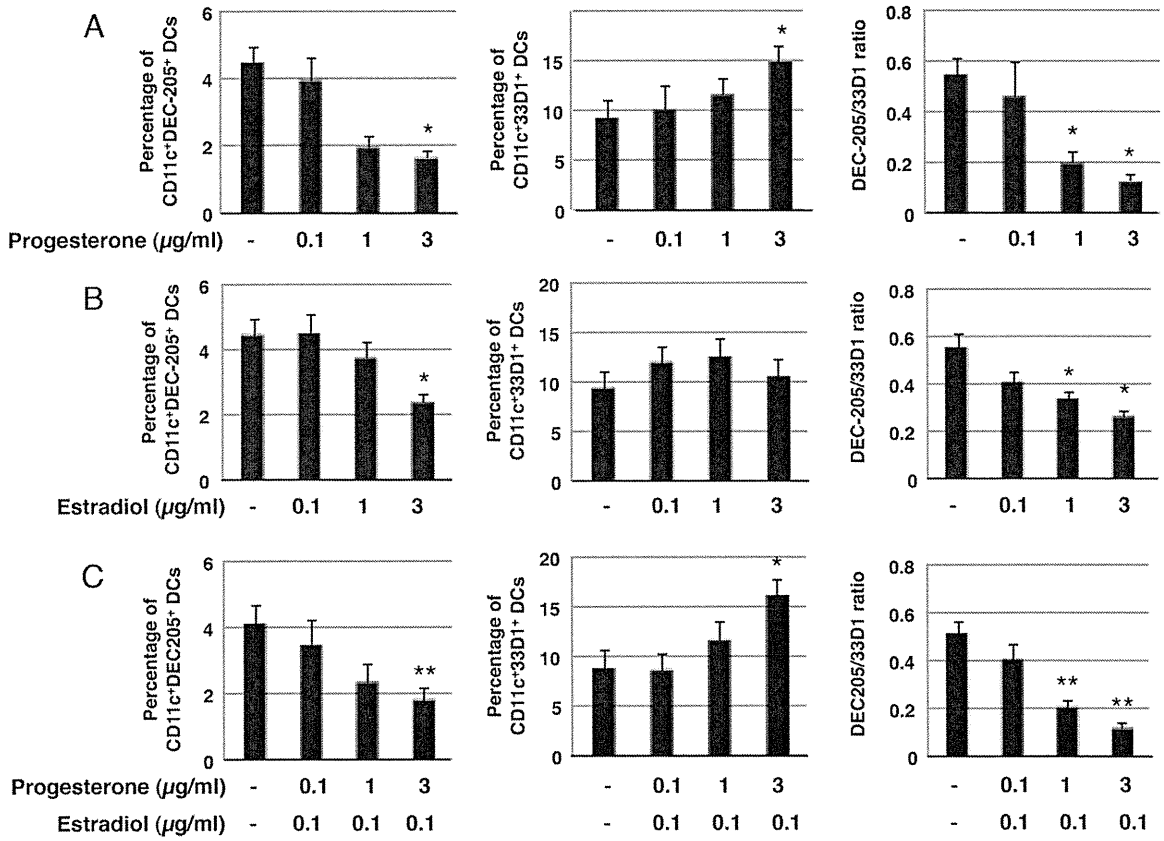

Fig. 1. Kinetics of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs derived from bone marrow cells co-cultured with progesterone and/or estradiol. The data were obtained from cytometric analysis. (A) Kinetics of CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs co-cultured with progesterone (μg/ml), (B) with estradiol (μg/ml), and (C) with progesterone (μg/ml) and estradiol (0.1 μg/ml). Data are shown as the mean ± SEM of $n = 7$–9 mice per group. $^*p < 0.05$ and $^{**}p < 0.01$.

Analysis for the expression of progesterone receptors (PRs) and estrogen receptor alpha (ERα) in DCs

The expression of progesterone receptors (PRs) and estrogen receptor alpha (ERα) in DCs was analyzed by intracellular staining of DCs. First, cells were stained with FITC- or PE-conjugated anti-CD11c to mark DCs and the marked cells were fixed with Fixation/Permeabilization solution (BD Biosciences) at 4 °C for 20 min. Permeabilized DCs were further stained with PE-conjugated 33D1 antibody or anti-progesterone receptor (PR)-specific antibody (clone: PR-AT 4.14; Abcam, Cambridge, UK) or anti-estrogen receptor alpha (ERα)-specific antibody (clone: 33; Abcam) at 4 °C for 60 min. These antibodies were labeled with Alexa Fluor' dyes (Zenon mouse IgG labeling kits) (Invitrogen, Eugene, OR). After being washed twice, cells were re-suspended in FACS buffer solution and analyzed by FACScanto II (BD Biosciences) with FlowJo software (Tree Star, Ashland, OR).

Induction of fetal loss by 33D1 antibody or by recombinant murine IL-12p70 injection

To investigate the effect of the imbalance of DC subsets on fetal loss, CD11c$^+$33D1$^+$ DCs were depleted from pregnant mice by three intraperitoneal (i.p.) injections on Gd 5.5, 6.5, and 7.5 with 0.5 mg 33D1 antibody (mAb against DCIR2) purified by ion-exchange chromatography using diethylaminoethyl (DEAD) cellulose (DE52; Whatman, Maidstone, UK). As controls, pregnant mice were i.p. injected with 0.5 mg control isotype-matched rat IgG2b (Jackson Immuno Research Laboratories, West Grove, PA). The depletion of CD11c$^+$33D1$^+$ DCs in the spleen was confirmed using FACScan. For IL-12 administration, pregnant mice were i.p. injected with murine recombinant IL-12p70 (0.2 μg/mouse) (R & D Systems, Minneapolis, MN) twice on Gd 9.5 and 10.5. Miscarriage on Gd 16.5 was identified macroscopically as a dark, small fetus. The percentage of fetal loss was calculated as follows: (resorbed implantation numbers/sum of viable and resorbed implantation numbers) × 100%. To examine the effect of progesterone on protection against miscarriage, mice were treated with i.p. injection of 3 μg progesterone (Sigma–Aldrich) on Gd 2.5, 3.5, and 4.5, followed by i.p. injection of 0.5 mg 33D1 antibody on Gd 5.5, 6.5, and 7.5.

Effect of IL-13 injection on rescue of fetal loss induced by IL-12p70 injection

To clarify the role of IL-13, recombinant IL-13 (0.02 μg/mouse) (R & D Systems) was i.p. injected simultaneously with IL-12p70 injection and the percentage of fetal loss was calculated.

Cytokine detection

Cytokine production in the sera from the heart of each pregnant mouse was measured at various time points. The IL-2, IL-4, IL-5, IL-6, IL-10, and IL-13 levels of each sample were measured using a FlowCytomix Simplex Kit (eBioscience). Also, the levels of IL-12p40 as well as IL-12p70 were measured using specific ELISA kits (R & D Systems).

Statistical analysis

The results were analyzed using Student's *t*-test and the results are presented as the mean ± SEM. Differences at $p < 0.05$ were considered significant.

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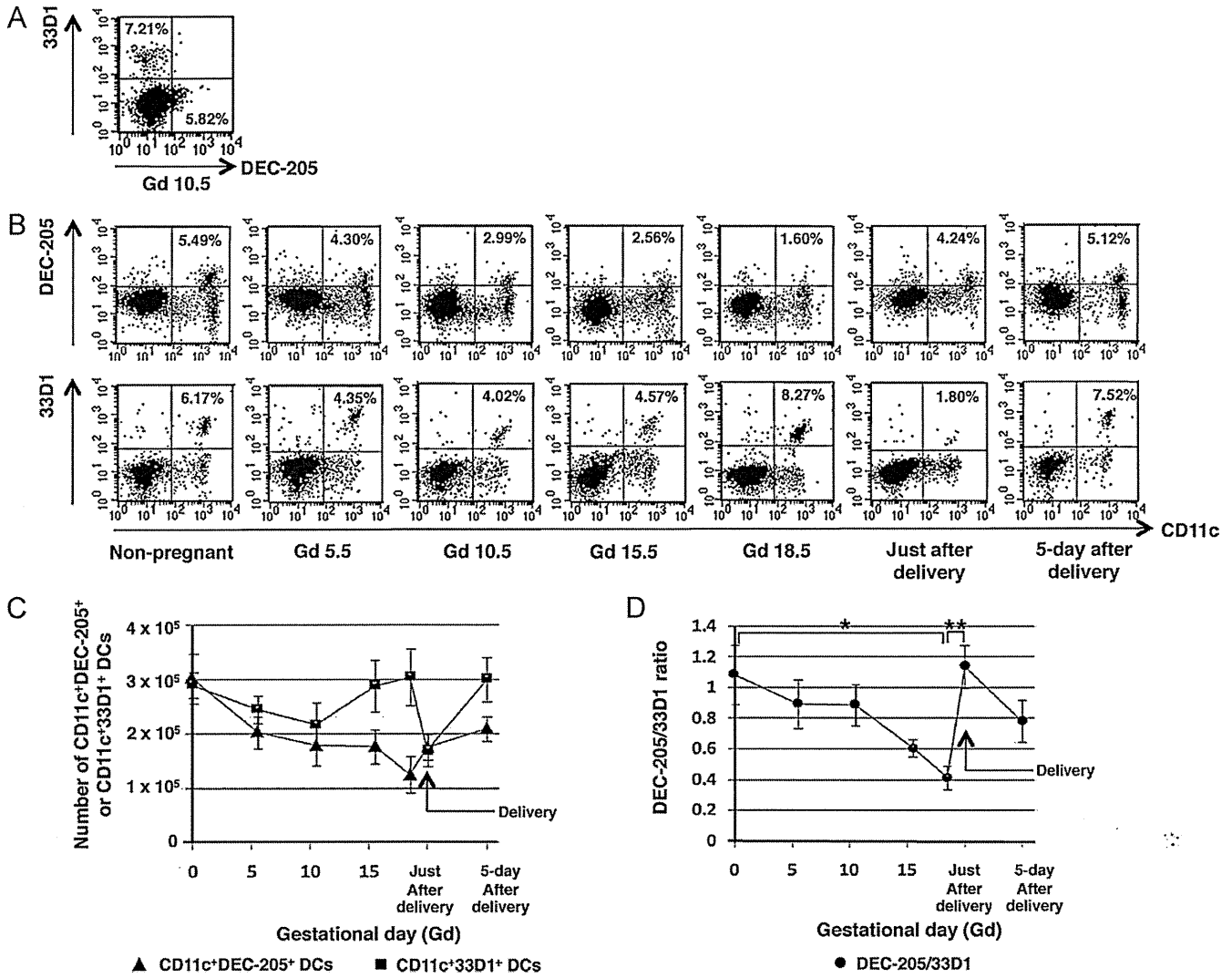

Fig. 2. Flow cytometric analysis of CD11c⁺DEC-205⁺ and CD11c⁺33D1⁺ DCs during perinatal period in the spleen. (A) Distribution of DEC-205⁺ (FITC-labeled) and 33D1⁺ (PE-labeled) DCs in spleen on Gd 10.5. Double-positive DCs, DEC-205⁺33D1⁺ DCs, were not detected in spleen. (B) Typical flow cytometric analysis of CD11c⁺DEC-205⁺ and CD11c⁺33D1⁺ DCs during perinatal period. Divided by the cross in each figure, the upper right region was double-stained for FITC- and PE-conjugated mAb. DCs were gated according to their FSC/SSC characteristics. (C) Number of CD11c⁺DEC-205⁺ and CD11c⁺33D1⁺ DCs during perinatal period. Delivery occurred on Gd 19.5 or 20.5 for all mice. (D) Alteration of DEC-205/33D1 ratio during perinatal period. Values in (D) were calculated from the values in (C). Data are shown as the mean ± SEM of $n = 6–10$ mice per group. *$p < 0.05$ and **$p < 0.01$.

