the same time, an obvious reduction in $CD4^+$ T lymphocytes was not found in the PB. In an attempt to compensate for the loss of $CCR5^+$ T_{EM}, $CCR5^-$ T_{CM} was persistently activated and divided in order to prevent the collapse of the T_{EM} compartment (Brenchley, Price, and Douek, 2006). However, $CCR5^-$ T_{CM} lose their regenerative capability after prolonged period of proliferation, leading to decrease in both T_{CM} and T_{EM} compartments (Brenchley, Price, and Douek, 2006). This continuous shortage in $CCR5^+$ T_{EM} and accompanying $CCR5^-$ T_{CM} exhaustion are thought to play an important role in the progression to AIDS (Centlivre et al., 2007). Although the overloading of $CD4^+$ memory T lymphocyte homeostasis serves a compelling model of immunodeficiency in SIV infection, its relevance in HIV-1 infection is still poorly defined. Therefore, it is necessary that memory T lymphocyte infection is studied in an experimental animal model reconstituted with competent human immune cells.

To investigate the dynamics of $CD4^+$ T lymphocyte depletion following HIV-1 infection and the status of HIV-1-producing cells *in vivo*, we infected human $CD34^+$ cells-transplanted newborn NOG mice (NOG-hCD34 mice) with HIV-1_{JR-CSF} (R5 HIV-1) or HIV-1_{NL4-3} (X4 HIV-1). Our findings indicate that X4 HIV-1 infection can cause the depletion of $CD4^+$ thymocytes which results in the reduction in both naïve and memory T lymphocytes, while R5 HIV-1 infection can selectively deplete memory $CD4^+$ T lymphocytes. Further analyses indicate that R5 HIV-1 preferentially infects $CCR7^-$ T_{EM} and that the infected cells are predominantly activated and in an actively proliferating state. These results suggest that preferential infection in the activated T_{EM} leads to selective depletion of memory $CD4^+$ T lymphocytes in R5 HIV-1-infected patients.

Results

Kinetics of PB $CD4^+$ T lymphocyte depletion in R5 and X4 HIV-1-infected mice

NOG-hCD34 mice were generated by human $CD34^+$ hematopoietic stem cell transplantation into neonatal NOG mice as described previously (Baenziger et al., 2006; Traggiai et al., 2004). A significant level of human leukocytes was maintained in the whole PB of 13–44

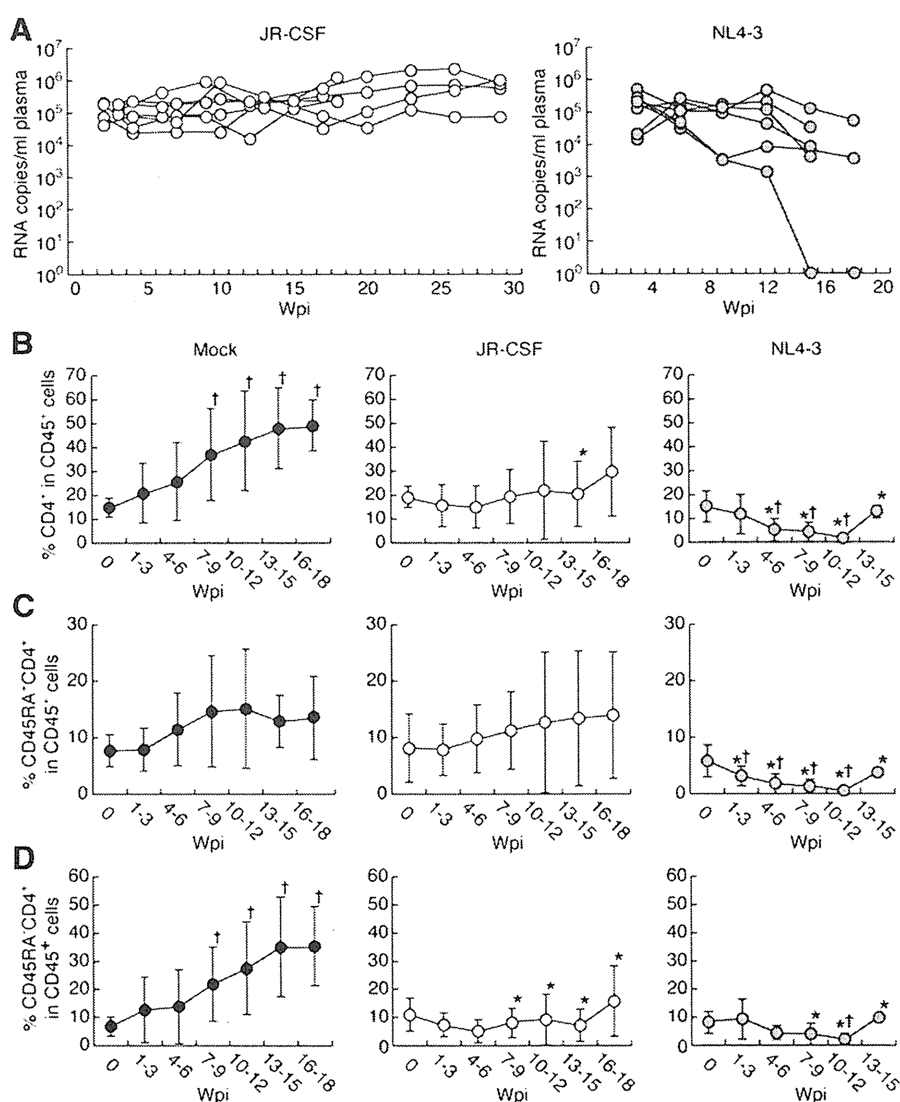

Fig. 1. Longitudinal analysis on plasma viral load and $CD4^+$ T lymphocytes in the PB of R5 and X4 HIV-1-infected mice. NOG-hCD34 mice were intraperitoneally injected with 1×10^5 $TCID_{50}$ of HIV-1_{JR-CSF} ($n = 7$) or HIV-1_{NL4-3} ($n = 8$) between 12 and 13 weeks olds. (A) The longitudinal analysis on the plasma viral load of HIV-1_{JR-CSF}-infected (left) and HIV-1_{NL4-3}-infected (right) mice. (B–D) PB was routinely sampled and analyzed for CD45RA expression in $CD4^+$ T lymphocytes from mock-infected ($n = 8$), HIV-1_{JR-CSF}-infected ($n = 7$), and HIV-1_{NL4-3}-infected ($n = 8$) mice. We assigned data into 7 periodic groups (data taken at 0, between 1–3, 4–6, 7–9, 10–12, 13–15, and 16–18 wpi), and the average percentage and standard deviation were calculated using data obtained from each mouse during each time period. The percentages of $CD4^+$ (B), $CD45RA^+CD4^+$ (C), and $CD45RA^-CD4^+$ (D) T lymphocytes in the peripheral $CD45^+$ cells are shown. Error bars show standard deviations. Daggers represent statistical difference ($P < 0.05$) when compared to the value at 0 wpi, and asterisks represent statistical difference when compared to the value obtained from the mock-infected mice.

week old mice (Supplemental Fig. 1A). Hematopoietic and lymphoid organs such as thymus, bone marrow, spleen and lymph nodes were highly repopulated with human mononuclear cells (Supplemental Figs. 1B–E). The expression of CCR5 was mainly restricted within the $CD45RA^-$ memory subset in $CD4^+$ T lymphocytes, and CXCR4 was broadly expressed on both naïve and memory T lymphocytes (Fig. 4C and data not shown) as observed in humans (Ebert and McColl, 2001).

NOG-hCD34 mice were inoculated with either an R5 HIV-1 (HIV-1_{JR-CSF}) or an X4 HIV-1 (HIV-1_{NL4-3}) between 12 and 13 weeks old. HIV-1 RNA was detected in the plasma of these mice as early as 3 weeks post-infection (wpi) and was maintained at high levels (1×10^4 to 10^6 copies per milliliter) until 28 wpi or until sacrificed (Fig. 1A). PB of these mice was then analyzed for longitudinal changes in $CD4^+$ T lymphocytes by flow cytometry. In the PB of both HIV-1_{JR-CSF}-infected and HIV-1_{NL4-3}-infected mice, depletion of human $CD4^+$ T lymphocytes was consistently found (Fig. 1B). In HIV-1_{NL4-3}-infected mice, both $CD4^+CD45RA^+$ naïve and $CD4^+CD45RA^-$ memory T lymphocytes were depleted, whereas in HIV-1_{JR-CSF}-infected mice, $CD4^+CD45RA^-$ memory T lymphocytes were specifically depleted (Figs. 1C and D). These data indicate that the infection with HIV-1_{NL4-3} caused faster and more severe depletion of both naïve and memory subsets of $CD4^+$ T lymphocytes and the infection with HIV-1_{JR-CSF} preferentially depleted memory $CD4^+$ T lymphocytes.

Thymopathy in X4 HIV-1-infected mice

To investigate the effect of HIV-1 infection on the thymopoiesis in NOG-hCD34 mice, the thymocytes from HIV-1-infected and mock-infected mice were isolated and were analyzed with flow cytometry. In mock-infected and HIV-1_{JR-CSF}-infected mice, CD4 and CD8 double positive (DP) thymocytes were predominant (Fig. 2A). CD4 single positive (SP) and CD8 SP thymocytes together made up a major fraction of the thymocyte population, and double negative (DN) thymocytes were only a minor fraction. In contrast, thymi from HIV-1_{NL4-3}-infected mice were severely depleted of both CD4 SP thymocytes and DP thymocytes (Figs. 2A–C). Furthermore, thymi from HIV-1_{NL4-3}-infected mice had greatly reduced number of all subsets of thymocytes (Fig. 2D). CD4 SP and DP thymocytes showed the greatest (approximately 100-fold) reduction, while CD8 SP thymocytes showed relatively milder (approximately 10-fold) reduction (Fig. 2D). These data indicate that infection with HIV-1_{NL4-3} led to disturbed thymopoiesis and that HIV-1_{JR-CSF} infection did not affect thymopoiesis.

Histological detection of p24-positive cells

HIV-1 p24-positive cells productively produce HIV-1 virions. Since human $CD45^+$ mononuclear cells were very few or absent in HIV-1_{NL4-3}-infected mice when sacrificed, they were not further analyzed (data not shown). As presented in Fig. 3, the immunohistological staining showed the presence of HIV-1 p24-positive cells in all of the bone marrow, spleen, and lymph nodes. HIV-1 p24 staining co-localized with CD4 staining. Also, a larger percentage of cells seemed to be productively infected with HIV-1 in the spleen and lymph nodes.

Depletion of splenic memory $CD4^+$ T lymphocytes

We isolated mononuclear cells from the spleen of HIV-1_{JR-CSF}-infected and mock-infected mice and then analyzed them by flow cytometry. As shown in Fig. 4A, the percentage of $CD4^+$ T lymphocytes in the spleen of HIV-1_{JR-CSF} mice was smaller than that of mock-infected mice by 2.7-fold ($P=0.003$), showing that HIV-1_{JR-CSF}-infected mice had significantly fewer splenic $CD4^+$ T lymphocytes. Moreover, the percentage of splenic $CD4^+CD45RA^-$ memory T lymphocytes in HIV-1_{JR-CSF}-infected mice was smaller than that in the mock-infected mice ($P=0.007$), whereas the percentages of splenic $CD4^+CD45RA^+$ naive T lymphocytes were indifferent ($P=0.17$) (Fig. 4B). In mock-infected mice, a significant fraction of $CD4^+CD45RA^-$ T lymphocytes were $CCR5^+$ memory T lymphocytes (Fig. 4C). In contrast, in HIV-1_{JR-CSF}-infected mice, we found approximately 20-fold reduction in the percentage (Fig. 4C) and 100-fold reduction in the number of $CD4^+CD45RA^-CCR5^+$ memory T lymphocytes (data not shown). These results suggest that the CCR5-expressing memory $CD4^+$ T lymphocytes are depleted by direct R5 HIV-1 infection and that such reduction of CCR5-expressing $CD4^+$ T lymphocytes would lead to the decrease in whole memory $CD4^+$ T lymphocytes.

