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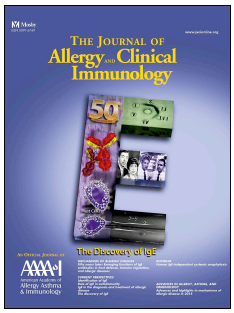

β-lactam hypersensitivity involves expansion of circulating and skin-resident Th22 cells

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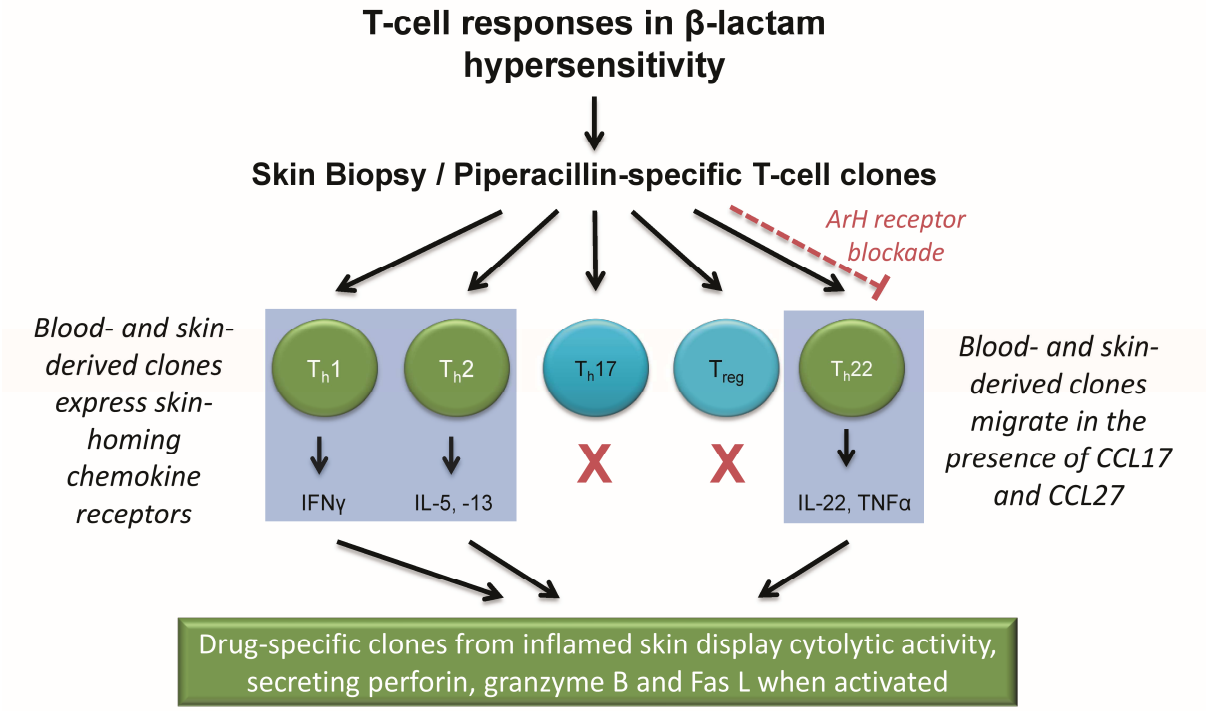
T-cell responses in β-lactam hypersensitivity
Skin Biopsy / Piperacillin-specific T-cell clones
ArH receptor blockade
Blood- and skin-derived clones express skin-homing chemokine receptors
Th1
Th2
Th17
Treg
Th22
IFNγ
IL-5, -13
IL-22, TNFα
Blood- and skin-derived clones migrate in the presence of CCL17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

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

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Running title: T-cell responses to low molecular weight compounds

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Abstract

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

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

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

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

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

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

Keywords: Human, T-cells, drug hypersensitivity.

Abbreviations: Stimulation index, SI; peripheral blood mononuclear cells, PBMC.

Introduction

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

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

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

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

Methods

Human subjects and cell isolation/separation

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

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

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

Medium for T-cell culture and cloning

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

Lymphocyte transformation test and PBMC ELIspot

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

Priming of naïve T-cells from healthy volunteers

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

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 ($1-5x10^6$/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

scintillation counting. Clones with a stimulation index (mean cpm drug-treated wells / mean cpm in control wells) of greater than 2 were expanded and analysed further.

T-cells from inflamed patient skin and healthy volunteer PBMC after priming were cloned using the same procedure.

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

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

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

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

Statistics

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

Results

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

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

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

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

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

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

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

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

measuring IFN-γ and IL-22 secretion in culture supernatants using specific ELISA. Piperacillin-treatment of IL-22^{high} resulted in the secretion of IFN-γ and IL-22 into culture supernatant. In contrast, with the IL-22^{low} clones only IFN-γ was detected (Figure E3).

Skin-derived piperacillin-specific clones secrete perforin, granzyme B and Fas L

Clones deriving from blood (n=24) and skin (n=24) secreted high levels of granzyme B (Figure 3D). However, significantly higher levels of perforin and Fas L were detected from the piperacillin-treated skin-derived clones. Interestingly, piperacillin-specific CD4+ clones secreted the highest levels of FasL and perforin (Figure 3E).

Chemokine receptor expression on piperacillin-specific T-cell clones

Expression of 12 chemokine receptors, CLA, CD69 and E-CAD were compared on piperacillin-specific blood- and skin-derived clones (n=24-36 per group). Piperacillin-specific blood and skin-derived clones expressed high levels of CD69 (Figure E4). Skin-derived clones that were not activated with piperacillin were used as an additional comparator. Piperacillin-specific skin and blood-derived clones were found to express multiple chemokine receptors including CCR4, 5, 8, 9 and 10 and CXCR1, 3 and 6 when flow cytometry data were analysed as MFI (Figure 4) or % positive cells (Figure E5) Skin-derived piperacillin-specific clones expressed significantly higher levels of CCR1 and CLA, compared with non-specific clones. Blood-derived piperacillin-specific clones expressed higher levels of CCR9 when compared with the skin-derived piperacillin-specific and non-specific clones. In contrast, CCR2, CXCR1, and CLA were expressed at higher levels on the piperacillin-specific skin-derived clones, when the two populations of piperacillin-specific clones were compared. (Figure 4; Figure E5). Representative dot plot images showing the panel of receptors expressed and analysed in terms of MFI and % positive cells are shown in Figure

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

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

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

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

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

hypersensitive patients. Moreover, piperacillin-primed cells were not activated with the control antigen nitroso sulfamethoxaozle. Similarly, nitroso sulfamethoxaozle-primed T-cells were not activated with piperacillin (results not shown).

Piperacillin stimulation of the primed cells resulted in the secretion of IFN-γ, IL-13 and IL-22, but IL-17 was not detected (Figure 6B). The priming experiment was repeated in the presence of cytokine cocktails known to polarize T-cells towards Th17 and Th22 subsets. No qualitative difference in the profile of cytokines secreted from piperacillin-specific T-cells was observed. In particular, IL-17 was not detected (results not shown).

Sixty-four piperacillin-responsive clones were generated from the 4 volunteers, out of a total of 526 tested (response rate: 12.2%) (Figure 6C and 6D). Piperacillin-specific CD4+ and CD8+ clones were detected; however, in contrast to clones from hypersensitive patients, most expressed CD8+. Thirty seven well-growing clones were tested and found to secrete IFN-γ, IL-13 and IL-22, but not IL-17, following treatment with piperacillin (Figure 6E).

Analysis of the profile of cytokines secreted from individual clones revealed that most CD4+ and CD8+ clones secreted high levels of IFN-γ (Figure 7A). The few clones that secreted high levels of IL-22 were all CD8+ and did not secrete other cytokines (i.e., IFN-γ, IL-13 and IL-17) (Figure 7B-D).

Signalling through the aryl hydrocarbon receptor is critical for the generation of piperacillin-specific IL-22 secreting T-cells

To investigate whether the generation of piperacillin-specific IL-22 secreting T-cells is regulated through the ArH receptor, naïve T-cell priming was studied in the presence of an

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

Discussion

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

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

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

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

data shows that IL-22 signalling participates in maculopapular drug eruptions; however, additional studies are required to delineate whether IL-22 is acting to promote disease progression or alternatively regulate the severity of tissue injury.

Fas L, perforin and granzyme B are T-cell secretory molecules that act in unison to induce apoptosis in tissue cells. Early studies by Posadas et al[31] reported upregulated levels of perforin and granzyme B in PBMC isolated from patients with acute mild and severe forms of drug-induced skin injury. Moreover, a strong correlation was observed between the level of cytolytic molecules and severity of the disease. In contrast, Fas L was only observed in patients with Stevens Johnson syndrome and toxic epidermal necrolysis. More recently, Zawodiniak et al[32] utilized a granzyme B ELIspot to detect cytotoxic T-cells in blood of patients with various forms of drug-induced skin injury. Accordingly, we utilized drug-specific clones isolated from blood and skin of the same hypersensitive patients to measure and compare Fas L, perforin and granzyme B secretion. Blood and skin-derived clones secreted high levels of granzyme B when activated with piperacillin, which to some extent explains the findings of Zawodiniak et al.[32] In contrast, perforin and Fas L secretion was largely restricted to the skin-derived clones. These data show that (1) drug-specific skin resident T-cells are the most likely mediators of tissue injury and (2) analysis of blood-derived T-cells alone underestimates the importance of cytolytic molecules in the disease pathogenesis.

A subset of T-cells expressing the skin homing chemokine receptors CCR4 and CCR10 have previously been shown to secrete IL-22 and hence are thought to be important mediators of skin pathophysiology.[33] Furthermore, ligands for CCR4 (CCL17) and CCR10 (CCL27), which contribute towards the recruitment of IL-22 secreting cells, have been found at high

levels in the lesional skin of patients with atopic dermatitis.[34,35] Thus, the next component of our study was to characterize expression of chemokine receptors on blood- and skin-derived piperacillin-specific IL-22 secreting clones and to measure migratory potential of the clones towards relevant chemokines. Blood and skin-derived CD4+ and CD8+ clones were found to express multiple chemokine receptors including CCR4, 5, 8, 9 and 10 and CXCR1, 3 and 6. Interestingly, CLA was expressed in higher levels on the skin-derived clones. Comparison of chemokine receptor expression and IL-22 secretion revealed that skin-derived IL-22 secreting clones had a tendency to express higher levels of CCR4 and the opposite was true for blood-derived clones. Most importantly, both skin- and blood-derived clones migrated towards CCL17 and CCL27 (i.e., ligands for the 2 chemokine receptors). Interestingly, the blood derived clones expressed high levels of CCR9, a receptor more traditionally associated with homing towards the gastrointestinal tract,[36] and migrated in the presence of the CCR9 ligand, CCL25, which suggests that drug-specific T-cells in blood have the capacity to migrate to different locations around the body.

