T cells. This suggests that there is a saturation of langerin at higher virus concentrations that overwhelms the protective mechanism of action, so that langerin is ultimately unable to completely prevent LC infection. This might explain the recent in vivo findings that demonstrated that molecules targeting CCR5 were able to protect against mucosal transmission of SIV,[23,24] whereas the CLR inhibitor, mannan, could not prevent SIV mucosal transmission in female macaques.[24] When taken together with the human epidemiologic data cited above, the results suggest that CD4/CCR5-mediated de novo infection of LCs is the major pathway involved in sexual transmission of HIV.

When encountering pathogens, LCs and dendritic cells (DCs) recognize pathogen-associated molecular patterns (PAMP) by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs). Subsequently, this leads to production of inflammatory cytokines that ultimately promote innate responses.[25] In addition, 2 members of the nucleotide-binding oligomerization domain (NOD) family, NOD1 and NOD2, mediate recognition of special bacterial components, and function as cytosolic sensors for innate recognition of these microorganisms.[26] Many studies have shown that ligands of the NOD family can synergize with many of the TLR ligands, including TLR2 ligands, and cause induction of cytokine production.[25] For example, lipopolysaccharide (LPS) is recognized by TLR4; lipoproteins and peptidoglycans (PGNs) are recognized by TLR1/TLR2, TLR2/TLR6, NOD1, or NOD2; and flagellin is recognized by TLR5. Alternatively, viral compounds can trigger signaling through endosome-associated receptors, such as TLR3 by dsRNA, TLR7 and TLR8 by ssRNA, and TLR9 by unmethylated cytosine guanine dinucleotide DNA.

Recent studies have suggested that there is involvement of PRRs in STD and BV immune responses, and among the specific PRRs, TLR2 signaling is considered as a critical stimulus for enhanced HIV acquisition during STD and BV infections, because it has been shown to promote HIV replication in various immune cells.[27-31] Thus, we hypothesized that when STDs or BV occur, stimulation of PRRs on LCs by bacterial components may augment HIV susceptibility in LCs, thereby leading to enhanced sexual transmission of HIV. In the current study, we examined whether specific agonists for PRRs, microbial components, or whole bacteria could modulate HIV infection levels in LCs and DCs.

Methods

Reagents

Cells were stimulated with synthetic or bacteria-derived TLR and NOD agonists for 24 hours at the following concentrations: $Pam_3CysSerLys_4$ (Pam_3CSK_4, TLR1/2; 0.2-5 μg/mL), heat-killed *Listeria monocytogenes* (HKLM, TLR2; 0.2-5 $\times 10^8$/mL), poly(I:C) (TLR3; 20 μg/mL), *Escherichia coli* K12 LPS (TLR4; 10 ng/mL), flagellin from *Salmonella typhimurium* (TLR5; 2.5 μg/mL), Pam_2CGDPKHPKSF (FSL1, TLR2/6; 2-50 μg/mL), loxoribine (TLR7; 500 μM), ssRNA40/LyoVec (TLR8; 10 μg/mL), CpG oligonucleotide type B (OPN2006, TLR9; 5 μM), lipoteichoic acid (LTA; TLR2; 0.4-10 μg/mL), PGN (TLR2 and NODs; 0.2-5 μg/mL), γ-D-Glu-mDAP (iE-DAP, NOD1; 100 μg/mL), and muramyl dipeptide (MDP, NOD2; 10 μg/mL; all from InvivoGen, San Diego, CA, except for PGN from Sigma-Aldrich, St Louis, MO, and MDP from Calbiochem, Madison, WI). For the blocking experiment, the following monoclonal antibody (mAb) and reagent were used: anti-TLR2 (eBioscience, San Diego, CA) and myeloid differentiating factor 88 (MyD88) homodimerization inhibitory peptide (Imgenex, San Diego, CA). Both *Staphylococcus aureus* and *S typhimurium* were gifts from Naoki Yamamoto (University of Osaka, Osaka, Japan). *Lactobacillus spp*, group B streptococcus, *Prevotella bivia*, and *Bacteroides fragilis* were collected from HIV-seronegative women after informed consent was obtained in accordance with the Declaration of Helsinki, using an Institutional Review Board–approved protocol from the University of Yamanashi. All the bacteria were heat-inactivated for 1 hour at 57°C.

Preparation of monocyte-derived LCs and mDCs

Monocyte-derived LCs (mLCs) and mDCs were cultured from peripheral blood mononuclear cells (PBMCs), as described previously.[32] Briefly, monocytes were isolated by depletion of magnetically labeled nonmonocytes (Monocyte Isolation Kit II; Miltenyi Biotec, Auburn, CA) from plastic-adherent PBMC obtained from healthy blood donors. Monocytes were cultured in RPMI 1640 (Invitrogen Life Technologies, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Cell Culture Technologies, Gravesano, Switzerland), 100 U/mL penicillin (Invitrogen Life Technologies), 100 μg/mL streptomycin (Invitrogen Life Technologies), 2 mM L-glutamine (Invitrogen Life Technologies; complete medium) supplemented with 1000 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; R&D Systems, Minneapolis, MN), 1000 U/mL recombinant human interleukin (IL)–4 (R&D Systems), and with mLCs or without mDCs and 10 ng/mL human platelet-derived transforming growth factor (TGF)–β1 (R&D Systems) for 7 days.

HIV infection of mLCs and mDCs in vitro

Purified, pelleted, and titered HIV-1Ba-L, an R5 HIV laboratory isolate (stock at 50% tissue culture–infective dose of $10^{7.17}$/mL and 1.8×10^{10} virus particles/mL), was purchased from Advanced Biotechnologies (Columbia, MD). For some experiments, 2×10^5 mLCs and mDCs were preincubated with various agonists or inhibitors, and then HIVBa-L at a 1/100 final dilution was added for 2 hours at 37°C. After incubation, cells were harvested, washed 3 times, resuspended in complete medium supplemented with GM-CSF and IL-4 and/or TGF-β1, and cultured for an additional 7 days at the same cellular concentration. Half of the total volume of the medium was replaced with fresh complete medium and GM-CSF and IL-4 every other day. HIV-infected cells were assessed by HIV p24 intracellular staining. HIV infection levels were expressed as a normalized percentage of the positive cells for HIV p24 using a calculated fold difference compared with the mean percentage of the positive cells for HIV p24 in untreated cells.

HIV infection of skin explants ex vivo

Epithelial sheets were obtained from suction blister roofs from HIV-negative healthy donors. Droplets (50 μL) containing HIVBa-L at a 1/100 final dilution were placed on the inside surfaces of sterile plastic culture dish covers. Explants were draped over droplets with the basal epithelial cell surface facing downward. Virus and explants were incubated together in this manner at 37°C for 2 hours. Explants were washed and then floated with the basal epithelial cell sides down. The emigrating cells from the epidermal sheets were collected 3 days after the HIV exposure. HIV-infected LCs were assessed using HIV p24 staining. In some experiments, 1×10^4 emigrated LCs were cocultured with 2×10^6 allogeneic $CD4^+$ T cells for 12 days. Supernatants were harvested every third day and examined for HIV p24 protein content by enzyme-linked immunosorbent assay (ELISA).

Flow cytometry

Single-cell suspensions were stained using the following anti–human mAbs: anti-CD83 (BD Biosciences, San Jose, CA), anti-CD86 (BD Biosciences), anti-CD4 (Beckman Coulter, Fullerton, CA), anti-CCR5 (R&D Systems), anti–DC-SIGN (R&D Systems) directly conjugated to fluorescein isothiocyanate (FITC), antilangerin (Immunotech, Fullerton, CA), anti–TLR-1, -2, -4, -3, -9 (eBioscience) directly conjugated to phycoerythrin (PE), and anti-CD11c (BD Biosciences) and anti-CD1a (BD Biosciences) directly conjugated to allophycocyanin. Biotinylated anti–TLR-6 (eBioscience), and in some experiments, biotinylated antilangerin (R&D Systems) were stained by streptavidin-FITC or allophycocyanin conjugate (BD Biosciences), respectively, and purified anti–E-cadherin (R&D Systems) and anti–TLR-7, -8 (eBioscience) were detected by

FITC-conjugated goat anti–mouse or FITC-conjugated rabbit F(ab′)$_2$, respectively. Cells were incubated with Abs for 30 minutes at 4°C, washed 3 times in staining buffer, and examined by FACSCalibur. For measurement of intracellular TLR-3, -9, -7, and -8, a fixation and permeabilization procedure was performed (Cytofix/Cytoperm; BD Biosciences). For HIV p24 intracellular staining, cells were incubated with 10 μg/mL PE-conjugated mouse anti–human langerin mAb and allophycocyanin-conjugated mouse anti–human CD11c mAb for 30 minutes at 4°C. Cells were then fixed and permeabilized with Cytofix/Cytoperm reagents for 20 minutes at 4°C. Cells were then incubated with 10 μg/mL FITC-conjugated mouse anti-HIV p24 mAb (Beckman Coulter) diluted for 30 minutes at 4°C, with the quantified numbers of HIV-infected cells determined by FACSCalibur. mLCs were gated on langerin$^+$ CD11c$^+$ cells, and mDCs were gated on CD11c$^+$ cells.

Real-time quantitative reverse transcription–polymerase chain reaction analysis

Relative mRNA expression was determined by real-time polymerase chain reaction (PCR) using an ABI PRISM 5500 Sequence Detection System (Applied Biosystems, Foster City, CA) with SYBR Green I dye (QIAGEN, Valencia, CA), according to the manufacturer's instructions. Total RNA was isolated using TRIzol (Invitrogen Life Technologies), and cDNA was synthesized using the SuperScript system (Invitrogen Life Technologies). Primers corresponding to human TLR-3, TLR-9, NOD1, NOD2, APOBEC3G, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) were designed by Takara Bio (Shiga, Japan). Cycle threshold numbers (Ct) were derived from the exponential phase of the PCR amplification. Fold differences in the expression of gene x in the cell populations y and z were derived by $2k$, where $k = (Ctx - CtG3PDH)y - (Ctx - CtG3PDH)z$.

ELISA

mLCs and mDCs were (10^6 cells/mL) stimulated for 24 hours with TLR and NOD ligands. The culture supernatants were collected after centrifugation, and stored at −80°C for cytokine measurements. The concentrations of cytokines (IL-6, IL-8, tumor necrosis factor (TNF)–α, and IL-10) in the culture supernatants were measured using cytometric bead array (CBA) assays (BD Biosciences). After acquisition of sample data by flow cytometry, results were analyzed using the BD Biosciences CBA analysis software. For measurement of HIV p24 protein levels, supernatants were collected, inactivated with Triton X-100 (Sigma-Aldrich; 2% final concentration), and kept frozen until measurements of HIV p24 protein levels were performed by ELISA (ZeptoMetrix, Buffalo, NY).

Western blot analysis

Proteins of the cells were extracted using a 15-minute incubation in complete lysis buffer containing a protease inhibitor. Equal amounts of protein were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto a transfer membrane (Daiichikagaku, Tokyo, Japan). Western blot was performed to detect human APOBEC3G and used 2.5 μg/mL rabbit immunoglobulin (Ig) G anti-APOBEC3G polyclonal antibody (Abcam, Cambridge, MA). Blots were incubated with the horseradish peroxidase (HRP)–linked secondary antibody, goat anti–rabbit IgG (Pierce, Rockford, IL). Analyses were performed using the HRP Western blot detection system (Pierce). To test for the A3G subcellular localization in some experiments, we separated the pellet (P) fraction, as has been described previously.[33] Briefly, cells were lysed with ice-cold lysis buffer (Sigma-Aldrich) for 30 minutes and centrifuged at 35 000 rpm (TLA-120.2 rotor in Optima TLX, Beckman Coulter) for 1.5 hours. Equal volumes of P and supernatant (SN) were loaded on gel and analyzed by Western blotting.