Preferential HIV-1 productive infection in CD4-negative effector memory T lymphocytes

To characterize the immunophenotypes of HIV-1 productively infected cells in NOG-hCD34 mice, splenic mononuclear cells from

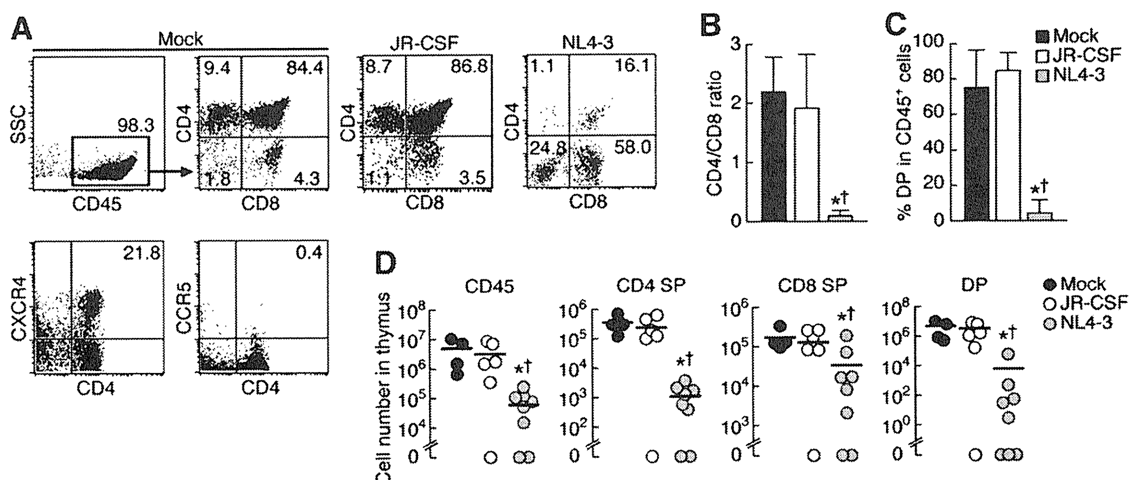

Fig. 2. Thymopathy in X4 HIV-1-infected mice. (A) Representative profile of flow cytometric analysis in thymi of mock-, HIV-1_{JR-CSF}-, and HIV-1_{NL4-3}-infected mice. The numbers in dot plots indicate the percentage of cells in $CD45^+$ thymocytes. (B and C) CD4/CD8 ratio (B) and the percentages of DP cells in $CD45^+$ thymocytes (C) in mock-infected ($n=4$), HIV-1_{JR-CSF}-infected ($n=5$), and HIV-1_{NL4-3}-infected ($n=6$) mice. (D) Number of $CD45^+$, CD4 SP, CD8 SP, and DP cells in thymi of mock-infected ($n=4$), HIV-1_{JR-CSF}-infected ($n=6$), and HIV-1_{NL4-3}-infected ($n=8$) mice. The horizontal bars in D show the average values, and the error bars in B and C show standard deviations. Asterisks indicate statistical significance ($P<0.05$) when compared to mock-infected mice, and daggers indicate statistical significance when compared to HIV-1_{JR-CSF}-infected mice.

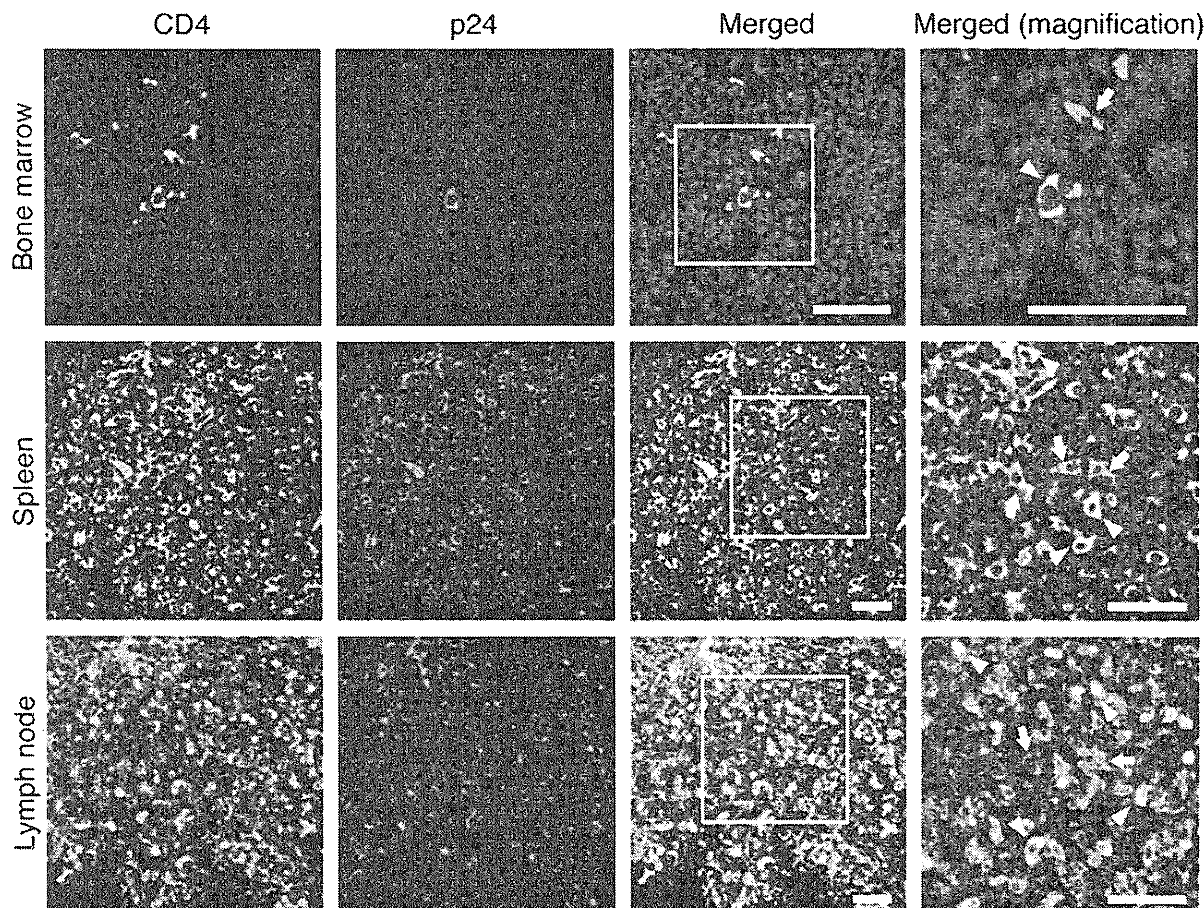

Fig. 3. Histological analysis on R5 HIV-1-infected mice. Representative immunohistological analysis of CD4 (green) and HIV-1 p24 (red) in the slices of bone marrow, spleen, and lymph nodes of HIV-1_{JR-CSF}-infected NOG-hCD34 mice. The low magnification images of the bone marrow slices were taken at ×80, and the high magnification images were taken at ×160. The low magnification images of the spleen and lymph nodes slices were taken at ×40, and the high magnification images were taken at ×80. The areas enclosed with the white squares were enlarged. The arrows point at representative $CD4^+$ cells and the arrowheads point at representative $CD4^+p24^+$ cells. Scale bars, 50 μm.

HIV-1_{JR-CSF}-infected mice were further analyzed for HIV-1 antigen p24 and the expression of lymphocyte surface markers. The anti-p24 antibody that we used did not react with any of the cells isolated from the mock-infected mice (Fig. 5A), as reported previously (Okuma et al., 2008). A significant fraction of splenic leukocytes expressed HIV-1 p24 and thus was productively infected with HIV-1 (Fig. 5A). The productively infected cells expressed surface CD3 but lacked surface CD4 (Figs. 5B and C). On average, over 90% of the $p24^+$ cells were $CD3^+$, yet only about 5% of these cells expressed surface CD4 (Fig. 5C). Also, p24-expressing cells were positive for CD45RO but not for CD45RA, suggesting that they were memory T lymphocytes (73.7±24.3% for $CD45RO^+CD45RA^-$ in $p24^+$ cells; Figs. 5D and E). Central memory T lymphocyte (T_{CM}) can be defined as a memory T lymphocyte that expresses CCR7, and effector memory T lymphocyte (T_{EM}) can be defined as a memory T lymphocyte that lacks CCR7 (Sallusto, Geginat, and Lanzavecchia, 2004). In p24-positive cells, 88.0±3.75% was negative for CCR7 (Figs. 5D and F), suggesting that T_{EM} dominantly and productively infect with HIV-1.

Productive HIV-1 infection in activated and dividing lymphocytes

To investigate the activation status of the productively infected cells, splenic mononuclear cells were stained with anti-p24, anti-Ki67, and anti-CD69 antibodies. Ki67 antigen is exclusively expressed in proliferating cells, and CD69 is expressed on the surface of the activated cells at the early phase (Sereti et al., 2007; Vatakis et al., 2007). In splenic $CD4^+$ T lymphocytes from mock-infected mice or p24-negative splenocytes from HIV-1_{JR-CSF}-infected mice, only a minor fraction of the cells expressed either Ki67 or CD69 (Figs. 6A and B). In contrast, the majority of p24-positive splenocytes from HIV-1_{JR-CSF}-infected mice expressed Ki67 and/or CD69 (Figs. 6A and B). Also, the percentage of cells positive for both Ki67 and CD69 were higher in p24-positive cells than in p24-negative splenocytes from HIV-1_{JR-CSF}-infected mice and in splenic $CD4^+$ T lymphocytes from mock-infected mice (Fig. 6B). These results indicate that a significantly higher frequency of p24-positive cells is activated and/or proliferating cells. Notably, although the frequency was significantly low, we could detect $Ki67^-CD69^-$ resting T lymphocytes in p24-positive cells (Figs. 6A and B).

