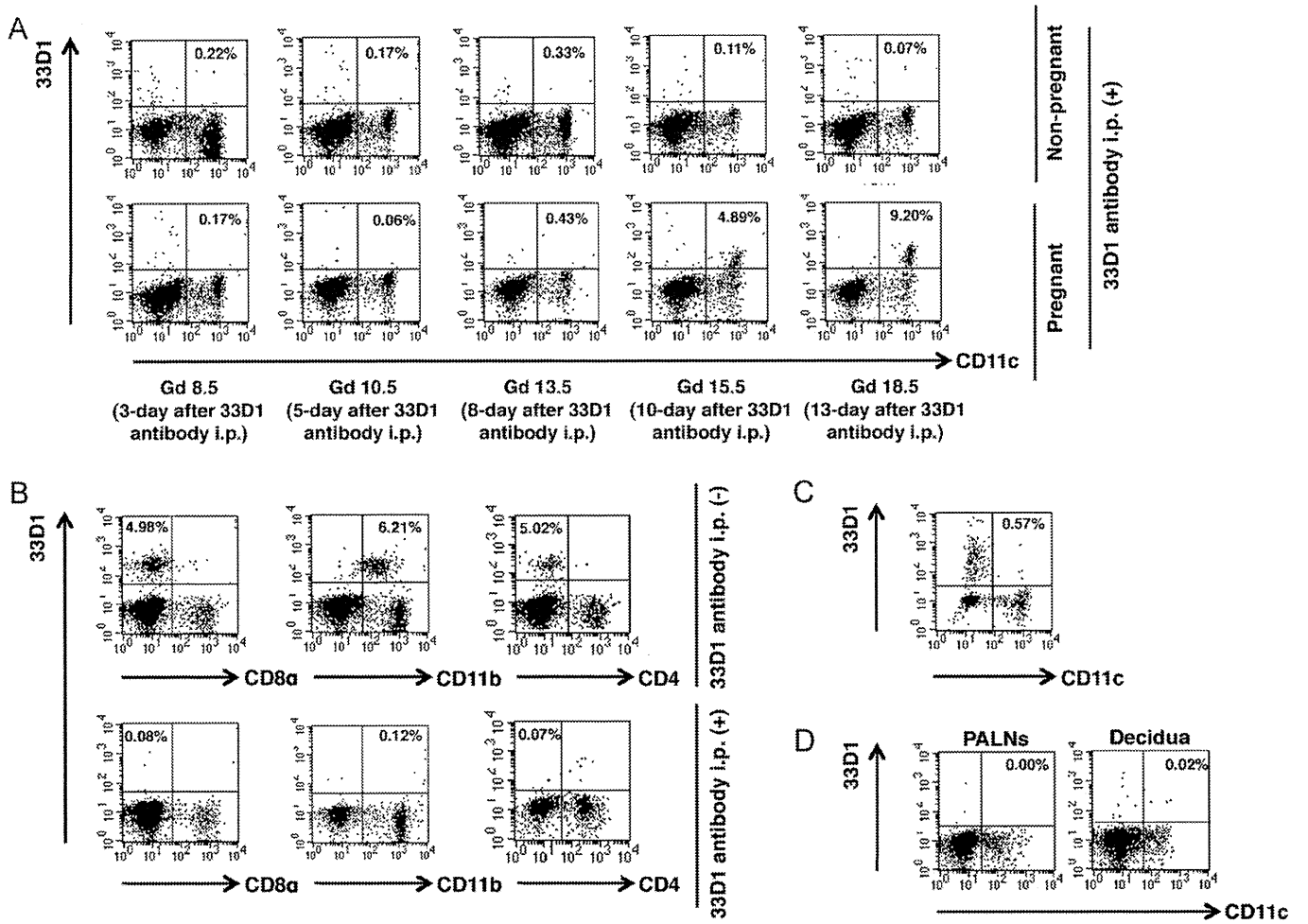

Fig. 4. Depletion of CD11c$^+$33D1$^+$ DCs in the spleen of non-pregnant and pregnant mice by i.p. administration of 33D1 antibody. (A) Kinetics of CD11c$^+$33D1$^+$ DCs in the spleen of non-pregnant (upper panel) and pregnant mice (lower panel) during pregnancy. Mice were administrated i.p. with 0.5 mg purified 33D1 antibody for 3 consecutive days (Gd 5.5, 6.5, and 7.5). For non-pregnant mice, the timings of Gd 10.5 and Gd 15.5 correspond to 5 and 10 days after starting 33D1 antibody injection. (B) Expression of CD8α, CD11b, and CD4 on 33D1$^+$ DCs with/without 33D1 antibody injection. (C) Intracellular staining of splenic DCs with 33D1 antibody treatment on Gd 10.5. (D) Analysis of 33D1$^+$ DCs in PALNs and decidua with 33D1 antibody treatment on Gd 10.5.

CD11c$^+$33D1$^+$ DCs could be achieved from around Gd 8.5–13.5 or 14.5 in pregnant mice treated with the above procedure. To confirm the depletion of CD11c$^+$33D1$^+$ DCs, several surface markers on 33D1$^+$ DCs were examined. As shown in the upper panels of Fig. 4B, although CD8α and CD4 were negative, CD11b was positive (CD11b^{intermediate}) on 33D1$^+$ DCs, which is consistent with previous observations (Dudziak et al. 2007; Liu and Nussenzweig 2010; Paharkova-Vatchkova et al. 2004; Shortman and Liu 2002). After i.p. administration of 33D1 antibody, the CD11b^{intermediate} 33D1$^+$ DCs were completely depleted (Fig. 4B, lower panels). Also, 33D1$^+$ DCs were not detected by intracellular staining in the spleen cells of pregnant mice treated i.p. with 33D1 antibody (Fig. 4C). Moreover, those 33D1$^+$ DCs were also depleted in the cells of PALNs and decidua on Gd 10.5 (Fig. 4D). These findings clearly indicate that CD11c$^+$33D1$^+$ DCs were actually depleted *in vivo* by the procedure.

Induction of fetal loss by depletion of 33D1$^+$ DCs in pregnant mice and the effect of progesterone on preventing fetal loss

Macroscopic appearance of the uterus of a typical case on Gd 16.5 is shown in Fig. 5A. In comparison with control pregnant mice injected with isotype-matched control rat IgG (Fig. 5A, first lane), a number of missing fetuses, indicated by arrows, were observed in 33D1$^+$ DC-depleted pregnant mice i.p. administrated with 0.5 mg purified 33D1 antibody for 3 consecutive days (Gd 5.5, 6.5, and 7.5) (Fig. 5A, second lane). The number of total implantation sites (open bars) and missing fetuses (closed bars) per mouse are shown in Fig. 5B. There was no statistical difference in total implantation sites among groups. We performed similar experiments several times and the percentage of fetal loss was demonstrated (Fig. 5C, first and second lanes). The percentage of fetal loss in mice treated with 33D1 antibody was statistically far higher than with control rat IgG. These results suggest that the systemic imbalance of DC subsets induced by the depletion of 33D1$^+$ DCs by injecting with 33D1 antibody caused apparent fetal loss in pregnant mice.

Because pregnancy is maintained by maternal hormones, such as progesterone and estrogen, and we have found that progesterone might induce a Th2-dominant state by reducing DEC-205$^+$ and increasing 33D1$^+$ DC subsets, particularly in the presence of estradiol, we examined whether progesterone played a protective role against fetal loss induced by the depletion of 33D1$^+$ DCs. To our surprise, we found that we could prevent miscarriages when pregnant mice were administrated 3 μg progesterone for 3 consecutive days (Gd 2.5, 3.5, and 4.5) before injecting with 33D1 antibody (Fig. 5A, third lane), although miscarriages could not be prevented by progesterone administration at the same time as 33D1 antibody injection (Fig. 5A, fourth lane). We performed similar experiments several times and confirmed the effect of prior progesterone

Please cite this article in press as: Negishi, Y., et al., Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. Immunobiology (2012), doi:10.1016/j.imbio.2012.01.011

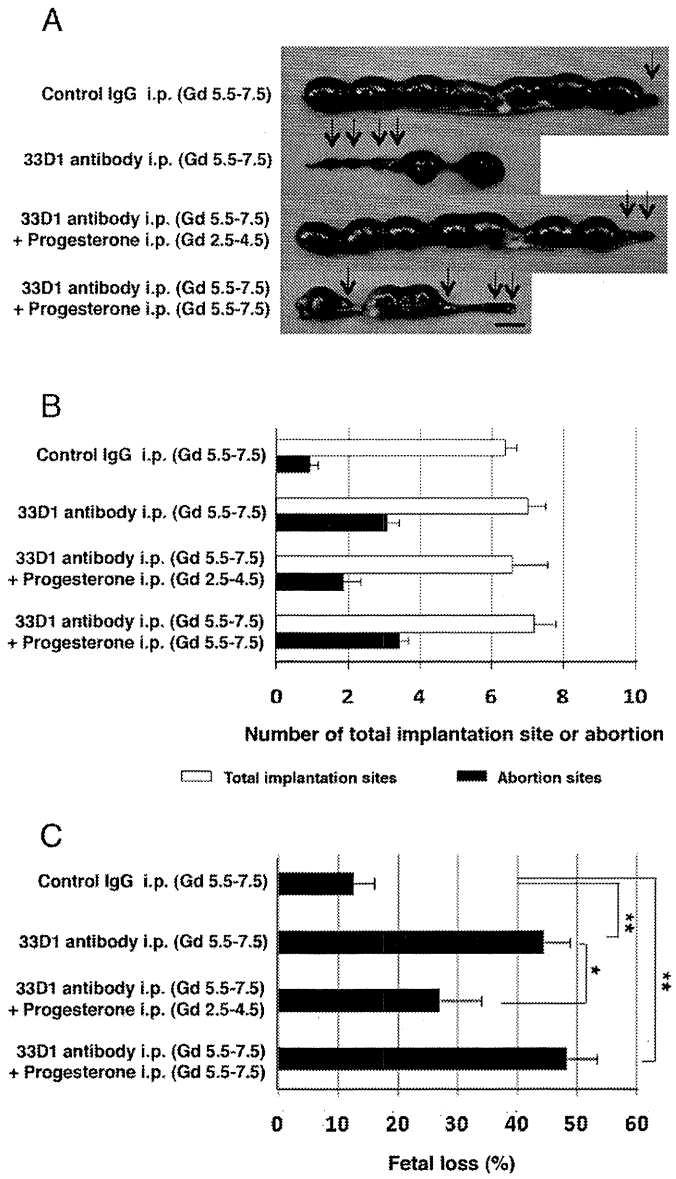

Fig. 5. Depletion of 33D1$^+$ DCs and administration of progesterone. For each figure, mice were injected i.p. either with 33D1 antibody or with control isotype-matched IgG on three consecutive days (Gd 5.5–7.5). Progesterone was also administrated i.p. on Gd 2.5, 3.5, and 4.5 or Gd 5.5, 6.5, and 7.5, consecutively. (A) Representative macroscopic appearances of uterus on Gd 16.5 are shown. Miscarriage on Gd 16.5 was identified macroscopically as a dark, small fetus (see arrows). The miscarriage on Gd 16.5 was around 3–5 mm in diameter. Scale bar: 1 cm. (B) Number of total implantation sites (open bars) and missing fetuses (abortion sites) (closed bars) per mouse. (C) Percentage of fetal loss with control IgG treatment, 33D1 antibody treatment, 33D1 antibody with progesterone (Gd 2.5–4.5) treatment and 33D1 antibody with progesterone (Gd 5.5–7.5) treatment on Gd 15.5. Data are shown as the mean ± SEM of $n = 19$ mice for control IgG treatment, $n = 18$ mice for 33D1 antibody treatment, $n = 8$ mice for 33D1 antibody with progesterone (Gd 5.5–7.5) treatment and $n = 8$ mice for 33D1 antibody with progesterone (Gd 2.5–4.5) treatment per group. $^*p < 0.05$ and $^{**}p < 0.01$.

administration on preventing fetal loss induced by the depletion of 33D1$^+$ DCs. Summarized results of the percentage of fetal loss are shown in Fig. 5C. These findings suggest that the strong Th2 dominancy established by progesterone in a relatively early stage of pregnancy seemed to prevent the Th1-mediated imbalance induced by 33D1$^+$ DC depletion.

The serum IL-12p40 production level on Gd 10.5 was enhanced by 33D1 antibody injection in comparison with control IgG-inoculated pregnant mice (Fig. 6A, first and second lanes). This enhanced serum IL-12p40 production level, which may reflect Th1 dominancy, disappeared with prior progesterone treatment but not at the same time as 33D1 antibody injection (Fig. 6A, third and fourth lanes). The results suggest that 33D1$^+$ DC depletion may shift the Th1/Th2 balance to a Th1-dominant state via activation of the remaining DEC-205$^+$ DCs that cause miscarriages during pregnancy, and progesterone might have a beneficial effect on preventing fetal loss induced by the imbalance of DC subsets through down-modulation of IL-12p40 secretion. The serum IL-12p40 production level in each group was almost identical on Gd 15.5 (Fig. 6B), indicating that the effect of 33D1 antibody on the enhancement of IL-12p40 was temporary. There was a statistical difference in the number of CD11c$^+$DEC-205$^+$ DCs in spleen cells on Gd 10.5 between progesterone-treated and -untreated mice i.p. administrated with 33D1 antibody (Fig. 6C). Moreover, when pregnant mice were i.p. injected with IL-12p70 (0.2 μg/mouse) on Gd 9.5 and 10.5, similar miscarriages were also observed (Fig. 6D), suggesting that the major cause of fetal loss may be initiated by the elevation of IL-12p70. These results indicate that progesterone may have the ability to save miscarriages induced by 33D1 antibody injection by suppressing temporal IL-12 secretion on Gd 10.5. Because temporal elevation of IL-13 following the IL-12 secretion was always observed in the normal pregnancy, we examined the effect of IL-13 injection on the miscarriages of mice induced by IL-12p70 administration. As expected, percentages of miscarriages induced by IL-12p70 were decreased by simultaneous i.p. injection of IL-13 (0.02 μg/mouse) on Gd 9.5 and 10.5 (Fig. 6D, third lane). Thus, IL-13, as well as progesterone, might have a capacity to prevent fetal loss.

Effect of 33D1-positive DC-depletion on C57BL/6 pregnant mice

To generalize the findings observed in BALB/c mating, we also studied the effects of 33D1-postive DC-depletion by inoculation of 33D1 antibody to C57BL/6 × C57BL/6 syngeneic mating combination during pregnancy. Based on our previous observation (Moriya et al. 2010), we used 33D1 antibody for CD11c$^+$33D1$^+$ DC depletion in C57BL/6 mice. The timing for injection of 33D1 antibody and progesterone were the same as for BALB/c mating combination as described above. The number of total implantation site (open bars) and missing fetuses (closed bars) per mouse are shown in Fig. 7A. As expected, miscarriage could also be induced by 33D1 antibody injection (Fig. 7B, second lane) and prior administration of 3 μg progesterone for 3 consecutive days (Gd 2.5, 3.5, and 4.5) before injecting with 33D1 antibody showed preventable tendency of the miscarriages (Fig. 7B, third lane). These results were consistent with the case of BALB/c mating combination. IL-12p40 was also detected in C57BL/6 on Gd 10.5 and 15.5 (Fig. 7C and D).

The activated CD4+ or CD8+ T cells were not be involved in the process of abortion induced by 33D1$^+$ DC-depletion or IL-12p70 injection

Finally, to examine whether the fetal loss was caused by the activated T cells, we analyzed the activation markers on the splenic T cells obtained from BALB/c mice having aborted fetus. Unexpectedly, as far as we have investigated extensively, we could not see any enhancement of the T cell activation markers such as CD69 and CD25 on both CD4+ and CD8+ splenic T cells of the mice treated with either 33D1 antibody or IL-12p70 administration (Fig. 8A and B). These findings suggest that acquired T cell-associated immunity was not involved in the induction of abortion as shown in the present study. Further investigation will be needed to clarify the actual mechanisms for fetal loss in the syngeneic mating system.

