% FCS in HBSS.
219 Chemokine receptor expression is presented as median fluorescence intensity of the whole
220 population of each clone and percentage of cells expressing each receptor. For intracellular
221 cytokine analysis clones ($1x10^5$) were cultured with irradiated antigen presenting cells
222 ($0.2x10^5$) and piperacillin (2mM) for 24 h. GolgiStop was added for the last 4 hours of
223 culture. Cells were then fixed and permeabilized using the BD Cytofix/Cytoperm Kit (BD
224 Biosciences, Oxford, UK) and incubated with anti-cytokine antibodies for 30 min on ice and
225 washed once. All cells were acquired using a FACSCanto II (BD Biosciences, Oxford, UK)
226 and data analyzed by Cyflogic. A minimum of 50,000 lymphocytes were acquired using
227 FSC/SSC characteristics. ELISA was conducted using human IFN-γ, IL-13 and IL-22 Ready-
228 SET-Go kits (eBioscience, Ltd) according to the manufacturer's instructions.

229

230 Chemotaxis assays were performed using 5µm-pore containing 24-well transwell plates. T-
231 cells ($1x10^5$) were added to the upper chambers and placed in medium containing different

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232 chemotactic molecules (CCL2, CCL4, CCL17, CCL25, CCL27, CXCL9, CXCL16; 100nM).
233 Time-dependent chemotactic migration was recorded using a haemocytometer between 1-
234 24h.
235

236 **Statistics**

237 All statistical analysis (One-way ANOVA unless stated otherwise) was performed using
238 SigmaPlot 12 software (*P<0.05).
239
240

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241 **Results**

242 **PBMC from piperacillin hypersensitive patients proliferate and secrete IFN-γ, IL-13**
243 **and IL-22 following drug stimulation**

244 PBMC from 4 hypersensitive patients were stimulated to proliferate with piperacillin in a
245 dose-dependent manner (0.25-4mM). At the optimal concentration of 2mM piperacillin, the
246 SI ranged from 20-62. Proliferative responses were also detected with the positive control
247 tetanus toxoid (Figure 1A). In contrast, PBMC from drug tolerant controls proliferated in the
248 presence of tetanus toxoid, but not piperacillin (SI less than 1.5; results not shown).

249

250 Cytokine secretion from piperacillin-stimulated hypersensitive patient PBMC were analysed
251 using ELIspot. Significant levels ($P<0.05$) of IFN-γ, IL-13 and IL-22 were secreted from
252 piperacillin (and PHA) treated cultures, when compared with cultures containing medium
253 alone (Figure 1B). Although PHA treatment of hypersensitive patient PBMC was associated
254 with the secretion of IL-17, piperacillin-specific IL-17 secretion was not detected.

255

256 **Piperacillin-specific CD4+ and CD8+ clones from PBMC and inflamed skin of**
257 **hypersensitive patients secrete IL-22**

258 A total of 570 clones responsive to piperacillin were generated from PBMC of the four
259 hypersensitive patients (39.5% response rate [clones with an SI of 2 or more]; Figure 1C and
260 D). The piperacillin-responsive clones were predominantly CD4+; however, drug-specific
261 CD8+ clones were isolated from each patient. Proliferative responses were concentration-
262 dependent with optimal responses detected using a concentration of 2mM piperacillin (results
263 not shown). Following expansion of the clones, 43 CD4+ and CD8+ well-growing clones,
264 randomly selected from the 4 patients, were used for analysis of cytokine secretion. Clones
265 expressed single, but varied VCR Vβ chains.

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266

267 Activation of the clones with piperacillin resulted in the secretion of IFN-γ, IL-13 and IL-22;
268 however, IL-17 was not detected (Figure 1E). Clones were also isolated from the skin of
269 hypersensitive patients 1 and 2 following a positive intradermal test. Ninety six piperacillin-
270 responsive clones were generated from a total of 690 tested (response rate, 13.9%) (Figure
271 2A and B). Analogous to the experiments with PBMC, the majority of drug-specific clones
272 were CD4+. Twenty four well-growing clones were selected for analysis of cytokine
273 secretion. Drug-treatment was associated with the release of IFN-γ, IL-13 and IL-22, but not
274 IL-17 (Figure 2C).

275

276 Figure 3A shows the levels of IFN-γ and IL-13 secreted from individual clones. Clones
277 secreting Th1 (IFN-γ^{high}, IL-13^{low}), Th2 (IFN-γ^{low}, IL-13^{high}), and Th1/2 (IFN-γ^{high}, IL-13^{high})
278 cytokines following drug stimulation were detectable with blood- and skin-derived clones.
279 However, with the skin-derived clones the response was much more polarized and clones
280 secreting low-moderate levels of cytokines were rarely seen.

281

282 Figure 3B shows the mean levels of IL-22 secretion from individual clones. Figure 3C shows
283 the level of IFN-γ and IL-13 secreted from the IL-22^{high} clones (i.e., clones forming at least
284 100 sfu above control values after piperacillin treatment). These data illustrate that (1) CD4+
285 and CD8+ clones secrete IL-22, (2) most blood-derived IL-22^{high} clones secrete IFN-γ and IL-
286 13, (3) most skin-derived IL-22^{high} clones secrete IFN-γ alone and (4) the response of the
287 skin-derived clones is highly polarized. Flow cytometry was used to measure intracellular
288 expression of IFN-γ and IL-22 with a panel of IFN-γ^{high}, IL-22^{high} and IFN-γ^{high}, IL-22^{low}
289 clones. For the IL-22^{high} clones, all cells in the culture secreted IFN-γ and IL-22, whereas the
290 IL-22^{low} clones only expressed IFN-γ (Figure E2). Protein secretion was confirmed by

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291 measuring IFN-γ and IL-22 secretion in culture supernatants using specific ELISA.
292 Piperacillin-treatment of IL-22^{high} resulted in the secretion of IFN-γ and IL-22 into culture
293 supernatant. In contrast, with the IL-22^{low} clones only IFN-γ was detected (Figure E3).
294
295 **Skin-derived piperacillin-specific clones secrete perforin, granzyme B and Fas L**
296 Clones deriving from blood (n=24) and skin (n=24) secreted high levels of granzyme B
297 (Figure 3D). However, significantly higher levels of perforin and Fas L were detected from
298 the piperacillin-treated skin-derived clones. Interestingly, piperacillin-specific CD4+ clones
299 secreted the highest levels of FasL and perforin (Figure 3E).
300
301 **Chemokine receptor expression on piperacillin-specific T-cell clones**
302 Expression of 12 chemokine receptors, CLA, CD69 and E-CAD were compared on
303 piperacillin-specific blood- and skin-derived clones (n=24-36 per group). Piperacillin-specific
304 blood and skin-derived clones expressed high levels of CD69 (Figure E4). Skin-derived
305 clones that were not activated with piperacillin were used as an additional comparator.
306 Piperacillin-specific skin and blood-derived clones were found to express multiple chemokine
307 receptors including CCR4, 5, 8, 9 and 10 and CXCR1, 3 and 6 when flow cytometry data
308 were analysed as MFI (Figure 4) or % positive cells (Figure E5) Skin-derived piperacillin-
309 specific clones expressed significantly higher levels of CCR1 and CLA, compared with non-
310 specific clones. Blood-derived piperacillin-specific clones expressed higher levels of CCR9
311 when compared with the skin-derived piperacillin-specific and non-specific clones. In
312 contrast, CCR2, CXCR1, and CLA were expressed at higher levels on the piperacillin-
313 specific skin-derived clones, when the two populations of piperacillin-specific clones were
314 compared. (Figure 4; Figure E5). Representative dot plot images showing the panel of
315 receptors expressed and analysed in terms of MFI and % positive cells are shown in Figure

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316 E6. Finally, it was possible to use quadrant analysis too obtain data from double-positive
317 populations for chemokine receptors CXCR1 and CXCR 3, and CCR8 and CCR10. The
318 representative traces shown in Figure E7 illustrate that the majority of piperacillin-specific T-
319 cells express both chemokine receptors.

320

321 No significant correlation between chemokine receptor expression and IL-22 secretion by
322 piperacillin-stimulated clones was observed (Figure E8). However, skin- and blood-derived
323 IL-22 secreting clones had a tendency to express CCR4 and lower levels of CCR4,
324 respectively.

325

326 All clones were found to migrate across transwell membranes, in a time-dependent manner,
327 in the presence of the CCR4 and CCR10 ligands, CCL17 and CCL27, respectively (Figure 5).
328 Furthermore, the blood-derived clones, which expressed significantly higher levels of CCR9,
329 migrated in the presence of CCL25, a ligand for CCR9. Interestingly, all clones migrated to a
330 similar extent, which suggests that threshold levels of chemokine receptor expression
331 determine whether a clone will migrate in the presence of the relevant ligand.