Results

Effects of progesterone and estrogen (estradiol) on the development of DC subsets derived from bone marrow cells

Various surface markers on DCs enabled us to classify their subpopulations. Among them, two major non-overlapping distinct subsets of DCs expressing their own C-type lectin receptors, 33D1 and DEC-205, have recently been found to regulate internal immune responses (Figdor et al. 2002). DEC-205⁺ DCs establish a Th1 predominant state while 33D1⁺ DCs, which recognize DCIR2, induce Th2 polarization (Dudziak et al. 2007). Also, the role of such DCs in the regulation of proper Th1/Th2 balance during pregnancy has been addressed (Laskarin et al. 2007). Pregnancy is established and maintained by maternal hormones, such as progesterone and estrogen, particularly progesterone. Indeed, the serum progesterone concentration increases up to 0.3 μM during pregnancy (Virgo and Bellward 1974).

To clarify the effect of progesterone and estradiol on the development of DCs, we analyzed the expressions of DEC-205 and 33D1 on CD11c⁺ DCs generated from murine bone marrow cells co-cultured with progesterone and/or estradiol. When bone marrow cells were stimulated with murine GM-CSF and IL-4 in the presence of progesterone, the percentage of CD11c⁺DEC-205⁺ DCs was markedly reduced, while that of CD11c⁺33D1⁺ DCs was increased, and thus the DEC-205/33D1 ratio was apparently decreased (about 0.54–0.13, $p < 0.05$) in a dose-dependent manner (Fig. 1A); however, when bone marrow cells were co-cultured in the presence of estradiol, the percentage of CD11c⁺33D1⁺ DCs was almost unchanged, although the percentage of CD11c⁺DEC-205⁺ DCs was partially reduced (Fig. 1B), and the DEC-205/33D1 ratio was decreased to some extent (about 0.54–0.26, $p < 0.05$).

In general, the serum concentration of both progesterone and estradiol increases during pregnancy, with the former much higher than the latter (Virgo and Bellward 1974). Therefore, we examined the synergistic effect of progesterone and less estradiol on DC development. When bone marrow cells were co-cultured with progesterone together with 0.1 μM estradiol, the DEC-205/33D1 ratio was more stably and significantly decreased (0.51–0.11, $p < 0.01$) with an apparent reduction of CD11c⁺DEC-205⁺ DCs and increase

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of CD11c$^+$33D1$^+$ DCs in a dose-dependent manner (Fig. 1C). To determine the mechanism of how DCs are differently affected by the addition of progesterone and/or estrogen, we examined the intracellular expression of progesterone receptor (PR) and estrogen receptor alpha (ERα) in bone marrow-derived DCs and found that PR and ERα were weakly but almost equally expressed in both CD11c$^+$DEC-205$^+$ and CD11c$^+$33D1$^+$ DCs (data not shown). The results indicate that the difference in DC growth by progesterone and/or estrogen might not have been caused by the amount of expression in their receptors.

Collectively, progesterone has strong potential to reduce the DEC-205/33D1 ratio in the presence of a small amount of estrogen, such as estradiol, to firmly suppress Th1 dominancy through DEC-205$^+$ DC reduction and 33D1$^+$ DC increase, particularly at more than 1 μg/ml.

Kinetics of DC subsets during pregnancy in the spleen

Among maternal hormones, the serum progesterone concentration increases during pregnancy in the presence of a small amount of estrogen. Thus, we examined the alterations in the number of murine splenic CD11c$^+$ DCs expressing either DEC-205 or 33D1 during pregnancy.

As has been previously demonstrated (Bozzacco et al. 2007; Moriya et al. 2010), CD11c$^+$ murine splenic DEC-205$^+$ or 33D1$^+$ DCs are non-overlapping distinct subsets (Fig. 2A). Based on this notion, we performed flow cytometric analysis to identify CD11c$^+$DEC-205$^+$ (Fig. 2B, upper panels) or CD11c$^+$33D1$^+$ DCs (Fig. 2B, lower panels) in the spleen on successive gestational days (Gds) 5.5, 10.5, 15.5, 18.5, and just after delivery, as well as 5 days after delivery.

As indicated in the figures, the number of CD11c$^+$DEC-205$^+$ DCs decreased during pregnancy and ended with a sudden increase that was determined just after delivery. In contrast, the number of CD11c$^+$33D1$^+$ DCs gradually decreased in the early phase of pregnancy until around Gd 10.5, increased in the late phase of pregnancy, and sharply declined around the delivery date. The sharp reduction in the percentage of CD11c$^+$33D1$^+$ DCs recovered rather quickly after delivery.

To clarify the alteration of the Th1/Th2 balance during pregnancy, the kinetics of the number of either CD11c$^+$DEC-205$^+$ DCs (closed triangle) or CD11c$^+$33D1$^+$ DCs (closed square) was plotted on the basis of flow cytometric analysis (Fig. 2C). The DEC-205/33D1 ratio was calculated on the basis of the above data. As shown in Fig. 2D, although the DEC-205/33D1 ratio (closed circle) rapidly decreased toward the end of pregnancy, a sharp augmentation of the ratio was seen just after delivery, which might have been initiated by the marked increase of CD11c$^+$DEC-205$^+$ DCs together with the rapid decrease of CD11c$^+$33D1$^+$ DCs.

Kinetics of DC subsets during pregnancy in the zone associated with the uterus

The kinetics of DC subpopulations in the PALNs and decidua associated with uterus was also examined. In contrast to the spleen, the number of CD11c$^+$DEC-205$^+$ DCs in PALNs increased before delivery and thus the DEC-205/33D1 ratio was significantly augmented before delivery. Although a number of DC-lineage CD11c$^+$ cells were observed in the decidua, only a few CD11c$^+$DEC-205$^+$ DCs were detected and CD11c$^+$33D1$^+$ DCs were hardly seen (data not shown). We performed similar experiments several times; however, it was difficult to evaluate the kinetics of DC subsets in the decidua quantitatively. Nevertheless, the number of CD11c$^+$DEC-205$^+$ DCs in the PALNs tended to decrease during pregnancy. Such reduction of CD11c$^+$DEC-205$^+$ DCs in PALNs might be beneficial for maintaining pregnancy. Further analysis of DC subsets in the zone associated with the uterus will be needed to clarify the actual roles of DCs in pregnancy.

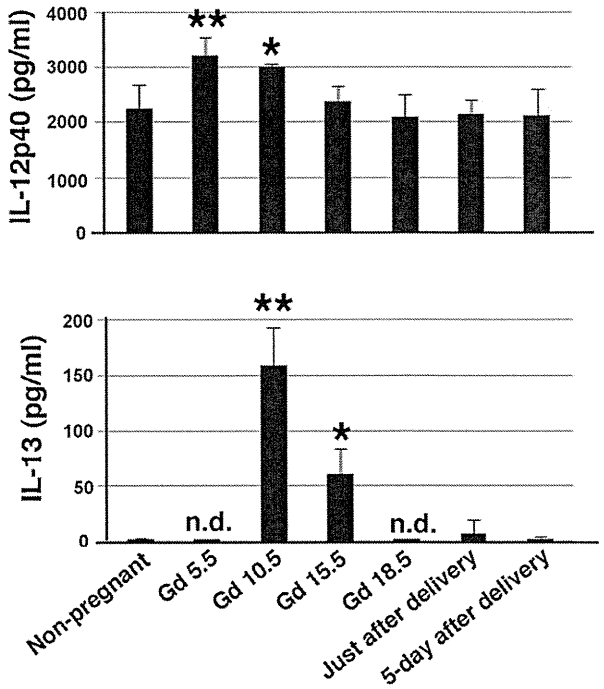

Fig. 3. IL-12p40 (pg/ml) and IL-13 (pg/ml) production in the sera during perinatal period. Data are shown as the mean ± SEM of $n = 6$–10 mice per measurement.

Kinetics of cytokine production in the sera of pregnant mice

Kinetics of the secretion of various cytokines in the sera during pregnancy was determined by FlowCytomix or ELISA. We measured serum IL-2 and IL-12 as Th1 cytokines and IL-4, IL-5, IL-6, IL-10, and IL-13 as Th2 cytokines. Because we hardly detect IL-12p70, a bioactive form of IL-12, in the standard ELISA kit (R & D Systems) in the obtained murine sera, we measured IL-12p40, a sub component of IL-12, instead. As far as we have examined, temporal elevation of IL-12p40 in the early phase of pregnancy followed by IL-13 elevation could be observed in the sera (Fig. 3). IL-2, IL-4, IL-5, IL-6, and IL-10 were not seen in the sera of pregnant mice (data not shown). The results indicate that cytokines produced mainly by innate cells such as DCs (names as "innate cytokines") were dominantly secreted in the syngeneic mating system. It should be noted that a similar secretion pattern of "innate cytokines" was also observed in the allogeneic mating system (BALB/c (female) × C57BL/6 (male)), in which a higher amount of IL-13 was detected during pregnancy (Y.N, M.S., and H.T., unpublished observation).

Depletion of 33D1$^+$ DCs in pregnant mice

The above findings strongly suggest that the sharp augmentation of the DEC-205/33D1 ratio is associated with the initial signaling to halt a pregnancy. This finding prompted us to examine the effect of CD11c$^+$33D1$^+$ DC depletion on fetal loss in pregnant mice. Based on our previous findings (Moriya et al. 2010), pregnant mice were i.p. administrated with 0.5 mg purified 33D1 antibody for 3 consecutive days (Gd 5.5, 6.5, and 7.5). Although CD11c$^+$33D1$^+$ splenic DCs were completely depleted *in vivo* at least two weeks after injection in normal non-pregnant mice (Moriya et al. 2010), such DCs could be observed in pregnant mice less than ten days after administration (Gd 15.5, Fig. 4A), indicating that CD11c$^+$33D1$^+$ DCs seem to recover earlier in pregnant mice. The depletion of