Results

Because ex vivo purification of epidermal LCs from human skin results in a significant spontaneous maturation, it is difficult to monitor PAMP-induced maturation of fresh isolated LCs.[34,35] Most of the phenotypic characteristics of LCs depend upon the presence of TGF-β1 in the epidermal microenvironment,[36,37] because TGF-β1 promotes the development of LC-like DCs from peripheral blood monocytes.[32,38] In general, epidermal LCs can be discriminated from DCs by the expression of E-cadherin and langerin. In 5 experiments, we generated monocyte-derived LCs (mLC) and found expression levels of E-cadherin$^+$ cells and langerin$^+$ cells to be 90.7 + 9.5% (mean + SD) and 35.1 + 14.5%, respectively (data not shown). Based on these findings, we used fluorescence-activated cell sorter (FACS) to isolate highly purified langerin-positive mLCs that could be used as surrogate cells for epidermal LCs. Alternatively, mLCs were identified by gating langerin-positive cells in FACS analyses. To test activation of LCs and DCs after TLR stimulation, mLCs and mDCs were challenged with Pam_3CSK_4 for TLR1/2, heat-killed *L monocytogenes* (HKLM) for TLR2, FSL1 for TLR2/6, poly(I:C) for TLR3, LPS for TLR4, flagellin for TLR5, loxoribine for TLR7, ssRNA40 for TLR8, and ODN2006 for TLR9 for 24 hours. Subsequently, we examined expression of CD86, a marker of LC/DC activation. Similar to previously reported findings,[35,39,40] there was significant up-regulation of CD86 on langerin$^+$ mLCs by Pam_3CSK_4, HKLM, FSL1, and poly(I:C), respectively (Figure 1A). These ligands also induced up-regulation of another activation marker, CD83, and stimulated IL-6 and IL-8 production by langerin$^+$ mLCs (data not shown). Bacteria-derived TLR2 agonists, LTA and PGN, also up-regulated expression levels of these activation markers in a dose-dependent manner, whereas the TLR4, 5, 7, 8, and 9 agonists did not (Figures 1A, 2A, and data not shown). These results indicated that mLCs express functional TLR1, TLR2, TLR3, and TLR6 proteins. We also used anti-TLR–specific mAb labeling, which made it possible to confirm the expression of these TLRs on mLCs (data not shown).

To determine the effects of TLR signaling on HIV susceptibility or HIV replication of mLCs or mDCs, cells were stimulated with TLR agonists for 24 hours before and after exposure to HIV. To specifically identify HIV-infected cells on a single-cell level, mLCs or mDCs were collected 7 days after HIV exposure and then double-stained with anti-HIV p24 mAb along with other antibodies directed against surface markers for mLCs or mDCs. This methodology made it possible to determine the number of HIV p24$^+$ cells within langerin$^+$/CD11c$^+$ mLCs and within CD11c$^+$ mDCs. Uninfected cells stained with anti-p24 mAb and HIV-infected cells stained with isotype control antibody always showed less than 0.10% positivity, thereby confirming the specificity of the p24 staining (data not shown). Surprisingly, incubation with the TLR2 agonists, including HKLM, Pam_3CSK_4, and FSL1, before and after HIV exposure dramatically enhanced HIV infection levels in mLCs, whereas incubation with the other agonists did not (Figure 1B,C). Similar effects were observed with the bacteria-derived TLR2 agonists, LTA and PGN, in a dose-dependent manner (Figure 2B,C). We also used ELISA to determine HIV p24 protein levels in mLC culture supernatants. ELISA results correlated with the single-cell flow cytometric data (data not shown). As was seen with LCs, preincubation with TLR2 or TLR3 agonists induced maturation of mDCs (Figure 1D). By contrast to the mLC findings, however, there was partial inhibition of HIV infection in mDCs when these cells were preincubated with TLR2 agonists (Figure 1E,F). Furthermore, TLR3 ligation by poly(I:C) before and after HIV exposures dramatically decreased HIV infection levels in mDCs. Thus, these data suggest that TLR signaling effects on HIV susceptibility and productive infection are differently regulated in LCs and DCs.

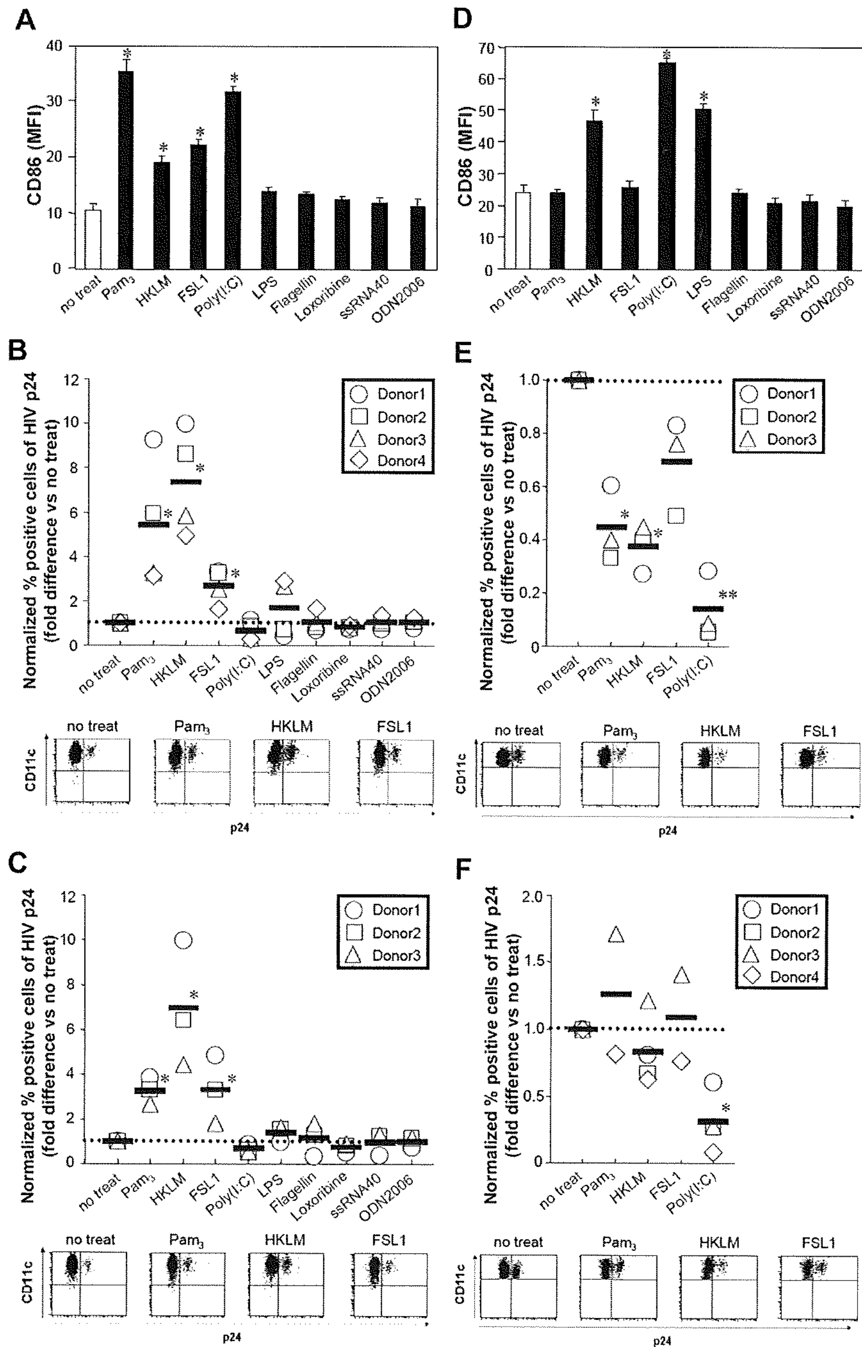

Figure 1. TLR stimuli modulate CD86 expression, HIV susceptibility, and HIV replication in mLCs and mDCs. mLCs and mDCs were cultured in medium alone or with 5 μg/mL Pam_3CSK_4, 5 × 10^8/mL HKLM, 20 μg/mL poly(I:C), 10 ng/mL LPS, 2.5 μg/mL flagellin, 50 μg/mL FSL1, 500 μM loxoribine, 10 μg/mL ssRNA40, or 5 μM ODN2006 for 24 hours. The expression of CD86 was examined (A, mLC; D, mDC; MFI, mean fluorescence intensity). Results are shown as means plus or minus SD ($*P < .05$). mLCs (B,C) or mDCs (E,F) were stimulated with the indicated TLR agonists for 24 hours before (B,E) and after (C,F) HIV exposure. To identify HIV infection levels, mLCs or mDCs were collected 7 days after the HIV exposure, and HIV $p24^+$ cells were assessed in $langerin^+$ $CD11c^+$ mLCs or $CD11c^+$ mDCs (expressed as normalized percentage of positive cells for HIV p24, as has been described in "HIV infection of mLCs and mDCs in vitro"). Mean values in mDCs and mLCs obtained from different donors are shown as horizontal marks. Representative FACS analyses of CD11c and p24 mAb double-stained cells after TLR2 stimulations are shown.

To understand how HIV traverses skin and genital mucosa, we recently developed an ex vivo model whereby resident LCs within epithelial tissue explants are exposed to HIV and then allowed to emigrate from tissue, thus mimicking conditions that occur after mucosal exposure to HIV.[11,41] Using this model, we examined whether TLR2 stimulation enhanced HIV infection of epidermal LCs, and whether TLR2 stimulation enhanced subsequent HIV transmission from LCs to $CD4^+$ T cells. Similar to the findings observed within mLCs, preincubation of epithelial sheets with HKLM significantly enhanced HIV infection levels in epidermal LCs as well as HIV transmission from emigrated LCs to $CD4^+$ T cells (Figure 2D,E). As controls, epithelial sheets preincubated with poly(I:C) failed to enhance HIV infection levels in LCs, and led to decreased HIV transmission from LCs to $CD4^+$ T cells.