Please cite this article in press as: Negishi, Y., et al., Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. Immunobiology (2012), doi:10.1016/j.imbio.2012.01.011

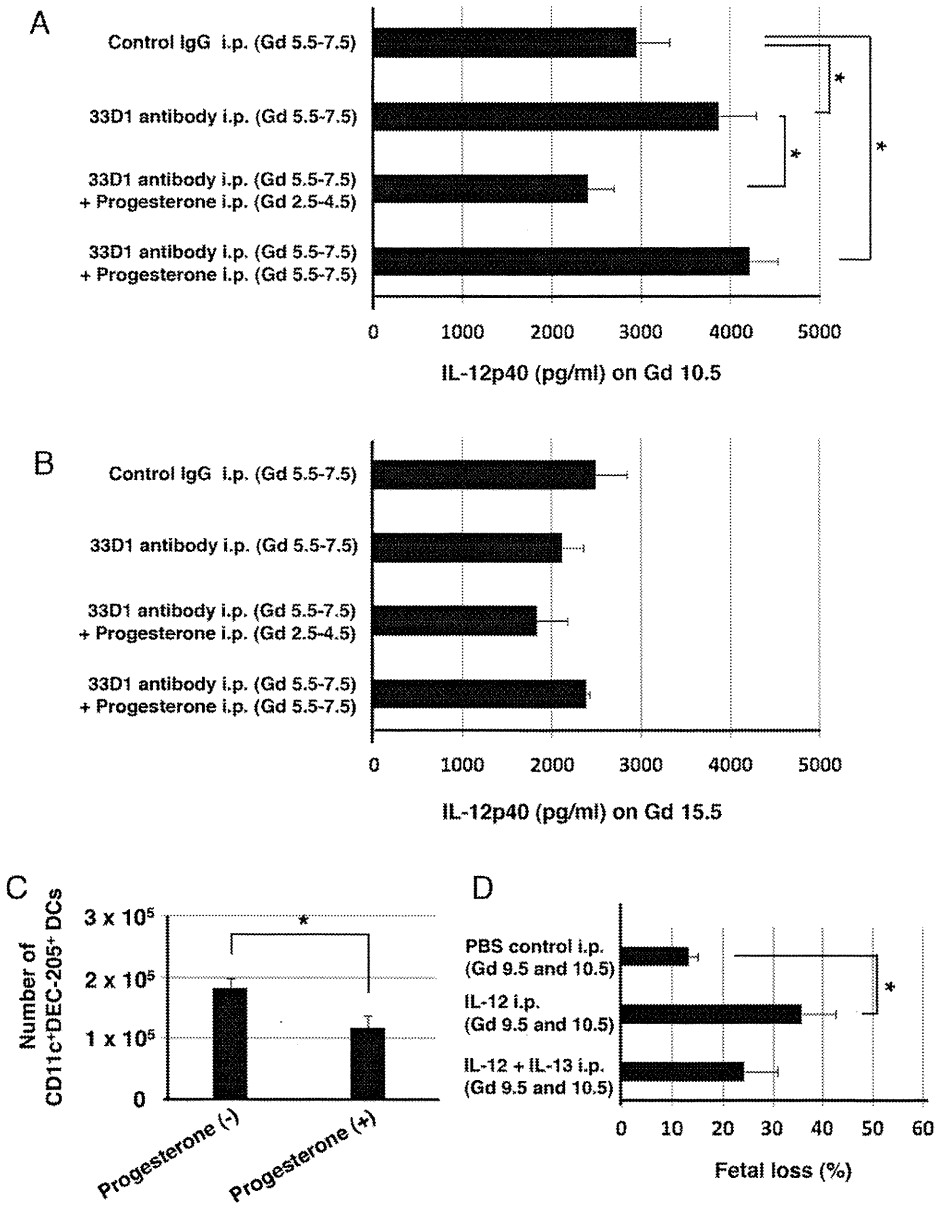

Fig. 6. Effects of 33D1 antibody injection and progesterone treatment on serum IL-12p40 production, number of CD11c$^+$DEC-205$^+$ DCs, and induction of miscarriages in pregnant mice. Serum IL-12p40 production with control isotype-matched IgG treatment, 33D1 antibody treatment, 33D1 antibody with progesterone (Gd 2.5–4.5) treatment, and 33D1 antibody with progesterone (Gd 5.5–7.5) treatment are shown either on Gd 10.5 (A) or on Gd 15.5 (B). (C) Number of CD11c$^+$DEC-205$^+$ DCs in pregnant mice injected with 33D1 antibody with/without progesterone (Gd 2.5–4.5) on Gd 10.5. Data of (A), (B), and (C), are shown as the mean ± SEM of $n = 7$ mice for each group. $^*p < 0.05$. (D) Induction of miscarriages in the pregnant mice by administrated i.p. with 0.2 μg/mouse recombinant IL-12p70 on Gd 9.5 and 10.5. Percentage of fetal loss induced by IL-12p70 (0.2 μg/mouse) injection (second lane) was decreased by simultaneous i.p. injection of recombinant IL-13 (0.02 μg/mouse) on Gd 9.5 and 10.5 (third lane). Control pregnant mice were inoculated twice with PBS on Gd 9.5 and 10.5 (first lane). Data are shown as the mean ± SEM of $n = 5$ mice. $^*p < 0.05$.

Discussion

In this study, we studied the kinetics of murine DC subsets, DEC-205$^+$ and 33D1$^+$ DCs, during pregnancy and found a sharp augmentation of the DEC-205/33D1 ratio reflecting Th1 dominancy just after delivery, although gradual reduction of the DEC-205/33D1 ratio was observed in the spleen as well as in the PALNs, indicating the requirement for the maintenance of Th2 predominance during pregnancy. This sharp augmentation was confirmed by the rapid decrease of 33D1$^+$ DCs and the prompt increase of DEC-205$^+$ DCs. We also observed that the depletion of 33D1$^+$ DCs during the perinatal period caused IL-12 secretion, by which fetal loss might be mediated. These findings strongly suggest that pregnancy and the onset of delivery are strictly regulated by an appropriate Th1/Th2 balance mediated through two distinct DC subsets, DEC-205$^+$ and 33D1$^+$ DCs, in the uterus where fetal/maternal interactions are initiated. In general, DEC-205$^+$ DCs are known as CD8α$^+$ cells and 33D1$^+$ DCs are known as CD8α$^-$ cells (Liu and Nussenzweig 2010; Maldonado-Lopez et al. 1999). Moreover, CD8α$^+$ DCs tend to induce a Th1-biased cytokine response and CD8α$^-$ DCs tend to induce Th2-biased cytokines (Langenkamp et al. 2000; Laskarin et al. 2007; Maldonado-Lopez et al. 1999; Shortman and Liu 2002). These findings support our speculation that the balance between DEC-205$^+$ and 33D1$^+$ DCs might affect the Th1/Th2 balance in systemic or regional areas.

Around the decidua, maternal immature DCs phagocytose apoptotic syncytiotrophoblast debris containing intracellular fetus class I and class II MHC molecules. Such phagocytotic DCs, particularly 33D1$^+$ DCs activated during pregnancy, may provide Th2-type humoral immunity and thus present fetal antigens in association

Please cite this article in press as: Negishi, Y., et al., Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. Immunobiology (2012), doi:10.1016/j.imbio.2012.01.011

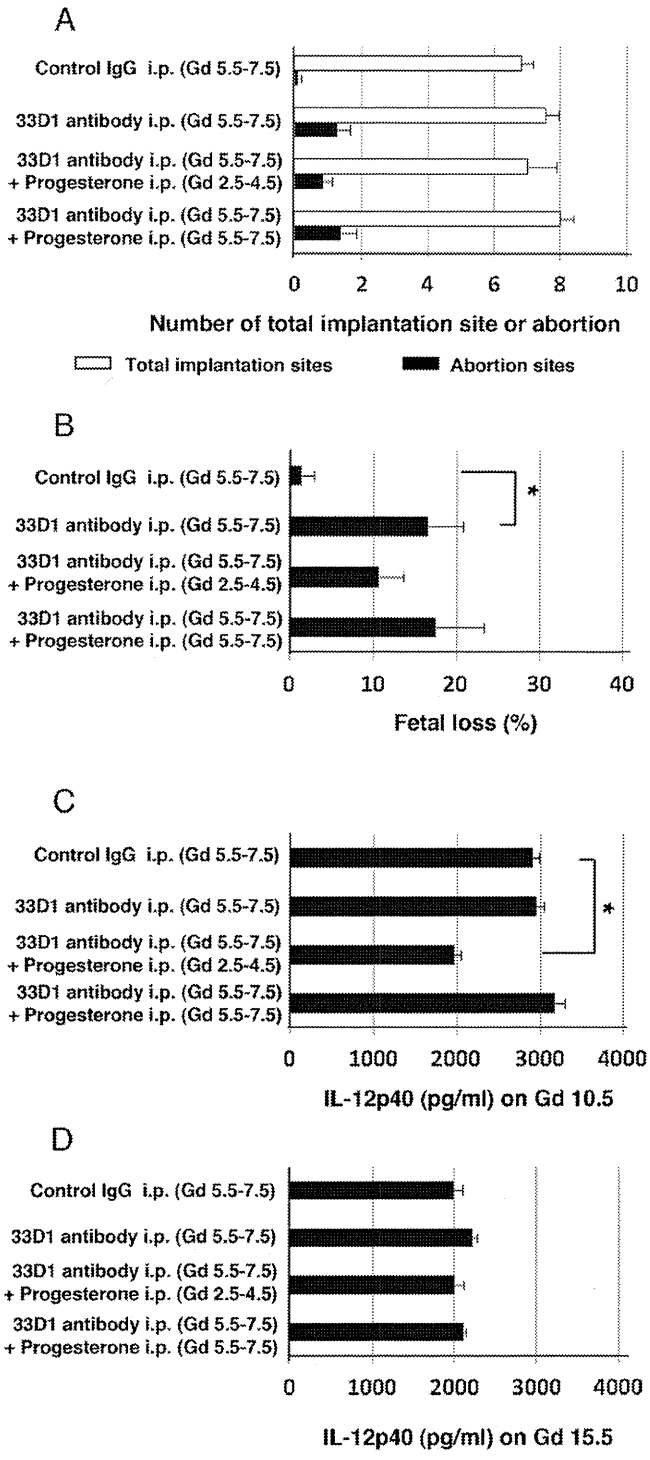

Fig. 7. 33D1 antibody and/or progesterone administration for C57BL/6 × C57BL/6 mating combination. For each figure, mice were injected i.p. either with 33D1 antibody or with control isotype-matched IgG on three consecutive days (Gd 5.5–7.5). Progesterone was also administrated i.p. on Gd 2.5, 3.5, and 4.5 or Gd 5.5, 6.5, and 7.5, consecutively. (A) Number of total implantation sites (open bars) and missing fetuses (abortion sites) (closed bars) per mouse. (B) Percentage of fetal loss with control IgG treatment, 33D1 antibody treatment, 33D1 antibody with progesterone (Gd 2.5–4.5) treatment and 33D1 antibody with progesterone (Gd 5.5–7.5) treatment on Gd 15.5. Serum IL-12p40 production with control isotype-matched IgG treatment, 33D1 antibody treatment, 33D1 antibody with progesterone (Gd 2.5–4.5) treatment, and 33D1 antibody with progesterone (Gd 5.5–7.5) treatment are shown either on Gd 10.5 (C) or on Gd 15.5 (D). Data are shown as the mean ± SEM of $n = 10$ mice for control IgG treatment, $n = 6$ mice for 33D1 antibody treatment, $n = 7$ mice for 33D1 antibody with progesterone (Gd 5.5–7.5) treatment and $n = 6$ mice for 33D1 antibody with progesterone (Gd 2.5–4.5) treatment per group. $^*p < 0.05$.

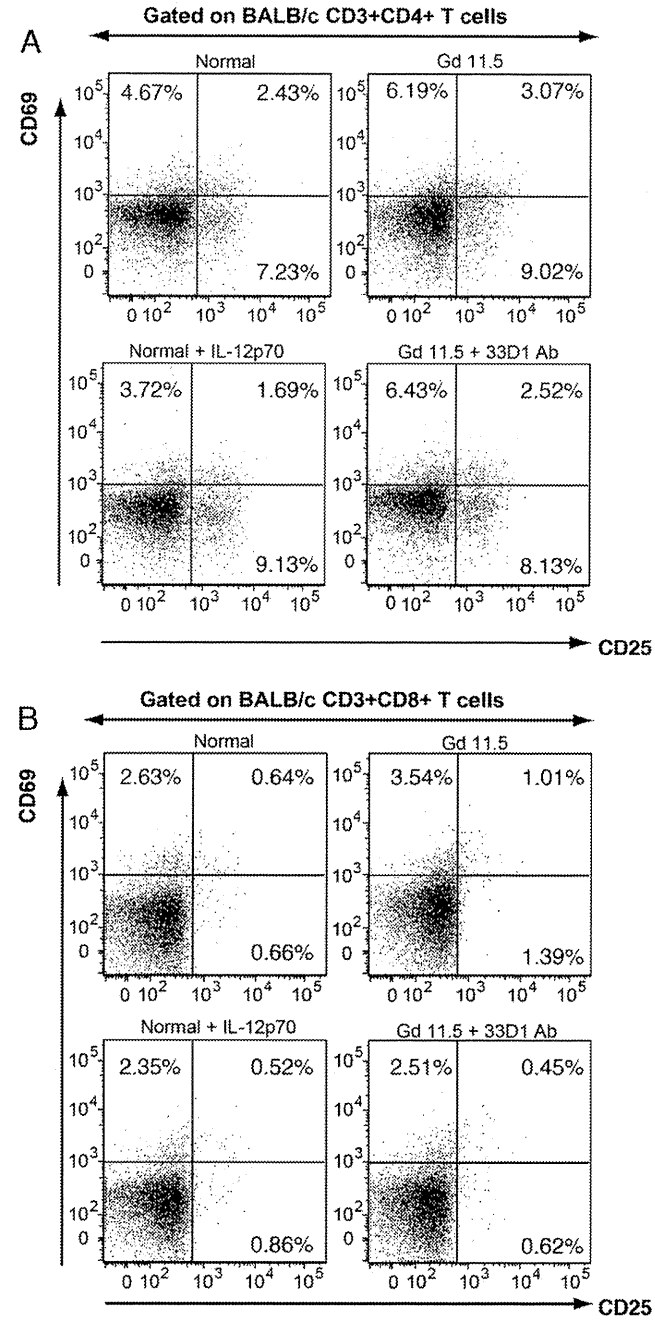

Fig. 8. Analysis of the activation markers on the splenic T cells obtained from BALB/c mice. Spleen cells, obtained from normal untreated BALB/c mice (upper left), from pregnant mice on Gd 11.5 (upper right), from pregnant mice on Gd 11.5 having miscarriages by IL-12p70 i.p. inoculation on Gd 9.5 and 10.5 (lower left), and from pregnant mice on Gd 11.5 having aborted fetus by i.p. injection of 33D1 antibody from Gd 5.5–7.5 (lower right), were stained with anti-CD3, anti-CD4, anti-CD8 mAb as well as anti-CD69 and anti-CD25 ($n = 5$ of each). By gating either CD3+CD4+ T cells (A) or CD3+CD8+ T cells (B), stained T cells were analyzed for their expression of activation markers, CD69 and CD25 by FACScanto II with FlowJo software.

with class II MHC molecules to CD4+ T cells. However, if phagocytotic APCs are DEC-205$^+$ DCs, having cross-presenting capacity (Dudziak et al. 2007; Shortman and Liu 2002), externally taken fetus-derived antigens will be presented in conjunction with class I MHC molecules that stimulate Th1-type cellular immunity, by which fetus-derived semi-allogeneic antigens will be recognized and the pregnancy terminated. Therefore, the proportion of DC subtypes in the decidual zone determines the continuity of pregnancy and thus several virus infections during pregnancy will shift the

Please cite this article in press as: Negishi, Y., et al., Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. Immunobiology (2012), doi:10.1016/j.imbio.2012.01.011

appropriate Th1/Th2 balance into the Th1-dominant state, which causes premature delivery. Indeed, a recent study reported that cross-presentation, an important mechanism leading to Th1 polarization, was mediated by DEC-205$^+$ DCs in HIV infection (Bozzacco et al. 2007; Moriya et al. 2010; Takahashi 2010; Trumpfheller et al. 2008).

The temporary enhancement of IL-12p40 production, eliciting Th1 dominancy, was seen in 33D1 antibody-injected pregnant mice on Gd 10.5. The fact that the size of miscarriages was approximately 3–5 mm, corresponding to the size of a live fetus around Gd 10.5, suggests that the onset of fetal loss induced by 33D1 antibody injection may occur around Gd 10.5 when Th1 dominancy started. The internal Th1 polarization at decidual sites mediated by the remaining DEC-205$^+$ DCs might initiate a miscarriage around Gd 10.5. Indeed, we observed miscarriages in pregnant mice inoculated with IL-12 around Gd 10.5 (Fig. 6D, second lane), although a higher amount of IL-12 administration around Gd 2-6 did not generate any miscarriages (Reina et al. 2004). Thus, the timing of IL-12 secretion seems to be critical for the induction of miscarriage. Also, as reported recently (Plaks et al. 2008; Pollard 2008), miscarriage may be associated with decidual angiogenesis mediated by the depletion of uterine DCs in a T cell-independent manner. Moreover, IL-13 administration improved the fetal loss induced by IL-12 injection (Fig. 6D, third lane), suggesting that IL-13 may have the capacity to maintain the pregnant state like progesterone. Further study will be needed to clarify the precise mechanism of miscarriage induced by transient elimination of 33D1 DCs *in vivo* in pregnant mice.