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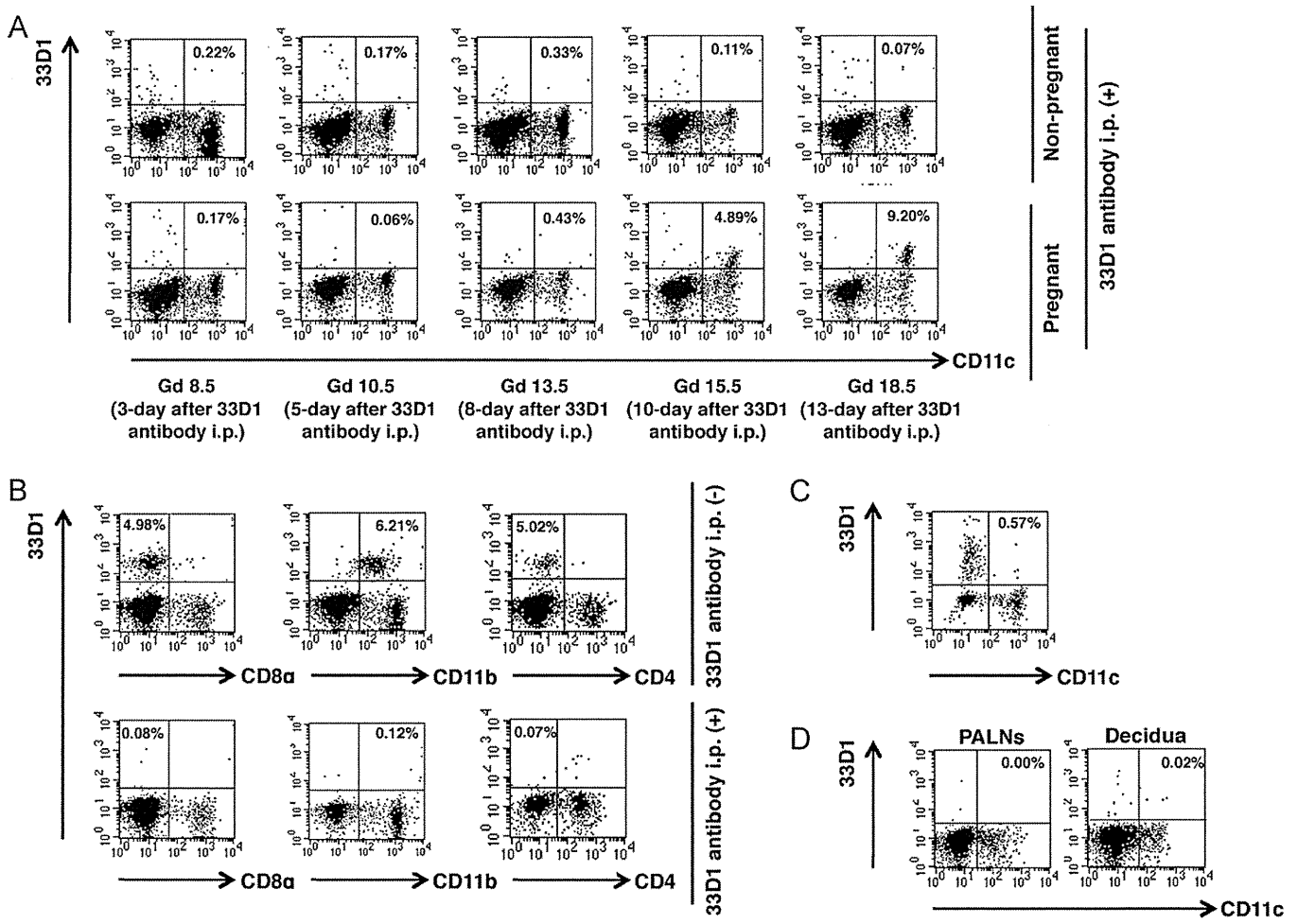

Fig. 4. Depletion of CD11c$^+$33D1$^+$ DCs in the spleen of non-pregnant and pregnant mice by i.p. administration of 33D1 antibody. (A) Kinetics of CD11c$^+$33D1$^+$ DCs in the spleen of non-pregnant (upper panel) and pregnant mice (lower panel) during pregnancy. Mice were administrated i.p. with 0.5 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

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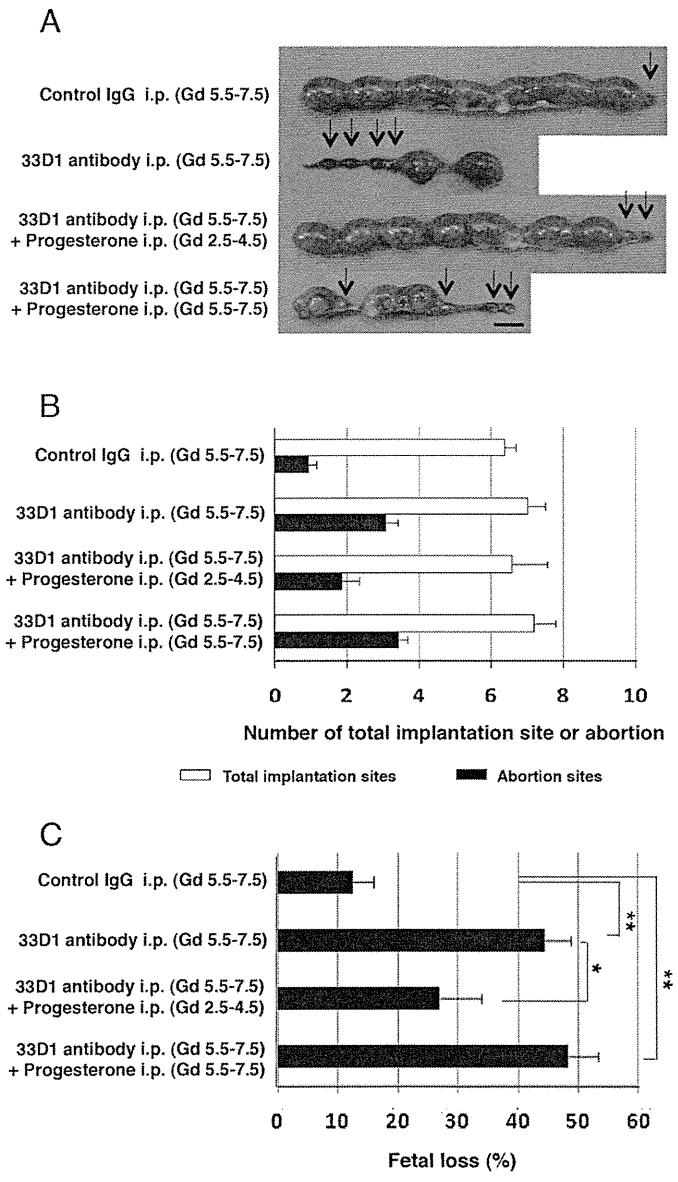

Fig. 5. Depletion of 33D1$^+$ DCs and administration of progesterone. For each figure, mice were injected i.p. either with 33D1 antibody or with control isotype-matched IgG on three consecutive days (Gd 5.5–7.5). Progesterone was also administrated i.p. on Gd 2.5, 3.5, and 4.5 or Gd 5.5, 6.5, and 7.5, 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.

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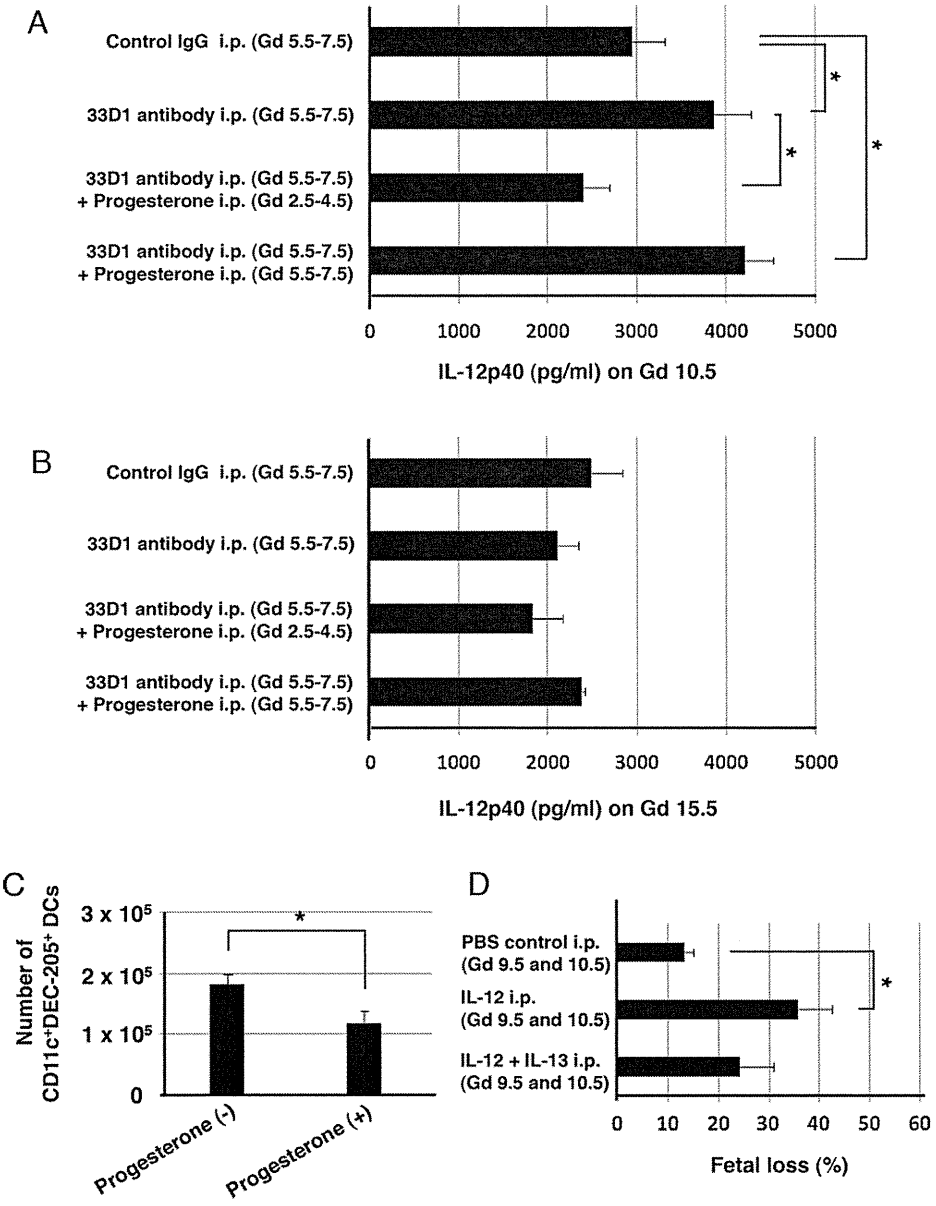

Fig. 6. Effects of 33D1 antibody injection and progesterone treatment on serum IL-12p40 production, number of CD11c$^+$DEC-205$^+$ DCs, and induction of miscarriages in pregnant mice. Serum IL-12p40 production with control isotype-matched IgG treatment, 33D1 antibody treatment, 33D1 antibody with progesterone (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

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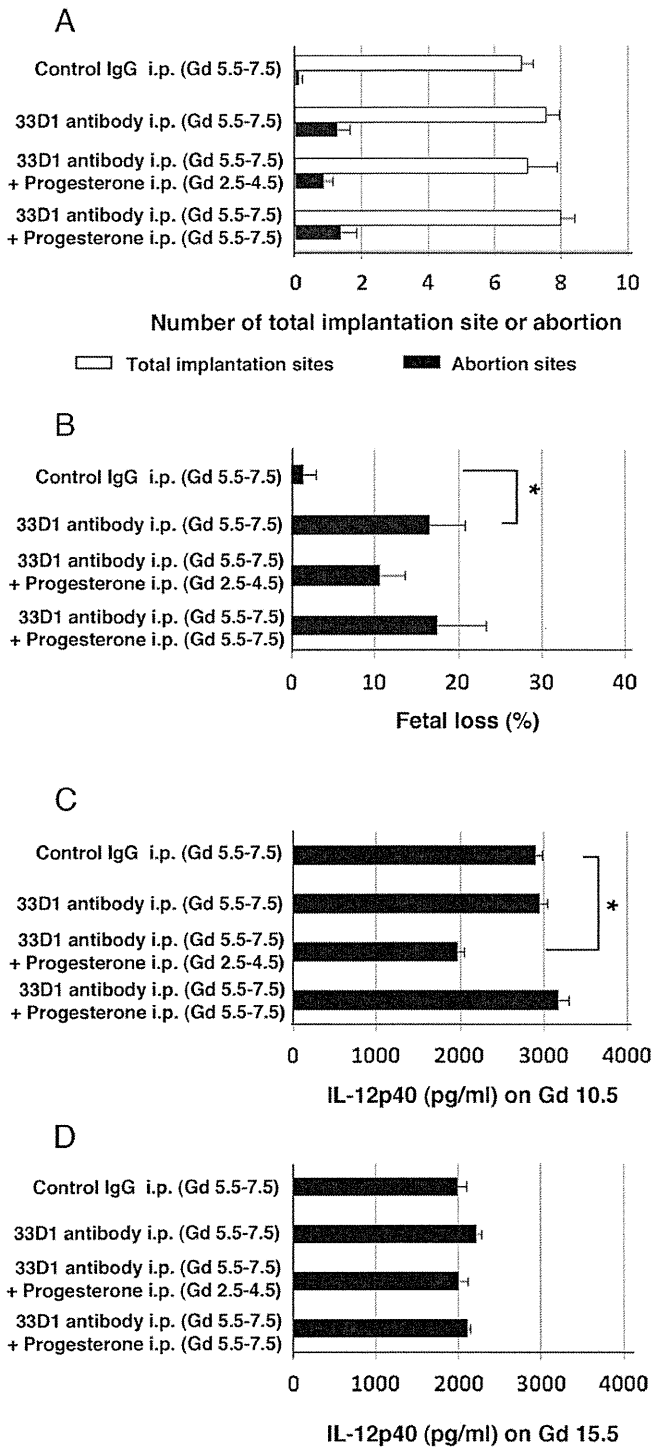

