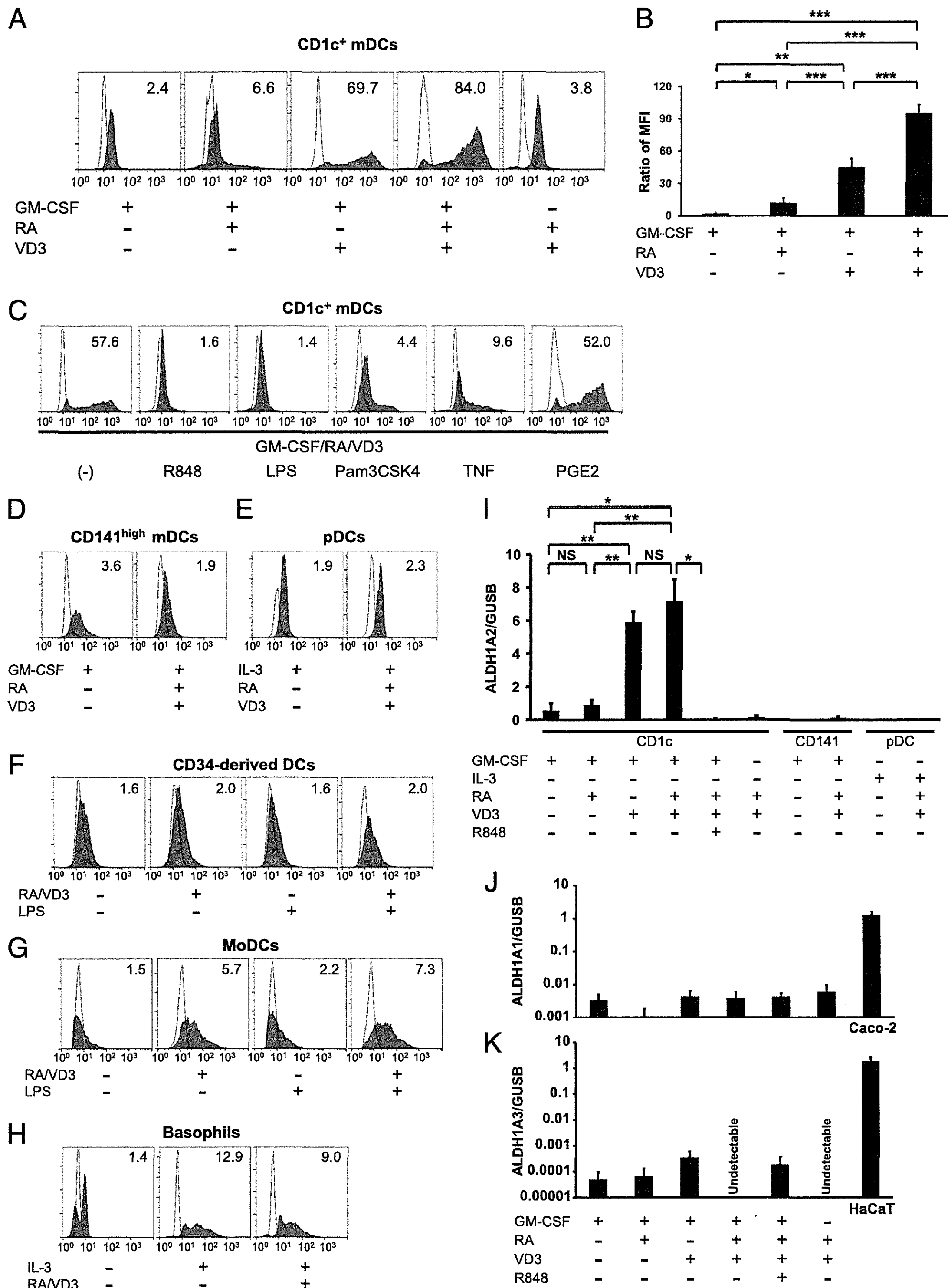



**FIGURE 1.** ALDH activity and ALDH1A2 mRNA expression in human blood DC subsets and basophils. CD1c$^+$ mDCs (**A–C**), CD141$^{high}$ mDCs (**D**), pDCs (**E**), and basophils (**H**) were cultured in the absence or presence of the indicated reagents for 2 d. CD34$^+$ progenitor cell–derived dendritic cells (CD34-derived DCs) (**F**) and MoDCs (**G**) were cultured as indicated in *Materials and Methods*. The cells were incubated with Aldefluor in the absence (solid histograms) or presence (open histograms) of an ALDH inhibitor DEAB and were analyzed by flow cytometry. The numbers shown with each histogram represent ratios of mean fluorescence intensity of Aldefluor in the absence of DEAB to that in the presence of DEAB. (**I**) ALDH1A2 mRNA expression was measured by real-time RT-PCR. CD1c$^+$ mDCs, CD141$^{high}$ mDCs, and pDCs were cultured with the indicated stimuli for 24 h. The expression levels were normalized to those of GUSB. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ALDH1A1 (**J**) and ALDH1A3 (**K**) mRNA expressions were measured by real-time RT-PCR. CD1c$^+$ mDCs were cultured with the indicated stimuli for 24 h. Human colon cancer cell line Caco-2 and human keratinocyte cell line HaCaT were used as positive controls for ALDH1A1 and ALDH1A3, respectively. The expression levels were normalized to those of GUSB. Note the low levels of the scales. The data are representative of three (A, D–G) or two (H) independent experiments and are shown as the mean ± SE of 8 (B), 4 (I), or 3 (J, K) independent experiments.

Downloaded from http://www.jimmunol.org/ at KYOTO UNIVERSITY on September 11, 2013

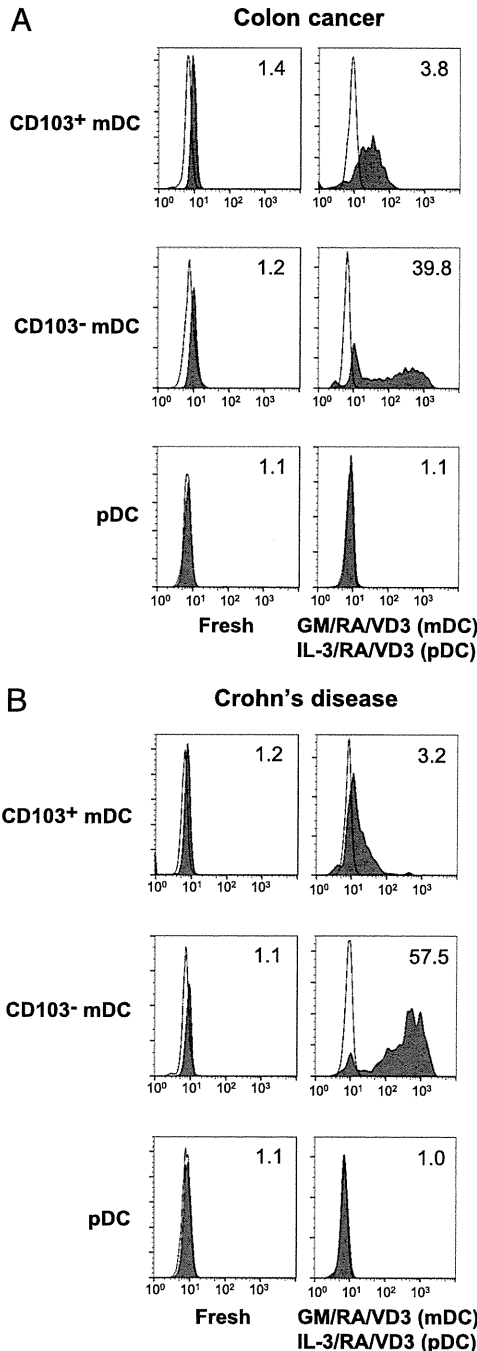



**FIGURE 2.** ALDH activity in human MLN DC subsets. CD103$^+$ mDCs, CD103$^-$ mDCs, or pDCs were purified from MLNs of patients with colon cancer (**A**) or Crohn's disease (**B**) and were analyzed without culture or after culture with the indicated stimuli for 24 h. Histograms and the numbers shown with them are presented as in Fig. 1. The data are representative of three (A) or two (B) independent experiments.

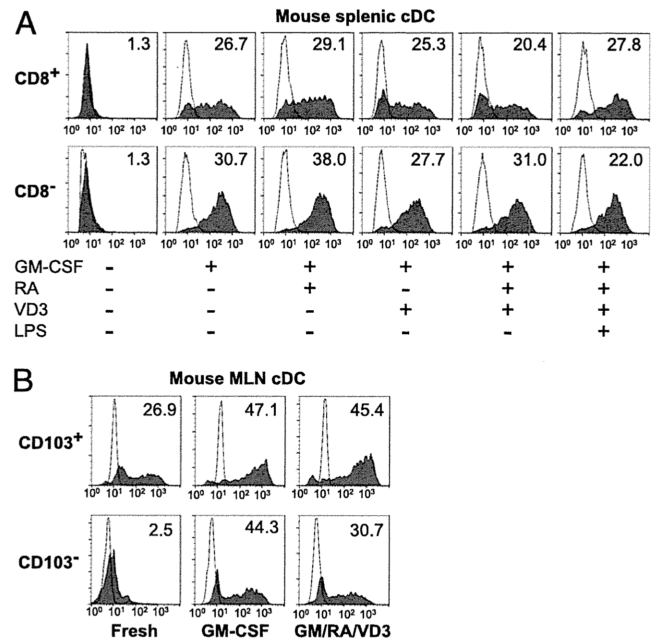



**FIGURE 3.** ALDH activity in mouse splenic and MLN cDC subsets. (**A**) CD8$^+$ and CD8$^-$ mouse splenic cDCs were cultured without or with the indicated stimuli for 24 h. (**B**) CD103$^+$ and CD103$^-$ mouse MLN cDCs were analyzed without culture or were cultured with the indicated stimuli for 24 h. Histograms and the numbers shown with them are presented as in Fig. 1. The data are representative of three independent experiments.

Collectively, the RA-producing DC subsets and the stimulation to induce RA production are different between human and mouse DCs, in that 1) both of the cDC subsets in mouse spleen (CD8$^+$ and CD8$^-$) and MLNs (CD103$^+$ and CD103$^-$) exhibit ALDH activity in response to GM-CSF alone, 2) $VD_3$ does not induce or increase the activity, and 3) TLR signaling does not suppress the activity in mice.

*Engagement of RA receptor is necessary for the high level of RALDH2 expression induced by $VD_3$*

We investigated the mechanisms by which GM-CSF, RA, and $VD_3$ induce a high level of RALDH2 in human CD1c$^+$ mDCs. Exogenous RA augmented the expression of RALDH2 induced by $VD_3$ (Fig. 1A, 1B). Thus, we examined whether endogenous RA is responsible for the induction of RALDH2 by $VD_3$. A pan-RA receptor (RAR) antagonist LE540 diminished the induction of ALDH activity by $VD_3$ (Fig. 4A), indicating that endogenous RA or possibly RAR agonists contained in the serum are necessary for the induction of a high level of RALDH2 by $VD_3$. However, the induction of RALDH2 by RA alone was much weaker than the induction by $VD_3$ (Fig. 1A, 1B), indicating that combined signals by RA and $VD_3$ are necessary for the full expression of RALDH2.

When cultured with GM-CSF, RA, and $VD_3$, CD1c$^+$ mDCs expressed only a low level of mRNA for CYP27B1, the enzyme that converts 25-hydroxyvitamin $D_3$ into its bioactive form $VD_3$ (27), although CD1c$^+$ mDCs expressed a high level of CYP27B1 mRNA in the presence of R848 (Supplemental Fig. 3A). Thus, endogenous $VD_3$ does not appear to participate in the induction of RALDH2.

CD1c$^+$ mDCs, CD141$^{high}$ mDCs, and pDCs expressed similar levels of mRNA for the nuclear VDR, consistent with a previous report (Supplemental Fig. 3B) (25). Thus, the marked effect of $VD_3$ on the induction of RALDH2 in CD1c$^+$ mDCs is not likely to be determined by differential expression of VDR among the DC subsets, but CD1c$^+$ mDCs may have distinctive molecular machineries to express RALDH2 in response to $VD_3$.

*Activation of p38 is necessary for the induction of RALDH2*

We investigated signaling pathways involved in the induction of RALDH2 in CD1c$^+$ mDCs. After stimulation with GM-CSF, RA, and $VD_3$, the DCs started to express ALDH1A2 mRNA at 6 h, and the expression reached its peak at 24 h (Fig. 4B). This slow kinetics indicates that the induction of ALDH1A2 mRNA is not due to direct transcriptional activity of VDR. Instead, secondary signals downstream of VDR and RAR are likely to mediate the induction of ALDH1A2 mRNA.

A pan-JAK inhibitor strongly blocked the induction of ALDH activity (Fig. 4C) in accord with the dependence of the induction

Downloaded from http://www.jimmunol.org/ at KYOTO UNIVERSITY on September 11, 2013

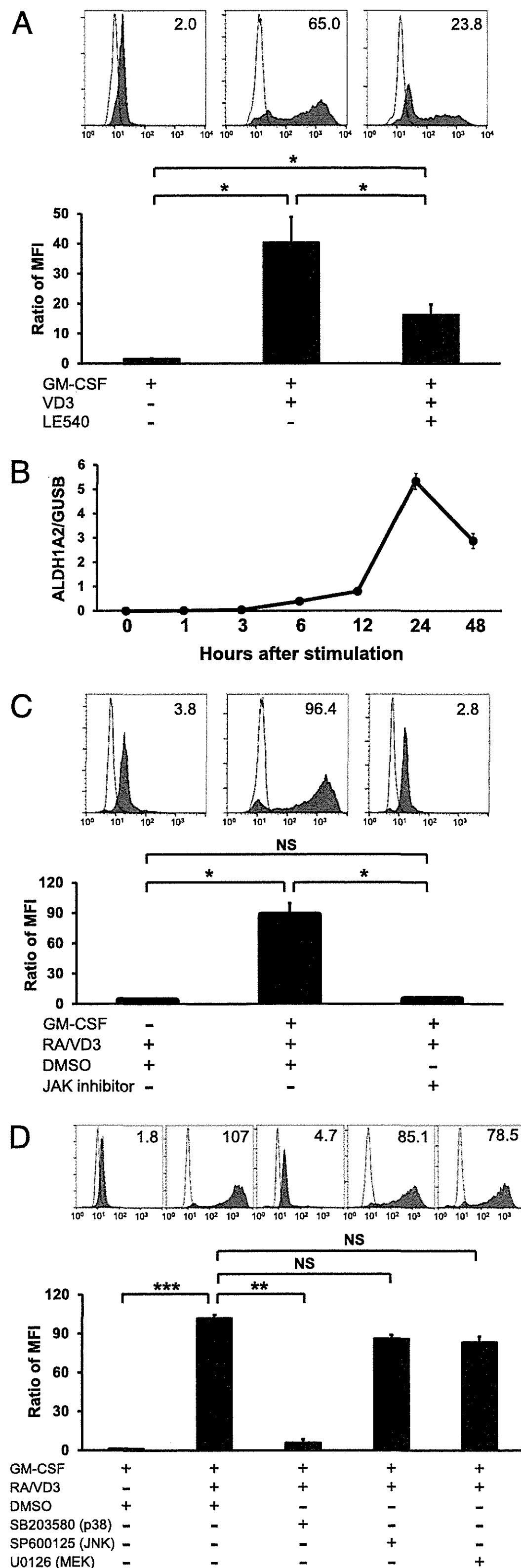



**FIGURE 4.** The induction of ALDH activity in CD1c$^+$ mDCs is dependent on RAR, JAK, and p38 signaling. (**A**, **C**, and **D**) CD1c$^+$ mDCs were cultured with the indicated reagents for 2 d. Histograms and the numbers shown with them are presented as in Fig. 1. The histograms are representative data, and the graphs show the mean ± SE of four (A) or three (C, D) independent experiments. (**B**) CD1c$^+$ mDCs were cultured with GM-CSF, RA, and $VD_3$ for the indicated time periods. ALDH1A2 mRNA expressions were measured by real-time RT-PCR. The expression levels were normalized to those of GUSB. The data are presented as the mean ± SD of duplicate samples in one of two independent experiments.

on GM-CSF. Three p38 MAPK inhibitors, SB203580 (Fig. 4D), SB239063 (28), and VX-745 (29) (data not shown), also blocked the induction. In contrast, inhibitors against JNK (SP600125) or MEK 1/2 (U0126) did not do so (Fig. 4D). There were no substantial differences in cell viability between different culture conditions (data not shown). Thus, RALDH2 in CD1c$^+$ mDCs is induced in a p38-dependent manner.