During pregnancy mediated by syngeneic mating, a transient increase of Th1-type cytokine IL-12 secretion in serum followed by temporal production of IL-13 was observed, despite cytokines released by cells of acquired immunity such as IL-2, IL-4, and IL-6 were not detected. Although the precise mechanisms remain to be elucidated, cytokines secreted mainly by innate DCs such as IL-12 and IL-13 may correlate with the unique kinetics of the DEC-205/33D1 ratio in our study. Many different cytokine profiles in various tissues for pregnancy have been demonstrated (Bizargity and Bonney 2009; Karsten et al. 2009). Th2-type cytokines are crucial at the feto-maternal interface and the production of cytokines varies according to the tissue and timing of gestation. Also, it has recently been reported that IL-15 production from uterine DCs is important for lymphoid cell homeostasis (Bizargity and Bonney 2009) and the proliferation of uterine NK cells (Karsten et al. 2009). These findings suggest that pregnancy seems to be controlled mainly by innate immune cells, such as DCs and NK cells, through "innate cytokines".

We have also demonstrated here the *in vitro* effects of maternal hormones on the development of DCs. Although some reports have shown the effect of progesterone and estradiol on the maturation and function of DCs (Segerer et al. 2009), the present study is the first to analyze the effect of progesterone and estradiol on DC development by focusing on two non-overlapping subpopulations, DEC-205$^+$ and 33D1$^+$ DCs. We found an apparent reduction of CD11c$^+$DEC-205$^+$ DCs and increase of CD11c$^+$33D1$^+$ DCs with a significant decrease of the DEC-205/33D1 ratio in a dose-dependent manner when these DCs were co-cultured with progesterone with/without 0.1 μM estradiol. Progesterone produced by the corpus luteum of the ovary and the placenta is known to be essential for the maintenance and establishment of pregnancy (Lydon et al. 1995), and our findings indicate that progesterone has a strong effect on suppressing Th1 dominancy by reducing the proportion of DEC-205$^+$ DCs, as well as increasing 33D1$^+$ DCs, particularly in the presence of estrogen. Similar to estradiol, human chorionic gonadotropin (hCG), secreted vigorously in the early stage of pregnancy for implantation but not in the late stage for its maintenance, can also affect the development of mouse and human DCs (Segerer et al. 2009; Wan et al. 2008). We are currently analyzing the effect of hCG on the differentiation and function of DEC-205$^+$ and 33D1$^+$ DCs.

The importance of DCs has been reported in human pregnancy (Laskarin et al. 2007). However, in humans, although DEC-205 is an important marker for DCs that induce Th1 type of immunity, it is also expressed on many other cells such as monocytes, T and B lymphocytes, and NK cells (Kato et al. 2006). Thus, to examine the effect of DEC-205$^+$ DCs during human pregnancy needs additional markers to pursue. It has been reported that C-type lectin called DC-specific intracellular adhesion molecule (ICAM)-3-grabbing nonintegrin (DC-SIGN) (Geijtenbeek et al. 2000) is observed in human decidualized endometrium DCs (Kammerer et al. 2003). Also, the number of DEC-205$^+$ DC-SIGN$^+$ DCs was up-regulated in placenta of patients fallen into hemolysis-elevated liver enzymes-low platelet (HELLP)-syndrome that sometimes causes serious complications in pregnant women (Scholz et al. 2008). Therefore, in the case of human pregnancy, it is recommended to examine both DC-SIGN and DEC-205 expression for targeting DCs.

It is unclear whether the marked enhancement of the DEC-205/33D1 ratio results in or from delivery; however, delivery, as well as pregnancy complications, miscarriage, HELLP-syndrome, and pre-eclampsia, may require a Th1-dominant condition. We thus experimentally induced Th1 dominancy during pregnancy by depleting 33D1$^+$ DCs required for Th2 predominance. To our surprise, macroscopically as well as statistically significant fetal loss was observed by elimination of 33D1$^+$ DCs. Progesterone is known to be an important hormone for the maintenance of successful pregnancy and is used to support luteal insufficiency in patients (Nardo and Sallam 2006). We injected progesterone into pregnant mice to see whether we could prevent fetal loss generated through the depletion of 33D1$^+$ DCs. Such fetal loss was actually prevented when progesterone was administered before the injection of 33D1 antibody.

Although the precise mechanisms for understanding the effect of progesterone need to be elucidated, temporary elevation of IL-12 on Gd 10.5 seems to be the key event to initiate miscarriage in pregnant mice treated with 33D1 antibody injection. Indeed, similar miscarriages were also observed when pregnant mice were i.p. injected twice with 0.2 μg/mouse IL-12p70 on Gd 9.5 and 10.5. Moreover, prior inoculation with progesterone suppressed enhanced serum IL-12 production in mice treated with 33D1 antibody. Taken together, our findings strongly indicate that progesterone might inhibit temporal IL-12 secretion on Gd 10.5 and thus the miscarriages induced by 33D1 antibody injection were saved. Further analysis of innate immunity, particularly in DC subsets together with their products, IL-12 and IL-13, may provide valuable and important information for understanding pregnancy.

Acknowledgments

This study was supported in part by grants from the Ministry of Education, Science, Sport, and Culture, from the Ministry of Health and Labor and Welfare, Japan, and from the Japanese Health Sciences Foundation, and by the Promotion and Mutual Aid Corporation for Private Schools of Japan.

References

Bizargity, P., Bonney, E.A., 2009. Dendritic cells: a family portrait at mid-gestation. Immunology 126, 565–578.
Blois, S.M., Joachim, R., Kandil, J., Margni, R., Tometten, M., Klapp, B.F., Arck, P.C., 2004. Depletion of CD8+ cells abolishes the pregnancy protective effect of progesterone substitution with dydrogesterone in mice by altering the Th1/Th2 cytokine profile. J. Immunol. 172, 5893–5899.
Blois, S.M., Kammerer, U., Alba Soto, C., Tometten, M.C., Shaikly, V., Barrientos, G., Jurd, R., Rukavina, D., Thomson, A.W., Klapp, B.F., et al., 2007. Dendritic cells: key to fetal tolerance? Biol. Reprod. 77, 590–598.

Please cite this article in press as: Negishi, Y., et al., Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. Immunobiology (2012), doi:10.1016/j.imbio.2012.01.011

Bozzacco, L., Trumpfheller, C., Siegal, F.P., Mehandru, S., Markowitz, M., Carrington, M., Nussenzweig, M.C., Piperno, A.G., Steinman, R.M., 2007. DEC-205 receptor on dendritic cells mediates presentation of HIV gag protein to CD8+ T cells in a spectrum of human MHC I haplotypes. Proc. Natl. Acad. Sci. U.S.A. 104, 1289–1294.

Collins, M.K., Tay, C.S., Erlebacher, A., 2009. Dendritic cell entrapment within the pregnant uterus inhibits immune surveillance of the maternal/fetal interface in mice. J. Clin. Invest. 119, 2062–2073.

Doria, A., Iaccarino, L., Arienti, S., Ghirardello, A., Zampieri, S., Rampudda, M.E., Cutolo, M., Tincani, A., Todesco, S., 2006. Th2 immune deviation induced by pregnancy: the two faces of autoimmune rheumatic diseases. Reprod. Toxicol. 22, 234–241.

Doria, A., Tincani, A., Lockshin, M., 2008. Challenges of lupus pregnancies. Rheumatology (Oxford) 47 (Suppl. 3), iii9–12.

Dudziak, D., Kamphorst, A.O., Heidkamp, G.F., Buchholz, V.R., Trumpfheller, C., Yamazaki, S., Cheong, C., Liu, K., Lee, H.W., Park, C.G., et al., 2007. Differential antigen processing by dendritic cell subsets in vivo. Science 315, 107–111.

Figdor, C.G., van Kooyk, Y., Adema, G.J., 2002. C-type lectin receptors on dendritic cells and Langerhans cells. Nat. Rev. Immunol. 2, 77–84.

Geijtenbeek, T.B., Kwon, D.S., Torensma, R., van Vliet, S.J., van Duijnhoven, G.C., Middel, J., Cornelissen, I.L., Nottet, H.S., KewalRamani, V.N., Littman, D.R., et al., 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell 100, 587–597.

Kammerer, U., Eggert, A.O., Kapp, M., McLellan, A.D., Geijtenbeek, T.B., Dietl, J., van Kooyk, Y., Kampgen, E., 2003. Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. Am. J. Pathol. 162, 887–896.

Karsten, C.M., Behrends, J., Wagner, A.K., Fuchs, F., Figge, J., Schmudde, I., Hellberg, L., Kruse, A., 2009. DC within the pregnant mouse uterus influence growth and functional properties of uterine NK cells. Eur. J. Immunol. 39, 2203–2214.

Kato, M., McDonald, K.J., Khan, S., Ross, I.L., Vuckovic, S., Chen, K., Munster, D., MacDonald, K.P., Hart, D.N., 2006. Expression of human DEC-205 (CD205) multilectin receptor on leukocytes. Int. Immunol. 18, 857–869.

Langenkamp, A., Messi, M., Lanzavecchia, A., Sallusto, F., 2000. Kinetics of dendritic cell activation: impact on priming of TH1, TH2 and nonpolarized T cells. Nat. Immunol. 1, 311–316.

Laskarin, G., Kammerer, U., Rukavina, D., Thomson, A.W., Fernandez, N., Blois, S.M., 2007. Antigen-presenting cells and materno-fetal tolerance: an emerging role for dendritic cells. Am. J. Reprod. Immunol. 58, 255–267.

Liu, K., Nussenzweig, M.C., 2010. Origin and development of dendritic cells. Immunol. Rev. 234, 45–54.

Lydon, J.P., DeMayo, F.J., Funk, C.R., Mani, S.K., Hughes, A.R., Montgomery Jr., C.A., Shyamala, G., Conneely, O.M., O'Malley, B.W., 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. Genes Dev. 9, 2266–2278.

Maldonado-Lopez, R., De Smedt, T., Michel, P., Godfroid, J., Pajak, B., Heirman, C., Thielemans, K., Leo, O., Urbain, J., Moser, M., 1999. CD8alpha+ and CD8alpha-subclasses of dendritic cells direct the development of distinct T helper cells in vivo. J. Exp. Med. 189, 587–592.

Moriya, K., Wakabayashi, A., Shimizu, M., Tamura, H., Dan, K., Takahashi, H., 2010. Induction of tumor-specific acquired immunity against already established tumors by selective stimulation of innate DEC-205(+) dendritic cells. Cancer Immunol. Immunother. 59, 1083–1095.

Nagler, A., Ohana, M., Alper, R., Doviner, V., Sherman, Y., Rabbani, E., Engelhardt, D., Ilan, Y., 2003. Induction of oral tolerance in bone marrow transplantation recipients suppresses graft-versus-host disease in a semiallogeneic mouse model. Bone Marrow Transplant. 32, 363–369.

Nardo, L.G., Sallam, H.N., 2006. Progesterone supplementation to prevent recurrent miscarriage and to reduce implantation failure in assisted reproduction cycles. Reprod. Biomed. Online 13, 47–57.

Paharkova-Vatchkova, V., Maldonado, R., Kovats, S., 2004. Estrogen preferentially promotes the differentiation of CD11c+ CD11b(intermediate) dendritic cells from bone marrow precursors. J. Immunol. 172, 1426–1436.

Piccinni, M.P., Beloni, L., Livi, C., Maggi, E., Scarselli, G., Romagnani, S., 1998. Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions. Nat. Med. 4, 1020–1024.

Plaks, V., Birnberg, T., Berkutzki, T., Sela, S., BenYashar, A., Kalchenko, V., Mor, G., Keshet, E., Dekel, N., Neeman, M., Jung, S., 2008. Uterine DCs are crucial for decidua formation during embryo implantation in mice. J. Clin. Invest. 118, 3954–3965.

Pollard, J.W., 2008. Uterine DCs are essential for pregnancy. J. Clin. Invest. 118, 3832–3835.

Reina, M., Broccia, M.L., Menegola, E., Di Blasio, A.M., Vigano, P., Giavini, E., 2004. Effects of interleukin-12 administration during the pre- and peri-implantation period on mouse embryofetal development. Am. J. Reprod. Immunol. 51, 345–351.

Scholz, C., Toth, B., Santoso, L., Kuhn, C., Franz, M., Mayr, D., Jeschke, U., Friese, K., Schiessl, B., 2008. Distribution and maturity of dendritic cells in diseases of insufficient placentation. Am. J. Reprod. Immunol. 60, 238–245.

Segerer, S.E., Muller, N., van den Brandt, J., Kapp, M., Dietl, J., Reichardt, H.M., Rieger, L., Kammerer, U., 2009. Impact of female sex hormones on the maturation and function of human dendritic cells. Am. J. Reprod. Immunol. 62, 165–173.

Shortman, K., Liu, Y.J., 2002. Mouse and human dendritic cell subtypes. Nat. Rev. Immunol. 2, 151–161.

Steinman, R.M., 1991. The dendritic cell system and its role in immunogenicity. Annu. Rev. Immunol. 9, 271–296.

Takahashi, H., 2010. Species-specific CD1-restricted innate immunity for the development of HIV vaccine. Vaccine 28 (Suppl. 2), B3–B7.

Takahashi, H., Nakagawa, Y., Leggatt, G.R., Ishida, Y., Saito, T., Yokomuro, K., Berzofsky, J.A., 1996. Inactivation of human immunodeficiency virus (HIV)-1 envelope-specific CD8+ cytotoxic T lymphocytes by free antigenic peptide: a self-veto mechanism. J. Exp. Med. 183, 879–889.

Trumpfheller, C., Caskey, M., Nchinda, G., Longhi, M.P., Mizenina, O., Huang, Y., Schlesinger, S.J., Colonna, M., Steinman, R.M., 2008. The microbial mimic poly IC induces durable and protective CD4+ T cell immunity together with a dendritic cell targeted vaccine. Proc. Natl. Acad. Sci. U.S.A. 105, 2574–2579.

Virgo, B.B., Bellward, G.D., 1974. Serum progesterone levels in the pregnant and postpartum laboratory mouse. Endocrinology 95, 1486–1490.

Wan, H., Versnel, M.A., Leijten, L.M., van Helden-Meeuwsen, C.G., Fekkes, D., Leenen, P.J., Khan, N.A., Benner, R., Kiekens, R.C., 2008. Chorionic gonadotropin induces dendritic cells to express a tolerogenic phenotype. J. Leukoc. Biol. 83, 894–901.

Please cite this article in press as: Negishi, Y., et al., Disruption of maternal immune balance maintained by innate DC subsets results in spontaneous pregnancy loss in mice. Immunobiology (2012), doi:10.1016/j.imbio.2012.01.011

Human Immunology 72 (2011) 783-790

Contents lists available at SciVerse ScienceDirect

journal homepage: www.elsevier.com/locate/humimm

Induction of CD56$^+$ T cells after prolonged activation of T cells *in vitro:* A possible mechanism for CD4$^+$ T-cell depletion in acquired immune deficiency syndrome patients

Megumi Takahashi*, Jiro Matsumura, Shinichiro Inagaki, Hidemi Takahashi

Department of Microbiology and Immunology, Nippon Medical school, Tokyo, Japan

ARTICLE INFO

Article history:
Received 13 April 2011
Accepted 8 June 2011
Available online 7 July 2011

Keywords:
CD56$^+$ T cells
HIV-1
CD4$^+$ T cell depletion
Immune activation

ABSTRACT

The pathogenic mechanisms responsible for depletion of CD4$^+$ T cells in aquired immune deficiency syndrome (AIDS) are not fully understood. Systemic immune activation mediated by persistent infection of human immunodeficiency virus (HIV) seems to be one of the predictors of disease progression. We predicted that certain lymphocytes responsible for CD4$^+$ T-cell depletion could be induced in patients during prolonged activation of lymphocytes. Therefore, we have established an *in vitro* long-term culture system for peripheral blood mononuclear cells with PHA-P stimulation and Herpesvirus saimiri infection, and examined what types of cells having strong cytotoxic activity to be emerged under the activated conditions. We observed that percentage of CD56$^+$ T cells was gradually increased in cultures from 30 days after stimulation and exhibited a cytotoxic activity against both autologous and allogeneic targets. Interestingly, HIV-1 infection enhanced the susceptibility of CD4$^+$ T cells to their cytotoxic effectors, and CD4$^+$ T cells from HIV-1–infected individuals showed decreased survival rate in the presence of autologous CD56$^+$ T cells. These findings raised the possibility that induction of autoreactive CD56$^+$ T cells in consequence of immune activation might be contributed to the depletion of CD4$^+$ T cells in HIV-1–infected patients.