Cell culture platforms have been developed in recent years to study the priming of naïve T-cells against chemical and drug antigens.[19,37,38] In these assays, naïve T-cells from healthy donors are cultured with autologous dendritic cells and the chemical/drug for 7-14 days. The newly primed T-cells are then restimulated with a second batch of dendritic cells and a range of compounds to assess antigen specificity. We utilized these methods to characterize the panel of cytokines secreted by naïve T-cells (from 4 healthy volunteers) primed against piperacillin. Following restimulation, the piperacillin-primed cells proliferated vigorously and secreted IFN-γ, IL-13 and IL-22. Importantly, IL-22 was seen in the absence of polarizing cytokines such as IL-6 and TNF-α, which promote the differentiation of naive T-cells into IL-22 secreting effectors.[33] T-cells cloned from the piperacillin-primed naïve T-cells were

skewed towards a CD8+ phenotype and drug stimulation resulted in the secretion of high levels of IFN-γ and low-moderate levels of IL-13 and IL-22. Analysis of individual clones revealed that the three clones secreting high levels of IL-22 were CD8+. They did not secrete Th1 or Th2 cytokines. Antigen-specific T-cells with this phenotype are rarely reported in the literature[16,39] and their role in allergic disease needs to be investigated further. The reason why CD8+ clones were detected in a high frequency from healthy volunteers is not clear, but possibly relates to (1) differences in drug concentration during priming or (2) a different mechanism of drug presentation as we have recently reported with the β-lactam flucloxacillin.[11,40]

The transcription factor ArH recptor regulates the differentiation of naïve T-cells into IL-22 secreting cells. Selective ArH receptor antagonists have been shown to perturb the production of IL-22 secreting cells from naïve precursors, whereas memory T-cells are refractory to ArH receptor regulation.[41,42] In the final component of the project, naïve T-cells were primed against piperacillin in the presence of an ArH receptor agonist (VAF347) and antagonist (CH-223191). T-cells primed to piperacillin in the presence of VAF347 secreted IFN-γ, IL-13 and IL-22 alongside cytolytic molecules perforin, granzyme B and Fas L following restimulation with the drug. In parallel experiments with CH-223191, the differentiation of naïve T-cells into piperacillin-specific IL-22 secreting cells was blocked, while all other secretory molecules were detected at essentially the same level. Thus, ArH receptor signalling is critical for the generation of drug-specific IL-22 secreting T-cells.

In conclusion, our study shows that circulating and skin-resident CD4+ and CD8+ T-cells that secrete IL-22, but not IL-17, alongside cytolytic molecules are important mediators of β-lactam hypersensitivity in humans. Differentiation of naïve T-cells into drug-specific IL-22

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

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509

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630

631

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^{o}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^{o}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-

derived T-cell clones. Filled circles depict CD8+ clones. (B) Mean ± SD IL-22 secretion from Th1, Th2 and Th1/2 clones. (C) Comparison of the level of IFN-γ and IL-13 secreted by individual IL-22h^{high} blood- and skin-derived T-cell clones. (D) Cytolytic molecules secreted by piperacillin-specific clones. (E) Comparison of the level of perforin and Fas L secreted by individual blood- and skin-derived T-cell clones.

Figure 4. Chemokine receptor expression on piperacillin-specific T-cell clones derived from blood and inflamed skin of hypersensitive patients. Piperacillin-specific T-cell clones deriving from blood and skin of hypersensitive patients were analysed for chemokine receptor expression by flow cytometry. The box plot shows the median and 25th/75th percentiles, with error bars showing the 10th/90th percentiles.

Figure 5. Migration of piperacillin-specific T-cell clones derived from blood and inflamed skin of hypersensitive patients towards chemokines. Time-dependent chemotactic migration was recorded by counting using a haemocytometer between 0.5-24h. Each line shows the response of an individual clone.

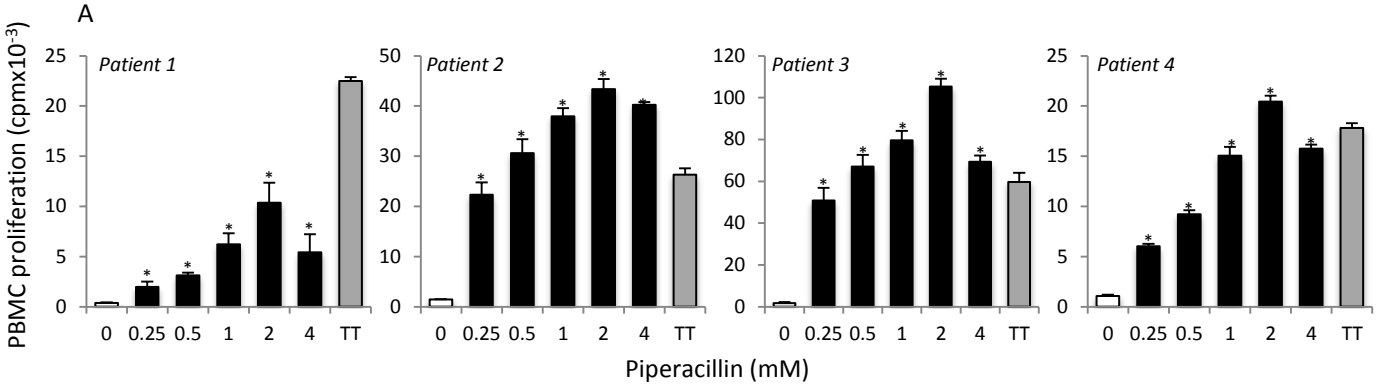
Figure 6. Piperacillin-specific priming of naïve T-cells from healthy volunteers. (A) Drug-specific T-cell proliferative responses were measured by [^{3}H]thymidine uptake. The data show mean ± SD of triplicate cultures. (B) Antigen-specific T-cell responses measured by IFN-γ, IL-13, IL-17 and IL-22 ELIspot. (C) Table shows the number of piperacillin-responsive T-cell clones generated from blood of healthy volunteers following priming and their CD phenotype. (D) T-cell clones were cultured with irradiated antigen presenting cells and piperacillin and proliferative responses were measured. (E) T-cell clones were cultured

with irradiated antigen presenting cells and piperacillin (2mM) and cytokine secretion was quantified.

Figure 7. Profile of cytokines secreted from piperacillin-specific T-cell clones derived from healthy volunteers. T-cell clones were cultured with irradiated antigen presenting cells and piperacillin and cytokine secretion was quantified. (A) Comparison of IFN-γ and IL-13 secreted by individual T-cell clones. Filled circles depict CD8+ clones. (B) Mean ± SD IL-22 secretion from Th1, Th2 and Th1/2 clones. (C) Comparison of IFN-γ/IL-22 and IL-13/IL-22 secretion by T-cell clones.

Figure 8. Piperacillin-specific priming of naïve T-cells from healthy volunteers in the presence of an AhR agonist and antagonist. Antigen-specific T-cell responses were measured by IFN-γ, IL-13, IL-17, IL-22, granzyme B, perforin and Fas L ELIspot. (A) Piperacillin concentration-dependent secretion of cytokines/cytolytic molecules. (B) Images from one representative donor.

A



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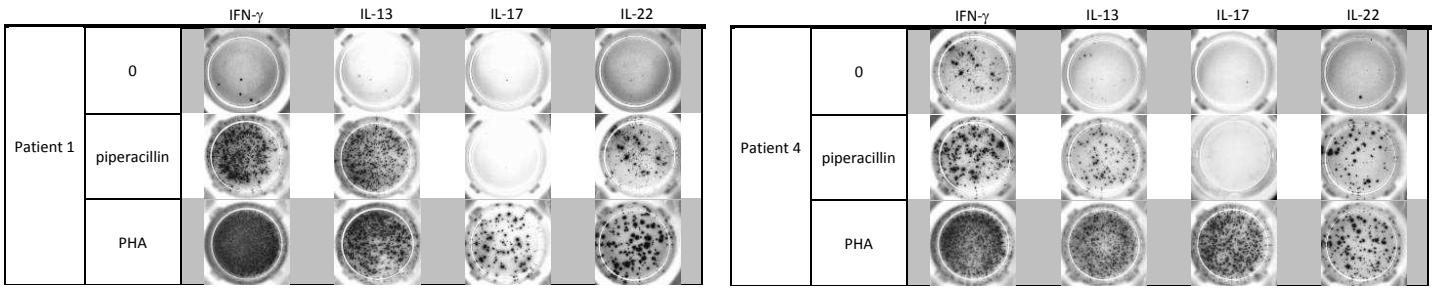
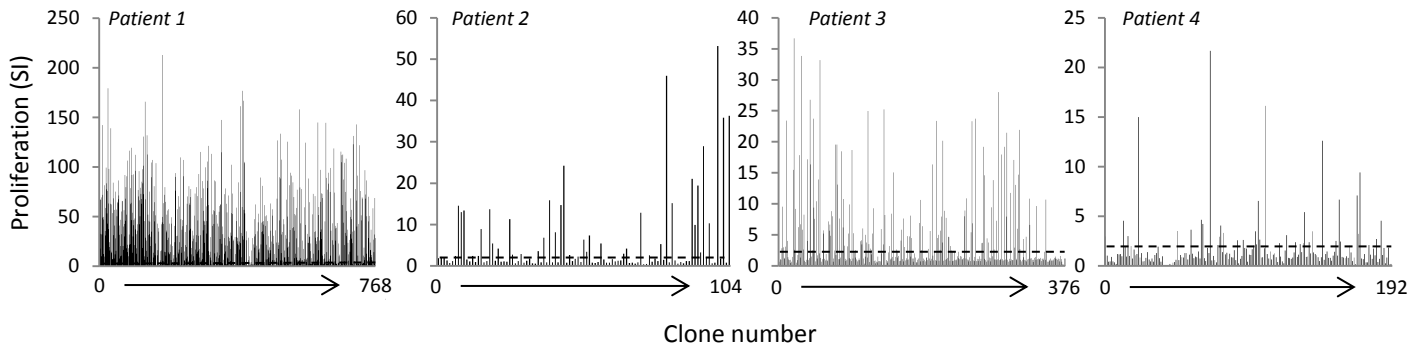