332

333 **Priming of naïve T-cells against piperacillin generates antigen-specific Th22 secreting**
334 **clones**

335 Naïve T-cells from four volunteers were cultured in the presence of autologous monocyte-
336 derived dendritic cells (CD11a^{high} CD11c^{high} CD14^{neg} CD80^{high} CD86^{high} MHC class II^{high})
337 and piperacillin for 8 days. Primed T-cells were then cultured with a second batch of
338 dendritic cells and the drug and antigen specificity was assessed. Upon restimulation,
339 piperacillin concentration-dependent proliferative responses were clearly detectable (Figure
340 6A). The strength of the induced response was similar to that seen with cells from

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341 hypersensitive patients. Moreover, piperacillin-primed cells were not activated with the
342 control antigen nitroso sulfamethoxaozle. Similarly, nitroso sulfamethoxaozle-primed T-cells
343 were not activated with piperacillin (results not shown).
344
345 Piperacillin stimulation of the primed cells resulted in the secretion of IFN-γ, IL-13 and IL-
346 22, but IL-17 was not detected (Figure 6B). The priming experiment was repeated in the
347 presence of cytokine cocktails known to polarize T-cells towards Th17 and Th22 subsets. No
348 qualitative difference in the profile of cytokines secreted from piperacillin-specific T-cells
349 was observed. In particular, IL-17 was not detected (results not shown).
350
351 Sixty-four piperacillin-responsive clones were generated from the 4 volunteers, out of a total
352 of 526 tested (response rate: 12.2%) (Figure 6C and 6D). Piperacillin-specific CD4+ and
353 CD8+ clones were detected; however, in contrast to clones from hypersensitive patients, most
354 expressed CD8+. Thirty seven well-growing clones were tested and found to secrete IFN-γ,
355 IL-13 and IL-22, but not IL-17, following treatment with piperacillin (Figure 6E).
356
357 Analysis of the profile of cytokines secreted from individual clones revealed that most CD4+
358 and CD8+ clones secreted high levels of IFN-γ (Figure 7A). The few clones that secreted
359 high levels of IL-22 were all CD8+ and did not secrete other cytokines (i.e., IFN-γ, IL-13 and
360 IL-17) (Figure 7B-D).
361
362 **Signalling through the aryl hydrocarbon receptor is critical for the generation of**
363 **piperacillin-specific IL-22 secreting T-cells**
364 To investigate whether the generation of piperacillin-specific IL-22 secreting T-cells is
365 regulated through the ArH receptor, naïve T-cell priming was studied in the presence of an

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366 ArH receptor agonist or antagonist. Restimulation of T-cells primed in the presence of the
367 ArH receptor agonist resulted in the secretion of IFN-γ, IL-13, IL-22, perforin, granzyme B
368 and Fas L. T-cells primed in the presence of the ArH receptor antagonist secreted IFN-γ, IL-
369 13, perforin, granzyme B and Fas L following restimulation with piperacillin; however, IL-22
370 secretion was no longer detected (Figure 8A and 8B).
371

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372 **Discussion**

373 To develop an effective classification of drug hypersensitivity, one must identify factors that
374 contribute to and discriminate between the spectrum of diseases that fall under that definition.
375 Classifying by drug is not effective since most that are associated with a high frequency of
376 reactions cause a range of adverse events. Similarly, classifying by HLA risk alleles doesn't
377 work as the same MHC molecule might predispose individuals to different forms of
378 hypersensitivity reaction (e.g., HLA-B*57:01; abacavir hypersensitivity & flucloxacillin-
379 induced liver injury).[23] The existing classification of delayed-type drug hypersensitivity
380 categorizes reactions based on the phenotype and function of drug-specific T-cells;[5] however,
381 new T-cell subsets have never been considered. With this in mind, the current study focussed
382 on IL-17 and IL-22 as these cytokines are now believed to be important mediators of allergic
383 reactions in the skin,[16,24] but have not been studied extensively in the context of drug
384 hypersensitivity. Piperacillin was selected as the study drug as β-lactam hypersensitivity is
385 the most common form of allergic drug reaction. To obtain a detailed assessment of the drug-
386 specific response, T-cells were cloned from blood and inflamed skin of the same
387 hypersensitive patients. Furthermore, naïve T-cells from healthy volunteers were primed
388 against piperacillin to study factors that govern the development of a drug-specific T-cell
389 response.

390

391 The lymphocyte transformation test and PBMC ELIspot are used routinely for
392 hypersensitivity diagnosis and to characterize the culprit drug.[25-26] In agreement with our
393 previous studies,[20,22] PBMC from hypersensitive patients, but not tolerant controls, were
394 stimulated in the presence of piperacillin to proliferate and secrete the Th1/2 cytokines IFN-γ
395 and IL-13. Moreover, PBMC from hypersensitive patients secreted high levels of IL-22,
396 while IL-17 was not detected. IL-17 secretion was, however, detected from mitogen-

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397 stimulated PBMC. Thus, its absence from drug-treated PBMC suggests that piperacillin-
398 specific IL-17 secreting T-cells do not circulate in the peripheral blood of hypersensitive
399 patients. The failure to detect piperacillin-specific responses from the tolerant patient group
400 (and drug-naïve donors) may relate to differences in the abundance of precursor cells at the
401 time of drug exposure. Future studies should attempt to quantify the difference using
402 established culture methods.[27].

403

404 To determine the source of the secreted cytokines, over 500 piperacillin-specific T-cell clones
405 were generated; drug treatment was associated with the secretion of Th1 (IFN-γ^{high}), Th2 (IL-
406 13^{high}) or a mixed phenotype panel of cytokines (IFN-γ^{high}, IL-13^{high}). Given that human skin
407 is protected by skin-resident and recirculating T-cells with distinct functional activity[18] it was
408 important to compare the piperacillin-specific blood-derived T-cells with T-cells isolated
409 from inflamed skin. These comparative studies gained further credence when Gaide *et al*[28]
410 demonstrated, using a mouse model of contact dermatitis, that skin resident and central
411 memory T-cells derive from a common origin. The authors reported that skin resident cells
412 responded rapidly following antigen challenge, whereas central memory cells were recruited
413 to the tissue more slowly to mediate a delayed response. Similar to the blood-derived clones,
414 approximately 85% of piperacillin-responsive clones isolated from inflamed skin were CD4+
415 and drug treatment resulted in the secretion of the same cytokines, namely IFN-γ, IL-13 and
416 IL-22, but not IL-17. Most of the skin-derived clones secreting IL-22 were of the Th1
417 phenotype. IL-22 binds to receptors expressed on cells such as keratinocytes to mediate
418 innate responses in skin. Although IL-22 has been described as a protective cytokine, it also
419 promotes pathogenic responses when secreted, as we have found, in the presence of other
420 cytokines.[29] For example, CD4+ and CD8+ T-cells that secrete IFN-γ and IL-22 are
421 implicated in the pathogenesis of chronic skin conditions such as psoriasis.[30] Collectively, our

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422 data shows that IL-22 signalling participates in maculopapular drug eruptions; however,
423 additional studies are required to delineate whether IL-22 is acting to promote disease
424 progression or alternatively regulate the severity of tissue injury.
425
426 Fas L, perforin and granzyme B are T-cell secretory molecules that act in unison to induce
427 apoptosis in tissue cells. Early studies by Posadas et al[31] reported upregulated levels of
428 perforin and granzyme B in PBMC isolated from patients with acute mild and severe forms
429 of drug-induced skin injury. Moreover, a strong correlation was observed between the level
430 of cytolytic molecules and severity of the disease. In contrast, Fas L was only observed in
431 patients with Stevens Johnson syndrome and toxic epidermal necrolysis. More recently,
432 Zawodiniak et al[32] utilized a granzyme B ELIspot to detect cytotoxic T-cells in blood of
433 patients with various forms of drug-induced skin injury. Accordingly, we utilized drug-
434 specific clones isolated from blood and skin of the same hypersensitive patients to measure
435 and compare Fas L, perforin and granzyme B secretion. Blood and skin-derived clones
436 secreted high levels of granzyme B when activated with piperacillin, which to some extent
437 explains the findings of Zawodiniak et al.[32] In contrast, perforin and Fas L secretion was
438 largely restricted to the skin-derived clones. These data show that (1) drug-specific skin
439 resident T-cells are the most likely mediators of tissue injury and (2) analysis of blood-
440 derived T-cells alone underestimates the importance of cytolytic molecules in the disease
441 pathogenesis.
442
443 A subset of T-cells expressing the skin homing chemokine receptors CCR4 and CCR10 have
444 previously been shown to secrete IL-22 and hence are thought to be important mediators of
445 skin pathophysiology.[33] Furthermore, ligands for CCR4 (CCL17) and CCR10 (CCL27),
446 which contribute towards the recruitment of IL-22 secreting cells, have been found at high

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447 levels in the lesional skin of patients with atopic dermatitis.[34,35] Thus, the next component of
448 our study was to characterize expression of chemokine receptors on blood- and skin-derived
449 piperacillin-specific IL-22 secreting clones and to measure migratory potential of the clones
450 towards relevant chemokines. Blood and skin-derived CD4+ and CD8+ clones were found to
451 express multiple chemokine receptors including CCR4, 5, 8, 9 and 10 and CXCR1, 3 and 6.
452 Interestingly, CLA was expressed in higher levels on the skin-derived clones. Comparison of
453 chemokine receptor expression and IL-22 secretion revealed that skin-derived IL-22 secreting
454 clones had a tendency to express higher levels of CCR4 and the opposite was true for blood-
455 derived clones. Most importantly, both skin- and blood-derived clones migrated towards
456 CCL17 and CCL27 (i.e., ligands for the 2 chemokine receptors). Interestingly, the blood
457 derived clones expressed high levels of CCR9, a receptor more traditionally associated with
458 homing towards the gastrointestinal tract,[36] and migrated in the presence of the CCR9 ligand,
459 CCL25, which suggests that drug-specific T-cells in blood have the capacity to migrate to
460 different locations around the body.
461
462 Cell culture platforms have been developed in recent years to study the priming of naïve T-
463 cells against chemical and drug antigens.[19,37,38] In these assays, naïve T-cells from healthy
464 donors are cultured with autologous dendritic cells and the chemical/drug for 7-14 days. The
465 newly primed T-cells are then restimulated with a second batch of dendritic cells and a range
466 of compounds to assess antigen specificity. We utilized these methods to characterize the
467 panel of cytokines secreted by naïve T-cells (from 4 healthy volunteers) primed against
468 piperacillin. Following restimulation, the piperacillin-primed cells proliferated vigorously and
469 secreted IFN-γ, IL-13 and IL-22. Importantly, IL-22 was seen in the absence of polarizing
470 cytokines such as IL-6 and TNF-α, which promote the differentiation of naive T-cells into IL-
471 22 secreting effectors.[33] T-cells cloned from the piperacillin-primed naïve T-cells were