To further analyze the cell cycle of HIV-1 productively infected cells (i.e., p24-positive cells), we carried out Hoechst staining, which quantifies DNA content of the cells. Ki67 staining in combination with the Hoechst staining will sort cells into those in G_0/G_{1a}, G_{1b}, and $S/G_2/M$ phases of the cell cycle (Wilpshaar et al., 2000). As shown in Fig. 6C, non-stimulated human peripheral blood leukocytes (PBLs) predominantly exist in G_0/G_{1a} phases ($Ki67^-Hoechst^{low}$, lower left in the quadrant), while PHA-activated human PBLs predominantly exist in cycling G_{1b} phase ($Ki67^+Hoechst^{low}$, upper left in the quadrant) and $S/G_2/M$ phases ($Ki67^+Hoechst^{high}$, upper right in the quadrant). By using this method, we observed that p24-positive cells contained a significantly higher frequency of cells in the G_{1b} phase. In addition, the percentage of p24-positive cells in $S/G_2/M$ phases was significantly higher than $CD4^+$ splenocytes from mock-infected mice (Figs. 6D and E). These findings indicate that the majority of HIV-1-producing cells in the spleen of R5 HIV-1-infected mice are activated and in cycling phase. On the other hand, we detected the p24-

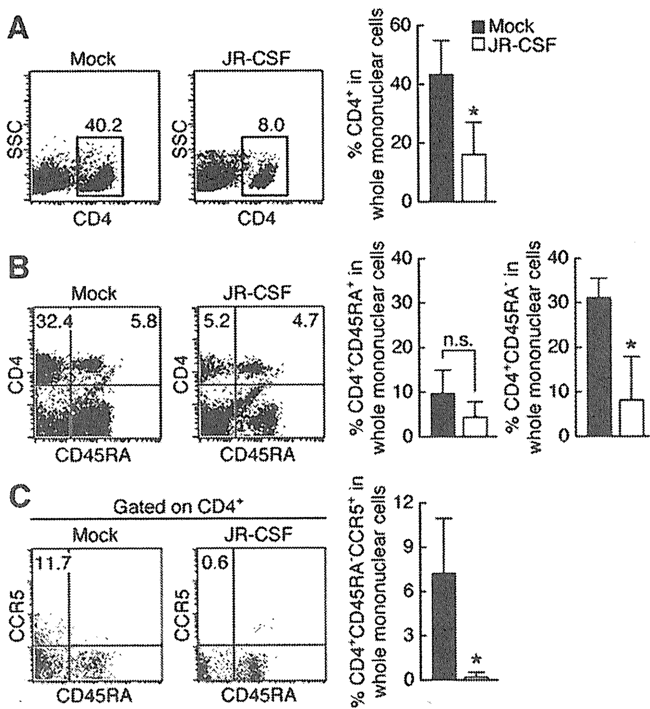

Fig. 4. The effect of R5 HIV-1 infection on the splenic $CD4^+$ T lymphocyte population. (A–C) Staining of splenic nucleated cells from the spleen of mock-infected ($n = 4$) and HIV-1_{JR-CSF}-infected ($n = 4$) mice were stained with CD4 (A), CD4 and CD45RA (B), and CCR5, CD4, and CD45RA (C). Representative profiles are shown, and the numbers in dot plots indicate the percentage of cells in $CD45^+$ splenic human leukocytes (A and B) or in $CD4^+$ cells (C). The graphs show the percentages of cells possessing each phenotype in whole mononuclear cells. The error bars show standard deviations. Asterisks indicate statistical significance ($P<0.05$) when compared to mock-infected mice.

positive splenocytes in G_0/G_{1a} phases, although the frequency was significantly lower than p24-negative splenocytes or $CD4^+$ splenocytes from mock-infected mice (Figs. 6D and E). These data suggest that a fraction of resting cells productively infects HIV-1. Moreover, we detected the significantly higher percentage of cells in $S/G_2/M$ phases in splenic p24-negative cells of HIV-1_{JR-CSF}-infected mice when comparing to that in splenic $CD4^+$ T lymphocytes of mock-infected mice (Figs. 6D and E).

Discussion

To investigate the mechanisms of $CD4^+$ T lymphocyte depletion by HIV-1 infection, we utilize human $CD34^+$ cells-transplanted NOG mice (Ito et al., 2002) and demonstrate that human $CD4^+$ T lymphocytes were differentially affected by X4 and R5 HIV-1 infection (Figs. 1–4). X4 virus induced immediate depletion of both naïve and memory $CD4^+$ T lymphocytes in periphery, while R5 virus gradually depleted memory $CD4^+$ T lymphocytes in the PB (Fig. 1) and spleen (Fig. 4). Our data suggest that distinctive pathogenesis of X4 and R5 viruses in NOG-hCD34 mice was caused by thymopathy (Fig. 2) and preferential infection of activated and dividing T_{EM} (Figs. 5 and 6), respectively. This is the first report addressing the mechanisms and dynamics of HIV-1-induced $CD4^+$ T lymphocyte depletion *in vivo*.

As previously shown in X4 SHIV-infected macaques (Ho et al., 2005; Nishimura et al., 2004), we observed the drastic loss of both naïve and memory T lymphocytes by X4 HIV-1-infected NOG-hCD34 mice (Fig. 1). We also found that $CD4^+$ thymocytes in NOG-hCD34 mice abundantly express CXCR4 (Fig. 2A) and that the $CD4^+$ thymocytes including DP and CD4 SP were preferentially reduced in HIV-1_{NL4-3}-infected mice (Fig. 2). It has been reported that intrathymic infection by X4 HIV-1 can lead to severe T lymphocytopenia (Berkowitz et al., 1998; Schnittman et al., 1990; Ye, Kirschner, and Kourtis, 2004). Therefore, our results suggest that the primary mechanism for naïve and memory T lymphocyte depletion in X4 virus infection can be attributed to impaired thymopoiesis caused by intrathymic infection.

In contrast to X4 HIV-1 infection, the depletion of PB $CD4^+$ T lymphocytes was more gradual and less intense in R5 HIV-1 infection and was confined to $CD45RA^-$ memory $CD4^+$ T lymphocytes (Fig. 1D). The selective depletion of memory $CD4^+$ T lymphocytes by R5 infection was also found in the spleen (Fig. 4). On the other hand, thymopathy was not detected in HIV-1_{JR-CSF}-infected mice (Fig. 2). These findings suggest that the selective depletion of memory $CD4^+$ T lymphocytes in PB and spleen of HIV-1_{JR-CSF}-infected mice caused through a different mechanism from HIV-1_{NL4-3}, and the mechanisms are further discussed below.

To investigate the mechanisms of memory $CD4^+$ T lymphocyte depletion in R5 HIV-1 infection in-depth, a series of flow cytometric analyses was carried out. The majority of $p24^+$ productively infected cells in the spleen were $CD3^+$ T lymphocytes (Figs. 5B and C). However, these infected cells were negative for surface CD4 (Figs. 5B

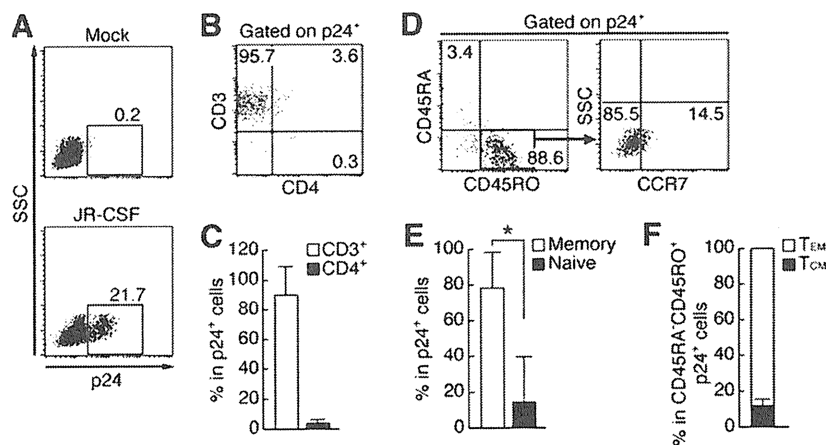

Fig. 5. Phenotype of productively infected $p24^+$ cells in the spleen of R5 HIV-1-infected mice. (A) Representative profiles of flow cytometric p24 staining in splenic nucleated cells of mock-infected ($n = 4$) and HIV-1_{JR-CSF}-infected ($n = 6$) mice. The numbers indicate the percentage of cells in splenic nucleated cells. (B and C) Staining of splenic nucleated cells of HIV-1_{JR-CSF}-infected mice for p24, CD3, and CD4. Representative profiles are shown in B, and the numbers in each quadrant indicate the percentage of cells in $p24^+$ cells. The percentages of each population in $p24^+$ cells are shown in C. (D–F) Staining of splenic nucleated cells of HIV-1_{JR-CSF}-infected mice for p24, CD45RA, CD45RO, and CCR7. Representative profiles are shown in D, and the numbers in each quadrant indicate the percentage of cells in $p24^+$ cells (left) or in $p24^+CD45RA^-CD45RO^+$ cells (right). The percentages of memory ($CD45RA^-CD45RO^+$) and naïve ($CD45RA^+CD45RO^-$) phenotyped cells in $p24^+$ cells are shown in E. The percentages of T_{EM} ($CCR7^-CD45RA^-CD45RO^+$) and T_{CM} ($CCR7^+CD45RA^-CD45RO^+$) in $p24^+$ cells are shown in F. The error bars in C, E, and F show standard deviations. Asterisks indicate statistical significance ($P<0.05$).

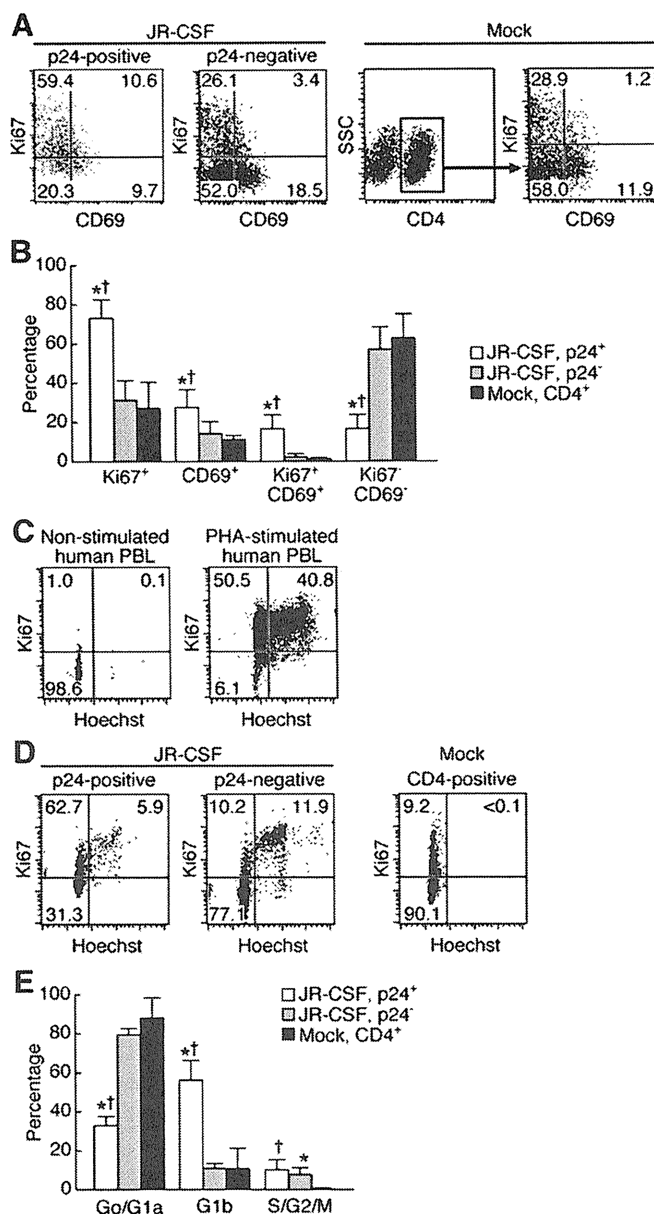

Fig. 6. Cell cycle analyses on productively infected p24$^+$ cells in the spleen of R5 HIV-1-infected mice. (A and B) Staining of splenic nucleated cells of mock-infected ($n=3$) and HIV-1_{JR-CSF}-infected ($n=4$) mice for Ki67, CD69, and either CD4 or p24. Representative profiles are shown in A, and each number indicates the percentage of cells in each quadrant. The graph in B shows the average percentages of cells possessing each population. (C) A representative profile of cell cycle analysis on non-stimulated human PBL (left panel) and PHA-activated human PBL (right panel) by using anti-Ki67 antibody and Hoechst. Each number indicates the percentage of cells in each quadrant. Ki67$^-$Hoechst^{low} (lower left in quadrant) indicates G_0/G_{1a} phase, while Ki67$^+$Hoechst^{low} (upper left in quadrant) and Ki67$^+$Hoechst^{high} (upper right in quadrant) indicate G_{1b} and $S/G_2/M$ phases, respectively. (D and E) Staining of splenic nucleated cells of mock-infected mice ($n=4$) for CD4, Ki67, and Hoechst, and HIV-1_{JR-CSF}-infected mice ($n=4$) for p24, Ki67, and Hoechst. Representative profiles are shown in D, and each number indicates the percentage of cells in each quadrant. The graph in E shows the average percentages of cells in each population. The error bars in B and E show standard deviations. Asterisks indicate statistical significance ($P<0.05$) when compared to the value of p24-negative cells in HIV-1_{JR-CSF}-infected mice, and daggers indicate statistical significance when compared to the value of CD4-positive cells in mock-infected mice.

and C), although immunohistological analysis revealed that splenic p24$^+$ cells expressed CD4 molecules (Fig. 3). These results suggest that surface CD4 molecules are severely down-regulated following infection. In fact, it has been well documented that HIV-1 gene products such as *Nef* (Fackler, Alcover, and Schwartz, 2007; Roeth and Collins, 2006), *Env* (Crise, Buonocore, and Rose, 1990), and *Vpu* (Bour and Strebel, 2003; Geleziunas, Bour, and Wainberg, 1994) have the potential to down-regulate CD4 molecules from the surface of infected cells (Lindwasser, Chaudhuri, and Bonifacino, 2007). Similar down-regulation of surface CD4 has also been reported in lymph nodes and PB of HIV-1-infected patients (Cheney et al., 2006; Kaiser et al., 2007; Marodon et al., 1999). Therefore, this down-regulation of surface CD4 molecules in HIV-1_{JR-CSF}-infected mice is physiologically relevant and can play a role in the reduction of CD4$^+$ T lymphocytes.