To test whether LCs or DCs express NOD1 and NOD2, mLCs and mDCs were challenged with PGN, the NOD1-specific agonist iE-DAP, and the NOD2-specific agonist MDP. These stimuli caused significant up-regulation of CD86 on mLCs and mDCs (Figure 3B,F). Quantitative reverse transcription (RT)–PCR confirmed the expression of NOD1 and NOD2 by these cells (Figure 3A,E). To determine effects of these NOD stimulators on HIV infection, cells were incubated with PGN, iE-DAP, or MDP for 24 hours before and after HIV exposure. Incubation with iE-DAP or MDP before and after HIV exposure significantly reduced HIV infection levels in mDCs (Figure 3G,H), but not in mLCs. Instead, these stimuli caused slight increases in HIV infection in mLCs (Figure 3C,D). Compared with iE-DAP and MDP, PGN significantly increased HIV infection levels in mLCs, suggesting that the

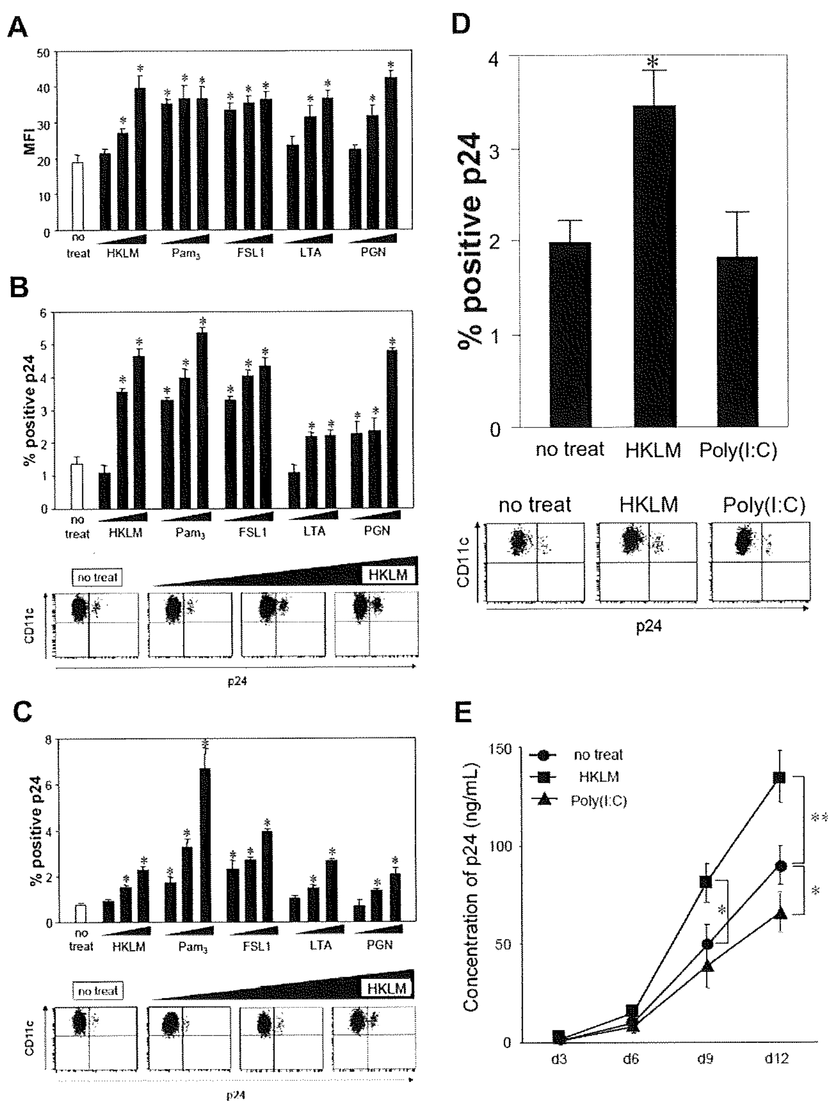

Figure 2. TLR2 stimuli enhance HIV susceptibility in mLCs and resident epidermal LCs. mLCs were stimulated via TLR2 using heat-killed Gram$^+$ bacteria (HKLM; 0.2, 1, 5 $\times$ 10^8/mL), synthetic agonists (Pam_3CSK_4, 0.2, 1, 5 μg/mL; FSL1, 2, 10, 50 μg/mL), or Gram$^+$ bacterial components (LTA, 0.4, 2, 10 μg/mL; PGN, 0.2, 1, 5 μg/mL) for 24 hours at various concentrations, as has been described in "Methods" (A-C). Cells were stimulated via TLR2 before (B) and after (C) HIV exposure. The expression of CD86 (A) and percentage of positive cells for HIV p24 (B,C) were assessed in langerin$^+$ CD11c$^+$ mLCs or CD11c$^+$ mDCs (MFI, mean fluorescence intensity). Epithelial sheets obtained from suction blister roofs were preincubated with 5 $\times$ 10^8/mL HKLM or 20 μg/mL poly(I:C) for 2 hours, and then exposed to R5 HIV (D). The emigrating cells from the epidermal sheets were collected 3 days after HIV exposure, and HIV-infected epidermal LCs were assessed by HIV p24 intracellular staining (D). Representative FACS analyses of CD11c and p24 mAb double-stained cells are shown. For the assessment of HIV transmission from LCs to CD4$^+$ T cells, emigrated LCs were collected 3 days after HIV exposure and washed, and then 10^4 LCs were cocultured with 2 $\times$ 10^6 allogeneic CD4$^+$ T cells for 12 days. p24 protein levels in culture supernatants were assessed by ELISA on the indicated days (E). Results are shown as means plus or minus SD (n = 3). *$P < .05$; **$P < .01$. All data shown represent at least 2 separate experiments.

infection-enhancing effects of PGN are primarily mediated by a TLR2 signaling pathway. This assertion is also supported by the fact that anti-TLR2 mAb significantly blocked the ability of PGN to enhance HIV infection in mLCs (Figure 4A).

In contrast to the findings observed for Gram$^+$ bacteria-derived or synthetic agonists for TLR2, LPS failed to enhance HIV infection levels in mLCs (Figure 1). This is not surprising, because LCs have been shown to lack expression of the functional TLR4 that is involved in the recognition of Gram$^-$ bacterial components.[35,39,42] We were also unable to detect expression of TLR4 on mLCs (data not shown). The distinct responsiveness to TLR2 and TLR4 agonists suggests that Gram$^+$ or Gram$^-$ bacteria may have differential effects on HIV infection levels in LCs. Therefore, we tested effects of whole Gram$^+$ and Gram$^-$ bacteria on HIV susceptibility and HIV replication in LCs. As expected, incubation with *S aureus* before and after the HIV exposures dramatically enhanced HIV infection levels in langerin$^+$/CD11c$^+$ mLCs, whereas *S typhimurium* showed little or no effect (Figure 5A,B). Similarly, preincubation with group B streptococcus significantly enhanced HIV infection levels in mLCs, whereas *Listeria* spp did not (Figure 5C). In contrast, incubation with *S aureus* or *S typhimurium* decreased HIV infection levels in mDCs (Figure 5D,E).

We next tested the capacity of mAbs against TLR2 and a MyD88 inhibitor for their ability to inhibit TLR2-enhanced HIV replication in mLCs. Anti-TLR2 mAb demonstrated a significant blocking capacity for HKLM- and PGN-enhanced HIV replication in mLCs, whereas isotype control IgG did not (Figure 4A). Furthermore, when mLCs were stimulated by HKLM or PGN in the presence of a MyD88 inhibitor peptide, we did not observe TLR2-enhanced infection (Figure 4B), confirming that increased HIV replication that occurs after exposure of mLC to Gram$^+$ bacteria is mediated by TLR2/MyD88 signaling.

We have previously reported that mLCs and mDCs express CD4 and CCR5 with expression levels for these HIV receptors lower on mLCs compared with mDCs.[32] Similar to previous DC studies,[32,43] mDCs matured in the presence of HKLM or PGN decreased CCR5 surface expression, whereas CCR5 levels on mLCs were not significantly down-regulated after exposure to these stimuli (Figure 6B,E). Interestingly, these stimuli significantly up-regulated CD4 expression on mLCs, whereas CD4 levels on mDCs were unchanged (Figure 6A,D). Further investigation of the expression levels for the surface CLRs determined that langerin expression on mLCs remained unchanged, whereas DC-SIGN levels on mDCs were markedly down-regulated after HKLM- or PGN-induced maturation (Figure 6C,F). Thus, there was a partial

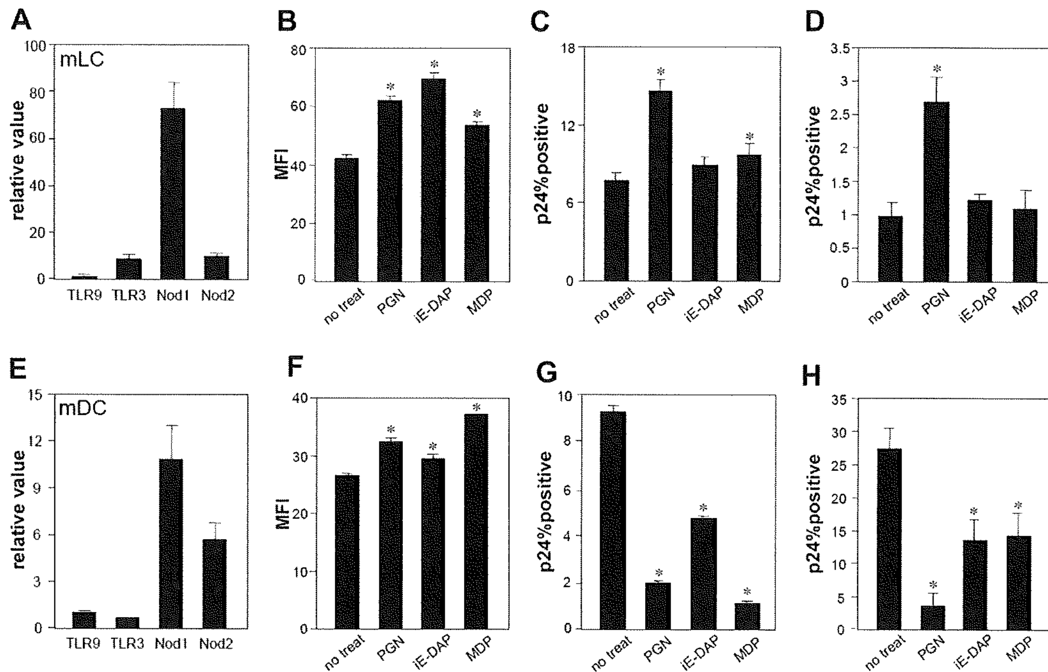

Figure 3. mLCs and mDCs express functional NOD1 and NOD2. Expression of NOD1 and NOD2, and TLR3 and TLR9, which were used as controls, in mLCs and mDCs was assessed using real-time quantitative RT-PCR analysis (qPCR; A, mLCs; E, mDCs). mLCs and mDCs were stimulated by PGN (5 μg/mL), iE-DAP (for NOD1, 100 μg/mL), and MDP (for NOD2, 10 μg/mL) for 24 hours. mLCs (B-D) or mDCs (F-H) were stimulated via NOD receptors before (C,G) and after (D,H) HIV exposure. The expression of CD86 (B,F) and percentage of positive cells for HIV p24 (C,D,G,H) was assessed in langerin$^+$ CD11c$^+$ mLCs or CD11c$^+$ mDCs (MFI, mean fluorescence intensity). Results are shown as means plus or minus SD (*$P < .05$). All data shown represent at least 2 separate experiments.

correlation between cell surface expression levels of the HIV receptors and CLRs with the HIV susceptibility phenotype observed in mLCs and mDCs stimulated by bacterial components via TLR2 (Figure 1).

