Low expression of activation and inhibitory molecules on NK cells and CD4+ T-cells is associated with viral control

Taborda NA1., Hernández JC2., Lajoie J3., Juno JA3., Kimani J3,4., Rugeles MT1., Fowke KR3,4

1Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia UdeA, Medellín, Colombia
2Infettare, Facultad de Medicina, Universidad Cooperativa de Colombia, Medellín, Colombia
3Department of Medical Microbiology, University of Manitoba, Winnipeg, Manitoba, Canada
4Department of Medical Microbiology, University of Nairobi, Nairobi, Kenya

Address correspondence to:

María T. Rugeles.
Grupo Inmunovirología, Facultad de Medicina, Universidad de Antioquia UdeA, Calle 70 No. 52-21, Medellín, Colombia
Telephone: 57-4-2196551
Fax: 57-4-2196482
(email: maria.rugeles@udea.edu.co).

Running title
Immunological markers in HIV-1 resistance
ABSTRACT

Chronic HIV-1 infection induces severe immune alterations, including hyperactivation, exhaustion and apoptosis. In fact, viral control has been associated with low frequencies of these processes. Here, we evaluated the expression of activation and inhibitory molecules on NK and CD4$^+$ T-cells in individuals exhibiting viral control: a cohort of HIV-1-exposed-seronegative individuals (HESN) and a cohort of HIV-controllers. There was lower expression of CD69, LAG-3, PD-1 and TIM-3 in both cohorts when compared to a low-risk population or HIV-progressors. These findings suggest that individuals exhibiting viral control have lower basal expression of markers associated with cellular activation and particularly immune exhaustion.
Similar to other pathogens, exposure to human immunodeficiency virus type-1 (HIV-1) does not always result in infection. In fact, there are individuals who have been repeatedly exposed to HIV-1 but do not exhibit clinical or serological evidence of infection, who are known as HIV-1-exposed seronegative individuals (HESN) \(^1\). One of the most recently proposed models to explain HESN is the immune quiescence phenotype, which is characterized by a low baseline immune activation that reduces HIV target cell availability, as was previously reported \(^3-5\). In addition, several studies indicate that NK and CD4\(^+\) T-cells play an important role in controlling or inhibiting viral replication, not only for their direct antiviral activity, but also for their interaction with other immune cells \(^6-9\).

However, when the HIV-1 infection is established, the virus induces severe alterations in the gut associated lymphoid tissue, favoring microbial translocation from the intestinal lumen to systemic circulation, inducing a persistent state of immune activation \(^10\). This state is characterized by expression of activation markers on immune cells, including T and NK cells, as well as high circulating levels of pro-inflammatory cytokines, such as IP-10 and TNF-\(\alpha\) \(^11,12\). During HIV-1 infection the persistent inflammatory environment finally triggers the immune exhaustion phenomenon, favoring alterations of CD4\(^+\) T-cell functions, decreases in cytokines, and low cytotoxic activity of NK and CD8\(^+\) T cells \(^13,14\). The molecules CD69, PD-1 (programmed-death 1), Tim-3 (T-cell immunoglobulin domain and mucin domain-3) and LAG-3 (lymphocyte-activation gene-3), associated with activation and exhaustion states, alter the anti-viral immune response modulating the susceptibility to HIV-1 infection and progression \(^15,16\). Interesting, clinical evolution of HIV-1 infected individuals is
variable, as there individuals as HIV-controllers who exhibit a spontaneous and sustained control of viral replication (<2000 copies/mL) at least for one year in the absence of antiretroviral therapy \(^2\).