### *RALDH2$^{high}$ mDCs induce T cells to acquire gut-homing capacities in an RA-dependent manner*

RA derived from mouse intestinal DCs endows T cells with the expression of gut-homing molecules, $\alpha_4\beta_7$ integrin and CCR9 (1). Furthermore, intestinal DCs reciprocally suppress the expression of skin-homing molecules, P- and L-selectin ligands, on T cells (30). Thus, we examined homing properties of T cells stimulated with GM-CSF/RA/$VD_3$–treated RALDH2$^{high}$CD1c$^+$ mDCs (hereafter referred to as RALDH2$^{high}$ mDCs). Naive CD4$^+$ T cells stimulated with allogeneic RALDH2$^{high}$ mDCs expressed a higher level of $\alpha_4\beta_7$ integrin (Fig. 5A, 5B) and reciprocally a lower level of a P- and L-selectin ligand CLA (Fig. 5C) than T cells stimulated with GM-CSF–treated RALDH2$^{low}$CD1c$^+$ mDCs (hereafter referred to as RALDH2$^{low}$ mDCs). The upregulation of $\alpha_4\beta_7$ integrin and downregulation of CLA by RALDH2$^{high}$ mDCs were abrogated by LE540. These data indicate that RALDH2$^{high}$ mDCs induce gut-homing and reduce skin-homing properties of T cells in an RA-dependent manner. RALDH2$^{high}$ mDCs did not induce allogeneic naive CD4$^+$ T cells or total CD8$^+$ T cells to express a detectable level of CCR9 (data not shown).

### *RALDH2$^{high}$ mDCs induce naive CD4$^+$ T cells to acquire Th2 cytokine–producing capacities in an RA-dependent manner*

We examined cytokine-producing properties of naive CD4$^+$ T cells stimulated with allogeneic mDCs. CD4$^+$ T cells stimulated with RALDH2$^{high}$ mDCs secreted significantly higher levels of Th2 cytokines IL-4, IL-5, and IL-13 than those stimulated with RALDH2$^{low}$ mDCs (Fig. 6A). This effect was abrogated by LE540 (Fig. 6A), but not by anti–IL-4 neutralizing mAb (Fig. 6B). CD4$^+$ T cells stimulated with RALDH2$^{high}$ mDCs secreted a similar level of IFN-$\gamma$, compared with those stimulated with RALDH2$^{low}$ mDCs (Fig. 6C). CD4$^+$ T cells stimulated with RALDH2$^{high}$ mDCs secreted a significantly higher level of IL-10 than those stimulated with RALDH2$^{low}$ mDCs, but the induction of IL-10 was not abrogated by LE540 (Fig. 6C). These data indicate that RALDH2$^{high}$ mDCs induce naive CD4$^+$ T cells to acquire the ability to produce high levels of Th2 cytokines in an RA-dependent and IL-4–independent manner.

We also examined whether naive CD4$^+$ T cells stimulated with RALDH2$^{high}$ mDCs acquire regulatory activity. Although CD4$^+$ T cells stimulated with RALDH2$^{high}$ mDCs slightly suppressed proliferation of concomitant T cells, the effect was much weaker than that exhibited by resting Treg cells directly purified from blood (20) (data not shown). Thus, RALDH2$^{high}$ mDCs do not have an unambiguous regulatory T cell–inducing ability detectable by our assay.

## Discussion

RA plays a critical role in maintaining immune homeostasis in the intestine (27). Human DCs that produce a high level of RA remained unknown. The present study identifies blood CD1c$^+$ mDCs as a DC subset that potently produces RA in response to $VD_3$ in humans. RALDH2$^{high}$ CD1c$^+$ mDCs induced T cells to preferentially express gut-homing molecules and Th2 cytokines in an RA-dependent manner. This study reveals a novel component in

Downloaded from http://www.jimmunol.org/ at KYOTO UNIVERSITY on September 11, 2013

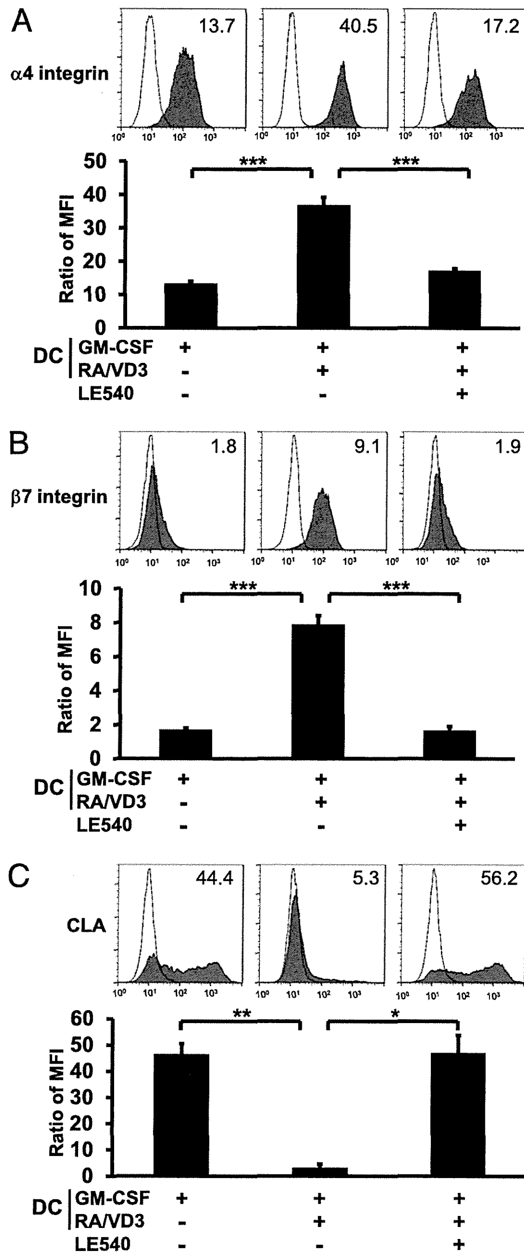



FIGURE 5. RALDH2$^{high}$ mDCs induce gut-homing and suppress skin-homing molecules on CD4$^+$ T cells in an RA-dependent manner. CD1c$^+$ mDCs were cultured with GM-CSF alone (RALDH2$^{low}$ mDCs) or GM-CSF, RA, and $VD_3$ (RALDH2$^{high}$ mDCs) for 2 d. The DCs were collected and extensively washed. Then allogeneic naive CD4$^+$ T cells were stimulated with RALDH2$^{low}$ mDCs or RALDH2$^{high}$ mDCs in the absence or presence of LE540 for 6 d. The T cells were stained for $\alpha_4$ integrin (**A**), $\beta_7$ integrin (**B**), or CLA (**C**). Open histograms represent cells stained with isotype-matched control mAbs. The numbers shown with each histogram represent ratios of mean fluorescence intensity (MFI) of each surface molecule to that of isotype-matched control. The histograms are representative data, and the graphs show the mean ± SE from six (A, B) or three (C) independent experiments. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

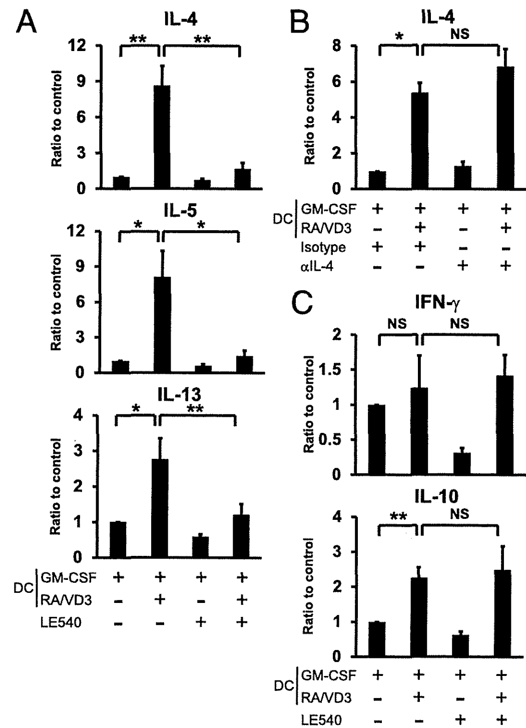



FIGURE 6. RALDH2$^{high}$ mDCs induce CD4$^+$ T cells to produce Th2 cytokines in an RA-dependent manner. CD1c$^+$ mDCs were cultured as in Fig. 5 and were collected and extensively washed. Then allogeneic naive CD4$^+$ T cells were cocultured with RALDH2$^{low}$ mDCs or RALDH2$^{high}$ mDCs in the absence or presence of LE540 for 6 d (**A**, **C**) or in the presence of rat IgG1 (isotype) or anti–IL-4 mAb (**B**). The stimulated T cells were restimulated for 24 h, and the supernatants were analyzed for cytokines by ELISA. The data are normalized to the value obtained from CD4$^+$ T cells cocultured with RALDH2$^{low}$ mDCs in the absence of LE540 or anti–IL-4 mAb. The data are shown as the mean ± SE of eight (A, C) or three (B) independent experiments. $*p < 0.05$, $**p < 0.01$. The mean and ranges of absolute cytokine concentrations from T cells cocultured with RALDH2$^{low}$ mDCs in the absence of LE540 or anti–IL-4 mAb were as follows: (A) IL-4, 79.5 pg/ml (46.8–145 pg/ml); IL-5, 57.9 pg/ml (15.6–122 pg/ml); IL-13, 1720 pg/ml (321–4547 pg/ml); (B) IL-4, 63.9 pg/ml (42.9–104 pg/ml); and (C) IFN-γ, 28.5 ng/ml (9.48–80.6 ng/ml), and IL-10, 219 pg/ml (93.6–416 pg/ml).

the immune system in humans, that is, a "vitamin D – CD1c$^+$ mDC – RA" axis for immune regulation.

Which DCs produce RA will be determined by two factors: 1) environmental signals DCs receive, and 2) intrinsic nature of each DC subset. In mice, RA (4–8), GM-CSF (4), IL-4 (4, 9), and TLR ligands (2, 4, 5, 10–12) induce DCs to express RALDH2. In humans, RA (5), $Pam_3CSK_4$ (5, 12), and a peroxisome proliferator–activated receptor γ ligand (rosiglitazone) (31) augment the expression of RALDH2 in MoDCs. In this study, to our knowledge, we demonstrated for the first time that $VD_3$ induces CD1c$^+$ mDCs to express a high level of RALDH2 in the presence of GM-CSF. Whereas exogenous RA moderately augmented the induction, neither IL-4 nor various immunomodulatory reagents including rosiglitazone augmented it. Notably, proinflammatory factors, TLR ligands and TNF, strongly suppressed ALDH activity. These data suggest that $VD_3$ is a key factor to induce human CD1c$^+$ mDCs to express RALDH2 in the steady state.

It has been shown that $VD_3$ inhibits the expression of RALDH2 in mouse DCs (32). In addition, $VD_3$ represses RA-transcriptional activity via VDR in human myeloid cells (33). These findings indicate that $VD_3$ antagonizes the activity of RA. Thus, the cooperation between exogenous $VD_3$ and endogenously induced RA for the induction of RALDH2 in human CD1c$^+$ mDCs was unexpected. These results indicate that signaling pathways triggered by RA and $VD_3$ may antagonize or synergize, depending on cell types, coexisting factors, and/or species.

Among the human DC subsets we examined, CD1c$^+$ mDCs was the only subset that expresses a high level of RALDH2 in response to $VD_3$. Human CD141$^{high}$ mDCs and their equivalent, mouse CD8$^+$CD11b$^-$ cDCs in lymphoid tissues (18) and CD103$^+$ cDC in

Downloaded from http://www.jimmunol.org/ at KYOTO UNIVERSITY on September 11, 2013

nonlymphoid tissues (17), share capacities to efficiently cross-present Ags to $CD8^+$ T cells (14–17). In contrast, distinctive functions of $CD1c^+$ mDCs, an equivalent of mouse $CD8^-CD11b^+$ cDCs (18), have been elusive. The present study suggests that, in contrast to $CD141^{high}$ mDCs, $CD1c^+$ mDCs may function as immunoregulatory DCs by preferentially producing RA upon exposure to $VD_3$.

Although several studies have shown that human MoDCs express RALDH2 (5, 12, 19, 31), gene expression profiling has shown that MoDCs markedly differ from the three subsets of human DCs in blood and lymphoid tissues and are more similar to macrophages (18). In addition, it remains to be determined to what extent monocytes differentiate into DCs in vivo in humans. Thus, the RALDH2 expression in $CD1c^+$ mDCs is likely to be more relevant to DC biology in vivo than that in MoDCs. Intriguingly, monocytes but not MoDCs exhibited a substantial level of ALDH activity in response to GM-CSF, RA, and $VD_3$, suggesting that $CD1c^+$ mDCs and monocytes may have similar machinery to express RALDH2.

Whereas freshly isolated mouse intestinal $CD103^+$ DCs but not $CD103^-$ DCs produce RA (3), we showed that 1) freshly isolated human MLN DCs do not have ALDH activity and that 2) $CD103^-$ mDCs but not $CD103^+$ mDCs in MLNs gain a high level of ALDH activity in response to the $VD_3$-containing stimulus. Although our data does not clarify the relationship between mouse and human DC subsets in MLNs, the data suggest that CD103 may not be a marker of DCs that preferentially produce RA in human MLNs. Jaesson et al. (34) reported that $CD103^+$ DCs from human MLNs induce T cells to express $\alpha_4\beta_7$ integrin and CCR9 in an RAR signaling–dependent manner. However, such DCs neither exhibited ALDH activity (Fig. 2A, 2B) nor induced T cells to express these gut-homing molecules (data not shown) in our experiments. Because Jaesson et al. (34) did not directly examine ALDH activity of MLN DCs, the reason for the discrepancy between the two studies is not clear.

Furthermore, GM-CSF alone was sufficient to induce high levels of ALDH activity in both of the cDC subsets in mouse spleen ($CD8^+$ and $CD8^-$) and MLNs ($CD103^+$ and $CD103^-$), and $VD_3$ did not augment the activity. Thus, DC subsets capable of acquiring ALDH activity and the stimulation to induce DCs to acquire the activity appear to be significantly different between humans and mice.

RA (5) or zymosan (11) induces mouse splenic DCs to express RALDH2 through RAR or TLR2, respectively, in an ERK-dependent manner. $Pam_3CSK_4$ induces mouse splenic DCs to express RALDH2 in a JNK-dependent manner (12). In contrast, we showed that p38 but not MEK or JNK is necessary to induce human $CD1c^+$ mDCs to express RALDH2 in response to $VD_3$. Thus, although MAPK is important for the induction of RALDH2 in DCs, it appears that which MAPK is involved depends on the type of stimuli and/or species.

Although $RALDH2^{high}$ $CD1c^+$ mDCs induced the expression of a higher level of $\alpha_4\beta_7$ integrin and reciprocally suppressed the expression of CLA on T cells in an RA-dependent manner, we could not observe the induction of CCR9 in the culture conditions we used. Spiegl et al. (26) also reported no CCR9 induction on human T cells by RA. Thus, the induction of CCR9 on human T cells may be more tightly regulated than that on mouse T cells.

$VD_3$ directly acts on T cells to induce skin-homing receptors (35). The present study showed that $VD_3$ induces T cells to express gut-homing receptors through inducing RA production by DCs. It appears to be difficult to reconcile these two phenomena. A possible scenario is that $CD1c^+$ mDCs are exposed to $VD_3$ in peripheral tissues, migrate into regional lymph nodes, and present RA to T cells. Indeed, tissue-resident cells such as epithelial cells and macrophages (36) express CYP27B1. As such, exposure of DCs to $VD_3$ may be spatially and chronologically separated from T cell stimulation by the DCs.