We found that TLR2 stimulation enhanced HIV infection levels in mLCs, even when mLCs were incubated with TLR2 agonists after the HIV exposures. We therefore considered postentry cellular restriction factors for HIV. Recently, Pion et al have reported that between the 2 families that have been shown to restrict HIV infection (tripartite motif [TRIM]/APOBEC),[33] T5α and A3F were not strong restriction factors in mDCs.[44] However, A3G did function as a potent postentry cellular restriction factor for HIV in mDCs. To analyze whether TLR stimulation modulated A3G activity in mLCs and mDCs, we activated these cells with TLR agonists and then quantified A3G expression. Interestingly,

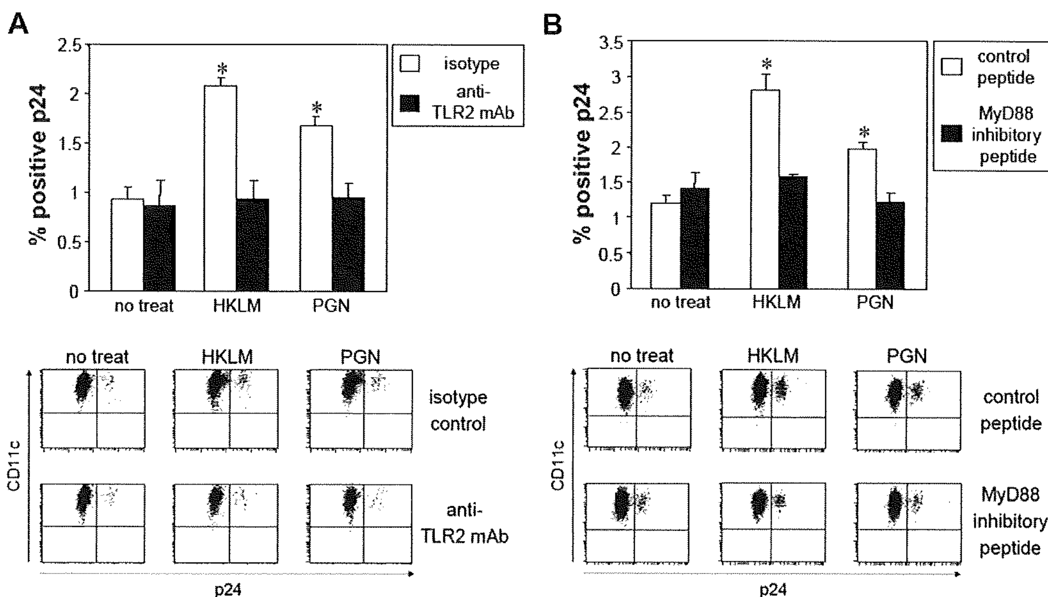

Figure 4. Enhancement of HIV replication by TLR2 ligation in mLCs is dependent on the TLR2-MyD88 signal pathway. After HIV exposure, mLC were preincubated with anti-TLR2 mAb (10 μg/mL) for 30 minutes or MyD88 inhibitory peptide (100 μM) for 24 hours, followed by coculturing with 5×10^8/mL HKLM, or 5 μg/mL PGN for 24 hours. Isotype control or control peptide experiments were performed using the same conditions. HIV-infected mLCs were assessed 7 days later by HIV p24 intracellular staining. (A) Anti-TLR2 mAb; (B) MyD88 inhibitory peptide. Summary of experiments and representative FACS analyses of CD11c and p24 mAb double-stained cells are shown. Results are shown as means plus or minus SD (n = 3). *$P < .05$. All data shown represent at least 2 separate experiments.

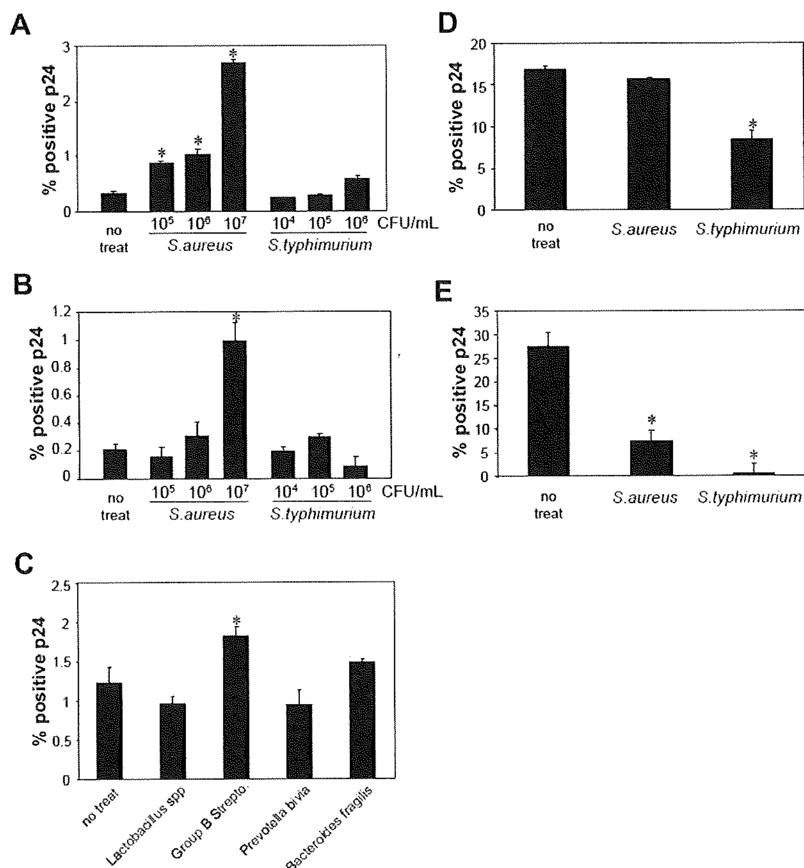

Figure 5. Gram+ bacteria enhance both HIV susceptibility and replication in the mLCs. mLCs and mDCs were incubated with heat-killed whole bacteria, including *S aureus* (10^{5-7} CFU/mL), *S typhimurium* (10^{4-6} CFU/mL), *L spp* (10^6 CFU/mL), group B streptococcus (10^6 CFU/mL), *P bivia* (10^6 CFU/mL), or *B fragilis* (10^6 CFU/mL) for 24 hours before (A,C,D) and after (B,E) HIV exposure. The percentage of positive cells for HIV p24 was assessed in langerin+ CD11c+ mLCs (A-C) or CD11c+ mDCs (D,E). Results are shown as means plus or minus SD (*$P < .05$). All data shown represent at least 2 separate experiments.

higher levels of A3G were expressed by mLCs versus mDCs (Figure 7). Consistent with a previous report,[33] A3G levels were significantly increased in mDCs by LPS (data not shown). In addition, A3G levels were increased by HKLM, Pam_3CSK_4, and poly(I:C) in mDCs (Figure 7A,B). Surprisingly, the same TLR2 ligation by HKLM and Pam_3CSK_4 resulted in a significant reduction of A3G levels in mLCs. Poly(I:C) increased A3G levels in mLCs, but not in mDCs.

Cellular A3G, which resides as a low molecular mass (LMM) active form, can function as a postentry restriction factor for HIV during reverse transcription in mDCs. To evaluate whether A3G was present in a LMM active form in mLCs, we used a previously reported method for mDCs that used high-speed centrifugation to separate the cytosolic proteins from the pellet (P) fraction (which contains heavy membranes, endosomes, and nuclei).[33] Using this method, cytosolic A3G was extracted in the SN fraction (A3G SN),

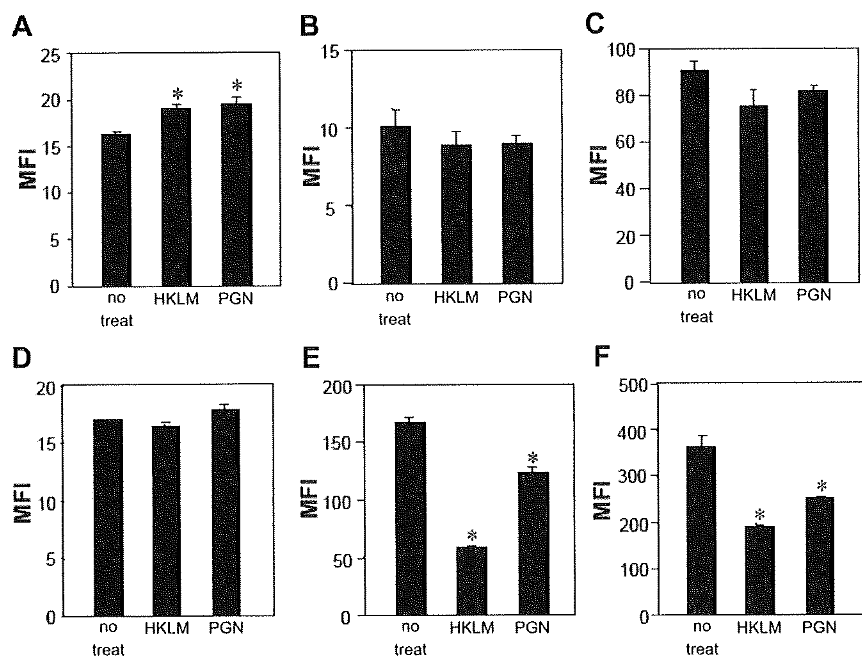

Figure 6. HKLM and PGN modulate surface expression of HIV receptors on mLCs and mDCs. mLCs and mDCs were cultured in medium alone or with 5 × 10^8/mL HKLM or 5 μg/mL PGN for 24 hours, and the expression of CD4 (A,D), CCR5 (B,E), langerin (C), or DC-SIGN (F) was then examined (A-C, mLCs; D-F, mDCs; MFI). Results are shown as means plus or minus SD (*$P < .05$). All data shown represent at least 2 separate experiments.

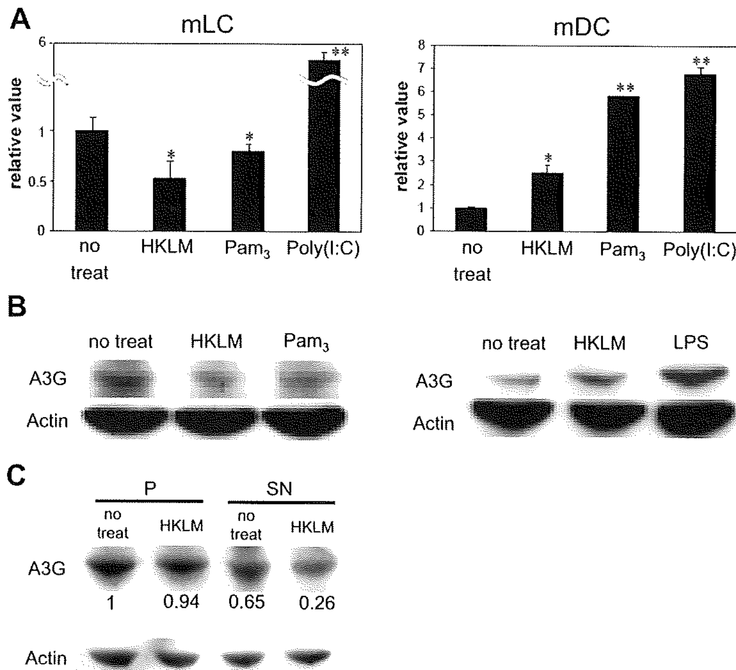

Figure 7. TLR2, but not TLR3, stimulation negatively modulates A3G expression levels in mLCs. mLCs and mDCs were cultured in medium alone or with 5×10^8/mL HKLM, 5 μg/mL Pam_3CSK_4, or 20 μg/mL poly(I:C) for 24 hours before HIV exposure. Expression of A3G in mLCs and mDCs was assessed using qPCR (A) or Western blotting (B). Results are shown as means plus or minus SD (*$P < .05$; **$P < .01$). Alternatively, cells were lysed and centrifuged at high speed, with equal volumes of the P fraction and the SN fraction, and then analyzed for A3G expression by Western blotting. A3G expression was quantified, with a value of 1 assigned to the pellet fraction in nontreated cells (C). All data shown represent at least 2 separate experiments.

whereas the higher molecular forms of A3G were left in the P fraction. Our results indicated that A3G SN was present in mLCs, although it was significantly down-regulated after the HKLM stimulation (Figure 7C). When taken together, these results suggest that the proportion of the A3G SN is well correlated with the HIV restriction phenotype in mLCs (Figures 1, 7A,B).