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472 skewed towards a CD8+ phenotype and drug stimulation resulted in the secretion of high
473 levels of IFN-γ and low-moderate levels of IL-13 and IL-22. Analysis of individual clones
474 revealed that the three clones secreting high levels of IL-22 were CD8+. They did not secrete
475 Th1 or Th2 cytokines. Antigen-specific T-cells with this phenotype are rarely reported in the
476 literature[16,39] and their role in allergic disease needs to be investigated further. The reason
477 why CD8+ clones were detected in a high frequency from healthy volunteers is not clear, but
478 possibly relates to (1) differences in drug concentration during priming or (2) a different
479 mechanism of drug presentation as we have recently reported with the β-lactam
480 flucloxacillin.[11,40]
481
482 The transcription factor ArH recptor regulates the differentiation of naïve T-cells into IL-22
483 secreting cells. Selective ArH receptor antagonists have been shown to perturb the production
484 of IL-22 secreting cells from naïve precursors, whereas memory T-cells are refractory to ArH
485 receptor regulation.[41,42] In the final component of the project, naïve T-cells were primed
486 against piperacillin in the presence of an ArH receptor agonist (VAF347) and antagonist
487 (CH-223191). T-cells primed to piperacillin in the presence of VAF347 secreted IFN-γ, IL-13
488 and IL-22 alongside cytolytic molecules perforin, granzyme B and Fas L following
489 restimulation with the drug. In parallel experiments with CH-223191, the differentiation of
490 naïve T-cells into piperacillin-specific IL-22 secreting cells was blocked, while all other
491 secretory molecules were detected at essentially the same level. Thus, ArH receptor
492 signalling is critical for the generation of drug-specific IL-22 secreting T-cells.
493
494 In conclusion, our study shows that circulating and skin-resident CD4+ and CD8+ T-cells
495 that secrete IL-22, but not IL-17, alongside cytolytic molecules are important mediators of β-
496 lactam hypersensitivity in humans. Differentiation of naïve T-cells into drug-specific IL-22

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497 secreting cells is dependent on ArH receptor signalling. The reason why β-lactam
498 hypersensitivity reactions are so common in patients with cystic fibrosis might relate to
499 clinical practice: drugs are administered at high intravenous concentrations for a long
500 duration and on a repeated basis. Moreover, enhanced risk might relate to the inflammatory
501 response in patients with cystic fibrosis. The lungs of a patient with cystic fibrosis exist in a
502 chronic acute responsive state with high numbers of neutrophils and cytokines such as IL-8,
503 IL-1β, IL-6, and TNF-α. In fact, the local cytokine environment might polarize the drug-
504 specific T-cell response towards the Th22 profile identified herein.

505

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508 to donate blood and tissue samples.

509

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632 **Figure legends**

633 **Figure 1. PBMC and T-cell clones from blood of hypersensitive patients proliferate and**
634 **secrete IFN-γ, IL-13 and IL-22 when stimulated with piperacillin.** (A) PBMC from
635 hypersensitive patients were cultured with piperacillin for 5 days at 37°C. [^{3}H]thymidine was
636 added for the final 16h to measure PBMC proliferation. (B) PBMC from hypersensitive
637 patients were cultured with piperacillin for 2 days at 37°C. Cytokine secretion was visualized
638 by ELIspot. Images from 2 representative patients are shown. (C) Table shows the number of
639 piperacillin-responsive T-cell clones generated from blood of hypersensitive patients and
640 their CD phenotype. (D) T-cell clones were cultured with irradiated antigen presenting cells
641 and piperacillin and proliferative responses were measured. (E) T-cell clones were cultured
642 with irradiated antigen presenting cells and piperacillin and cytokine secretion was
643 quantified..

644

645 **Figure 2. T-cell clones from inflamed skin of hypersensitive patients proliferate and**
646 **secrete IFN-γ, IL-13 and IL-22 when stimulated with piperacillin.** (A) Table shows the
647 number of piperacillin-responsive T-cell clones generated from skin of hypersensitive
648 patients and their phenotype. (B) T-cell clones were cultured with irradiated antigen
649 presenting cells and piperacillin and proliferative responses were measured. (C) T-cell clones
650 were cultured with irradiated antigen presenting cells and piperacillin and cytokine secretion
651 was quantified.

652

653 **Figure 3. Profile of cytokine and cytolytic molecules secreted from piperacillin-specific**
654 **T-cell clones derived from blood and inflamed skin of hypersensitive patients.** T-cell
655 clones were cultured with irradiated antigen presenting cells and piperacillin and secretions
656 were quantified. (A) Comparison of IFN-γ and IL-13 secreted by individual blood- and skin-

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657 derived T-cell clones. Filled circles depict CD8+ clones. (B) Mean ± SD IL-22 secretion from
658 Th1, Th2 and Th1/2 clones. (C) Comparison of the level of IFN-γ and IL-13 secreted by
659 individual IL-22h^{high} blood- and skin-derived T-cell clones. (D) Cytolytic molecules secreted
660 by piperacillin-specific clones. (E) Comparison of the level of perforin and Fas L secreted by
661 individual blood- and skin-derived T-cell clones.
662
663 **Figure 4. Chemokine receptor expression on piperacillin-specific T-cell clones derived**
664 **from blood and inflamed skin of hypersensitive patients.** Piperacillin-specific T-cell
665 clones deriving from blood and skin of hypersensitive patients were analysed for chemokine
666 receptor expression by flow cytometry. The box plot shows the median and 25th/75th
667 percentiles, with error bars showing the 10th/90th percentiles.
668
669 **Figure 5. Migration of piperacillin-specific T-cell clones derived from blood and**
670 **inflamed skin of hypersensitive patients towards chemokines.** Time-dependent
671 chemotactic migration was recorded by counting using a haemocytometer between 0.5-24h.
672 Each line shows the response of an individual clone.
673
674 **Figure 6. Piperacillin-specific priming of naïve T-cells from healthy volunteers.** (A)
675 Drug-specific T-cell proliferative responses were measured by [^{3}H]thymidine uptake. The
676 data show mean ± SD of triplicate cultures. (B) Antigen-specific T-cell responses measured
677 by IFN-γ, IL-13, IL-17 and IL-22 ELIspot. (C) Table shows the number of piperacillin-
678 responsive T-cell clones generated from blood of healthy volunteers following priming and
679 their CD phenotype. (D) T-cell clones were cultured with irradiated antigen presenting cells
680 and piperacillin and proliferative responses were measured. (E) T-cell clones were cultured

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681 with irradiated antigen presenting cells and piperacillin (2mM) and cytokine secretion was
682 quantified.
683
684 **Figure 7. Profile of cytokines secreted from piperacillin-specific T-cell clones derived**
685 **from healthy volunteers.** T-cell clones were cultured with irradiated antigen presenting cells
686 and piperacillin and cytokine secretion was quantified. (A) Comparison of IFN-γ and IL-13
687 secreted by individual T-cell clones. Filled circles depict CD8+ clones. (B) Mean ± SD IL-22
688 secretion from Th1, Th2 and Th1/2 clones. (C) Comparison of IFN-γ/IL-22 and IL-13/IL-22
689 secretion by T-cell clones.
690
691 **Figure 8. Piperacillin-specific priming of naïve T-cells from healthy volunteers in the**
692 **presence of an AhR agonist and antagonist.** Antigen-specific T-cell responses were
693 measured by IFN-γ, IL-13, IL-17, IL-22, granzyme B, perforin and Fas L ELIspot. (A)
694 Piperacillin concentration-dependent secretion of cytokines/cytolytic molecules. (B) Images
695 from one representative donor.
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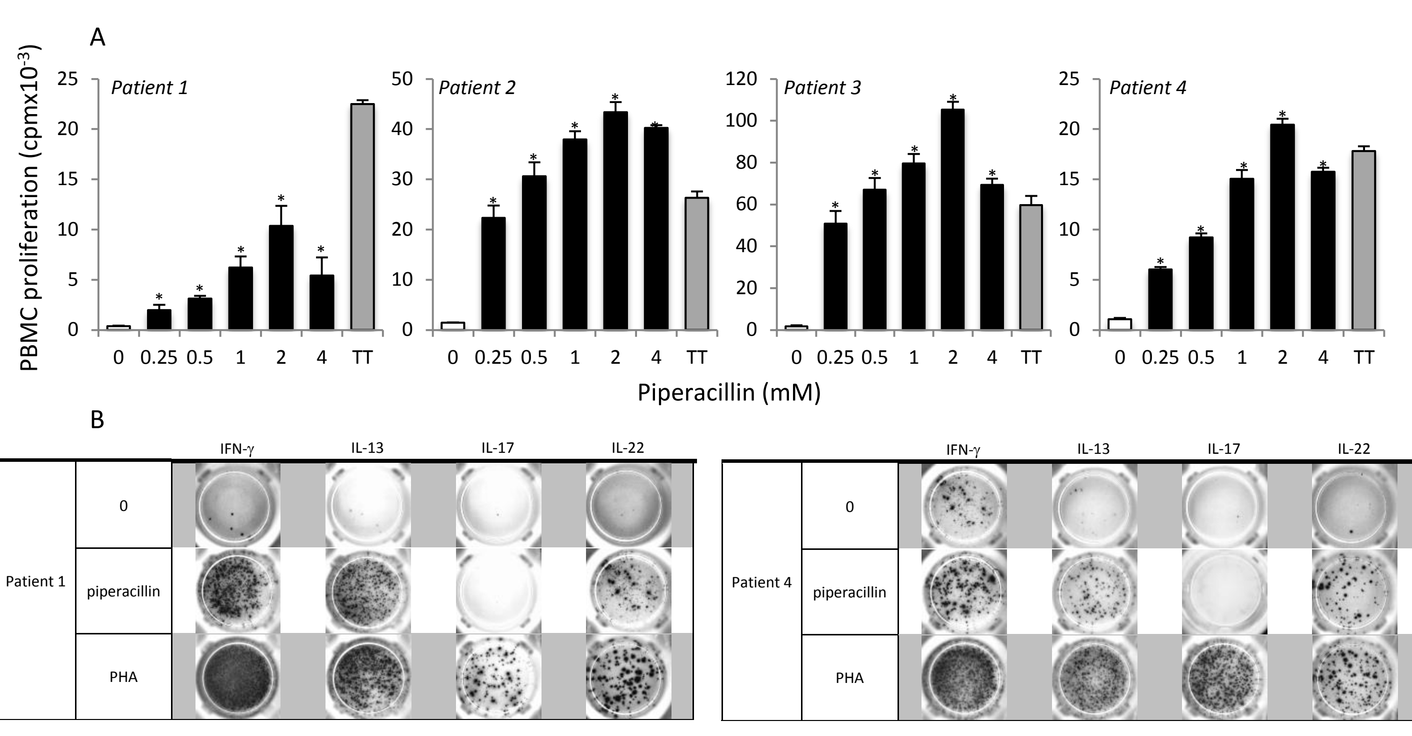
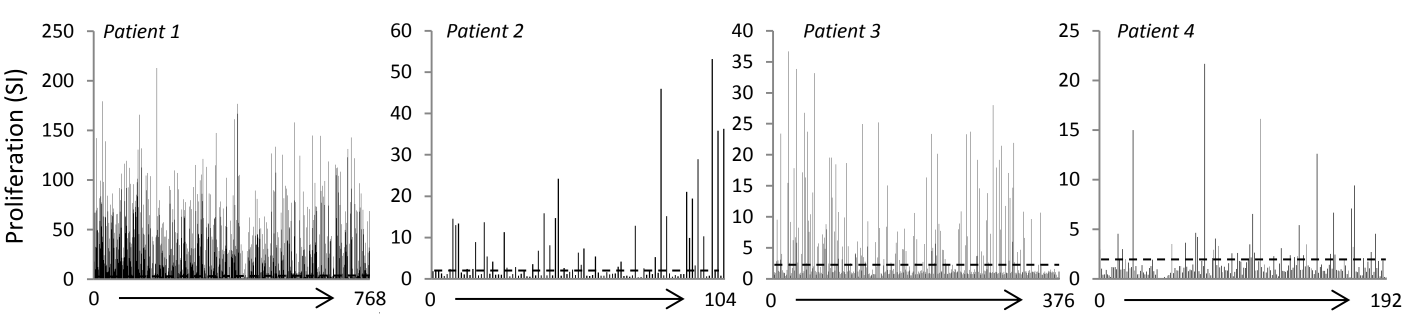
Figure 1