C

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

D



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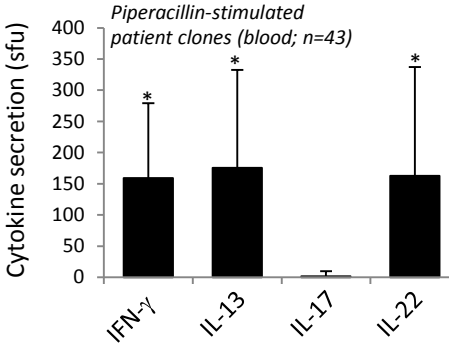
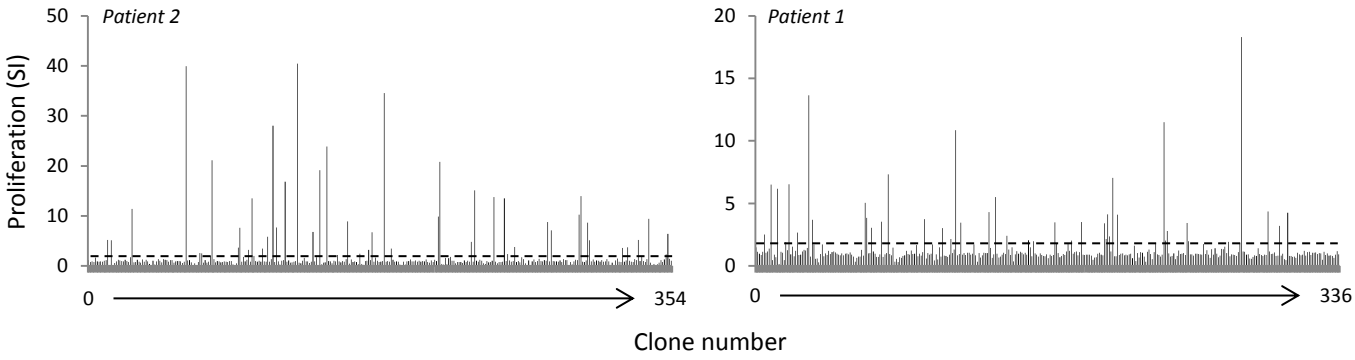


A

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

B



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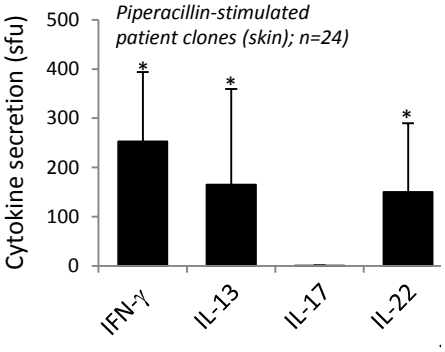
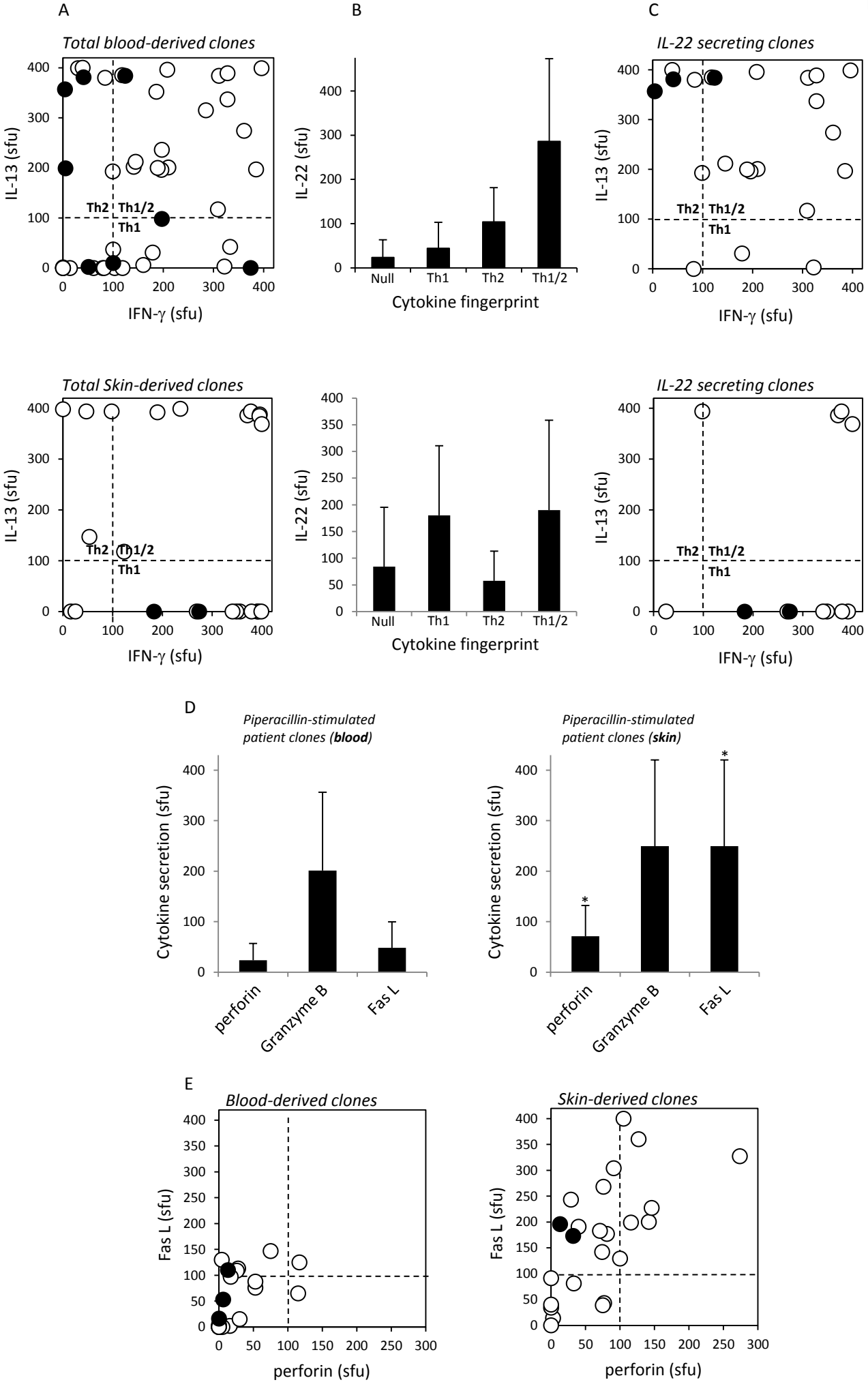
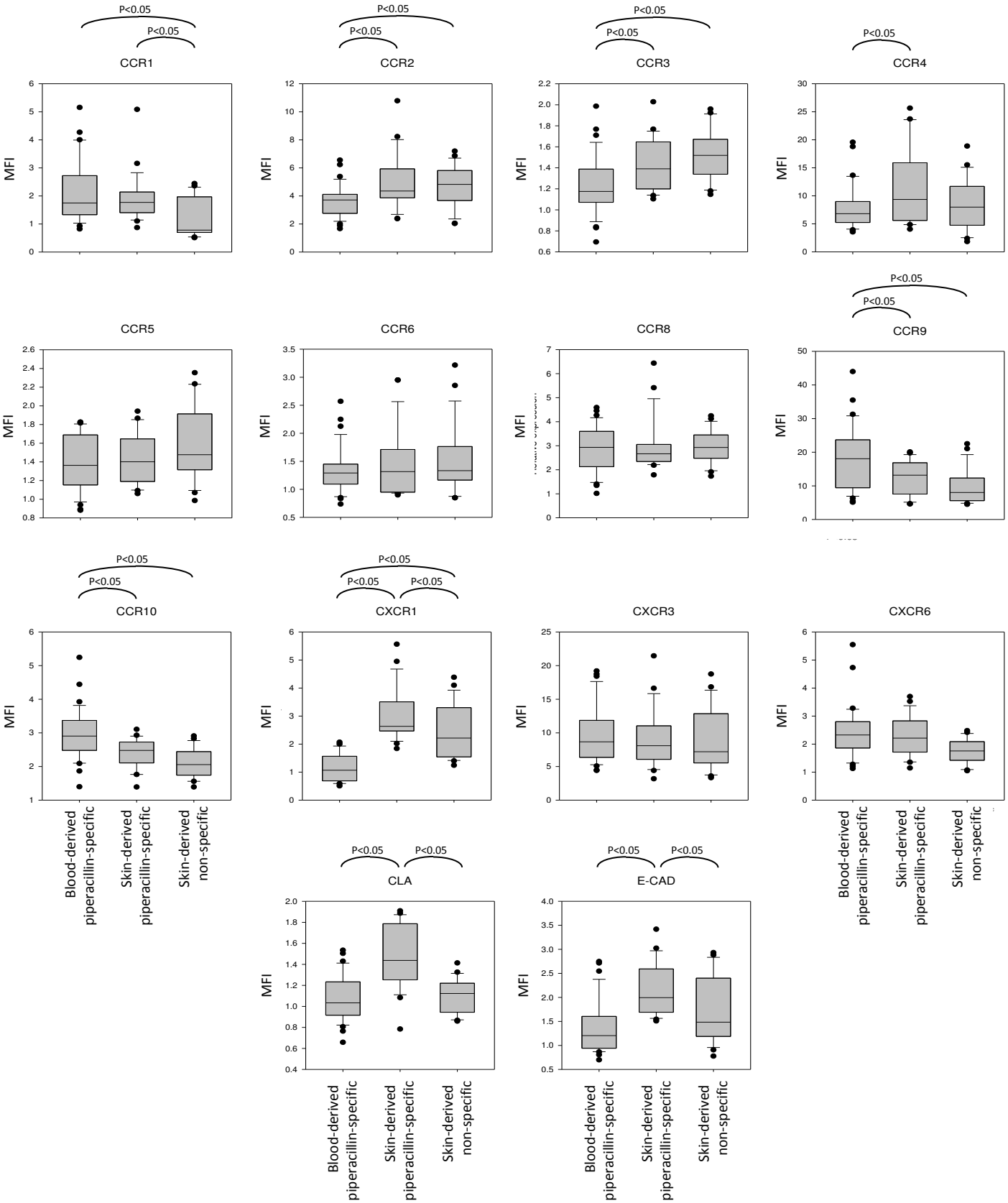
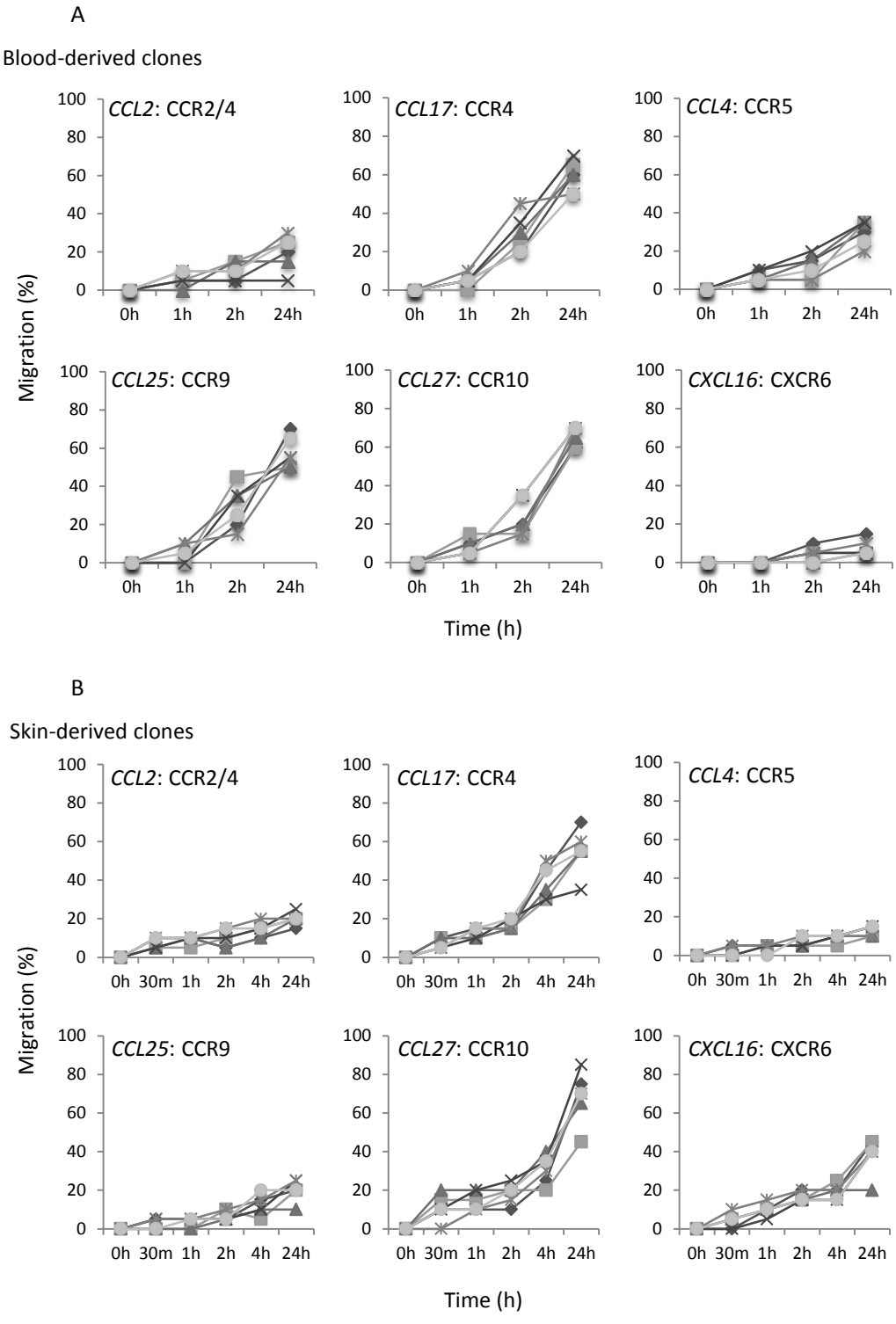
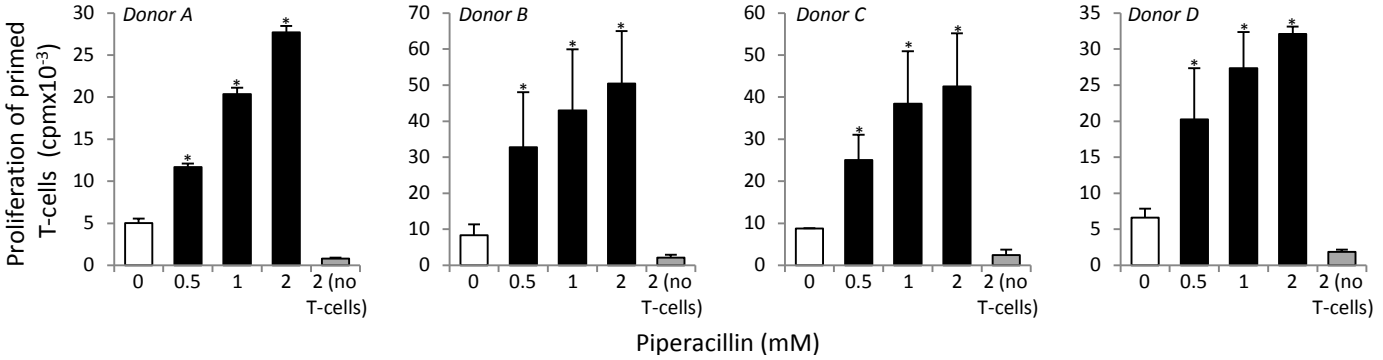



