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1 **Natural resistance to HIV infection: lessons learned from**
2 **HIV-exposed uninfected individuals**

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1 **Footnote page**

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14

1 **Abstract**

2 We explored potential mechanisms of resistance to HIV-1 infection in different groups of
3 uninfected individuals exposed by systemic or mucosal routes: intravascular drug-users in
4 Vietnam, spouses of HIV-infected individuals in Cambodia and Central African Republic.
5 Our main findings were: reduced susceptibility of PBMC to HIV-1 infection *in vitro*,
6 associated with low levels of CD4+T cell activation *in vivo* and/or cell restriction of viral
7 replication; enhanced NK cell activity associated with increased activating/inhibitory NK cell
8 receptor ratios. These results support a contribution of innate responses to resistance against
9 HIV-1 infection. Scientific and ethical issues encountered during research on EU are
10 discussed.

11

12 Key words:

13 HIV, Exposed uninfected, risk of infection, natural resistance, viral restriction, NK cells,
14 immune activation, Africa, South-East Asia, ethics.

15

1 INTRODUCTION

2 A small percentage of individuals who have been repeatedly exposed to HIV-1 through sexual
3 or systemic routes over long periods of time remain free of any detectable sign of infection.
4 These naturally resistant individuals have been referred to as “Exposed Seronegative” (ESN)
5 or “Exposed Uninfected” (EU). Since the overwhelming majority of them are HIV-negative
6 by both serology and PCR we will use here the EU definition.

7 While some EU individuals may remain free of infection by chance (for example, the
8 probability of transmission by sexual intercourse is low [1]), there is strong evidence that host
9 mechanisms are involved in resistance to HIV-1 infection [2]. Both genetic polymorphisms,
10 including the CCR5 Δ 32 mutation in Caucasian populations, and HIV-specific T and B cell
11 responses have been detected in EU subjects [3-6] (reviewed in [7]) raising the question of
12 whether EU are resistant to HIV-1 infection or whether they are protected by specific
13 immunity. This question is obviously relevant to the possibility that helpful clues for vaccine
14 research could be inferred from studies of EU.

15 Acquired immune responses to infectious agents require time to develop, and these may be
16 not able to block primary HIV infection and the consequent integration of the viral DNA into
17 host genome. Innate mechanisms of resistance and/or immediate/early immune defenses may
18 be needed to fight the incoming virus and to help to establish efficient adaptive responses. We
19 explored these potential mechanisms in EU from South-East Asia and Central Africa, notably
20 through analyzing target cell susceptibility to HIV-1 infection and NK cell function. We will
21 briefly summarize our main findings and draw conclusions from the whole body of our
22 studies.

23

1 **Study populations**

2 We studied three groups of EU exposed to HIV-1 by either systemic or mucosal routes:
3 intravascular drug users from Hô Chi Minh City (HCMC), Vietnam, (N: 45; 38 M, 7 F; drug
4 use time: 13-31 yr, risk factor: shared needles); heterosexual partners of HIV+ individuals
5 from Phnom Penh, Cambodia (N: 48; 7 M, 41 F; risk factor: unprotected sexual intercourse >
6 1.5 yr beyond seroconversion), and Bangui, Central African Republic (CAR) (N: 45; 20 M,
7 25 F; risk factor: unprotected sexual intercourse, communal life: med 8 yr). The populations
8 studied have been described in detail elsewhere [8-10]. Informed consent was obtained by all
9 the participants. The exposure to HIV-1 and the risk of infection differed for the three groups.
10 Vietnamese EU were recruited from a group of IDU, amongst whom 86% were HIV-
11 seropositive. High seroprevalences of HBV (82%), HCV (100%) and HTLV (80%) in the
12 EU IDU supported a high risk of infection by HIV-1. The cumulative risk of being HIV-
13 infected by their infected spouse for EU from Cambodia and CAR was estimated by the
14 equation $1-(1-p)^n$, where p designates the probability of infection per unprotected
15 heterosexual intercourse with an infected individual (0.0008 for men, and 0.0012 for women;
16 adapted from [11] to take into account the 50% increased risk in women compared to men),
17 and n the number of reported unprotected sexual intercourses during the period of communal
18 life. In CAR (2 to 3 intercourses per week), the risk of infection per year was 9.1% for men
19 and 13.4% for women, and the cumulative risk over 8 years of communal life was 53.5% for
20 men and 68.3% for women. In Cambodia (1-2 intercourses per week), the cumulative risk of
21 infection was 6.1% for men and 8.9% for women during one and a half years, and 22.1% for
22 men and 31.2% for women over 3 years. These data suggest that the probability of remaining
23 HIV-uninfected owing to some mechanism of resistance or protection, and not by chance, was
24 highest in the Vietnamese EU. Accordingly, although some mechanisms of resistance