C

ID	Origin	# Tested	# Specific	% CD4+	% CD8+
Donor 1	Blood	768	361	87	13
Donor 2	Blood	104	34	84	16
Donor 3	Blood	376	134	89	11
Donor 4	Blood	192	41	77	23



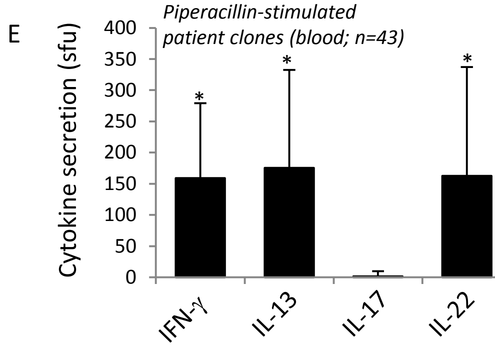
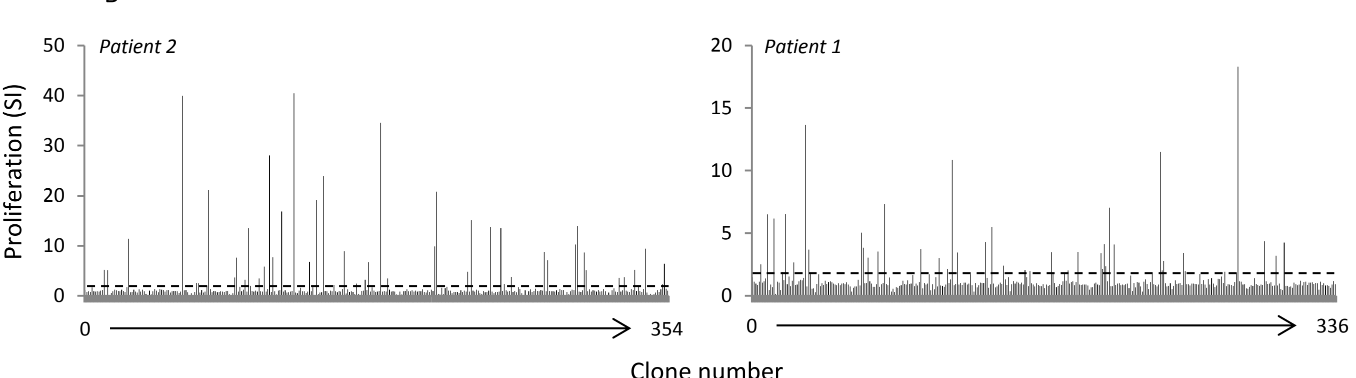


Figure 2

A

ID	Origin	# Tested	# Specific	% CD4+	% CD8+
Donor 1	Skin	354	48	89	11
Donor 2	Skin	336	48	82	18

B



C

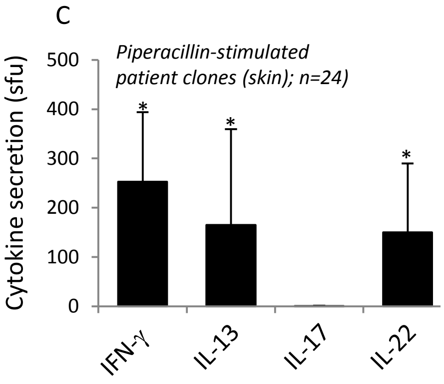
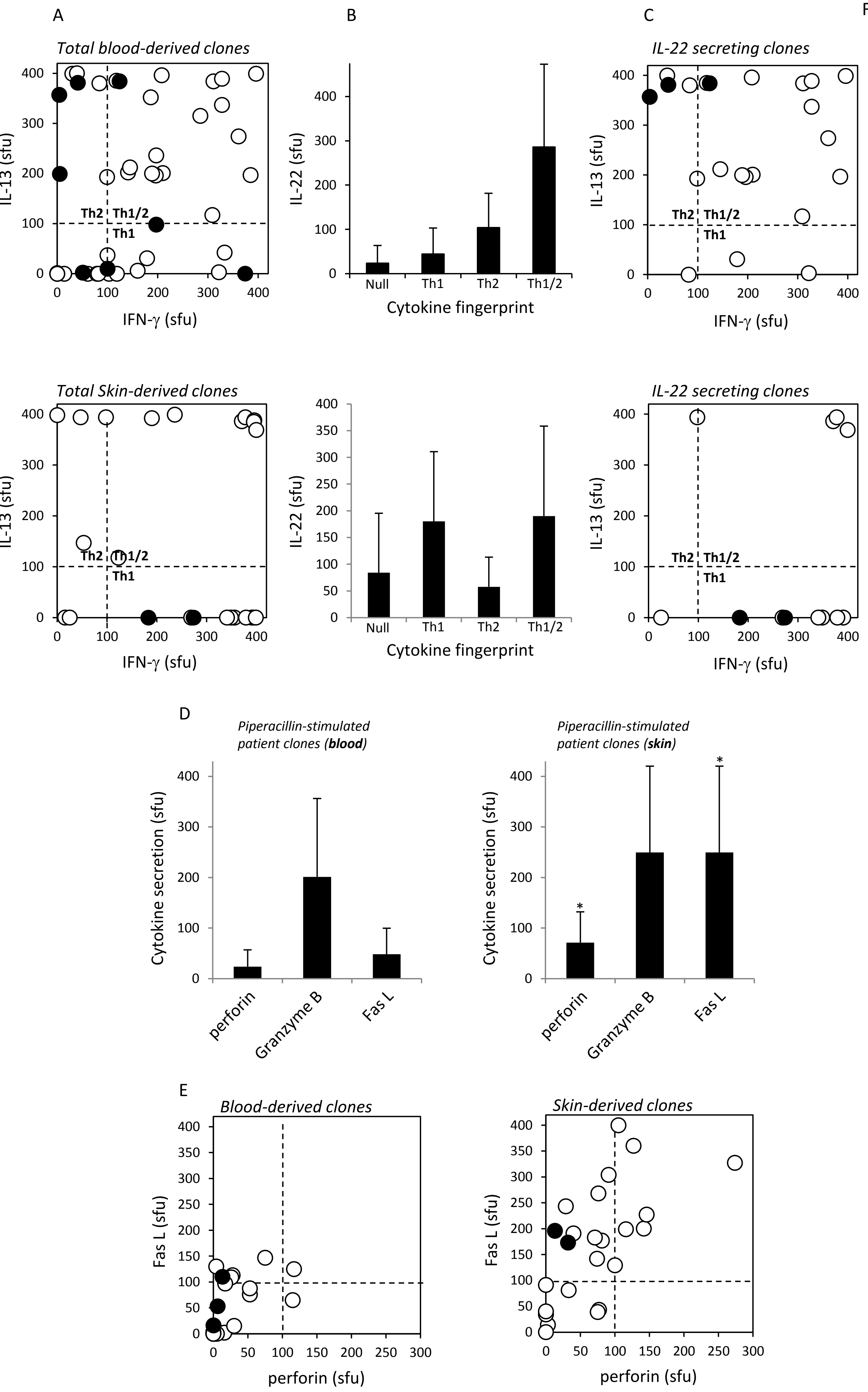
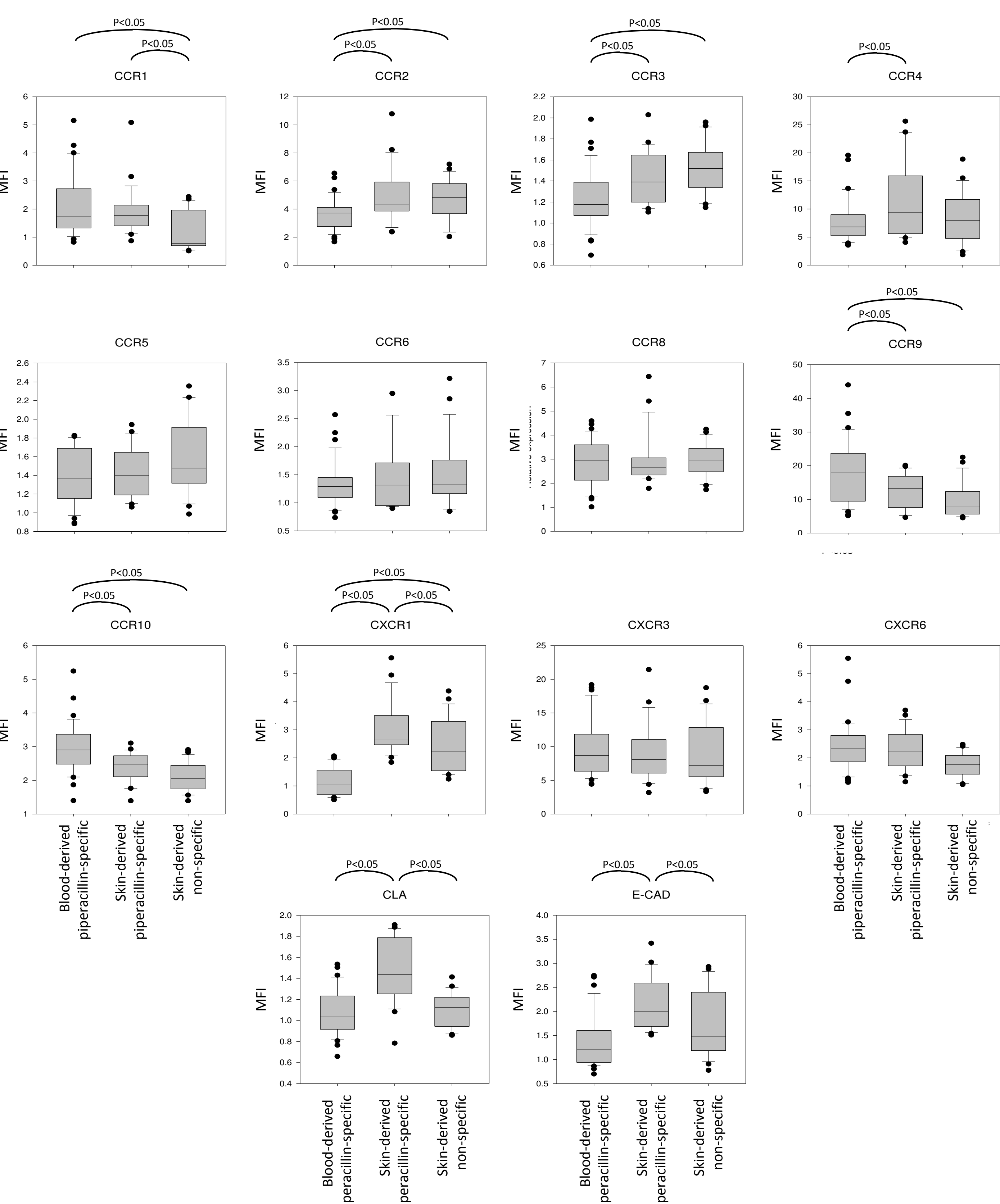
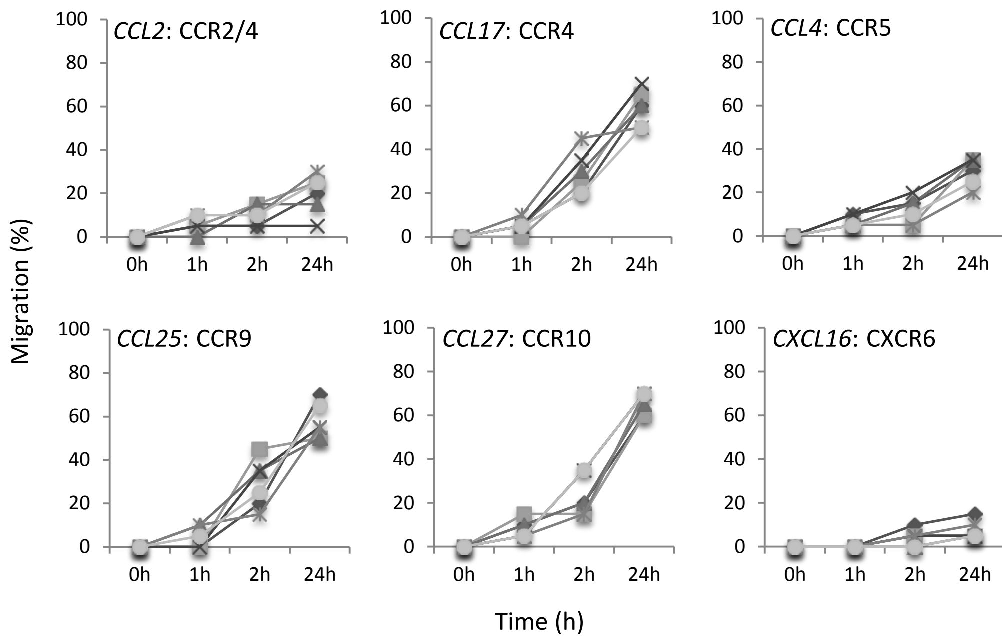