A
Total blood-derived clones
IL-13 (sfu)
IFN-γ (sfu)
Th2
Th1/2
Th1
B
IL-22 (sfu)
Null
Th1
Th2
Th1/2
Cytokine fingerprint
C
IL-22 secreting clones
Total Skin-derived clones
D
Piperacillin-stimulated patient clones (blood)
Piperacillin-stimulated patient clones (skin)
Cytokine secretion (sfu)
perforin
Granzyme B
Fas L
E
Blood-derived clones
Skin-derived clones
Fas L (sfu)
perforin (sfu)


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


A
Blood-derived clones
CCL2: CCR2/4
CCL17: CCR4
CCL4: CCR5
CCL25: CCR9
CCL27: CCR10
CXCL16: CXCR6
Migration (%)
Time (h)
0h 1h 2h 24h
B
Skin-derived clones
0h 30m 1h 2h 4h 24h

A



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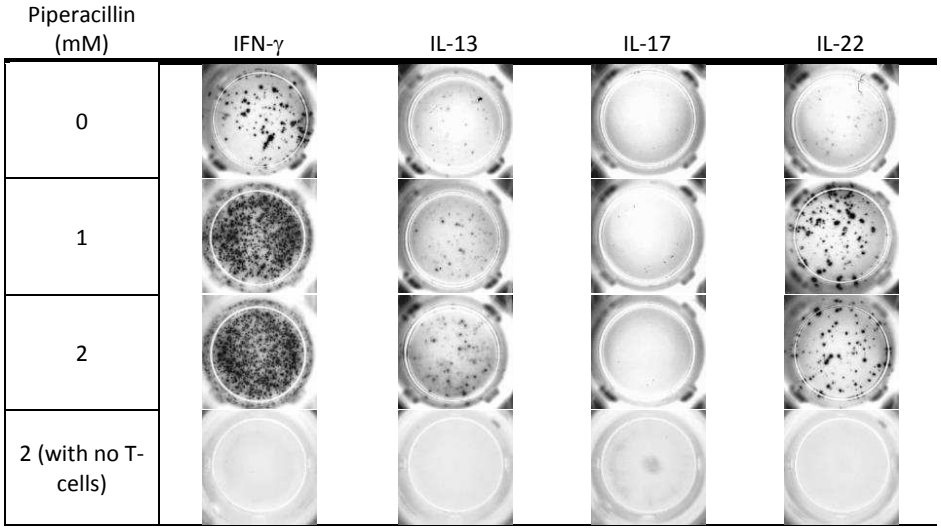
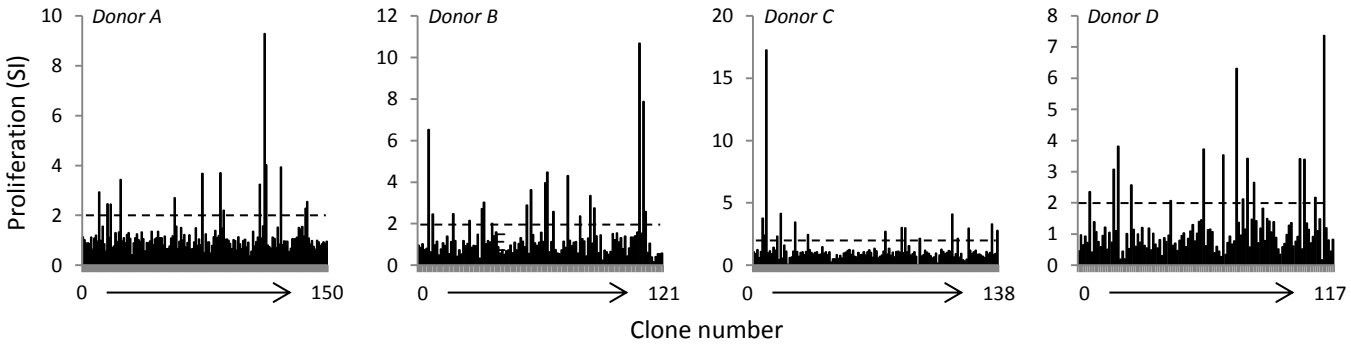