In HIV-1_{JR-CSF}-infected mice, more than 80% of the productively infected cells were activated and in cycling phase (Fig. 6). It has been well known that activated cells massively produce HIV-1 virions (Ho et al., 1995). Therefore, this result suggests that the persistent viremia in R5 infection (Fig. 1A) is primarily due to the productive infection in the activated and proliferating cells. On the other hand, a fraction of infected cells were quiescent T lymphocytes negative for both activation and proliferation markers (Fig. 6). It is thought that HIV-1 cannot manifest productive infection in quiescent cells (Stevenson et al., 1990; Zack et al., 1990). However, studies on *ex vivo* infected human tonsil histocultures (Eckstein et al., 2001; Kinter et al., 2003), small intestines and cervix of SIV-infected rhesus macaques (Li et al., 2005; Zhang et al., 1999; Zhang et al., 2004), and HIV-1-infected patients (Zhang et al., 1999) have established that quiescent T lymphocytes residing in the lymphoid tissues are capable of supporting productive SIV or HIV-1 infection. Our results provide further support that productive infection of HIV-1 can take place in non-dividing cells, presumably resting T lymphocytes, in NOG-hCD34 mice. Productive infection not only in proliferative cells but also in quiescent cells may be an important factor in CD4$^+$ T lymphocyte depletion and persistent virus infection.

The preferential infection of CD45RO$^+$CD45RA$^-$ memory T lymphocytes with R5 HIV-1 is also the evidence supportive for the selective depletion of memory CD4$^+$ T lymphocytes (Figs. 5D and E). Nevertheless, only $16.3\pm9.7\%$ of splenic CD4$^+$ T lymphocytes expressed CCR5 in NOG-hCD34 mice, and the severe depletion of memory T lymphocytes in R5 HIV-1 infection cannot be explained by cell death caused by infection solely. In this regard, it has been reported that memory CD4$^+$ T lymphocyte reduction in SIV-infected macaques can be initiated by specific disruption of T_{EM} due to its preferential infection in the acute phase (Centlivre et al., 2007; Mattapallil et al., 2005; Okoye et al., 2007). In response to the T_{EM} reduction, T_{CM} proliferates and supplies *de novo* T_{EM} (Sallusto, Geginat, and Lanzavecchia, 2004). However, Brenchley et al. (2004) and Okoye et al. (2007) have reported that R5 virus infection induces chronic immune activation in macaques, which leads to the attenuation of regenerative capacity of T_{CM} (Brenchley et al., 2004; Okoye et al., 2007). In addition to the depletion of T_{EM} by direct infection, the attenuation of regenerative potential of T_{CM} causes not only the loss of T_{CM} but also the shortage of T_{EM} and eventually leads to the reduction of whole memory T lymphocytes (Brenchley et al., 2004; Okoye et al., 2007). This hypothesis of the dynamics of CD4$^+$ T lymphocyte depletion has been helpful for explaining the memory CD4$^+$ T lymphocyte in effector sites of SIV-infected macaques. Our observations in HIV-1_{JR-CSF}-infected mice, the severe depletion of memory T lymphocytes despite the limited availability of CCR5-expressing CD4$^+$ T lymphocytes and the preferential infection in T_{EM}, can be explained by the aforementioned hypothesis proposed in the previous literature (Brenchley et al., 2004; Okoye et al., 2007). Notably, we found that the frequency of cells in $S/G_2/M$ phases elevated in splenic p24-negative cells of HIV-1_{JR-CSF}-infected mice when comparing to that in splenic CD4$^+$ T lymphocytes of mock-infected mice (Figs. 6D and E). These data may explain that HIV-1 pathophysiology is caused by accelerated cells division, ultimately leading to the exhaustion of CD4$^+$ T lymphocytes. Taken together, our findings suggest that the selective infection of T_{EM} may be an important event that governs CD4$^+$ T lymphocyte depletion not

only in the effector sites of macaques during SIV infection but also in lymphoid organs during HIV-1 infection.

In summary, we showed differential $CD4^+$ T lymphocyte reduction in R5 and X4 HIV-1 infection. We report for the first time the selective depletion of memory $CD4^+$ T lymphocytes and the preferential infection of T_{EM} in an experimental model of R5 HIV-1 infection. Our data suggest that HIV-1 infection in T_{EM} can be an important step leading to $CD4^+$ T lymphocyte decline. Our findings confirm the applicability of NOG-hCD34 mice as a useful model to study the dynamics of HIV-1 pathogenesis including $CD4^+$ T lymphocytes depletion *in vivo*.

Materials and methods

Mice

NOD/SCID/IL-2Rγ^{null} (NOG) mice (Ito et al., 2002) were obtained from the Central Institute for Experimental Animals (Kanagawa, Japan). The mice were maintained under specific pathogen-free conditions and were handled in accordance with the Regulation on Animal Experimentation at Kyoto University.

Purification and transplantation of cord blood-derived CD34 cells

The purification of cord blood-derived CD34 cells was conducted as described previously (Ishikawa et al., 2005; Ito et al., 2002). Fresh human cord blood was obtained with parent written informed consent from healthy full-term newborns and CD34 MicroBead Kit (Miltenyi Biotec Inc, Auburn, CA) was used to isolate $hCD34^+$ cells according to the manufacturer's instructions. Cells were either stored at −80 °C or immediately transplanted when newborn mice were available. $CD34^+$ cells ($5–12 \times 10^4$) were intrahepatically injected into newborn mice of ages between 0 and 2 days after total radiation of 10 cGy per mouse in MBR-1520 x-ray irradiator (Hitachi Medico, Tokyo, Japan).

Peripheral blood collection and isolation of nucleated cells from organs

PB was routinely taken from NOG-hCD34 mice under ether anesthesia via retro-orbital venousplexus as described previously (Ishikawa et al., 2005). The red blood cells in the PB were lysed in preparation for flow cytometric analysis in 1× BD lysis buffer (BD Pharmingen, San Diego, CA). When the mice were sacrificed, PB was taken by cardiac puncture. Lymph nodes, thymi, spleen, and bone marrow were taken from HIV-1-infected and mock-infected mice upon sacrifice for histological or flow cytometric analysis. Lymph nodes and thymi were gently homogenized using a homogenizer pestle and spleens were crushed and rubbed on a steal mesh with 1-mm grids to generate single cell suspensions in RPMI 1640 supplemented with 4% fetal calf serum (FCS). To collect bone marrow, thigh bones were dissected at both ends and the interior was flushed with RPMI 1640 supplemented with 4% FCS. The cells were immediately used for flow cytometric analysis or stored in Cell Banker (Juji Field Inc., Tokyo, Japan) at −80 °C until use. As there are some variations in the combination of antibodies used to study the human cell population in each mouse, the number of data collected for each surface marker may differ. Data used for any longitudinal analysis were taken from identical mice.

Flow cytometric analysis of human blood cells in transplanted mice

The staining for flow cytometric analysis was done with some modifications to the protocol previously described (Sato et al., 2008). Briefly, for the surface staining, the cells were blocked with FcR blocker (Miltenyi Biotec Inc) for 5 min at room temperature (RT) and then incubated with the appropriate antibodies at optimum concentration in 1× phosphate-buffered saline (PBS) containing 2% FCS for 30 min at 4 °C. Fluorescein isothiocyanate-conjugated (FITC-conjugated) anti-human CD19 (HD37; Dako, Tokyo, Japan), CD8 (DK25; Dako), CD14 (TUK4; Miltenyi Biotec Inc), CD4 (L3T4; eBioscience, San Diego, CA), CD3 (UCHT1; BD Pharmingen, San Diego, CA), CCR5 (3A9; BD Pharmingen), and CD303/BDCA2 (AC144; Miltenyi Biotec Inc) mouse IgG monoclonal antibodies (mAb); phycoerythrin-conjugated anti-human CD3 (UCHT1; Dako), CD4 (MT310; Dako), CD34 (AC136; Miltenyi Biotec Inc), CD11c (B-ly6; BD Pharmingen), CXCR4 (12G5; BD Pharmingen), CCR7 (FAB197; R&D systems, Abingdon, UK), and CCR5 (3A9; BD Pharmingen) mouse IgG mAb; biotinylated anti-human CD45 (H130; eBioscience), CD45RA (HI-100; BD Pharmingen), CD8 (RPA-T8; BD Pharmingen), CD4 (RPA-T4; BD Pharmingen), and mouse IgG mAb; peridinin-chlorophyll-conjugated (PerCP-conjugated) anti-human CD69 (L78; BD Immunocytometry Systems, San Jose, CA) mouse IgG mAb; PE-Cy5-conjugated anti-human HLA-DR (G46-6; BD Pharmingen) mouse IgG mAb; allophycocyamin-conjugated anti-human CD45RO (UCHL1; BD Pharmingen) and CD8 (DK25; Dako) mouse IgG mAb were used. Each antibody was controlled with appropriate isotype antibodies purchased from Dako and BD Pharmingen. Streptavidin-PerCP (SA-PerCP) was purchased from BD Immunocytometry Systems. Following the incubation, the cells were washed and further incubated with SA-PerCP for 30 min at 4 °C, if needed. For the intracellular staining, the cells were permeabilized and fixed by treatment with BD Cytoperm/Cytofix solution (BD Pharmingen) and were stained with FITC-conjugated anti-HIV-1 p24 (clone 2C2) (Okuma et al., 2008) and anti-human Ki67 (B56; BD Pharmingen) mouse IgG mAb for 30 min at 4°C in 1× BD PermWash buffer (BD Pharmingen). For DNA staining to analyze cell cycle, the cells were incubated with Hoechst33342 (Invitrogen, Carlsbad, CA) for 30 min at 4 °C as described previously (Wilpshaar et al., 2000). Data collection was performed on BD FACSCan (BD Biosciences) for 3-color staining, BD FACSCalibur (BD Biosciences) for 4-color staining, and BD FACSCanto (BD Biosciences) for cell cycle analyses using Hoechst33342, and the obtained data were analyzed with CellQuest software (BD Immunocytometry System, San Jose, CA).

HIV-1 infection

NOG mice were injected intraperitoneally with RPMI 1640 ($n = 8$) or 1×10^5 50% tissue culture infective doses ($TCID_{50}$) of HIV-$1_{JR\text{-}CSF}$ ($n = 7$) or HIV-$1_{NL4\text{-}3}$ ($n = 8$) between 12 and 13 weeks of ages. The viruses used were prepared by transfection as previously described (Sato et al., 2008). Infectious titers in the form of $TCID_{50}$ of each virus stock were determined by endpoint dilution with phytohemagglutinin-activated PBMCs as described (Koyanagi et al., 1997).