Fig. 7. 33D1 antibody and/or progesterone administration for C57BL/6 × C57BL/6 mating combination. For each figure, mice were injected i.p. either with 33D1 antibody or with control isotype-matched IgG on three consecutive days (Gd 5.5–7.5). Progesterone was also administrated i.p. on Gd 2.5, 3.5, and 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. $^*p < 0.05$.

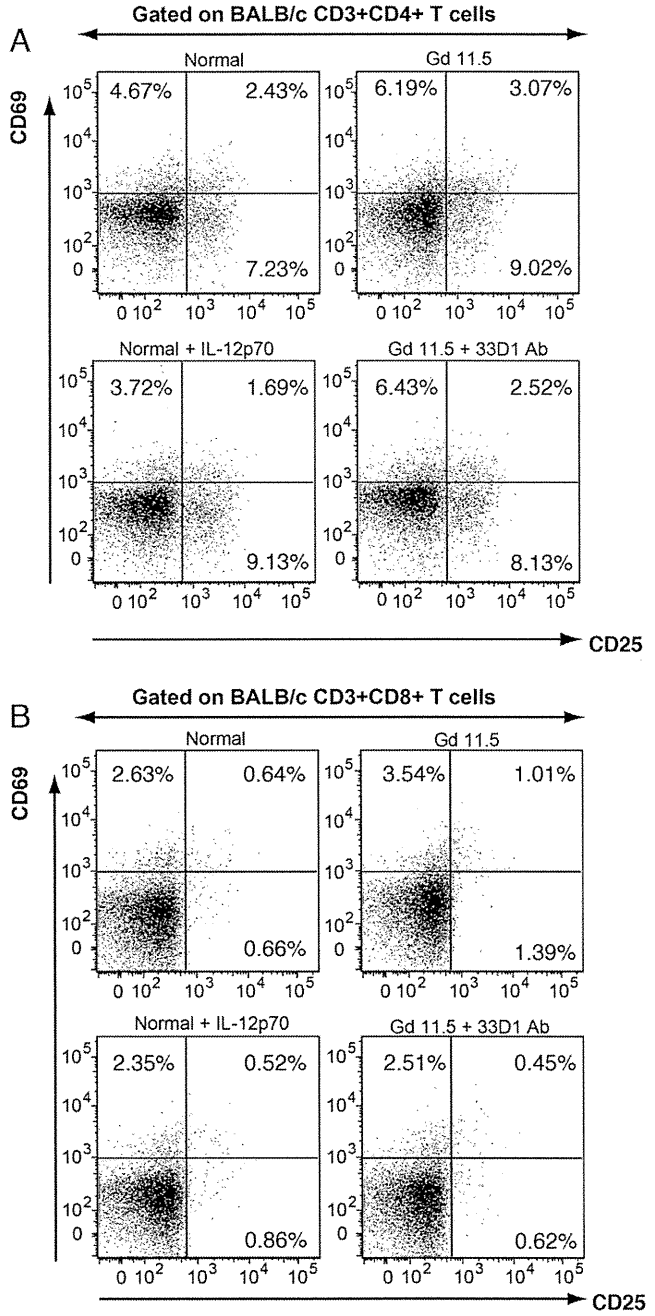

Fig. 8. Analysis of the activation markers on the splenic T cells obtained from BALB/c mice. Spleen cells, obtained from normal untreated BALB/c mice (upper left), from pregnant mice on Gd 11.5 (upper right), from pregnant mice on Gd 11.5 having miscarriages by IL-12p70 i.p. inoculation on Gd 9.5 and 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

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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

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RETROVIROLOGY

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Identification of an unique CXCR4 epitope whose ligation inhibits infection by both CXCR4 and CCR5 tropic human immunodeficiency type-I viruses

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Abstract

Background: Small chemical compounds which target chemokine receptors have been developed against human immunodeficiency virus type 1 (HIV-1) and are under investigation for use as anti-HIV-1 microbicides. In addition, monoclonal antibodies (mAbs) against chemokine receptors have also been shown to have anti-HIV-1 activities. The objective of the present study was to screen a panel of three anti-CXCR4 specific monoclonal antibodies (mAbs) for their ability to block the HIV-1 infection using *in vitro* activated primary peripheral blood mononuclear cells (PBMCs).

Results: PBMCs from normal donors were pre-activated with anti-CD3 and anti-CD28 mAbs for 1 day, and aliquots were infected with a low dose of CCR5-tropic (R5), CXCR4 tropic (X4) or dual tropic (X4R5) HIV-1 isolates and cultured in the presence of a panel of anti-CXCR4 mAbs. The panel included clones A145 mAb against the N-terminus, A120 mAb against a conformational epitope consisting of extracellular loops (ECL)1 and ECL2, and A80 mAb against ECL3 of CXCR4. Among these mAbs, the A120 mAb showed the most potent inhibition of infection, by not only X4 but surprisingly also R5 and X4R5 HIV-1. The inhibition of R5 HIV-1 was postulated to result from the novel ability of the A120 mAb to induce the levels of the CCR5-binding β-chemokines MIP-1α, MIP-1β and/or RANTES, and the down modulation of CCR5 expression on activated $CD4^+$ T cells. Neutralizing anti-MIP-1α mAb significantly reversed the inhibitory effect of the A120 mAb on R5 HIV-1 infection.

Conclusions: The data described herein have identified a unique epitope of CXCR4 whose ligation not only directly inhibits X4 HIV-1, but also indirectly inhibits R5 HIV-1 infection by inducing higher levels of natural CCR5 ligands.

Background

CXCR4 and CCR5 belonging to the family of G-protein coupled receptors (GPCR) serve as receptors for the CXC-chemokine stromal derived factor 1 (SDF-1) and the CC-chemokines MIP-1α, MIP-1β and RANTES, respectively. The ligation of these chemokine receptors transmits a number of intracellular signals, and the receptors also serve as co-receptors for HIV-1 [1-5]. Under normal physiological conditions, CXCR4 molecules form closely linked dimers [6] and heterodimers with other chemokine receptors including CCR5 [7]. CXCR4 is expressed extracellularly, consisting of an N-terminal (NT) region and extracellular loops (ECL) 1, ECL2 and ECL3. Several lines of evidence indicate that the interaction between CXCR4 and SDF-1 or HIV-1 involves multiple domains of the receptor. For example, while the NT and the ECL2 domains appear to be critical for SDF-1 binding and signaling, the regions contiguous to the ECL2 and ECL3 have been implicated in HIV-1 co-receptor activity and homologous cell adhesion [8-11]. Studies with CXCR4 mutants have revealed that the HIV-1 co-receptor activity of CXCR4 is

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independent of its ability to function as a chemokine receptor and/or transduce intracellular signaling [11,12].

Current and prospective anti-HIV-1 therapy includes the use of small chemical compounds which target chemokine receptors that are termed viral occupancy inhibitors (VIROC) [13]. In addition, mAbs against chemokine receptors have also been shown to have a potential for HIV-1 inhibition. For example, an anti-human CCR2 mAb that is neither an agonist nor an antagonist blocks both X4 and R5 HIV-1, due to oligomerization of CCR2 with CCR5 and CXCR4, but not receptor down-modulation [14]. In addition, an unique mAb with specificity for the N-terminus region of CCR5 that does not block the interaction between HIV-1 gp120 and CCR5, blocks R5 HIV-1 infection by inducing CCR5 dimerization [15].

Herein, we examined a series of three rat IgG anti-human CXCR4 mAbs made by our laboratory [16], and we demonstrate that clone A120, that recognizes a conformational epitope encompassing the ECL1 and ECL2 domains of CXCR4, has a unique functional property. Thus, the interaction of the A120 mAb with CXCR4 inhibits not only X4, but also R5 HIV-1 infection of in vitro activated PBMCs, via mechanisms detailed herein. The novel anti-CXCR4 mAb function described in this study potentially provides a unique adjunct to conventional anti-HIV-1 chemotherapy with activity against not only CXCR4 but also CCR5 and dual tropic HIV-1.

Results

Suppressive effects of anti-CXCR4 mAbs on HIV-1 infection in primary activated PBMCs

We first tested our 3 different anti-CXCR4 mAb clones (A145, A120 and A80) for their potential to inhibit the infection of the prototype X4 HIV-1_{NL4-3} and for purposes of controlling the prototype R5 HIV-1_{JR-FL} in *in vitro* activated primary PBMC cultures. None of these anti-human CXCR4 mAbs cross-reacts with human CCR5, and only the A120 mAb can block the SDF-1-mediated Ca^{2+} influx [16]. Thus, the PBMCs infected with low levels of HIV-1 (at a multiplicity of infection of lower than 0.01) were cultured for 5 days in the presence or absence of 10 μg/ml of either anti-CXCR4 mAb or isotype control. As shown in Figure 1a, while the A145 mAb had minimal inhibitory effect, the A120 and A80 mAbs markedly inhibited the infection of the