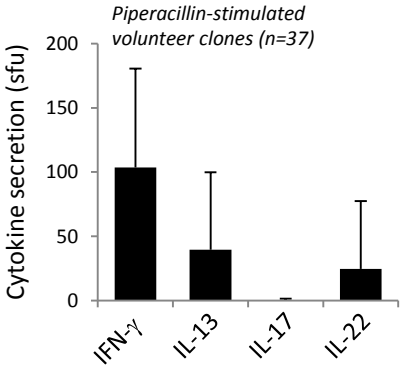
C

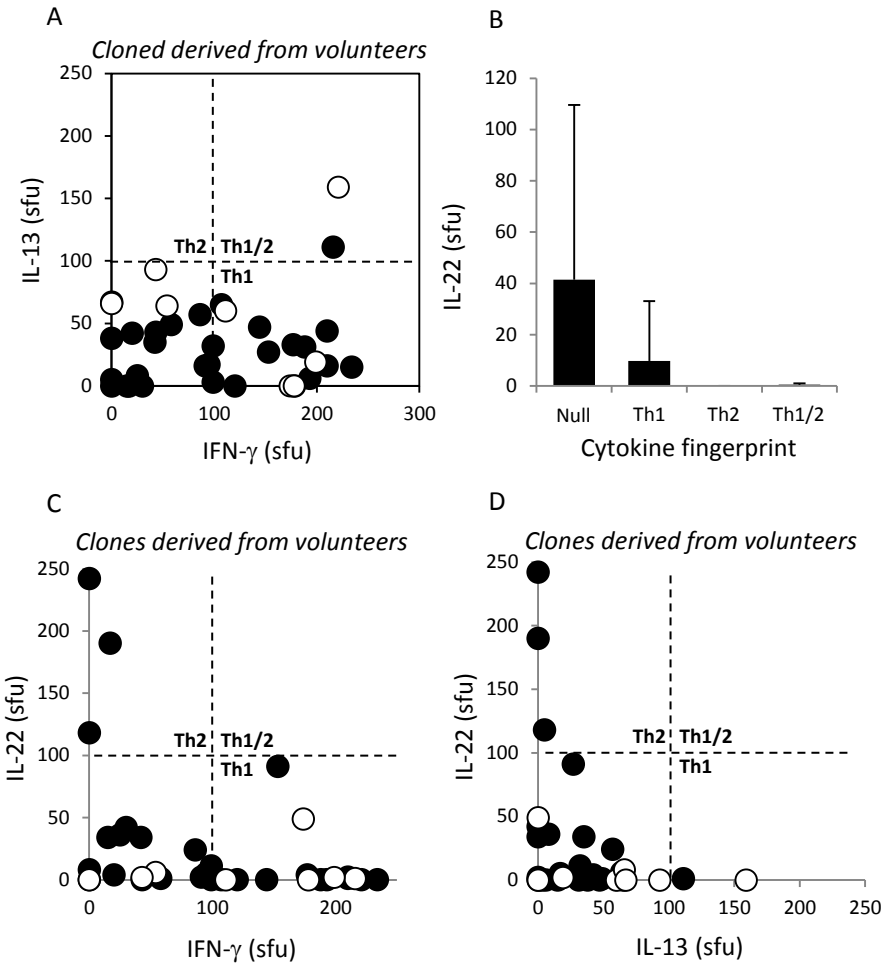
ID	Origin	# Tested	# Specific	% CD4+	% CD8+
Donor A	Naïve T-cells	150	14	36	64
Donor B	Naïve T-cells	121	19	37	63
Donor C	Naïve T-cells	138	16	6	94
Donor D	Naïve T-cells	117	15	13	87