$VD_3$ and RA have been thought to reciprocally control immune responses in the skin and intestine (27). Thus, the induction of RALDH2 by $VD_3$ is counterintuitive. However, such dichotomy between $VD_3$ and RA are becoming blurred. On the one hand, $VD_3$ locally produced by epithelial cells and macrophages in various organs and lymphoid tissues (36) likely has an immunomodulatory effect in a paracrine manner (37). On the other hand, RA-producing DCs exist in extraintestinal as well as intestinal tissues and their corresponding draining lymph nodes (2). In addition, a wide variety of cells can produce GM-CSF. Thus, $VD_3$, RA, and GM-CSF are likely to have opportunities to collaborate and to stimulate $CD1c^+$ mDCs in various tissues, and such DCs may induce gut-homing T cells by producing RA in extraintestinal as well as intestinal compartments.

$RALDH2^{high}$ mDCs induced naive $CD4^+$ T cells to acquire the ability to produce Th2 cytokines in an RA-dependent and IL-4–independent manner. The apparently direct effect of RA on Th2 induction is consistent with our previous report with mice (38). It has been shown that RA derived from basophils also induces Th2 cells (26). Thus, RA derived from $CD1c^+$ mDCs as well as basophils may contribute to Th2 polarization.

It has been proposed that Th2-type allergic responses may constitute an important asset of the immune system to maintain tissue homeostasis by ameliorating inflammation and promoting tissue repair (39, 40). The present study suggests that locally produced $VD_3$ may induce RA-producing $CD1c^+$ mDCs that promote a "type 2" environment, thus contributing to maintaining tissue homeostasis. Inflammation caused by infections, exemplified by the stimulation of $CD1c^+$ mDCs with TLR ligands and TNF, may extinguish the Th2-inducing RA production, and turn on type 1 inflammation. Taken together with a recent report that $VD_3$-stimulated $CD1c^+$ blood mDCs produce IL-10 and induce Treg cells (41), $CD1c^+$ mDCs may represent a DC subset that maintains immune homeostasis. Furthermore, epidemiological studies have shown that a poor vitamin D status is associated with an increased risk of autoimmune diseases (37). Thus, it is intriguing to speculate that RA production by $CD1c^+$ mDCs stimulated with $VD_3$ contributes to prevention of autoimmune diseases in the steady state.

In conclusion, this study reveals a novel link between two key immunomodulatory vitamins (vitamin A and D) via a distinctive human DC subset, that is, $CD1c^+$ mDCs. This may constitute a previously unrecognized immune component for maintaining tissue homeostasis. Exploiting immunomodulatory activity of this component may lead to novel therapies or prevention of various autoimmune or inflammatory disorders.

## Acknowledgments

We thank Keiko Fukunaga for excellent technical assistance.

## Disclosures

The authors have no financial conflicts of interest.

## References

1. Iwata, M., A. Hirakiyama, Y. Eshima, H. Kagechika, C. Kato, and S.-Y. Song. 2004. Retinoic acid imprints gut-homing specificity on T cells. *Immunity* 21: 527–538.
2. Guilliams, M., K. Crozat, S. Henri, S. Tamoutounour, P. Grenot, E. Devilard, B. de Bovis, L. Alexopoulou, M. Dalod, and B. Malissen. 2010. Skin-draining lymph nodes contain dermis-derived $CD103^-$ dendritic cells that constitutively produce retinoic acid and induce $Foxp3^+$ regulatory T cells. *Blood* 115: 1958–1968.
3. Coombes, J. L., K. R. R. Siddiqui, C. V. Arancibia-Cárcamo, J. Hall, C.-M. Sun, Y. Belkaid, and F. Powrie. 2007. A functionally specialized population of mu-

Downloaded from http://www.jimmunol.org/ at KYOTO UNIVERSITY on September 11, 2013

cosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-β and retinoic acid-dependent mechanism. *J. Exp. Med.* 204: 1757–1764.
4. Yokota, A., H. Takeuchi, N. Maeda, Y. Ohoka, C. Kato, S.-Y. Song, and M. Iwata. 2009. GM-CSF and IL-4 synergistically trigger dendritic cells to acquire retinoic acid-producing capacity. *Int. Immunol.* 21: 361–377.
5. Villablanca, E. J., S. Wang, J. de Calisto, D. C. O. Gomes, M. A. Kane, J. L. Napoli, W. S. Blaner, H. Kagechika, R. Blomhoff, M. Rosemblatt, et al. 2011. MyD88 and retinoic acid signaling pathways interact to modulate gastrointestinal activities of dendritic cells. *Gastroenterology* 141: 176–185.
6. Feng, T., Y. Cong, H. Qin, E. N. Benveniste, and C. O. Elson. 2010. Generation of mucosal dendritic cells from bone marrow reveals a critical role of retinoic acid. *J. Immunol.* 185: 5915–5925.
7. Molenaar, R., M. Knippenberg, G. Goverse, B. J. Olivier, A. F. de Vos, T. O'Toole, and R. E. Mebius. 2011. Expression of retinaldehyde dehydrogenase enzymes in mucosal dendritic cells and gut-draining lymph node stromal cells is controlled by dietary vitamin A. *J. Immunol.* 186: 1934–1942.
8. Jaensson-Gyllenbäck, E., K. Kotarsky, F. Zapata, E. K. Persson, T. E. Gundersen, R. Blomhoff, and W. W. Agace. 2011. Bile retinoids imprint intestinal CD103+ dendritic cells with the ability to generate gut-tropic T cells. *Mucosal Immunol.* 4: 438–447.
9. Elgueta, R., F. E. Sepulveda, F. Vilches, L. Vargas, J. R. Mora, M. R. Bono, and M. Rosemblatt. 2008. Imprinting of CCR9 on CD4 T cells requires IL-4 signaling on mesenteric lymph node dendritic cells. *J. Immunol.* 180: 6501–6507.
10. Uematsu, S., K. Fujimoto, M. H. Jang, B.-G. Yang, Y.-J. Jung, M. Nishiyama, S. Sato, T. Tsujimura, M. Yamamoto, Y. Yokota, et al. 2008. Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5. *Nat. Immunol.* 9: 769–776.
11. Manicassamy, S., R. Ravindran, J. Deng, H. Oluoch, T. L. Denning, S. P. Kasturi, K. M. Rosenthal, B. D. Evavold, and B. Pulendran. 2009. Toll-like receptor 2-dependent induction of vitamin A-metabolizing enzymes in dendritic cells promotes T regulatory responses and inhibits autoimmunity. *Nat. Med.* 15: 401–409.
12. Wang, S., E. J. Villablanca, J. De Calisto, D. C. O. Gomes, D. D. Nguyen, E. Mizoguchi, J. C. Kagan, H.-C. Reinecker, N. Hacohen, C. Nagler, et al. 2011. MyD88-dependent TLR1/2 signals educate dendritic cells with gut-specific imprinting properties. *J. Immunol.* 187: 141–150.
13. Ziegler-Heitbrock, L., P. Ancuta, S. Crowe, M. Dalod, V. Grau, D. N. Hart, P. J. M. Leenen, Y.-J. Liu, G. MacPherson, G. J. Randolph, et al. 2010. Nomenclature of monocytes and dendritic cells in blood. *Blood* 116: e74–e80.
14. Jongbloed, S. L., A. J. Kassianos, K. J. McDonald, G. J. Clark, X. Ju, C. E. Angel, C.-J. J. Chen, P. R. Dunbar, R. B. Wadley, V. Jeet, et al. 2010. Human CD141+ (BDCA-3)+ dendritic cells (DCs) represent a unique myeloid DC subset that cross-presents necrotic cell antigens. *J. Exp. Med.* 207: 1247–1260.
15. Poulin, L. F., M. Salio, E. Griessinger, F. Anjos-Afonso, L. Craciun, J.-L. Chen, A. M. Keller, O. Joffre, S. Zelenay, E. Nye, et al. 2010. Characterization of human DNGR-1+BDCA3+ leukocytes as putative equivalents of mouse CD8α+ dendritic cells. *J. Exp. Med.* 207: 1261–1271.
16. Bachem, A., S. Güttler, E. Hartung, F. Ebstein, M. Schaefer, A. Tannert, A. Salama, K. Movassaghi, C. Opitz, H. W. Mages, et al. 2010. Superior antigen cross-presentation and XCR1 expression define human CD11c+CD141+ cells as homologues of mouse CD8+ dendritic cells. *J. Exp. Med.* 207: 1273–1281.
17. Haniffa, M., A. Shin, V. Bigley, N. McGovern, P. Teo, P. See, P. S. Wasan, X. N. Wang, F. Malinarich, B. Malleret, et al. 2012. Human tissues contain CD141hi cross-presenting dendritic cells with functional homology to mouse CD103+ nonlymphoid dendritic cells. *Immunity* 37: 60–73.
18. Robbins, S. H., T. Walzer, D. Dembélé, C. Thibault, A. Defays, G. Bessou, H. Xu, E. Vivier, M. Sellars, P. Pierre, et al. 2008. Novel insights into the relationships between dendritic cell subsets in human and mouse revealed by genome-wide expression profiling. *Genome Biol.* 9: R17.
19. Stock, A., S. Booth, and V. Cerundolo. 2011. Prostaglandin $E_2$ suppresses the differentiation of retinoic acid-producing dendritic cells in mice and humans. *J. Exp. Med.* 208: 761–773.
20. Miyara, M., Y. Yoshioka, A. Kitoh, T. Shima, K. Wing, A. Niwa, C. Parizot, C. Taflin, T. Heike, D. Valeyre, et al. 2009. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. *Immunity* 30: 899–911.
21. Iyoda, T., S. Shimoyama, K. Liu, Y. Omatsu, Y. Akiyama, Y. Maeda, K. Takahara, R. M. Steinman, and K. Inaba. 2002. The CD8+ dendritic cell subset selectively endocytoses dying cells in culture and in vivo. *J. Exp. Med.* 195: 1289–1302.
22. Ito, T., M. Inaba, K. Inaba, J. Toki, S. Sogo, T. Iguchi, Y. Adachi, K. Yamaguchi, R. Amakawa, J. Valladeau, et al. 1999. A CD1a+/CD11c+ subset of human blood dendritic cells is a direct precursor of Langerhans cells. *J. Immunol.* 163: 1409–1419.
23. Penna, G., and L. Adorini. 2000. 1α,25-Dihydroxyvitamin D3 inhibits differentiation, maturation, activation, and survival of dendritic cells leading to impaired alloreactive T cell activation. *J. Immunol.* 164: 2405–2411.
24. Piemonti, L., P. Monti, M. Sironi, P. Fraticelli, B. E. Leone, E. Dal Cin, P. Allavena, and V. Di Carlo. 2000. Vitamin $D_3$ affects differentiation, maturation, and function of human monocyte-derived dendritic cells. *J. Immunol.* 164: 4443–4451.
25. Penna, G., S. Amuchastegui, N. Giarratana, K. C. Daniel, M. Vulcano, S. Sozzani, and L. Adorini. 2007. 1,25-Dihydroxyvitamin $D_3$ selectively modulates tolerogenic properties in myeloid but not plasmacytoid dendritic cells. *J. Immunol.* 178: 145–153.
26. Spiegl, N., S. Didichenko, P. McCaffery, H. Langen, and C. A. Dahinden. 2008. Human basophils activated by mast cell-derived IL-3 express retinaldehyde dehydrogenase-II and produce the immunoregulatory mediator retinoic acid. *Blood* 112: 3762–3771.
27. Mora, J. R., M. Iwata, and U. H. von Andrian. 2008. Vitamin effects on the immune system: vitamins A and D take centre stage. *Nat. Rev. Immunol.* 8: 685–698.
28. Barone, F. C., E. A. Irving, A. M. Ray, J. C. Lee, S. Kassis, S. Kumar, A. M. Badger, R. F. White, M. J. McVey, J. J. Legos, et al. 2001. SB 239063, a second-generation p38 mitogen-activated protein kinase inhibitor, reduces brain injury and neurological deficits in cerebral focal ischemia. *J. Pharmacol. Exp. Ther.* 296: 312–321.
29. Davis, M. I., J. P. Hunt, S. Herrgard, P. Ciceri, L. M. Wodicka, G. Pallares, M. Hocker, D. K. Treiber, and P. P. Zarrinkar. 2011. Comprehensive analysis of kinase inhibitor selectivity. *Nat. Biotechnol.* 29: 1046–1051.
30. Mora, J. R., G. Cheng, D. Picarella, M. Briskin, N. Buchanan, and U. H. von Andrian. 2005. Reciprocal and dynamic control of CD8 T cell homing by dendritic cells from skin- and gut-associated lymphoid tissues. *J. Exp. Med.* 201: 303–316.
31. Szatmari, I., A. Pap, R. Rühl, J.-X. Ma, P. A. Illarionov, G. S. Besra, E. Rajnavolgyi, B. Dezso, and L. Nagy. 2006. PPARγ controls CD1d expression by turning on retinoic acid synthesis in developing human dendritic cells. *J. Exp. Med.* 203: 2351–2362.
32. Chang, S.-Y., H.-R. Cha, J.-H. Chang, H.-J. Ko, H. Yang, B. Malissen, M. Iwata, and M.-N. Kweon. 2010. Lack of retinoic acid leads to increased langerin-expressing dendritic cells in gut-associated lymphoid tissues. *Gastroenterology* 138: 1468–1478, e1–e6.
33. Bastie, J. N., N. Balitrand, F. Guidez, I. Guillemot, J. Larghero, C. Calabresse, C. Chomienne, and L. Delva. 2004. 1α,25-Dihydroxyvitamin D3 transrepresses retinoic acid transcriptional activity via vitamin D receptor in myeloid cells. *Mol. Endocrinol.* 18: 2685–2699.
34. Jaensson, E., H. Uronen-Hansson, O. Pabst, B. Eksteen, J. Tian, J. L. Coombes, P.-L. Berg, T. Davidsson, F. Powrie, B. Johansson-Lindbom, and W. W. Agace. 2008. Small intestinal CD103+ dendritic cells display unique functional properties that are conserved between mice and humans. *J. Exp. Med.* 205: 2139–2149.
35. Sigmundsdottir, H., J. Pan, G. F. Debes, C. Alt, A. Habtezion, D. Soler, and E. C. Butcher. 2007. DCs metabolize sunlight-induced vitamin D3 to 'program' T cell attraction to the epidermal chemokine CCL27. *Nat. Immunol.* 8: 285–293.
36. Zehnder, D., R. Bland, M. C. Williams, R. W. McNinch, A. J. Howie, P. M. Stewart, and M. Hewison. 2001. Extrarenal expression of 25-hydroxyvitamin d(3)-1 α-hydroxylase. *J. Clin. Endocrinol. Metab.* 86: 888–894.
37. Hewison, M. 2012. An update on vitamin D and human immunity. *Clin. Endocrinol.* 76: 315–325.
38. Iwata, M., Y. Eshima, and H. Kagechika. 2003. Retinoic acids exert direct effects on T cells to suppress Th1 development and enhance Th2 development via retinoic acid receptors. *Int. Immunol.* 15: 1017–1025.
39. Allen, J. E., and T. A. Wynn. 2011. Evolution of Th2 immunity: a rapid repair response to tissue destructive pathogens. *PLoS Pathog.* 7: e1002003.
40. Palm, N. W., R. K. Rosenstein, and R. Medzhitov. 2012. Allergic host defences. *Nature* 484: 465–472.
41. Chu, C.-C., N. Ali, P. Karagiannis, P. Di Meglio, A. Skowera, L. Napolitano, G. Barinaga, K. Grys, E. Sharif-Paghaleh, S. N. Karagiannis, et al. 2012. Resident CD141 (BDCA3)+ dendritic cells in human skin produce IL-10 and induce regulatory T cells that suppress skin inflammation. *J. Exp. Med.* 209: 935–945.