Detection of HIV-1 RNA in the plasma of infected mice

The detection of HIV-1 RNA in the plasma of the infected mice was routinely carried out using Amplicor HIV-1 monitor v1.5 according to the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).

Immunohistological analysis

Organs were fixed in 1× PBS containing 4% paraformalhyde and embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan) after immersion in 10%–20% gradient sucrose. The OCT embedded organs were then sliced and were permeabilized with 0.1% Triton-X at RT for 10 min, incubated three times with 10 mM glycine for 5 min and blocked with 5% normal goat serum at RT for 1 hr. The sections were then incubated with mouse anti-HIV-1 p24 (Kal-1; Dako) IgG mAb at 4°C overnight, followed by incubation with Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen) at RT for 2 hr. The sections were further incubated with biotinylated mouse anti-

human CD4 IgG mAb (RFT-4g; Southern Biotech, Birmingham, AL) at 4°C overnight, followed by incubation with Streptavidin–Alexa Fluor 647 (Invitrogen) and Hoeschst33342 at RT for 2 hr. All the antibody staining was performed in blocking solution. Images were acquired with a Leica TCS SP2 AOBS confocal laser microscope (Leica Microsystems, Heidelberg, Germany).

Statistical analysis

Data were expressed as an average with standard deviation. Significant differences between data groups were determined by Student's t test or paired t test. A P value less than 0.05 was considered significantly different.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.virol.2009.08.011.

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The 'immunologic advantage' of HIV-exposed seronegative individuals

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Introduction

In 1989, a curious phenomenon was described: HIV-specific T-cell responses to the viral envelope and core proteins could be detected in antibody-positive and antigen-negative sexual partners of known HIV-positive men [1]. Two other reports confirmed that initial observation on a total of six exposed seronegative (ESN) individuals, and the author raised the possibility that exposure to HIV that did not result in seroconversion and infection could be associated with the exclusive priming of T lymphocytes [2,3]. Analyses performed in different cohorts of individuals at high risk of HIV infection, including healthcare workers parenterally exposed to HIV and healthy newborns of HIV-infected mothers, revealed that HIV-specific $CD4^+$ T helper cells, but not antibodies, were present in these persons [4,5]. These observations led to the hypothesis that viral exposure resulting in the exclusive priming of HIV-specific T cells could be associated with protection against the establishment of HIV infection [6].

This hypothesis was greatly strengthened by the independent observations that although the majority of commercial sex workers in Nairobi (the Pumwani cohort) became HIV-infected within a year, a sizable minority, subsequently estimated to be around 15% of the individuals tested, showed resistance to infection [7]; and that HIV-specific cytotoxic T lymphocytes (CTLs) could be isolated from healthy uninfected newborns of HIV-infected mothers [8]. The novel concept of 'resistance' to HIV infection in HIV-exposed individuals was proposed, and the search for immune correlates of such protection against HIV infection was initiated at that point.

Subsequent pivotal reports showed that in HIV-exposed but uninfected individuals a particular genetic background, epitomized by the $\Delta 32$ deletion in the CCR5 receptor gene, can be detected [9], the production of soluble factors, including the $CD8^+$ cell antiviral factor (CAF) and beta-chemokines, is increased [10–12], secretory HIV-specific IgA as well as T helper cells and CTLs can be observed in cervico-vaginal fluids and ejaculates [13,14], and natural killer (NK) cell activity is particularly potent [15]. Thus, 15 years after the first description of the detection of HIV-specific T helper cells in seronegative individuals, the 'immunologic advantage' possibly conferring resistance to HIV infection can be

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summarized as being correlated with the elicitation of systemic and mucosal cell-mediated immunity, presumably within favorable genetic and innate immunity settings. The suggested multiple components of this 'immunologic advantage' are summarized in Fig. 1 and will be discussed in detail within this review.

Terminology

Some methodological notes are needed: a consensus on how to define individuals with reduced susceptibility to HIV infection has not been reached. None of the definitions so far proposed is fully satisfactory. Thus, the definitions of ESN individuals and that of highly exposed persistently seronegatives overlook the possible presence of mucosal IgA in these individuals. The exposed uninfected and the multiple (or highly) exposed but uninfected definitions are based on the possibly erroneous assumption that these individuals have never undergone a subclinical and time-limited infection [6]. Noticeably, there is not the final proof that the immune and genetic correlates described herein confer an absolute resistance to HIV infection; rather, we believe that these correlates are associated with a robust down-modulation of the susceptibility to such infection.

Lacking better definitions, the historic ESN acronym will be used in this review; we will define the immune and genetic correlates of this clinical state as associated with 'reduced susceptibility' to HIV infection. It should be noted, however, that the ESN individuals must not be confused with either long-term non-progressive (LTNP) patients, who have not developed AIDS for an extended period after HIV seroconversion, or with elite suppressors (or controllers), who show very low set-point viral load after acute infection. The latter groups are productively infected with HIV, whereas the HIV genome or proviruses are rarely, if ever, detectable in ESN individuals [2–5,7–13]. We will also concentrate on observations

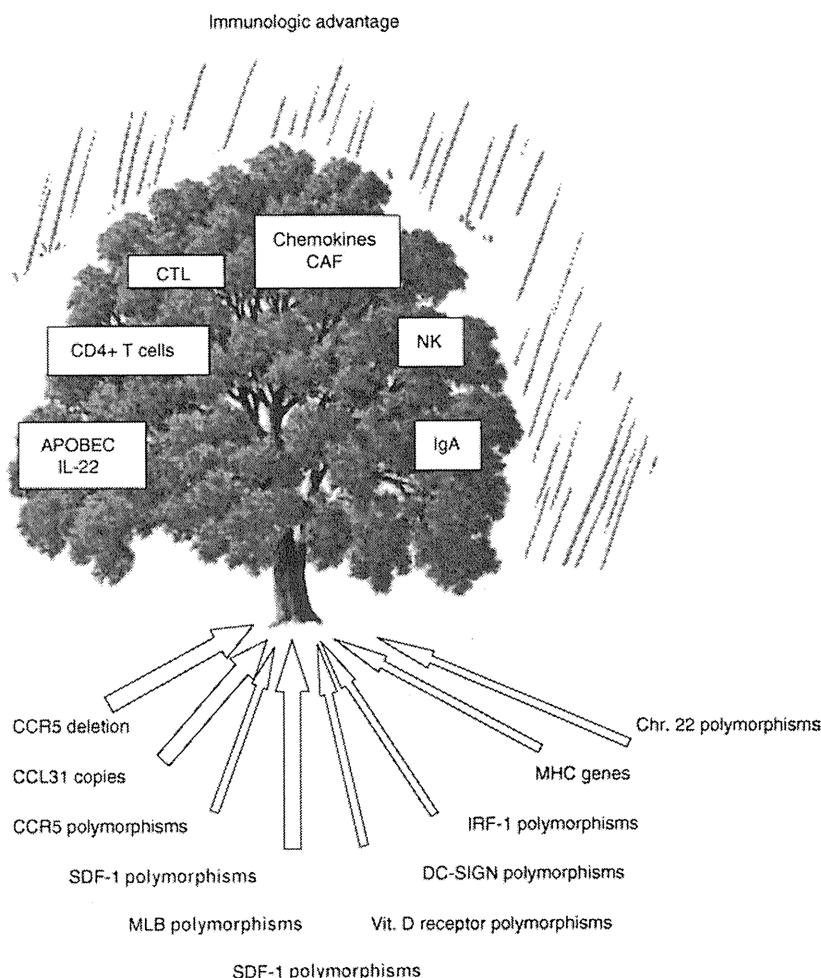

Fig. 1. The 'immunologic advantage' tree. Multiple genetic characteristics are the roots of the peculiar exposed seronegative (ESN) phenotype. These properties result in the activation of multiple immune effector mechanisms that allow the ESN immune system to handle HIV as if HIV was a 'normal' virus and prevent the establishment of infection. This response prevents the initiation of a chronic infection, $CD4^+$ cell depletion, and the development of AIDS. In this figure, $CD4^+$ T cells and cytotoxic T lymphocytes are HIV-specific lymphocytes.

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and studies conducted in humans. Notably, some of these observations are still controversial; the points that are still not fully clarified will be highlighted in the manuscript. Additionally, these studies are often based on small numbers of individuals, and reported results are sometimes almost anecdotic; we will summarize how many ESN individuals have been investigated to draw the main conclusions summarized herein. Finally, attempts to compare results obtained in different cohorts of ESN individuals have often limited success for a number of reasons, and in particular because a clear definition of who should be classified as lacking ESN. A clear definition of who should be classified as ESN is still required.

The small number of ESN individuals analyzed notwithstanding, the overall observations done on ESN individuals have allowed the creation of a quilt whose design is getting more and more complex, but from which a recognizable pattern is slowly emerging.

Immune correlates of reduced susceptibility to HIV infection: cell-mediated immunity

HIV-specific $CD4^+$ T cell responses were initially described in heavily HIV-exposed but seronegative (ESN) men enrolled from the MACS cohort ($n = 5/5$) [3]. These data were confirmed in other ESN groups including healthcare workers ($n = 8/12$) and healthy neonates born of HIV-infected mothers who did not receive antivirals ($n = 8/23$) [4,5]. HIV-specific CTLs in ESN individuals were first described some years later [8,16–19] in a dozen individuals. The lag was probably due to the fact that in the early 1990s, it was technically easier to measure $CD4^+$ T cell than CTL responses.

$CD4^+$ T cells

HIV-specific $CD4^+$ T lymphocytes isolated from ESN individuals were found to produce interleukin (IL)-2 and to proliferate after stimulation with HIV peptides [20–22]. The HIV-specific T cells of ESN individuals were subsequently shown to produce low quantities of IL-10 in comparison with those from HIV-infected individuals [13]. HIV envelope (env)-specific $CD4^+$ T cells of ESN individuals were also shown to generate high levels of CC chemokines, in particular RANTES and MIP-1β [11,23] (RANTES observed in 9/12 ESN individuals enrolled; MIP-1β observed in 24/25 ESN individuals studied), and to be capable of suppressing *in vitro* the replication of macrophage-tropic HIV strains [11]. Given the pivotal role of CC chemokines in modulating receptor binding and replication of HIV [24,25], these results are particularly important in connecting the ESN status and $CD4^+$ T cell responses. Finally, higher levels of tumor necrosis factor (TNF)-α and TNF-β mRNA were detected in ESN individuals than in HIV-infected patients and healthy controls, both at the systemic level in peripheral blood lymphocytes and at the mucosal level in biopsies of the genital tract [26] ($n = 9$). No clear trends were identified regarding interferon (IFN)-γ production, as some groups reported that this cytokine is reduced [27–30] and other research indicated that IFN-γ is increased [12,31] in ESN individuals compared with HIV-infected individuals (Table 1). Notably, recent data suggested that HIV-specific T cell responses in ESN individuals could be dampened by an excess of regulatory T (Treg) cells. Consequently, the removal of $CD4^+CD25^+$ Treg revealed the presence of previously undetected strong HIV-specific T-cell responses in ESN newborns and neonates [35].

Table 1. $CD4^+$ T lymphocytes in exposed seronegative individuals: functional and phenotypic characterization.