Figure 3




CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR8
CCR9
CCR10
CXCR1
CXCR3
CXCR6
CLA
E-CAD
P<0.05
MFI
Blood-derived piperacillin-specific
Skin-derived piperacillin-specific
Skin-derived non-specific



B

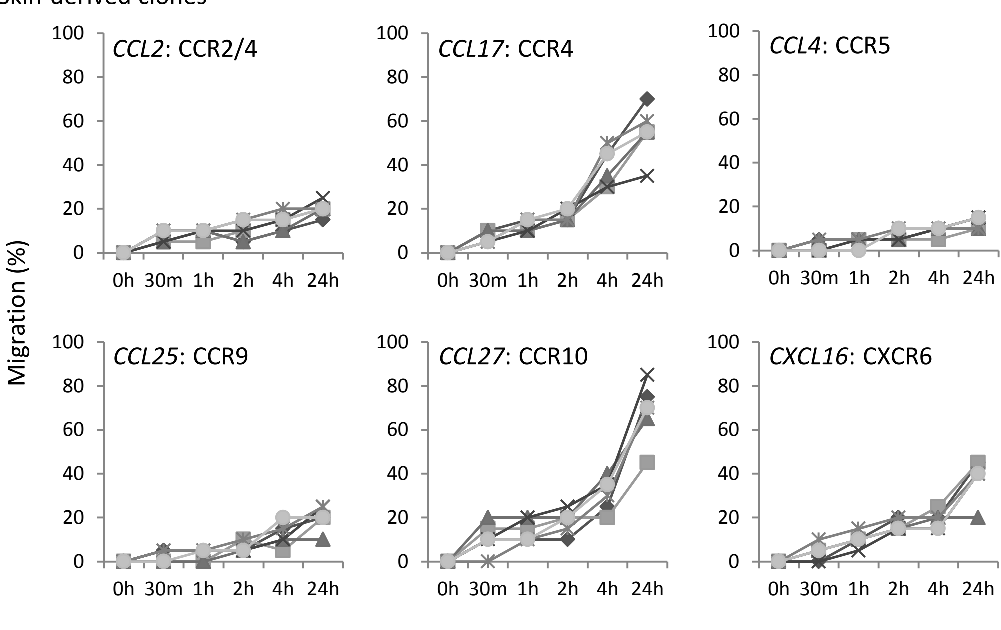


Figure 6

A

Donor A, Donor B, Donor C, Donor D

Proliferation of primed T-cells (cpmx10^{-3})

0 | 0.5 | 1 | 2 | 2 (no T-cells)

Piperacillin (mM)

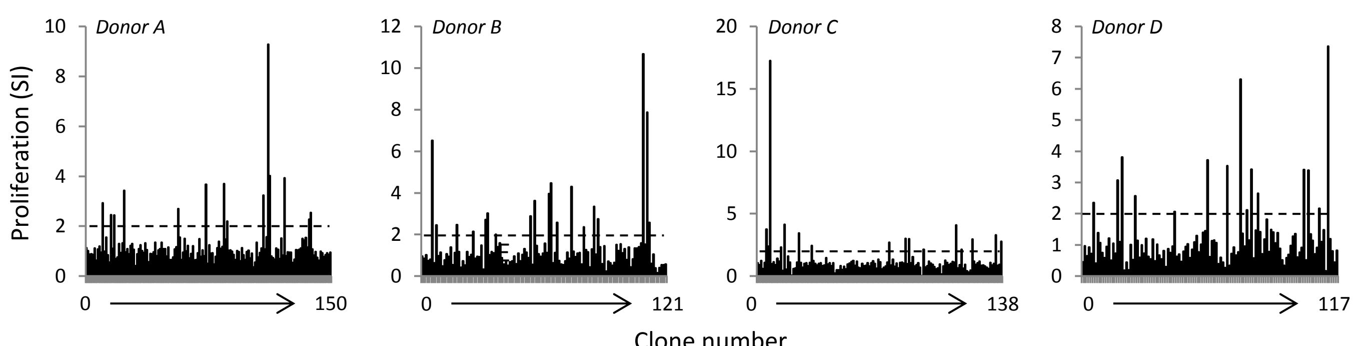
B

Piperacillin (mM)	IFN-γ	IL-13	IL-17	IL-22
0				
1				
2				
2 (with no T-cells)				

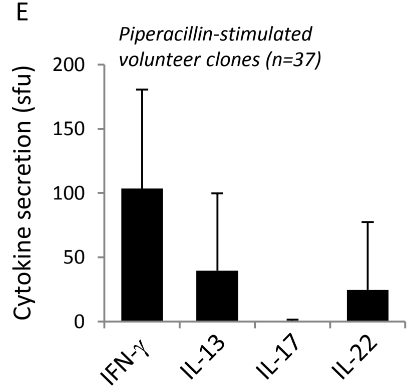
C

ID	Origin	# Tested	# Specific	% CD4+	% CD8+
Donor A	Naïve T-cells	150	14	36	64
Donor B	Naïve T-cells	121	19	37	63
Donor C	Naïve T-cells	138	16	6	94
Donor D	Naïve T-cells	117	15	13	87