Downloaded from http://www.jimmunol.org/ at KYOTO UNIVERSITY on September 11, 2013

OPEN ACCESS Freely available online 

# A Novel Protective MHC-I Haplotype Not Associated with Dominant Gag-Specific CD8$^+$ T-Cell Responses in SIVmac239 Infection of Burmese Rhesus Macaques

**Naofumi Takahashi[1,2], Takushi Nomura[1,2], Yusuke Takahara[1,2], Hiroyuki Yamamoto[1], Teiichiro Shiino[1], Akiko Takeda[1], Makoto Inoue[3], Akihiro Iida[3], Hiroto Hara[3], Tsugumine Shu[3], Mamoru Hasegawa[3], Hiromi Sakawaki[4], Tomoyuki Miura[4], Tatsuhiko Igarashi[4], Yoshio Koyanagi[4], Taeko K. Naruse[5], Akinori Kimura[5], Tetsuro Matano[1,2]***

**1** AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan, **2** The Institute of Medical Science, The University of Tokyo, Tokyo, Japan, **3** DNAVEC Corporation, Tsukuba, Japan, **4** Institute for Virus Research, Kyoto University, Kyoto, Japan, **5** Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

## Abstract

Several major histocompatibility complex class I (MHC-I) alleles are associated with lower viral loads and slower disease progression in human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) infections. Immune-correlates analyses in these MHC-I-related HIV/SIV controllers would lead to elucidation of the mechanism for viral control. Viral control associated with some protective MHC-I alleles is attributed to CD8$^+$ T-cell responses targeting Gag epitopes. We have been trying to know the mechanism of SIV control in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level. Here, we found a protective MHC-I haplotype, *90-010-Id* (D), which is not associated with dominant Gag-specific CD8$^+$ T-cell responses. Viral loads in five D$^+$ animals became significantly lower than those in our previous cohorts after 6 months. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses after SIV challenge. Further analyses suggested two Nef-epitope-specific CD8$^+$ T-cell responses exerting strong suppressive pressure on SIV replication. Another set of five D$^+$ animals that received a prophylactic vaccine using a Gag-expressing Sendai virus vector showed significantly reduced viral loads compared to unvaccinated D$^+$ animals at 3 months, suggesting rapid SIV control by Gag-specific CD8$^+$ T-cell responses in addition to Nef-specific ones. These results present a pattern of SIV control with involvement of non-Gag antigen-specific CD8$^+$ T-cell responses.

**Citation:** Takahashi N, Nomura T, Takahara Y, Yamamoto H, Shiino T, et al. (2013) A Novel Protective MHC-I Haplotype Not Associated with Dominant Gag-Specific CD8$^+$ T-Cell Responses in SIVmac239 Infection of Burmese Rhesus Macaques. PLoS ONE 8(1): e54300. doi:10.1371/journal.pone.0054300

**Editor:** Douglas F. Nixon, University of California San Francisco, United States of America

**Received** November 13, 2012; **Accepted** December 10, 2012; **Published** January 14, 2013

**Copyright:** © 2013 Takahashi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology, and grants-in-aid from the Ministry of Health, Labor, and Welfare. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** Makoto Inoue, Akihiro Iida, Hiroto Hara, Tsugumine Shu and Mamoru Hasegawa are employed by DNAVEC Corporation. There are no patents, products in development or marketed products to declare. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials, as detailed online in the guide for authors.

* E-mail: tmatano@nih.go.jp

## Introduction

Virus-specific CD8$^+$ T-cell responses play a central role in the control of human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) replication [1,2,3,4,5]. Genetic diversities of HLA or major histocompatibility complex class I (MHC-I) result in various patterns of CD8$^+$ T-cell responses in HIV-infected individuals. Cumulative studies on HIV infection have indicated the association of MHC-I genotypes with higher or lower viral loads [6,7,8,9,10]. In some MHC-1 alleles associating with lower viral loads and slower disease progression, certain CD8$^+$ T-cell responses restricted by these MHC-I molecules have been shown to be responsible for HIV control [11,12,13]. In rhesus macaque AIDS models, *Mamu-A*01*, *Mamu-B*08*, and *Mamu-B*17* are known as protective alleles, and macaques possessing these alleles tend to show slower disease progression after SIVmac251/SIVmac239 challenge [14,15,16,17].

Recent studies have indicated great contribution of CD8$^+$ T-cell responses targeting Gag epitopes to reduction in viral loads in HIV/SIV infection [18,19,20,21]. Viral control associated with some protective MHC-I alleles is attributed to Gag epitope-specific CD8$^+$ T-cell responses [22,23,24]. For instance, CD8$^+$ T-cell responses specific for the HLA-B*57-restricted Gag$_{240-249}$ TW10 and HLA-B*27-restricted Gag$_{263-272}$ KK10 epitopes exert strong suppressive pressure on HIV replication and frequently select for an escape mutation with viral fitness costs, leading to lower viral loads [22,24,25,26,27]. On the other hand, CD8$^+$ T-cell responses targeting SIV antigens other than Gag, such as Mamu-B*08- or Mamu-B*17-restricted Vif and Nef epitopes, have been indicated to exert strong suppressive pressure on SIV replication [28,29,30,31,32,33]. Accumulation of our knowledge on the potential of these non-Gag-specific as well as Gag-specific CD8$^+$ T-cell responses for HIV/SIV control should be encouraged for elucidation of viral control mechanisms.

We have been examining SIVmac239 infection in multiple groups of Burmese rhesus macaques sharing MHC-I genotypes at the haplotype level and indicated an association of MHC-I haplotypes with AIDS progression [21,34]. In our previous study, a group of macaques sharing MHC-I haplotype *90-120-Ia* (A) induced dominant Gag-specific CD8$^+$ T-cell responses and tended to show slower disease progression after SIVmac239 challenge [21]. Prophylactic immunization of these A$^+$ macaques with a DNA vaccine prime and a Gag-expressing Sendai virus (SeV-Gag) vector boost resulted in SIV control based on Gag-specific CD8$^+$

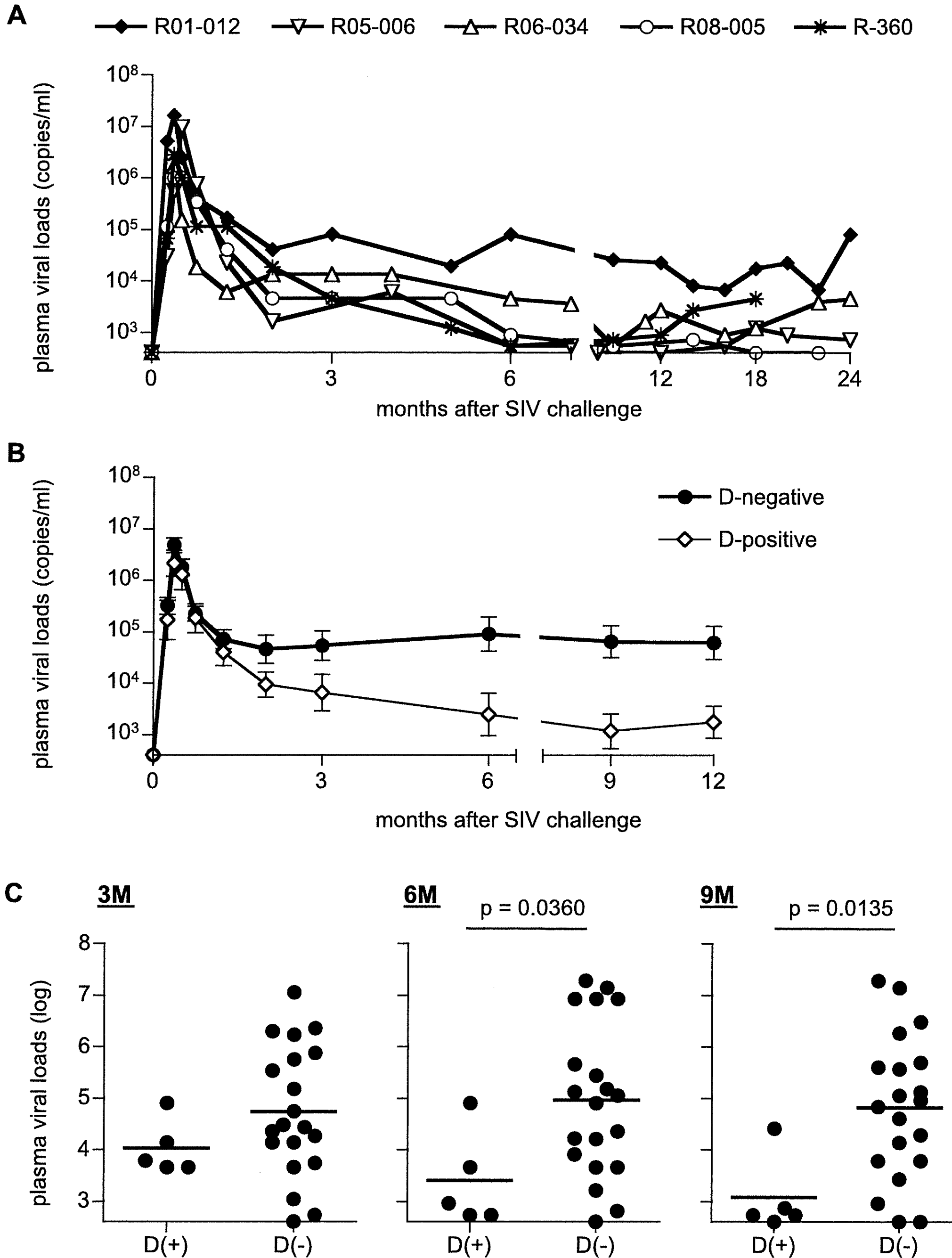



**Figure 1. Plasma viral loads after SIVmac239 challenge in unvaccinated macaques.** Plasma viral loads (SIV *gag* RNA copies/ml plasma) were determined as described previously [35]. The lower limit of detection is approximately $4\times10^2$ copies/ml. (A) Changes in plasma viral loads after challenge in unvaccinated macaques possessing MHC-I haplotype D. (B) Changes in geometric means of plasma viral loads after challenge in five unvaccinated D$^+$ animals in the present study and twenty D$^-$ animals in our previous cohorts [21]. Three of twenty D$^-$ animals were euthanized because of AIDS before 12 months, and we compared viral loads between D$^+$ and D$^-$ animals until 12 months. (C) Comparison of plasma viral loads at 3 months (left panel), 6 months (middle panel), and 9 months (right panel) between the unvaccinated D$^+$ and the D$^-$ animals. Viral loads at 6 months and 9 months in D$^+$ animals were significantly lower than those in the latter D$^-$ animals ($p = 0.0360$ at 6 months and $p = 0.0135$ at 9 months by t-test).
doi:10.1371/journal.pone.0054300.g001

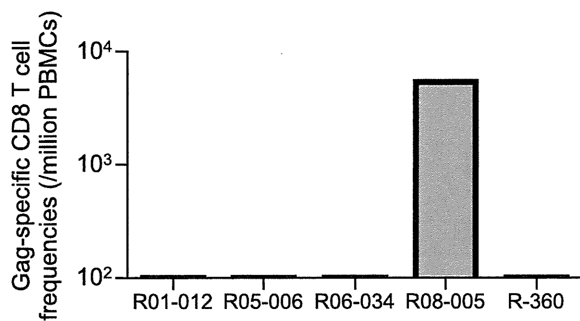



**Figure 2. SIV Gag-specific CD8$^+$ T-cell responses in unvaccinated D$^+$ macaques at week 2 after SIVmac239 challenge.**
doi:10.1371/journal.pone.0054300.g002

T-cell responses [35,36]. Accumulation of data on interaction between virus replication and T-cell responses in multiple groups of macaques sharing individual MHC-I haplotypes would provide great insights into our understanding of the mechanism for HIV/SIV control.

In the present study, we investigated SIVmac239 infection of a group of Burmese rhesus macaques possessing the MHC-I haplotype *90-010-Id* (D), which was not associated with dominant Gag-specific CD8$^+$ T-cell responses. These animals had persistent viremia in the early phase but showed significant reduction of viral loads around 6 months after SIV challenge. Most D$^+$ animals showed predominant Nef-specific but not Gag-specific CD8$^+$ T-cell responses. This study presents a protective MHC-I haplotype, indicating the potential of non-Gag antigen-specific CD8$^+$ T-cell responses to contribute to SIV control.

## Materials and Methods

### Ethics Statement

Animal experiments were carried out in National Institute of Biomedical Innovation (NIBP) and Institute for Virus Research in Kyoto University (IVRKU) after approval by the Committee on the Ethics of Animal Experiments of NIBP and IVRKU in accordance with the guidelines for animal experiments at NIBP, IVRKU, and National Institute of Infectious Diseases. To prevent viral transmission, animals were housed in individual cages allowing them to make sight and sound contact with one another, where the temperature was kept at 25°C with light in 12 hours per day. Animals were fed with apples and commercial monkey diet (Type CMK-2, Clea Japan, Inc. Tokyo). Blood collection, vaccination, and SIV challenge were performed under ketamine anesthesia. The endpoint for euthanasia was determined by typical signs of AIDS including reduction in peripheral CD4$^+$ T-cell counts (less than 200 cells/µl), 10% loss of body weight, diarrhea, and general weakness. At euthanasia, animals were deeply anesthetized with pentobarbital under ketamine anesthesia, and then, whole blood was collected from left ventricle.

### Animal Experiments

We examined SIV infections in a group of Burmese rhesus macaques ($n = 10$) sharing the MHC-I haplotype *90-010-Id* (D). The determination of MHC-I haplotypes was based on the family study in combination with the reference strand-mediated conformation analysis (RSCA) of *Mamu-A* and *Mamu-B* genes and detection of major *Mamu-A* and *Mamu-B* alleles by cloning the reverse transcription (RT)-PCR products as described previously [21,34,37]. Macaques R01-012 and R01-009 used in our previous report [35] and macaques R03-021 and R03-016 used in an unpublished experiment were included in the present study. Five macaques R01-009, R06-020, R06-033, R03-021, and R03-016 received a prophylactic DNA prime/SeV-Gag boost vaccine (referred to as DNA/SeV-Gag vaccine) [35]. The DNA used for the vaccination, CMV-SHIVdEN, was constructed from an *env*-deleted and *nef*-deleted simian–human immunodeficiency virus SHIVMD14YE [38] molecular clone DNA (SIVGP1) and has the genes encoding SIVmac239 Gag, Pol, Vif, and Vpx, and HIV Tat and Rev. At the DNA vaccination, animals received 5 mg of CMV-SHIVdEN DNA intramuscularly. Six weeks after the DNA prime, animals received a single boost intranasally with $6 \times 10^9$ cell infectious units (CIUs) of F-deleted replication-defective SeV-Gag [39,40]. All animals were challenged intravenously with 1,000 $TCID_{50}$ (50 percent tissue culture infective doses) of SIVmac239 [41]. At week 1 after SIV challenge, macaque R03-021 was inoculated with nonspecific immunoglobulin G (IgG) and macaques R03-016 with IgG purified from neutralizing antibody-positive plasma of chronically SIV-infected macaques in our previous experiment [42].

### Analysis of SIV Antigen-specific CD8$^+$ T-cell Responses

SIV antigen-specific CD8$^+$ T-cell responses were measured by flow-cytometric analysis of gamma interferon (IFN-γ) induction as described previously [43]. Autologous herpesvirus papio-immortalized B-lymphoblastoid cell lines (B-LCLs) were established from peripheral blood mononuclear cells (PBMCs) which were obtained from individual macaques before SIV challenge [44]. PBMCs obtained from SIV-infected macaques were cocultured with autologous B-LCLs pulsed with peptides or peptide pools using panels of overlapping peptides spanning the entire SIVmac239 Gag, Pol, Vif, Vpx, Vpr, Tat, Rev, Env, and Nef amino acid sequences. Alternatively, PBMCs were cocultured with B-LCLs infected with a vaccinia virus vector expressing SIVmac239 Gag for Gag-specific stimulation. Intracellular IFN-γ staining was performed using CytofixCytoperm kit (BD, Tokyo, Japan). Fluorescein isothiocianate-conjugated anti-human CD4 (BD), Peridinin chlorophyll protein (PerCP)-conjugated anti-human CD8 (BD), allophycocyanin Cy7 (APC-Cy7)-conjugated anti-human CD3 (BD), and phycoerythrin (PE)-conjugated anti-human IFN-γ antibodies (Biolegend, San Diego, CA) were used. Specific T-cell levels were calculated by subtracting non-specific IFN-γ$^+$ T-cell frequencies from those after peptide-specific stimulation. Specific T-cell levels less than 100 cells per million PBMCs were considered negative.

### Sequencing Analysis of Plasma Viral Genomes

Viral RNAs were extracted using High Pure Viral RNA kit (Roche Diagnostics, Tokyo, Japan) from macaque plasma samples. Fragments of cDNAs encoding SIVmac239 Gag and Nef were amplified by nested RT-PCR from plasma RNAs and subjected to direct sequencing by using dye terminator chemistry and an automated DNA sequencer (Applied Biosystems, Tokyo, Japan) as described before [45]. Predominant non-synonymous mutations were determined.