HESN and HIV-controllers are very interesting cohorts, as their characterization might give insights into mechanisms associated with viral control. We hypothesize that HESN and HIV-controllers exhibit a lower basal expression of activation and inhibitory molecules on CD4\(^+\) T-cells and NK cells, as well as lower levels of plasma pro-inflammatory cytokines, when compared to low-risk or HIV-progressor individuals, respectively. This study was conducted to evaluate differences in the expression of CD69, LAG-3, PD-1 and TIM-3 on NK and CD4\(^+\) T-cells, and the plasma levels of IP-10 and TNF-\(\alpha\) in the following populations: i) Kenyan HESN vs. low-risk individuals, and ii) Colombian HIV-controllers vs. HIV-progressors.

HESN (n=5, GENDER, AGE => JULIE!!) were recruited from the Pumwani Commercial Sex Worker Cohort (Nairobi, Kenya). Low-risk individuals (n=5, GENDER, AGE => JULIE!!) from the same socioeconomic district of Nairobi from the Pumwani Mother child health clinic. The HIV status was determined by enzyme-linked immunosorbent assay (ELISA) and rapid test. Those two groups were previously described \(^17\).

HIV-infected patients, -controllers and –progressors, were recruited from health programs in Medellín, Colombia. These individuals were HAART naïve and classified as: i) patients with spontaneous control of viral replication (designated as HIV-controllers \(^2\), n=5, 3 men vs. 2 female; median age: 21 years), who had a diagnosis confirmed at least one year before the enrollment (median time of diagnosis: 43 months, range = 12–102 months),
and exhibited a spontaneous and stable plasma viral load below 2000 copies/mL (median: 162 copies/mL, range min-max: 20 - 412 copies/mL) and levels of CD4\(^+\) T-cells (936, range min-max: 635 - 1367 cells/µL) during the last year. HIV-progressors (n=6, 4 men vs. 2 females; median age: 24 years) were individuals HAART-naïve with a CD4\(^+\) T-cells count between 300 - 500 cells/µL (365, range min-max: 306 - 443 cells/µL), plasma viral load between 10,000 - 100,000 viral RNA copies/mL (37,784, range min-max: 16,656 – 63,715 copies/mL) and median time of diagnosis of 48 months (range = 15 - 88 months).

Ethical Review Boards from University of Manitoba, University of Nairobi and Universidad de Antioquia approved the study. A clear explanation of the objectives and implications of results were given to the participants; subsequently, an institution-approved informed consent was signed.

The following fluorochrome-labeled mouse monoclonal antibodies were used: anti-CD3 and anti-CD4 (Becton Dickinson, San Jose, CA); anti-CD16, anti-CD56 and anti-CD69 (eBioscience, San Diego, CA); anti-LAG-3 (Enzo Life Sciences, Farmingdale, NY); anti-PD-1 (Biolegend, San Diego, CA) and anti-Tim-3 (R&D Systems, Minneapolis, MN).

The expression of activation and inhibitory molecules was determined in peripheral blood mononuclear cells (PBMC) by flow cytometry. Briefly, 1x10\(^6\) PBMC were incubated with specific monoclonal antibodies for 25 min at room temperature in darkness. Cells were washed twice with cold phosphate-buffered saline (PBS) at 250 \(x\) g for 5 min and fixed with 2% paraformaldehyde.
The gate of lymphocytes was used to analyze the cells populations: NK cells (defined as CD3⁻/CD16⁺/CD56⁺), and CD4⁺ T-cells (CD3⁺/CD4⁺). The expression of CD69, LAG-3, PD-1 and TIM-3 was determined in both NK and CD4⁺ T-cells using appropriate isotype-matched control antibodies and fluorescence minus one strategy. Acquisition was performed using the BD™ LSR II flow cytometer and analyzed by using FlowJo software, version 9.3.3.

The concentrations of IP-10, TNF-α, IFN-γ, IL-12, IL-13, IL-1β and G-CSF were determined in plasma samples using a custom multiplex bead array kit (Millipore Corporation, Burlington, MA). Data were acquired on a Bioplex200 and analyzed by using Bioplex Manager software, version 5.0 (BioRad Corporation, Mississauga, Canada). The detection limits were 8.6 pg/mL for IP-10 and 0.7 pg/mL for TNF-α.