1. Introduction

The progressive depletion of CD4$^+$ T cells in human immunodeficiency virus (HIV) –infected patients is still a major fundamental and controversial question in acquired immunde deficiency syndrome (AIDS) research. HIV selectively infects and kills cells expressing the CD4 molecule on their surface, particularly T lymphocytes and cells of the monocyte/macrophage lineage. Direct killing of infected cells appears to contribute to the loss of CD4$^+$ T cells in primary HIV-1 infection [1,2]. However, the percentage of HIV-1–infected CD4$^+$ T cells is too small to account for the extensive depletion of T-helper cells in patients who are progressing to AIDS through chronic infection with HIV-1 [3,4]. Thus, several other mechanisms have been proposed to explain the deletion of CD4$^+$ T cells [5,6].

HIV infection leads to sustained immune activation and causes profound alterations in T-cell homeostasis [7]. HIV-infected patients display elevated expression of activation markers, such as CD38, CD25, and CD69, on CD4$^+$ and CD8$^+$ T cells. High levels of proinflammatory cytokines and chemokines, such as tumor necrosis factor–α (TNF-α), interleukin-6 (IL-6), interleukin-1β (IL-1β), and macrophage inflammatory protein (MIP-1) are also observed in both plasma and lymph nodes in these patients [8,9]. Although the actual mechanisms underlying such an immune activation remain unclear, higher T-cell activation levels are associated with CD4$^+$ T-cell depression in untreated patients independent of plasma HIV RNA [10,11].

Previously, it has been reported that HIV-1–infected individuals had circulating cytotoxic T cells that were cytotoxic for noninfected CD4$^+$ T cells, and it was suggested that autoimmune phenomena contributed to the depletion of CD4$^+$ T cells [12,13]. It is interesting which types of cells are induced in consequence of immune activation and exhibit cytotoxicity against autologous CD4$^+$ T cells. In most *in vitro* studies, effector cells were prepared from peripheral blood mononuclear cells (PBMC) stimulated with mitogen followed by short-time culture in the presence of cytokines. We wanted to find effector cells in activated PBMC without adding any specific cytokines as well as antigens after long-term culture, because the presence of cytokines and antigens result in cytokine dependent expansions or antigen specific T-cell responses, and chronic immune activation persist for long time after HIV-1 infection *in vivo*. Therefore, we have established an *in vitro* long-term culture system for PBMC in the absence of cytokines and examined what types of cells are induced under the activated culture condition.

We found that CD56$^+$ T cells were gradually increased from long-cultured PBMC at approximately 30 days after stimulation, and we observed their cytotoxic activities against various tumor

* Corresponding author.
E-mail address: megumi@nms.ac.jp (M. Takahashi).

0198-8859/11/$32.00 - see front matter © 2011 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.humimm.2011.06.001

cells in a non–major histocompatibility complex (MHC)–restricted manner as well as autologous cells. It should be noted that HIV-1 infection enhanced the susceptibility of $CD4^+$ T cells to be killed by $CD56^+$ T cells. Moreover, $CD56^+$ T cells from HIV-1–infected patients exhibited a potent cytotoxicity against autologous $CD4^+$ T cells by *in vitro* activation. Therefore, if such autoreactive $CD56^+$ T cells will be expanded in HIV-1–infected patients, both non–HIV-1–infected and infected $CD4^+$ T cells might be strongly killed and depleted. Thus, we presented here a possible involvement of $CD56^+$ T cells in the depletion of $CD4^+$ T cells in HIV-1 infection.

2. Subjects and methods

2.1. Preparation of cells

Blood samples were obtained from healthy donors and highly active antiretroviral therapy (HAART)–treated or untreated asymptomatic HIV-1–infected individuals with peripheral $CD4^+$ T lymphocytes counts of $>300/\mu l$. All subjects gave informed consent under a protocol approved by the Institutional Review Board of the Nippon Medical School. PBMC were purified over a Ficoll density gradient and stimulated with 10 μg/ml of phytohemagglutinin-P (PHA-P: Sigma-Aldrich, St. Louis, MO) for 24 hours and subsequently inoculated with the Herpesvirus saimiri (HVS) strain C-488; kindly provided by M. Yasukawa at moi 10. Stimulated PBMC were cultured with T-cell culture medium (CTM) [14], composed of RPMI 1640 medium supplemented with 2 mmol/l of L-glutamine, 1 mmol/l of sodium pyruvate, 0.1 mmol/l of nonessential amino acids, a mixture of vitamins, 1 mmol/l of HEPES, 100 U/ml of penicillin, 100 μg/ml of streptomycin, 50 μmol/l of 2-mercaptoethanol, and heat-inactivated 10% fetal calf serum (FCS). Approximately 50 days later, stimulated PBMC, termed long-cultured PBMC (LC-PBMC), were used as effector cells in cell-mediated cytotoxicity assays. MOLT-4, MOLT-4/HIV-1_{IIIB} (kindly provided by S. Harada), Jurkat, K562 cells were cultured in CTM. Human glioblastoma cells, A172 and A172-448 [15], were grown in Eagle's minimum essential medium (MEM) supplemented with 10% FCS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. To establish the HVS transformed T-cell line, PBMC were stimulated with PHA-P and infected with HVS at moi 0.1. Stimulated cells were cultured in CTM with 100 U/ml of IL-2 for more than 3 months.

2.2. Antibodies and flow-cytometric analysis

Cells were pelleted and resuspended at a concentration of $<5 \times 10^5$ cells in 50 μl of PBS with 0.1% NaN_3 and 1% human serum containing each monoclonal antibody (mAb). FITC- or PE-labeled mouse anti-human CD3, CD4, CD8, CD56, CD1d, $\gamma\delta$, and Fas mAb were purchased from BD Biosciences (San Diego, CA). APC-labeled mouse anti-human CD3 and PE-labeled mouse anti-human MICA/B mAb were purchased from Biolegend (San Diego, CA). FITC-labeled mouse anti-human Vα24 mAb was purchased from Beckman Coulter, Inc. (La Brea, CA). PE-labeled mouse anti-human ULBP-2 mAb was purchased from R&D Systems, Inc. (Minneapolis, MN). After 30 minutes' incubation with each antibody at 4°C, cells were washed and resuspended in PBS for analysis by FACScan (BD Biosciences). Neutralizing antibodies against Fas ligand and NKG2D were purchased from R&D Systems, and CD1d were obtained from BD Biosciences.

2.3. Cytotoxicity assay

Cytotoxicity was assessed in a standard 4-hour ^{51}Cr-release assay with ^{51}Cr-labeled targets at various E:T ratios in 96well, U-bottomed culture plates. After incubation, the plates were centrifuged, and 100 μl of cell-free supernatants was collected to measure the radioactivity using a γ-counter. The percentage of specific ^{51}Cr release was calculated as follows: (experimental release- − spontaneous release)/(maximum release- − spontaneous release) × 100. In our laboratory, we cannot perform the ^{51}Cr-release assay using HIV-1–infected cells because of the constraint of Infectious Diseases Control Law in Japan. Therefore, we have established a method to measure the cytotoxic activity by flow-cytometric analysis using carboxyfluorescein succinimidyl ester (CFSE; Life Technology Corporation, Carlsbad, CA) staining [16]. Target cells were labeled with 0.8 nmol/l CFSE to differentiate from effector cells. Effectors and targets were mixed at a ratio of 1:1 and incubated at 37°C for 24 hours. After incubation, cells were harvested and resuspended in 4% paraformaldehyde in PBS and then stored at 4°C in the dark before cytometry. Figure 1A shows the cell size and granularity profiles of target cell alone (upper) and co-culture of targets and effectors (lower). High fluorescence intensity of CFSE in target cells was indicated by R1 region (R1 gate), and viable cells were gated from nonviable cells by their distinct forward scatter (FSC) versus side scatter (SSC) (R2 gate). R1 and R2 gates were used for estimation of survival target cell number. The percentage of survival target cells was calculated as follows: (survival target cells in co-culture of targets and effectors)/(survival targets in target cell alone) × 100.

2.4. Cell purification or depletion

Cells were incubated with biotinylated monoclonal antibody for 5 minutes at room temperature and washed three times with PBS. The labeled cells were then further incubated with streptoavidin microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), followed by magnetic column selection or depletion, respectively. Biotynylated mouse anti-human CD4 and CD8 were purchased from BD Biosciences, and CD56 were obtained from Biolegend.

2.5. Granzyme B activity

Granzyme B activity was determined by granzyme B (GrB)–specific hydrolysis of substrates. A 180-μl volume of the reaction mixture (200 mmol/l acetyl-Ile-Glu–Pro–Asp-paranitroanilide in reaction buffer containing 50 mmol/l HEPES, pH 7.5, 10% sucrose, 0.05% 3-(3-cholamidepropyl)dimethylammonio-1-propanesulphonate (CHAPS), and 5 mmol/l dithiothreitol (DTT)) was added to 20 μl of cell lysates. The absorbance at 405 nm was measured after incubation for 24 hours at 37°C. Concentration of samples were determined using a standard curve with recombinant mouse GrB (Sigma-Aldrich, St. Louis, MO).

2.6. Detection of HVS DNA

DNA was isolated from LC-PBMC and HVS transformed T-cell line using Blood & Tissue Genome Mini (Viogene, Sunnyvale, CA). HVS genome was detected by polymerase chain reaction (PCR) using the following primers for the STP gene from HVS subgroup C strain 488 to generate a 783-bp fragment: 5′-CTCTAAGCA-CAGGGGCACA-3′ and 5′-CTACGCAGAAGTCGGAAGCC-3′.

2.7. Statistical analysis

Student's *t* test was used to determine the significance of differences in means. A p value of <0.05 was considered significant.

3. Results

3.1. Expression of cytotoxic activity in LC-PBMC

To examine which types of cells to be emerged *in vitro* cultures of PBMC under the activated conditions, we have established a unique *in vitro* long-term culture system for PBMC without adding any exogenous cytokines as well as specific antigens, as the treatments lead to initiation of cytokine production or antigen-specific T-cell responses. To carry out the experiments, we cultured PBMC stimulated with PHA-P and subsequently infected with HVS. HVS, an oncogenic tumor virus of New World monkeys, has been reported to infect and to immortalize human CD4 and CD8 T cells

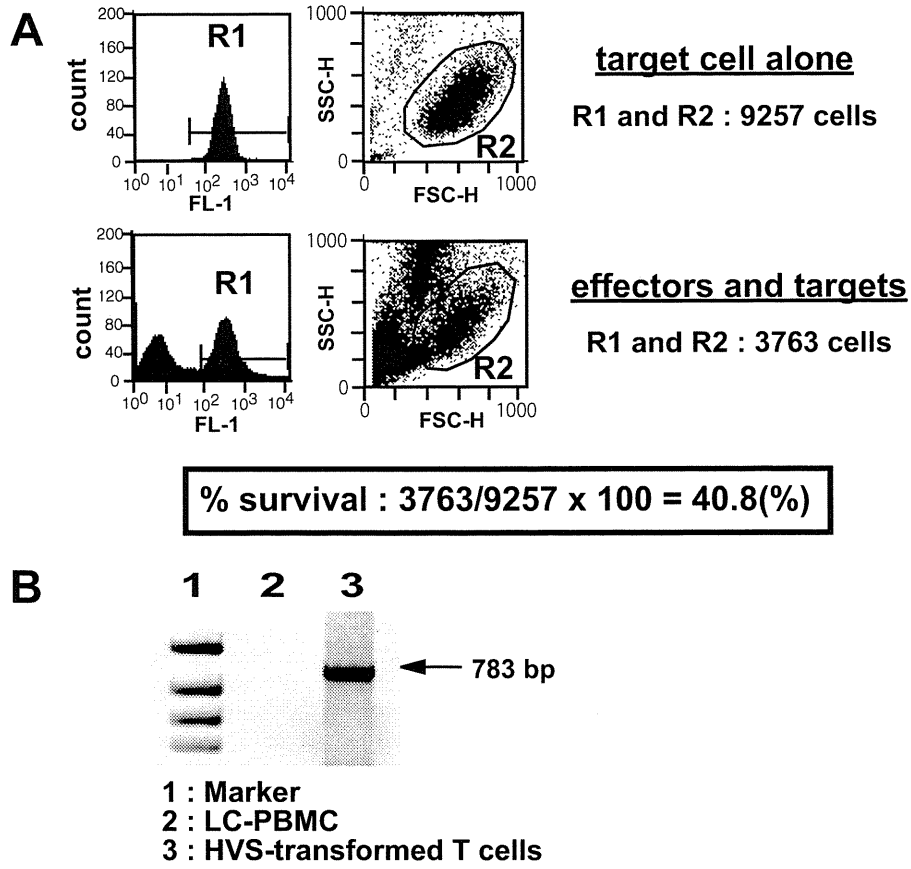

Fig. 1. Induction of long-cultured PBMC (LC-PBMC) and detection of their cytotoxic activity. (A) Method for measurement of cytotoxic activity by flow-cytometric analysis using carboxyfluorescein succinimidyl ester (CFSE) staining. R1, high fluorescence intensity of CFSE in target cells. R2, viable cells gated from nonviable cells by their distinct FSC versus SSC. A total of 10,000 cells gated in R1 were acquired, and R1 and R2 gates were used for estimation of survival target cell number. (B) PCR amplification of the Herpesvirus saimiri (HVS) genome. PCR products of the expected size were detected in HVS-infected owl monkey kidney cells and no PCR product was detected in LC-PBMC.

expressing $\alpha\beta$ and $\gamma\delta$ T cells [17,18]. We found that when PHA-stimulated PBMC were infected with HVS at high moi and then cultured in the absence of IL-2, T cells could maintain an activated state more than 30 days after stimulation. However, these treatments scarcely yielded HVS-transformed T-cells line. Indeed, as shown in Fig. 1B, we could not detect the PCR amplification of HVS gene in 60 days of cultured PBMC. We defined here such stimulated T cells as LC-PBMC, which could actively replicate more than 30 days after stimulation and maintain the cultures for more than 50 days without adding of any specific antigens and cytokines, but were not immortalized.

When we examined the cytotoxic potential of LC-PBMC after culturing them for more than 50 days in a ^{51}Cr -release cytotoxity assay, they showed significant killing activity against various T-cell lines, K562 cells, and A172 cells in a non–MHC-restricted manner. Moreover, LC-PBMC showed weak cytotoxicity against autologous PBMC (Fig. 2A). Next, we examined whether virus infection would affect the susceptibility to cytotoxic activity of LC-PBMC, by using persistently infected cell lines. Although MOLT-4 were efficiently killed by LC-PBMC, HIV-1-producing counterpart cells MOLT-4/HIV-1 were more sensitive to LC-PBMC. However, measles virus (MV)–infected A172-448 or HVS-transformed T cells were not susceptible compared with noninfected cells (Fig. 2B). These findings suggest that HIV-1 infection might enhance the susceptibility of infected cells to LC-PBMC.

3.2. $CD3^+CD56^+$ cells might be major effectors in LC-PBMC

We wanted to address the question of which types of cells among LC-PBMC are responsible for producing such cytotoxic activities. Therefore, aliquots of the cultures were removed on days 0, 3, 10, 20, 30, 40, and 50 of cultures for phenotypic analysis and measurement of cytotoxic activity against both MOLT-4 and MOLT-4/HIV-1. Time course analysis showed that until 30 days after, LC-PBMC exhibited little cytotoxic activity against both MOLT-4 and MOLT-4/HIV-1. However, after 40 days of stimulation, LC-PBMC mediated approximately 75% and 50% survival of MOLT-4 and MOLT-4/HIV-1, respectively, and another 10 days later LC-PBMC mediated less than 50% and 20% survival (Fig. 3A). The percentage of $CD3^-CD56^+$ cells was decreased by 20 days after *in vitro* stimulation, whereas that of $CD3^+CD56^+$ cells was increased gradually from 30 days after stimulation. Changes in the relative percentage of $\gamma\delta$T cells were minimal (Fig. 3B). These results suggested that $CD3^+CD56^+$ cells might be the major effectors in LC-PBMC, and natural killer (NK) cell and $\gamma\delta$ T-cell contribution was limited.