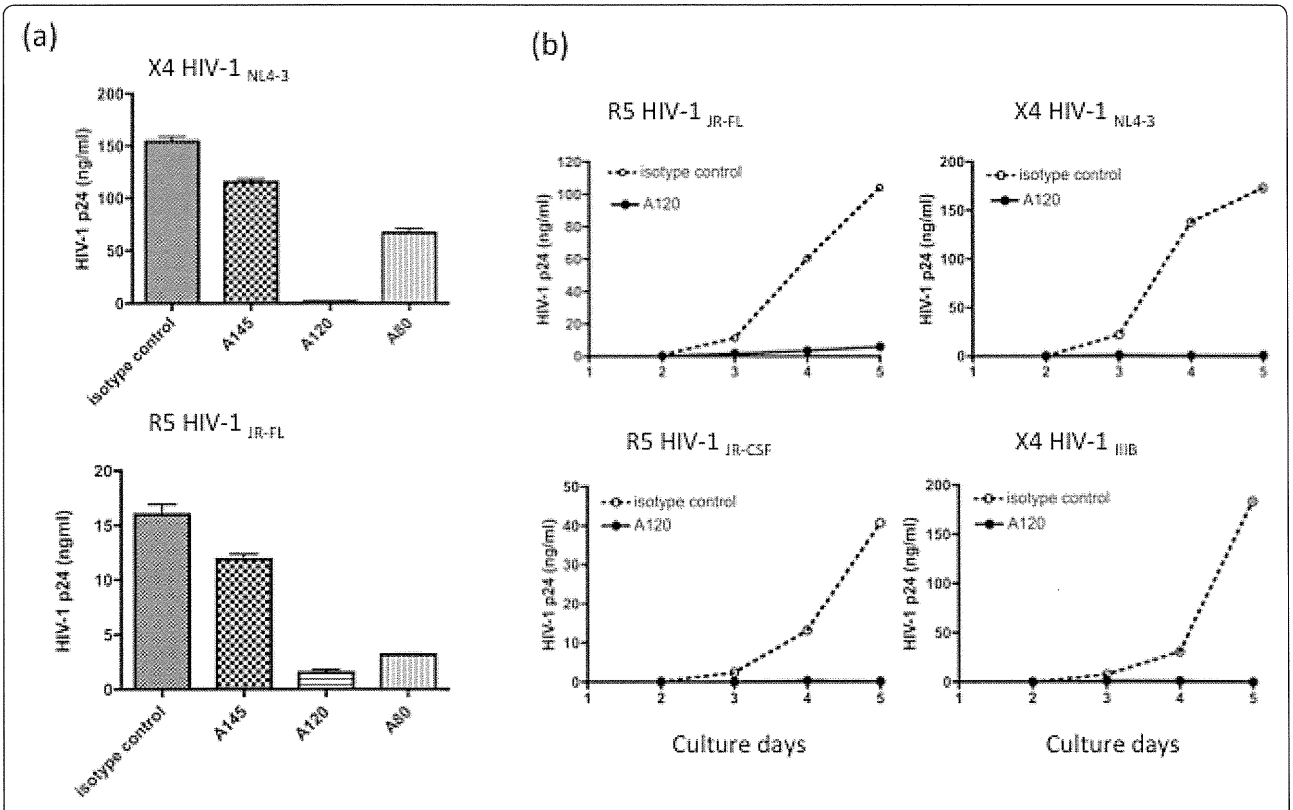

Figure 1 Inhibition of HIV-1 infection in activated PBMCs by anti-CXCR4 mAbs. (a) PBMCs activated with anti-CD3/CD28 for 1 day were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours, washed and then cultured in the presence of 10 μg/ml of the A145, A120, A80 rat IgG mAbs or isotype control rat IgG mAb mixture. After 5 days, virus production in the culture supernatants was determined by p24 ELISA. (b) Activated PBMCs infected with R5 HIV-1_{JR-FL}, R5 HIV-1_{JR-CSF}, X4 HIV-1_{NL4-3} or X4 HIV-1_{IIIB} were aliquoted and cultured in the presence of 10 μg/ml of the A120 mAb or isotype control mAb. The p24 levels in the culture supernatants were monitored daily by ELISA. Data shown for both (a) and (b) are representative of 3 independent experiments using PBMCs from different donors.

X4, but to our surprise, also the R5 HIV-1 strain. Since the inhibitory potential of the A120 mAb was the highest among these mAbs, we selected the A120 mAb for further characterization. Although the production of HIV-1 from activated PBMCs was influenced by culture conditions, mostly cell concentration at time of infection and cultivation steps, as shown in Figure 1b, the inhibitory effect of A120 mAb was further confirmed using an additional R5 (JR-CSF) and X4 (IIIB) HIV-1 strains.

To examine tPBMC donor variabilities, the ability of the A120 mAb to inhibit R5 HIV-1_{JR-FL} and X4 HIV-1_{NL4-3} in activated PBMCs from 6 different unrelated donors was also studied. Viral production was quantitated by measuring both the levels of p24 and the frequency of infected cells using flow cytometry as outlined in the methods section. As seen in Figure 2a, whereas there was indeed considerable variability in the relative susceptibility of *in vitro* activated PBMCs from different donors to support R5 and X4 HIV-1 infection, the addition of the A120 mAb to the cultures showed variable levels of moderate to significant inhibition in each case (differences in the ability of PBMCs from different donors to support R5 versus X4 HIV-1 is an interesting subject that is currently under study). In addition, the fact that the addition of the A120 mAb also inhibited the increase in the frequency of infected

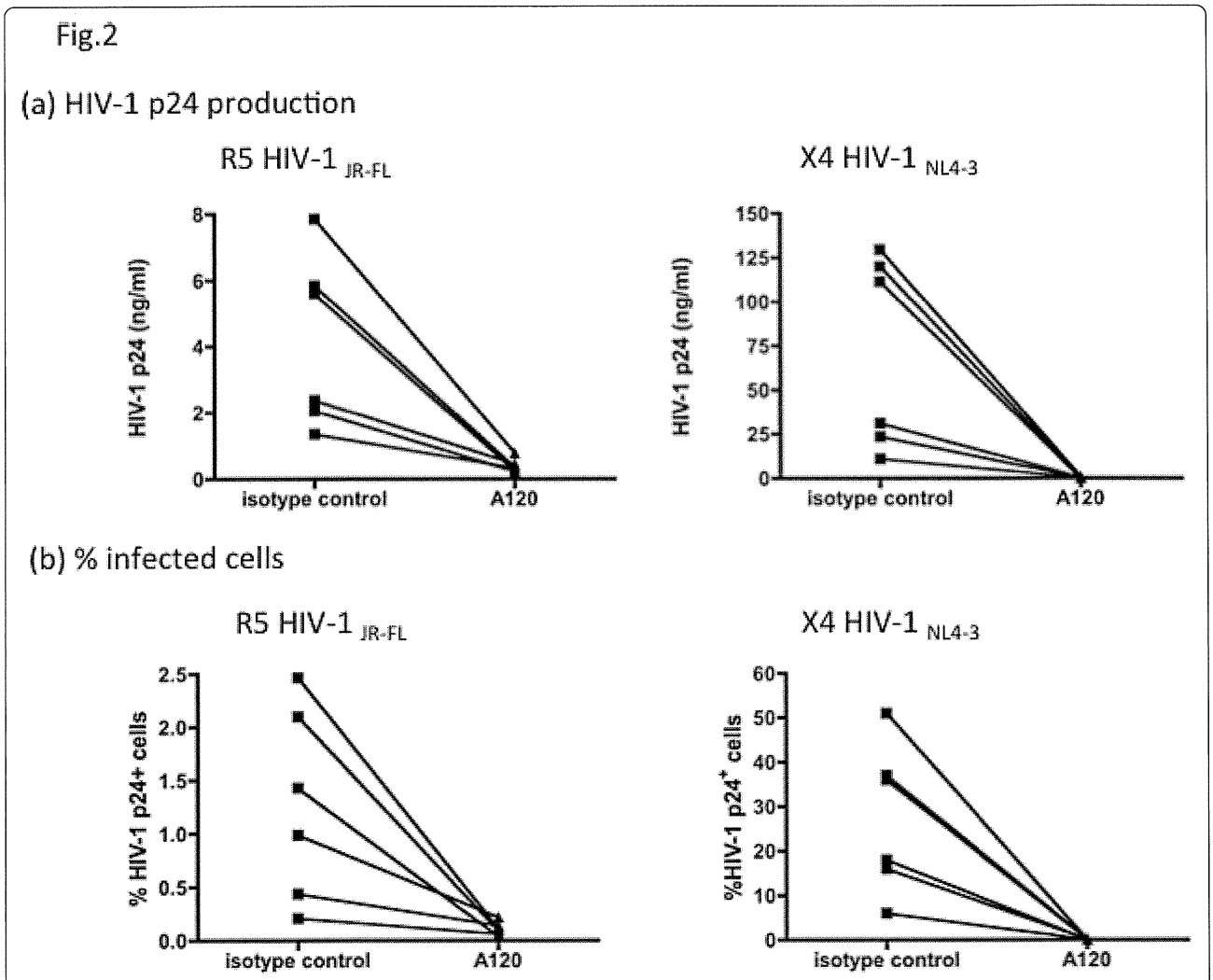

Figure 2 The A120 mAb-mediated inhibition of HIV-1 infection in activated PBMCs from different donors. Activated PBMCs from 6 different donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3} for 2 hours. After extensive washing, the PBMCs were aliquoted and cultured in the presence of A120 or isotype control IgG at 10 μg/ml. (a) After 3~5 days, virus production was determined by p24 ELISA in the culture supernatants, and values obtained on day 4 are shown as representative. P values were 0.007 and 0.032 for R5 HIV-1 and X4 HIV-1, respectively. (b) The PBMC samples obtained on day 4 after infection were fixed and permeabilized, and then stained with anti-HIV-1 p24 mAb labeled with Alexa Fluor 488 and examined by flow cytometry. The frequencies (percentages) of $p24^+$ cells were plotted. P values were 0.026 and 0.031 for R5 HIV-1 and X4 HIV-1, respectively. Representative data from 3 independent experiments are shown.

cells as determined by flow cytometry (Figure 2b) suggests that the A120 mAb inhibits new infection in the cultures. To our knowledge, this is the first report of an anti-CXCR4 mAb that inhibits infection of both X4 and R5 HIV-1 strains in activated PBMCs.

Dose response studies were conducted next to determine whether differences exist in the inhibition of R5 as compared with X4 HIV-1. As seen in Figure 3, maximum inhibition was achieved at a concentration of more than 5 μg/ml and 0.6 μg/ml for R5 and X4 HIV-1, respectively. The difference noted in the titration curves indicates that the potential mechanisms for A120 mAb-mediated R5 and X4 HIV-1 suppression are likely to be distinct from each other. The inhibition of virus replication by the addition of the A120 mAb in these cultures was not secondary to the presence of non-specific inhibitors in the A120 mAb preparation since the addition of the same A120 mAb preparation to the CXCR4 expressing HIV-1 producing Molt-4/IIIB cell line and the HTLV-1 producing MT-2 cell line had no detectable effect on virus production (Figure 4). Because the two cell lines express high levels of CXCR4 that readily binds the A120 mAb, it appears that the mere ligation of CXCR4 via A120 mAb epitope does not interfere with the virus production from these cell lines.

One of the trivial explanations for the R5 HIV-1 suppression by the anti-CXCR4 mAb could be ascribed to the potential presence of LPS in the A120 mAb preparation. However, it is highly unlikely, because (1) the A120 mAb preparation contained little LPS since it was repeatedly passed through a polymyxin B column to eliminate possible LPS contamination, (2) exogenously added LPS at 0.1 μg/ml did not inhibit R5 HIV-1 infection in the same culture conditions, and (3) the inclusion of anti-human CD14 mAb that blocks the binding of LPS failed to interfere with the A120 mAb-mediated R5 HIV-1 inhibition (Figure 5). As seen in Figure 5a, while the addition of the A120 mAb clearly inhibited the generation of syncytia by R5 HIV-1_{JR-FL} and p24 production, there was no detectable inhibition with the addition of LPS. The facts that LPS at 0.1 μg/ml failed to inhibit HIV-1 production (unlike the A120 mAb) and that the addition of anti-CD14 mAb (which blocks LPS binding to its receptor, CD14) did not reverse the inhibition of R5 HIV-1 infection suggest that the activity of the A120 mAb is not due to LPS contamination.

Altogether, these data document that the anti-human CXCR4 mAb, clone A120, which ligates CXCR4 molecules via the ECL1/ECL2 domains potently inhibited not only X4 but also R5 HIV-1 strains in freshly *in vitro* activated primary PBMC cultures.