### Statistical Analysis

Statistical analysis was performed using Prism software version 4.03 with significance levels set at a P value of $<0.050$ (GraphPad Software, Inc., San Diego, CA). Plasma viral loads were log transformed and compared by an unpaired two-tailed t test.

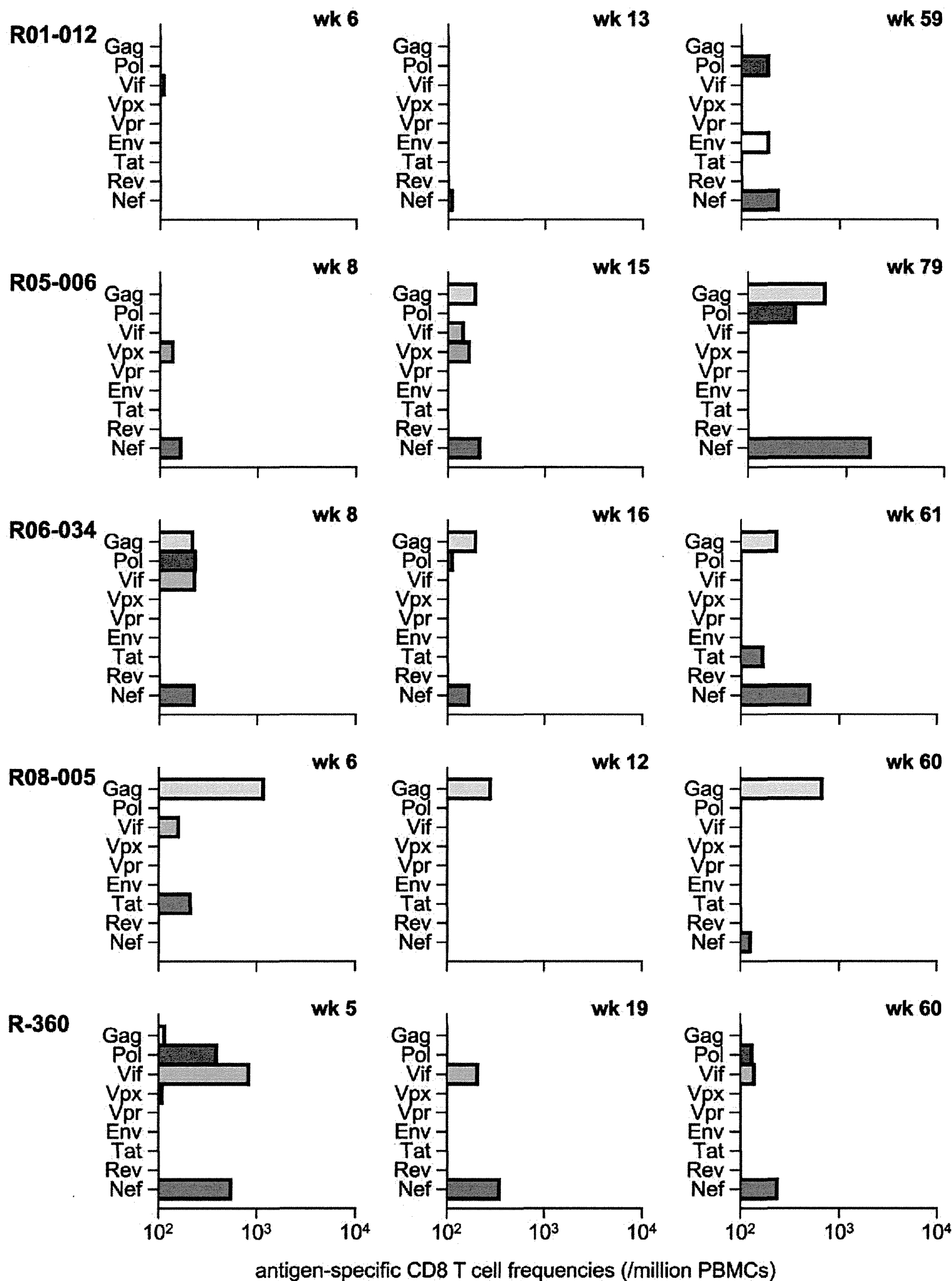



**Figure 3. SIV antigen-specific CD8⁺ T-cell responses in unvaccinated D⁺ macaques.** Responses were measured by the detection of antigen-specific IFN-γ induction in PBMCs obtained at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g003

## Results

### Lower Viral Loads in D⁺ Macaques in the Chronic Phase of SIV Infection

We first investigated SIVmac239 infection of five unvaccinated Burmese rhesus macaques sharing the MHC-I haplotype D (referred to as D⁺ macaques). Confirmed MHC-I alleles consisting of this haplotype is *Mamu-A1*032:02*, *Mamu-B*004:01*, and *Mamu-B*102:01:01*. These animals showed lower set-point plasma viral loads (Fig. 1). Comparison of plasma viral loads between these five animals and our previous cohorts of SIVmac239-infected Burmese D-negative (D⁻) rhesus macaques (n = 20) [21] revealed no

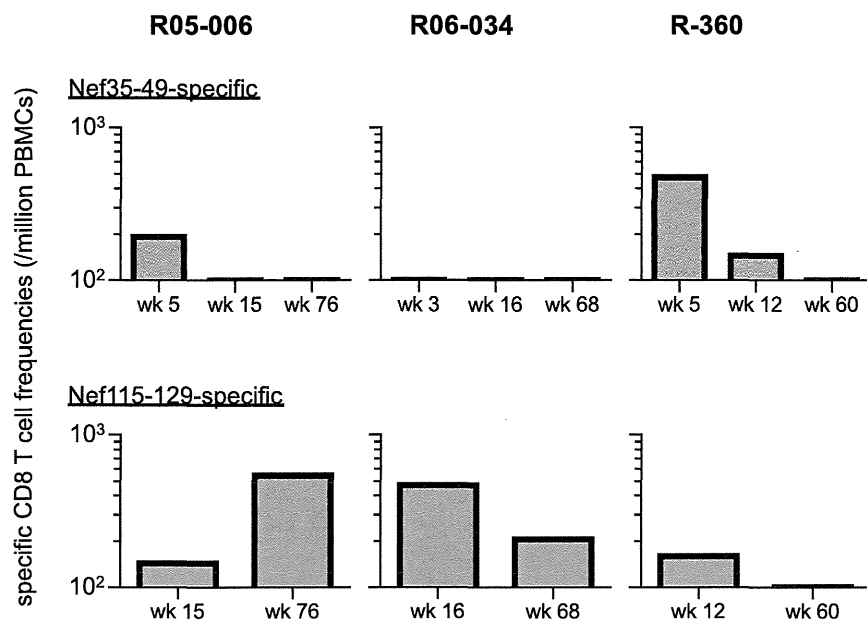



**Figure 4. SIV Nef-specific CD8+ T-cell responses in macaques R05-006, R06-034, and R-360.** $Nef_{35-49}$-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8+ T-cell responses were examined at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g004

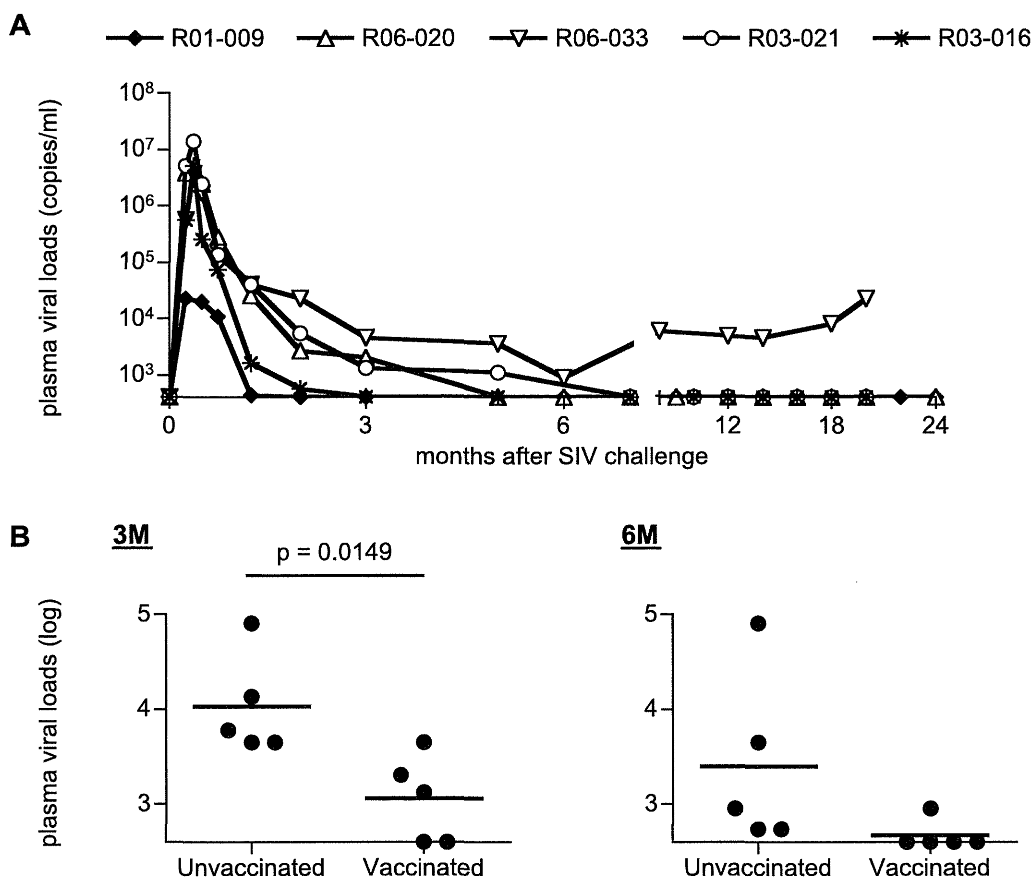



**Figure 5. Plasma viral loads after SIVmac239 challenge in vaccinated D+ macaques.** (A) Changes in plasma viral loads after challenge vaccinated macaques possessing MHC-I haplotype D. (B) Comparison of plasma viral loads at 3 months (left panel) and 6 months (right panel) between five unvaccinated D+ and five vaccinated D+ animals. Viral loads at 3 months in vaccinated animals were significantly lower than those in the unvaccinated ($p = 0.0149$ by t-test).
doi:10.1371/journal.pone.0054300.g005

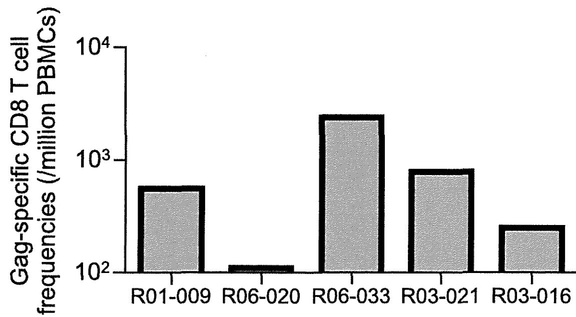



**Figure 6. SIV Gag-specific CD8⁺ T-cell responses in vaccinated D⁺ macaques at week 2 after SIVmac239 challenge.**
doi:10.1371/journal.pone.0054300.g006

significant difference at 3 months after SIV challenge ($p = 0.2436$ by t-test), but viral loads in the former $D^+$ animals became significantly lower than the latter after 6 months ($p = 0.0360$ at 6 months and $p = 0.0135$ at 9 months by t-test; Fig. 1). Four of these five macaques sharing MHC-I haplotype D showed low viral loads, less than $5 \times 10^3$ copies/ml, after 6 months, whereas macaque R01-012 maintained relatively higher viral loads.

## Predominant Nef-specific CD8⁺ T-cell Responses

We examined SIV antigen-specific $CD8^+$ T-cell responses by detection of antigen-specific IFN-γ induction. In the very acute phase, we did not have enough PBMC samples for measurement of individual SIV antigen-specific $CD8^+$ T-cell responses and focused on examining Gag-specific $CD8^+$ T-cell responses in most animals. At week 2 after challenge, Gag-specific $CD8^+$ T-cell responses were undetectable in four of five animals (Fig. 2).

**Figure 7. SIV antigen-specific CD8⁺ T-cell responses in vaccinated D⁺ animals after SIVmac239 challenge.** Samples for this analysis were unavailable in macaque R01-009.
doi:10.1371/journal.pone.0054300.g007

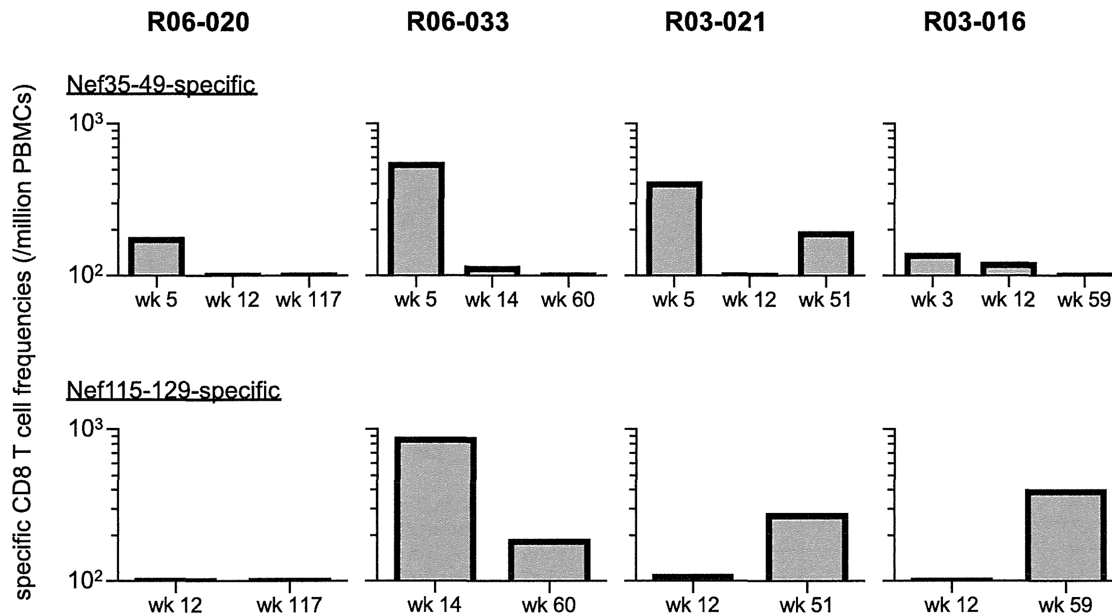



**Figure 8. SIV Nef-specific CD8$^+$ T-cell responses in macaques R06-020, R06-033, R03-021, and R03-016.** $Nef_{35-49}$-specific (upper panels) and $Nef_{115-129}$-specific (lower panels) CD8$^+$ T-cell responses were examined at indicated time points after SIVmac239 challenge.
doi:10.1371/journal.pone.0054300.g008

We then examined CD8$^+$ T-cell responses specific for individual SIV antigens in the early and the late phases (Fig. 3). Nef-specific but not Gag-specific CD8$^+$ T-cell responses were predominant in most D$^+$ animals. Gag-specific CD8$^+$ T-cell responses were dominantly induced in macaque R08-005 showing very low set-point viral loads. Macaque R01-012 having higher viral loads showed poor CD8$^+$ T-cell responses in the early phase.

Among four D$^+$ animals controlling SIV replication with less than $5 \times 10^3$ copies/ml of plasma viral loads after 6 months, Gag-specific CD8$^+$ T-cell responses were dominant only in macaque R08-005, while efficient Nef-specific CD8$^+$ T-cell responses were induced in the remaining three, suggesting possible contribution of Nef-specific CD8$^+$ T-cell responses to SIV control in these three controllers (R05-006, R06-034, and R-360). We then attempted to localize Nef CD8$^+$ T-cell epitopes shared in these animals and found $Nef_{35-49}$-specific and $Nef_{115-129}$-specific CD8$^+$ T-cell responses (Fig. 4), although we did not have enough samples for mapping the exact epitopes.

