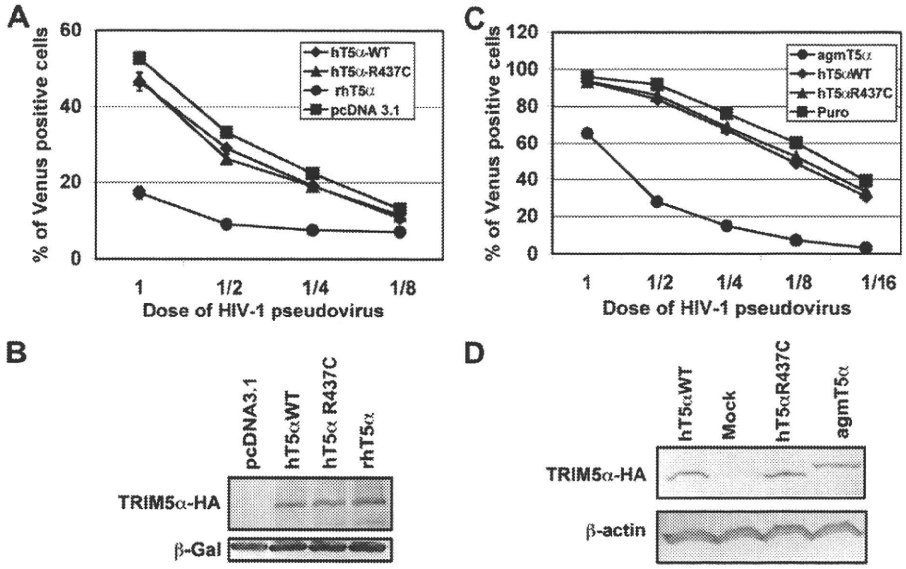

Fig. 4. Effect of various TRIM5αs on HIV-1 entry. 293T cells were transfected with TRIM5α_{hu}-HA (hT5αWT or R437C) or TRIM5α_{rh}-HA (rhT5α) expression plasmid, and then infected with VSV-G pseudotyped HIV-1-Venus (A). HeLa cells that had been transduced with various TRIM5α encoding retrovectors were infected with VSV-G pseudotyped HIV-1-Venus (C). Forty-eight hours after infection, the cells were harvested and the Venus-positive cells were counted by FACS. (A and C)shows a typical result of three independent experiments. (B and D) The expression of TRIM5α was examined by immunoblot assay.

which is consistent with the published data [37]. These results indicate that the amino acid 437 in the SPRY domain is involved in the ability of TRIM5α_{hu} to suppress both HIV-1 production and N-MLV infection.

4. Discussion

Sakuma et al. reported an inhibitory effect of TRIM5α_{rh} at a late phase of HIV-1 replication [17]. In this study we extended this finding to TRIM5α_{hu} and showed that this effect is specific, using a loss of function point mutant (TRIM5α_{hu}R437C). Although TRIM5α_{hu} has weaker ability to block the HIV-1 production than TRIM5α_{rh}, the knockdown experiment clearly showed that endogenous TRIM5α_{hu} do inhibit the HIV-1 production. The ability of TRIM5α_{hu} to reduce the production of HIV-1 some extent has also been reported previously [Supplementary Fig. 2 of 17,18]. Accordingly our results suggest that endogenous TRIM5α_{hu} functions as an innate immunity molecule that reduces HIV-1 production.

The RING, B-box2 and coiled-coil motifs (RBCC) of TRIM5α_{rh} have been reported to be essential for blocking

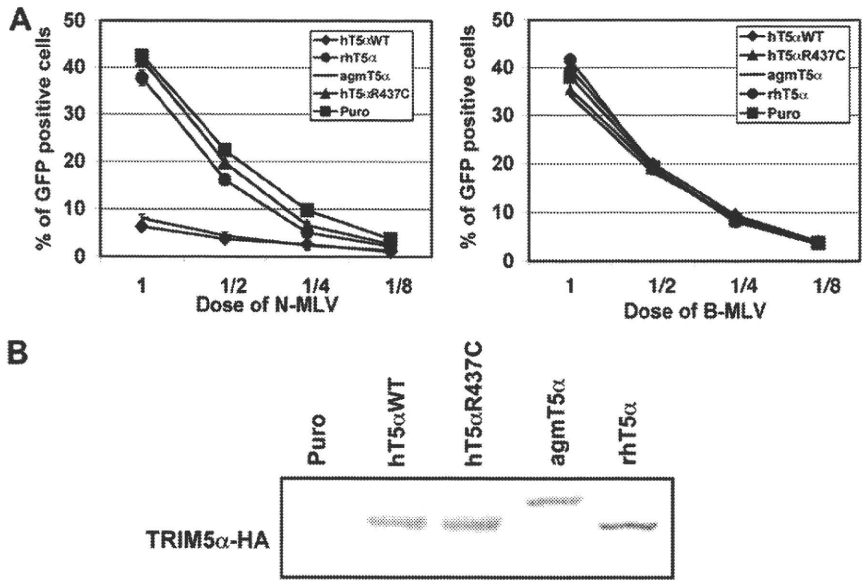

Fig. 5. Effect of various TRIM5αs on N-MLV infection. The 293T cells transduced with various TRIM5α encoding retrovectors were infected with VSV-G-pseudotyped GFP encoding N and B-tropic MLVs. Forty-eight hours after infection, the cells were harvested and GFP-positive cells were counted by FACS. (A) The left panel includes the means ± S.D, which was calculated based on three independent experiments. The right panel represents a typical result of 2 independent experiments. (B) The expression of TRIM5αs was examined by immunoblot assay.

HIV-1 production. Particularly RING and B-box domains have been identified to regulate the interaction between TRIM5α_{rh} and HIV-1 Gag, while the coiled-coil domain determines the late restriction activity [21]. Our results suggest that the SPRY domain of TRIM5α_{hu} is also involved in this restriction and that arginine at reside 437 is important, since the arginine to cysteine mutation severely abolished HIV-1 inhibition (Fig. 1B and C). However, its importance is limited to TRIM5α_{hu}, as introduction of corresponding mutation into TRIM5α_{rh} did not alter the restriction activity of TRIM5α_{rh}. TRIM5α_{hu} specific effect of the Arg to Cys mutation is concordant with the reduction of affinity between TRIM5α and Gag, which was demonstrated by the encapsidation of TRIM5α into VLP. Since the affinity of TRIM5α_{hu} to Gag was naturally weaker than that of TRIM5α_{rh}, the effect of the mutation in TRIM5α_{hu}, but not TRIM5α_{rh}, may become phenotypically apparent.

The critical motif on the restriction at early stage of retroviral infection has been reported to lie between residues 332–340 of TRIM5α_{hu}, which shows the greatest sequence diversity among human, rhesus and African green monkey TRIM5αs [36,38,39]. For example, a single amino acid substitution (R332P) conferred the ability to restrict HIV-1 to TRIM5α_{hu} [39,40]. A change of tyrosine 336 to alanine or lysine TRIM5α_{hu} enabled restriction of B-MLV, NB-tropic Moloney MLV and SIVmac [41,42]. The arginine at residue 437 of TRIM5α_{hu} is located outside the motif and conserved among human, rhesus and rodent Trim5s (accession numbers: NM_001014023.1, NM_175677.4, and NP_001014045). Therefore, our results reveal that the C-terminal conserved region of the SPRY domain is also involved in the interaction of TRIM5α to HIV-1 Gag so as to play an important role in restriction of both HIV-1 production and human resistance to MLV infection. Since rodents are susceptible to MLV infection, the other portion besides Arg437a.a. of TRIM5α_{hu} should be also involved in the restriction effect on N-MLV infection.

Although Sakuma et al. reported that overexpression of TRIM5α_{rh} reduced both HIV-1 p55 and p24 levels, we observed reduction only of p24, while p55 levels remained constant (Fig. 1C). Instead, we noted TRIM5α_{hu} dependent reduction of p38, a processing intermediate of the HIV-1 Gag protein. This difference may be ascribed to more efficient Gag processing in our system, or insensitive detection of p55 by the anti Gag monoclonal antibody [34] used in this study. These results are not inconsistent with the proposed hypothesis that TRIM5α reduces HIV-1 production by degradation of HIV-1 Gag polyproteins. However, we cannot rule out the possibility that TRIM5α_{hu} may inhibit HIV-1 production by a different mechanism, because only low incorporation of TRIM5α_{hu} was observed in HIV-1 VLPs in contrast to the abundant incorporation of TRIM5α_{rh}. TRIM5α_{hu} might be involved in the HIV-1 Gag maturation rather than degradation of the polyproteins.