D



E



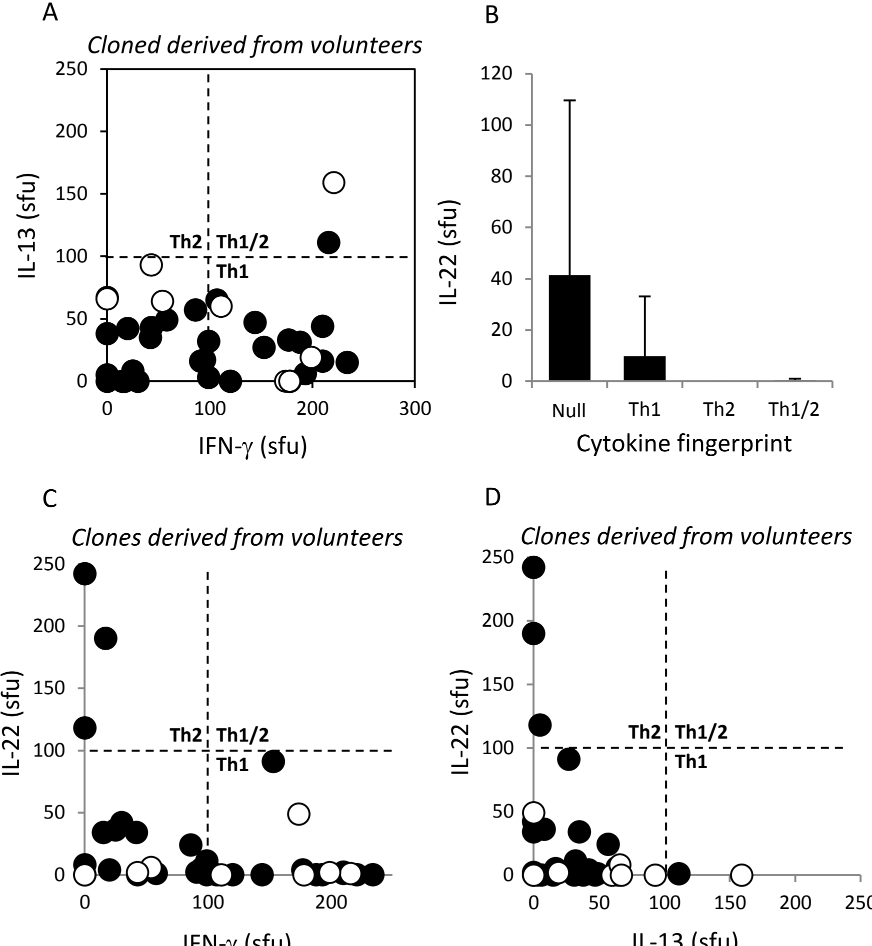
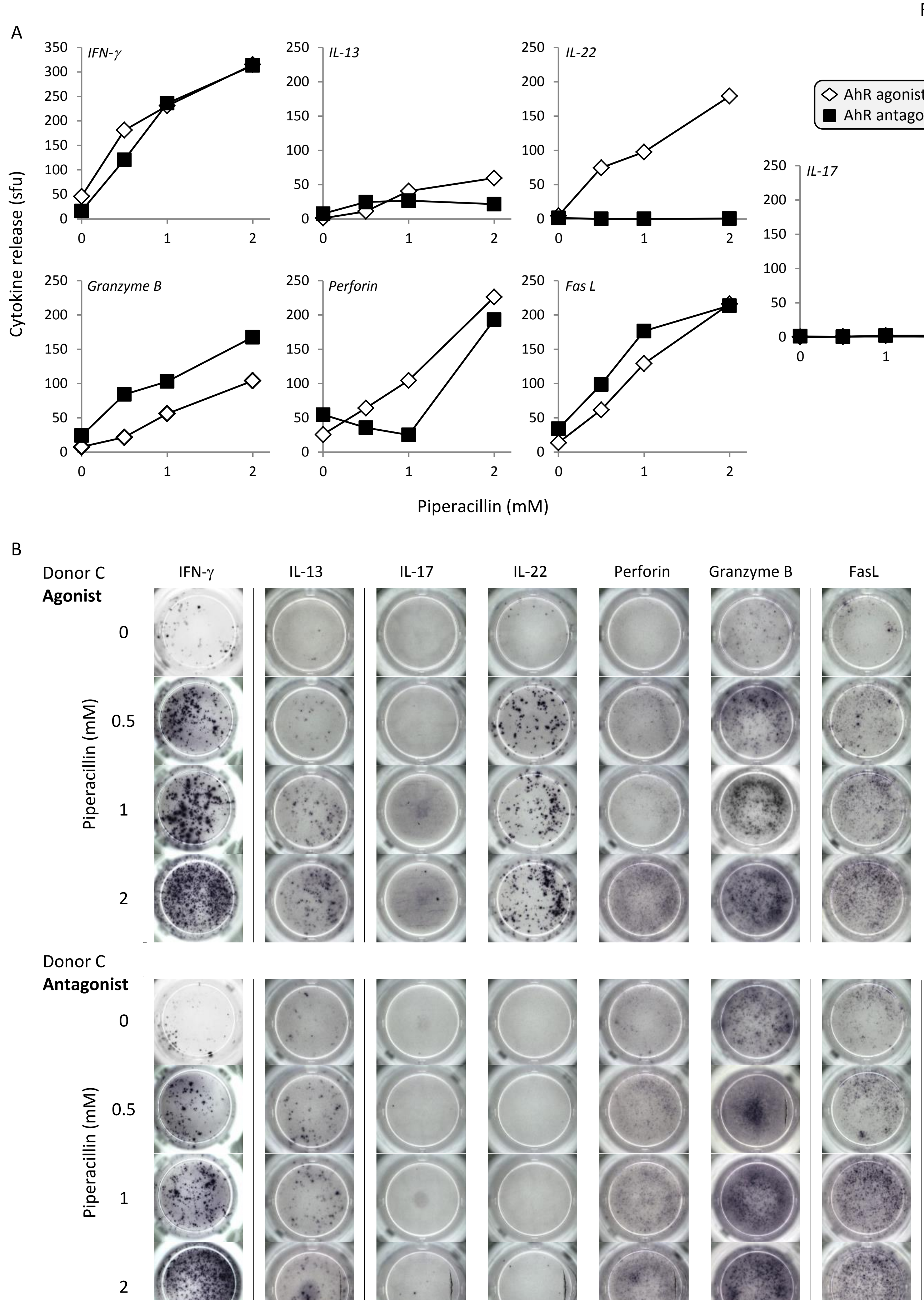
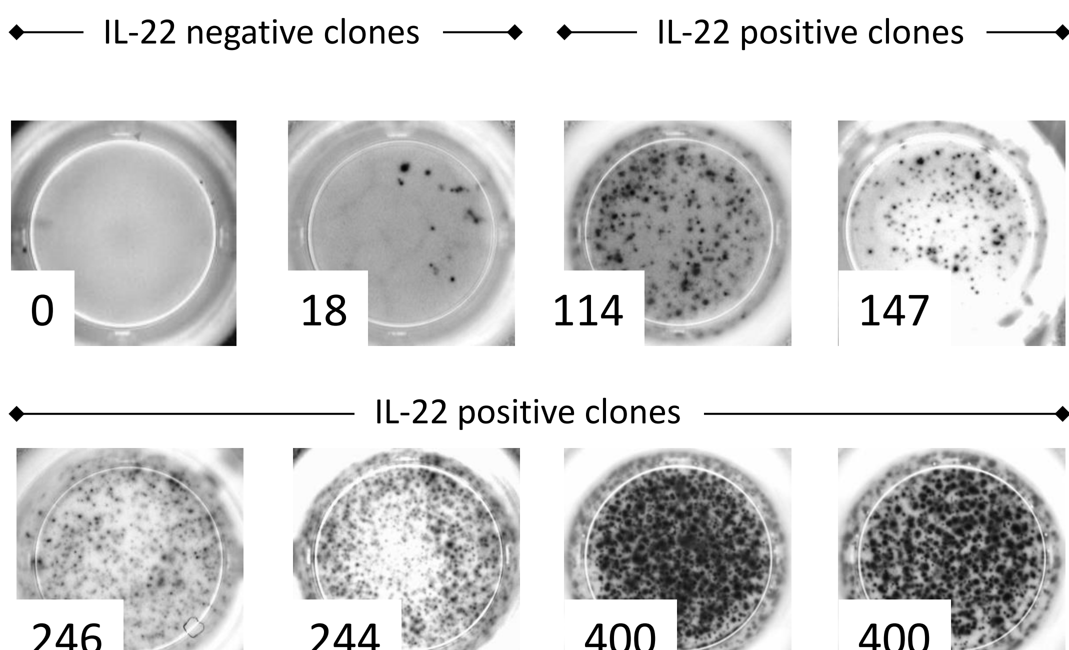

A
Cloned derived from volunteers
IL-13 (sfu)
IFN-γ (sfu)
Th2
Th1/2
Th1
250
200
150
100
50
0
0
100
200
300
B
IL-22 (sfu)
120
100
80
60
40
20
0
Null
Th1
Th2
Th1/2
Cytokine fingerprint
C
Clones derived from volunteers
IL-22 (sfu)
IFN-γ (sfu)
Th2
Th1/2
Th1
250
200
150
100
50
0
0
100
200
D
Clones derived from volunteers
IL-22 (sfu)
IL-13 (sfu)
Th2
Th1/2
Th1
250
200
150
100
50
0
0
50
100
150
200
250

Figure 8




IL-22 negative clones
IL-22 positive clones
0
18
114
147
IL-22 positive clones
246
244
400
400