3.3. Characterization of cytotoxic cells in LC-PBMC

When the effector and target cells were separated by permeable filter, LC-PBMC showed minimal killing (data not shown). Therefore, cell–cell contact between effectors and targets are required for efficient killing by LC-PBMC. Figure 3 demonstrates that cytotoxic activity was elevated in parallel with increase of $CD3^+CD56^+$ cells. Thus, to examine the possible engagement of $CD3^+CD56^+$ cells in this cytotoxicity, $CD56^+$ cells were depleted from LC-PBMC by magnetic bead selection. An apparent reduction in cytotoxic activity was observed against MOLT-4/HIV-1 (Fig. 4A). In addition, depletion of $CD8^+$ cells had a significant effect on the cytotoxicity of LC-PBMC, as two-thirds of $CD3^+CD56^+$ cells were CD8 positive (Fig. 4B). Taken together, these results suggested that $CD3^+CD8^+CD56^+$

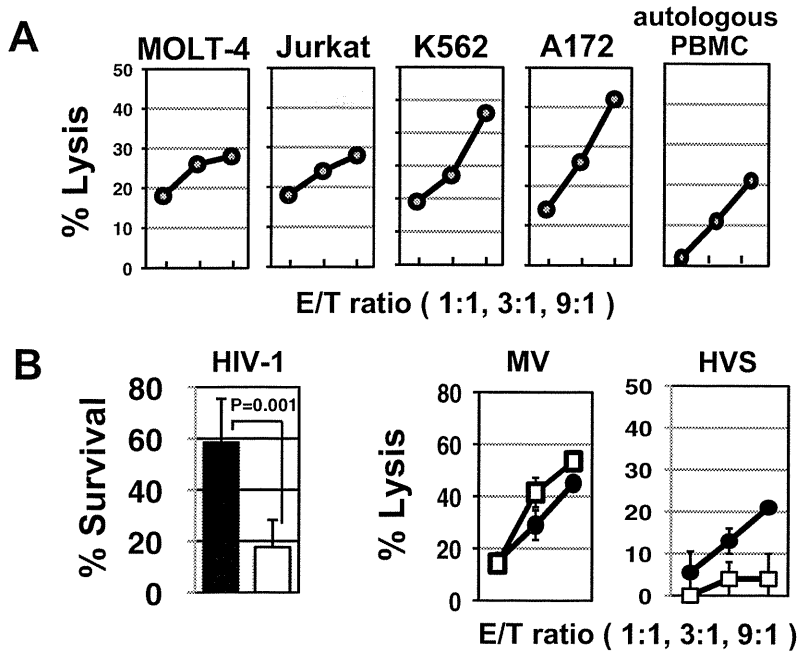

Fig. 2. Cytotoxic activity of long-cultured PBMC (LC-PBMC) against MOLT-4, Jurkat, K562, A172, and autologous PBMC (A). Cytotoxicity was assessed in ^{51}Cr-release assay. (B) Effect of virus infection on the susceptibility of target cells to cytotoxic activity of LC-PBMC. Assessment of cytotoxic activity was evaluated as survival rate in HIV-1 infection (closed bar; MOLT-4, open bar; MOLT-4/HIV-1) and was done as killing rate in measles virus (MV) (filled circle, A172; open square, A172-448) or Herpesvirus saimiri (HVS) infection (filled circle, PHA-P–stimulated T cells; open squares, HVS-transformed T cells).

cells might be the predominant effectors in LC-PBMC. We then assessed the contribution of FasL and NKG2D to LC-PBMC–mediated cytotoxicity using the neutralizing antibodies to FasL and NKG2D. Figure 4C demonstrates that anti-NKG2D antibody significantly affected the cytotoxic activity of LC-PBMC against MOLT-4/HIV-1, whereas anti-FasL antibody blocked the cytotoxicity only slightly. Moreover, increased granzyme B activity was observed when LC-PBMC were incubated with target cells (Fig. 4D).

Type 1 NKT cells is characterized by a semi-invariant T-cell receptor (TCR) using a unique TCR Vα24Jα18 chain in human and by its recognition of the glycolipid α-galactosylceramide (α-GalCer) loaded onto CD1d molecules. We tested the expression of Vα24 on CD3$^+$CD56$^+$ cells in LC-PBMC, but they did not express this molecule (Fig. 4E, left panel). We also examined whether cytotoxicity of target cells by LC-PBMC was in a CD1d-restricted manner, target cells were incubated with anti-CD1d neutralizing antibody [19] before co-culturing with effector cells. As shown in Fig. 4E, cytotoxic activity of LC-PBMC was not affected, suggesting that LC-PBMC-mediated cytotoxicity was not CD1d restricted.

3.4. HIV-1–infected CD4$^+$ T cells are more susceptible to cytotoxic activity of LC-PBMC

We then asked why HIV-1–infected cells are more susceptible to LC-PBMC–mediated killing than are noninfected cells. Because the killing of target cells was mostly NKG2D mediated, we examined the cell surface expression of ligands for NKG2D on both MOLT-4 and MOLT-4/HIV-1. Phenotypic analysis showed that the level of CD1d and MICA/B expression on MOLT-4/HIV-1 were lower than MOLT-4, whereas that of ULBP-2 was higher (Fig. 5A). These findings agree with previous report that HIV-1-Vpr up-regulated the expression of ligands for the activating NKG2D receptor, including ULBP-1, −2, and −3 but not MICA/B in infected cells [20]. However, as treatment of targets cells with anti–ULBP-2 antibody slightly blocked the cytotoxiciy of LC-PBMC (data not shown), all other ULBPs might be involved in enhancing susceptibility of HIV-1–infected cells to LC-PBMC–mediated killing.

Finally, to confirm the susceptibility of HIV-1–infected CD4$^+$ T cells to the cytotoxic activity of LC-PBMC, we infected primary CD4$^+$ T cells from healthy or HIV-1–infected donors with HIV-1 IIIB at moi of 0.1 for 4 days, followed by co-culturing with autologous LC-PBMC for 1 day. As shown in Fig. 5B, LC-PBMC were cytotoxic against autologous CD4$^+$ T cells and became more cytotoxic against HIV-1–infected CD4$^+$ T cells. Of note, although the percentages of CD3$^+$CD56$^+$ cells in LC-PBMC from HIV-1–infected individuals were little less than from healthy donors (data not show), CD4$^+$ T cells from HIV-1–infected patients showed a decreased survival rate in the presence of autologous LC-PBMC compared with healthy donors.

4. Discussion

Chronic immune activation is one of the characteristic features in HIV infection [21]. Polyclonal B-cell activation, increased T-cell turnover, increased frequencies of T cells with an activated phenotype, and increased serum levels of proinflammatory cytokines and chemokines were observed in HIV-1–infected patients [22]. In the absence of antiretroviral treatment, markers of T-cell activation and T-cell turnover predicted the rate of disease progression and CD4$^+$ T-cell depletion [10,23]. Chronic simian immunodeficiency virus (SIV) infection in macaques provides a relevant and useful model to explore the mechanisms for progressive CD4$^+$ T-cell decline in AIDS pathogenesis. Rhesus macaques, which develop progressive CD4$^+$ T-cell depletion and progression to AIDS upon SIV infection, are characterized by strong T-cell activation. In contrast, SIV-infected sooty mangabeys and African green monkeys, the natural hosts of SIV, which do not develop any immunodeficiency, exhibit minimal T-cell activation despite evident viral replication [24]. Although the underlying causes of immune activation have remained elusive, several mechanisms have been proposed. For example, CD4$^+$ T-cell depletion during the acute stage of HIV-1 infection occurs rapidly within the first few weeks of infection and is predominantly localized to the gastrointestinal tract. Therefore, in the context of a compromised gastrointestinal mucosal surface,

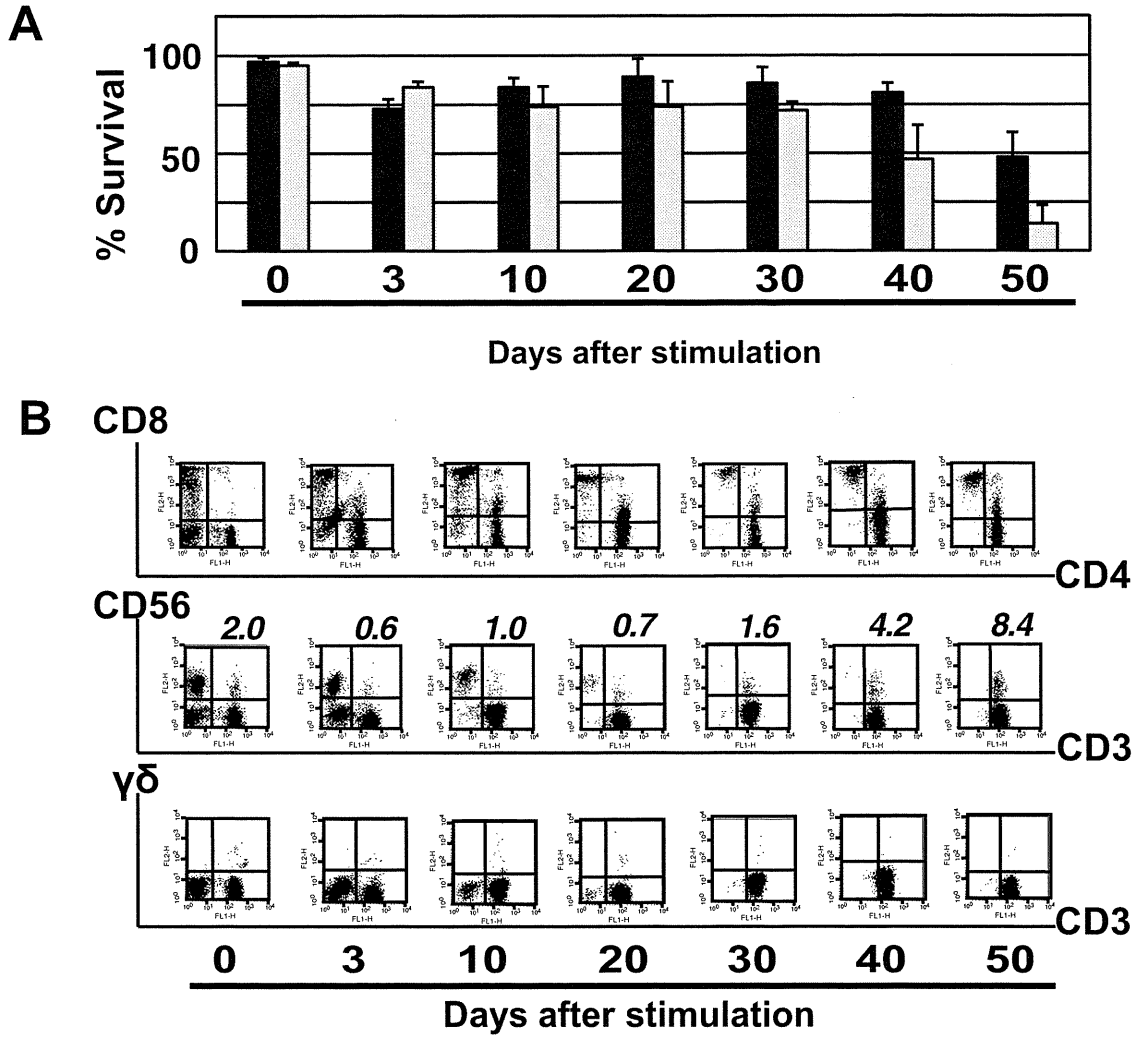

Fig. 3. $CD3^+CD56^+$ cells might be the major effectors in long-cultured PBMC (LC-PBMC). (A) Time course analysis of cytotoxic activity in LC-PBMC. PBMC were stimulated with PHA-P and subsequently infected with Herpesvirus saimiri (HVS). Aliquots of the cultures were removed on days 0, 3, 10, 20, 30, 40, and 50 of culture for measurement of cytotoxic activity against both MOLT-4 (filled bar) and MOLT-4/HIV-1 (open bar). Assessment of cytotoxic activity was evaluated as survival rate of target cells in triplicates (mean ± SD). (B) Time course analysis of phenotype in LC-PBMC. PBMC were stimulated with PHA-P and subsequently infected with HVS. Aliquots of the cultures were removed on days 0, 3, 10, 20, 30, 40, and 50 of culture for phenotypic analysis. The indicated numbers represent the percentages of $CD3^+CD56^+$ cells.

circulating microbial products (*e.g.*, lipopolysaccharide [LPS]) might be a possible cause of HIV-related systemic immune activation [25]. Furthermore, HIV-1 gene products such as gp120 or Nef are able to activate lymphocytes or to enhance their responsiveness to activation [26].

Activation of T cells includes their turnover, differentiation from naive to memory cells, and various type of cytokine production. These events might cause the alterations of constitution in immune cell populations. In this study, we applied an alternative approach using PHA-P and HVS infection to maintain an activated condition of T cells and examined which types of cells would emerge *in vitro* in cultures. We observed that $CD56^+$ T cells were gradually increased in the long-term culture conditions from 30 days after stimulation and exhibited a potent cytotoxic activity against both autologous and allogeneic targets. In human beings, innate and acquired immune responses are thought to be mediated in part by NK cells, γδ T cells, and T cells that express both NK cell–associated markers and TCR. Among NK-like T cells, $CD56^+$ T cells are well characterized for their phenotype and function [27–30]. $CD56^+$ T cells are a heterogeneous population that include $CD4^+$, $CD8^+$, and $CD4^-CD8^-$ cells expressing αβ or γδ TCR and various combinations of NK cell receptors. They account for a small percentage (~5%) of PBMC, but are present in remarkably high numbers in the liver and bone marrow of healthy adults, accounting for 15–55% of all T cells in these organs [31]. $CD56^+$ T cells possess dual innate and adaptive immune functions displaying properties of both T and NK cells capable of both MHC-restricted and non–MHC-restricted cytotoxicity and secretion of cytokines, including IFN-γ, TNF-α, and IL-4 [32]. These properties provide a role for $CD56^+$ T cells to regulate the immune responses against microorganisms and tumors. Indeed, decreased numbers of $CD56^+$ T cells have been reported to occur in the livers of chronically hepatitis C virus (HCV) –infected patients, and failure to eliminate HCV is thought to result from a deficiency of such innate lymphocytes [33,34].

Previous studies reported that invariant NKT (iNKT) cells, which comprise a small portion of peripheral $CD56^+$ T cells, expressing an invariant Vα24Vβ11 and recognizing glycolipid antigens presented by CD1d, were highly susceptible to HIV-1 infection, and that selective depletion of these cells might contribute to developments in HIV pathogenesis [35]. Because $CD56^+$ T cells observed in this study did not show the expression of Vα24 and CD1d-mediated cytotoxicity, they were not iNKT cells. There are few studies on the expres-