Discussion

LCs are thought to be the initial cellular targets that play a pivotal role in the viral dissemination during sexual transmission of HIV. To understand the biologic mechanisms by which STDs and BV contribute to increase in HIV acquisition, we tested the hypothesis that bacterial recognition pathways are responsible for modulating LC susceptibility to HIV. We demonstrated that Gram+ bacteria (eg, HKLM) enhance HIV susceptibility in langerin+ mLCs as well as in epidermal LCs. Among the TLR1-9-specific agonists, only TLR2 and TLR3 agonists were able to induce significant maturation and cytokine production in mLCs. Interestingly, TLR2 agonists enhanced HIV susceptibility in mLCs, whereas other TLR-specific agonists, including TLR3, did not. Thus, our findings suggest that TLR2 plays a central role in Gram+ bacteria-enhanced infection that occurs in LCs. This statement is also supported by our findings that TLR2 mAb exhibited a significant ability to block the enhancement of HIV infection in LCs by Gram+ bacteria. It should be noted that the stimulation of TLR2 and TLR3 decreased HIV susceptibility in mDCs. Although it is unlikely that submucosal DCs are directly infected with HIV in the sexual transmission of HIV, this may occur in the presence of ulcerating STD infections. In these instances, our data suggest that TLR2 stimulation by bacterial components may act to protect DC from HIV infection. Overall, the current findings suggest that TLR2 stimulation might induce a cell type–specific pathway in LCs that enhances HIV susceptibility of these cells.

There are several possible mechanisms that could account for enhancement of HIV infection in LCs via Gram+ bacteria/TLR2. We demonstrated that HKLM and PGN significantly increased surface expression of CD4 in mLCs, which may be involved in enhanced HIV susceptibility of LCs. In addition, TLR2 ligation may also affect HIV infection in mLC postviral entry, because the enhancement of HIV infection was observed even if the cells were stimulated after HIV exposure. Recent studies have revealed a crucial contribution for A3G in the susceptibility of various cell types to HIV. In mDCs, it has been shown that A3G functions as an especially strong postentry restriction factor, and its expression level is significantly increased by TLR4 ligation, which further restricts HIV infection.[33] Our results demonstrated that TLR2 ligation significantly reduced A3G levels in mLCs, and the proportion of A3G SN was inversely correlated with HIV infection levels in these cells (Figures 1, 7), suggesting that Gram+ bacteria enhance HIV infection in LCs by decreasing A3G expression. Interestingly, there was no significant difference between HIV infection levels in mLCs treated by TLR2 ligands before infection compared with treatment after infection (Figure 1B,C). In contrast, mDC treated by HKLM or Pam_3CSK_4 demonstrated significantly higher infection levels with pretreatment compared with posttreatment (Figure 1E,F, and data not shown). These results suggest that TLR2-mediated regulation of cell surface HIV receptor expression may contribute to decreased HIV infection in mDCs, but that regulation of cell surface HIV receptor expression contributes relatively little to TLR2-mediated enhancement of HIV infection in mLCs.

We found that A3G expression is strongly induced in mDCs upon TLR2 stimulation; however, it failed to limit HIV replication even if cells were stimulated after infection (Figures 1F, 7A). Because other factors (eg, APOBEC family other than A3G, TRIM family, chemokines, or cytokines) can affect HIV infection levels in macrophages,[45] these conditions may supercede restriction effects controlled by A3G in HIV-infected TLR2-stimulated DCs. Recent studies have shown that interferons are strong inducers of A3G, and interferon-β expression is induced in mLCs by activation with TLR3, but not TLR2, agonists.[45,46] In our study, TLR3, but not TLR2, agonists significantly increased A3G levels in mLCs (Figure 7A), suggesting that interferons produced by LCs may be involved in the regulation of A3G by TLRs. Thus, mucosal application of

TLR3 agonists or interferons may be considered as alternative approaches to decrease sexual transmission of HIV.

Recently, de Jong et al reported that TNF-α stimulated HIV transmission from LCs to T cells by increasing HIV replication in LCs, whereas Pam_3CSK_4 increased LC capture of HIV and subsequent *trans* infection of T cells.[47] In contrast to our findings, they showed Pam_3CSK_4 alone did not increase HIV infection in LCs. Considering the indirect effect of TNF-α, we found that TLR2 (but not TLR3) agonists induced abundant TNF-α production by mLCs (data not shown), consistent with previous findings.[40,46] It is possible that the enhancement of HIV infection in LCs by TLR2 stimuli observed in our study might be due, at least in part, to increased production of TNF-α by LCs.

Bacterial components are either liberated from live bacteria or released upon bacterial lysis. In the current study, we were able to demonstrate for the first time that mLCs express functional NOD1 and NOD2. Although their ligands induced significant maturation of mLCs, effects on HIV infection were quite limited compared with PGN. Furthermore, TLR2 mAb significantly blocked the capacity of PGN to enhance HIV infection, suggesting that PGN is modulating HIV infection levels in LCs via TLR2-MyD88 rather than through NOD pathways.

In most healthy women, the vaginal microflora comprises large quantities of a limited number of lactobacilli species, which appears to confer some protection against HIV acquisition.[1] BV is characterized by a reduction in the number of lactobacilli and the growth of several species of anaerobic bacteria. Interestingly, in this regard, we found that *Listeria* spp as well as anaerobic bacteria did not significantly affect HIV infection levels in langerin$^+$ mLCs. The failure of anaerobic bacteria to enhance HIV susceptibility in LCs might be because anaerobic bacteria, including *P bivia* and *B fragilis*, are Gram$^-$ bacteria recognized mainly by TLR4.[50] In addition, it has been recently reported that *Listeria* spp did not induce TNF-α secretion and HIV LTR activation in THP-1 cells that express TLR2.[37] Taken together, in addition to bacterial components, we also demonstrated that whole Gram$^+$ bacteria, including *L monocytogenes*, *S aureus*, and group B streptococcus, enhanced HIV susceptibility in langerin$^+$ mLCs. Nonulcerative STDs, such as *Neisseria gonorrhoeae*, *Chlamydia trichomatis*, and *Candida* spp, have been shown to stimulate cells via TLR2,[28,48,49] suggesting that these organisms may also enhance HIV infections in LCs like in other immune cells.[27-31]

We demonstrated that Gram$^+$ bacterial components directly augmented HIV infection in LCs by activating TLR2. It is possible that TLR2 stimulation of LC infection underlies, at least in part, enhanced sexual transmission of HIV in settings where there is concomitant STD infection. Based on this theory, blockade of TLR2 signaling might be considered as an alternate approach to decrease sexual transmission of HIV.

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Authorship

Contribution: Y.O. performed experiments and analyzed data; T. Kawamura directed and performed experiments, analyzed data, and wrote the manuscript; T. Kimura performed experiments and analyzed data; M.I. contributed analytical tools; A.B. codirected experiments and wrote the manuscript; and S.S. codirected experiments.

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Gene Corner

Novel and recurrent nonsense mutation of the *SLC39A4* gene in Japanese patients with acrodermatitis enteropathica

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Acrodermatitis enteropathica (AE, OMIM 201100) is a rare autosomal recessive disease characterized by hypozincaemia derived from the inability to absorb intestinal zinc. The clinical manifestations of AE include growth retardation, diarrhoea, alopecia, repeated infections due to immune dysfunction, and characteristic skin lesions on acral and periorificial areas. The hypozincaemia in AE is believed to be caused by a defect in a zinc transporting protein. Recently, the gene encoding this zinc transporting protein was identified on chromosomal region 8q24.3,[1] and subsequently *SLC39A4* located on the chromosome was identified as the AE gene.[2,3] *SLC39A4* encodes the ZIP4 zinc transporter, which transports zinc into the cytoplasm, mainly in the intestine. Here, we report two Japanese families with AE, in which a novel nonsense mutation in the *SCL39A4* gene was identified.

Cases and methods

Family 1

A 47-year-old Japanese man was previously diagnosed with AE and treated with oral zinc sulphate at other clinics since he was 1 year old. He visited the Department of Dermatology, University of Yamanashi Hospital with severe eruptions and diarrhoea because he had recently stopped taking his prescribed medicine. Physical examination revealed symmetrical erythema, erosions, blisters and crusts in perioral and anogenital lesions and acral sites of the extremities (Fig. 1a–d). In addition, he had nail dystrophy (Fig. 1a) and alopecia (Fig. 1c,d). Skin biopsy from an erythematous area on his eyebrow region showed confluent parakeratosis, scattered dyskeratotic cells, spongiotic change of epidermal keratinocytes and perivascular lymphocyte infiltration. Laboratory examinations, including a full blood count and liver and kidney function tests, were within normal range. The serum zinc level was only 6 $\mu g\ dL^{-1}$ (normal 60–120) and the alkaline phosphatase level was 100 IU L^{-1} (normal 441–825). No other affected individuals and no consanguinity were reported in the family. The proband showed marked clinical improvement after a week of administering oral zinc supplements.

Family 2

A 9-month-old Japanese girl was referred to the Department of Dermatology, Hirosaki University Hospital for evaluation of exanthema on the face, hands, feet and anogenital regions, which had developed approximately 8 months after birth. Examination revealed encrusted erosive lesions associated with inflammatory erythema in the periorificial (Fig. 1e) and anogenital regions and the fingers and toes. Tense blisters were also seen on the toes (Fig. 1f). Laboratory data were within normal limits except for reduced serum zinc (11 $\mu g\ dL^{-1}$) and alkaline phosphatase (89 IU L^{-1}) levels. The parents were healthy and reportedly not related. The mother was in her third month of pregnancy when she first visited our clinic.

After obtaining written informed consent, we performed a mutational analysis of the *SLC39A4* gene using genomic DNA extracted from peripheral blood leucocytes as described previously.[4] The study protocol was approved by the Ethics Committee of Hirosaki University Hospital (No. 2007-067) and conducted according to the Declaration of Helsinki Principles.