1 appeared to be shared by the three groups of EU, the clearest results were obtained from
2 studies on Vietnamese EU.

3 **Reduced cell susceptibility to HIV-1 infection in vitro**

4 To assess whether the absence of infection could be associated with cell resistance to HIV-1,
5 we first compared the susceptibility to HIV-1 infection *in vitro* of PBMC from EU to those
6 from local low risk populations (healthy blood donors). In each of the three groups, PBMC
7 from EU were more resistant to infection, by one or more viral isolates, than were control
8 PBMC [8-10]. While infectivity assays were generally performed on PHA-activated PBMC,
9 in CAR we also evaluated the susceptibility of PBMC to HIV-1 by exposing unstimulated
10 PBMC to the virus inoculum and activating the cells after infection, to allow the virus to
11 replicate. This experiment was done to evaluate PBMC susceptibility *ex vivo*, at a baseline
12 activation state. In this condition, both the R5 and X4 HIV-1 isolates tested replicated to a
13 significantly lesser extent in PBMC from EU than in control PBMC [10]. Interestingly, the
14 expression of the activation marker HLA-DR was lower *in vivo* on CD4+ T cells from Central
15 African EU than of those from controls (p= 0.0001), suggesting decreased activation of
16 peripheral CD4+ T cells [10]. Vietnamese EU also exhibited a decreased expression of HLA-
17 DR on their CD4+ T cells compared to controls (absolute number and percentage
18 <0.001)[12]. Taken together these observation suggest that lowered levels of activated CD4+
19 T cells may contribute to protection in EU, decreasing the size of the pool of HIV-1 target
20 cells able to replicate the virus efficiently. It is tempting to speculate that a low level of
21 CD4+ T cell activation may result in a reduced permissivity to HIV-1 replication *in vivo*, as
22 suggested by the *ex vivo* PBMC infectivity assays. The causes of the reduced CD4+ T cell
23 activation in EU have not been elucidated. In another study, a low CD4+ T cell activation in
24 EU sex workers from Kenya has been associated with an increased frequency of regulatory T
25 cells [13].

1 Additional factors, including specific mechanisms of restriction of viral replication, may
2 contribute to the PHA-activated PBMC resistance to HIV-1 infection in EU [14]. We
3 investigated a potential intrinsic resistance of CD4⁺ T cells in Vietnamese EU. In several
4 cases (7 out of 14 EU analyzed), CD4⁺ T cells were resistant to HIV-1 infection [8]. We
5 followed 5 of these EU for 2-5 years, and found that the CD4 T cell resistance persisted over
6 time [15]. Different restriction phenotypes were identified in the CD4⁺ T cells from these 5
7 EU [15] (Fig. 1A). In four EU the restriction affected R5 tropic HIV-1 inhibiting viral entry
8 and was linked either to a low/absent expression of CCR5 co-receptor variants (G106R,
9 C178R) on cell surface in two individuals or to an enhanced sensitivity to the inhibitory
10 action of endogenous produced β -chemokines on viral entry in the other two [15].
11 Remarkably, in one EU the restriction was independent of HIV-1 tropism and suppressed
12 early reverse transcription products. The restriction also affected other retroviruses such as
13 SIVmac, SIVagm (Fig 1B) and MoMLV (not shown), and was not overcome at high HIV-1
14 doses, suggesting that a saturable species-specific inhibitory factor such as TRIM5 α was not
15 involved [15]. Some CD4⁺ T cell clones derived from this EU retained the restriction
16 phenotype observed in the parental cells (Fig 1B). Infectivity experiments with heterokaryons
17 obtained by fusion of a resistant T cell clone with a replication competent T cell line
18 suggested that the restriction was due to the lack of a cellular co-factor needed for viral
19 replication (Fig. 1C).