Although HIV-1 is known to replicate well in human cells, HIV-1 infection generally progresses to a latent stage that shows few or no symptoms and that can persist for decades. Adaptive immune responses such as those mediated by cytotoxic T cells (CTL) and the humoral system suppress viral replication during chronic infection. Aspects of the innate immune system can also contribute to viral suppression during chronic infection. For example, Apobec3G is induced in macrophages by IFN-α and reduces HIV-1 production even if the virus expresses the anti-Apobec3G factor Vif [9]. The ability of TRIM5α_{hu} to restrict HIV-1 production suggests that it may also constitute an innate immunity factor that functions to lower virus replication levels and elicit a long nonsymptom phase.

A considerable number of polymorphisms in TRIM5α_{hu} have been documented. Although the majority of them are not associated with susceptibility to HIV-1 infection [43–45], one common nonsynonymous single nucleotide polymorphism (SNP), R136Q, affects acquisition of HIV-1 infection [45]. A recent report by Torimiro et al. [46] revealed that about 4% of Baka pygmies in Cameroon were heterozygous for a truncation mutant of TRIM5α (R332X), which completely loses the ability to restrict HIV-1 infection. The Trim5α_{hu}R437C mutant in our study was amplified from the cDNA from peripheral blood mononuclear cells of an individual, suggesting the existence of another SNP related to retrovirus infection susceptibility although it cannot be ruled out that the mutation was introduced during PCR.

Taken together, our data provides evidence that endogenous human TRIM5α possesses suppressive activity at the step of HIV-1 progeny production, supporting the hypothesis that it comprises part of the innate immune system that limits HIV-1 replication. This notion is in line with data showing IFN-α treatment increases the levels of TRIM5α_{hu} mRNA and enhances antiviral activity against N-MLV infection [47]. IFN-α treatment also up-regulates TRIM5α mRNA in rhesus monkey cells, which correlates with enhanced TRIM5α-mediated pre- and post-integration restriction of HIV-1 replication [8]. Further investigation of the mechanisms by which TRIM5α_{hu} prevents production of HIV-1 may provide valuable information for antiviral immune therapy.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micinf.2010.05.004.

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Species-specific CD1-restricted innate immunity for the development of HIV vaccine

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ABSTRACT

The human immune defense system is composed of two distinct elements: innate immunity located primarily at body surfaces restricted by species-specific CD1 molecules and acquired immunity found mainly in internal compartments associated with individually restricted MHC molecules. Historically, effective vaccines have focused on eliciting pathogen epitope-specific acquired immune responses to protect against infectious diseases; however, such traditional approaches to developing HIV vaccines have been unsuccessful. This review addresses the importance of activating host species-restricted innate immunity to enhance the virus epitope-specific acquired immunity that is needed for HIV vaccines.

1. Introduction

Our internal defense system is composed of two distinct elements. One is local innate immunity principally arranged on surface areas, such as skin or mucous membrane to establish barriers against various pathogens, and the other is systemic acquired immunity, mainly found in systemic components, for example, circulating blood or lymphoid organs, such as lymph nodes and spleen, to survey and control internal damage and disorders. The former innate arm is chiefly regulated via species-restricted CD1 antigen-presenting molecules and the latter acquired arm is orchestrated by individually restricted MHC molecules (Fig. 1).

In vaccine development for both the prevention of pathogen intrusion and suppression of its expansion as well as tumor growth, we have been focusing on the induction of acquired immune responses composed of MHC molecule-restricted peptide epitope-specific T cells and antibodies that bind specifically to the particular epitopes on pathogens or tumors through their definite receptors created by gene re-arrangements. Thus, the main work to advance vaccine development has been focusing on the identification of epitopes and the establishment of a powerful and non-toxic adjuvant for the induction of epitope-specific immunity. However, because pathogen- or tumor-derived epitopes vary among diverse MHC molecules, the analysis and discovery of cross-reactive immuno-dominant epitope(s) should be considered to overcome MHC diversity [1,2].

Under these conditions, a lack of correlation between acquired virus-specific immunity and resistance to infection with simian immunodeficiency virus (SIV) in rhesus monkeys has been reported recently [3]. Also, most exposed, uninfected commercial sex workers eventually became infected after quitting their jobs to limit mucosal human immunodeficiency virus type-1 (HIV-1) exposure, although virus-specific cell-mediated immunity and immunoglobulin A (IgA) antibody responses had been confirmed [4,5], suggesting that continuous mucosal virus stimulation may be required to maintain protective acquired immunity against persistently infected pathogens. Moreover, the reservoir for HIV-1 in persistently infected patients with no free virus particles in the circulating blood after highly active anti-retroviral treatment (HAART) has been identified as innate CD4-positive dendritic cells (DC) or natural killer T (NKT) cells in the small intestine (J.M. and H.T.; unpublished observation). In the present review, based on our recent progress, the importance of activating species-restricted local innate immunity to develop and HIV-1 vaccine rather than individually restricted systemic acquired immunity will be addressed.

2. Species-specific antigen-presenting molecule CD1s

Species-specific CD1 molecules are further divided into two sub-classes, group I CD1 (CD1a–CD1c) and group II CD1 (CD1d) [6]. These CD1s have been found to present lipid/glycolipid antigens to the appropriate T cells bearing relatively invariant

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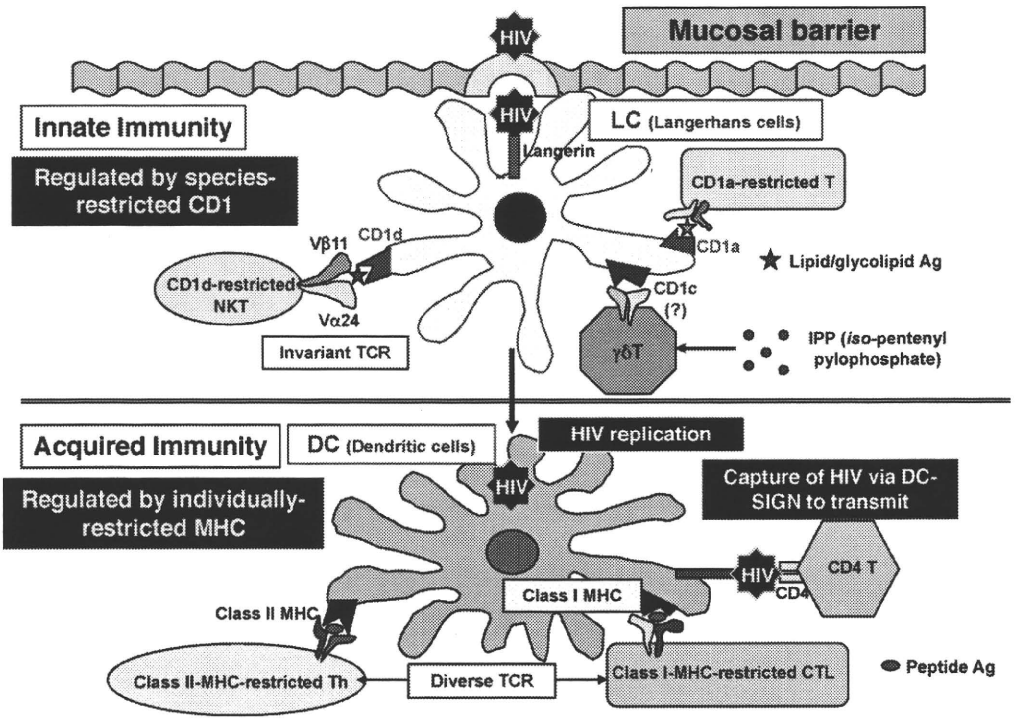

Fig. 1. Innate immunity and acquired immunity. Our internal defense system is composed of two distinct elements. One is innate immunity composed of γδT cells and NKT cells as effectors expressing fixed invariant receptors controlled mainly by species-restricted CD1 molecules on Langerhans cells and (LC) dendritic cells (DC), and the other is systemic acquired immunity composed of helper T cells (Th), cytotoxic T lymphocytes (CTL), and antibodies bearing diverse receptors from re-arranged genes orchestrated by individually restricted MHC molecules.