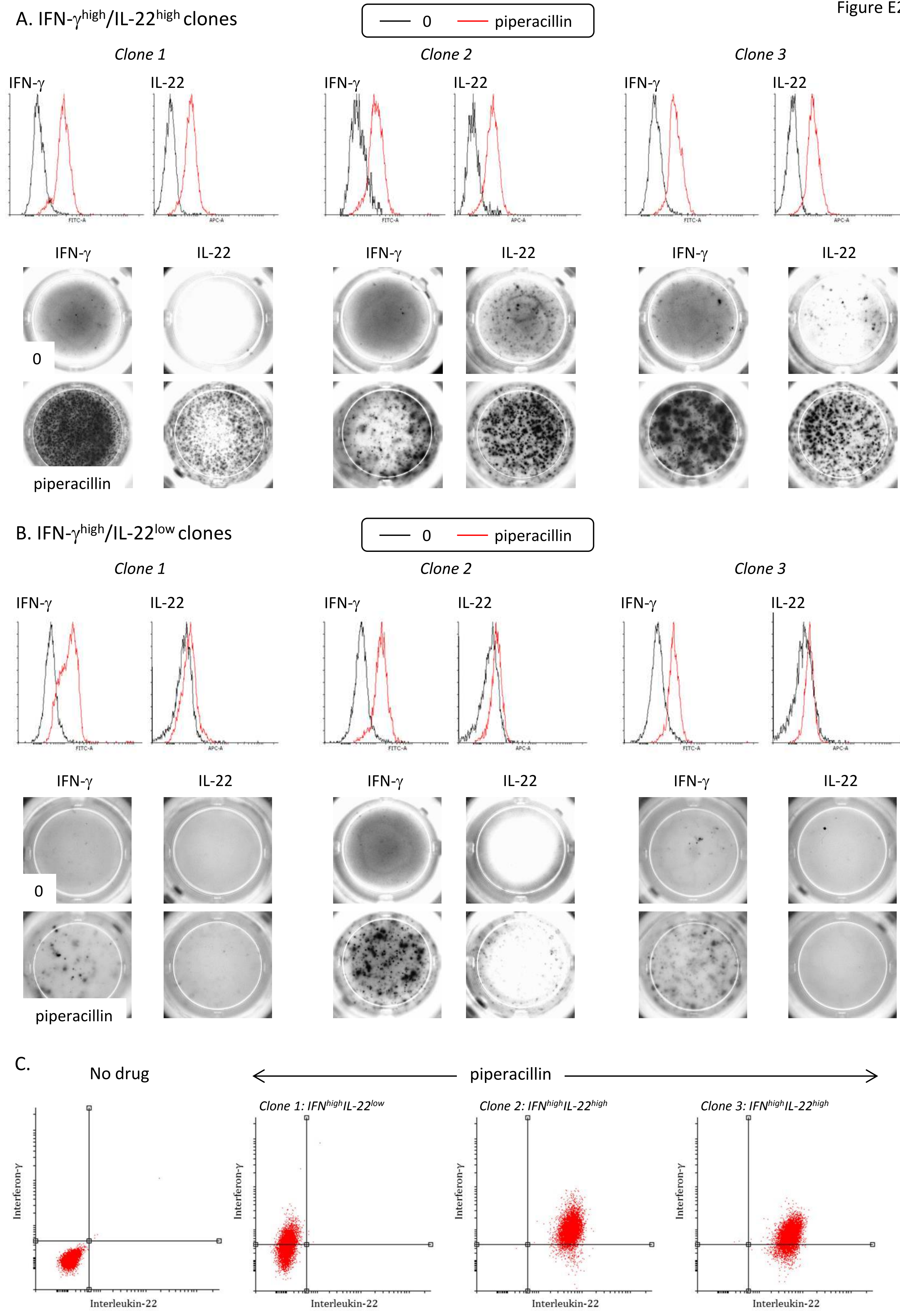
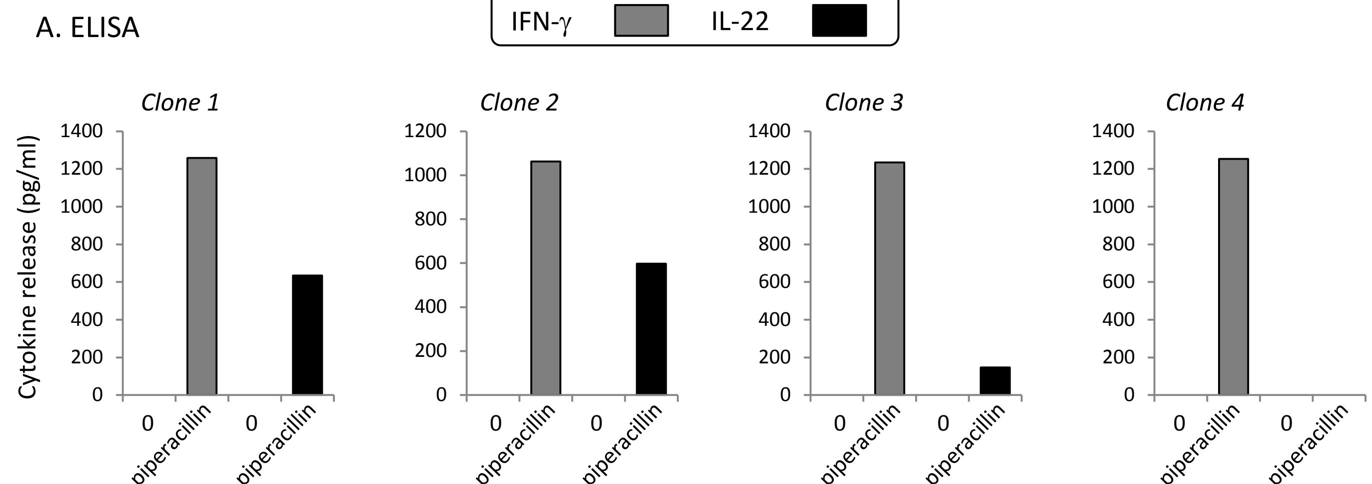


Figure E2
A. IFN-γ^high/IL-22^high clones
0
piperacillin
Clone 1
Clone 2
Clone 3
IFN-γ
IL-22
FITC-A
APC-A
0
piperacillin
B. IFN-γ^high/IL-22^low clones
C.
No drug
piperacillin
Clone 1: IFN^high IL-22^low
Clone 2: IFN^high IL-22^high
Clone 3: IFN^high IL-22^high
Interferon-γ
Interleukin-22

Figure E3

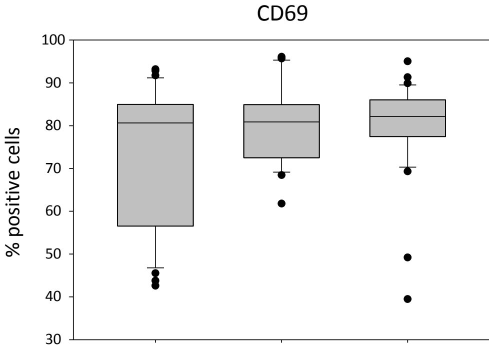
A. ELISA



B. ELIspot

		Clone 1	Clone 2	Clone 3	Clone 4
IFN-γ	Control				
	Drug				
IL-22	Control				
	Drug				

Figure E4



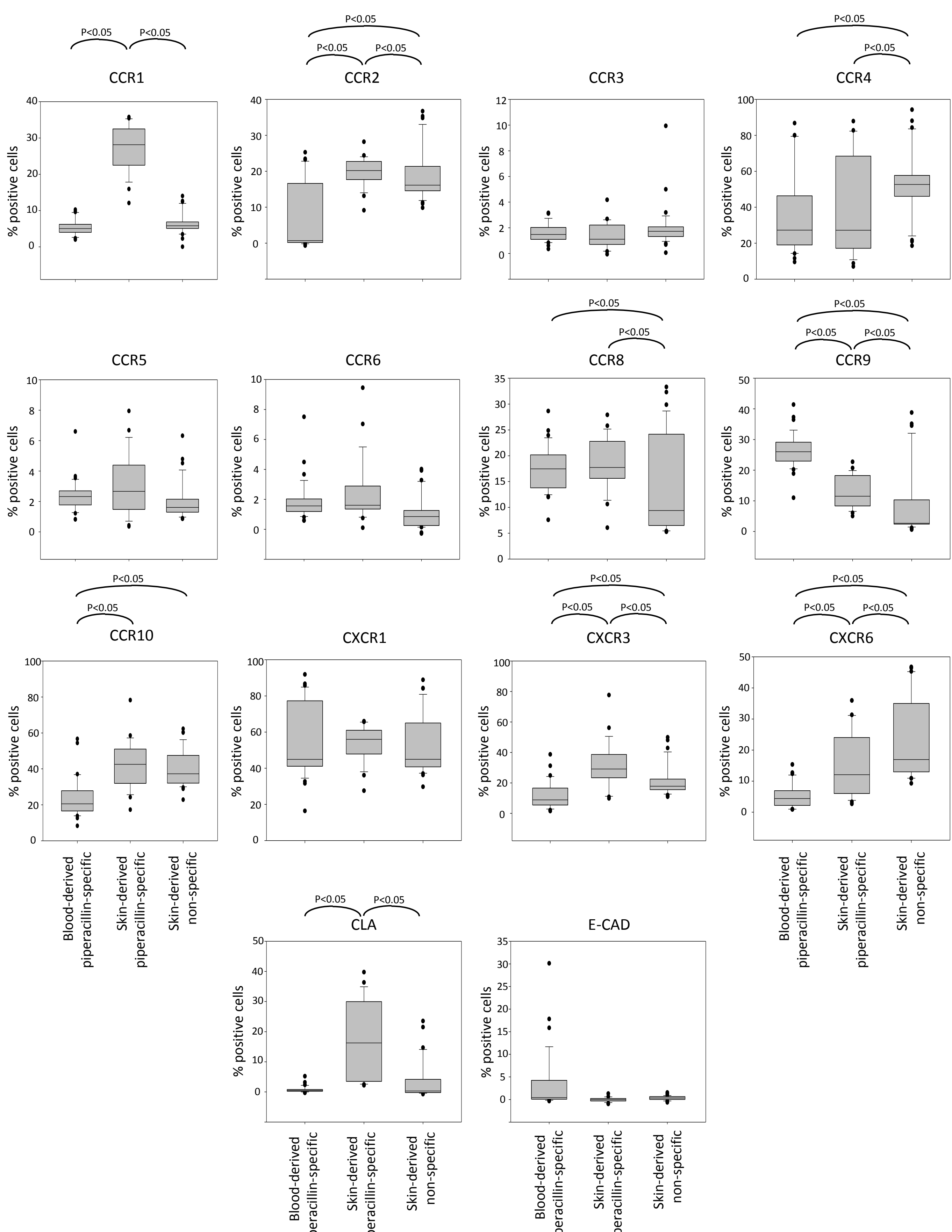

CCR1
CCR2
CCR3
CCR4
CCR5
CCR6
CCR8
CCR9
CCR10
CXCR1
CXCR3
CXCR6
CLA
E-CAD
P<0.05
% positive cells
Blood-derived piperacillin-specific
Skin-derived piperacillin-specific
Skin-derived non-specific