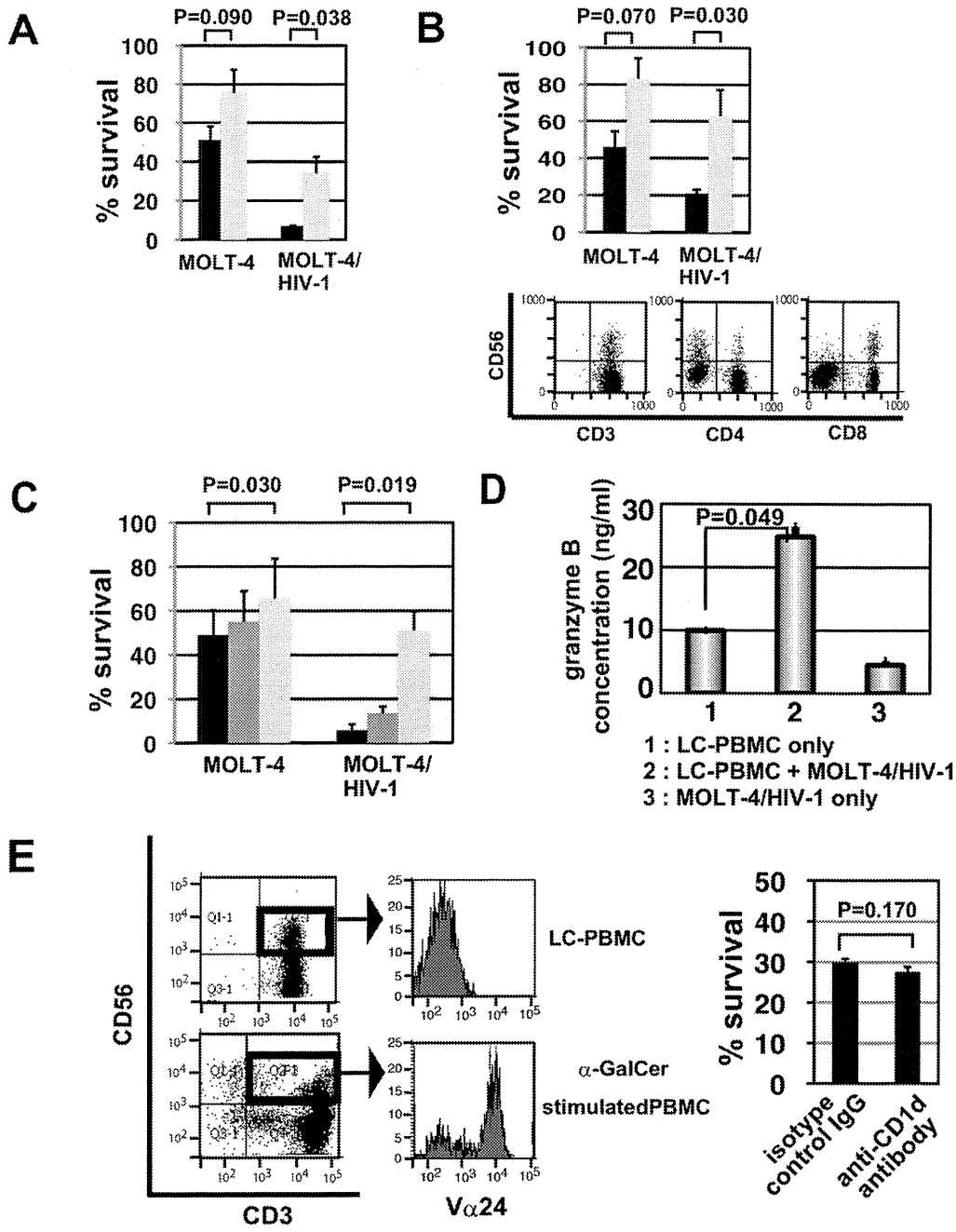

Fig. 4. Characterization of effector cells in long-cultured PBMC (LC-PBMC). Identification of effector cells in LC-PBMC. $CD56^+$ cells (A) or $CD8^+$ cells (B) were depleted from LC-PBMC by magnetic beads selection and subjected to cytotoxicity assay. (C) Effect of anti-FasL or NKG2D neutralizing antibody on cytotoxic activity of LC-PBMC. Effector cells and target cells were co-cultured in the presence of neutralizing antibodies and subjected to cytotoxicity assay. (A–C); Assessment of cytotoxic activity was evaluated as survival rate of target cells in triplicate (mean ± SD). (D) Granzyme B activity of LC-PBMC. LC-PBMC were cultured with or without MOLT-4/HIV-1. After 1 day, cells were harvested and subjected to measurement of granzyme B activity. (E) Examination of Vα24 expression on $CD3^+CD56^+$ cells. LC-PBMC and α-galactodemide stimulated PBMC were stained with APC (anti-CD3), PE, (anti-D56) and FITC (anti-Vα24) labeled antibodies (left panel). Target cells were incubated with anti-CD1d neutralizing antibody before coculturing with effector cells (right panel).

sion and function of $CD56^+$ T cells in HIV infection besides iNKT cells. Tarazona et al. observed a decreased number of $CD8^{bright}$ T cells expressing CD56 in non–HAART-treated HIV-1 patients [36]. Parsons et al. reported that the phenotypic changes of CD56 from positive to negative on $CD8^+$ T cells with non–MHC-restricted cytotoxicity occurred in progressive HIV infection, and suggested that these changes might reflect autoreactive and pathogenic diversion of the $CD8^+$ T-cell repertoire [37].

Induction of $CD56^+$ T cells by PHA-P stimulation and HVS infection do not necessarily reflect cytotoxic T cells *in vivo*. However, when we examined the cytotoxic activity of $CD56^+$ T cells from HIV-1–infected or noninfected donors, $CD56^+$ T cells were cytotoxic against autologous $CD4^+$ T cells and became more cytotoxic against HIV-1–infected $CD4^+$ T cells. It should be noted that $CD4^+$ T cells from HIV-1–infected patients showed significant susceptibility against autologous $CD56^+$ T cells. The percentages of $CD3^+CD56^+$ cells in LC-PBMC from HIV-1–infected patients were little less than from healthy donors. These findings suggest that $CD56^+$ T cells from HIV-1–infected patients are more likely to acquire a potent cytotoxicity under the activated culture condition.

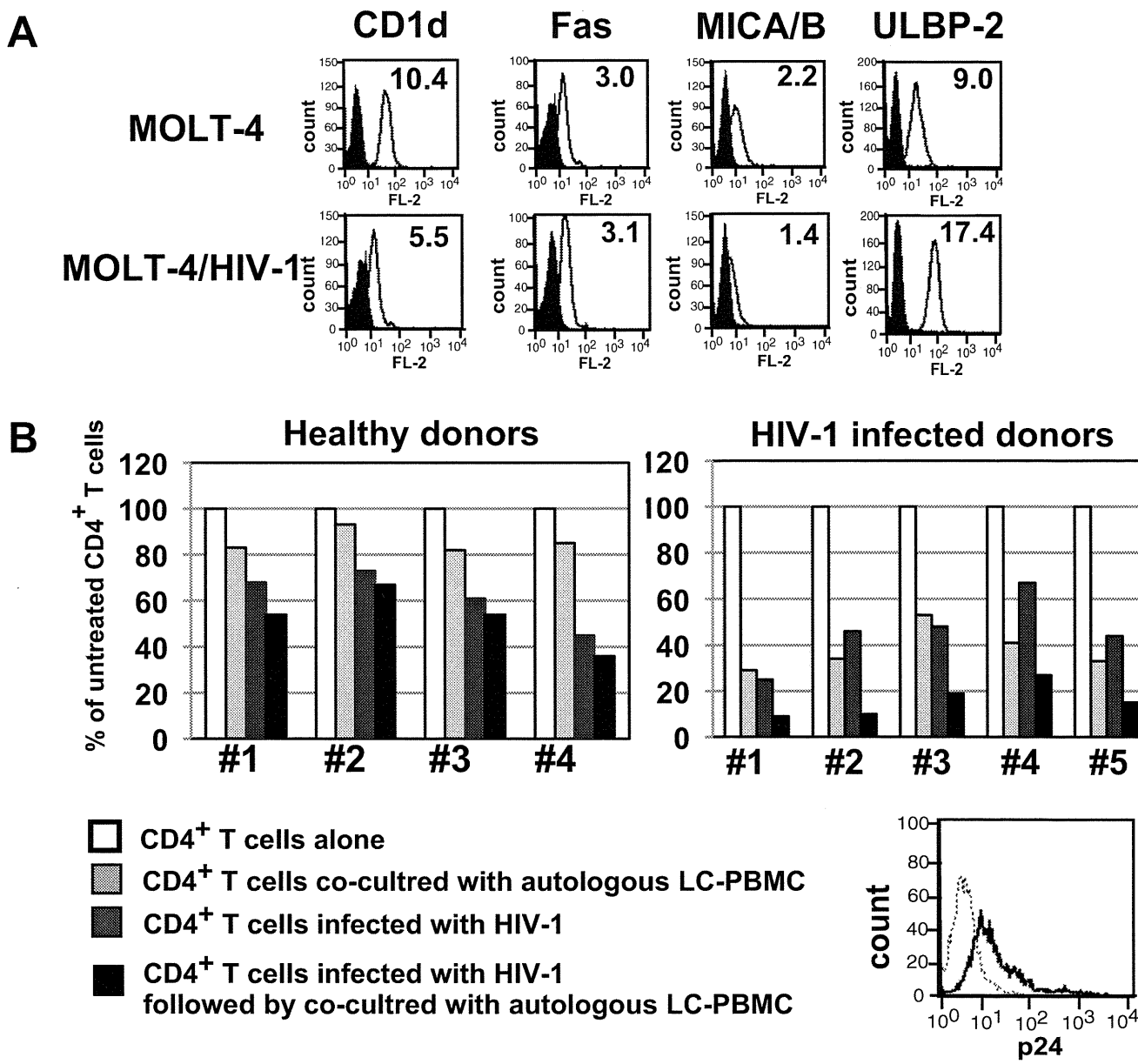

Fig. 5. $CD4^+$ T cells from HIV-1–infected individuals are more susceptible to cytotoxic activity of autologous long-cultured PBMC (LC-PBMC). (A) Cell surface expression of ligands for NKG2D on both MOLT-4 and MOLT-4/HIV-1. Both cells were stained with anti-CD1d, Fas, MICA/B, or ULBP-2 antidody. MFI values are indicated in the upper right corner of histogram. (B) Enhancement of susceptibility of $CD4^+$ T cells against autologous LC-PBMC by HIV-1 infection. PBMC from healthy and HIV-1–infected donors were stimulated with PHA-P followed by HVS infection. After ~50 days' incubation, cells were harvested and used as LC-PBMC. $CD4^+$T cells were purified from PHA-P–stimulated PBMC by magnetic beads selection and infected with HIV-1 IIIB at 0.1 moi for 4 days, and then co-cultured with autologous LC-PBMC for 1 day. Assessment of cytotoxic activity was evaluated as survival rate of target cells. HIV-1 infection was confirmed by intracellular staining of HIV-1 gag antigen p24. Results were normalized and are expressed as percentages of intact $CD4^+$ T cells.

We assumed that if the level of immune activation was highly elevated, autoreactive $CD56^+$ T cells might be up-regulated and expanded in HIV-1–infected patients. Therefore, we present here our findings on the possible involvement of $CD56^+$ T cells in the depletion of $CD4^+$ T cells.

Some HIV-1–infected individuals have autoreactive antibodies directed CD4 molecules, human leukocyte antigen (HLA) class II, and myelin basic protein [38,39]. Recently Kuwata et al. demonstrated that increased autoreactive antibodies correlated with the extent of $CD4^+$ T-cell depletion in an SIV/macaque model [40]. Induction and development of autoreactive antibodies is also likely to be caused by immune activation in HIV-1–infected patients. Therefore, it is necessary to block or to minimize immune activation and inflammation to prevent such unfavorable responses. Antiretroviral therapy exerts a suppressive effect on T-cell activation and apoptosis through its potent and prolonged inhibition of HIV replication. However, there are several limitations to application of HIV therapy for HIV-1–infected individuals, such as drug resistance and dose-limiting side effects. Use of immunosuppressive agents or inhibitors of proinflammatory cytokines might also be effective for suppression of immune activation. However, because the immune system also has to cope with other persisting or exogenous pathogens, casual use of these agents may cause progression toward severe infectious disease. Therefore, a better understanding of the mechanisms by which HIV-1 infection causes immune activation is required for the development of strategies for control of immune activation, that is, what types of agents we can use, or under what conditions these agents can be used.

References

[1] Brenchley JM, Schacker TW, Ruff LE, et al. CD4+ T-cell depletion during all stages of HIV disease occurs predominantly in the gastrointestinal tract. J Exp Med 2004;200:749-59.

[2] Mehandru S, Poles MA, Tenner-Racz K, et al. Primary HIV-1 infection is associated with preferential depletion of CD4+ T lymphocytes from effector sites in the gastrointestinal tract. J Exp Med 2004;200:761-70.

[3] Harper ME, Marselle LM, Gallo RC, Wong-Staal F. Detection of lymphocytes expressing human T-lymphotropic virus type III in lymph nodes and peripheral blood from infected individuals by in situ hybridization. Proc Natl Acad Sci U S A 1986;83:772-6.

[4] Schnittman SM, Psallidopoulos MC, Lane HC, et al. The reservoir for HIV-1 in human peripheral blood is a T-cell that maintains expression of CD4. Science 1989;245:305-8.

[5] Gougeon ML, Piacentini M. New insights on the role of apoptosis and autophagy in HIV pathogenesis. Apoptosis 2009;14:501-8.

[6] Herbeuval JP, Grivel JC, Boasso A, et al. CD4+ T-cell death induced by infectious and noninfectious HIV-1: Role of type 1 interferon-dependent, TRAIL/DR5-mediated apoptosis. Blood 2005;106:3524-31.

[7] Hazenberg MD, Stuart JW, Otto SA, Borleffs JC, Boucher CA, de Boer RJ, et al. T-cell division in human immunodeficiency virus (HIV)-1 infection is mainly due to immune activation: A longitudinal analysis in patients before and during highly active antiretroviral therapy (HAART). Blood 2000;95:249-55.

[8] Hazenberg MD, Otto SA, van Benthem BH, et al. Persistent immune activation in HIV-1 infection is associated with progression to AIDS. AIDS 2003;17:1881-8.

[9] Lederman MM, Kalish LA, Asmuth D, Fiebig E, Mileno M, Busch MP. "Modeling" relationships among HIV-1 replication, immune activation and CD4+ T-cell losses using adjusted correlative analyses. AIDS 2000;14:951-8.

[10] Deeks SG, Kitchen CM, Liu L, et al. Immune activation set point during early HIV infection predicts subsequent CD4+ T-cell changes independent of viral load. Blood 2004;104:942-7.

[11] Hunt PW, Brenchley J, Sinclair E, et al. Relationship between T-cell activation and CD4+ T-cell count in HIV-seropositive individuals with undetectable plasma HIV RNA levels in the absence of therapy. J Infect Dis 2008;197:126-33.

[12] Zarling JM, Ledbetter JA, Sias J, Fultz P, Eichberg J, Gjerset G, et al. HIV-infected humans, but not chimpanzees, have circulating cytotoxic T lymphocytes that lyse noninfected CD4+ cells. J Immunol 1990;144:2992-8.

[13] Grant MD, Smaill FM, Rosenthal KL. Lysis of CD4+ lymphocytes by non-HLA-restricted cytotoxic T lymphocytes from HIV-infected individuals. Clin Exp Immunol 1993;93:356-62.

[14] Takahashi M, Osono E, Nakagawa Y, Wang J, Berzofsky JA, Margulies DH, et al. Rapid induction of apoptosis in CD8+ HIV-1 envelope-specific murine CTLs by short exposure to antigenic peptide. J Immunol 2002;169:6588-93.

[15] Takahashi M, Watari E, Shinya E, Shimizu T, Takahashi H. Suppression of virus replication via down-modulation of mitochondrial short chain enoyl-CoA hydratase in human glioblastoma cells. Antivir Res 2007;75:152-8.

[16] Nakagawa Y, Watari E, Shimizu M, Takahashi H. One-step simple assay to determine antigen-specific cytotoxic activities by single-color flow cytometry. Biomed Res 2011; 32:159-66.

[17] Yasukawa M, Inoue Y, Kimura N, Fujita S. Immortalization of human T cells expressing T-cell receptor gamma delta by Herpesvirus saimiri. J Virol 1995;69:8114-7.

[18] Takahashi M, Ido E, Uesaka H, Fukushima T, Ibuki K, Miura T, et al. Comparison of susceptibility to SIVmac239 infection between CD4+ and CD4+ 8+ T cells. Arch Virol 2005;150:1517-28.

[19] Galli G, Nuti S, Tavarini S, Galli-Stampino L, De Lalla C, Casorati G, et al. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. J Exp Med 2003;197:1051-7.

[20] Richard J, Sindhu S, Pham TN, Belzile JP, Cohen EA. HIV-1 Vpr up-regulates expression of ligands for the activating NKG2D receptor and promotes NK cell-mediated killing. Blood 2010;115:1354-63.

[21] Appay V, Sauce D. Immune activation and inflammation in HIV-1 infection: Causes and consequences. J Pathol 2008;214:231-41.

[22] Biancotto A, Grivel JC, Iglehart SJ, et al. Abnormal activation and cytokine spectra in lymph nodes of people chronically infected with HIV-1. Blood 2007;109:4272-9.

[23] Giorgi JV, Lyles RH, Matud JL, et al. Predictive value of immunologic and virologic markers after long or short duration of HIV-1 infection. J Acquir Immune Defic Syndr 2002;29:346-55.

[24] Silvestri G, Sodora DL, Koup RA, Paiardini M, O'Neil SP, McClure HM, et al. Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia. Immunity 2003;18:441-52.

[25] Brenchley JM, Price DA, Schacker TW, et al. Microbial translocation is a cause of systemic immune activation in chronic HIV infection. Nat Med 2006;12:1365-71.

[26] Manninen A, Renkema GH, Saksela K. Synergistic activation of NFAT by HIV-1 nef and the Ras/MAPK pathway. J Biol Chem 2000;275:16513-7.

[27] Mingari MC, Vitale C, Cambiaggi A, Schiavetti F, Melioli G, Ferrini S, et al. Cytolytic T lymphocytes displaying natural killer (NK)-like activity: Expression of NK-related functional receptors for HLA class I molecules (p58 and CD94) and inhibitory effect on the TCR-mediated target cell lysis or lymphokine production. Int Immunol 1995;7:697-703.

[28] Norris S, Doherty DG, Collins C, McEntee G, Traynor O, Hegarty JE, et al. Natural T cells in the human liver: Cytotoxic lymphocytes with dual T-cell and natural killer cell phenotype and function are phenotypically heterogenous and include Valpha24-JalphaQ and gammadelta T-cell receptor bearing cells. Hum Immunol 1999;60:20-31.

[29] Campbell JJ, Qin S, Unutmaz D, Soler D, Murphy KE, Hodge MR, et al. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. J Immunol 2001;166:6477-82.