20 **Enhanced NK cell activity**

21 NK cells may exert antiviral activity during the earliest phases of HIV-1 infection before the
22 induction of adaptive immune responses, and may influence the efficiency of the adaptive
23 immune responses. NK cell activity is regulated by the integration of target cell signals
24 transmitted through inhibitory or activating NK-cell receptors (NKR), including the
25 polymorphic Killer Immunoglobulin-like Receptors (KIR), the c-type lectin NKG2 receptors,

1 the natural cytotoxicity receptors (NCR) and Fc γ IIIa (CD16). KIR expression is controlled
2 both at the genetic level and by regulatory mechanisms that lead to a stochastic expression of
3 KIR in a single NK cell. Several studies have unraveled the impact of particular HLA and
4 KIR genotypes with regard to HIV infection [16, 17](reviewed in [18]). We investigated NK
5 cell receptor repertoire and function in Vietnamese EU [19, 20]. NK cell cytotoxic activity
6 against K562 target cells was strongly enhanced in EU, as compared to exposed infected
7 individuals who eventually seroconverted or to low-risk controls (Fig 2A). Interestingly, in
8 contrast to the control groups, significant cytolytic activity was observed against the NK-
9 resistant Daudi cell line, suggesting that EU NK cells are activated *in vivo*. NK cell from EU
10 were also able to produce cytokines (IFN- γ and TNF- α) and chemokines (CCL3-5), even in
11 the absence of any exogenous stimulation (TNF- α and CCL3 are shown in Fig. 2B) [19].
12 Accordingly, NK cytotoxic granule exocytosis, as evaluated by the measurement of CD107a
13 cell surface expression, showed that NK cell activation could be detected in EU even in the
14 absence of target cell stimulation (Fig 2C) [20]. Remarkably, the kinetics of unstimulated NK
15 cell degranulation was more rapid in EU than in controls, indicating an ability of EU to exert
16 NK effector functions within a very short delay (Fig 2D).

17 Analysis of KIR transcript in EU showed enhanced KIR3DS1/KIR3DL1 and
18 NKG2C/NKG2A ratios in favor of an activated profile of NK cells [20]. We also evidenced a
19 higher representation of EU individuals expressing the inhibitory KIR2DL3 receptor in the
20 absence of its KIR2DL2 allelic counterpart in individuals bearing the HLA class I C1 allele
21 [20]. Since KIR2DL3 has lower affinity than KIR2DL2 for the HLA-C1 ligand, this should
22 confer lower HLA inhibitory signaling. Interestingly, homozygosis for KIR2DL3 in
23 combination with HLA-C1 has been reported to be protective in hepatitis C infection [21].
24 The balance of expression patterns in favor of activating NKR expression and of KIR
25 combinations with lower inhibitory potential may underlie the enhanced NK cell activity

1 observed in EU. These NKR expression patterns in EU may be the result of a selection of NK
2 receptor-ligand combination repertoires driven by other viral challenges including HCV and
3 HBV to which EU were highly exposed.

4 **Concluding remarks**

5 The reduced susceptibility to HIV-1 infection *in vitro*, associated with low levels of CD4+ T
6 cell activation and to genuine mechanisms of viral restriction, as well as the enhanced NK cell
7 activity that we found in different groups of EU sustains the concept of a contribution of
8 innate anti-viral responses to the resistance against HIV infection. The intrinsic resistance of
9 CD4+ T cells in some EU, associated or not to mutations of the CCR5 receptor genes,
10 indicates a prominent role of the genetic background in these individuals. This is also
11 suggested by the association of the constitutive activation of NK cells in Vietnamese EU with
12 particular patterns of NKR expression. Although NKR expression may be epigenetically
13 regulated by environmental factors, including prior infectious challenges, different repertoires
14 were found in EU and in HIV-1 infected IDU, with similar prevalence of viral infections,
15 pointing out the role of genetic differences in providing EU with a better armed immune
16 response to resist encounters with HIV. Other studies have also reported increased ratios of
17 activating vs inhibitory NKR expression in EU or KIR profiles consistent with enhanced NK
18 cell function, including higher representation of KIR3DS1 and KIR2DL3 homozygosity [20-
19 23]. It should be underlined, however, that the heterogeneity of NKR repertoires and of the
20 mechanisms of resistance to HIV-1 in CD4+ T cells, as well as of other immune mechanisms
21 of protection observed among EU [7] are not in favor of a single, general mechanism of
22 resistance.