Function/phenotype	References
Increased production of IL-2	[20–22]
Increased production of CC chemokines	[11]
Increased TNF-α and TNF-β mRNA	[26]
Increased IFN-γ production	[12,31]
Reduced IFN-γ production	[27–30]
Diminished IL-10 production	[13]
Recognition of rare, conserved epitopes of Env	[32]
Decreased percentage of naive T lymphocytes	[33]
Increased percentage of Gag-specific central memory cells	[33]
Increased percentage of $CD4^+CD25^+$ T cells	[22,25,33,34]

$CD8^+$ T cells

HIV-specific CTLs have been described in several different ESN cohorts and many authors claim that these cells make a fundamental contribution to modulating resistance to HIV infection. HIV-specific CTLs have been observed and characterized in the Pumwani Kenyan cohort of sex workers both at systemic [19,36] and mucosal levels [37]; in injecting drug users [38,39]; and in sexual partners of HIV-infected patients [14,22,26]. These studies have analyzed a total of approximately 100 ESN individuals; HIV-specific CTLs were observed in the majority (>70%) of such ESN (Table 2).

Table 2. $CD8^+$ T lymphocytes in exposed seronegative individuals: functional and phenotypic characterization.

Function/phenotype	References
Increased intracellular concentration of perforin	[40]
Increased intracellular concentration of granzymes	[40]
Recognition of peculiar epitopes	[32]
Increased production of CAF	[10,12]
Reduced IFN-γ production	[27–30]
Increased percentage of Gag-specific central memory cells	[33]
Decreased percentage of Gag-specific effector memory cells	[33]
Increased percentage of $CD8^+/CD38^+$ T cells	[22,25,33,34]
Increased percentage of $CD8^+/CD28^+$ T cells	[22,33,34]

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The detection of HIV-specific CTLs in ESN individuals raises important scientific questions: only the successful infection of host cells, that is, infection resulting in at least one complete cycle of viral replication, allows the effective presentation of viral peptides within a binary complex with a human leukocyte antigen (HLA) class I molecule [41]. The detection of HIV-specific CTLs in ESN individuals thus seems to indicate that HIV has managed to infect the host, but that its further propagation has been contained by immune mechanisms. The recent description of an alternative mechanism of processing and presentation by HLA class I molecules of exogenous antigens known as cross-priming could, nevertheless, explain the presence of CTLs in ESN individuals in the absence of actual infection. According to this mechanism, dendritic cells can process the virus and present it to CTL precursors in the absence of viral replication [42]. If this is the case, the presence of HIV-specific CTLs in ESN individuals would be the consequence, not necessarily of an infection, but of a different and presumably more efficient processing pathway of HIV antigens within dendritic cells.

T-cell responses

Comparison of HIV-specific T lymphocyte responses in ESN and HIV-infected individuals also suggests that the differences in susceptibility to HIV infection between these two groups resides in the quality rather than in the quantity of their immune responses. Thus, HIV-specific $CD8^+$ CTLs of the ESN individuals recognize HIV epitopes that are different from those recognized by cells of HIV-infected patients [43]; rare conserved $CD4^+$ T-cell epitopes within the HIV Env protein are immunodominant in ESN individuals, but are rarely recognized by HIV-infected patients [32]; and Gag-stimulated $CD8^+$ T lymphocytes isolated from ESN individuals are characterized by higher levels of intracellular perforin and granzymes than those isolated from the HIV-infected partners [40] ($n = 30$ ESN individuals studied).

Role of continual exposure

The persistence of both HIV-specific $CD4^+$ T cell and CTLs in ESN individuals seems to be strictly dependent on continuous virus exposure. In fact, various studies using different cohorts of ESN individuals that were followed longitudinally confirm that repeated exposure to HIV is necessary to maintain protective immunity. To summarize, HIV-specific $CD4^+$ T cell and CTLs responses disappeared within 6–9 months after cessation of exposure to the virus in uninfected newborns of HIV-infected women [4] ($n = 8/23$) and in healthcare workers having reported a single professional exposure to HIV-infected body fluids [5] ($n = 8/12$); the concentration of HIV-specific IgA was significantly diminished in ESN women who underwent counseling and reported the adoption of safe-sex procedures [44] ($n = 14/15$); late seroconversion concomitant with the waning of HIV-specific $CD8^+$ T cell responses occurred in two Kenyan HIV-resistant sex workers who interrupted commercial sex work for a period of time, probably due to reduced antigenic exposure [45]; and $CD8^+$ cell non-cytotoxic responses (CNARs), observed in nearly half of 35 ESN individuals examined, were shown to decline in time after the last exposure to HIV [10]. Subsequent reports also showed that both HIV-specific $CD4^+$ T cell and CTL responses are more frequent in ESN women with more recent sexual exposure [26,40,46]; the magnitude of $CD4^+$ T cell responses correlates with the frequency rather than with the duration of virus exposure in sex workers [32]; and an inverse correlation is detected between exposure to virus and in-vitro susceptibility of peripheral blood mononuclear cells (PBMCs) to HIV infection [39]. These studies involved more than 100 ESN individuals recruited in different parts of the world.

The observation that maintenance of possibly protective HIV-specific immunity in both the systemic and mucosal compartments might be contingent upon repeated antigen-specific immune stimulation suggests that exposure to HIV does not result in the generation of long-lasting memory cells. It will be important to analyze this issue in depth. Available data on naive and memory T lymphocyte subpopulations in ESN individuals show the presence of a low naive/memory cell ratio: an observation that resembles what has been seen in HIV infection [33,34]. Additionally, Gag-specific central memory $CD4^+$ and $CD8^+$ T cells, as well as terminally differentiated $CD8^+$ T cells were augmented, whereas $CD8^+$ effector memory cells were found to be reduced in ESN individuals compared with HIV-infected individuals (studies performed in 15 ESN individuals). The increase in terminally differentiated lymphocytes was suggested to play a role in determining the resistant phenotype [33].

Immune activation

With few notable exceptions [47,48] ($n = 45$ and $n = 20$ ESN individuals analyzed, respectively), a consensus seems to emerge that indicates that ESN individuals are characterized by a generalized immune activation. This observation was made when $CD4^+/CD25^+$, $CD8^+/CD38^+/CD45RO$, and both HLADR-expressing $CD4^+$ and $CD8^+$ activated T lymphocytes, were analyzed in the peripheral blood of ESN individuals [26,34,49] and mucosally in commercial sex workers and their heterosexual partners [32] (a total of 93 individuals was investigated). It is also important to underline that recent results by Suy *et al.* [49] indicate that peripheral

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lymphocytes expressing CCR5 and CXCR4, the major HIV coreceptors, are upregulated on peripheral blood $CD4^+$ T lymphocytes of sexually exposed ESN individuals (21 heterosexual couples were analyzed). Finally, some authors have also observed an increase of $CD8^+/CD28^+$ cells in ESN women [26]. This finding is very intriguing, given that these cells produce CAF, a soluble factor considered to be responsible for the non-cytotoxic antiviral response exerted by $CD8^+$ cells via the inhibition of HIV RNA transcription [50,51]. The expression of CAF has been previously described to be present in nearly half of 35 studied ESN individuals [10].

The observation that generalized immune activation, including an increase of lymphocytes bearing the major HIV coreceptors, might be associated with reduced susceptibility to HIV infection is curious given that T-cell activation facilitates spreading of HIV infection [52–54]. A plausible explanation could be that immune activation is a favorable factor in inducing an immune resistance to primary HIV infection in ESN individuals, whereas, during the course of HIV propagation, it facilitates the replication of the virus and the consequent progression of the disease.

Immune correlates of reduced susceptibility to HIV: humoral immunity

ESN individuals also display some unconventional humoral immune responses that may play a role in HIV neutralization: a very effective protective mechanism against viral infections. Two kinds of HIV-related humoral immune responses have been described in ESN individuals so far: antibodies to cellular proteins involved in the HIV infection/entry process, and HIV-specific mucosal antibodies.

The induction of anticell immune responses is rather common, and antilymphocyte antibodies have been observed early in sera from HIV-infected patients [55–57]. As the majority of these antibodies recognize HLA or CD4 molecules, their appearance had first been attributed to the cytopathic effect of HIV infection or to the long-lasting exposure to blood derivatives, as in the case of haemophiliacs [58,59]. In this regard, HIV-blocking IgG directed against HLA class I and CD4 molecules have been found in sera of ESN individuals [60–63] ($n = 14/31$). Anti-CD4 antibodies of ESN individuals recognize epitopes exposed by gp120 binding, suggesting that such antibody response is generated after repeated, long-lasting exposure to HIV in either horizontal or vertical transmission [61,63]. Although anti-CD4 antibodies were also found in some HIV-seropositive individuals and in some healthy blood donors, such anti-CD4 antibodies recognized epitopes different from those seen by antibodies found in sera of ESN individuals [61,62,64,65].

Another cellular protein, CCR5, is also targeted in ESN individuals by IgA and IgG antibodies at the mucosal and systemic levels. These antibodies are directed toward a conformational epitope corresponding to the second extracellular loop of CCR5 (YAAAQWDFGNTMCQ), which is not involved in HIV binding (6/48 ESN individuals analyzed). Thus, anti-CCR5 antibodies are likely to exert their possibly protective role through the downregulation of the CCR5 protein [66]. The effect of anti-CCR5 antibodies could be due to the recognition and the interaction with specific epitopes or, alternatively, to steric hindrance. Recent data showing that the mechanism of action of these antibodies is mediated by the internalization of the receptor through a clathrin-dependent pathway [67] seem to suggest that CCR5-specific antibodies mediate their effect secondarily to epitope-specific recognition. Anti-CCR5 antibodies do not affect physiologic immune functions, due to the redundancy in chemokine receptor family, but could possibly play an initial role in protection [68]. Notably, both anti-CD4 and anti-CCR5 antibodies have been described as specific markers of HIV-exposure in Asian and Caucasian but not in African ESN individuals [69] (anti-CCR5 and anti-CD4 antibodies were detected in 10.7 and 5.5% of the 149 enrolled ESN individuals, respectively). This discrepancy could be due to differences in the genetic background, in the route of exposure, or in the different environmental conditions, which can modulate immune responses to microbes [52,70–72].

The generation of anti-CCR5 antibodies has been attributed to several mechanisms: Ditzel *et al.* [73] showed that CCR5 can act as an alloantigen in *CCR5Δ32* homozygous individuals. Anti-CCR5 antibodies directed toward epitopes different from those seen by antibodies of ESN individuals have been also observed in healthy individuals not previously exposed to HIV [74–76]. This finding could be explained by autoimmune phenomena triggered by membrane perturbations unrelated to HIV stimuli, such as exogenous or endogenous viruses or local inflammation. Alternatively, ESN individuals could have undergone priming with other (possibly cross-reactive) viruses or proteins, and once exposed to HIV, they could possibly mount a secondary response, directed toward allo-antigen and self-antigens associated with viral particles [77].

Allo-immune and auto-immune responses have been found in HIV-infected patients [58,61,78,79] and such potentially autoimmunity-associated immune responses could play a protective role in preventing HIV infection [75,80]. For instance, the HIV-neutralizing human monoclonal antibodies 2F5 and 4E10 are produced in natural infection, recognize conserved gp41 epitopes [80–82], and can bind membrane phospholipids with

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kinetics comparable with those of anticardiolipin immunoglobulins generated in autoimmune syndromes [81]. The ability of these antibodies to bind cardiolipin has nevertheless recently been questioned by two independent groups [83,84]. These authors did not confirm the cardiolipin-binding properties of 2F5 and 4E10 and showed that, even if 4E10 can bind phospholipids, such binding has a much lower affinity compared with the one for gp41. These discrepancies seem to reflect technical difficulties that could be explained by a low affinity of the 2F5/4E10–phospholipid interaction or, alternatively, by the fact that the anionic lipids could be only a small portion of the antigen-binding paratope.