αβT-cell receptors (TCR), most of which are conserved among species; for example, highly conserved CD1d molecules present α-galactosyl ceramide (α-GalCer) to natural killer T (NKT) cells of their own species. Indeed, human NKT cells generally express unique combinations of TCRs that consist of an invariant Vα24 chain preferentially paired with a Vβ11 [7], while murine α-GalCer-reactive CD1d-restricted NKT cells express invariant Vα14 paired with various Vβ combinations [8].

The structures of CD1 molecules are similar to those of class I MHCs bearing non-covalently bound β2-microglobulin that may regulate the antigen-binding capacity of the presenting molecules; however, CD1s show limited polymorphism and do not map to MHC genes [9]. Also, CD1-encoding genes are highly conserved and their structures are shared among species [10]. We have confirmed recently that the genetic structure of CD1d molecules is very tightly conserved among species, such as rhesus macaques, African green monkeys (AGMs), and chimpanzees, and would not be affected by long-term environmental stimulation [11]. It is important to note that, unlike rhesus macaques and AGMs, both α1 and α2 domains of the CD1d of chimpanzees were identical to those of humans, although 4 amino acids in α3 domain differed [11]. Since the α2 domain of CD1d molecules with a hydrophobic ligand-binding pocket critical for antigen presentation [12], changes of amino acids in the α2 domain may alter the capacity of the presented glycolipid/lipid antigens to effector NKT cells. It is widely known that both humans and chimpanzees are susceptible to HIV-1 [13,14] but very weakly to SIV, to which rhesus macaques and AGMs are susceptible. These findings suggest an evolutionary relationship between species-specific CD1d molecules and retrovirus susceptibility through the activation of innate effector NKT cells.

3. Individually restricted antigen-presenting molecule MHC

In contrast, both class I and class II MHC molecules are extremely diverse among species with self-restricted elements that can present internally processed peptide antigens only to the same MHC molecule-bearing cells. Such individually restricted peptide epitopes will be recognized by highly diverse αβTCRs established via suitable intracellular gene re-arrangements that create antigen-specificity. In general, CD8αβ-positive T cells recognize the processed epitope peptide presented by class I MHC molecules, whereas CD4-positive T cells recognize epitope peptide in association with class II MHC [15]. Both class I and class II MHC molecule-restricted T cells can be elicited by individual class I and class II MHC molecule-expressing DC that capture antigenic proteins and select to present specific epitopes with their MHC molecules; therefore, the epitope-specific rearranged αβTCR-expressing T cells in the acquired arm seem to be controlled by individual antigen-captured DC *in vivo*.

In viral infection, various viral proteins and genetic components are disseminated throughout the body. The former viral proteins may be captured by immature DC (iDC) and the latter genetic components may stimulate antigen-loading iDC to mature via Toll-like receptors (TLR), inducing virus-specific cellular immunity, in particular, cytotoxic T lymphocytes (CTL) that eliminate virus-infected cells. Double-strand RNA, polyriboinosinic polyribocytidylic acid (poly(I:C)), which reflects a natural genetic product from a variety of viruses during replication, has recently been identified as one of the critical stimuli of TLR3 [16]. We and others have shown that iDC could present processed antigen from captured purified protein in association with class I MHC molecules via a cross-presentation mechanism when iDC were stimulated with poly(I:C) [17,18]. Also, such cross-presentation of externally added purified proteins can be achieved by a saponin-associated adjuvant like ISCOMs [19] or cholera toxin (CT) [20]. Taken together, virus-specific acquired immunity restricted by individual MHCs can be spontaneously elicited by the appropriate activation of innate iDC that capture viral antigenic molecules during the course of infection.

4. Interaction between HIV-1 and DC

DC at the mucosal site appear to be a natural reservoir for HIV-1, whose Nef protein is known as a key factor in disease progression. Indeed, nef-deficient HIV-1 as well as SIV markedly slowed the clinical manifestation of AIDS [21]. We and others have recently reported that the surface expression of CD1a and CD1d was selectively down-regulated among CD1 molecules as well as class I MHC on HIV-1 infected iDC by Nef [22,23], indicating that iDC may lose their function to present virus-associated antigens to both innate and acquired effectors, which may cause disease progression. Thus, stimulation of iDC or deletion of the Nef effect in HIV-1 infected DC may alter the immunological state of HIV-1-infected individuals.

Two distinct types of innate DC are observed at the local mucosal site. One is sentinel Langerhans cells (LC) that are present in the surface epithelial compartment to survey antigens, including viruses, via the LC-specific sampling receptor, Langerin; langerin-bound antigens are internalized into Birbeck granules and degraded. Recently de Witte et al. [24] proposed that LCs provide a barrier to HIV-1 infection by demonstrating that HIV-exposed skin-derived LCs captured HIV via Langerin and degraded the virus. However, evidence contradicting the capacity of LCs to protect against HIV was obtained using human vaginal explant cultures, a more direct, biologically relevant model of HIV-sexual transmission [25]. Hladik et al. showed that after HIV-1 exposure of vaginal explants, LCs were rapidly virus-penetrated primarily by endocytosis via multiple receptors and that endocytosed virions could persist in the cytoplasm. Also, DC-SIGN (CD209) and langerin (CD207) apparently had a negligible role in mediating endocytotic HIV infection by vaginal LCs. Thus, depending on the tissue source of LCs and the precise experimental conditions, human LCs are capable of degrading HIV-1 or being infected and harboring HIV-1. It is important to note that Haldik et al.'s results support prior observations of rapid vaginal LC infection in macaques after experimental SIV vaginal inoculation [26]. We have reported that DC-SIGN-positive macrophages in the early colostrum (breast milk macrophages: BrMMØ) and their DC-SIGN expression were markedly enhanced by externally added interleukin (IL)-4 [27]. IL-4-treated BrMMØ showed strong capacity to transmit HIV-1 to $CD4^+$ cells via DC-SIGN [28]. Therefore, evidence from different human tissue culture model systems indicates that both LCs and DC-sign-positive DCs can be reservoirs for HIV-1.

5. Selective activation of innate DC lineage cells for the induction of HIV-1-specific acquired immunity

The two major distinct subsets of DC are arranged to regulate immune responses *in vivo*; 33D1-positive and DEC-205-positive DC. Using anti-33D1-specific monoclonal antibody (mAb), 33D1-positive DC were successfully depleted from C57BL/6 mice *in vivo*. When the remaining DEC-205^+ DC in $33D1^+$ DC-depleted mice were stimulated with LPS, serum IL-12 but not IL-10 secretion was markedly enhanced, which may induce Th1 dominancy upon TLR signaling. After implanting various syngeneic tumor cells into the dermis of $33D1^+$ DC-depleted mice, subcutaneous injection with LPS resulted in significant suppression of tumor growth *in vivo*. Moreover, apparent proliferation of class I MHC molecule-restricted epitope-specific $CD8^+$ CTL among tumor infiltrating lymphocytes (TIL) against already established syngeneic tumors was observed in the LPS-stimulated $33D1^+$ DC-deleted mice administered intraperitoneally with very small unaffected amount of melphalan (L-phenylalanine mustard; L-PAM) (K.M., A.W., and H.T.; unpublished observation).

These findings indicate the importance and effectiveness of selective targeting of a specific subset of innate DC, such as DEC-205^+ DC alone or with very small amount of anti-cancer drugs to activate functional acquired epitope-specific $CD8^+$ CTL without externally added antigen stimulation *in vivo*. This may be true for HIV-1 intrusion in the local mucosal area, in which selective activation of suitable DC with or without small amount of anti-HIV-1 drugs will induce effective acquired immunity specific for the pathogen.

6. Concluding remarks

Most vaccine work to block pathogens has focused on how to artificially elicit acquired individual MHC molecule-restricted effectors specific for pathogens or pathogen-infected cells before pathogen invasion by using attenuated pathogens or pathogen-derived immunogenic molecules containing epitopes with a strong adjuvant. However, to establish the most suitable pathogen-specific acquired immunity before intrusion in individuals may be very difficult and sometimes harmful since some deteriorate products, such as CTL and antibodies, may spread in the blood before pathogen intrusion. Thus, the acquired products circulating throughout the body may attack or bind the pathogens, their destructive products, or pathogen-infected cells, having negative effects on the body.