Results are presented as median. To compare data from HESN vs. Low risk individuals, and HIV-controllers vs. HIV-progressors, a non-parametric test (Mann-Whitney U - two-tailed test) was used. A p value <0.05 was considered statistically significant. The statistical analysis was performed using the Graph-Pad Software version 5.00.

Compared to low risk individuals, the HESN exhibited a lower percentage of NK cells expressing CD69 (p=0.0079), LAG-3 (p=0.008), PD-1 (p=0.0079), and Tim-3 (p=0.0079). Similar results were obtained on CD4⁺ T-cells, where HESN had lower expression of LAG-3 (p=0.007), PD-1 (p=0.0079) and Tim-3 (p=0.0119) (Figure 1A and B). In addition, as an approximation of the expression of these molecules on CD8+ T cells, we evaluated the gate of
CD3+ CD4− T cells, where the expression of PD-1 and LAG-3 was lower in the HESN group compared to low risk.

Regarding the HIV-controller population, the expression of activation and exhaustion molecules was decreased on NK cells from HIV-controllers, who exhibited lower expression of CD69 ($p=0.0303$), LAG-3 ($p=0.0303$), PD-1 ($p=0.0135$) and Tim-3 ($p=0.0087$) compared to HIV-progressors (Figure 1 C). The expression of HLA-DR and CTLA-4 was also evaluated, but no significant differences were observed (data not shown). The expression of activation and exhaustion molecules on CD4+ T-cells from HIV-controllers and HIV-progressors was not measured due to sample restrictions.

When plasma level of pro-inflammatory cytokines was measured, decreased levels of TNF-α ($p=0.0224$) and IP-10 ($p=0.0470$) were observed in HIV-controllers compared to HIV-progressors (Figure 2A and B). Plasmatic concentrations of IFN-γ, IL-12, IL-13, IL-1β and G-CSF were undetectable. This determination was only carried-out in HIV-infected patients.

The study of mechanisms involved in resistance to HIV-1 infection or to AIDS progression is important to clarify viral pathogenesis to facilitate the development of new immunological therapies. Here, we evaluated the expression of CD69 as an activation marker, and LAG-3, PD-1 and Tim-3 as immune regulatory molecules on NK cells and CD4+ T cells in cohorts of HESN and HIV-controllers who were compared to low risk population and HIV-progressors, respectively. Our data shown that both, HESN and HIV-controllers exhibited lower expression of all molecules evaluated, compared with their respective control group. In addition, HIV-controllers exhibited decreased
plasma levels of the pro-inflammatory cytokines TNF-α and IP-10. It is important to note that the two cohorts of HESN/Low risk vs. HIV-controllers/progressors were not comparable as they have genetic and geographical differences.

The initial immune response during sexual exposure to HIV-1 is critical, because it defines whether the virus is eliminated at the mucosal site or if the infection is established. Several investigators have demonstrated the presence of genetic and immunological mechanisms that can reduce the risk of acquiring HIV-1 infection or limit its progression, including the presence of genetic polymorphisms in the viral co-receptors, innate and adaptive immune cells with particular phenotypic and functional features, and molecules such as antibodies and soluble factors that play an important role in defense against HIV-1 infection. Moreover, some reports indicate that HESN individuals had a lower basal expression of activation molecules, as well as lower production of proinflammatory cytokines, compared to healthy controls. Using microarray assays, it was demonstrated that HESN exhibit a low level of expression in genes encoding activation molecules, suggesting a quiescent cellular state. These data supports our observation of low expression of CD69 on NK cells in HESNs. Low activated immune cells are less prompted to become infected and less susceptible to produce viral particles.

There is limited information on the relevance of the expression of inhibitory molecules during HIV-1 exposure. One study reported that dendritic cells and monocytes exposed in vitro to HIV-1 showed a significant increase in PD-1 ligand (PD-L1) expression, which in consequence reduced the response of the interacting T cells, suggesting that during HIV-1 exposure the basal
expression of inhibitory molecules could be influencing the immune response, therefore the establishment of the infection.