Results and discussion

Direct sequencing of all exons and their flanking intronic regions of the *SLC39A4* gene, which was polymerase chain reaction (PCR)-amplified from genomic DNA of the proband in family 1, identified a homozygous c.1438G>T mutation in exon 9 (Fig. 2a). This transversion was predicted to create a premature termination codon (PTC) at amino acid residue 480 (p.Glu480Stop). To our knowledge, this mutation has not been previously reported in the literature. Interestingly, the same homozygous mutation was also detected in the proband of family 2. In the normal allele, *NciI* cleaved the 206-bp PCR product spanning exon 9 into fragments of 80, 59, 45 and 22 bp, whereas digestion of the mutated allele resulted in fragments of 104, 80 and 22 bp. Restriction enzyme fragment length polymorphism analysis using *NciI* demonstrated that the parents in family 2 were heterozygous for the mutation (Fig. 2b). The two families described in this report originate from different districts in Japan and are apparently unrelated. To test this hypothesis, we genotyped five short tandem repeat (STR) markers, D8S1727, D8S1744, D8S1836, D8S373 and D8S2334[1] flanking the *SLC39A4* gene, by capillary electrophoresis of denatured PCR products harbouring the STR markers

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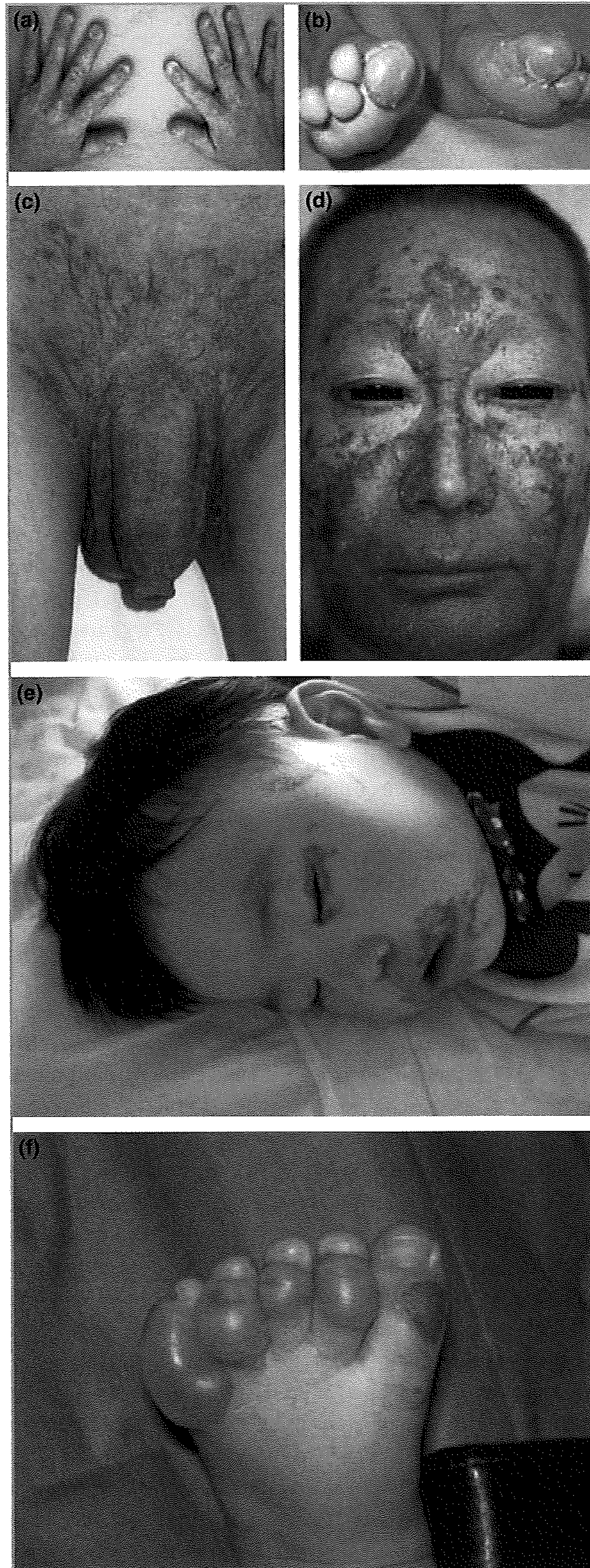
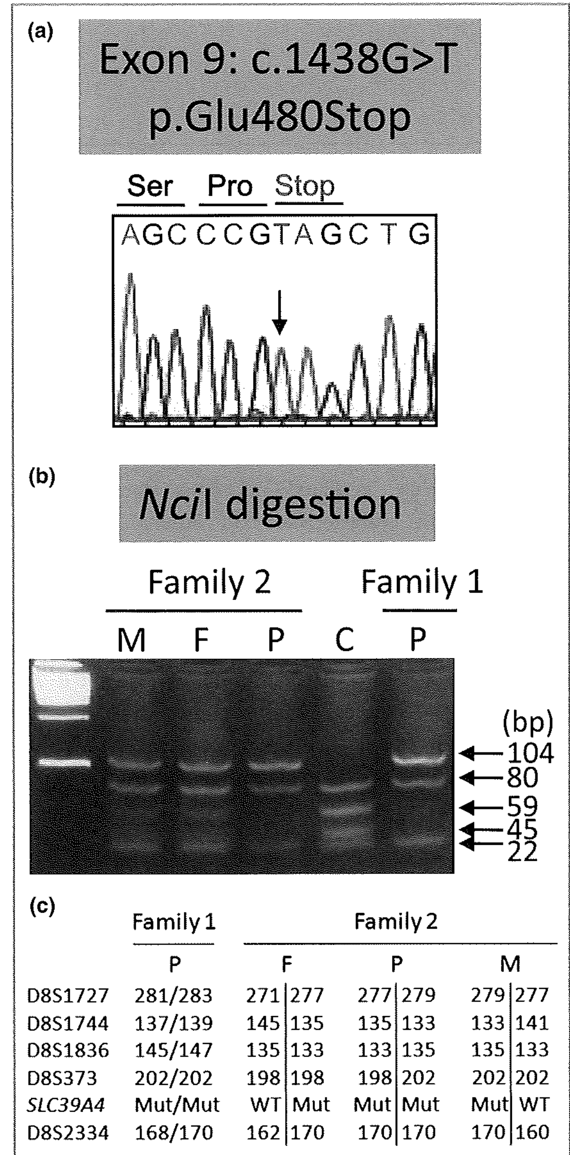


Fig 1. Clinical findings of the probands in families 1 (a–d) and 2 (e, f). Encrusted erythema of acral sites of the extremities in association with nail dystrophy (a, b). Symmetrical erythema, erosions, blisters and crusts in genital and perioral lesions. Alopecia affecting the pubic hair (c) and eyebrows (d). Erosive erythema with yellowish crust in periorificial areas (e). Tense blisters and erosion on the toes (f).



	Family 1	Family 2		
	P	F	P	M
D8S1727	281/283	271 | 277	277 | 279	279 | 277
D8S1744	137/139	145 | 135	135 | 133	133 | 141
D8S1836	145/147	135 | 133	133 | 135	135 | 133
D8S373	202/202	198 | 198	198 | 202	202 | 202
SLC39A4	Mut/Mut	WT | Mut	Mut | Mut	Mut | WT
D8S2334	168/170	162 | 170	170 | 170	170 | 160

Fig 2. A homozygous c.1438G>T (p.Glu480Stop) mutation in exon 9 detected in the proband of family 1 (a). Restriction fragment length polymorphism analysis: the parents in family 2 were heterozygous for c.1438G>T, while the proband was homozygous (b). M, mother; F, father; P, proband; C, normal control. Far left lane, molecular marker. (c) Haplotype analysis of the two Japanese families with acrodermatitis enteropathica. Short tandem repeat allele sizes are shown in base pairs. M, mother; F, father; P, proband; Mut, mutation; WT, wild-type.

using an ABI PRISM 3100 genetic analyzer (Applied Biosystems Japan, Tokyo, Japan). The STR haplotypes of the paternal and maternal alleles in the proband of family 1 were different from those in the proband of family 2, suggesting that the two families are unrelated (Fig. 2c). We further examined 30 intragenic single nucleotide polymorphisms (SNPs) throughout the *SLC39A4* gene including the 5′ flanking region. The two probands were homozygous for all SNPs examined and had identical genotypes (data not shown). Based on these

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findings, we cannot exclude the possibilities of a founder effect for the mutation identified in our patients or consanguinity in both families. To date, no recurrent mutation has been identified in Japanese patients with AE. We could not determine whether the homozygous mutation detected in the proband of family 1 resulted from partial uniparental isodisomy, as genomic DNA samples from the parents were not available. Our results suggest that exon 9 in the SLC39A4 gene encompassing c.1438G should be screened first in the molecular diagnosis of Japanese patients with AE. The truncated ZIP4 protein presumably lacks the last five of eight transmembrane domains, which may result in the inability of ZIP4 to transport zinc into the cytoplasm. However, we could not confirm whether the truncated protein was virtually translated or if SLC39A4 mRNA transcripts harbouring the PTC were degraded to an undetectable level by nonsense-mediated mRNA decay.

The pathogenic mechanism underlying the development of characteristic cutaneous lesions remains to be elucidated. Northern blot analysis demonstrated that the *SLC39A4* gene, encoding ZIP4, was specifically expressed in the stomach, small intestine, colon and kidney in human tissues, while transcripts of this gene were minimally detected in the skin.[2,5] These findings imply that ZIP4 protein expression in the skin could be quite low or undetectable and that the cutaneous lesions of AE may not be primarily caused by a defect in ZIP4. Of note, patients with AE have been reported to have a decreased number of epidermal Langerhans cells (LC), potentially due to apoptosis.[6] In addition, patients with necrotizing migratory erythema, an acquired inflammatory skin disease with eruptions similar to those in AE and reduced serum zinc levels, also have significantly reduced epidermal LC.[7] Together, these findings suggest that a reduction in epidermal LC and serum zinc concentrations may play a pivotal role in the pathogenesis of characteristic eruptions in AE.

Patients with AE require lifelong zinc supplementation, and discontinuation of this essential trace metal inevitably leads to a relapse of the clinical manifestations of AE, as exemplified in the proband of family 1. For this proband, confirming the clinical diagnosis by a mutational analysis was quite useful in improving adherence to zinc therapy without discontinuation. The mother in family 2 was pregnant with her second child. Thus, *SLC39A4* genotyping the newborn will quickly determine if the newborn is affected by AE and enable zinc therapy to begin early, if necessary.

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ORIGINAL ARTICLE

A clinical study of Henoch-Schönlein Purpura associated with malignancy

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Abstract

Background Malignancy has been reported as a causative factor of cutaneous vasculitis, although only two retrospective epidemiological studies have analysed the association between Henoch-Schönlein purpura (HSP) and malignancy to date.

Objective To analyse the association between adult HSP and malignancy.

Methods We retrospectively reviewed the medical records of patients and found 103 cases of HSP over the past 20 years. Fifty-three cases (aged ≥ 41 years) were categorized to two groups including 'with malignancy' or 'without malignancy', so that we could analyse the differences of clinical features between them. We also compared our study to previous reports.

Results Twenty-three cases out of 53 patients exhibited underlying malignant tumours. We focused on nine patients in which malignant tumours were thought to be strongly associated. Seven of nine patients exhibited new metastatic lesions or died due to underlying cancer within 1–32 months.

Conclusions An association between HSP and malignant disease might have important diagnostic and pathophysiologic implications.

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Keywords

adult, Henoch-Schönlein Purpura, malignant disease, metastasis, paraneoplastic vasculitis

Conflicts of interest

None declared

Introduction

Henoch-Schönlein Purpura (HSP) is a systemic vasculitis that involves the small vessels, most notably those in the skin, gastrointestinal tract, and glomeruli, accompanied by arthralgia or arthritis.[1] Its main histopathologic features are leukocytoclastic vasculitis (LCV) mainly in papillar dermis with haematoxylin-eosin staining, and vascular deposits of Immunoglobulin A (IgA) and complement 3 (C3) with direct immunofluorescence (DIF).[2] HSP occurs more commonly in children but can also affect adults. In adults, HSP is usually considered uncommon and can occur at any stage. Nevertheless, the frequency of HSP in adults has been reported to be as high as 15% of all cases of cutaneous vasculitis.[3]

Abbreviations used
C3: complement 3; DIF: direct immunofluorescence; H.C.C.: hepatocellular carcinoma; HL: Hodgkin lymphoma; HSP: Henoch-Schönlein purpura; IgA: immunoglobulin A; LCV: leukocytoclastic vasculitis; NHL: non-Hodgkin lymphoma; S.C.C.: squamous cell carcinoma

While the exact cause of HSP remains unknown, various triggers, including bacteria or virus infection, and drugs, have been hypothesized. Malignancy has also been reported as a rare causative factor of HSP, although only two retrospective epidemiological studies have analysed the association between HSP and malignancy.[4,5] Herein, we report the analysis of the association between HSP and malignancy from 103 cases of HSP managed in our hospital over the past 20 years.