As indicated above, it should be considered that pathogens will attack species but not individuals like HIV-1, in humans and chimps, while SIV in monkeys. Humans have survived battled against various pathogens for a long period probably as a result of species-specific CD1-restricted innate immunity rather than individually restricted acquired immunity. Species-restricted innate cells are mainly arranged on the surface area where pathogens invade from outside. Here, an innovative vaccination strategy against various pathogens or tumors is suggested. Namely, similar to tumors, selective activation of innate immunity with slight damage of the pathogens using a potent drug or an antibody will spontaneously achieve to establish most favorable acquired immunity in each individual.

However, innate immune cells, such as DC, do not usually keep long, persistent memories, and thus have to be stimulated constantly to maintain their activities. Under the conditions in which selective and constant activation of DC is performed, attenuated or killed pathogens or their components, or even live infectious pathogens themselves, should be addressed to establish immunity. Such a procedure may help to generate the most suitable acquired immunity to control pathogens spontaneously. Also, as suggested in exposed, uninfected commercial sex workers, constant activation of innate DC is required to maintain the most favorable acquired immunity [29].

Intravesical bacillus Calmette-Guerin (BCG) therapy against human bladder carcinoma is considered the most successful immunotherapy against solid tumors [30]. During the course of determining the actual effector cells activated by intravesical BCG therapy to inhibit the growth of bladder carcinoma, we found that innate alert cells, such as $V\gamma2V\delta2$ T cells and NKT cells derived from peripheral mononuclear cells (PBMC) activated by live BCG-pretreated DC, appear to inhibit the proliferation of T24 tumor cells as well as eliminate them [31]. These findings strongly suggest that some products in live BCG or live BCG itself must stimulate suitable DC for tumor surveillance and such DC will help to induce and maintain most effective acquired effectors against the tumor. We are currently searching for the substances from live BCG.

Collectively, to develop an ideal vaccine, the activation of species-restricted innate immunity located at the surface compartment should be the focus to establish more favorable individual-restricted acquired immunity against external pathogens, such as HIV-1, or internal tumors. The fact that one can

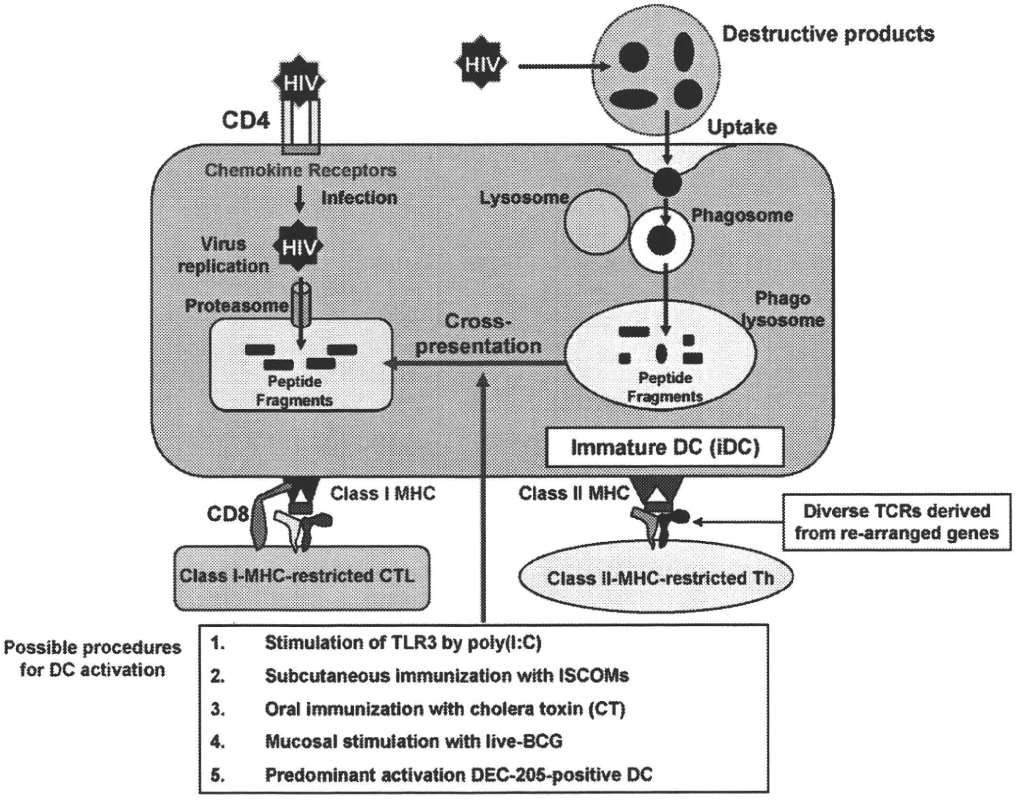

Fig. 2. Antigen-presentation by class I and class II MHC molecules. Intracellular antigens are degraded by proteasomes into peptides that are loaded into class I MHC and displayed on the cell surface to CD8-positive CTL. In contrast, extracellular antigens are taken up by phagosomes fused with lysosome containing various enzymes and processed into peptides that bind to class II MHC molecules to activate CD4-positive Th. Such extracellular peptide antigens can be displayed in association with class I MHC molecules to prime CD8-CTL when antigen-presenting DC are treated with various stimuli.

usually obtain a suitable protective acquired immunity including MHC molecule-restricted CTL after a pathogen infection indicates that some factors that activate innate DC to generate suitable acquired immunity via cross-presentation seem to be hidden in the pathogen itself (Fig. 2).

Conflict of interest statement

The author states that they have no conflict of interest.

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Transformation of breast milk macrophages by HTLV-I: implications for HTLV-I transmission via breastfeeding

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ABSTRACT

Human T cell leukemia virus type I (HTLV-I), a causative agent of adult T-cell leukemia (ATL), is transmitted from mother to child predominantly by breastfeeding. The source of HTLV-I-infected cells in breast milk has been thought to be T cells, however, the majority of cells in breast milk are $CD14^+$ macrophages but not $CD3^+$ T lymphocytes, and no data are available regarding HTLV-I transmission through breast milk macrophages (BrMMΦ). To explore the potential of BrMMΦ as a possible source of infection in mother to child transmission (MTCT) of HTLV-I, an immortalized cell line (HTLV-BrMMΦ) has been established from BrMMΦ by infection with HTLV-I. HTLV-BrMMΦ retained macrophage characteristics and did not express a complete dendritic cell (DC) phenotype; nevertheless, HTLV-BrMMΦ efficiently promoted T cell proliferation in primary allogeneic mixed lymphocyte reaction (MLR) like DC. Moreover, HTLV-I infection could be transmitted from HTLV-BrMMΦ to activated T cells in the peripheral blood. These findings suggested that BrMMΦ might be an appropriate HTLV-I reservoir involved in MTCT transmission via breastfeeding.

Human T cell leukemia virus type I (HTLV-I) is a delta-retrovirus that infects 10–20 million people worldwide. It is estimated that 2–5% of infected individuals develop adult T cell leukemia/lymphoma (ATL) during their lifetime after a long latent asymptomatic period (1). Further, another small proportion (0.1–2%) will develop a clinically distinct progressive neurological disease known as HTLV-I-associated myelopathy or tropical spastic paraparesis (21, 22). HTLV-I predominantly exists as a cell-associated provirus and can infect various types of cells, such as T cells, B cells, monocytes, and fibroblasts (7). Cell-to-cell contact seems to be required for transmission because naturally infected cells with HTLV-I produce very few amount of cell-free virions (2). HTLV-I is transmitted primarily in three ways: mother to child (8), man to woman (29), and blood transfusion (4). Among these routes, mother-to-child transmission (MTCT) of HTLV-I is the most important issue to stop, because infection early in life is associated with a subsequent risk of ATL (8). The risk of infection in infant from a seropositive mother correlates with provirus load in breast milk (17, 18), the concordance of HLA class I type between mother and child (3), and the duration of breastfeeding (25). So far, the source of HTLV-I-infected cells in breast milk has been thought to be $CD4^+$ T cells (16, 27), however, the majority of cells in breast milk particularly in early colostrum turned out to be $CD14^+$ macrophages but not $CD3^+$ T cells (23), and there has been no report regarding HTLV-I transmission via breast milk macrophages

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(BrMMΦ). Therefore, we have developed experimental systems for studying the biological roles of breast milk macrophages in MTCT of HTLV-I.