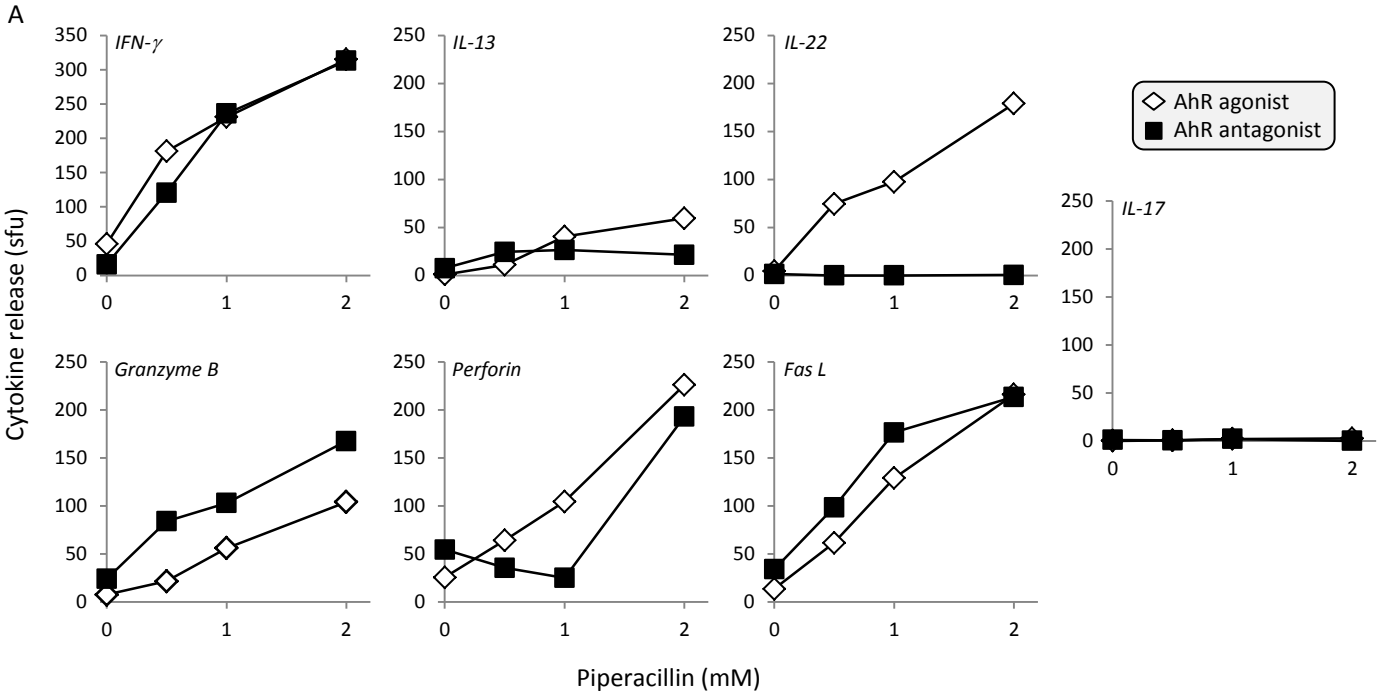
D

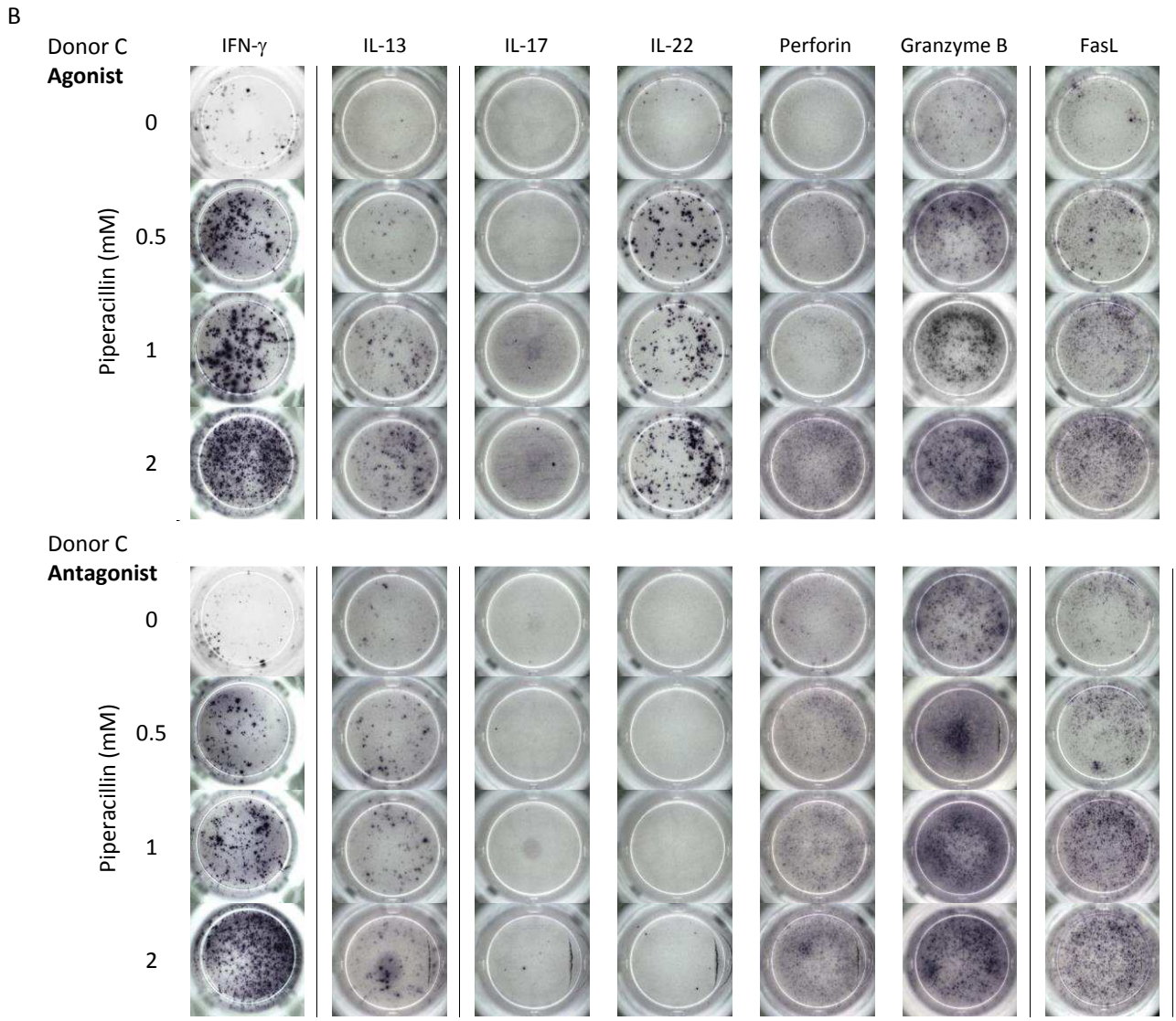


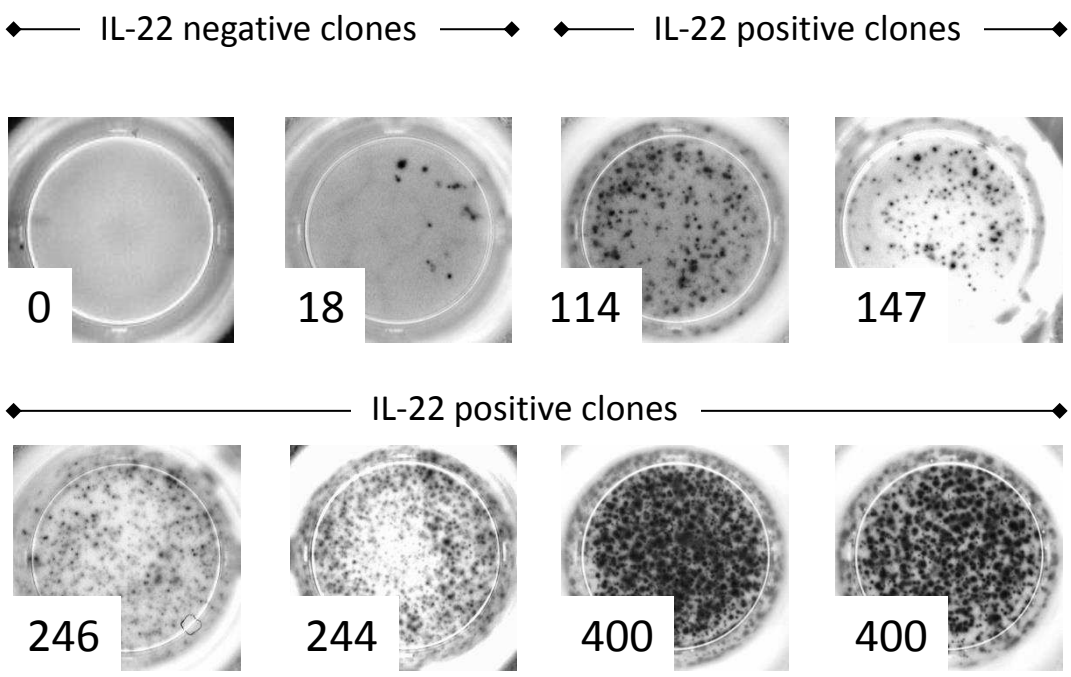
E

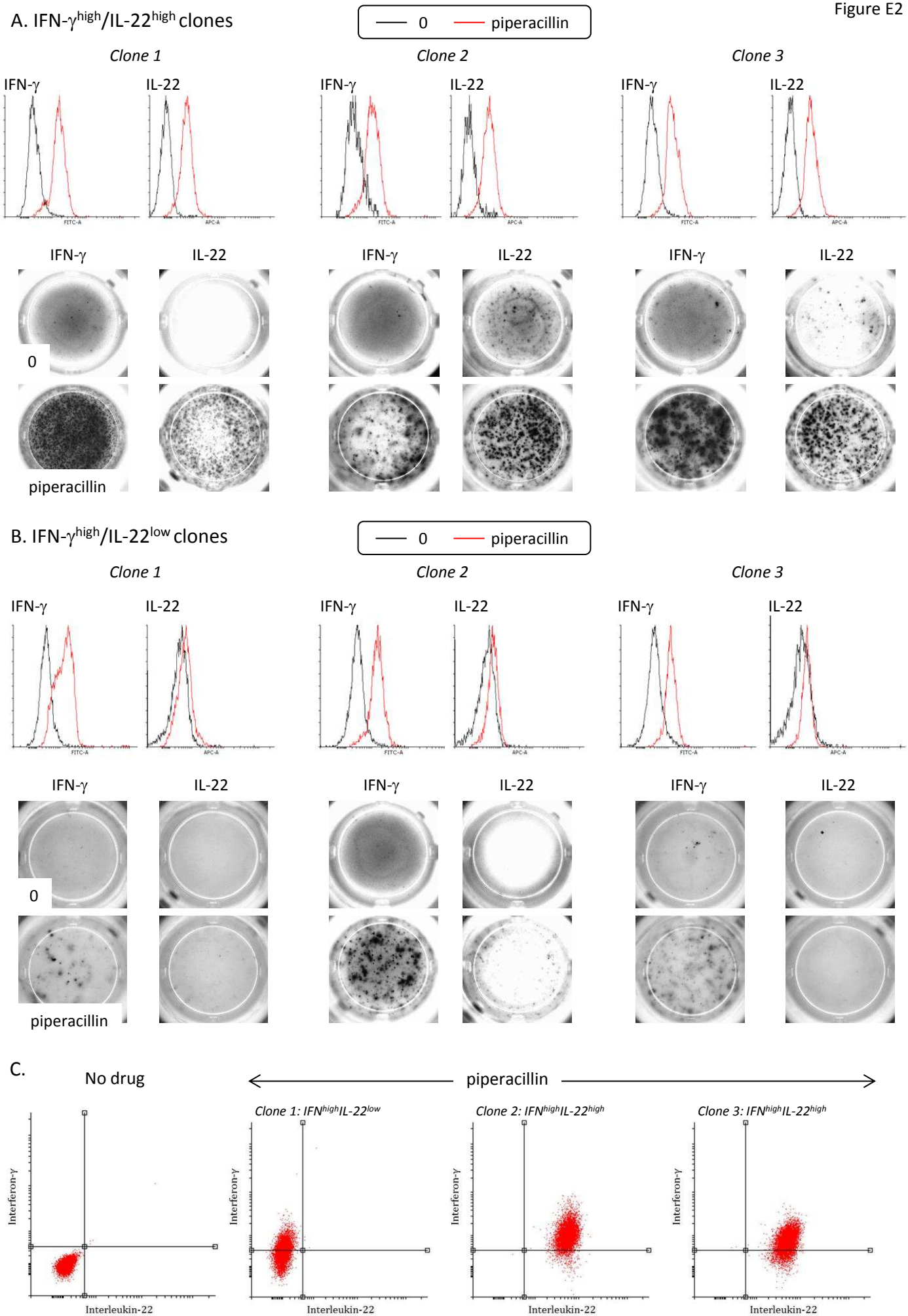



A
Cloned derived from volunteers
IL-13 (sfu)
IFN-γ (sfu)
Th2
Th1/2
Th1
B
IL-22 (sfu)
Null
Th1
Th2
Th1/2
Cytokine fingerprint
C
Clones derived from volunteers
IL-22 (sfu)
IFN-γ (sfu)
D
Clones derived from volunteers
IL-22 (sfu)
IL-13 (sfu)

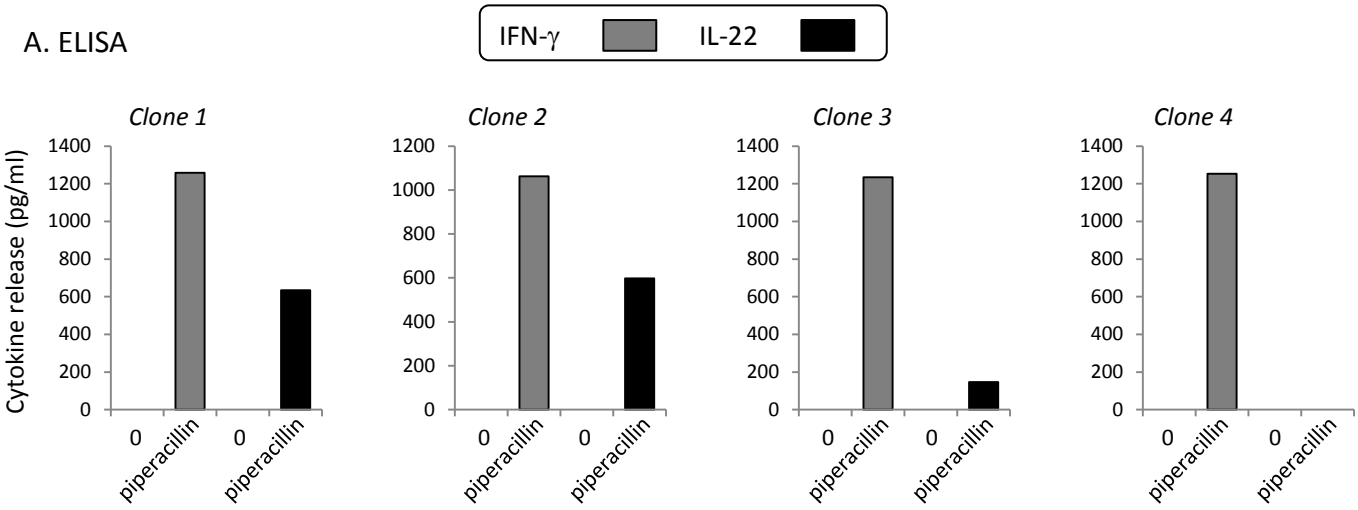

A
IFN-γ
IL-13
IL-22
IL-17
Granzyme B
Perforin
Fas L
AhR agonist
AhR antagonist
Cytokine release (sfu)
Piperacillin (mM)


B
Donor C
Agonist
IFN-γ
IL-13
IL-17
IL-22
Perforin
Granzyme B
FasL
Piperacillin (mM)
0
0.5
1
2
Donor C
Antagonist