## Reduction of Viral Loads in the Early Phase of SIV Infection by Prophylactic Vaccination

We also investigated SIVmac239 infection of additional five, vaccinated Burmese rhesus macaques sharing the MHC-I haplotype D. These animals received a prophylactic DNA/SeV-Gag vaccination. In four of these five vaccinated macaques, plasma viremia became undetectable after 6 months, while macaque R06-033 showed persistent viremia (Fig. 5A). Difference in viral loads between unvaccinated and vaccinated D$^+$ animals was unclear in the acute phase, but the latter vaccinees showed significant reduction in viral loads compared to those in the former unvaccinated at 3 months ($p = 0.0360$; Fig. 5B). After 6 months, unvaccinated animals also showed reduced viral loads, and the difference in viral loads between unvaccinated and vaccinated became unclear.

In contrast to unvaccinated D$^+$ animals, all five vaccinated animals elicited Gag-specific CD8$^+$ T-cell responses at week 2 after challenge (Fig. 6), reflecting the effect of prophylactic vaccination.

We then examined CD8$^+$ T-cell responses specific for individual SIV antigens in these vaccinated animals (Fig. 7). Samples for this analysis were unavailable in vaccinated macaque R01-009. Vaccinated animals except for macaque R06-020 showed dominant Gag-specific CD8$^+$ T-cell responses even at 1–2 months. However, Gag-specific CD8$^+$ T-cell responses became not dominant after 1 year, while Nef-specific or Vif-specific CD8$^+$ T-cell responses became predominant, instead, in most vaccinees except for macaque R03-016.

Like three unvaccinated macaques (R05-006, R06-034, and R-360), vaccinated D$^+$ animals induced $Nef_{35-49}$-specific and $Nef_{115-129}$-specific CD8$^+$ T-cell responses after SIV challenge (Fig. 8). In analyses of three unvaccinated (Fig. 4) and four vaccinated animals (Fig. 8), $Nef_{35-49}$-specific CD8$^+$ T-cell responses were induced in the early phase in six animals but mostly became undetectable in the chronic phase. $Nef_{115-129}$-specific CD8$^+$ T-cell responses were also induced in most animals except for macaque R06-020 which showed $Nef_{112-126}$-specific ones in the chronic phase (data not shown). Macaques R05-006, R03-021, and R03-016 showed efficient $Nef_{115-129}$-specific CD8$^+$ T-cell responses not in the early phase but in the chronic phase. In contrast, vaccinated animal R06-033 that failed to control viremia showed higher $Nef_{115-129}$-specific CD8$^+$ T-cell responses in the early phase than those in the chronic phase.

## Selection of Mutations in Nef CD8$^+$ T-cell Epitope-coding Regions

To see the effect of selective pressure by Nef-specific CD8$^+$ T-cell responses on viral genome mutations, we next analyzed nucleotide sequences in viral *nef* cDNAs amplified from plasma RNAs obtained at several time points after SIV challenge. Nonsynonymous mutations detected predominantly in $Nef_{35-49}$-coding and $Nef_{115-129}$-coding regions were as shown in Fig. 9. Remarkably, all the unvaccinated and vaccinated D$^+$ animals showed rapid selection of mutations in the $Nef_{35-49}$-coding region in 3 months. On the other hand, mutations in the $Nef_{115-129}$-coding region were observed in the late phase in all the three

| | | Nef35-49 | | | | | Nef115-129 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Nef | | 36 | 37 | 41 | 42 | 44 | 119 | 122 | 124 | 125 | 126 |
| | | E | D | Q | S | G | M | F | K | E | K |
| R01-012 | 1M | | *G | | | | | | | | |
| | 3M | | *G | | | | | | | | |
| | 14M | *G | *G | | | | | | | | |
| | 24M | *G | *G | | | *E | | | | | |
| R05-006 | 1M | | | | | *E | | | | | |
| | 3M | | | R | | | | | | | |
| | 16M | | | R | | | | | | | |
| | 24M | | | R | | | | | | | R |
| R06-034 | 1M | | | | | | | | | | |
| | 3M | *G | | | | | | | | | |
| | 10M | *G | *G | | | *E | | | | | *R |
| | 18M | G | | | | E | | | | | R |
| R08-005 | 1M | | | | | | | | | | |
| | 3M | | | | *F | | | | | | |
| | 6M | G | | | | | | | | | |
| | 14M | | | | F | | | | | | |
| | 24M | *G | | | F | | | | | | |
| R-360 | 1M | | | | | | | | | | |
| | 3M | | *G | | | | | | | | |
| | 6M | | G | | | | | | | | |
| | 12M | | G | | | | T | L | | | |
| | 20M | | G | | | | T | L | | | |
| R06-020 | 1M | | | | | | | | | | |
| | 3M | *K | | | | | | | | | |
| | 11M | | G | R | | | | | | | |
| R06-033 | 1M | | | | | | | | | | |
| | 3M | *G | | | | | | | | | |
| | 6M | | *G | | | | | | | | |
| | 14M | | G | | | *E | | | | K | E |
| R03-021 | 1M | | | | | | | | | | |
| | 3M | | | | *F | | | | | | |
| | 14M | G | | | | | | | R | | |
| R03-016 | 1M | *K | | *R | | | | | | | |
| | 4M | K | | | | | | | | | |
| | 12M | K | | | | | | | | | |

**Figure 9. Predominant non-synonymous mutations in $Nef_{35-49}$-coding and $Nef_{115-129}$-coding regions of viral cDNAs in $D^+$ animals after SIVmac239 challenge.** Amino acid substitutions are shown. Detection of similar levels of wild-type and mutant sequences at the residue is indicated by asterisks. Samples for this analysis were unavailable in macaque R01-009.
doi:10.1371/journal.pone.0054300.g009

unvaccinated animals eliciting $Nef_{115-129}$-specific $CD8^+$ T-cell responses. These mutations were also detected in two of three vaccinated animals eliciting $Nef_{115-129}$-specific $CD8^+$ T-cell responses.

We also analyzed viral *gag* sequences to see the effect of Gag-specific $CD8^+$ T-cell pressure on viral genome mutations in vaccinated animals (data not shown). Our previous study [35] showed rapid selection of a mutation leading to a glutamine (Q)-to-lysine (K) change at the 58th residue in Gag (Q58K) at week 5 in vaccinated macaque R01-009, although no more samples were available for this sequencing analysis. This Q58K mutation results in escape from $Gag_{50-65}$-specific $CD8^+$ T-cell recognition. In the present study, macaque R03-016 showed rapid selection of a mutation leading to a K-to-asparagine (N) change at the 478th residue in Gag in 1 month. These results may reflect rapid disappearance of detectable plasma viremia in 1 or 2 months in these two vaccinees. Macaque R06-020 showed selection of a *gag*

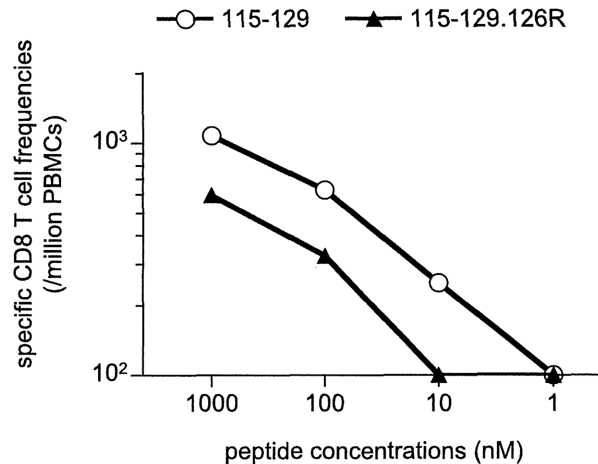



**Figure 10. IFN-γ induction in $CD8^+$ T cells after stimulation with the wild-type or the mutant peptide.** PBMCs obtained at week 31 from macaque R06-033 were stimulated by coculture with B-LCL pulsed with indicated concentrations of the wild-type $Nef_{115-129}$ peptide (open circles, 115–129, LAIDMSHFIKEKGGL) or the mutant $Nef_{115-129}$ peptide with a K126R alteration (closed triangles, 115–129.126R, LAIDMSHFI-KERGGL).
doi:10.1371/journal.pone.0054300.g010

mutation in 3 months, while other two vaccinees (R06-033 and R03-021) selected no *gag* mutation in the early phase.

## Discussion

HIV infection in humans with polymorphic MHC-I genotypes induces various patterns of viral antigen-specific $CD8^+$ T-cell responses. Previous studies have found several protective MHC-I alleles associated with lower viral loads and slower disease progression in HIV/SIV infection [7,13,14,16,17]. Elucidation of the mechanisms of viral control associated with individual protective MHC-I alleles would contribute to HIV cure and vaccine-based prevention. Because $CD8^+$ T-cell responses specific for some MHC-I-restricted epitopes can be affected by those specific for other MHC-I-restricted epitopes due to immunodominance [29,46,47], macaque groups sharing MHC-I genotypes at the haplotype level are useful for the analysis of cooperation of multiple epitope-specific $CD8^+$ T-cell responses. Previously, we reported a group of Burmese rhesus macaques sharing MHC-I haplotype *90-120-Ia* (A), which dominantly induce Gag-specific $CD8^+$ T-cell responses and tend to show slower disease progression after SIVmac239 challenge [21]. In the present study, we presented another type of protective MHC-I haplotype, which is not associated with dominant Gag-specific $CD8^+$ T-cell responses. Significant reduction of viral loads in unvaccinated macaques possessing this D haplotype compared to those in $D^-$ macaques was observed after 6 months. Analysis of SIV infection in macaques sharing this protective MHC-I haplotype would lead to understanding of $CD8^+$ T-cell cooperation for viral control.

Analyses of antigen-specific $CD8^+$ T-cell responses after SIVmac239 challenge indicate that this MHC-I haplotype D is associated with predominant Nef-specific $CD8^+$ T-cell responses. Nef-specific $CD8^+$ T-cell responses were efficiently induced in all SIV controllers, whereas Gag-specific $CD8^+$ T-cell responses were dominant in only one of them. We found $Nef_{35-49}$-specific and $Nef_{115-129}$-specific $CD8^+$ T-cell responses shared in $D^+$ animals. We were unable to determine the MHC-I alleles restricting these epitopes, but these responses are not usually induced in our

previous $D^-$ cohorts and considered to be associated with this MHC-I haplotype D.

Sequencing analysis of viral genomes showed rapid selection of mutations in the $Nef_{36-44}$-coding region within 3 months in all the $D^+$ animals. This is consistent with our results that $Nef_{35-49}$-specific $CD8^+$ T-cell responses were mostly induced in the early phase but undetectable in the chronic phase. These mutations were not consistently selected in our previous $D^-$ cohorts and thus considered as MHC-I haplotype D-associated mutations. This suggests strong selective pressure by $Nef_{35-49}$-specific $CD8^+$ T-cell responses in the acute phase of SIVmac239 infection in $D^+$ macaques, although it remains undetermined whether these mutations result in viral escape from $Nef_{35-49}$-specific $CD8^+$ T-cell recognition.

$Nef_{115-129}$-specific $CD8^+$ T-cell responses were detected in six $D^+$ animals. In five of them, nonsynonymous mutations in the $Nef_{119-126}$-coding region were observed in the chronic phase. At least, we confirmed viral escape from $Nef_{115-129}$-specific $CD8^+$ T-cell recognition by a mutation leading to a K-to-arginine (R) (K126R) substitution at Nef residue 126 (Fig. 10). The number of nonsynonymous substitutions per the number of sites estimated to be nonsynonymous (dN) exceeded that estimated to be synonymous (dS) during the evolution process of $Nef_{115-129}$-coding region, but the value did not show statistically significant difference from that of neutral selection. Among three unvaccinated animals that controlled SIV replication without dominant Gag-specific $CD8^+$ T-cell responses, amino acid substitutions in the $Nef_{119-126}$-coding region were observed in a year in macaques R06-034 and R-360 but after 2 years in macaque R05-006. The former two animals tended to show earlier increases in plasma viral loads in the chronic phase, while the latter R05-006 maintained higher frequencies of $Nef_{115-129}$-specific $CD8^+$ T-cell responses. $Nef_{115-129}$-specific $CD8^+$ T-cell responses were efficient in the chronic phase in vaccinated controllers R03-021 and R03-016 but decreased in R06-033 that failed to contain SIV replication. Although a possible effect of this haplotype-associated factors other than $CD8^+$ T-cell responses such as NK activity on SIV infection [48,49,50] remains undetermined, these results imply involvement of Nef-specific $CD8^+$ T-cell responses in the SIV control associated with MHC-I haplotype D.

Unvaccinated macaque R08-005 dominantly elicited Gag antigen-specific $CD8^+$ T-cell responses and showed rapid selection of a mutation encoding Gag 257 residue, which was not observed in any other $D^+$ animals. Nef-specific $CD8^+$ T-cell responses were detectable only at week 2 in the acute phase (data not shown) and a mutation in the $Nef_{42}$-coding region was rapidly selected. It is speculated that those dominant Gag-specific $CD8^+$ T-cell responses associated with the second, non-D MHC-I haplotype were effective in this animal. $Nef_{35-49}$-specific $CD8^+$ T-cell responses may not be efficient due to immunodominance but exert some suppressive pressure on viral replication.

DNA/SeV-Gag vaccination resulted in earlier reduction of viral loads after SIV challenge. Vaccinees showed significantly lower viral loads at 3 months than those in unvaccinated animals. Gag-specific $CD8^+$ T-cell responses were elicited at week 2 in all the vaccinees but not in the unvaccinated except for one animal R08-005. No *gag* mutations were shared in the vaccinees in the acute phase, but three of them showed rapid selection of individual non-synonymous mutations in *gag*. Rapid selection of mutations in the $Nef_{36-44}$-coding region was consistently detected even in these vaccinees. These results suggest broader $CD8^+$ T-cell responses consisting of dominant vaccine antigen Gag-specific and inefficient naive-derived Nef-specific ones in the acute phase. In three vaccinated animals, Gag-specific $CD8^+$ T-cell responses became lower or undetectable, and instead, Nef-specific $CD8^+$ T-cell responses became predominant in the chronic phase.

In summary, we found a protective MHC-I haplotype not associated with dominant Gag-specific $CD8^+$ T-cell responses in SIVmac239 infection. Our results in $D^+$ macaques suggest suppressive pressure by $Nef_{35-49}$-specific and $Nef_{115-129}$-specific $CD8^+$ T-cell responses on SIV replication, contributing to reduction in set-point viral loads. DNA/SeV-Gag-vaccinated $D^+$ animals induced Gag-specific $CD8^+$ T-cell responses in addition to Nef-specific ones after SIV challenge, resulting in earlier containment of SIV replication. This study presents a pattern of SIV control with involvement of non-Gag antigen-specific $CD8^+$ T-cell responses, contributing to accumulation of our knowledge on HIV/SIV control mechanisms.

## Acknowledgments

We thank F. Ono, K. Oto, K. Hanari, K. Komatsuzaki, M. Hamano, H. Akari, and Y. Yasutomi for their assistance in animal experiments.

## Author Contributions

Performed animal experiments: HS TM TI YK. Performed MHC-I typing: TKN AK. Conceived and designed the experiments: NT TM. Performed the experiments: NT TN YT HY AT. Analyzed the data: NT HY T. Shiino TM. Contributed reagents/materials/analysis tools: MI AI HH T. Shu MH. Wrote the paper: NT TM.