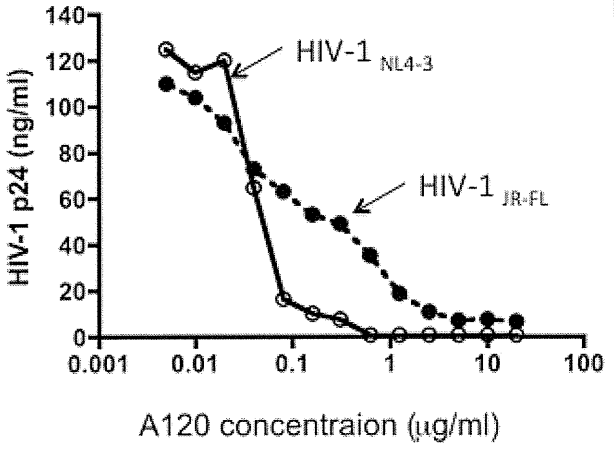

Figure 3 Dose responses of the A120 mAb-mediated inhibition of R5 and X4 HIV-1 infection in activated PBMCs. Activated PBMCs from the donors were infected with either R5 HIV-1_{JR-FL} or X4 HIV-1_{NL4-3}. After washing, the PBMCs were aliquoted and cultured in the presence of graded concentrations of the A120 mAb for 4 days. Virus production in the culture supernatant was determined by p24 ELISA. Representative data from 3 independent experiments using 3 different donors' PBMCs are shown.

Enhancement of the production of the CCR5 binding β-chemokines and reduction of CCR5 expression by A120 mAb treatment

The present observations that the anti-CXCR4 A120 mAb inhibited the production of R5 HIV-1 in *a*ctivated PBMCs prompted us to examine whether CCR5 binding β-chemokines were involved. Thus, we tested whether neutralizing mAbs against human MIP-1α, MIP-1β and RANTES could reverse the effects of the A120 mAb on virus infection. As shown in Figure 6, indeed the A120 mAb-mediated inhibition of R5 HIV-1 infection was significantly reversed by anti-MIP-1α mAb and partially by anti-MIP-1β but not anti-RANTES mAb. These data suggest that MIP-1α and possibly MIP-1β were likely the major factors involved in the inhibition of R5 HIV-1 infection. As expected, the addition of these anti-β-chemokine mAbs did not reverse A120 mAb-mediated blocking of X4 HIV-1 infection (data not shown). However, this β-chemokine dependent mechanism for the inhibition of R5 HIV-1 by the addition of the A120 mAb is donor-dependent. Notably, the addition of the anti-chemokine mAbs failed to reverse the A120 mAb mediated inhibition of R5 HIV-1 in cultures of PBMCs from 2 of the 6 donors. The reason(s) for this resistance in these donors remains to be studied.

To confirm that the β-chemokines were indeed produced by the ligation of CXCR4 by the A120 mAb in activated PBMCs, we quantitated the concentration of these chemokines. Figure 7a shows that the A120 mAb enhanced the synthesis of MIP-1α and MIP-1β in most if not all the cases. Although enhanced RANTES production was seen in 3 out of the 6 donors, it is unlikely

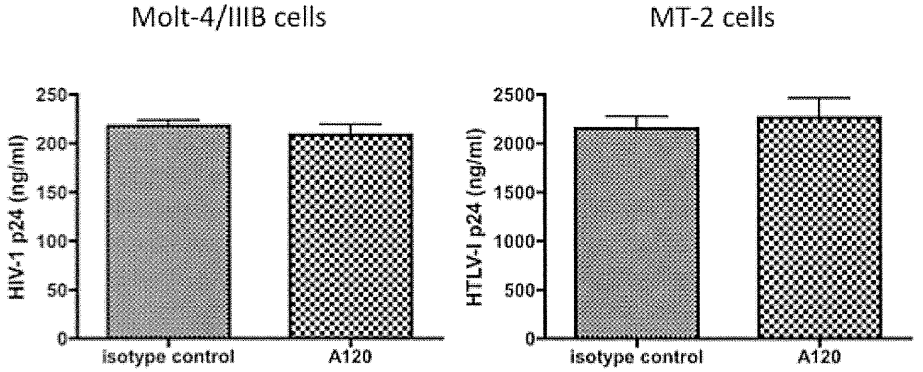

Figure 4 The A120 mAb does not affect HIV-1 and HTLV-I production from producer cell lines. The X4 HIV-1_{IIIB} producer cell line (Molt-4/IIIB) and the HTLV-I producer cell line (MT-2) cells were cultured in the presence of 10 μg/ml of A120 or control mAb for 3 days. The culture supernatants were assayed for HIV-1 p24 and HTLV-I p24 by standard ELISA.

that RANTES is involved in the A120 mAb-mediated R5 HIV-1 inhibition as shown in Figure 6. As expected, treatment of activated PBMCs with the A120 mAb led to a significant reduction in the frequency of cells expressing CCR5 (Figure 7b and 7c). In contrast, there appeared to be a slight increase in the frequency of CXCR4 expressing $CD4^+$ T cells (Figure 7b and 7c). Therefore, these results indicate that the incubation of

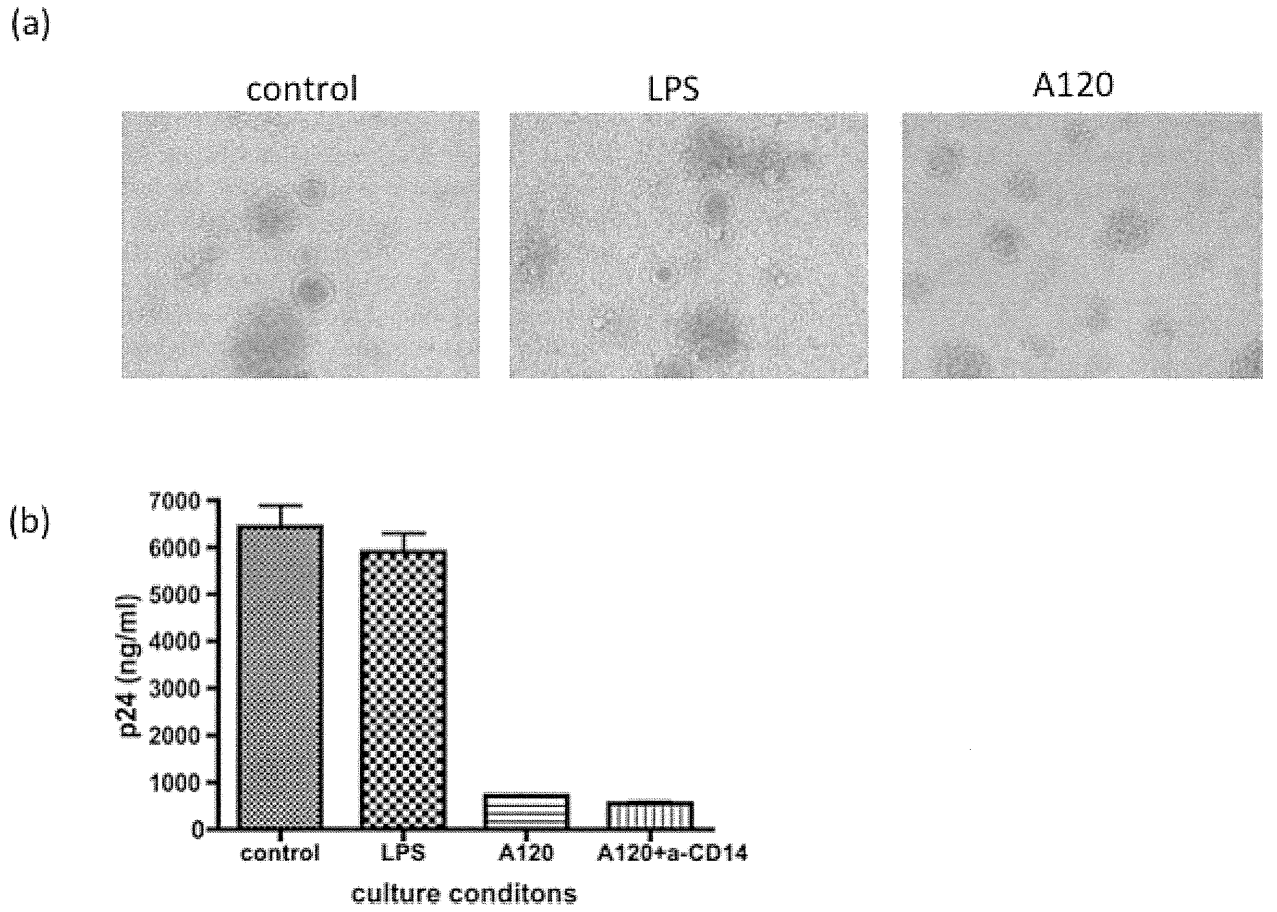

Figure 5 LPS is not involved in the A120 mAb-mediated inhibition of HIV-1 infection. Activated PBMCs infected with R5 HIV-1_{JR-FL} were cultured in the presence or absence of LPS (0.1 μg/ml) or the A120 mAb with or without anti-CD14 mAb. After 4 days, syncytium formation and virus production in the culture supernatants were determined microscopically (a) and using a p24 ELISA kit (b), respectively.

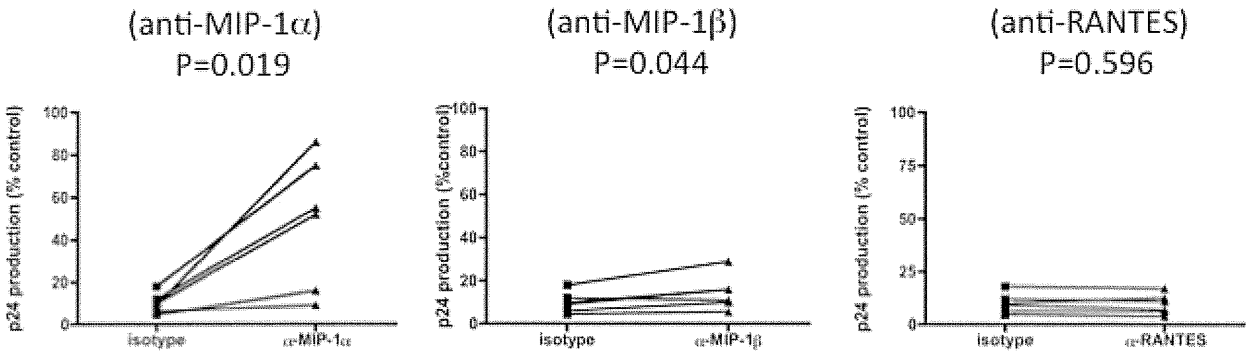

Figure 6 Reversal of the A120 mAb-mediated inhibition of R5 HIV-1 infection in activated PBMCs with anti-CCR5 ligand-neutralizing mAbs. Activated PBMCs from 6 donors were infected with R5 HIV-1_{JR-FL} and cultured in the presence of 10 μg/ml A120 mAb or isotype control mAb together or without anti-chemokine mAbs including anti-MIP-1α, anti-MIP-1β or anti-RANTES at 10 μg/ml for 4 days. Virus production in the culture supernatants was determined by p24 ELISA. The p24 levels were plotted as percent of control values obtained in cultures incubated in each anti-β-chemokine mAb for each donor.

activated PBMCs in the presence of the A120 mAb inhibited R5 HIV-1 infection primarily via the blockade of the co-receptor function of CCR5, most likely due to its ability to induce the synthesis of CCR5-binding β-chemokines. It is important to note that the levels of MIP-1α induced by the A120 mAb showed a typical dose response curve (Additional file 1), and the level of R5 HIV-1 inhibition was inversely-correlated with levels of MIP-1α detected.