23 Our researches carried out on limited numbers of EU from Africa and South East Asia
24 havebrought to light some informative issues concerning the mechanisms that may contribute
25 to resistance in such high-risk individuals. However such studies remain difficult to conduct

1 and may even raise ethical concerns associated with the reduction of risk behaviors that
2 hopefully result from better medical counseling linked with such protocols in developing
3 countries. In Cambodia, for example, 93% of serodiscordant couples declared as having safe
4 sex at their second visit 6 months after recruitment, which is an achievement in terms of
5 research protocol impact [9]. IDU in HCMC finally declared that they used disposable
6 syringes or stopped drug use. Therefore, EU become unexposed, and, unless genetically
7 determined, the mechanisms of resistance may vanish with time [22, 23]. Some groups of EU,
8 such as sex workers or drug users, are vulnerable populations and particular ethical care has
9 to be taken at recruitment and during follow-up, because these individuals may be easily
10 influenced and may be the object of various environmental pressures that may lead to their
11 loss during follow-up. Problems related to specific socio-political conditions of the country in
12 which the study is carried out may also jeopardize the study itself. For example, political
13 instability in CAR (especially a political coup in March 2003) caused the flight of urban
14 populations, including many families recruited in our study, and hindered and finally blocked
15 the research.

16 Nevertheless, the study of cohorts of individuals who develop mechanisms to resist HIV
17 infection remains a major challenge to understand the basis of « natural resistance » and may
18 orient the design of novel therapeutic or vaccine approaches, based on manipulating host
19 defenses to provide better protection against HIV. Vaccine strategies that induce target cell
20 resistance to HIV by stimulating β -chemokine production and/or increasing intracellular
21 levels of restriction factors have been proposed [24]. NK activating receptor triggering or
22 inhibitory ligand/receptor blockade may constitute potential ways to modulate NK cell
23 activity. Alternatively, vectors that have an impact on the NK cell repertoire or cytokines,
24 such as IL-21 or IL-15, as adjuvant may be used to induce or improve NK cell response [25,
25 26].