Recently, we have reported that BrMMΦ were capable of producing granulocyte-macrophage colony-stimulating factor (GM-CSF) spontaneously and differentiating into $CD1^+$ dendritic cells (DCs) by stimulation with exogenously added interleukin-4 (IL-4) (11). DCs are essential for the initiation of primary acquired/adaptive immune responses against viral infections by taking up antigen, migrating to lymphoid organs, and presenting selected antigens to T cells in association with their surface MHC molecules. Conversely, some viruses, like human immunodeficiency virus type 1 (HIV-1), hijack the trafficking properties of DCs to be transported them from the peripheral blood to lymph nodes (28). Also, we have found that DCs derived from BrMMΦ gained a strong capacity to transmit HIV-1 to permissible cells. These findings suggest that BrMMΦ, involved in the initiation and modulation of local immune responses, may provide an opportunity for the invaded pathogen to spread and transmit via breastfeeding.

Here, we have successfully established and characterized an immortalized macrophage cell line (HTLV-BrMMΦ) from BrMMΦ by infection with HTLV-I, and provide a possible mechanism of MTCT of HTLV-I by using the HTLV-BrMMΦ.

MATERIALS AND METHODS

Establishment of a BrMMΦ cell line. Early colostrum breast milk was collected from a healthy woman within 5 days of delivery after informed consent under a protocol approved by the Institutional Review Board of Nippon Medical School and in accordance with the human-experimental guidelines of the US Department of Health and Human Services. Fresh BrMMΦ were isolated from breast milk by Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation, followed by adherence to polystyrene tissue culture dishes for 1 h at 37°C. The adherent cells were then removed by incubation with 5 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 4°C. Freshly isolated BrMMΦ (1×10^5) were infected with 0.5 mL of supernatants containing 354 ng/mL of HTLV-I p19 from MT-2, an HTLV-I-producing T cell line, for 18 h at 37°C. After incubation, cells were washed with phosphate-buffered saline (PBS) to remove unabsorbed free virus and were further propagated in RPMI 1640 medium-based complete culture medium (10) supplemented with 10% fetal calf serum (FCS), 20 mmol/L HEPES (Invitrogen, Carlsbad, CA), 50 μmol/L 2-mercapto-ethanol (Sigma-Aldrich, St. Louis, MO), 2 mmol/L L-glutamine (Sigma), and 100 U penicillin-streptomycin solution (Sigma).

Cell preparation. Peripheral blood mononuclear cells (PBMCs) and cord blood mononuclear cells were isolated from heparinized blood using Ficoll-Hypaque density gradient centrifugation. For mixed lymphocyte reaction (MLR) assays, non-T cells were removed from PBMCs using the Lympho-kwik (One Lambda, Canoga Park, CA) according to the manufacturer's instructions.

Cell staining. Cytospin preparations of HTLV-BrMMΦ were fixed with 2% paraformaldehyde and then stained with May-Grünwald-Giemsa solution or non-specific esterase staining solution (1.1 mM α-naphthyl butyrate and 3.4 mM hexazotized pararosanilin in PBS, pH 5.0. For immunochemical analysis, cells were incubated with anti-HTLV-I p24 antibody (ZeptoMetrix Buffalo, NY). After washing, they were overlaid with Texas red-conjugated anti-mouse IgG antibody (Jackson Immuno Research Lab. Inc., Baltimore, MD). Fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 antibody (eBioscience Inc. San Diego, CA) was used to detect T cells. For electron microscopic analysis, cells were pelleted by centrifugation and fixed with modified Karnovsky's solution (2.5% glutaraldehyde and 1.6% paraformaldehyde in 0.1 M phosphate buffer). After fixed with 1% OsO_4, cells were dehydrated through a series of ethanol and n-butyl-glycidyl ether, and embedded in Epon 812 (TAAB, Berks, England). Ultrathin sections were cut and stained with uranyl acetate and lead citrate for examination by H-700 electron microscope (Hitachi, Tokyo, Japan). For flow cytometric analysis, FITC-conjugated antibodies to CD4, CD14, CD19, HLA-DR, CD40, CD80, CD1b, as well as phycoerythrin (PE)-conjugated CD11b, CD83, CD86, CXCR4, CCR5, CD1a, CD1d were purchased from BD Bioscience Pharmingen (Franklin Lakes, NJ). PE-conjugated antibody to CD1c was from Immunotech (Marseille, France) and PE-conjugated antibody to DC-SIGN was from R&D Systems (Minneapolis, MN). FITC-conjugated antibodies to mouse immunoglobulins were purchased from Beckman Coulter (Fullerton, CA). Cells were stained with the relevant antibody for 30 min on ice in PBS with 2% FCS and 0.01 mol/L sodium azide (PBS-based medium), washed twice, and resuspended in PBS-based medium. Labeled cells

were analyzed using a FACScan (Becton Dickinson, Mountain View, CA) with propidium iodide gating for viable cells.

Reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was extracted from HTLV-BrMMΦ using a commercial RNeasy kit (QIAGEN, Hilden, Germany), and first-strand DNA was synthesized using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA). Transcripts of HTLV-I-Tax were amplified by PCR reactions using primer: sense, 5'-tgtttggagactgtgta caaggcg-3'; antisense, 5'-gttgtatgattggcggggtaa-3'. After 35 cycles of PCR reactions, the PCR products were resolved by electrophoresis in agarose gels and visualized by ethidium bromide staining using a UV light source.

Southern hybridization analysis. Aliquots (15 μg) of high-molecular weight DNA were digested with EcoRI. The cleavage products were size-separated on 0.6% agarose gels and transferred to nylon-membranes. The HTLV-I-specific primer pair 5'-ccaccca gagaacctctaa-3' and 5'-gggtgtacaggttttgggc-3' was used to amplify 200 base pair fragment from MT-2 cells for using as a probe. Based on the previous report (24, 26), Southern hybridization was performed with digoxigenin labeled probe using DIG DNA labeling kit (Roche Applied Science, Mannheim, Germany) and the band pattern was visualized with DIG nucleic acid detection kit (Roche Applied Science).

MLR assays. For assessment of T cell stimulation in allogeneic MLR, both HTLV-BrMMΦ and freshly isolated BrMMΦ were irradiated (9000 rads) and used as antigen-presenting cells (APCs). Purified allogeneic T cells (5×10^4 cells/well) as well as allogeneic naïve cord blood T cells (5×10^4 cells/well) were cultured with the indicated numbers of irradiated APCs in 96-well U-bottomed tissue culture plates at 37°C for 5 days. Proliferation was measured by addition of 0.5 μCi ^{3}H-thymidine/well for the last 16 h, and the plates were harvested and counted using a β-counter (1450 Microbeta Trilux; Wallac, Gaithersburg, MD).

HTLV-I infection by HTLV-BrMMΦ. PBMC-derived lymphocytes (1×10^6/mL) were stimulated or unstimulated with either 10 μg/mL phytohemagglutinin (PHA)-P (Sigma) or 5 μg/mL concanavarin A (ConA) (Sigma), and co-cultured with HTLV-BrMMΦ (2×10^5/mL) or MT-2 cells (2×10^5/mL) for 4 days. The percentage of HTLV-I-infected T cells was determined by intracellular staining of HTLV-I-p24 gag antigen using BD Cytofix/Cytoperm (BD Bioscience Pharmingen). To expose HTLV-BrMMΦ to an acidic environment, HTLV-BrMMΦ were treated with culture medium adjusted to pH 3 for 3 min, followed by neutralization with 0.1 mol/L Tris-buffer (pH 8.0), and then co-cultured with either Con A-activated or PHA-activated T cells.