[30] Linn YC, Hui KM. Cytokine-induced NK-like T cells: From bench to bedside. J Biomed Biotechnol 2010 2010;2010:435745.

[31] Doherty DG, O'Farrelly C. Innate and adaptive lymphoid cells in the human liver. Immunol Rev 2000;174:5-20.

[32] Kelly-Rogers J, Madrigal-Estebas L, O'Connor T, Doherty DG. Activation-induced expression of CD56 by T cells is associated with a reprogramming of cytolytic activity and cytokine secretion profile in vitro. Hum Immunol 2006;67:863-73.

[33] Golden-Mason L, Madrigal-Estebas L, McGrath E, Conroy MJ, Ryan EJ, Hegarty JE, et al. Altered natural killer cell subset distributions in resolved and persistent hepatitis C virus infection following single source exposure. Gut 2008;57:1121-8.

[34] Deignan T, Curry MP, Doherty DG, Golden-Mason L, Volkov Y, Norris S, et al. Decrease in hepatic, CD56+ T cells and V alpha CD24+ natural killer T cells in chronic hepatitis C viral infection. J Hepatol 2002;37:101-8.

[35] Li D, Xu XN. NKT cells in HIV-1 infection. Cell Res 2008;18:817-22.

[36] Tarazona R, DelaRosa O, Casado JG, Torre-Cisneros J, Villanueva JL, Galiani MD, et al. NK-associated receptors on CD8 T cells from treatment-naive HIV-infected individuals: Defective expression of CD56. AIDS 2002;16:197-200.

[37] Parsons MS, Zipperlen K, Gallant M, Howley C, Grant M. Distinct phenotype of unrestricted cytotoxic T lymphocytes from human immunodeficiency virus-infected individuals. J Clin Immunol;30:272-9.

[38] Thiriart C, Goudsmit J, Schellekens P, Barin F, Zagury D, De Wilde M, et al. Antibodies to soluble CD4 in HIV-1-infected individuals. AIDS 1988;2:345-51.

[39] Mathiesen T, Sönnerborg A, Wahren B. Detection of antibodies against myelin basic protein and increased levels of HIV-IgG antibodies and HIV antigen after solubilization of immune complexes in sera and CSF of HIV infected patients. Viral Immunol 1989;2:1-9.

[40] Kuwata T, Nishimura Y, Whitted S, et al. Association of progressive CD4+ T-cell decline in SIV infection with the induction of autoreactive antibodies. PLoS Pathog 2009;5;e1000372.

Research Article

Journal of Innate Immunity

J Innate Immun
DOI: 10.1159/000329226

Received: August 26, 2010
Accepted after revision: May 10, 2011
Published online: July 22, 2011

HIV-1 Gag-Virus-Like Particles Induce Natural Killer Cell Immune Responses via Activation and Maturation of Dendritic Cells

Myint Oo Chang[a] Tomoyuki Suzuki[b] Hitoshi Suzuki[b] Hiroshi Takaku[a, b]

[a]High Technology Research Centre, and [b]Department of Life and Environmental Sciences, Chiba Institute of Technology, Narashino, Japan

Key Words

Gag-virus-like particles · Dendritic cells · Natural killer cells · HIV · Innate immunity

Abstract

Despite the extensive efforts that have been made to combat acquired immune deficiency syndrome (AIDS), the number of people infected each year with human immunodeficiency virus type 1 (HIV-1) is still increasing worldwide, and a safe and effective vaccine to control HIV infection is urgently needed. Recently, the natural killer (NK) cell-mediated innate immune response, which represents the first line of defense against infections, has attracted attention for its role in combating HIV infection and disease progression. In the present study, we investigated the immunogenic ability of HIV-1 Gag-virus-like particles (Gag-VLPs) to induce NK cell immune responses in vitro and in vivo. Gag-VLPs efficiently activated human monocyte-derived dendritic cells (MDDCs), eliciting MDDC maturation with an associated increase in the surface expression of CD80, CD86 and MHC classes I and II, MDDC proliferation and proinflammatory cytokine production. Gag-VLP-treated MDDCs subsequently activated autologous NK cells, leading to their proliferation and production of interferon-γ and to the upregulation of NK cell cytotoxicity against YAC-1 cells and HIV-1-infected $CD4^+$ T cells. In addition, we introduced a 2-phase immunization strategy in BALB/c mice to assess the role of DCs in the induction of NK cell immune responses by Gag-VLPs in vivo. Our findings reveal that Gag-VLPs efficiently activate DCs, which in turn induce innate and Gag-specific immune responses in NK cells.

Introduction

Control of human immunodeficiency virus (HIV) infection by highly active antiretroviral therapy has greatly decreased morbidity and mortality due to acquired immune deficiency syndrome (AIDS). However, despite enormous efforts, the number of HIV-1-infected individuals increases on a daily basis, and we still lack prophylactic and therapeutic vaccines against HIV-1. Several vaccine components such as live attenuated viruses, recombinant viral vectors and DNA vaccines have been proven to be effective in inducing cellular and humoral immune responses; however, serious safety concerns and the limited efficacy of some of these vaccine components prevent their clinical application as anti-HIV vaccines. The possible risks of live attenuated viruses and recombinant viral vectors, including (1) potential reversion to a virulent pathogenic form of the attenuated virus, especially in immunodeficient recipients, (2) possible recombination of the vaccine strain with wild-type pathogenic

Co-corresponding author:
Dr. Myint Oo Chang
E-Mail c_myintoo@hotmail.com

Prof. Hiroshi Takaku
Department of Life and Environmental Sciences, Chiba Institute of Technology
2-17-1, Tsudanuma, Narashino
Chiba 275-0016 (Japan)
Tel. +81 47 478 0407, Fax +81 47 471 8764, E-Mail hiroshi_takaku@it-chiba.ac.jp

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

1662–811X/11/0000–0000$38.00/0

Accessible online at:
www.karger.com/jin

virus in an infected individual, (3) dysregulation of the immune system by viral proteins and (4) ability of the proviral genome to integrate into the host genome, have prevented their approval for use in humans [1, 2]. Furthermore, several potential risks of DNA vaccines, including tumor induction due to chromosomal integration, autoimmune reactions as a result of the induction of anti-DNA antibodies or adverse reactions due to the biological activity of the vector or the expressed antigens have not yet been ruled out [3]. Because of these difficulties, a safe and effective HIV vaccine suitable for prophylactic and therapeutic use in humans is urgently needed.

Virus-like particles (VLPs) are stable, self-assembling, nonreplicating and noninfectious particles capable of inducing immune responses without containing any viral genome or other potentially toxic viral gene products [4–6]. Recent research in HIV vaccine strategies has focused on the use of VLPs as highly attractive HIV-1 vaccine candidates because of their ability to elicit humoral and cellular immunity more effectively than soluble proteins [7, 8].

VLPs are incorporated into dendritic cells (DCs) via actin-dependent macropinocytosis and endocytosis. VLP uptake is also induced by receptor-mediated endocytosis. Mannose-recognizing receptors, including the DC-SIGN receptor, which belongs to the C-type lectin family, are reportedly involved in this process. The DC-SIGN receptor, present on the DC surface, recognizes VLPs and enhances internalization for lysosomal degradation, antigen processing and cross-presentation on both MHC class I (MHC I) and MHC class II (MHC II) molecules. VLPs induce maturation and activation of monocyte-derived DCs (MDDCs), and this effect is partially mediated through Toll-like receptors [9–11]. Previous reports have shown that Gag-VLPs induce $CD4^+$ T cell activation and $CD8^+$ cytotoxic T lymphocyte (CTL) responses as well as B cell-mediated humoral immunity [9, 12–17]. Despite these findings, little is known about the ability of Gag-VLPs to elicit natural killer (NK) cell immune responses.

DCs are the most potent antigen-presenting cells of the immune system and play a pivotal role in the initiation and regulation of immune responses to various antigens. Antigen-loaded DCs acquire a mature phenotype associated with the production of proinflammatory cytokines and stimulate $CD4^+$ T cells and CTL responses [18–20]. The mature DCs migrate towards the lymphoid organs, where they interact with NK cells, inducing the activation and proliferation of these cells. The interaction between DCs and NK cells initiates innate signals that orchestrate subsequent adaptive immune responses [21–25].

NK cells are the primary effector cells of the innate immune system and are essential for the immune control of infections and tumors through production of cytokines such as interferon (IFN)-γ and the potent lysis of infected cells or tumor cells, without the need for prestimulation. Notably, other than activated T cells, NK cells are the only known source of IFN-γ which shapes the pattern of innate and adaptive immune responses [26–28]. The innate immune response of NK cells against HIV is crucial both for the subsequent course of the infection and for the induction of an efficient adaptive response. Both NK cell cytotoxicity and cytokine production are impaired in HIV-1 viremic patients. Preservation of NK cell activity and cell number correlates with lower plasma viral load and slower progression to AIDS [29, 30]. Moreover, because NK cell responses following vaccination correlate with clinical outcome more closely than do T cell responses, the effectiveness of an HIV vaccine should be evaluated by monitoring the potentially important NK cell responses after immunization [31].

In this article, we demonstrate that Gag-VLP-treated MDDCs (VLP-DCs) underwent activation and maturation characterized by the increased surface expression of costimulatory molecules, including CD80, CD86, MHC I and MHC II, and the production of proinflammatory cytokines, including IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ. MDDCs subsequently activated NK cells, inducing their proliferation, release of IFN-γ and enhanced cytotoxicity. Next, we immunized BALB/c mice with Gag-VLPs in a phase I immunization study to examine the activation of DCs and NK cells in vivo. These experiments were followed by a phase II immunization study in which mice were inoculated with splenic DCs of phase I-immunized mice to assess the contribution of DCs to the induction of NK cell immune responses.

In conclusion, we show that Gag-VLPs induce activation and maturation of DCs, which then mediate NK cell innate and Gag-specific immune responses. Our findings reveal that Gag-VLPs are an effective HIV-1 vaccine candidate for prophylactic and therapeutic purposes in humans.

Materials and Methods

Mice and Cell Culture

Six- to 8-week-old female BALB/c mice were purchased from Nippon SLC (Hamamatsu, Japan). All mice were maintained under specific-pathogen-free conditions. All animal experiments were carried out in compliance with institutional guidelines and approved by the animal experimentation committee of the Chiba

Institute of Technology. *Spodoptera frugiperda* (Sf9) insect cells were grown at 27°C in BD Gold serum-free medium containing 100 μg/ml kanamycin sulfate. HeLa cells and HEK-293T cells were maintained in DMEM culture medium (Sigma), and YAC-1 cells were maintained in RPMI-1640 culture medium (Sigma). Both DMEM and RPMI-1640 were supplemented with 10% fetal bovine serum (Life Technologies), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma).

Generation of Recombinant Baculovirus and Wild-Type HIV-1

The baculovirus transfer vector pAcCAG-gag containing the HIV-1 gag gene was generated by cloning of cDNA encoding the HIV-1 (NL4-3) gag gene into the multiple cloning site of the baculovirus transfer vector pAcCAG-MCS2, under control of the mammalian CAG promoter. HIV-1 gag was first cloned into the *Dra*III and *Nru*I sites of pcDNA3.1 and then excised with *Kpn*I and *Not*I endonucleases and ligated into the *Kpn*I and *Not*I sites of the pAcCAG-MCS2 vector. Recombinant baculovirus (AcCAG-gag) was generated by cotransfection of Sf9 cells with the baculovirus transfer vector pAcCAG-gag and AcMNPV DNA using a BD Gold Baculovirus transfection kit (BD Biosciences) according to the manufacturer's protocol. AcCAG-gag was expanded in Sf9 cells, and the titer was determined by plaque assay. Wild-type HIV-1 NL4-3 (X4-tropic) was generated by transfection of HEK-293T cells with HIV-1 plasmid (pNL4-3) using FuGENE 6, and culture supernatant was collected on day 2. Viral p24 content was measured by ELISA using Lumipulse.

Production of Gag-VLPs in HeLa Cells

HeLa cells were infected with recombinant baculovirus AcCAG-gag at a multiplicity of infection of 100 for 1 h at 37°C, washed twice with PBS to thoroughly remove the baculovirus and then replenished with fresh culture medium. After incubation for 3 days, the culture supernatant was clarified by centrifugation at 2,500 rpm for 20 min and filtered through a 0.45-μm filter. VLPs were then pelleted at 19,000 rpm for 2 h at 4°C with a Beckman NVT-100 rotor, resuspended in PBS, purified by 20–60% sucrose gradient centrifugation at 25,000 rpm for 90 min and again resuspended in PBS. Protein concentrations of purified Gag-VLP samples were determined using a BCA™ protein assay kit (Pierce). Gag-VLP preparations were determined to be free of endotoxin (<0.01 endotoxin units/ml) using a Pyrodick endotoxin kit (Seikagaku Co., Tokyo, Japan).

Preparation of Human MDDCs

Human peripheral blood mononuclear cells were separated from buffy coats using Ficoll-Paque density gradient centrifugation. Monocytes were allowed to adhere to plastic plates at 37°C for 2 h, and nonadherent cells (peripheral blood lymphocytes, PBLs) were removed. Monocytes were washed with medium and cultured for 6 days in DC culture medium consisting of RPMI-1640 (Sigma) supplemented with 2 mM L-glutamine (Sigma), 50 μM 2-mercaptoethanol (Sigma), 1% nonessential amino acids (Gibco) and 10% fetal calf serum in the presence of 20 ng/ml GM-CSF and IL-4 (PeproTech, London, UK). On days 3 and 5, one half of the volume of medium was replaced with fresh medium supplemented with GM-CSF and IL-4. On day 6, MDDCs were purified by positive selection using a CD1c$^+$ (BDCA-1) DC isolation kit (Miltenyi Biotec Inc., Auburn, Calif., USA).

MDDC Activation and Analysis

Human MDDCs (1×10^6) were incubated with Gag-VLPs (10 μg/ml), lipopolysaccharide (LPS; 1 μg/ml; Sigma) or medium alone (control) for 24 h. For phenotypic analysis, MDDCs were washed and stained with FITC-conjugated anti-human CD1a and PE-conjugated anti-human CD80, CD86, HLA-ABC or HLA-DR (eBioscience) and analyzed using a FACSCalibur flow cytometer and CellQuest software. Culture supernatants were collected to quantify IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ levels using ELISA kits (BD Biosciences, San Diego, Calif., USA). MDDC proliferation was evaluated using a CellTiter 96 proliferation assay kit (Promega) and carboxyfluorescein succinimidyl ester (CFSE; Invitrogen) labeling. For the proliferation assay, MDDCs treated with Gag-VLPs, LPS or medium alone were incubated in a 96-well plate (5×10^3 cells/well in 100 μl) for 5 days in triplicate wells. CellTiter 96 reagent containing MTS (20 μl) was added to each well, and samples were incubated for 4 h at 37°C. The absorbance of light released from formazin was recorded with a plate reader. For CFSE labeling, MDDCs (5×10^5) were loaded with 5 μM CFSE and cultured for 5 days. Proliferation was analyzed by measuring the decrease in CFSE fluorescence intensity by flow cytometry after staining cells with anti-CD1a-PE.

NK/DC Coculture and Analysis of NK Cell Activation

MDDCs were treated with Gag-VLPs, LPS or medium for 24 h and washed thoroughly with PBS before coculturing with NK cells. Autologous human NK cells were isolated from PBLs using a CD56$^+$/CD16$^+$ NK cell isolation kit (Miltenyi Biotec) and cultured for 12 h in the presence of IL-2. NK cells (5×10^5) were cocultured with MDDCs (1×10^5) at a ratio of 5:1 for 18 h. NK cell activation was analyzed by flow cytometry after staining cells with anti-CD56-PE and anti-CD69-FITC.

Intracellular Cytokine Staining

Intracellular cytokine staining for IFN-γ-producing NK cells from NK/DC cocultures was performed after 18 h of coculture. The transport inhibitor brefeldin A (10 μg/ml; Sigma-Aldrich, St. Louis, Mo., USA) was added to cocultures for the last 4 h. NK cells were surface stained with anti-CD56-PE and permeabilized with Cytofix/Cytoperm reagent (BD Pharmingen) according to the manufacturer's instructions. Cells were then stained with anti-IFN-γ-FITC and examined by FACS analysis.

NK Cell Proliferation

NK cell proliferation was analyzed using the CellTiter 96 proliferation assay and CFSE labeling. NK cells were cocultured with MDDCs treated with Gag-VLP or LPS or with untreated control MDDCs at a DC to NK ratio of 1:5 for 5 days. NK cell proliferation was analyzed using the CellTiter 96 proliferation assay kit (Promega) as described above. For CFSE labeling, NK cells were labeled with 5 μM CFSE and cocultured with MDDCs at a DC to NK ratio of 1:5 for 5 days. To assess the degree of proliferation, CFSE-labeled NK cells were then stained with anti-CD56-PE, and the decrease in CFSE fluorescence intensity was measured by flow cytometry.