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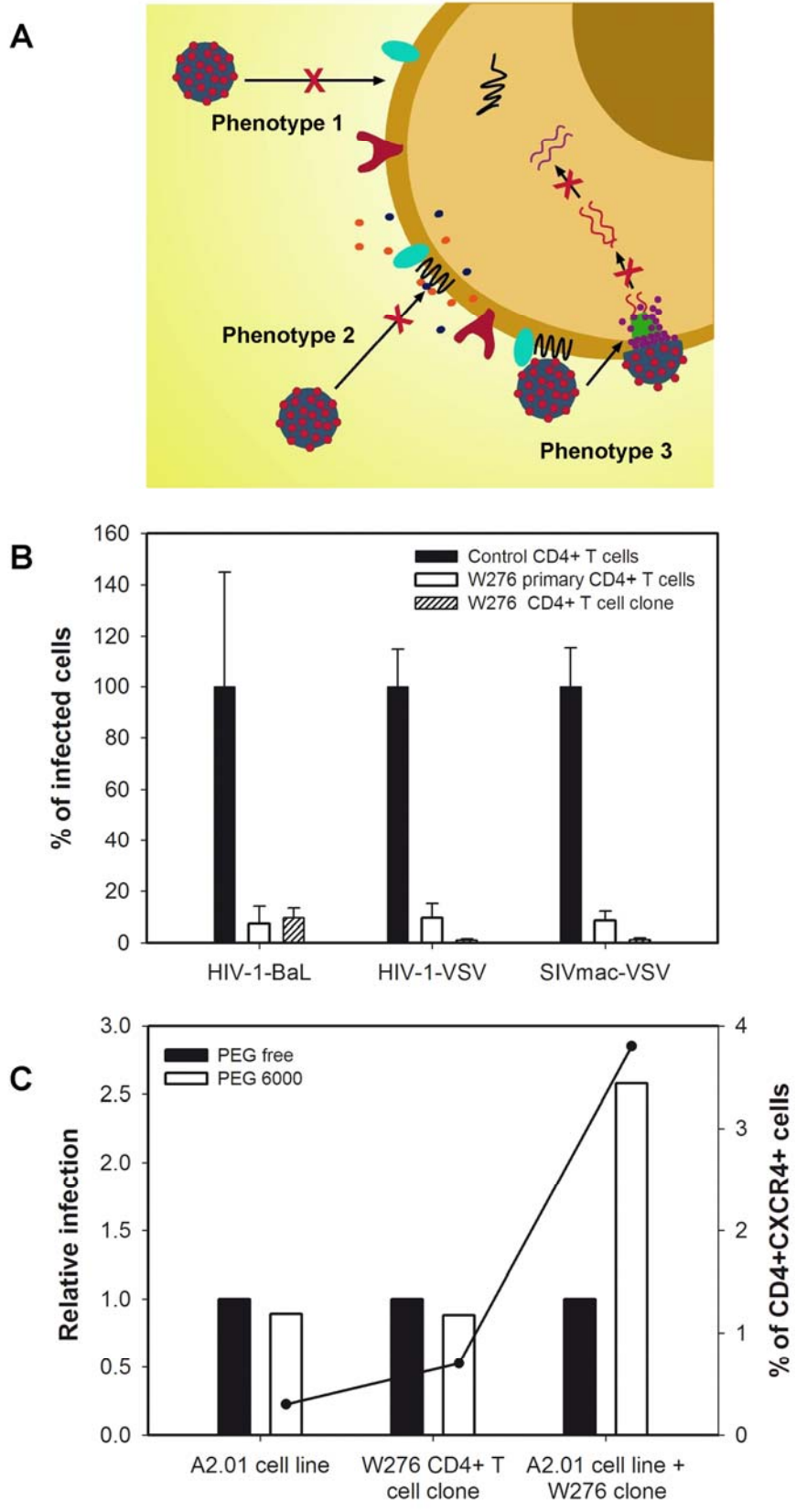
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Figure Legends

Figure 1. **A.** We identified 3 phenotypes of HIV-1 restriction in CD4⁺ T cells from Vietnamese EU: in three cases the restriction affected entry of R5 HIV-1 and was associated with low surface expression of heterozygous CCR5 co-receptor variants (G106R, C178R and C269F) (phenotype 1); in two other cases, R5 HIV-1 infection was inhibited due to an enhanced sensitivity to the blocking effect of endogenously produced β -chemokines (phenotype 2). In one case (W276) the restriction affected not only HIV-1 but also other retroviruses, and was due to a post-entry block of viral replication (phenotype 3). **B.** Primary CD4⁺ T cells from the W276 EU and a CD4⁺ T cell clone derived from these cells were highly resistant to infection with HIV-1 particles, pseudotyped with BaL or VSV-G fusion proteins, or VSV-G-pseudotyped SIVmac particles. **C.** The formation of heterokaryons (indicated by the appearance of double positive CD4⁺ CXCR4⁺ cells, circles) between a resistant T cell clone not expressing CXCR4 and a replication competent CD4^{neg} T cell line was accompanied by a significant increase of HIV-1 infection (bars), suggesting that a cellular co-factor needed for viral replication was lacking in the resistant T cell clone and was provided by the T cell line.