Patients and methods

We retrospectively reviewed the medical records of patients who were treated for HSP in University of Yamanashi Hospital from January 1986 to July 2005. We diagnosed HSP according to following criteria: (1) typical non-thrombocytopenic symmetric palpable purpura, mainly involving the lower extremities; (2) histologic findings of LCV in the upper dermis with IgA or C3 deposits in biopsy specimens. We excluded patients with the following diseases: microscopic polyangiitis, vasculitis allergica cutis,

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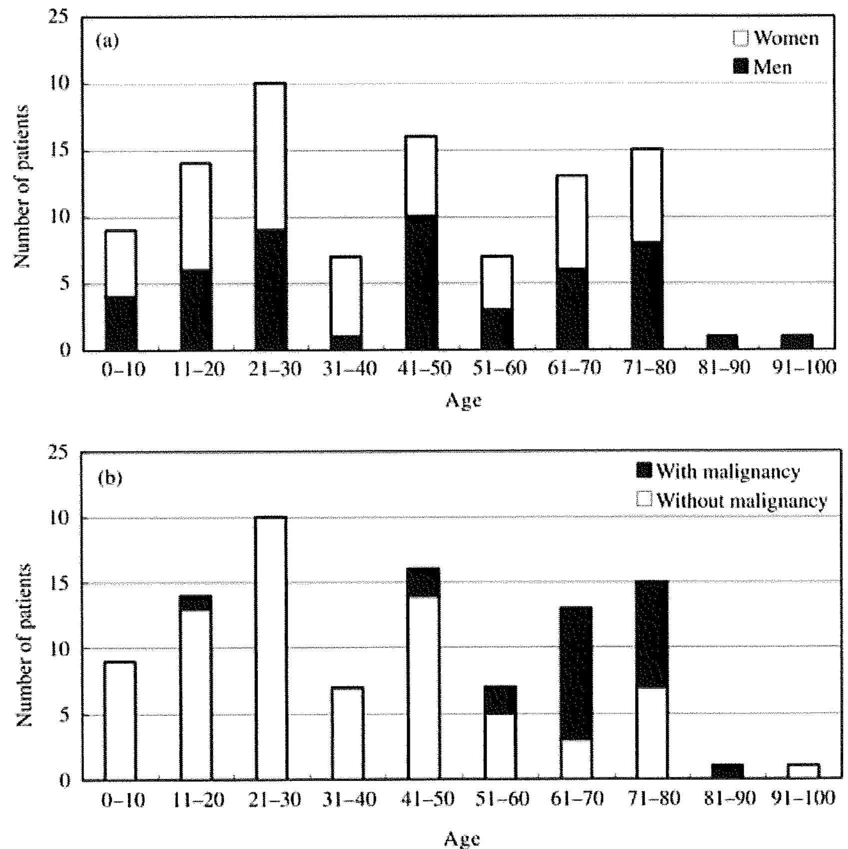

Figure 1 Number and distribution of HSP patients enrolled in this study. (a) One hundred and three patients (49 men and 54 women) visited our hospital with a mean age of 41.3 years (range, 5–92 years) from 1986 to 2005. (b) Among them 24 cases had underlying malignancies. 23 of these patients were over 41 years of age, and 43.4% had underlying malignancies at this age.

polyarteritis nodosa, hypergammaglobulinemic purpura, urticarial vasculitis, cryoglobulinemic purpura, idiopathic thrombocytopenic purpura, and vasculitis associated with collagen diseases.

Then we analysed the patients who had underlying malignancy regarding the kinds of malignancies, association with infectious diseases, symptoms of HSP, time course between HSP and malignant diseases, and mortality of malignancies, and compared them to previous reports.

Statistical analyses were performed using Student's *t*-test and Fisher's exact test. Because of the small sample size, Mann–Whitney-*U*-test was used for comparison of the erythrocyte sedimentation rate (ESR) between two groups of patients. $P < 0.05$ was considered significant.

Results

One hundred and three patients (49 men and 54 women) were enrolled, with a mean age of 41.3 years (range, 5–92 years; Fig. 1a). Out of all of these patients, we found 24 cases that exhibited underlying malignancies (Fig. 1b). Surprisingly, 23 of 53 HSP patients (43.4%) over 41 years of age had underlying malignancies. The patients associated with undergoing malignant tumour in our hospital are summarized in Table 1, and the previous reports of HSP associated with malignant tumour are summarized in Table 2.

We initially compared the kinds of malignant tumour between our study and current one. As shown in Table 3, 29 of 43 tumours (67.4%) were solid tumours in the previously reported patients. In addition, there were some tendencies that the organs affected by malignant tumours focused on lung (11 patients) and haematological diseases (14 patients). In our cases, 24 of 27 (88.9%) tumours were solid tumours, but we did not observe any significant differences among organs affected in our patients. We also found that four patients originally had skin cancer [cutaneous T-cell lymphoma, extramammary Paget's carcinoma, squamous cell carcinoma (S.C.C.) and malignant melanoma].

We next analysed the clinical features of HSP associated with malignancy. A comparison between our study and previous study (Table 4) showed that there was no significant difference in the average age of the patients (65.2 years: 62.2 years). Our patients showed no significant trend towards either gender (15 males: 9 females), whereas previous reports showed that HSP with malignancy tended to occur more often in men (39 males: 6 females). Interestingly, there was a significant difference regarding the onset of HSP during the ongoing disease course of these malignancies. In 20 out of 24

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Table 1 List of HSP patients with malignancy in our hospital

No.	Age/Gender	Malignancy	Timing	Infection
1	69/M	Liver (H.C.C.)	malignancy→AP	No
2	71/M	Skin (cutaneous T cell lymphoma)	malignancy→AP	operation site
3	74/F	Skin (extra-mammary Paget's Ca.)	malignancy→AP	No
4	44/M	Thyroid gland (papillary)	malignancy→AP	No
5	47/F	Rectum (adenoid)	malignancy→AP	No
		Ovary (adenoid)	malignancy→AP	
6	74/F	Maxilla (mucoepidermoid)	malignancy→AP	MRSA infection
7	73/M	Thyroid gland, neck LNs meta.	malignancy→AP	No
		Bile duct	malignancy→AP	
8	65/M	Skin (S.C.C.)	AP→malignancy	No
		left inguinal LNs meta		
9	65/F	Endometrial carcinoma	malignancy→AP	No
10	67/F	Lung (adenoid),	AP→malignancy	upper respiratory tract
		brain,bone,liver meta.		
11	69/M	Hypopharynx (S.C.C.)	malignancy→AP	No
		Lung	malignancy→AP	
12	71/F	Cervical cancer (S.C.C.)	malignancy→AP	No
13	69/M	Colon (adenoid), liver meta.	malignancy→AP	No
14	69/M	Maxilla (S.C.C.)	malignancy→AP	operation site
15	58/F	Skin (malignant melanoma)	malignancy→AP	No
16	73/M	Sigmoid colon	AP→malignancy	liver abscess
17	63/F	Kidney (transitional cell)	malignancy→AP	upper respiratory tract
		bladder meta.		
18	70/M	B cell lymphoma	malignancy→AP	No
19	53/M	Stomach	malignancy→AP	No
		Prostate (adenoid)	malignancy→AP	
20	71/F	Thyroid gland (follicular)	AP→malignancy	No
		LNs meta.		
21	78/M	Stomach	malignancy→AP	No
22	65/F	Malignant lymphoma	malignancy→AP	No
23	88/M	Stomach	malignancy→AP	upper respiratory tract
24	18/F	Cervical cancer (S.C.C.)	AP→malignancy	No

patients (83.3%) in our study, the onset of HSP occurred after the diagnosis of malignant tumour. On the other hand, previous studies showed that 30 of 40 patients (75.0%) were diagnosed with malignant diseases after the onset of HSP. Further analysis regarding the time course between HSP and malignant disease is shown in Fig. 2. Previous reports found that the diagnosis of malignant tumour occurred around 4 months after the onset of HSP (closed bar). In particular, 19 of 28 patients (67.9%) were diagnosed within 4 months after the onset of HSP, whereas in our cases, 20 patients had already been diagnosed and were being treated for their malignant tumour prior to the appearance of HSP (open bar).

We also analysed the differences of the clinical setting in our patients. Fifty-three patients who were over 41 years of age were divided into two groups. One included 23 patients that were associated with malignant tumour, while another 30 patients exhibited no correlation with malignancies, as shown in Table 5. Based on this stratification, the average age was higher in the malignancy-associated group compared to the no correlation group (67.2 – 57.8, $P = 0.012$). Physical examinations revealed that the percentage of patients involved with arthralgia were higher among the no malignancy-associated patients ($P = 0.016$). Blood examination showed that there were no significant differences in the levels of serum IgA and CRP, whereas ESR was higher in the malignancy-associated group compared with the no correlation group (90.7 mm/h: 36.2 mm/h, $P < 0.01$). We observed a tendency that the numbers of patients affected with infectious diseases and proteinuria were greater in patients without malignancy.

Finally, we focused on nine patients that we considered to be strongly associated with malignant tumours (Table 6). Three patients were diagnosed with malignant tumours after a crisis of

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Table 2 List of HSP patients previously reported in the literature

Author	Age/Gender	Malignancy	Timing
Cairns[6]	63/M	Bronchus (epidermoid)	AP→malignancy
Cairns[6]	73/M	Bronchus (epidermoid)	AP→malignancy
Maurice[7]	59/M	Bronchus (epidermoid)	AP→malignancy
Mitchell[8]	57/M	Bronchus (epidermoid)	AP→malignancy
Garcias[9]	60/M	Prostate (adenocarcinoma)	AP→malignancy
Vesole[10]	63/M	NHL (diffuse large B cell)	AP→malignancy
Ng[11]	39/M	HL (nodular sclerosis)	AP→malignancy
Pfitzenmeyer[12]	79/M	Bronchus (epidermoid)	AP→malignancy
Hughes[13]	58/F	Breast (adenocarcinoma)	malignancy→AP
Birchmore[14]	50/M	IgA multiple myeloma	AP→malignancy
Blanco R[15]	67/M	Bronchus (anaplastic small cell)	AP→malignancy
Blanco R[16]	43/M	Myelodysplasia	AP→malignancy
Chong[17]	67/M	Stomach (adenocarcinoma)	AP→malignancy
Hayem[18]	55/M	Small bowel	AP→malignancy
Blanco P[19]	29/M	HL (mixed cellularity)	AP→malignancy
Pertuiset[4]	68/M	NHL (diffuse large B cell)	AP→malignancy
Pertuiset[4]	46/F	Kidney (adenocarcinoma)	AP→malignancy
Pertuiset[4]	34/M	Essential thrombocythemia	AP→malignancy
Pertuiset[4]	77/M	Prostate (adenocarcinoma)	AP→malignancy
Carlos[20]	43/M	Myelodysplastic syndrome	AP→malignancy
Carlos[20]	67/M	Lung (small cell)	AP→malignancy
Nakano[21]	80/F	Lung	unknown
Nakano[21]	88/M	Rectal	unknown
Nakano[21]	63/M	Liver	unknown
Nakano[21]	67/F	Lung	unknown
Nakano[21]	73/F	Kidney	unknown
Uemura[22]	76/M	Large bowel	AP→malignancy
Miura[23]	72/M	Sigmoid colon	malignancy→AP
Onoyama[24]	60/M	Myelodysplastic syndrome	malignancy→AP
Osawa[25]	60/M	Thymus	malignancy→AP
Gutierrez[26]	78/M	Bronchus	AP→malignancy
Ponge T[27]	75/M	Bronchus	malignancy→AP
Couzi L[28]	86/M	Prostate	AP→malignancy
Fain O[29]	55/M	Schwannoma	AP→malignancy
Arrizabalaga P[30]	72/M	multiple myeloma	AP→malignancy
Conte G[31]	41/M	multiple myeloma	malignancy→AP
Helm-Van[32]	57/M	multiple myeloma	AP→malignancy
Zickerman[33]	50/M	multiple myeloma	AP→malignancy
Day C[34]	66/M	NHL	AP→malignancy
Zurada[5]	71/M	Prostate, Kidney	malignancy→AP
Zurada[5]	80/M	Prostate, LNs meta.	malignancy→AP
Zurada[5]	46/F	Anal (S.C.C.), meta.	malignancy→AP
Fain O[35]	over 50/M	solid tumor	AP→malignancy
Fain O[35]	over 50/M	solid tumor	simultaneously
Fain O[35]	over 50/M	solid tumor	malignancy→AP