Cell populations that produce the β-chemoknes

In an effort to identify the cell lineage that was involved in the synthesis of the β-chemokines following incubation of the activated PBMCs in the presence of the

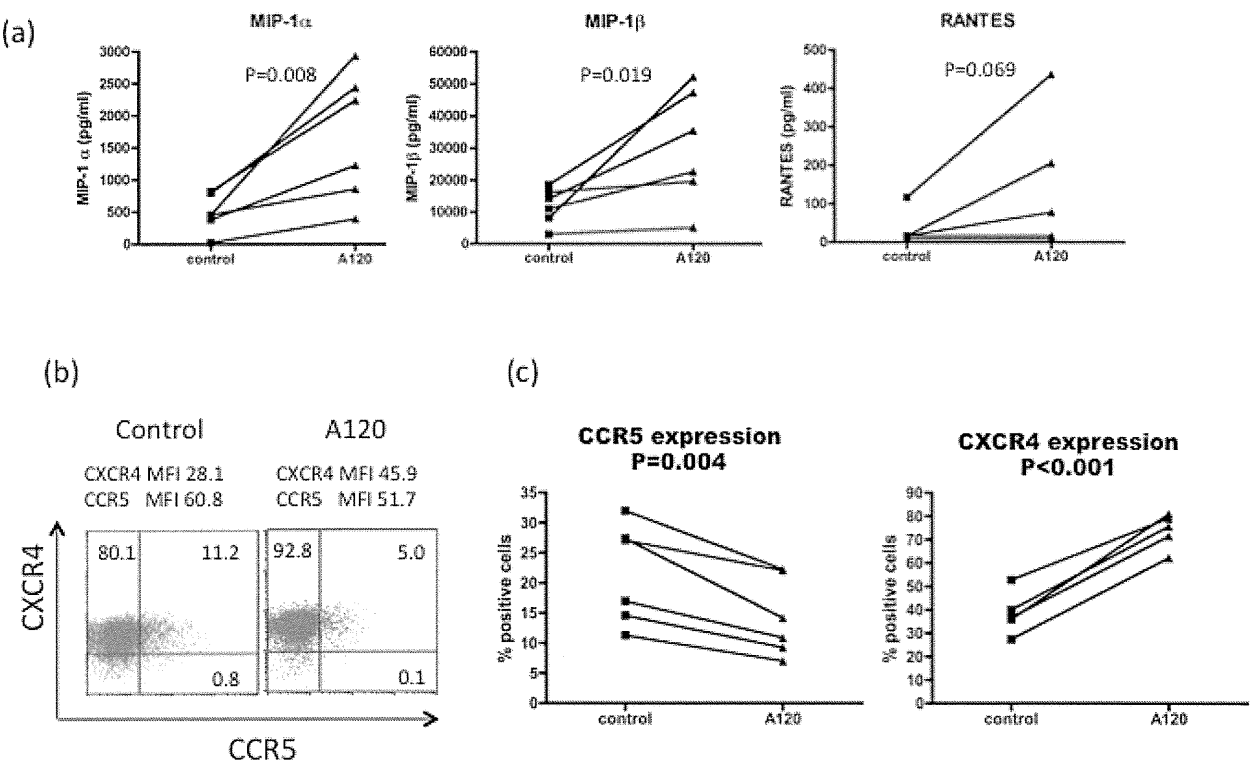

Figure 7 The A120 mAb-treatment induces the production of CCR5 binding β-chemokines and the down-modulation of CCR5 expression. PBMCs from 6 donors were activated with anti-CD3/28 mAbs for 1 day, washed, aliquoted and then incubated in the presence of 10 μg/ml A120 mAb or isotype control mAb for an additional day. (a) Changes in the concentrations of MIP-1α, MIP-1β and RANTES in the culture supernatants were assayed by ELISA. (b and c) Cells were analyzed for changes in the cell surface expression of CCR5 and CXCR4 on gated populations of $CD4^+$T cells (MFI denotes mean fluorescence intensity). A representative flow cytometry dot blot profile is shown.

A120 mAb, cell depletion experiments were carried out. Thus, aliquots of activated PBMCs were depleted of $CD19^+$ B cells, $CD4^+$ T cells, $CD8^+$ T cells or $CD14^+$ monocytes utilizing immune-beads conjugated with the appropriate lineage specific mAbs. Non-depleted (mock) and each cell lineage depleted PBMCs were cultured for 24 hours in the presence or absence of 10 μg/ml of A120 mAb. As shown in Figure 8, the most marked reduction in β-chemokine levels in the culture supernatants was noted in cultures depleted of $CD14^+$ monocytes followed by those depleted of $CD4^+$ T cells and $CD8^+$ T cells. However, B-cell depletion had minimal if any effect on the levels of β-chemokines synthesized. These results suggest that activated T cells along with monocytes were responding to the A120 mAb by secreting β-chemokines.

A120 mAb exhibits broad HIV-1 clade inhibition

Finally, the unique availability of a panel of HIV-1 with distinct co-receptor usage and clades prompted us to examine the breadth of inhibitory activity of the A120 mAb. Once again, aliquots of 1-day anti-CD3/28 activated PBMCs were infected with 15 different HIV-1 strains and then cultured in the presence of 10 μg/ml of the A120 mAb or control IgG, and the levels of p24 in the supernatant fluids were quantitated on day 5 after infection. As shown in Table 1, incubation of the cultures in the presence of the A120 mAb uniformly led to a marked decrease in the levels of p24 for all ten R5 HIV-1 strains, three X4 HIV-1 strains, and two dual R5/X4 tropic HIV-1 strains ($p = 0.0065$).

Discussion

The present study is the first report that documents the unique property of an anti-humanCXCR4 mAb (clone A120) which upon ligation of CXCR4 via the ECL1/ECL2 domains strongly blocks the infection of not only X4 but also R5 and dual tropic HIV-1 strains in freshly *in vitro* activated PBMC cultures. The mechanism for the inhibition of the X4 HIV-1 is likely due to direct interference and binding of gp120 to CXCR4 as reported previously. In addition, since A120 mAb treatment increases CXCR4 expression on $CD4^+$ T cells (Figure 7), it may also be possible that the A120 mAb may block X4 HIV-1 infection by interfering with CXCR4 trafficking. By contrast, the predominant mechanism for the inhibition of the R5 HIV-1 infection by the A120 mAb is most likely due to the production of the CCR5-binding β-chemokines, especially MIP-1α, from activated T cells and monocytes leading to down-modulation of CCR5 expression on $CD4^+$ T cells. The observations that the anti-CXCR4 N-terminus mAb (clone A145) showed little or no inhibition, and the anti-CXCR4 ECL3 mAb (clone A80) was not as potent in inhibiting HIV-1 infection, as compared with the A120 mAb, indicate that the ligation via the ECL1 and/or ECL2 domains is critical for the inhibition of R5 and X4 HIV-1 infection. This view is supported by the finding that a panel of commercially available murine mAbs, whose reactive sites were localized to the ECL1/ECL2 domains or the single ECL2 domain of CXCR4, also showed similar, but less effective suppressive effects on infection with both the X4 and R5 HIV-1 and enhanced MIP-1α and β production under the same culture conditions presented herein (data not shown).

Preliminary data indicate that chemically inactivated X4 HIV-1 (HIV-1IIIB) and recombinant SDF-1 did not induce the synthesis of such β-chemokines or inhibit R5 HIV-1 infection in activated PBMCs (data not shown). Thus, it is important to point out that ligation of CXCR4 by its natural ligand SDF-1 or HIV-1 gp120 is not sufficient for generating signals suitable for the synthesis of the CCR5 ligands, and that ligation of CXCR4 via specific domains is required for these unique anti-HIV-1 activities.

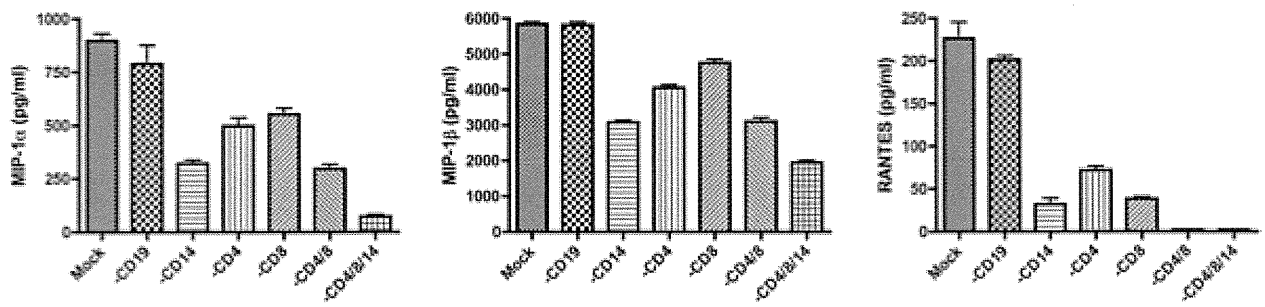

Figure 8 The A120 mAb stimulates T cells and monocytes to produce β-chemokines in activated PBMCs. One day-activated PBMCs were depleted of $CD19^+$B cells, $CD4^+$T cells, $CD8^+$T cells and/or $CD14^+$monocytes using immunobeads conjugated with appropriate lineage specific mAbs, and then cultured in the presence of A120 mAb or isotype control mAb for one day. Concentrations of MIP-1α, MIP-1β and RANTES in the culture supernatants were assayed by ELISA. Representative data from three independent experiments are shown.

Table 1 Suppressive effect of the A120 mAb on various clades of HIV-1 strains.

Member	HIV-1 Subtype	Isolate	Country of Origin	Syncytium	Co-receptor Usage	Percent inhibition of p24 production
PRD320-01	A	UG275	Uganda	NSI	CCR5	88.3%
PRD320-02	A	I-2496	Ghana	NSI	CCR5	99.8%
PRD320-03	CRF02_AG	DJ263	Djibouti	NSI	CCR5	94.7%
PRD320-04	CRF02_AG	POC44951	Liberia	NSI	CCR5	99.7%
PRD320-06	B	BZ167	Brazil	SI	CXCR4	97.2%
PRD3200-7	C	DJ259	Djibouti	NSI	CCR5	91.5%
PRD320-08	C	ZAM18	Zambia	NSI	CCR5	93.7%
PRD320-09	D	SE365	Senegal	SI	CXCR4	98.5%
PRD320-10	D	UG270	Uganda	SI	CXCR4	99.7%
PRD320-11	CRF01_AE	ID17	Indonesia	NSI	CCR5	81.0%
PRD320-12	CRF01_AE	NP03	Thailand	SI	CXCR4	94.5%
PRD320-14	F	BCI-R07	Romania	SI	CXCR4/CCR5	99.4%
PRD320-15	G	BCF-DIOUM	Zaire	NSI	CCR5	99.9%
PRD320-16	G	HH8793	Kenya	NSI	CCR5	83.3%
PRD320-17	H	BCF-KITA	Zaire	NSI	CCR5	92.5%
PRD320-18	O	BCF06	Cameroon	SI	CXCR4/CCR5	98.3%
PRD320-19	O	I-2478B	US	NSI	CCR5	65.6%

Anti-CD3/CD28 activated PBMCs were infected with each of 15 different HIV-1 strains belonging to various clades and with previously defined different CXCR4 and CCR5 usages. HIV-1 dose of 10 ng p24 value was added to 1 × 10^6 cells for infection. After washing, PBMCs were aliquoted and cultured in triplicate in the presence of 10 μg/ml of the A120 mAb or isotype control IgG for 5 days. Virus production was determined by quantitation of p24 in the culture supernatants by ELISA and the mean values calculated. Percent inhibition was calculated relative to the values obtained with the isotype control mAb alone. Representative data from three independent experiments are shown.