RESULTS

Establishment of a BrMMΦ cell line

Although BrMMΦ are thought to have an important role in MTCT of various pathogens, the precise roles of BrMMΦ remain unclear; therefore, we have established a BrMMΦ cell line persistently infected with HTLV-I, termed HTLV-BrMMΦ. The appearance of HTLV-BrMMΦ (Fig. 1A left) was different from freshly isolated BrMMΦ, which contained numerous lipid particles in the cytoplasm (Fig. 1A middle; see arrows) as reported previously (11). Moreover, HTLV-BrMMΦ constitutively expressed high levels of intracellular HTLV-I antigens p24 (Fig. 1A right). These virus particles stained with uranyl acetate and lead citrate could apparently be observed in thin-section electron micrographs of fixed HTLV-BrMMΦ (Fig. 1B). Moreover, induced HTLV-I infection was confirmed by RT-PCR using a primer to Tax of HTLV-I (Fig. 1C), and Southern blot hybridization analysis revealed the integrated-status of the HTLV-I provirus (Fig. 1D). To examine whether HTLV-I virions are released from HTLV-BrMMΦ into culture supernatants, we measured the extracellular HTLV-I antigen p19 by ELISA. Indeed, culture supernatants from HTLV-BrMMΦ harvested on day 2 contained higher amounts of p19 (623.6 ± 38.7 ng/mL) compared to that from MT-2 (354.1 ± 13.1 ng/mL).

Characterization of HTLV-BrMMΦ

To define the physiological properties of HTLV-BrMMΦ, we examined phagocytic activity of HTLV-BrMMΦ by flow cytometry and fluorescence microscopy, as well as by electron microscopy using fluorescent latex particles. The majority of HTLV-BrMMΦ displayed a remarkable phagocytosis (Fig. 2A, 2B). In addition, cytochemical staining of HTLV-BrMMΦ showed strong positive reactions for nonspecific esterase (Fig. 2C). These findings indicated that HTLV-BrMMΦ retained macrophage phagocytic activity to uptake and process foreign antigens.

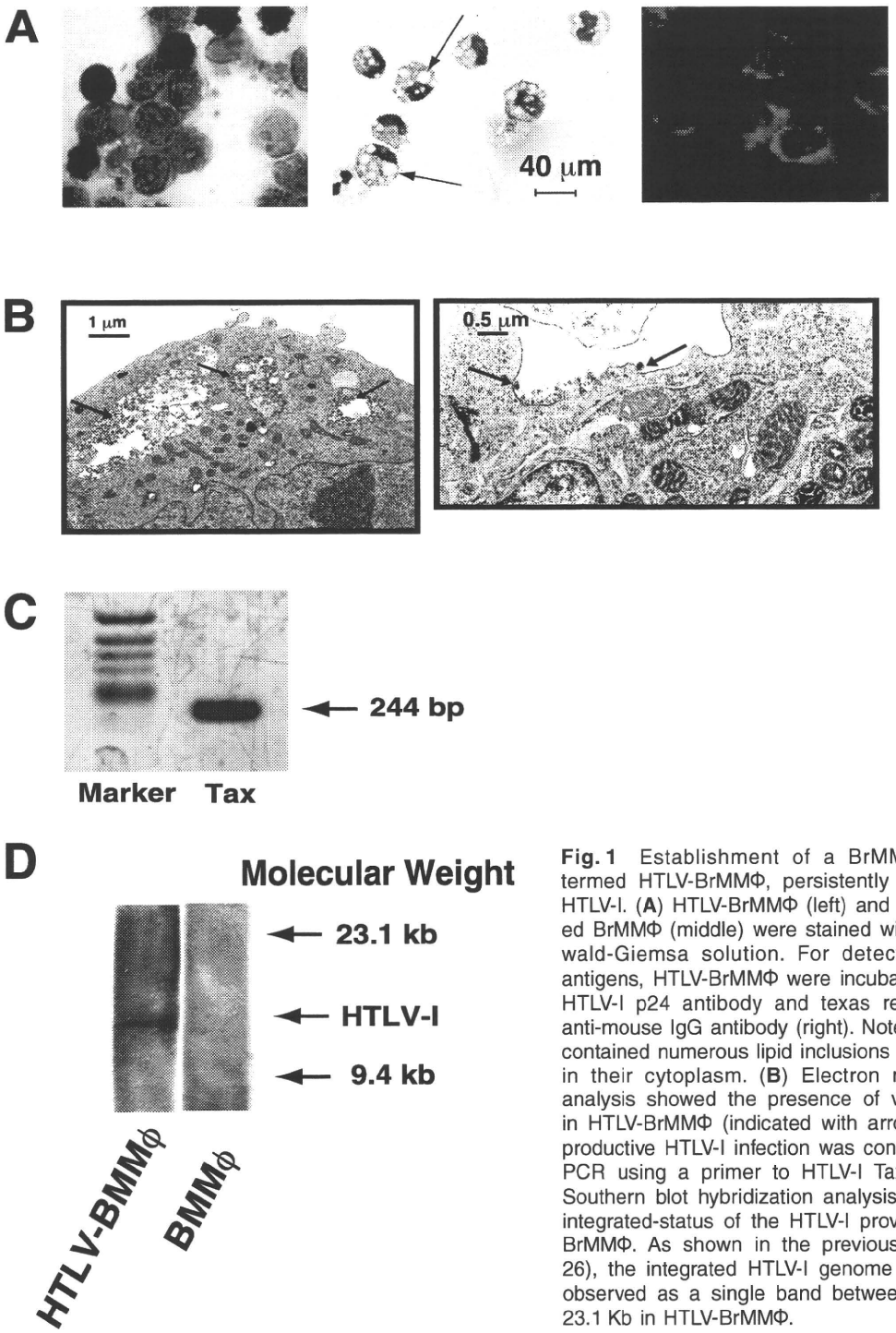

Fig. 1 Establishment of a BrMMΦ cell line, termed HTLV-BrMMΦ, persistently infected with HTLV-I. (**A**) HTLV-BrMMΦ (left) and freshly isolated BrMMΦ (middle) were stained with May-Grünwald-Giemsa solution. For detection of virus antigens, HTLV-BrMMΦ were incubated with anti-HTLV-I p24 antibody and texas red-conjugated anti-mouse IgG antibody (right). Note that BrMMΦ contained numerous lipid inclusions (arrows) within their cytoplasm. (**B**) Electron microscopical analysis showed the presence of virus particles in HTLV-BrMMΦ (indicated with arrows). (**C**) The productive HTLV-I infection was confirmed by RT-PCR using a primer to HTLV-I Tax mRNA. (**D**) Southern blot hybridization analysis revealed the integrated-status of the HTLV-I provirus in HTLV-BrMMΦ. As shown in the previous reports (24, 26), the integrated HTLV-I genome was similarly observed as a single band between 9.4 Kb and 23.1 Kb in HTLV-BrMMΦ.

Next, we analyzed the expression of surface antigens on HTLV-BrMMΦ and compared those on freshly isolated BrMMΦ. Both T and B cell markers, CD3 and CD19, were not expressed on HTLV-BrMMΦ as well as BrMMΦ. Monocyte/macrophage lineage markers, CD14 and CD11b, were expressed on original BrMMΦ but not on transformed HTLV-BrMMΦ. Nevertheless, both types of cells expressed antigen-presenting molecules, such as HLA-DR and class I MHC, as well as various co-stimulatory molecules, such as CD80, CD86 and CD40, involved in T cell stimulation (Fig. 3A). Moreover, CD83, a

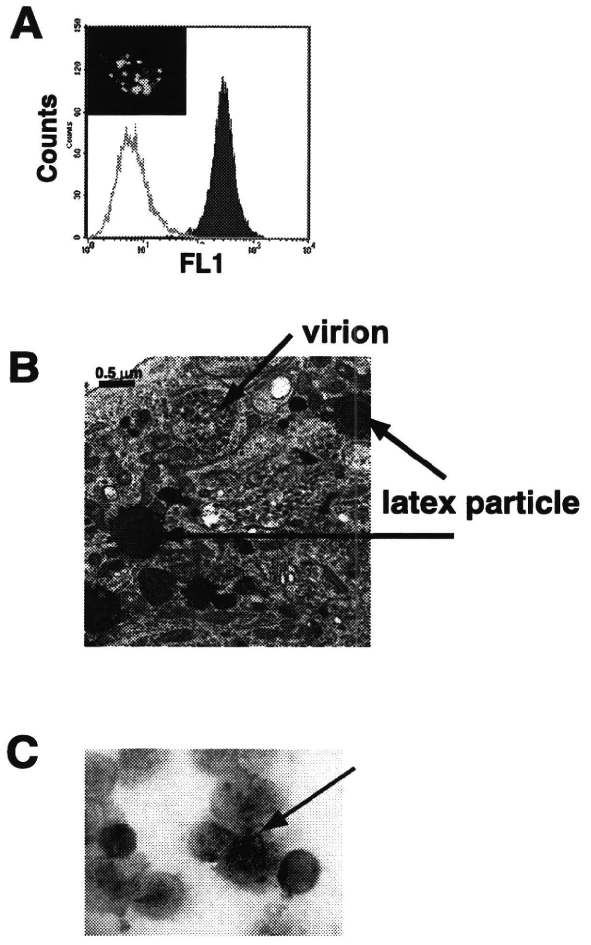

Fig. 2 HTLV-BrMMΦ retained macrophage characteristics. Flow cytometric analysis and fluorescence microscopic analysis using fluorescent latex particles for the phagocytic activity of HTLV-BrMMΦ. (**A**) HTLV-BrMMΦ were cultured with FITC-labeled latex beads for 1 h. After washing, flow cytometric and fluorescent microscopic observation indicated that the majority of HTLV-BrMMΦ displayed a remarkable phagocytosis. (**B**) Electron micrograph of latex-beads-treated HTLV-BrMMΦ. (**C**) Non-specific esterase staining of HTLV-BrMMΦ.