The lower expression of inhibitory molecules on immune cells from HESNs could be an intrinsic consequence of their lower expression of activation molecules, as it has been previously shown, in order to be able to become effector cells when needed \(^{28}\). This is supported by reports showing that despite the low level of expression of activation molecules, HESNs are able to mount an effective immune response upon stimulation \(^{27}\). Alternatively, HESNs could have genetically low baseline levels of activation/inhibitory molecules associated to polymorphisms in genes encoding for these proteins. Indeed, generic variations have been reported in PD-1, Tim-3 and CTLA-4 genes that can modulate the baseline expression of these molecules \(^{31–33}\). Interesting, the minor G allele at rs4704846 in the gene that encodes Tim-3 (HAVCR2), was more common in HIV-1 infected individuals than in HESN. This variation increases the expression of HAVCR2, and most likely reduces the response against HIV-1 during exposure \(^{34}\). However, one study reported a high expression of Tim-3 in mature NK cells that responded efficiently after the stimulation with cytokines \(^{35}\). This somehow contrasting data underlines the requirement of further studies exploring the role of these molecules during the viral challenge.

On the other hand, in HIV-1-infected patients, negative regulators of the immune response are associated to AIDS progression, as well as an inflammatory environment and immune activation \(^{15,36}\). In fact, the expression of HLA-DR and CD38 on CD4\(^+\) and CD8\(^+\) T cells is currently proposed as predictors of AIDS, as their expression indicates immune hyperactivation \(^{36}\). In
In this sense, the expression of inhibitory molecules has been related with low production of cytokines and cytotoxic molecules, like IFN-γ and perforin. In fact, the expression of PD-1, CTLA-4, LAG-3 and Tim-3 is positively correlated to viral load and negatively to CD4+ T cell counts. Supporting these previous evidences we found that HIV-controllers exhibited lower expression of activation and inhibitory molecules along with lower production of proinflammatory cytokines, compared to HIV-progressors. Previous studies in HIV-controllers suggest that the early control of viral replication is mediated by different effector mechanisms, including: i) increased HIV-specific IL-21+ CD4+ T-cell responses, associated with optimal cytotoxic responses mediated by CD8+ T-cells; ii) greater cytolytic activity of HIV-specific CD8+ T-cells, mainly directed to cells expressing different Gag epitopes; iii) effective production of cytokines and cytotoxic response of NK cells; iv) higher HIV–specific neutralizing antibody responses; and v) increased expression of soluble antiviral proteins. It seems that in HIV-1 controllers, low viral replication over time is a key factor to maintain in normal levels the expression of activation and inhibitory molecules, as well as a low proinflammatory environment that contributes to preservation of the immune function.

In conclusion, a reduced proportion of immune cells expressing activation and inhibitory molecules may contribute to the inhibition of HIV-1 infection in those individuals exposed to the virus, or with the control of viral replication in infected patients with low or undetectable viral load, as they can respond effectively, reducing the risk of infection or AIDS progression. Finally, our data support the immune quiescence hypothesis in HESN and expand it to disease control.
Acknowledgments

The authors thank patients and volunteers who kindly participated in this study. We also acknowledge to Carlos Julio Montoya and Walter Osorio for their support in patients recruitment.

Sources of support

This investigation was supported by CODI-Universidad de Antioquia convocatoria mediana cuantia 2011; Estrategia para Sostenibilidad de Grupos 2014-2015 Universidad de Antioquia; CIHR IID&GH Program of the University of Manitoba; Natalia Taborda received a scholarship from Colciencias “Convocatoria Nacional 511 para el estudio de Doctorado en Colombia año 2010” Support also from Canadian Institutes for Health Research grant MOP-86721.

Conflict of interest

The authors declare that they have no conflict of interests.
REFERENCES