## References

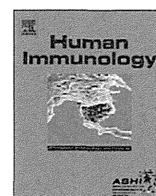
1. Borrow P, Lewicki H, Hahn BH, Shaw GM, Oldstone MB (1994) Virus-specific CD8+ cytotoxic T-lymphocyte activity associated with control of viremia in primary human immunodeficiency virus type 1 infection. J Virol 68: 6103–6110.
2. Koup RA, Safrit JT, Cao Y, Andrews CA, McLeod G, et al. (1994) Temporal association of cellular immune responses with the initial control of viremia in primary human immunodeficiency virus type 1 syndrome. J Virol 68: 4650–4655.
3. Matano T, Shibata R, Siemon C, Connors M, Lane HC, et al. (1998) Administration of an anti-CD8 monoclonal antibody interferes with the clearance of chimeric simian/human immunodeficiency virus during primary infections of rhesus macaques. J Virol 72: 164–169.
4. Jin X, Bauer DE, Tuttleton SE, Lewin S, Gettie A, et al. (1999) Dramatic rise in plasma viremia after CD8+ T cell depletion in simian immunodeficiency virus-infected macaques. J Exp Med 189: 991–998.
5. Schmitz JE, Kuroda MJ, Santra S, Sasseville VG, Simon MA, et al. (1999) Control of viremia in simian immunodeficiency virus infection by CD8+ lymphocytes. Science 283: 857–860.
6. Carrington M, Nelson GW, Martin MP, Kissner T, Vlahov D, et al. (1999) HLA and HIV-1: heterozygote advantage and B*35-Cw*04 disadvantage. Science 283: 1748–1752.
7. Migueles SA, Sabbaghian MS, Shupert WL, Bettinotti MP, Marincola FM, et al. (2000) HLA B*5701 is highly associated with restriction of virus replication in a subgroup of HIV-infected long term nonprogressors. Proc Natl Acad Sci USA 97: 2709–2714.
8. Tang J, Tang S, Lobashevsky E, Myracle AD, Fideli U, et al. (2002) Favorable and unfavorable HLA class I alleles and haplotypes in Zambians predominantly infected with clade C human im- munodeficiency virus type 1. J Virol 76: 8276–8284.
9. Kiepiela P, Leslie AJ, Honeyborne I, Ramduth D, Thobakgale C, et al. (2004) Dominant influence of HLA-B in mediating the potential co-evolution of HIV and HLA. Nature 432: 769–775.
10. Leslie A, Matthews PC, Listgarten J, Carlson JM, Kadie C, et al. (2010) Additive contribution of HLA class I alleles in the immune control of HIV-1 infection. J Virol 84: 9879–9888.
11. Altfeld M, Addo MM, Rosenberg ES, Hecht FM, Lee PK, et al. (2003) Influence of HLA-B57 on clinical presentation and viral control during acute HIV-1 infection. AIDS 17: 2581–2591.
12. Altfeld M, Kalife ET, Qi Y, Streeck H, Lichterfeld M, et al. (2006) HLA alleles associated with delayed progression to AIDS contribute strongly to the initial CD8+ T cell response against HIV-1. PLoS Med 3: e403.
13. Goulder PJ, Watkins DI (2008) Impact of MHC class I diversity on immune control of immunodeficiency virus replication. Nat Rev Immunol 8: 619–630.

14. Muhl T, Krawczak M, Ten Haaft P, Hunsmann G, Sauermann U (2002) MHC class I alleles influence set-point viral load and survival time in simian immunodeficiency virus-infected rhesus monkeys. J Immunol 169: 3438–3446.
15. Mothe BR, Weinfurter J, Wang C, Rehrauer W, Wilson N, et al. (2003) Expression of the major histocompatibility complex class I molecule Mamu-A*01 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol 77: 2736–2740.
16. Yant LJ, Friedrich TC, Johnson RC, May GE, Maness NJ, et al. (2006) The high-frequency major histocompatibility complex class I allele Mamu-B*17 is associated with control of simian immunodeficiency virus SIVmac239 replication. J Virol 80: 5074–5077.
17. Loffredo JT, Maxwell J, Qi Y, Glidden CE, Borchardt GJ, et al. (2007) Mamu-B*08-positive macaques control simian immunodeficiency virus replication. J Virol 81: 8827–8832.
18. Edwards BH, Bansal A, Sabbaj S, Bakari J, Mulligan MJ, et al. (2002) Magnitude of functional CD8+ T-cell responses to the gag protein of human immunodeficiency virus type 1 correlates inversely with viral load in plasma. J Virol 76: 2298–2305.
19. Zuniga R, Lucchetti A, Galvan P, Sanchez S, Sanchez C, et al. (2006) Relative dominance of Gag p24-specific cytotoxic T lymphocytes is associated with human immunodeficiency virus control. J Virol 80: 3122–3125.
20. Kiepiela P, Ngumbela K, Thobakgale C, Ramduth D, Honeyborne I, et al. (2007) CD8+ T-cell responses to different HIV proteins have discordant associations with viral load. Nat Med 13: 46–53.
21. Nomura T, Yamamoto H, Shiino T, Takahashi N, Nakane T, et al. (2012) Association of major histocompatibility complex class I haplotypes with disease progression after simian immunodeficiency virus challenge in Burmese rhesus macaques. J Virol 86: 6481–6490.
22. Schneidewind A, Brockman MA, Yang R, Adam RI, Li B, et al. (2007) Escape from the dominant HLA-B27-restricted cytotoxic T-lymphocyte response in Gag is associated with a dramatic reduction in human immunodeficiency virus type 1 replication. J Virol 81: 12382–12393.
23. Emu B, Sinclair E, Hatano H, Ferre A, Shacklett B, et al. (2008) HLA class I-restricted T-cell responses may contribute to the control of human immunodeficiency virus infection, but such responses are not always necessary for long-term virus control. J Virol 82: 5398–5407.
24. Miura T, Brockman MA, Schneidewind A, Lobritz M, Pereyra F, et al. (2009) HLA-B57/B*5801 human immunodeficiency virus type 1 elite controllers select for rare gag variants associated with reduced viral replication capacity and strong cytotoxic T-lymphocyte recognition. J Virol 83: 2743–2755.
25. Leslie AJ, Pfafferott KJ, Chetty P, Draenert R, Addo MM, et al. (2004) HIV evolution: CTL escape mutation and reversion after transmission. Nat Med 10: 282–289.
26. Martinez-Picado J, Prado JG, Fry EE, Pfafferott K, Leslie A, et al. (2006) Fitness cost of escape mutations in p24 Gag in association with control of human immunodeficiency virus type 1. J Virol 80: 3617–3623.
27. Crawford H, Prado JG, Leslie A, Hue S, Honeyborne I, et al. (2007) Compensatory mutation partially restores fitness and delays reversion of escape mutation within the immunodominant HLA-B*5703-restricted Gag epitope in chronic human immunodeficiency virus type 1 infection. J Virol 81: 8346–8351.
28. Friedrich TC, Valentine LE, Yant LJ, Rakasz EG, Piaskowski SM, et al. (2007) Subdominant CD8+ T-cell responses are involved in durable control of AIDS virus replication. J Virol 81: 3465–3476.
29. Loffredo JT, Bean AT, Beal DR, Leon EJ, May GE, et al. (2008) Patterns of CD8+ immunodominance may influence the ability of Mamu-B*08-positive macaques to naturally control simian immunodeficiency virus SIVmac239 replication. J Virol 82: 1723–1738.
30. Maness NJ, Yant LJ, Chung C, Loffredo JT, Friedrich TC, et al. (2008) Comprehensive immunological evaluation reveals surprisingly few differences between elite controller and progressor Mamu-B*17-positive simian immunodeficiency virus-infected rhesus macaques. J Virol 82: 5245–5254.
31. Valentine LE, Loffredo JT, Bean AT, Leon EJ, MacNair CE, et al. (2009) Infection with "escaped" virus variants impairs control of simian immunodeficiency virus SIVmac239 replication in Mamu-B*08-positive macaques. J Virol 83: 11514–11527.
32. Budde ML, Greene JM, Chin EN, Ericsen AJ, Scarlotta M, et al. (2012) Specific CD8+ T cell responses correlate with control of simian immunodeficiency virus replication in Mauritian cynomolgus macaques. J Virol 86: 7596–7604.
33. Mudd PA, Martins MA, Ericsen AJ, Tully DC, Power KA, et al. (2012) Vaccine-induced CD8+ T cells control AIDS virus replication. Nature 491: 129–133.
34. Naruse TK, Chen Z, Yanagida R, Yamashita T, Saito Y, et al. (2010) Diversity of MHC class I genes in Burmese-origin rhesus macaques. Immunogenetics 62: 601–611.
35. Matano T, Kobayashi M, Igarashi H, Takeda A, Nakamura H, et al. (2004) Cytotoxic T lymphocyte-based control of simian immunodeficiency virus replication in a preclinical AIDS vaccine trial. J Exp Med 199: 1709–1718.
36. Kawada M, Tsukamoto T, Yamamoto H, Iwamoto N, Kurihara K, et al. (2008) Gag-specific cytotoxic T-lymphocyte-based control of primary simian immunodeficiency virus replication in a vaccine trial. J Virol 82: 10199–10206.
37. Tanaka-Takahashi Y, Yasunami M, Naruse T, Hinohara K, Matano T, et al. (2007) Reference strand-mediated conformation analysis-based typing of multiple alleles in the rhesus macaque MHC class I Mamu-A and Mamu-B loci. Electrophoresis 28: 918–924.
38. Shibata R, Maldarelli F, Siemon C, Matano T, Parta M, et al. (1997) Infection and pathogenicity of chimeric simian-human immunodeficiency viruses in macaques: determinants of high virus loads and CD4 cell killing. J Infect Dis 176: 362–373.
39. Li HO, Zhu YF, Asakawa M, Kuma H, Hirata T, et al. (2000) A cytoplasmic RNA vector derived from nontransmissible Sendai virus with efficient gene transfer and expression. J Virol 74: 6564–6569.
40. Takeda A, Igarashi H, Nakamura H, Kano M, Iida A, et al. (2003) Protective efficacy of an AIDS vaccine, a single DNA priming followed by a single booster with a recombinant replication-defective Sendai virus vector, in a macaque AIDS model. J Virol 77: 9710–9715.
41. Kestler HW III, Ringler DJ, Mori K, Panicali DL, Sehgal PK, et al. (1991) Importance of the nef gene for maintenance of high virus loads and for development of AIDS. Cell 65: 651–662.
42. Yamamoto H, Kawada M, Takeda A, Igarashi H, Matano T (2007) Post-infection immunodeficiency virus control by neutralizing antibodies. PLoS One 2: e540.
43. Iwamoto N, Tsukamoto T, Kawada M, Takeda A, Yamamoto H, et al. (2010) Broadening of CD8+ cell responses in vaccine-based simian immunodeficiency virus controllers. AIDS 24: 2777–2787.
44. Voss G, Nick S, Stahl-Hennig C, Ritter K, Hunsmann G (1992) Generation of macaque B lymphoblastoid cell lines with simian Epstein-Barr-like viruses: transformation procedure, characterization of the cell lines and occurrence of simian foamy virus. J Virol Methods 39: 185–195.
45. Kawada M, Igarashi H, Takeda A, Tsukamoto T, Yamamoto H, et al. (2006) Involvement of multiple epitope-specific cytotoxic T-lymphocyte responses in vaccine-based control of simian immunodeficiency virus replication in rhesus macaques. J Virol 80: 1949–1958.
46. Tenzer S, Wee E, Burgevin A, Stewart-Jones G, Friis L, et al. (2009) Antigen processing influences HIV-specific cytotoxic T lymphocyte immunodominance. Nat Immunol 10: 636–646.
47. Ishii H, Kawada M, Tsukamoto T, Yamamoto H, Matsuoka S, et al. (2012) Impact of vaccination on cytotoxic T lymphocyte immunodominance and cooperation against simian immunodeficiency virus replication in rhesus macaques. J Virol 86: 738–745.
48. Flores-Villanueva PO, Yunis EJ, Delgado JC, Vittinghoff E, Buchbinder S, et al. (2001) Control of HIV-1 viremia and protection from AIDS are associated with HLA-Bw4 homozygosity. Proc Natl Acad Sci USA 98: 5140–5145.
49. Martin MP, Gao X, Lee JH, Nelson GW, Detels R, et al. (2002) Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. Nat Genet 31: 429–434.
50. Martin MP, Qi Y, Gao X, Yamada E, Martin JN, et al. (2007) Innate partnership of HLA-B and KIR3DL1 subtypes against HIV-1. Nat Genet 39: 733–740.

Human Immunology 74 (2013) 163–165

Contents lists available at SciVerse ScienceDirect

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



# Status of TIM-1 exon 4 haplotypes and CD4+T cell counts in HIV-1 seroprevalent North Indians

Gaurav Sharma [a], Hitoshi Ohtani [b], Gurvinder Kaur [a,*], Taeko K. Naruse [b], S.K. Sharma [c], Madhu Vajpayee [d], Akinori Kimura [b,*], Narinder Mehra [a]

[a] Department of Transplant Immunology and Immunogenetics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India
[b] Department of Molecular Pathogenesis, Tokyo Medical and Dental University, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8510, Japan
[c] Department of Medicine, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India
[d] Department of Microbiology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India

ARTICLE INFO

*Article history:*
Received 11 July 2012
Accepted 27 November 2012
Available online 5 December 2012

ABSTRACT

The *TIM* (T cell/transmembrane, immunoglobulin and mucin) proteins are crucial regulators of Th1/Th2 immune responses and have been implicated in several diseases including HIV-1/AIDS. The *TIM1* exon 4 that codes for mucin domain is highly diverse, with sequence variants associated with varying phenotypes. In this study, *TIM1* exon 4 was sequenced among 227 HIV-1 seroprevalent and 288 healthy non infected individuals from North Indian population and haplotypes established. A novel but rare haplotype D1* was identified among the healthy and differed from D1 by a synonymous substitution G>T at Thr208Thr. The *TIM1* haplotype diversity showed no association with susceptibility to HIV-1 infection. The seroprevalent individuals carrying D3A had relatively higher median CD4+T cell counts (368/μl) than those without (313/μl; $p = 0.02$). A comparison of CD4+T counts between D3-A individuals on ART or ART naïve did not show any significant difference plausibly due to confounding nature of ART and other factors.

© 2012 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.

## 1. Introduction

The human TIM (T cell/transmembrane, immunoglobulin and mucin) family consists of three type I cell surface glycoproteins (TIM1, TIM3 and TIM4) with N terminal immunoglobulin (Ig) like domains at distal end and highly polymorphic proximal mucin domains with N- and O-linked glycosylation sites [1–3]. The TIM family genes are located on chromosome 5q33.2 [4], and have been reported to be associated with asthma [5], atopy [6], autoimmunity [1], malaria [7] and viral infections [8].

The *TIM1* mucin domain, in particular, has been a hotspot of both positive and over-dominant selection with numerous non-synonymous substitutions and phenotypic variations [9]. It appears to have undergone a selective sweep in Chimpanzees caused by pathogens like SIV and is thus an important candidate gene for evaluating human immune responses against HIV-1 [8,9].

The TIM-1 receptor is preferentially expressed on Th2 cells and acts as a potent co-stimulatory activator of Th2 responses [3]. An imbalance of Th1/Th2 cell responses has been linked to poor prognosis and increased viral replication during HIV-1 infection [10]. The role of TIM gene family products and their variability on Th1/Th2 immune responses in HIV-1 infection, disease progression and immunopathogenesis are largely unknown. There has been only one study reported so far that has shown an association of *TIM1* haplotype (D3-A) with delayed progression to AIDS and better CD4 counts among HIV-1 infected Thai female cohort [8]. Therefore, the present study was planned with the aim of evaluating *TIM1* mucin domain polymorphisms among North Indian population infected with HIV-1.

## 2. Materials and methods

The study was conducted using materials collected from 227 HIV-1 positive (+ve) patients, enrolled from Department of Microbiology and antiretroviral treatment (ART) clinic of All India Institute of Medical Sciences, New Delhi and a control group of 288 healthy individuals, all unrelated and evenly distributed within the North Indian states of Delhi, Punjab, Haryana, Himachal Pradesh, Uttarakhand and Uttar Pradesh. The healthy and patient groups were age and gender matched; with median age of 28 and 32 years and male/female ratios of 1.28 and 1.7, respectively. Among patients, 121 were asymptomatic ART naïve while 106 were on ART. The patients were followed for clinical and immunological details including CD4 counts for more than 2 years from the date of enrollment.

* Corresponding authors.
*E-mail addresses:* gurvinder@hotmail.com (G. Kaur), akitis@mri.tmd.ac.jp (A. Kimura).