IgA antibodies are the most abundant isotypes found in mucosal secretions and epithelia and take part in several effector pathways that may protect the host from mucosal infection and clear the virus [85]. Soluble antibodies can compete with HIV for attachment to epithelial cells [86], participate in opsonization, activate complement-mediated cell lysis, induce antibody-dependent cell-mediated cytotoxicity (ADCC) [86–88], and inhibit transcytosis [89,90]. As HIV is transmitted mainly by sexual intercourse and the genital mucosa is the main site where initial host–virus contact takes place, it is noteworthy that HIV-specific antibodies could be detected in the mucosa of African ESN individuals [91] (antibodies were detected in 7.5% of the 342 ESN individuals enrolled in the study). In this regard, IgA reactive to the above-mentioned conformational epitope of CCR5 in ESN individuals were able to specifically block transcytosis of HIV across a tight epithelial cell layer, but monoclonal antibodies against other regions of CCR5 had no effect on HIV-mediated transcytosis. This finding likely reflects a different conformation of CCR5 at the mucosal level [92,93].

Possible immune protection (or resistance) in ESN individuals has also correlated with HIV-specific mucosal IgA antibodies [13]. These IgA have been observed in cervical secretions of ESN individuals from cohorts with different genetic background such as heterosexual women from Italy [13] ($n = 16$, IgA detected in the urine of 62% and in the cervical secretions of 81% of the enrolled ESN individuals) and India [94] ($n = 40$, IgA detected in 70% of individuals), as well as in female sex workers from Kenya [95,96] (two studies enrolled a total of 134 ESN individuals; IgA were observed in 76 and 74% of the two cohorts, respectively), Thailand [97] ($n = 13$, IgA detected in 76% of individuals); Cambodia [98] ($n = 48$, IgA detected in 39% of individuals); and Cote d'Ivoire [99] ($n = 342$, IgA detected in 7.3 and 29.8% of individuals using two different enzyme-linked immunosorbent assay (ELISA) methods]. These antibodies were also detected in the seminal fluid of male ESN partners of HIV-infected women [14] ($n = 14$, IgA detected in 78% of individuals). In addition, low levels of HIV-specific cervicovaginal IgG have also been found in female sexual partners of HIV-seropositive individuals with mixed ethnicity [100]. Finally, recent results of a study analyzing 145 infants of HIV-infected mothers indicate that HIV-specific salivary IgA can be observed in such infants (IgA in 13/145 cases). Notably all HIV-exposed infants in whom IgA were detected remained uninfected after 1 year of follow-up [101].

The detection of HIV-reactive IgA in mucosal secretions of ESN individuals in the absence of detectable HIV-specific IgG in their sera (seronegativity) might seem enigmatic, especially from the conventional view of antibody class-switching. However, strong antiviral IgA responses with neutralizing activities have been observed in the absence of virus-specific IgM and IgG in experimental conditions. Thus, Sangster *et al.* [102] demonstrated a $CD4^+$ T cell-dependent antiviral IgA response that is restricted exclusively to virus-specific B cells and is generated in the absence of B cell signaling via MHC class II or CD40. This phenomenon was observed in MHCII and CD40 knockout mice in which a reduction of virus-specific IgG and IgM, in the presence of strong virus-neutralizing IgA responses, could be detected. Recent experiments performed utilizing genetically modified mice have dissected the different molecular requirements for the induction of IgA and IgG antibody responses. In this latter study, Gärdby *et al.* [103] showed that differentiation to functional gut mucosal IgA responses against T-cell dependent antigens does not require signaling through CD28 and can be independent of germinal centers formations and isotype-switching in Peyer's patches. By contrast, serum IgA responses, similar to IgG responses, are dependent on germinal centers and CD28. Thus, they suggested a different costimulatory pathway response for the different immunoglobulin isotype. Moreover, costimulatory signals required for mucosal IgA responses are strikingly different from those for systemic antibody responses and conventional class-switching [104]. In ESN individuals ($n = 6$), resistance to HIV infection has been associated with HIV-neutralizing antibodies directed to a small area within the α helical region of the extramembrane portion of gp41 (QAR-ILAV epitope) [105]. This interacts with the C5 region in gp120 [106], a conserved neutralizing determinant. It is noteworthy that the α helical region of gp41 is not recognized by IgA in HIV-infected individuals. In other ESN cohorts, HIV-neutralizing IgA recognize a separate immunodominant region of gp41 (ELDKWA epitope) [89], which is frequently recognized by HIV-seropositive individuals. Possibly more than one HIV region can modulate the host's susceptibility to HIV.

HIV-specific IgA of ESN individuals have been shown to prevent HIV entry into $CD4^+$ T cells in in-vitro infection of PBMC [107,108] and to inhibit HIV transcytosis in an in-vitro model using colon cell lines [109,110]. IgA from ESN individuals were also shown to cross-neutralize primary HIV isolates from different clades (including A,

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B, C, and D clades) [108] (30 ESN individuals were analyzed overall). Finally, recent results showed that the saliva and breast milk concentration of CCL28, a chemokine that preferentially attracts IgA-secreting plasma cells in the epithelial lamina propria [111,112], is increased in the majority (78% of 50 individuals) of ESN individuals. A direct correlation between CCL28 and survival of HIV-infected and breastfed neonates was detected in a Zambian cohort [113]. Notably, administration of mice with a CCL28-expressing construct resulted in a significant increase of IgA-secreting plasma cells in the rectal/genital mucosa [113] (Table 3).

An important caveat regarding these results is that mucosal IgA antibodies have been observed in some but not all groups of ESN individuals. Thus, studies performed in some cohorts of African and North American ESN individuals did not show mucosal HIV-specific IgA [114,115] (J. Mestecky *et al.*, unpublished observations) ($n = 97$), whereas ambiguous results (IgA in 2/14 ESN individuals) were reported in another cohort [22]. Such discrepancy could be due to technical difficulties associated with detection of IgA in mucosal fluids, which can have very low concentrations of such antibodies [116–118]. In addition, although secretory IgA are only partially sensitive to proteolytic cleavage [119], mucosal immunoglobulin levels can vary due to different collection methods and to the concentrations of proeolytic enzymes. Nevertheless, when specimens collected in multiple ESN cohorts with different methods were analyzed in the same laboratory using the same ELISA methodology and the same source of antigen, HIV-specific IgAs were detected in some but not all such cohorts (J. Mestecky *et al.*, manuscript in preparation). These contradictory results suggest that currently unknown factors may be involved in induction and/or detection of HIV-specific mucosal immune responses. Finally, although mucosal HIV-specific antibodies might play a role in preventing horizontal HIV transmission at the mucosal level, it seems that these antibodies do not play any role in the prevention of infection transmitted through breast milk [120,121].

Table 3. Humoral immunity in exposed seronegative individuals: functional and phenotypic characterization.

Function/phenotype	References
HLA-specific antibodies with HIV-blocking ability	[60]
CD4-specific antibodies with HIV-blocking ability	[61–63,69]
CCR5-specific antibodies	[66,67,69]
Allo-specific antibodies[a]	
HIV-specific IgA	b

[a]Observed in [80,81]. Not confirmed in [83,84]. HLA, human leukocyte antigen.
[b]Observed in [13,94–101,107–109] (more than 600 exposed seronegative individuals). Not confirmed in [114,115] (118 exposed seronegative individuals). Ambigous results (IgA in 2/14 exposed seronegative individuals) in [22].

Innate immunity and the modulation of susceptibility to HIV infection

The role of innate immunity in the modulation of susceptibility to HIV infection is unclear and few reports describe a possible role for this arm of the immune response. The activation of NK cells in the early phase of lentiviral infections and the possible role of these cells in the containment of acute viral replication has been demonstrated in a monkey model of SIV infection [122]. A similar inverse correlation between the CC chemokine-producing activities of host NK cells and levels of viremia in HIV infection has also been reported [123]. Further, an increase in NK cell activity has been observed in 37 HIV-exposed but uninfected Vietnamese injecting drug users, suggesting a possible protective role of NK cells [15]. It has also been shown that low numbers of NK cells are associated with rapid progression to AIDS in HIV-infected individuals [124]. These results were confirmed by Jennes *et al.* [125], who also demonstrated that, although killer cell immunoglobulin-like receptors (KIRs) as a whole are expressed normally on NK cells of ESN individuals, HLA molecules that bind inhibitory KIR are downregulated in these individuals ($n = 41$). Further, a high KIR3DS1/KIR3DL1 ratio associated with downregulated KIR3DL1 transcript levels and an increased NKG2C/NKG2A ratio were detected in ESN individuals [126] ($n = 80$). The increased expression of the activating receptor KIR3DS1 was recently confirmed [127] ($n = 25$); these observations seem to support the notion that NK cell activity is indeed augmented in ESN individuals Finally, results of a very recent study that analyzed particular allele combinations in 46 ESN individuals showed that the coexpression of KIR3DL1*h/*y and B*57, which has been associated with a reduced risk of progressing to AIDS in HIV-infected individuals [128], also lowers the risk of infection in ESN individuals [129].

The possibility that innate mechanisms could play a role in protection against HIV infection was reinitiated by the exciting discovery of innate intracellular antiviral proteins. The apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like editing complex (APOBEC) proteins are the most important of these antiviral proteins. In particular, APOBEC3G shows a broad antiviral activity capable of reducing viral fitness. This is achieved by the introduction of by introducing detrimental levels of G-to-A hypermutations in the proviral genome, through the conversion of cytosine to uracil in the minus-sense single-strand DNA during reverse transcription. APOBEC3G can also prevent proviral integration into the cellular genome through a deamination-independent mechanism (review in [130]). The antiviral activity of human APOBEC3G is counteracted by the viral protein Vif; it could, therefore, be argued that the fate of exposure to HIV is determined by the relative balance between APOBEC3G and Vif. Recent results demonstrated that higher basal and IFN-α-induced APOBEC3G mRNA

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and protein levels are seen in monocyte/macrophage-lineage cells of ESN individuals [131] ($n = 30$). Other authors analyzed APOBEC3G in resting and activated $CD4^+$ and $CD8^+$ T cells of ESN individuals ($n = 8$) and found a slightly lower expression of APOBEC3G in resting $CD4^+$ T cells [132]; these authors did not analyze monocyte/macrophage-lineage cells and focused on resting lymphocytes alone. Overall, these results seem to suggest that endogenous antiviral factors could play a role in determining susceptibility to HIV (Table 4).

Additional data obtained by a transcriptome and proteome analyses on T cells and plasma or sera of 21 ESN individuals indicated that several innate immune genes are upregulated in activated T cells of ESN individuals. In particular, IL-22, a cytokine that induces the production of acute-phase proteins, was significantly increased in ESN individuals compared with the values observed in either HIV-infected patients or healthy controls [133]. Moreover, a higher quantity of the acute-phase amyloid A protein (A-SAA) was present in sera of ESN individuals. These high levels of A-SAA were shown to have an inhibitory activity on the in-vitro infection of dendritic cells by HIV [133]. This observation can probably be explained by binding of A-SAA to its formyl peptide receptor on the dendritic cell surface, which, in turn, mediates CCR5 phosphorylation and downregulation. Because IL-22 upregulates the production of acute-phase proteins, and in particular of A-SAA, these data suggest that an IL-22-induced pathway of protein production could contribute to reducing susceptibility to HIV infection. Subsequent experiments also indicated that the IL-22-mediated antiviral effects include the stimulation of the production of beta-defensins 2 and 3 by ectocervix epithelial cells as well (F. Veas, M. Clerici, unpublished observations). These innate cationic peptides have broad antiviral activities directed against a wide spectrum of viruses including HIV (for a recent review see [152]) and might play a role in the generation of the ESN status [153].

Table 4. Innate immunity and genetic characteristics of exposed seronegative individuals.