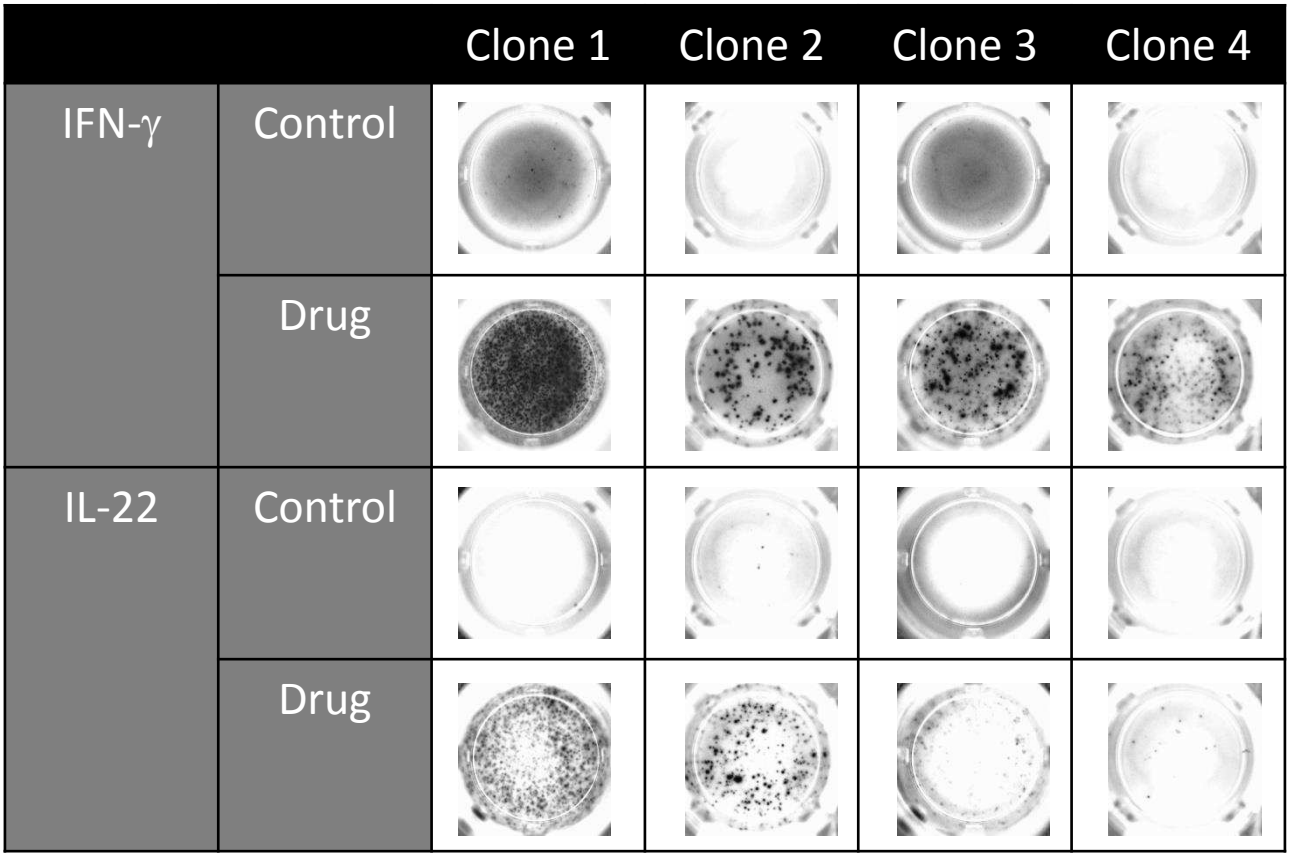

IL-22 negative clones
IL-22 positive clones
0
18
114
147
IL-22 positive clones
246
244
400
400

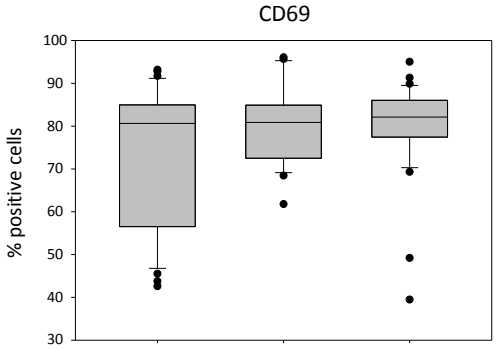

A. IFN-γ^high/IL-22^high clones
0
piperacillin
Clone 1
Clone 2
Clone 3
IFN-γ
IL-22
0
piperacillin
B. IFN-γ^high/IL-22^low clones
0
piperacillin
Clone 1
Clone 2
Clone 3
IFN-γ
IL-22
0
piperacillin
C.
No drug
piperacillin
Clone 1: IFN^high IL-22^low
Clone 2: IFN^high IL-22^high
Clone 3: IFN^high IL-22^high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