0198-8859/$36.00 - see front matter © 2012 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.humimm.2012.11.013

**Table 1**
Distribution of TIM-1 haplotypes in healthy and HIV +ve subjects ('ART naïve' and 'on ART') in North Indian population.

| TIM1 haplotype# | T>C (Thr158Met) | 3bp deletion (Thr160del) | 18bp deletion (6AA 161-166 del) | C>T (Pro180Leu) | 3bp deletion (Thr201del) | 1bp deletion (frameshift fs207) | A>G (Thr208Ala) | G>T (Thr208Thr) | Haplotype frequency | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | | Healthy controls($2n = 576$) ($n = 288$) Number (frequency) | Total HIV +ve ($2n = 454$) ($n = 227$) Number (frequency) | ART Naïve HIV +ve ($2n = 242$) ($n = 121$) Number (frequency) | On ART HIV +ve ($2n = 212$) ($n = 106$) Number (fFrequency) |
| D1 | T | del | w | T | w | w | A | T | 12 (0.021) | 10 (0.022) | 6 (0.025) | 4 (0.019) |
| D1* | T | del | w | T | w | w | A | G | 4 (0.007) | 0 (0) | 0 (0) | 0 (0) |
| D3-A | C | w | del | C | w | w | A | G | 274 (0.475) | 211 (0.465) | 112 (0.463) | 99 (0.467) |
| D3-C | C | w | del | C | w | w | G | G | 126 (0.219) | 109 (0.240) | 61 (0.252) | 48 (0.226) |
| D4 | T | w | w | C | del | w | A | G | 123 (0.214) | 99 (0.218) | 49 (0.202) | 50 (0.236) |
| W-A | T | w | w | C | w | w | G | G | 33 (0.057) | 23 (0.051) | 12 (0.050) | 11 (0.052) |
| W-C | T | w | w | C | w | w | A | G | 4 (0.007) | 2 (0.004) | 2 (0.008) | 0 (0) |

# Haplotypes as described by Nakajima et al. [9].

Ethical approval for the study was obtained from the Institutional Ethical Committee of All India Institute of Medical Sciences and study subjects were enrolled following their informed consent. The study protocol was also approved by the Ethics Reviewing Committee of Medical Research Institute, Tokyo Medical and Dental University. Ten ml of peripheral blood was collected by venipuncture into $Na_2$-EDTA coated vacutainers for DNA extraction and plasma isolation. Ammonium acetate salting out procedure was used for extraction of DNA from blood samples [11] and used for *TIM1* exon 4 sequencing.

Polymorphisms in the mucin domain of *TIM-1* encoded by exon 4 were analyzed by direct sequencing, as described previously [8]. Briefly, primers 5′-GGGCAATGACCAAGATTGAC-3′ and 5′-ACCTTGATACAATGCCCTGG-3′ were used to amplify a 470 bp fragment containing exon 4 of *TIM1*. The PCR products were sequenced by using the PCR primers and BigDye Terminator v 3.1 cycle sequencing kit (Applied Biosystems, California, USA) in ABI Prism 3130xl genetic analyzer. The *TIM-1* haplotypes were determined using Haploview software based on the previous report [8,9]. Haplotypes were compared among the healthy and HIV-1 infected groups by the chi square test or Fisher's exact test wherever applicable. Continuous variables (CD4+T cell counts) were stratified based on the haplotypic background and compared by non-parametric Wilcoxon Rank Sum (Mann Whitney *U*) test.

## 3. Results

Of the known eleven *TIM-1* haplotypes [9], six haplotypes namely W-A, W-C, D1, D3-A, D3-C and D4 were observed in the North Indian population (Table 1). Among these, D3-A was found to be the most prevalent haplotype and occurred with comparable frequencies in healthy (47.5%) as well as HIV +ve subjects (46.5%). The next most frequent haplotype was D3-C (21.9%) followed by D4 (21.4%), W-A (5.7%), D1 (2.1%) and W-C (0.7%). In addition, a novel haplotype D1* which differed from D1 by a synonymous G to T transversion (Thr 208 Thr) was found in 4 healthy individuals (0.7%).

A comparison of distribution of *TIM-1* haplotypes between healthy controls and HIV +ve subjects is shown in Table 1. There was no significant difference in *TIM-1* haplotypic distribution among these groups. In addition, the haplotypic distribution of *TIM-1* did not show any significant difference in asymptomatic ART naïve HIV +ve subjects versus symptomatic patients on ART (Table 1).

The median CD4+T cell counts of HIV +ve subjects were analyzed by stratifying the individuals according to the presence or absence of various *TIM-1* haplotypes (D1, D3-A, D3-C, D4 and W-A) as shown in Table 2. The patients carrying the D3-A haplotype had relatively higher CD4+T counts/μl (median 368/μl) as compared to those without it (median 313/μl; $p = 0.02$). However, when patients on ART or ART naïve were compared among each other, the level of significance of this difference was abolished. This could plausibly be attributed to the confounding nature of ART and other factors and could be further established among a larger cohort size.

## 4. Discussion

This is a first preliminary study on the role of *TIM1* sequence variations during HIV-1 infection in the North Indian population. The study has shown the presence of six *TIM1* haplotypes W-A, W-C, D1, D3-A, D3-C and D4, similar to those as described earlier [8,9] plus an additional novel D1* haplotype. The haplotype D3-A was found to be the most predominant one and showed a modest association with higher CD4+T cell counts among HIV +ve individ-

**Table 2**
Comparison of median CD4+T cell counts/μl blood in HIV +ve subjects (with or without symptoms/antiretroviral treatment) stratified on the basis of presence or absence of various TIM-1 haplotypes.

| Haplotype | D1 | | D3-A | | D3-C | | D4 | | W-A | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Present | Absent | Present | Absent | Present | Absent | Present | Absent | Present | Absent |
| ART naïve ($n$ = 99) | $n$ = 5 | $n$ = 94 | $n$ = 77 | $n$ = 22 | $n$ = 43 | $n$ = 56 | $n$ = 34 | $n$ = 65 | $n$ = 8 | $n$ = 91 |
| Median CD4+T counts | 448 | 439 | 448 | 400 | 425 | 457 | 410 | 468 | 451 | 440 |
| *p Value* | 0.96 | | 0.44 | | 0.76 | | 0.1 | | 0.81 | |
| On ART ($n$ = 101) | $n$ = 4 | $n$ = 97 | $n$ = 66 | $n$ = 35 | $n$ = 41 | $n$ = 60 | $n$ = 41 | $n$ = 60 | $n$ = 11 | $n$ = 90 |
| Median CD4+T counts | 284 | 239 | 262 | 211 | 219 | 253 | 222 | 261 | 225 | 250 |
| *p Value* | 0.99 | | 0.07 | | 0.39 | | 0.33 | | 0.96 | |
| Total HIV cohort ($n$ = 200) | $n$ = 9 | $n$ = 191 | $n$ = 143 | $n$ = 57 | $n$ = 84 | $n$ = 116 | $n$ = 75 | $n$ = 125 | $n$ = 19 | $n$ = 181 |
| Median CD4+T counts | 324 | 355 | 368 | 313 | 358 | 344 | 324 | 364 | 355 | 355 |
| *p Value* | 0.89 | | 0.02 | | 0.82 | | 0.07 | | 0.96 | |

Note: Haplotypes W-C and D1* were not evaluated since these were either absent or present in very low frequencies in the patients.

uals. Similar difference was also observed amongst ART naïve and on ART subgroups but could not reach statistical significance. This could plausibly be attributed to confounding nature of ART plus other factors and needs to be explored further among a larger cohort size. Recently, studies have shown that mucin 1 secreted in milk [12] and mucin 6 in seminal plasma [13] could bind to DC-SIGN and block viral transfer to CD4 cells and ultimately skew the mounted immune responses.

A similar study in a HIV-1 infected female cohort in Thailand also showed an association of *TIM1* D3-A haplotype with higher CD4+T cell counts and delayed disease progression to AIDS [8]. A possible link of these haplotypes has been suggested with relatively low levels of TIM-1 expression [8] and hence lower Th2 promotion and enhanced Th1 responses thereby facilitating enhanced CTL responses and better prognosis or delayed disease progression. On the contrary, enhanced Th1 could also favor proliferation of CCR5+CD4+ T cells and support viral replication.

The present study suffers from a major limitation of unavailability of dates of HIV-1 infection/seroconversion and regular viral load data. Hence, we could not assess the rates of progression among individuals with different *TIM1* haplotypes. A comparison of haplotype frequencies did not reveal any significant difference among healthy and HIV +ve subjects, suggesting a lack of direct association of *TIM1* haplotypes with the susceptibility to HIV infection in the Indian population, although their indirect effect via interaction with other genes cannot be ruled out.

The D3-A haplotype (and D3-C) of *TIM1* contain an 18 bp deletion due to which a 6 amino acid long stretch of MTTTVP is excluded and a shorter form of protein is expressed. It has been hypothesized that this shorter form evolved as a protective mechanism against the hepatitis A virus (HAV) since this receptor form does not bind to the virus as efficiently as long forms. It was shown that HAV-induced liver damage was associated with the insertion polymorphism [14], earlier shown to be associated with protection against asthma and allergic diseases. A relative analysis of presence of this deletion/insertion in the present study, however, did not reveal any significant correlation with the susceptibility to HIV-1 or to CD4+T cell counts (data not shown).

In conclusion, we report a possible influence of *TIM1* D3-A haplotype on HIV-1 infection in North Indians. Further studies are required to explore the influence of circulating *TIM1* and other TIM haplotypes on specific CD4+T cell subsets during HIV-1 infection and in the development of AIDS.

## Acknowledgements

We thank Dr. Toshiaki Nakajima for his contribution in the initial course of the study. This work was supported in part by grants for India-Japan Cooperative Science Program from Japan Society for the Promotion of Science (JSPS), Japan and Department of Science and Technology (DST), India and research grants from the Ministry of Health, Labor and Welfare, Japan, and a support for women researchers from the Tokyo Medical and Dental University.

## References

[1] Encinas JA, Kuchroo VK. Mapping and identification of autoimmunity genes. Curr Opin Immunol 2000;12:691–7.
[2] McIntire JJ, Umetsu DT, DeKruyff RH. TIM-1, a novel allergy and asthma susceptibility gene. Springer Semin Immunopathol 2004;25:335–48.
[3] Freeman GJ, Casasnovas JM, Umetsu DT, et al. TIM genes: a family of cell surface phosphatidylserine receptors that regulate innate and adaptive immunity. Immunol Rev 2010;235:172–89.
[4] Lee J, Phong B, Egloff AM, Kane LP. TIM polymorphisms – genetics and function. Genes Immunity 2011;12:595–604.
[5] Gao PS, Mathias RA, Plunkett B, et al. Genetic variants of the TIM-1 but not the TIM3 gene are associated with asthma in an African American population. J Allergy Clin Immunol 2005;115:982–8.
[6] Graves PE, Siroux V, Guerra S, et al. Association of atopy and eczema with polymorphisms in T cell immunoglobulin domain and mucin domain IL2 inducible T cell kinase gene cluster in chromosome 5q33. J Allergy Clin Immunol 2005;116:650–6.
[7] Nuchnoi P, Ohashi J, Kimura R, et al. Significant association between TIM1 promoter polymorphisms and protection against cerebral malaria in Thailand. Ann Hum Genet 2008;72:327–36.
[8] Wichukchinda N, Nakajima T, Saipradit N, Nakayama E, et al. TIM-1 haplotype may control the disease progression to AIDS in a HIV-1 infected female cohort in Thailand. AIDS 2010;24:1625–31.
[9] Nakajima T, Wooding S, Satta Y, Jinnai N, et al. Evidence for natural selection in the HAVCR1 gene: *high degree of amino acid variability in the mucin domain* of human HAVCR1 protein. Genes Immunity 2005;6:398–406.
[10] Romagnani S, DelPrete G, Manetti R, Ravina A, et al. Role of Th1/Th2 cytokines in HIV infection. Immunol Rev 1994;140:73–92.
[11] Kaur G, Kumar N, Nandakumar R, et al. Utility of saliva and hair follicles in donor selection for hematopoietic stem cell transplantation and chimerism monitoring. Chimerism 2012;3:1–9.
[12] Saeland E, de Jong MA, Nabatov AA, Kalay H, et al. MUC1 in human milk blocks transmission of human immunodeficiency virus from dendritic cells to T cells. Mol Immunol 2009;46:2309–16.
[13] Stax MJ, van Montfort T, Sprenger RR, Melchers M, et al. Mucin 6 in seminal plasma bindsDC-SIGN and potently blocks dendritic cell mediated transfer of HIV-1 to CD4(+)T-lymphocytes. Virology 2009;391:203–11.
[14] Kim HY, Eyheramonho MB, Pichavant M, et al. A polymorphism in TIM1 is associated with susceptibility to severe hepatitis A virus infection in humans. J Clin Invest 2011;121:1111–8.

# CRF01_AE-Specific Neutralizing Activity Observed in Plasma Derived from HIV-1-Infected Thai Patients Residing in Northern Thailand: Comparison of Neutralizing Breadth and Potency between Plasma Derived from Rapid and Slow Progressors

**Sompong Sapsutthipas[1], Naho Tsuchiya[2], Panita Pathipavanich[3], Koya Ariyoshi[2], Pathom Sawanpanyalert[4], Naokazu Takeda[1,5], Panasda Isarangkura-na-ayuthaya[4], Masanori Kameoka[1,5,6]***

**1** Thailand-Japan Research Collaboration Center on Emerging and Re-emerging Infections (RCC-ERI), Nonthaburi, Thailand, **2** Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan, **3** Lampang Hospital, Lampang, Thailand, **4** National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand, **5** Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, **6** Department of International Health, Kobe University Graduate School of Health Sciences, Hyogo, Japan

## Abstract

***Background:*** Development of a protective vaccine against human immunodeficiency virus type 1 (HIV-1) is an important subject in the field of medical sciences; however, it has not yet been achieved. Potent and broadly neutralizing antibodies are found in the plasma of some HIV-1-infected patients, whereas such antibody responses have failed to be induced by currently used vaccine antigens. In order to develop effective vaccine antigens, it is important to reveal the molecular mechanism of how strong humoral immune responses are induced in infected patients. As part of such studies, we examined the correlation between the anti-HIV-1 neutralizing antibody response and disease progression.

***Methodology/Principal Findings:*** We evaluated the anti-HIV-1 neutralizing activity of plasma derived from 33 rapid and 34 slow progressors residing in northern Thailand. The level of neutralizing activity varied considerably among plasmas, and no statistically significant differences in the potency and breadth of neutralizing activities were observed overall between plasma derived from rapid and slow progressors; however, plasma of 4 slow progressors showed neutralizing activity against all target viruses, whereas none of the plasma of rapid progressors showed such neutralizing activity. In addition, 21% and 9% of plasmas derived from slow and rapid progressors inhibited the replication of more than 80% of CRF01_AE Env-recombinant viruses tested, respectively. Neutralization of subtype B and C Env-recombinant viruses by the selected plasma was also examined; however, these plasma samples inhibited the replication of only a few viruses tested.

***Conclusions/Significance:*** Although no statistically significant differences were observed in the potency and breadth of anti-HIV-1 neutralizing activities between plasma derived from rapid and slow progressors, several plasma samples derived from slow progressors neutralized CRF01_AE Env-recombinant viruses more frequently than those from rapid progressors. In addition, plasma derived from HIV-1-infected Thai patients showed CRF01_AE-specific neutralizing activity.

**Citation:** Sapsutthipas S, Tsuchiya N, Pathipavanich P, Ariyoshi K, Sawanpanyalert P, et al. (2013) CRF01_AE-Specific Neutralizing Activity Observed in Plasma Derived from HIV-1-Infected Thai Patients Residing in Northern Thailand: Comparison of Neutralizing Breadth and Potency between Plasma Derived from Rapid and Slow Progressors. PLoS ONE 8(1): e53920. doi:10.1371/journal.pone.0053920

**Editor:** Welkin E. Johnson, Boston College United States of America

**Received** September 6, 2012; **Accepted** December 4, 2012; **Published** January 7, 2013

**Copyright:** © 2013 Sapsutthipas et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported by the program of the Japan Initiative for Global Research Network on Infectious Diseases (J-GRID) by the Ministry of Education, Culture, Sports, Science and Technology of Japan, and the research budget of the Department of Medical Sciences, Ministry of Public Health of Thailand. The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

* E-mail: mkameoka@port.kobe-u.ac.jp

## Introduction

More than 30 million individuals are infected with human immunodeficiency virus type 1 (HIV-1) worldwide, and 2.5 million new infections have been estimated to occur yearly; therefore, an HIV-1 vaccine is urgently required. Neutralizing antibodies are a critical component of the protective immunity required for developing an effective HIV-1 vaccine [1]. In addition, it is necessary to design vaccine antigens which induce a potent and broadly neutralizing antibody response against various HIV-1 strains [1,2]. Plasma of some HIV-1-infected patients contains potent and broadly reactive neutralizing antibodies, and human monoclonal antibodies with broad and potent neutralizing activity have been established [3,4,5,6,7,8]. It is believed that understand-