Innate immunity	References
$CD8^+$ non-cytotoxic antiviral response	[10,12,50,51]
Increased NK cells activity	[123,124]
Down-regulation of HLA molecules binding inhibitory KIR proteins	[125]
Up-regulation of activating KIR	[125–129]
Increased levels of APOBEC3G ($CD14^+$ cells)[a]	[131]
Increased concentrations of IL-22	[133]
CCR5 deletion	[9]
Polymorphisms in the CCR5 regulatory region	[134–138]
Polymorphisms in the SDF-1 gene	[139]
Polymorphisms in the MLB gene	[140,141]
Polymorphisms in the DC-SIGN gene	[142]
Polymorphisms in the vitamin D receptor	[143]
Polymorphisms in the IRF-1 gene	[144]
Increased copy numbers of CCL31	[145–148]
MHC genes	[149,150]
Polymorphisms in chromosome 22q12–13	[151]

[a]APOBEC3G is really an 'intrinsic cellular factor' that bridges innate immunity and genetic factors. HLA, human leukocyte antigen; KIR, killer cell immunoglobulin-like receptor; NK, natural killer.

Genetic correlates of reduced susceptibility to HIV infection

Host genetic factors can confer resistance to HIV acquisition at different steps in viral infection including the penetration of the virions through surface barriers of the body, their attachment to target cells, integration, and viral gene expression. In addition, host genetic factors also influence immune responses to HIV antigens. The molecularly best defined genetic factor that confers cellular resistance to HIV infection is the homozygous 32 base-pair deletion in the CCR5 chemokine receptor gene (the *CCR5Δ32* allele), which results in the lack of cell-surface expression of the coreceptor for macrophage-tropic HIV. Homozygosity for this mutation was first observed in two uninfected individuals with histories of multiple high-risk sexual exposure to HIV [9]; subsequent analyses of multiple cohorts have nevertheless revealed very low frequencies (1.7–12%) of *CCR5Δ32* homozygosity. These findings indicate the possible importance of other genetic factors (reviewed in [154]) (Table 4). Polymorphisms in the regulatory region of the *CCR5* gene have been identified, but no significant association of a particular genotype with HIV-exposed but uninfected status has been observed [134,135]. A genetic variant of CCR2 with a valine-to-isoleucine change at position 64 (*CCR2-64I*) is in strong linkage disequilibrium with a mutation in the *CCR5* regulatory region [136]. There are discrepant reports as to the possible association of this genotype with a reduced risk of HIV acquisition [137,138]. Similarly, an A-to-G substitution at position 801 within the 3′ untranslated region of the *SDF-1* chemokine gene was reported to be highly accumulated among a group of high-risk exposed uninfected individuals [139]. However, this genotype was not associated with resistance to HIV acquisition in separate studies [155].

Mannose-binding lectin (MBL) is a component of the soluble innate immune complement system and can bind HIV as well as surface oligosaccharides of other infectious microorganisms. Variant alleles of the *MBL* gene have been associated with susceptibility to HIV infection in Danish [140] ($n = 96$) and Gabonese [141] ($n = 188$) cohorts, but the frequencies of the variant are very low, and no association was observed in a Columbian cohort [156] ($n = 278$). Likewise, dendritic cells are among the first cell types to encounter HIV during sexual transmission, and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is involved in the

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CD4-independent binding of HIV gp120. A deletion in the 23 amino acid repeat units within the neck region of DC-SIGN was noted at higher frequency among ESN individuals in one cohort [139], but the reported frequency of the variant alleles was low (3.2%), and cannot account for the majority of the uninfected phenotype. DC-SIGN and its homologue DC-SIGNR (DC-SIGN related) play a crucial role in enhancing trans-infection of $CD4^+$ T cells from HIV-carrying dendritic cells in the regional lymph nodes. A haplotype consisting of a specific repeat number in exon 4 and a SNP allele in exon 5 of the DC-SIGNR gene has also been associated with the ESN status among 102 HIV-seronegative individuals of HIV-seropositive spouses in Thailand [142]. Variants of the human natural resistance-associated macrophage protein 1 were also associated with a reduced risk of HIV acquisition, but were not studied in exposed uninfected individuals [157].

Vitamin D receptor gene sequence variations and polymorphisms in the transcriptional regulator gene interferon regulatory factor 1 (IRF-1) have recently been shown to correlate with reduced susceptibility to HIV infection [143] in ESN individuals ($n = 125$). These results are particularly important considering that vitamin D acts as an immunoregulatory hormone by activating monocytes and stimulating cell-mediated immunity. The observation that the protective IRF-1 genotypes are associated with significantly lower basal IRF-1 expression and reduced responsiveness to exogenous IFN-γ stimulation [144] seems to suggest that the increased production of IFN-γ has a marginal role or might be an epiphenomenon in the modulation of susceptibility to HIV infection. Finally, recent results focusing on the gene encoding CCL3L1, a natural ligand for the HIV coreceptor CCR5 with a suppressive effect on HIV replication, showed, in a large cohort of individuals, that a *CCL3L1* copy number lower than the population average is associated with markedly enhanced rates of HIV infection and AIDS development [145,146]. Additional results obtained in a large cohort ($n = 849$) of HIV-infected South African pregnant women indicated that higher *CCL3L1* copy number is associated with reduced vertical HIV transmission [147]. Finally, a study performed in human T lymphotropic virus-2-infected individuals showed that the median copy number of the *CCL3L1* and the *CCL3L1/CCL3* mRNA ratios were increased in ESN individuals and in LTNP patients compared to those in healthy controls [148] ($n = 8$).

Immune responses to viral antigens are strictly controlled by host immune response genes. Major histocompatibility complex (MHC) genes are by far the most influential of such genes due to their function in binding and presenting antigenic epitopes to T lymphocytes. Effects of different MHC alleles on spontaneous and vaccine-induced immune resistance against fatal retroviral infection have been studied in detail using the mouse model of immunosuppressive Friend retrovirus infection [158–160]. Effects of human MHC genotypes on host immune responses to HIV have been recognized (reviewed in [161,162]): *HLA-B*57* is associated with particularly effective CTLs responses and lower viral load, *B*27* is involved in selective presentation of an immunodominant $p24^{gag}$ epitope, and *B*35* is most consistently associated with an earlier AIDS progression among whites.

Associations of particular HLA class II alleles and resistance to HIV acquisition have been reported (reviewed in [158–162]). In this regard, a study performed in the Pumwani Sex Worker cohort revealed that several DQB1 alleles are associated with resistance to HIV infection [149]. Additionally, studies performed in HIV-infected mothers showed that class I HLA concordance between mother and infant was independently associated with a stepwise increase in the risk of perinatal HIV transmission, suggesting that discordant HLA provide infants with a means of protection against HIV, possibly as a result of allogeneic infant antimaternal MHC immune responses [150].

Non-MHC genes are involved also in the regulation of host immune responses, the best documented example being the *Rfv3* locus described for Friend mouse retroviral infection [163,164]. *Rfv3* genotypes influence the duration of Friend virus viremia in mice after infection and this effect is mediated through the *Rfv3* gene's influence on the production of virus-neutralizing antibodies [164,165]. A recent study has indicated that a gene located within a segment of human chromosome 22 that is autologous to the region of mouse chromosome 15 that harbors the *Rfv3* locus is strongly associated with resistance to HIV acquisition [151] ($n = 42$) (Table 4). It is of particular interest that the *APOBEC3* locus is present in the middle of the above chromosomal segment [151]. Notably, very recent data indicate that polymorphisms in the *APOBEC3* locus indeed affect the susceptibility to Friend virus infection in mice [166], and that Rfv3 is encoded by APOBEC [167].

Conclusion

What emerges from this review of immunologic analyses performed over several years in different cohorts of ESN individuals is that, paradoxically and with few exceptions (e.g., immune activation), the 'immunologic advantage' of ESN individuals stems from their ability to react to HIV in a normal, physiological way. Challenged with a virus, the immune system of ESN individuals responds with the activation of multiple effector mechanisms, which are the same mechanisms normally triggered in response to viruses. The difference that makes the immune response to HIV of ESN individuals stand apart is the absence of an effective memory function. What

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creates the 'immune advantage' of ESN individuals? A number of speculations are possible: ESN individuals handle HIV in the same way as they would handle any other viruses, that is, the immune system of ESN individuals does not recognize the biological peculiarities of HIV that render HIV infection a chronic process; genetic differences stimulate the activation of slightly peculiar immune responses; stronger/better immune effector mechanisms are present in ESN individuals. Fifteen years ago, it was theorized that TH1 responses and CMI could be particularly intense in ESN individuals, and that these immunologic features would lead to protection [168,169]. If genetic factors could be identified that result in the activation of stronger/better TH1 responses and CMI, possibly in association with innate and mucosal immunity, the loop could be closed.

How can we use this body of knowledge to design a vaccine? An indispensable premise is that LTNP patients, the focus of many sophisticated analyses, are not ESN individuals. LTNP patients are extremely interesting HIV-infected patients, and results of in-depth analyses on these individuals are likely to shed light on the pathogenesis of HIV disease. Nevertheless, although LTNP patients, despite being able to avoid progression to AIDS for an extended period of time, are indeed infected, ESN individuals are not infected. It appears that the identification of the immune/genetic correlates in ESN individuals alone could clarify how to design preventive vaccine approaches. Another fundamental point is that any vaccine approach will have to include the triggering of a potent innate immune response. The results obtained in ESN individuals, and brute logic, indicate that it is very likely that without this component effective defense against HIV – a defense that will prevent the integration of viral genome into the host cell – will be nearly impossible to achieve. Unfortunately, we know much better how to manipulate acquired immunity than we do to innate immunity.

With all these possibilities considered, the data summarized here suggest that an effective preventive vaccine needs to block the transmission of HIV particularly through the mucosal surface. This result would allow the penetration of only small quantities of HIV into the lymph-nodes/peripheral blood. Innate and specific immune responses, including CTLs and CNAR/chemokines would be capable of handling the reduction in this HIV load, preventing established infection [170].

How to stimulate these responses? Vaccine constructs could include IFN-α, a cytokine with the ability to stimulate APOBEC, adjuvants/chemokines (IL-5, transforming growth factor-β, CCL28) to stimulate IgA-mediated mucosal immune responses, and adjuvant/cytokines (IL-7, IL-15), with the ability to elicit the generation of memory lymphocytes (Fig. 2). It will be important to verify whether this type of approach will be able to reproduce the 'immunologic advantage' that characterizes ESN individuals in the population at-large, possibly resulting in a true protection against HIV infection.

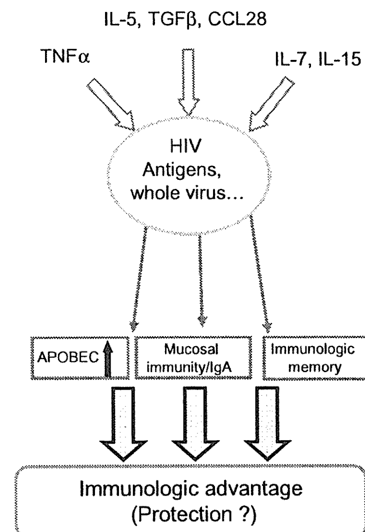

Fig. 2. An attempt to design preventive vaccines that would confer the 'immunologic advantage' of exposed seronegative people over those lacking ESN genotype, possibly resulting in protection against HIV infection. The use of IFN-α, IL-5 (and/or TGFβ and/or CCL28), and IL-7 (and/or IL-15) as multiple adjuvants could results in the stimulation of the expression of APOBEC, the elicitation of mucosal IgA, and generation of memory T lymphocytes and, ultimately, a possibly protective immune response mediated by an array of mechanisms.

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Authors' contribution

Masaaki Miyazawa reviewed the literature on genetic correlates of resistance and wrote this part of the manuscript.

Lucia Lopalco reviewed the literature on humoral immunity and wrote this part of the manuscript.

Francesco Mazzotta and Sergio Lo Caputo are responsible of the clinical coordination of the ESN cohorts that are at the basis of the ESN study group.

Francisco Veas reviewed the literature on innate immunity and wrote this part of the manuscript.

Mario Clerici reviewed the literature on cellular immunity, wrote, organized, edited, and finalized the whole manuscript.

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