Figure E6

A

	PE-A / APC-A	PE-A / FITC-A	APC-A / FITC-A
Unstained Isotype control	33-1.fcs TL – 0 TR – 0 BL – 100 BR – 0	33 -1.fcs TL – 0 TR – 0 BL – 100 BR – 0	33 -1.fcs TL – 0 TR – 0 BL – 100 BR – 0
Tube 1 **PE – CXCR6** **APC – CXCR3** **FITC – CCR5**	33 - 2.fcs TL – 8 TR – 16 BL – 33 BR – 43	33 - 2.fcs TL – 28 TR – 3 BL – 68 BR – 1	33 - 2.fcs TL – 1 TR – 2 BL – 41 BR – 56
Tube 2 **PE – CCR4** **APC – CXCR1** **FITC – CCR3**	33 - 3.fcs TL – 15 TR – 15 BL – 39 BR – 31	33 - 3.fcs TL – 34 TR – 2 BL – 63 BR – 1	33 - 3.fcs TL – 44 TR – 1 BL – 54 BR – 1
Tube 3 **PE – CCR8** **APC – CCR9** **FITC – CLA**	33 - 4.fcs TL – 27 TR – 22 BL – 32 BR – 19	33 - 4.fcs TL – 53 TR – 2 BL – 45 BR – 0	33 - 4.fcs TL – 27 TR – 22 BL – 32 BR – 19
Tube 4 **PE – CCR10** **APC – CCR6** **FITC – CD69**	33 - 5.fcs TL – 26 TR – 8 BL – 55 BR – 11	33 - 5.fcs TL – 0 TR – 42 BL – 0 BR – 58	33 - 5.fcs TL – 0 TR – 19 BL – 1 BR – 80
Tube 5 **PE – CCR1** **APC – CCR2** **FITC – E-CAD**	33 - 6.fcs TL – 12 TR – 11 BL – 49 BR – 28	33 - 6.fcs TL – 21 TR – 8 BL – 63 BR – 8	33 - 6.fcs TL – 33 TR – 6 BL – 56 BR – 5

B

	PE-A / SSC-A	FITC-A / SSC-A	APC-A / SSC-A
Unstained Isotype control	67-1.fcs TL – 0 TR – 0 BL – 100 BR – 0	67-1.fcs TL – 0 TR – 0 BL – 100 BR – 0	67-1.fcs TL – 0 TR – 0 BL – 100 BR – 0
Tube 1 **PE – CXCR3** **FITC – CCR5** **APC – CXCR1**	67-2.fcs TL – 18.4 TR – 0 BL – 81.6 BR – 0	67-2.fcs TL – 2.3 TR – 0 BL – 97.7 BR – 0	67-2.fcs TL – 43.2 TR – 0 BL – 56.8 BR – 0
Tube 2 **PE – CCR4** **FITC – CCR3** **APC –CCR9**	67-3.fcs TL – 30.2 TR – 0 BL – 69.8 BR – 0	67-3.fcs TL – 1.3 TR – 0 BL – 98.7 BR – 0	67-3.fcs TL – 20.3 TR – 0 BL – 79.7 BR – 0
Tube 3 **PE – CCR10** **FITC – E-CAD** **APC –CCR8**	67-4.fcs TL – 35.4 TR – 0 BL – 64.6 BR – 0	67-4.fcs TL – 0.1 TR – 0 BL – 99.9 BR – 0	67-4.fcs TL – 12.7 TR – 0 BL – 87.3 BR – 0
Tube 4 **PE – CCR1** **FITC – CD69** **APC –CCR6**	67-5.fcs TL – 12.5 TR – 0 BL – 87.5 BR – 0	67-5.fcs TL – 58.5 TR – 0 BL – 41.5 BR – 0	67-5.fcs TL – 1.4 TR – 0 BL – 98.6 BR – 0
Tube 5 **PE – CXCR6** **FITC – CLA** **APC –CCR2**	67-6.fcs TL – 10.1 TR – 0 BL – 89.9 BR – 0	67-6.fcs TL – 2.1 TR – 0 BL – 97.9 BR – 0	67-6.fcs TL – 18.0 TR – 0 BL – 82.0 BR – 0

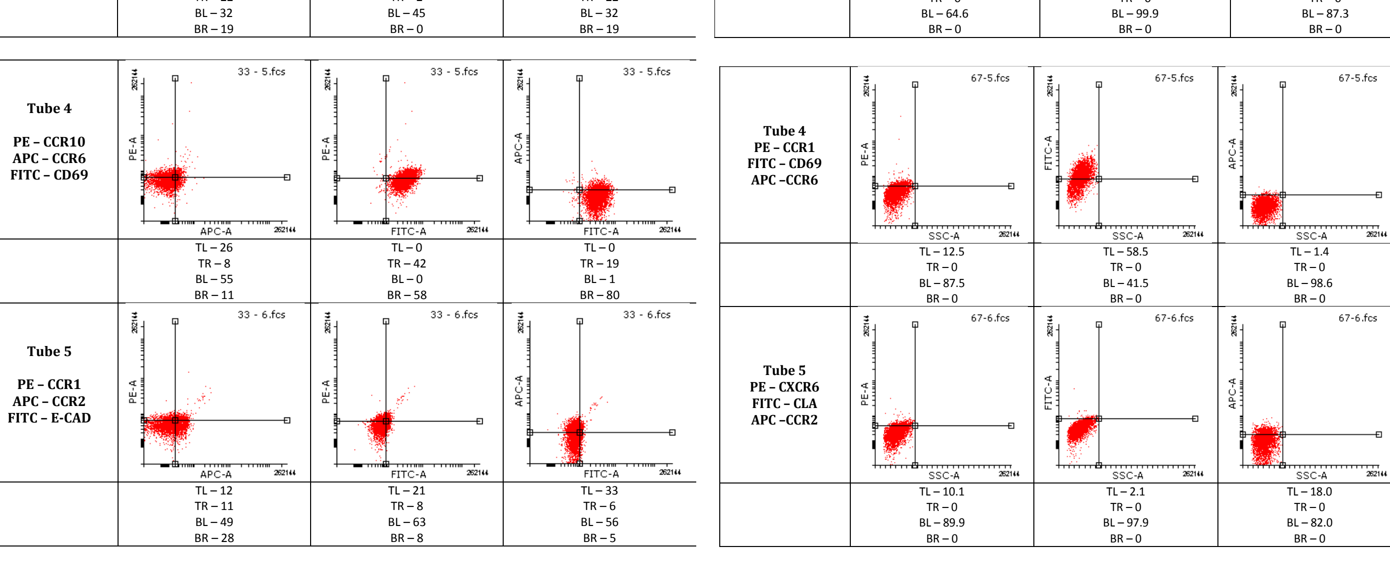
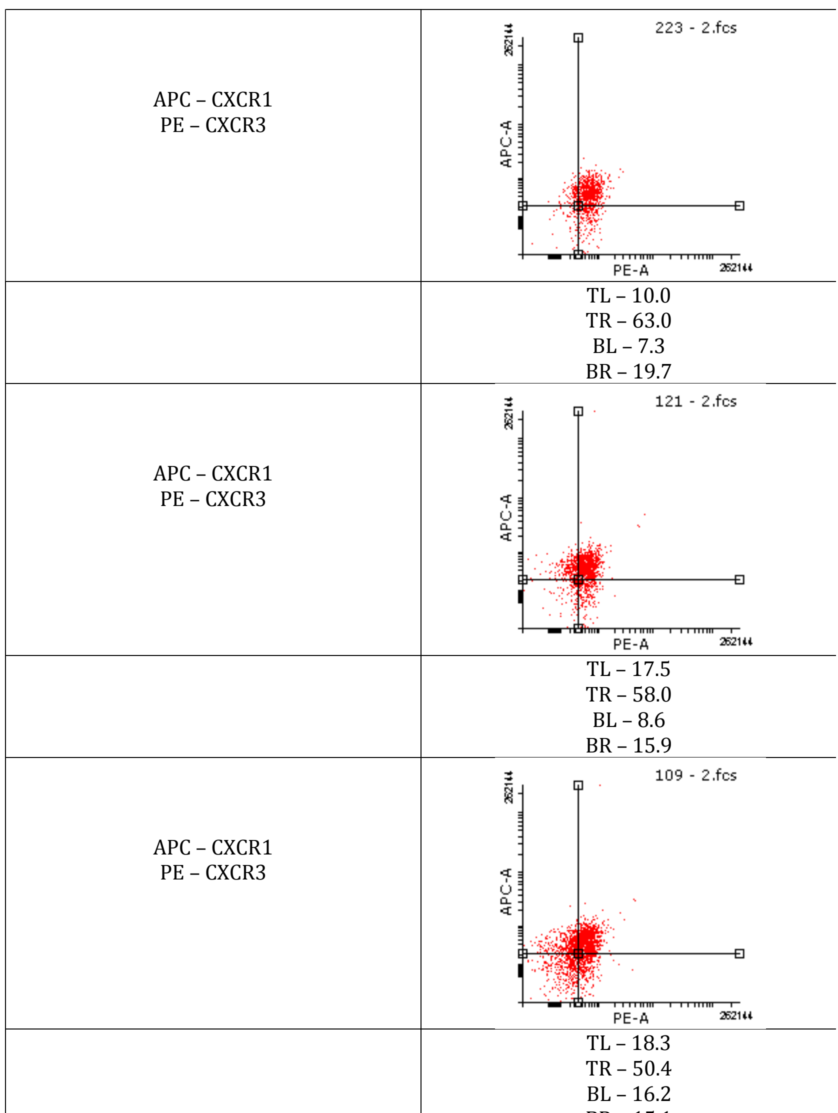


Figure E7



APC – CXCR1 PE – CXCR3	223 - 2.fcs APC-A PE-A 262144
	TL – 10.0 TR – 63.0 BL – 7.3 BR – 19.7
APC – CXCR1 PE – CXCR3	121 - 2.fcs APC-A PE-A 262144
	TL – 17.5 TR – 58.0 BL – 8.6 BR – 15.9
APC – CXCR1 PE – CXCR3	109 - 2.fcs APC-A PE-A 262144
	TL – 18.3 TR – 50.4 BL – 16.2 BR – 15.1

Figure E8