glycoprotein expressed on mature DCs was also expressed on both cells (Fig. 3A). These findings strongly suggested that, similar to uninfected BrMMΦ that showed strong capacity to induce allogeneic MLR as demonstrated previously, the virally transformed HTLV-BrMMΦ might also have the ability to stimulate T cells via allogeneic MLR.

Therefore, we examined whether virus-infected HTLV-BrMMΦ could stimulate T cells to proliferate in a primary allogeneic MLR. As responders for primary MLR, we used purified T cells from PBMCs and naïve cord blood T cells based on our previous findings, in which a higher number of uninfected BrMMΦ generated stronger proliferative responses (11). In contrast to our previous results, although an appropriate number of HTLV-BrMMΦ induced proliferation of allogeneic T cells, a higher number of HTLV-BrMMΦ did not (Fig. 3B). This might be because infected HTLV-BrMMΦ may secrete some growth inhibitory factors for T cells. We are currently investigating to compare cellular factors between uninfected BrMMΦ and HTLV-BrMMΦ.

Because freshly isolated uninfected BrMMΦ expressed the C-type lectin DC-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN) (9) that will tightly capture free HIV virions and transmit them to HIV-1-susceptible infant CD4-positive cells (23), we examined DC-SIGN expression on HTLV-BrMMΦ. Unexpectedly, DC-SIGN was not detected on transformed HTLV-BrMMΦ at all (Fig. 3C) and the lack of its expression was further confirmed at the transcriptional level by RT-PCR (data not shown), although CD4 and CXCR4 but not CCR5 were observed on their surface (Fig. 3C). These findings indicate that transformed HTLV-BrMMΦ had lost the capacity to capture HIV-1 virions while they may retain HIV-1 susceptibility to X4-type HIV-1.

Transmission of HTLV-I to T cells via HTLV-BrMMΦ

To confirm whether HTLV-I is transmittable from HTLV-BrMMΦ to PBMCs, viable HTLV-BrMMΦ were co-cultured with lectin-stimulated or un-stimulated PBMCs. As controls, PBMCs were also co-cultured with a known HTLV-I producing cell line, MT-2. FACS analysis showed the intracellular expression of HTLV-p24 gag antigen; about 9% positive for p24 antigen in stimulated T cells and below 1% positive in un-stimulated T cells at day 4. Thus, transmission of HTLV-I to PBMCs was mediated only to the activated T cells and the transmissibility by HTLV-BrMMΦ was more potent than that by MT-2 cells (Fig. 4A). Also, transmission of HTLV-p24 antigen was confirmed in the lectin-stimulated T cells by fluorescent laser microscopy (data not shown).

These results suggest that breast milk of the HTLV-I-infected mothers who may have virus-infected BrMMΦ might transmit the virions to gastro-intestinal targets in the newborn baby through breastfeeding. The ingested milk with virus-infected BrMMΦ must encounter gastric juice with low pH (pH 3.0–4.0) before meeting with intestinal target cells (19). Also, there is a possibility that BrMMΦ may die under the acidic condition. Therefore, we conducted an experiment in which HTLV-BrMMΦ

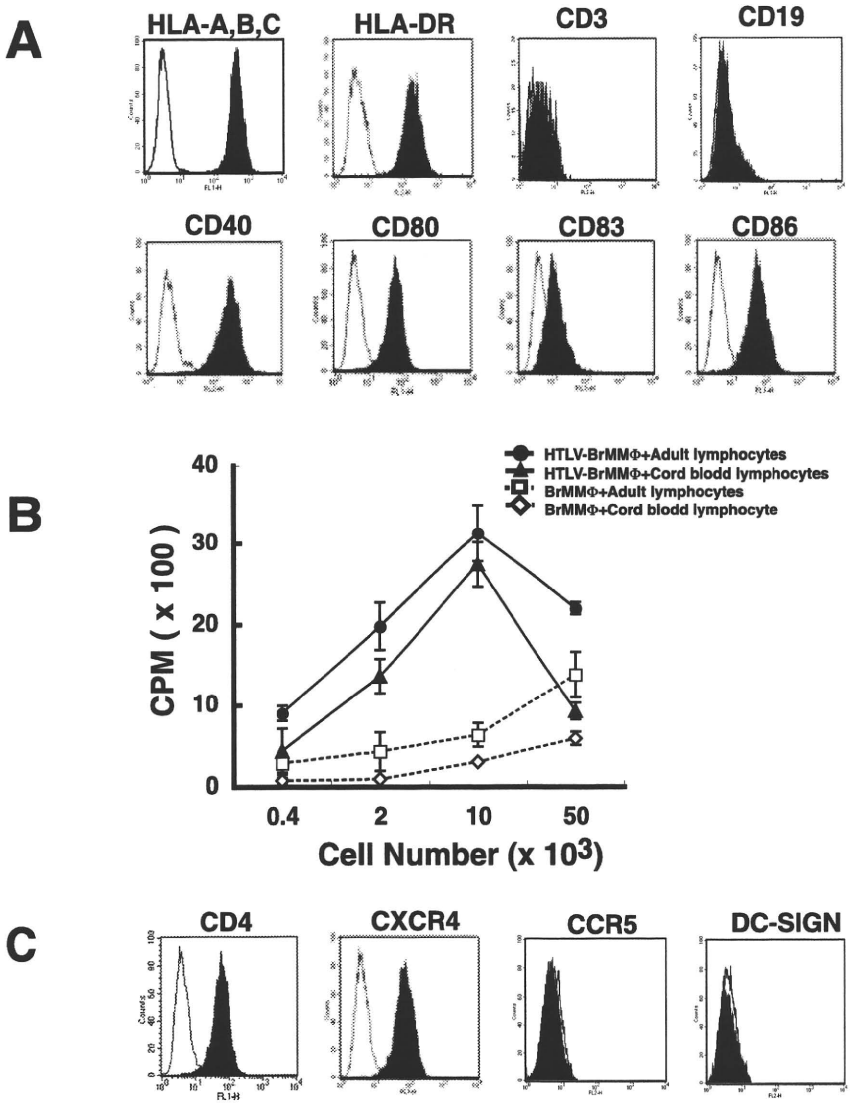

Fig. 3 Characterization of HTLV-BrMMΦ and their potency to promote T cell proliferation in an allogeneic MLR. (**A**) Analysis of antigen-presenting and co-stimulatory molecule-expression on HTLV-BrMMΦ by flow cytometry. (**B**) Purified allogeneic T cells (5×10^4/well) derived from PBMCs (● or □) or allogeneic naïve cord blood T lymphocytes (▲ or ◇) were co-cultured with the indicated numbers of irradiated (9,000 rad) HTLV-BrMMΦ or control BrMMΦ for 5 days. Proliferation was measured by addition of 0.5 μCi ^{3}H-thymidine/well for the last 16 h. (**C**) Analysis of CD4, chemokine receptors, and DC-SIGN expression on HTLV-BrMMΦ by flow cytometry.

pre-treated with culture medium adjusted to pH 3 for 3 min were further co-cultured with either concanavalin-A (Con A) or phytohemaggulutinin (PHA)-stimulated PBMCs (Fig. 4B). The results showed that medium acidity had little influence on HTLV-I transmissibility for lectin-stimulated PBMCs. Taken together, the HTLV-I-infected BrMMΦ in the colostrums milk of HTLV-I-infected mothers may stimulate mucosal infant T cells and transmit them the virions via breastfeeding.