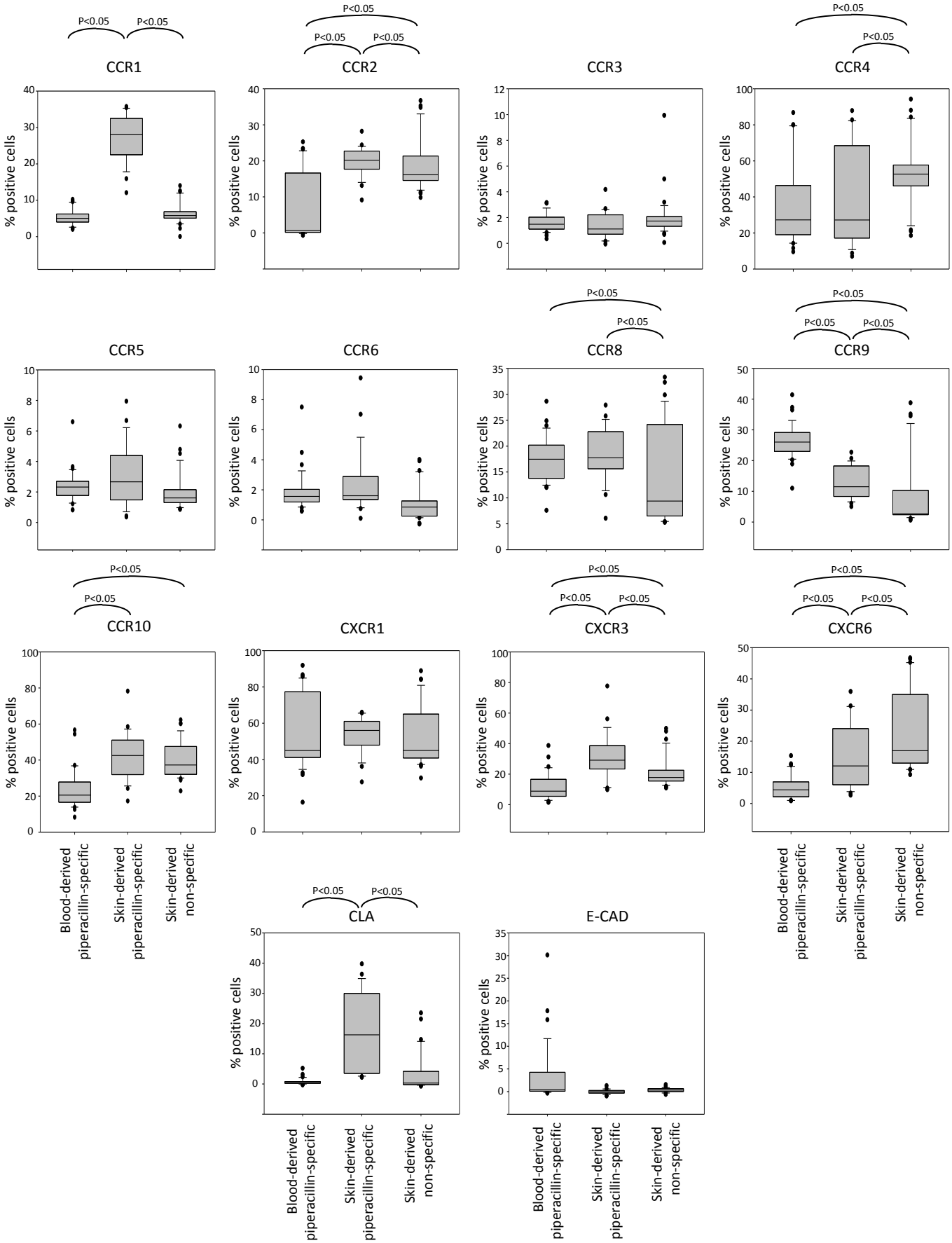
A. ELISA



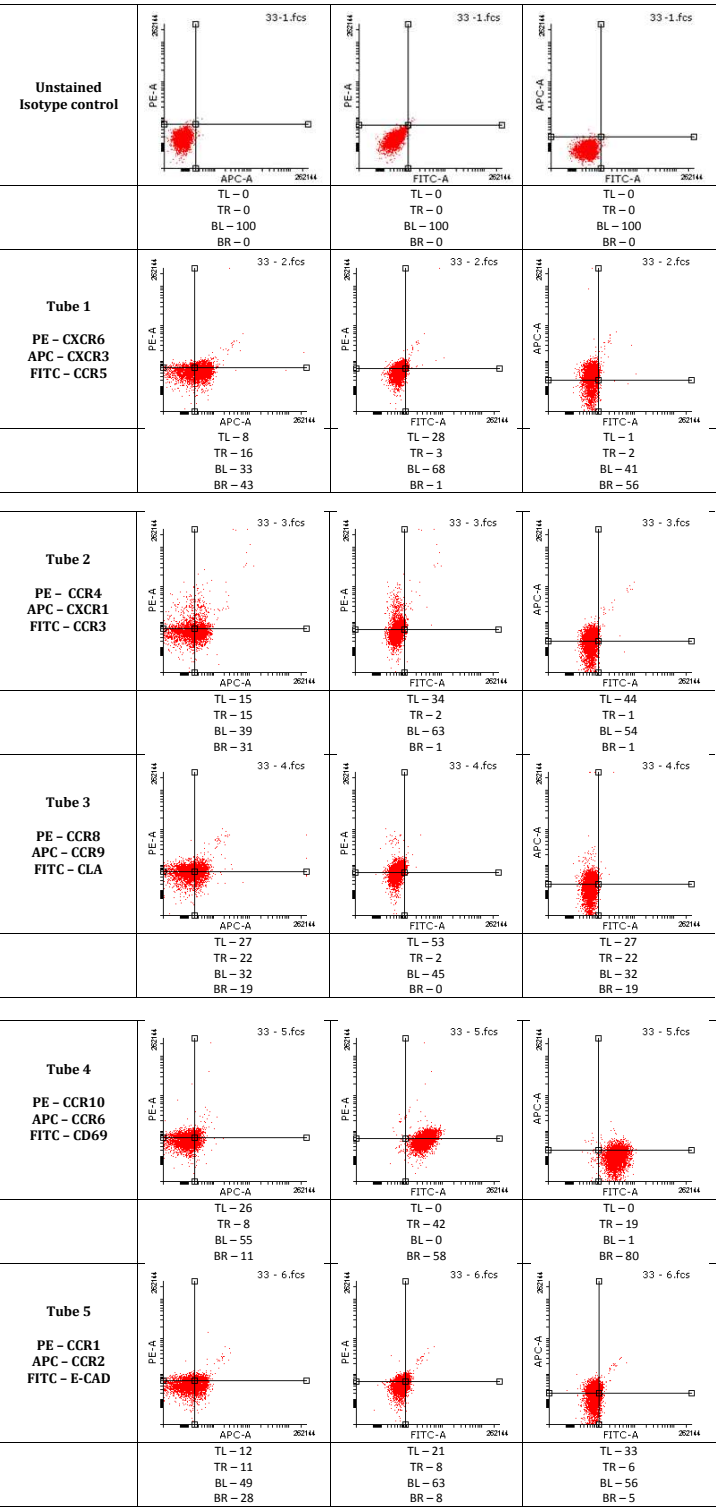
B. ELIspot



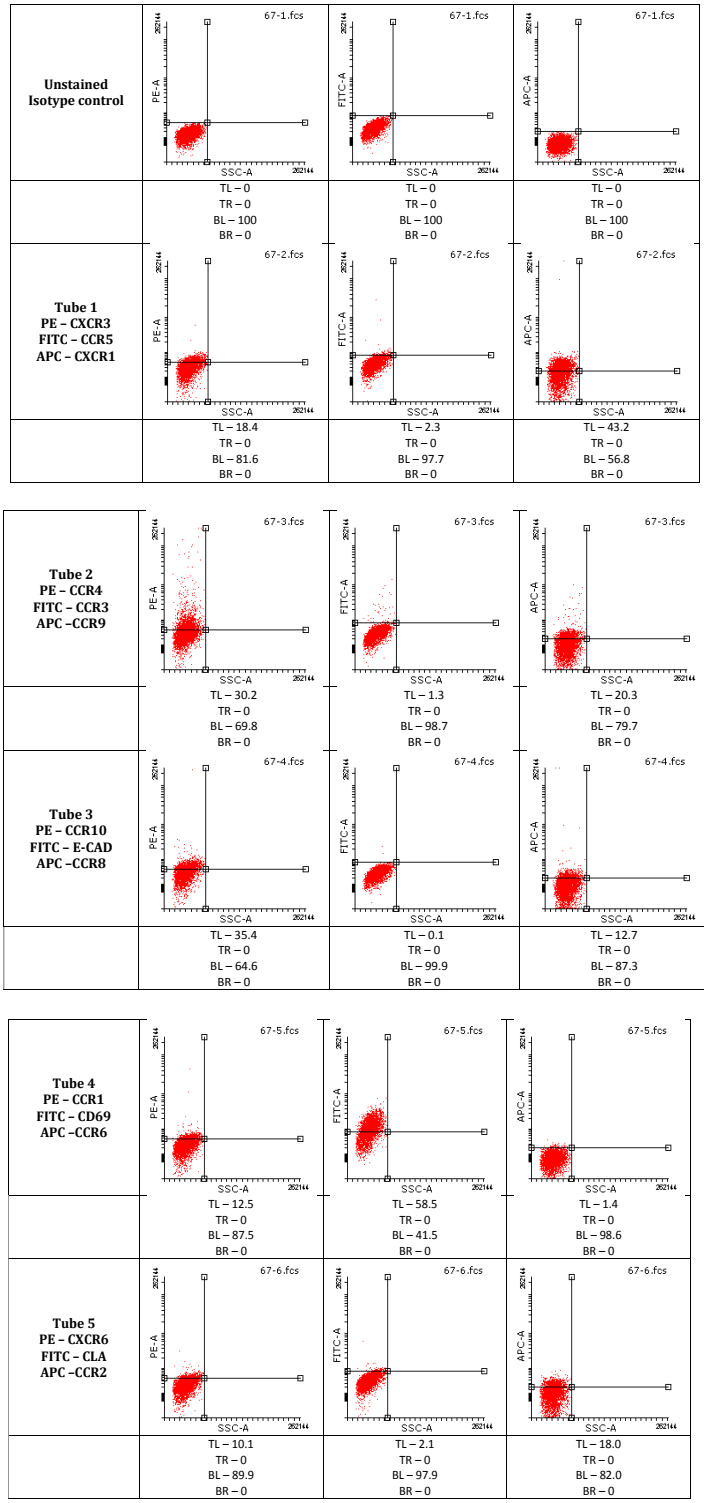

CD69
% positive cells
100
90
80
70
60
50
40
30


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

A


Unstained
Isotype control
33-1.fcs
PE-A
APC-A
FITC-A
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
TL – 28
TR – 3
BL – 68
BR – 1
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
TL – 34
TR – 2
BL – 63
BR – 1
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
TL – 53
TR – 2
BL – 45
BR – 0
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
TL – 0
TR – 42
BL – 0
BR – 58
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
TL – 21
TR – 8
BL – 63
BR – 8
TL – 33
TR – 6
BL – 56
BR – 5

B


Unstained
Isotype control
67-1.fcs
PE-A
FITC-A
APC-A
SSC-A
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
TL – 2.3
TR – 0
BL – 97.7
BR – 0
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
TL – 1.3
TR – 0
BL – 98.7
BR – 0
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
TL – 0.1
TR – 0
BL – 99.9
BR – 0
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
TL – 58.5
TR – 0
BL – 41.5
BR – 0
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
TL – 2.1
TR – 0
BL – 97.9
BR – 0
TL – 18.0
TR – 0
BL – 82.0
BR – 0

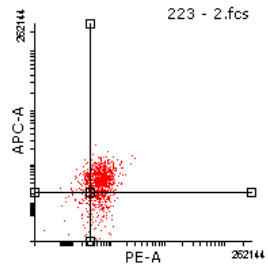
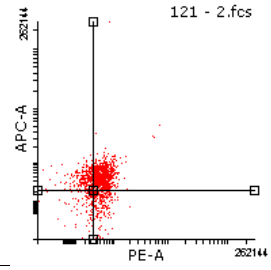
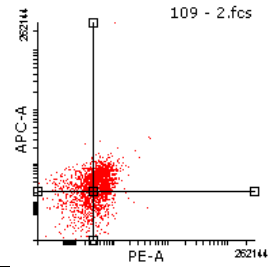
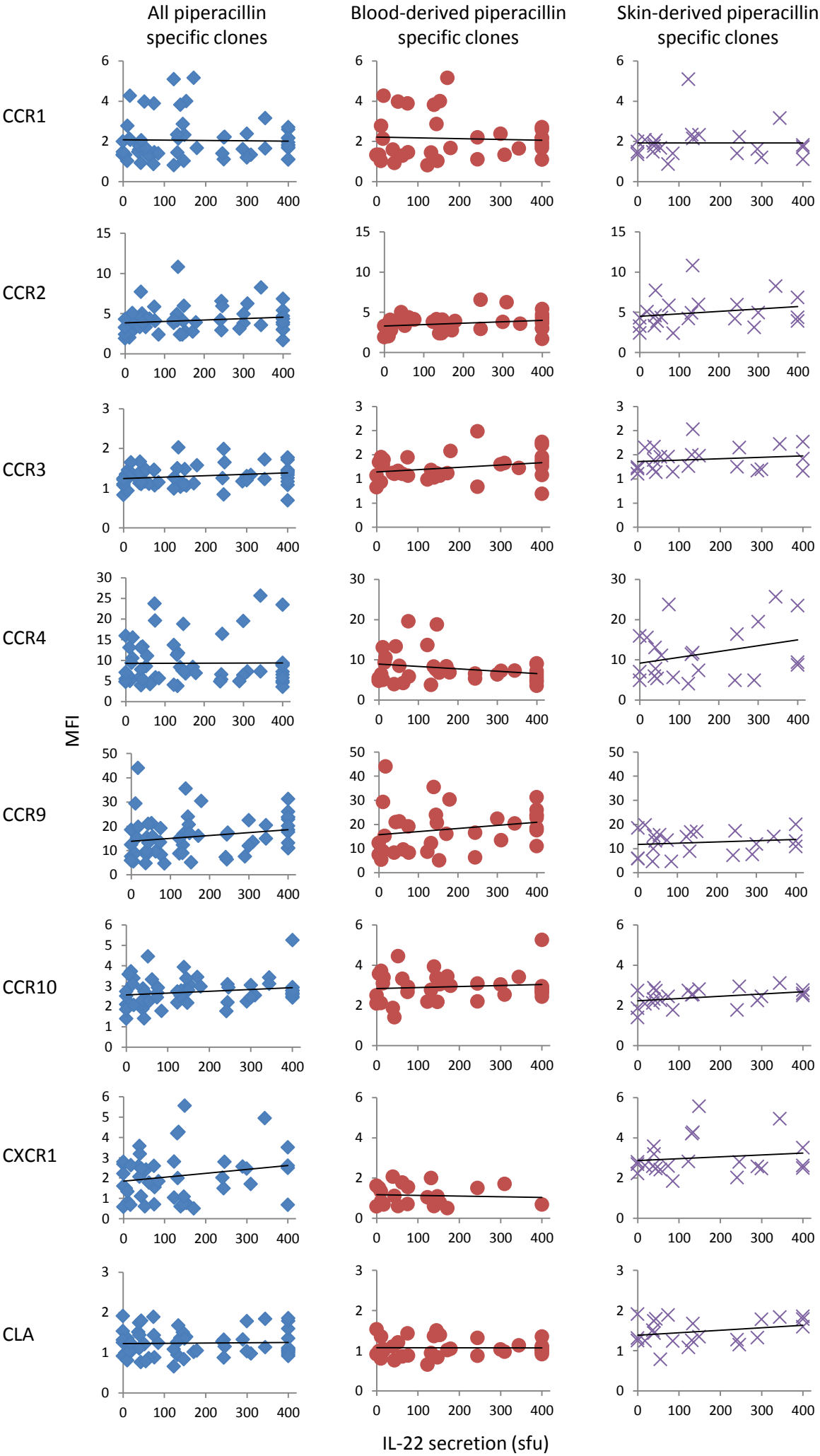
APC – CXCR1 PE – CXCR3	
	TL – 10.0 TR – 63.0 BL – 7.3 BR – 19.7
APC – CXCR1 PE – CXCR3	
	TL – 17.5 TR – 58.0 BL – 8.6 BR – 15.9
APC – CXCR1 PE – CXCR3	
	TL – 18.3 TR – 50.4 BL – 16.2 BR – 15.1

Figure E8


All piperacillin specific clones
Blood-derived piperacillin specific clones
Skin-derived piperacillin specific clones
CCR1
CCR2
CCR3
CCR4
CCR9
CCR10
CXCR1
CLA
MFI
IL-22 secretion (sfu)