Figure 2 : **A.** EU individuals have a higher cytotoxic potential to lyse K562 and DAUDI target cells compared to both healthy donors (HD) who are at low risk of HIV exposure and seroconverted IDU individuals tested before (EbSC) or after seroconversion (EaSC) . PBMCs from EU, HD or IDU were incubated with K562 or Daudi cell lines at an effector to target cell ratio of 50:1 and lysis was tested by chromium release assays. Significant differences are indicated. **B.** Spontaneous production of TNF- α and CCL33 by NK cells from EU, HD or IDU tested before seroconversion evaluated in the absence of an extrinsic stimulus are shown. Intracellular staining of CD3-CD16⁺CD56⁺ NK cells for TNF- α and CCL3 is shown for each group. Significant differences are indicated. **C.** CD107a cell surface expression was analyzed

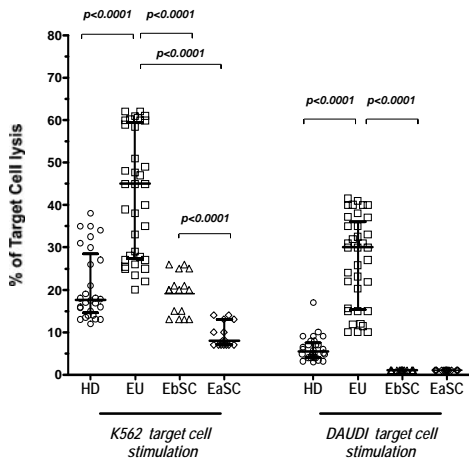
within the CD3-CD56⁺ NK cell subset both in the absence of stimulation or after 3 hours of K562 target cell stimulation (effector to target ratio =2). The percentage of CD3-CD56⁺ NK cells expressing the CD107a cell surface marker were used to compare NK cell granule exocytosis potential in HD, EU, and EI individuals. In the absence of exogenous stimulation, granule exocytosis could only be observed in NK cells from EU, and not from low-risk healthy donors (HD) or IDU seroconverters (exposed infected, EI). D. Kinetics of NK cell degranulation reveals early constitutive activation in EU. NK cell CD107a degranulating activity was measured at different time points (10 min, 60 min and 120 min) using unstimulated NK cells from EU , healthy donors (HD) and an HIV-infected IDU (exposed infected, EI).



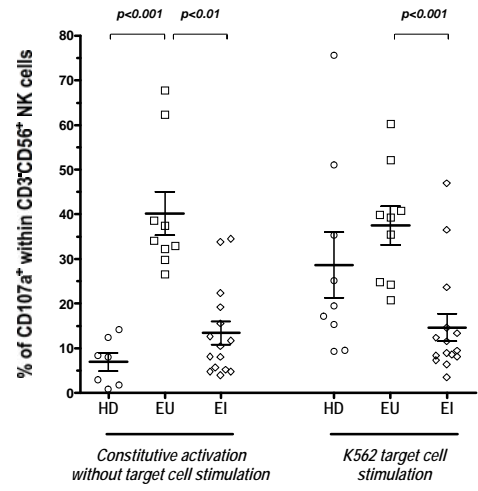
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Figure 1

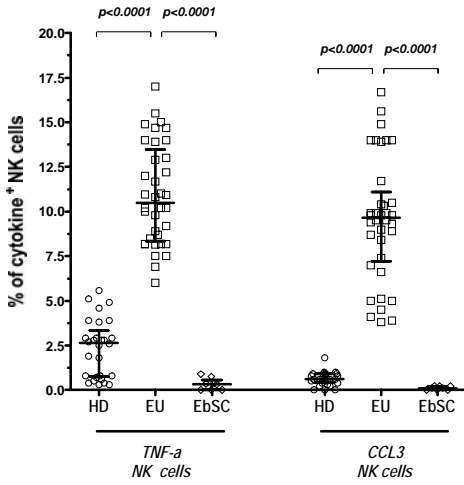
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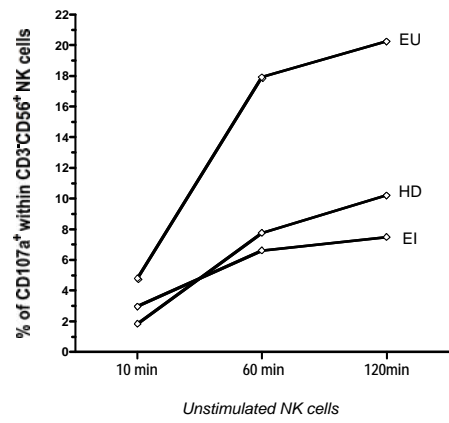


Figure 2