NK Cell Cytotoxicity Assays

Autologous CD4$^+$ T cells were separated from PBLs using a human CD4$^+$ T cell isolation kit (Miltenyi Biotec) and cultured for 12 h in the presence of IL-2. CD4$^+$ T cells were infected with

HIV-1 (NL4-3; 100 ng of p24 for 1 × 10^6 cells) for 2 h. $CD4^+$ T cells or YAC-1 cells were seeded into a 96-well round-bottomed tissue culture plate in triplicate (1 × 10^4 cells/well) and used as target cells. Effector NK cells were cocultured with Gag-VLP or LPS-treated MDDCs for 24 h and added to target cells at effector to target ratios of 10:1, 5:1, 2.5:1 and 1.25:1, in a total volume of 100 μl, and incubated for 4 h. Unstimulated NK cells were added as a control. Lysis of target cells was determined by measuring lactate dehydrogenase (LDH) release using the Cytotox 96 nonradioactive cytotoxicity assay kit (Promega Corp., Madison, Wisc., USA). Spontaneous release of LDH from target cells was less than 15% of the maximum release.

Immunization of Mice and Analysis of Immune Responses

Six-week-old female BALB/c mice were immunized using a 2-phase immunization procedure. In the phase I immunization, 4 groups of BALB/c mice, each consisting of 3 subgroups with 4 mice each, were injected intramuscularly with 50 μg of Gag-VLPs or 1 μg of LPS resuspended in 100 μl of sterile PBS without addition of adjuvants. Control mice were left untreated. Twenty-four hours after priming, mice from the first group were sacrificed, spleens were removed and activation of splenic DC and NK cells was assessed by flow cytometry. Seven days after priming, the first booster immunization was administered to the remaining 3 groups of mice using the same doses. Twenty-four hours after the first booster injection, mice from the second group were sacrificed and spleens were removed for flow cytometry. On day 14, the remaining 2 groups of mice were given a second booster injection using the same doses. After 24 h, mice from the third group (donor mice) were sacrificed, and blood samples were collected for ELISA quantification of serum IFN-γ. Spleens were removed for flow cytometry. At the same time, splenic DCs were separated from the remaining spleen cells using a mouse $CD11c^+$ DC isolation kit (Miltenyi Biotec) and subsequently used in phase II immunizations. Mice from the fourth group were sacrificed 3 weeks after the second booster injection, and spleens were collected. A portion of the spleen cells was used for intracellular staining of IFN-γ-producing NK cells without re-stimulation. The remaining spleen cells were re-stimulated overnight with 5 μg/ml Gag-VLPs and used for ex vivo cytotoxicity assays and intracellular staining of IFN-γ-producing NK cells. For intracellular staining, brefeldin A (10 μg/ml) was added, and samples were incubated for an additional 4 h and processed as described above.

In the phase II immunization, 4 groups of (recipient) BALB/c mice, each consisting of 5 animals, were intraperitoneally injected with purified splenic DCs (1 × 10^6 cells/mouse) from each group of mice from the phase I immunization. All mice were sacrificed after 24 h, serum samples were collected for the quantification of IFN-γ and spleens were removed for flow cytometry analysis. All immunizations were performed in 2 independent experiments in accordance with institutional animal experiment guidelines.

Ex vivo Cytotoxicity Assay

NK cells were separated from the spleens of the fourth group of phase I-immunized or control mice using a mouse NK cell isolation kit (Miltenyi Biotec) after restimulation with Gag-VLPs and used as effector cells. Spleen cells isolated from non-immunized BALB/c mice were used as target cells. Target spleen cells were pulsed with Gag-VLPs (5 μg/ml) for 2 h and seeded in a 96-well round-bottomed tissue culture plate (1 × 10^4 cells/well) in triplicate. Effector NK cells were then added at effector to target ratios of 10:1, 5:1, 2.5:1 and 1.25:1 and incubated for 4 h. Lysis of target cells was determined by measuring LDH release using the Cytotox 96 nonradioactive cytotoxicity assay kit as mentioned above.

Statistical Analysis

All data are presented as means ± SD. Statistical analysis was performed using Student's t test. A p value of <0.05 was considered a significant difference.

Results

Gag-VLPs Induce Activation and Proliferation of DCs

Given the crucial role of DCs in antiviral innate immunity, we sought to investigate whether HIV-1 Gag-VLPs could activate human DCs in vitro. Treatment of MDDCs with Gag-VLPs resulted in increased expression of the activation markers CD80, CD86, MHC I and MHC II. MHC II expression was especially elevated and reached levels higher than those observed upon treatment of MDDCs with LPS (fig. 1a). We next examined the secretion of IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ by VLP-DCs in the culture supernatant. The levels of cytokines induced by VLPs were comparable to those induced by LPS (fig. 1b). We then investigated the proliferation of MDDCs using a proliferation assay kit and CFSE labeling. The results of both experiments showed that VLP-DCs and LPS-stimulated DCs (LPS-DCs) proliferated to a similar extent (fig. 1c). These results indicate that Gag-VLPs induce activation and maturation of DCs, which are associated with the production of specific cytokines and proliferation in vitro.

Activation of NK Cells by VLP-DCs

To determine whether human NK cells were functionally modified by VLP-DCs in vitro, MDDCs were cocultured with autologous NK cells at a 1:5 ratio for 18 h, and NK cell activation was evaluated by measuring the expression of the NK cell activation marker CD69. NK cells cocultured with VLP-DCs exhibited a statistically significant 6-fold increase in CD69 expression over NK cells cocultured with control DCs (fig. 2a). We next assessed the amount of IFN-γ in cocultured supernatants by ELISA. NK cells cocultured with VLP-DCs produced higher levels of IFN-γ than did NK cells cocultured with control DCs (fig. 2b, left panel). This finding was further substantiated by intracellular staining of NK cells, which showed an increase in the frequency of IFN-γ-producing NK cells following coculture with VLP-DCs (fig. 2b, right panel).

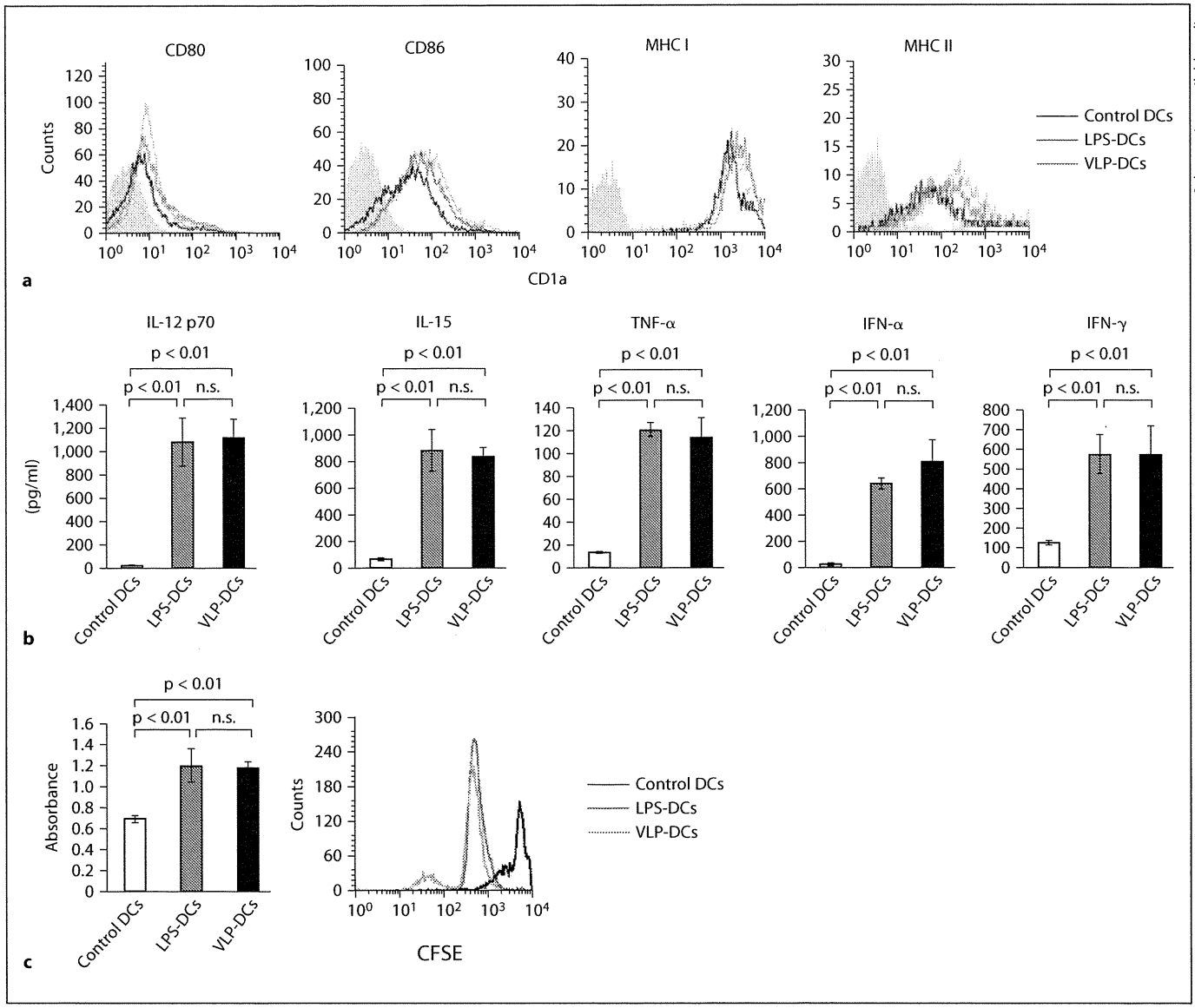

Fig. 1. Gag-VLPs induce activation and proliferation of MDDCs. **a** Expression of the costimulatory molecules CD80, CD86, MHC I and MHC II on human MDDCs treated with Gag-VLPs (10 μg/ml), LPS (1 μg/ml) or medium alone for 24 h was analyzed by flow cytometry. Gray curves represent isotype controls. Results are representative of 3 independent experiments using cells from 3 different donors. **b** Levels of the cytokines IL-12 p70, IL-15, TNF-α, IFN-α and IFN-γ in culture supernatants were measured by ELISA. All results are expressed as means ± SD of triplicate wells. Statistical analysis was performed using Student's t test. **c** MDDCs were treated with Gag-VLPs, LPS or medium and cultured for 5 days. MDDC proliferation was evaluated using a proliferation assay kit. The results are expressed as means ± SD of triplicate wells. For CFSE labeling, MDDCs were labeled with 5 μM CFSE and treated with Gag-VLPs, LPS or medium and cultured for 5 days. MDDC proliferation was evaluated by measuring the decrease in CFSE fluorescence intensity by flow cytometry. The results are representative of 3 independent experiments from 3 different donors.

We next examined NK cell proliferation after coculture with VLP-DCs. The results of both proliferation assays (fig. 2c, left panel) and CFSE labeling of NK cells (fig. 2c, right panel) demonstrated that NK cells cocultured with VLP-DCs proliferated at a rate comparable to that of NK cells cocultured with LPS-DCs after 5 days, while NK cells cocultured with control DCs showed only a basal level of proliferation. To investigate NK cell cytotoxicity, we used NK-sensitive YAC-1 cells (fig. 2d, left panel) and HIV-infected autologous $CD4^+$ T cells (fig. 2d,

right panel) as target cells and NK cells cocultured with VLP-DCs, LPS-DCs or control DCs as effector cells in cytotoxicity assays. Both the non-specific and the Gag-specific cytolytic activity of NK cells were shown to be strongest in NK cells cocultured with VLP-DCs (fig. 2d).

In vivo Activation of DC and NK Cells by Gag-VLPs

To investigate whether Gag-VLPs can induce immune responses in vivo, we introduced a 2-phase immunization strategy in BALB/c mice as illustrated in figure 3a. In the phase I immunization, we assessed the efficacy of Gag-VLPs in activating murine DCs and NK cells in vivo. To examine whether booster immunizations of mice with Gag-VLPs may be required to induce stronger NK cell activation and Gag-specific memory responses in vivo, we immunized 4 groups of mice and evaluated NK cell responses after each immunization.

Splenic DCs of Gag-VLP-immunized mice expressed higher levels of MHC I, MHC II and the DC activation markers CD80 and CD86 than splenic DCs of control mice, and the degree to which these markers were up-regulated was similar to that observed in splenic DCs of LPS-immunized mice (fig. 3b). In vivo activation of NK cells was examined by analyzing CD69 expression on splenic NK cells after each immunization. NK cells from Gag-VLP-immunized mice showed a significant increase in the expression of CD69 after the second booster injection (fig. 3c). We next investigated serum IFN-γ levels 24 h after the second booster injection (fig. 3d, left panel) and the frequency of IFN-γ-secreting splenic NK cells after restimulation with Gag-VLPs, 3 weeks after the second booster injection. Only immunization with VLPs resulted in a Gag-specific memory response, as evidenced by an increased frequency of IFN-γ-producing splenic NK cells only in VLP-immunized mice (fig. 3d, right panel).

Next, to investigate whether Gag-VLPs could induce Gag-specific NK cell cytolytic responses, splenic NK cells (effector cells) of the fourth group of phase I-immunized mice or control mice were restimulated and cocultured with Gag-VLP-loaded splenocytes (target cells) at various effector to target ratios, and cytotoxicity was analyzed by measuring the lysis of target cells. NK cells from Gag-VLP-immunized mice displayed a dose-dependent increase in Gag-specific cytolytic activity higher than that observed in control mice and LPS-immunized mice (fig. 3e). Together, these data demonstrate that HIV-1 Gag-VLPs induce not only NK cell innate immune responses but also Gag-specific memory responses in vivo.

DCs from Gag-VLP-Immunized Mice Trigger NK Cell Activation and IFN-γ Production in vivo

One of the major aims of this study was to investigate whether activation of DCs and NK cell immune responses could be induced by Gag-VLPs. Remarkably, immunization of mice with splenic DCs isolated from phase I, Gag-VLP-immunized mice resulted in NK cell activation in vivo to an extent nearly as great as that observed in mice immunized with DCs isolated from LPS-immunized mice. In contrast, NK cells of mice immunized with DCs isolated from control mice showed no increase in expression of CD69 (fig. 4a). Furthermore, ELISAs (fig. 4b, upper panel) and intracellular staining (fig. 4b, lower panel) demonstrated that inoculation of mice with DCs isolated from VLP- or LPS-immunized mice resulted in increased IFN-γ secretion in vivo.

Fig. 2. Activation of human NK cells cocultured with VLP-DCs. MDDCs were treated with Gag-VLPs, LPS or medium for 24 h. Purified autologous NK cells were added at a DC to NK ratio of 1:5 and incubated for 18 h. **a** NK cell activation was analyzed by flow cytometry after staining with anti-CD56-PE and anti-CD69-FITC. **b** Production of IFN-γ was detected by ELISA (left panel) and intracellular cytokine staining (right panel). IFN-γ in culture supernatants was measured using an ELISA kit. All results are expressed as means ± SD of triplicate wells. Statistical analysis was performed using Student's t test. For intracellular cytokine staining, NK cells were stained with anti-CD56-PE, permeabilized and stained with anti-IFN-γ-FITC. IFN-γ-producing NK cells were examined by FACS analysis. The results are representative of 3 independent experiments. **c** NK cell proliferation was analyzed using a lymphocyte proliferation assay kit (left panel) and CFSE labeling (right panel). NK cells were cocultured with MDDCs for 5 days, and NK cell proliferation was evaluated using a proliferation assay kit. The results are expressed as means ± SD of triplicate cultures from 3 independent experiments. For CFSE labeling, NK cells were loaded with 5 μM CFSE and cocultured with MDDCs for 5 days. Proliferation was assessed by measuring the decrease in the intensity of CFSE fluorescence by flow cytometry after staining cells with anti-CD56-PE. The results are representative of 3 independent experiments using cells from 3 different donors. **d** To examine nonspecific and Gag-specific cytotoxicity, NK cells were cocultured with MDDCs for 24 h. YAC-1 cells (left panel) and HIV-1 NL4-3-infected $CD4^+$ T cells (right panel) were then added to each well (1×10^4 cells/well), and samples were incubated for another 4 h. As a control, unstimulated NK cells were incubated with target cells. Lysis of target cells was determined by measuring LDH release using the Cytotox 96 non-radioactive cytotoxicity assay kit. The results are expressed as means ± SD of triplicate cultures. Comparable results were obtained in 2 independent experiments. E:T = Effector to target.