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Table 3 Differences of affected organs of malignant disease between our patients and previous reports

Affected organ	Our cases	Previous reports
Skin	3	0
Blood	3	14
Thyroid gland	3	1
Maxillary and pharynx	3	0
Stomach	3	1
Large intestine	3	4
Uterus and ovary	3	0
Lung	2	11
Liver	2	1
Kidney	1	2
Prostate gland	1	5
Small intestine	0	1
Mammary gland	0	1

We initially compared the kinds of malignant tumor between our study and current one.

Thirty-one of 45 tumors (68.9%) were solid tumors in the previously reported patients, furthermore 24 of 27 (88.9%) tumors were solid tumors in our cases.

Table 4 Comparison about clinical features of HSP associated with malignancy between our study and previous study

	Our cases	Previous reports
Age	65.2 y.o. (18–88)	62.2 y.o. (29–88)
Male to female	15 to 9	39 to 6
AP→malignancy	4	30
Malignancy→AP	20	10
Double cancer	4	1

HSP. One patient was revealed to have an early stage of sigmoid colon cancer by colonoscopy during the treatment of HSP. After resection of the tumour by colonoscopic polypectomy, HSP was cured and no recurrence was observed. Two patients were concurrently diagnosed with their underlying malignancies and HSP, but they already had metastatic lesions from their malignant diseases. The remaining six patients were diagnosed with HSP during the treatment of the initial malignant diseases. After the diagnosis of HSP, their underlying malignancy became worse. Two patients exhibited new metastatic lesions within 3 months. Surprisingly, 6 out of 9 patients died because of malignancy within 4–32 months.

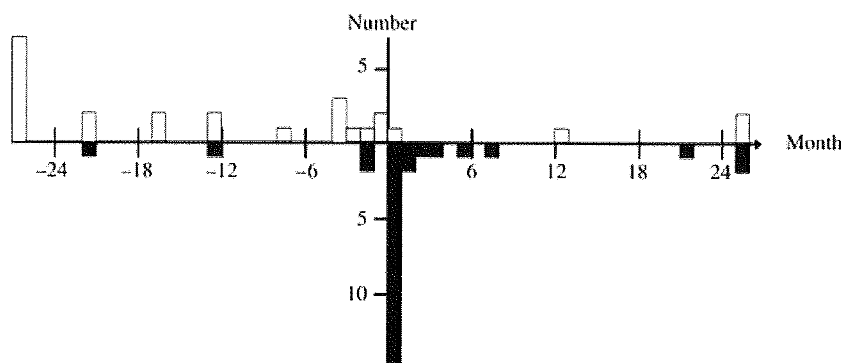

Figure 2 Time course of disease onset of HSP and malignancy. In 20 out of 24 patients (83.3%) in our study (□), the onset of HSP occurred after the diagnosis of malignant tumour. On the other hand, previous studies (■) showed that 30 of 40 patients (75.0%) were diagnosed with malignant diseases after the onset of HSP. +: Malignancy was found after crisis of HSP, −: Malignancy was already treated before crisis of HSP.

Table 5 Comparison of the clinical setting between HSP coexistence with malignancy and without malignancy

	With malignancy (n = 23)	Without malignancy (n = 30)	P
Average age	67.2 ± 9.8 (44–88)	57.8 ± 14.3 (41–92)	0.012*
Male : female	15 : 8	16 : 14	N.S.†
Infection	30.4%	53.3%	N.S.†
Renal involvment	44.4% (n = 18)	50% (n = 26)	N.S.†
Proteinurea (> 1 g/day)	0% (n = 18)	12.5% (n = 24)	N.S.†
Arthralgia	5.6% (n = 18)	37.5% (n = 24)	0.016†
Gastrointestinal symptoms	22.2% (n = 18)	21.7% (n = 23)	N.S.†
Serum iga (mg/dL)	423.6 ± 140.0 (n = 14)	438.5 ± 194.2 (n = 24)	N.S.*
CRP (mg/dL)	3.4 ± 4.4 (n = 19)	2.2 ± 3.5 (n = 24)	N.S.*
ESR (mm/1 h)	90.7 ± 32.9 (n = 6)	36.2 ± 29.1 (n = 18)	< 0.01‡

Fifty-three patients were divided into two groups. One included 23 patients that were associated with malignant tumour, while another 30 patients exhibited no correlation with malignancies.

*: Student t-test, †: Fisher's exact test, ‡: Mann–Whitney-U-test.

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Table 6 Patients list considered strong association with malignancy in our hospital

No.	Age/Gender	Malignancy	Timing	Prognosis
3	74/F	Skin (extra-mammary Paget's Ca.)	malignancy→AP	died after 32 months
5	47/F	Rectum (adenoid)	malignancy→AP	died after 6 months
		Ovary (adenoid)	malignancy→AP	
8	65/M	Skin (S.C.C.) left inguinal LNs meta.	malignancy→AP	liver and bone meta. after 3 months died after 4 months
9	65/F	Endometrial carcinoma	malignancy→AP	died after 9 months
10	67/F	Lung (adenoid), brain, bone, liver meta.	AP→malignancy	died after 17 months
11	69/M	Hypopharynx (S.C.C.) Lung	malignancy→AP malignancy→AP	bone meta. after 1 month
13	69/M	Colon (adenoid), liver meta.	malignancy→AP	died after 6 months
16	73/M	Sigmoid colon	malignancy→AP	
20	71/F	Thyroid gland (follicular) LNs meta.	AP→malignancy	

We focused on nine patients that we considered to be strongly associated with malignant tumours. Two patients were diagnosed with a malignant tumour after a crisis of HSP. The seven remaining patients were diagnosed with HSP during initial treatment for malignant diseases. Seven of nine patients exhibited new metastatic lesions or died due to underlying cancer within 1–32 months.

Discussion

Paraneoplastic syndromes are those syndromes associated with malignancy that occur at a distance from the primary tumour or metastases.[36] Cutaneous vasculitis is generally a benign and self-limited condition that is confined exclusively to the skin. However, cutaneous vasculitis may occur in patients with systemic necrotizing vasculitis or other entities, such as infections or connective tissue diseases. Cutaneous vasculitis may also be associated with malignant disorders and behave like a paraneoplastic syndrome.[37–40] The prevalence of malignancy in adults with cutaneous or systemic vasculitis has been estimated at 2.5% to 5.0%.[41,42] Although haematological malignancy has been shown to be three to five times more common than solid tumours in all types of vasculitis,[41,43,44] some previous reports have indicated that solid tumours were more common in association with HSP.[29,37,45] We have experienced 23 cases of HSP associated with malignancy, including four cases of double cancers. Twenty-four out of 27 tumours (88.9%) were solid tumours, whereas only 3 cases (11.1%) were haematological malignancies. Thus, similar to previous reports, the malignant tumours that coexisted with HSP tended to be solid tumours. According to Pertuiset's report,[4] in 18 of 19 cases, the malignant tumours existed concurrent with or after the diagnosis of HSP. However, Zurada *et al.*[5] reported that one patient was diagnosed concurrently with HSP with primary malignancy, whereas another two cases of HSP were observed when metastatic lesions were already present. More recently, Fain *et al.* also reported that one of three HSP patients showed the skin manifestation for HSP after diagnosis with malignancy.[35] Among our cases, 19 of 23 patients were diagnosed with HSP after the diagnosis of malignancy. On this point, our cases were quite different compared to those reported in the literature.

The clinical features in our patients associated with malignancy were slightly different from previous reports. The mean age was 67.2 years old (range: 44–88 years). There were no differences between gender (male : female = 15 : 8). Mild renal dysfunction was apparent in 44.4% of the patients, but severe proteinuria ($\geq$ 1.0 g/day) was not observed in our patients. Twenty-two per cent of these patients exhibited gastrointestinal symptoms and 5.6% exhibited arthralgias. According to previous reports, the average age was 61.2 years and was predominantly seen in males (94%). Physical examination revealed that gastrointestinal symptoms, renal involvement, and arthralgias occurred in 61%, 87%, and 77% of the patients, respectively. The reason for these differences of clinical features between our patients and those in the literature are not known, but further analysis of the clinical features of HSP with malignancy is necessary.

We focused on nine cases that we estimated to exhibit a strong correlation between HSP and malignancy. One patient was diagnosed concurrently with HSP and primary malignancy. Two patients were diagnosed concurrently with HSP and primary tumour, and metastatic lesions were found at the same time. The diagnosis of HSP was made after that of malignancy in six cases. Surprisingly, 7 of these 9 patients exhibited new metastatic lesions within 3 months or died due to the underlying cancer within 4–32 months. Many previous reports have noted that the importance of HSP with malignancy might be the opportunity for the diagnosis of malignant tumours, whereas according to our current analysis, we thought that the occurrence of HSP after the diagnosis of malignancy might indicate metastasis or growth of malignancy. As result of the experiences described herein, we strongly recommend that patients with a known history of malignant tumour should undergo re-evaluation for metastatic lesion.

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Several potential reasons have been proposed for the mechanism of paraneoplastic vasculitis including HSP:[9,39,42] (1) decreased immune complex clearance, (2) abnormal production of antibodies and tumour neoantigens leading to the formation of immune complexes that deposit within blood vessel walls, (3) similarities between tumour antigens and endothelial cell antigens, (4) dysregulated lymphocytes that cause a switch from IgM to IgA isotypes, and (5) aberrant inflammatory cytokines produced either by malignant tumour cells or through tumour microenvironments.[46] (6) It has also been reported that TGF-β secreted around many kinds of tumour induces IgA class switch.[47,48] (7) Malignancy might also contribute to the development of HSP by creating a hyperviscous state that can cause endothelial damage and increase the contact time for the deposition of immune complexes.[49] (8) The treatment of cancer also has been reported to trigger HSP.[13,14] Meanwhile, the pathophysiology of paraneoplastic vasculitis and also HSP remains unclear.

In summary, we again strongly recommend that adult HSP patients, especially men and women over 41 years of age, should be investigated for malignancies. Patients without identifiable triggers of HSP should be evaluated for underlying malignancy. Furthermore, patients with a known history of malignant tumour should undergo re-evaluation for metastatic lesion.

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