So far, similar suppression of both X4 and R5 HIV-1 infection has also been reported in a study utilizing anti-human CCR2 mAb that is neither agonistic nor antagonistic [14]. It was reasoned that this anti-CCR2 mAb functions by the induction of hetero-oligomerization of CCR2 with CCR5 and CXCR4, but not receptor down-modulation. Another report showed that a non-agonistic/antagonistic anti-CCR5 N-terminus specific mAb that is unable to block the binding of R5 HIV-1 gp120 to CCR5 interferes with R5 HIV-1 infection by induction of CCR5 dimerization rather than down-modulation of CCR5 [16]. It is of interest to note that this anti-CCR5 mAb does not inhibit X4 HIV-1. Thus, our finding that ligation of CXCR4 via the ECL1/ECL2 region on activated PBMCs results in the production of CCR5-binding β-chemokines followed by down-modulation of CCR5 expression is unique. However, it remains to be determined whether the ligation of CXCR4 with the A120 mAb similarly induces hetero-dimerization of CXCR4 with CCR5 or the other chemokine receptors or CCR5 homo-dimerization. Further studies are in progress using immunoprecipitation and Western blot techniques utilizing appropriate mAbs.

It is important to note that the addition of anti-chemokine mAbs did not show the same degree of reversal of the A120 mAb-induced inhibition of R5 HIV-1 infection in the cultures from 2 out of the 6 PBMC donors (Figure 6). In addition, there was a lack of correlation between enhanced β-chemokine levels and the reversing effects of the anti-β-chemokine antibodies on the A120-mediated R5 HIV-1 inhibition. We assume that the concentration of the β-chemokine antibodies (10 μg/ml) was sufficient to neutralize endogenously produced β-chemokines as the antibodies at this concentration could neutralize > 100 ng/ml of each of the recombinant β-chemokines (data not shown). While resistance of these donors was not due to the production of some other anti-HIV-1 factor such as $CD8^+$T lymphocyte antiviral factor (CAF) [17], it may be possible that treatment with the A120 mAb might induce the hetero-dimerization of CXCR4 and CCR5 which results in resistance to R5 HIV-1 infection. Further studies are in progress to address this issue. It is interesting to note that among the neutralizing mAbs against the β-chemokines, the anti-MIP-1α mAb was the most effective in reversing the A120 mAb-induced R5 HIV-1 inhibition. Since all the available anti-MIP-1α mAbs at present do not distinguish MIP-1α (LD78α) from its homologue CCR3L1 product (LD78β) [18], it is possible that CCR3L1 protein is also produced upon A120 mAb treatment and involved in the R5 HIV-1 inhibition. As CCR3L1 is known to be a potent factor that may delay the progression to clinical AIDS [19], it will be important to determine whether A120 mAb stimulates the production of CCR3L1 proteins. Such studies are also in progress.

The generation of resistance to CCR5 inhibitors involving either the selection of pre-existing CXCR4 tropic HIV-1 and/or due to the evolution of Env variants has been well documented [20]. Thus, in such cases, the availability of a reagent like the A120 mAb that has inhibitory properties for both CCR5 and CXCR4 tropic HIV-1 may provide a unique therapeutic tool worthy of consideration. Since the A120 mAb also inhibits the SIV-1 infection in activated PBMCs from rhesus macaques (Takahashi et al., unpublished), this hypothesis is currently being investigated using the nonhuman primate model.

Conclusions

Data described herein have identified a unique epitope of CXCR4 whose ligation not only directly inhibits CXCR4 tropic HIV-1, but also indirectly inhibits the infection of R5 tropic HIV-1 via the synthesis of natural CCR5 ligands.

Methods

Reagents

RPMI 1640 medium (Sigma-Aldrich. Inc. St. Louis, MO) supplemented with 10% fetal calf serum (FCS), 100 U/ml of penicillin and 100 μg/ml of streptomycin (hereinafter called RPMI medium) was utilized for the described studies. Anti-human CD3 (clone OKT-3) and anti-CD28 (clone 28.2) were obtained from the American Type Culture Collection (Rockville, MD) and BioLegend (San Diego, CA), respectively. Neutralizing mAbs against human RANTES, MIP-1α and MIP-1β were purchased from R&D systems (Minneapolis, MN). The rat anti-CXCR4 mAbs used were produced in our laboratory and included clones A145 (IgG1), A120 (IgG2b) and A80 (IgG1) [16]. Mapping of the epitopes recognized by these mAbs was reported previously [16]. Other rat mAbs used were IgG1 anti-CCR5, IgG2b anti-HTLV-I gp46 and IgG1 anti-HCV produced in our laboratory [16,21,22]. These mAbs were purified from CB.17-SCID mouse ascites fluids by ammonium sulfate precipitation followed by gel filtration using Superdex G-200 (GE), and passed through a polymyxin B column to remove potential LPS contamination. The fluorescent dye-labeled anti-human CD4, CD8, CD14 and CD19 mAbs were purchased from Beckman-Coulter or BioLegend. The anti-HIV-1 p24 mAbs used were also produced in our laboratory. Magnetic beads conjugated with mAbs against human CD4, CD8, CD14 or CD19 were purchased from Dynal and used according to the manufacturer's recommendation. Low endotoxin murine anti-CXCR4 mAbs including clone 12G5 and the other anti-CXCR4 ECL2 mAbs were purchased from BioLegend and R&D.

HIV-1 preparation

Virus stocks of R5 HIV-1_{JR-FL}, R5 HIV-1_{JR-CSF} and X4 HIV-1_{NL4-3} were produced by transfection of the 293T cells with the appropriate HIV-1 infectious plasmid DNAs utilizing the calcium phosphate method as described previously [23]. X4 HIV-1_{IIIB} was produced in the Molt-4/IIIB cell line. The other HIV-1 isolates used were from the HIV subtype infectivity panel PRD320 (BBI Diagnostics, West Bridgewater, MA, USA) which included clade A R5 HIV-1 (UG275, I-2496 isolates), clade CRF02AG R5 HIV-1 (DJ263, POC44951 isolates), clade B R5 (US2 isolate) and X4 HIV-1 (BZ167 isolate), clade C R5 HIV-1 (DJ259, ZAM18 isolates), clade D X4 HIV-1 (SE365, UG270 isolates), clade CRF01AE R5 (ID17 isolate) and X4 HIV-1 (NP03 isolate), clade F R5 (BZ163 isolate) and X4/R5 HIV-1 (BCI-R17 isolate), clade G R5 HIV-1 (BCF-DIOUM, HH8793 isolates), clade H R5 HIV-1 (BCF-KITA isolate), clade O R5 (I-2478B isolate) and X4/R5 HIV-1(BCF06 isolate). Each of these panel HIV-1 strains was grown in primary PHA-activated PBMCs and the levels of p24 determined and 10 ng of p24 used to infect PBMCs. These HIV-1 stocks were aliquoted and stored at -80°C until used.

In vitro stimulation of PBMCs and infection with HIV-1

PBMCs from healthy donors were obtained by density gradient centrifugation on HistoPAQUE-1077 (Sigma-Aldrich), suspended at 2×10^6 cells/ml in RPMI medium, dispensed into individual wells of 24-well plates (BD) (1 ml/well) pre-coated with 5 μg/ml anti-CD3 mAb (OKT-3) and cultured in the presence of soluble 0.1 μg/ml anti-CD28 mAb at 37°C in a 5% CO_2 humidified atmosphere for 24 hours. The activated PBMCs were collected, washed once and infected with HIV-1 at a multiplicity of infection (m.o.i.) of 0.005~0.01 or at 10 ng p24 per $1\sim2 \times 10^6$ cells for 2 hours. Infected PBMCs were washed three times, re-suspended at $0.5\sim1 \times 10^6$ cells/ml in RPMI medium containing 20 U/ml recombinant human IL-2 containing RPMI medium, dispensed into individual wells of 48-well plates (BD) (0.5 ml/well) and then cultured in the presence or absence of various concentrations of the anti-CXCR4 or control mAbs. Production of HIV-1 was determined by the measurement of HIV-1 core p24 levels by ELISA, and the number of HIV-1 $p24^+$ cells were determined by FCM as described previously [24]. For select experiments, activated PBMCs were cultured at 1×10^6 cells/ml in RPMI medium containing 20 U/ml IL-2 in the presence or absence of 10 μg/ml of A120 mAb for 24 hours, and the culture supernatants were collected, and the levels of β-chemokines were determined by ELISA. All the experiments in this study were performed in triplicate wells.

Cell lines

Molt-4/IIIB [25] and MT-2 [26] cells that were productively infected with HIV-1IIIB (Molt-4/IIIB) and human T cell leukemia virus type-I (HTLV-I), respectively, were cultured in RPMI medium. HIV-1 and HTLV-I production were determined by our in-house HIV-1 p24 and HTLV-I p24 sandwich ELISA kits (Tanaka et al., unpublished).

Flow Cytometry (FCM)

Cells to be analyzed were Fc-blocked with 2 mg/ml normal human pooled IgG on ice for 15 minutes, and aliquots of these cells were subjected to staining using pre-determined optimum concentrations of fluorescent dye-conjugated mAbs for 30 minutes on ice. The cells were then washed using FACS buffer (PBS containing 2% FCS and 0.1% sodium azide), fixed in 1% paraformaldehyde (PFA) in FACS buffer and analyzed using a FACS Calibur. The data obtained were analyzed using the Cell Quest software (BD). For detection of HIV-1 infected cells, cells were fixed with 4% PFA-containing PBS for 5 min at room temperature followed by washing with 0.1% Saponin-containing FACS buffer. These cells were then Fc-blocked with 2 mg/ml normal human pooled IgG on ice for 15 min, and aliquots of these cells were stained with Alexa Fluor 488-conjugated anti-HIV-1 p24 mAb (clone 2C2) for 30 min on ice. The cells were then washed using FACS buffer and the frequency and the absolute number of p24+ cells determined by FCM using a cell counting kit (BD) according to the manufacturer's protocol.

Statistical analysis

Data were tested for significance using the Student's *t* test using the Prism software (GraphPad Software).

Additional material

Additional file 1: Dose response of the A120 mAb-mediated MIP-1α production in activated PBMCs. As described in the legend for Figure 7, activated PBMCs were incubated in the presence of graded concentrations of the A120 mAb or isotype control mAb for an additional day. Changes in the concentrations of MIP-1α in the culture supernatants were assayed by ELISA. Isotype control mAbs did not enhance MIP-1α production at 0.5~20 μg/ml in these culture conditions (data not shown). Representative data are from 3 independent experiments using PBMCs from a single donor.

Lists of abbreviations used

HIV: human immunodeficiency virus; PBMC: peripheral blood mononuclear cells; mAb: monoclonal antibody; X4: CXCR4-tropic; R5: CCR5-tropic; ECL: extra-cellular loop.

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Authors' contributions

TA and RT performed research, analyzed data, and wrote the manuscript. AK, SM, and Takahashi contributed to experiments and analyzed data. AAA contributed to designing research and wrote the manuscript. YT designed and preformed research, wrote the manuscript and provided funding for this study. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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