DISCUSSION

The evidence of HTLV-I transmission via breast milk has been well documented since the 1980s and recent reports have also shown the importance of proviral load in breast milk as a predictor of HTLV-I transmission from mother to child (18). Particularly, in Japan, screening of pregnant women and avoiding breastfeeding by those infected resulted in profound reduction of the prevalence of HTLV-I (14). HTLV-I infection requires cell-to-cell contact be-

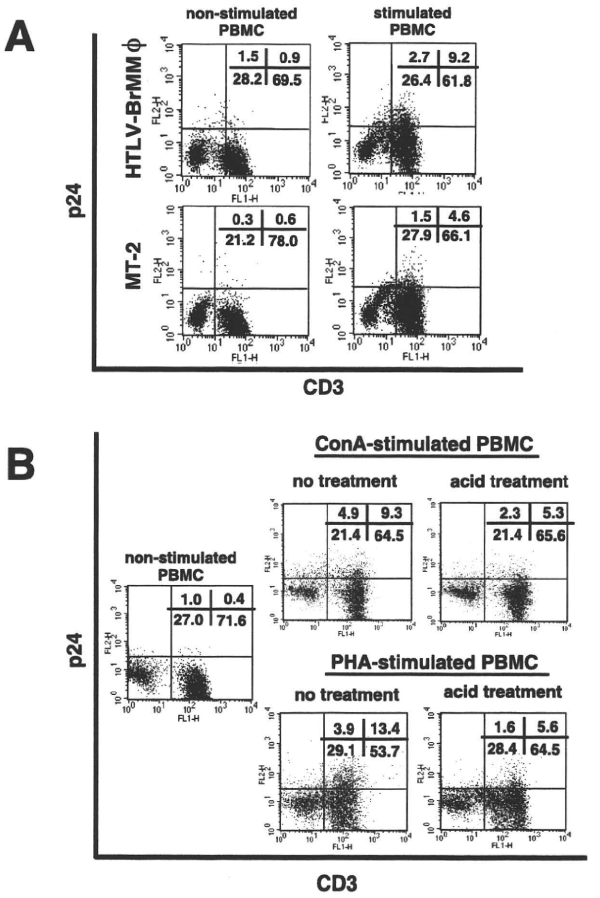

Fig. 4 Transmissibility of HTLV-I to T cells by HTLV-producing cells. (**A**) HTLV-BrMMΦ or MT-2 cells (2×10^5/mL) were co-cultured with stimulated or un-stimulated PBMCs (1×10^6/mL) for 4 days. The percentage of HTLV-I-infected T cells was determined by intracellular staining for HTLV-I-p24 gag antigen. HTLV-I infected T cells derived from PBMCs are expressed as double positive populations with HTLV-I p24 gag antigen (FL2) and CD3 antigen (FL1). (**B**) HTLV-BrMMΦ were treated with culture medium adjusted to pH 3 for 3 min, followed by neutralized with 0.1 mol/L Tris-buffer (pH 8.0), and then co-cultured with Con A-stimulated (top panels) or PHA-stimulated stimulated (bottom panels) PBMCs.

cause lymphocytes naturally infected with HTLV-I produce very few cell-free HTLV-I virions (6). HTLV-I-infected T cells in breast milk have been proposed as a source of HTLV-I infection. However, the following evidence has led us to hypothesize that BrMMΦ would be more likely to be responsible for MTCT of HTLV-I. First, HTLV antigen-positive T cells from seropositive mothers were not always detected in their breast milk (27). The majority of breast milk cells are $CD14^+$ macrophages (23). Also, it should be noted that the HTLV-I receptor is the glucose transporter 1 protein (GLUT1) (15) and surface heparin proteoglycan serves as a receptor for HTLV-I (12). Both GLUT1 and heparin proteoglycan are ubiquitously expressed on the surface of many types of cells, hence, HTLV-I can infect not only $CD4^+$ T cells but also macrophages. Second, we have previously reported that BrMMΦ were capable of producing GM-CSF spontaneously and differentiating into immature DCs by stimulation with exogenous IL-4 alone, indicating that BrMMΦ maintain the developmental potential to differentiate into DCs and efficiently respond to environmental stimuli. DCs act not only as sentinels for the detection of, but also as target cells for intruded viruses, and this can be important for viral transport and spread.

We have demonstrated that BrMMΦ stimulated with IL-4 showed a strong capacity to transmit HIV-I with enhanced expression of DC-SIGN (23). Moreover, Jones *et al.* demonstrated that HTLV-I can efficiently infect myeloid and plasmacytoid DCs, and DCs exposed to HTLV-I can rapidly, efficiently, and reproducibly transfer a virus to autologous primary $CD4^+$ T cells (13). Therefore, to explore the potential of BrMMΦ to be a HTLV-I reservoir involved in transmission during breastfeeding, we have established and characterized an immortalized cell line of HTLV-BrMMΦ, which lost monocyte/macrophage lineage markers CD14 and CD11b. Nevertheless, they expressed DC marker CD83, antigen-presenting molecules HLA-DR, and co-stimulatory molecules such as CD80, CD86, and CD40. These phenotypes were consistent with immature DCs (iDCs) derived from monocytes co-cultured with both GM-CSF and IL-4. However, DC-SIGN and human group 1 CD1 molecules (CD1a, CD1b, CD1c), expressed mainly on professional APCs, were not detected on HTLV-BrMMΦ. These findings indicate that HTLV-BrMMΦ were not equipped with a complete DC phenotype; however, an appropriate number of them efficiently induced T cell proliferation in an allogeneic MLR like DCs. The ability of HTLV-BrMMΦ to stimulate T cells is of considerable significance, because stimulated T cells are highly susceptible to virus infections; thus, HTLV-BrMMΦ can serve as both a T cell stimulator and a source of infection. Collectively, HTLV-BrMMΦ derived from colostrum milk seem to be an excellent reservoir as well as a transmitter for HTLV-I.

In this study, we showed the ability of HTLV-BrMMΦ to transmit HTLV-I virions to stimulated T cells derived from PBMCs but few un-stimulated T cells could be infected by contact with HTLV-BrMMΦ, which may be due to the inappropriate

culture conditions for T cell activation. It is possible that when HTLV-I-infected BrMMΦ are ingested with the maternal breast milk and arrive at the infant intestinal tracts through exposure to acidic gastric juice, they might encounter intestinal intraepithelial lymphocytes (IEL). Since most IELs at the surface mucosal compartment are 'partially activated' T cells waiting for appropriate signals to put them into a state of full activation (20), IELs might be more susceptible to HTLV-I infection. Indeed, Bourinbaiar *et al.* proposed that ATL might be derived from the intestinal lymphocytes of breastfed infant, because ATL-derived $CD4^+$ T cell lines were positive for HML-1 monoclonal antibody that recognizes human intestinal lymphocytes (5). Although we have not yet shown that BrMMΦ were actually infected with HTLV-I, if effective infection of intestinal lymphocytes with HTLV-I is necessary for the development of ATL, HTLV-I infected BrMMΦ might be an appropriate HTLV-I reservoir with regard to a potent T cell stimulator.

We showed here that BrMMΦ could be differentiated into DC-like cells by HTLV-I infection. Since breastfeeding allows the invasion of foreign antigens into breast milk, BrMMΦ may have an opportunity to encounter the antigens. In some cases, BrMMΦ might be stimulated and undergone phenotypic and functional modifications leading to a beneficial or deteriorate influence on infant immunity after breastfeeding. Therefore, to further clarify the biological roles of individual BrMMΦ may be helpful to understand the formation of immune systems of breastfed infant.

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IMMUNOLOGY ORIGINAL ARTICLE

Inhibition of DC-SIGN-mediated transmission of human immunodeficiency virus type 1 by Toll-like receptor 3 signalling in breast milk macrophages

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Summary

The majority of cells in early/colostrum milk are breast milk macrophages (BrMMø) expressing dendritic cell (DC)-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN), and the expression level of DC-SIGN on BrMMø will determine cell-to-cell human immunodeficiency virus type 1 (HIV-1) transmissibility. Thus, one of the strategies to prevent vertical transmission of HIV-1 through breast-feeding is to find a way to suppress DC-SIGN expression on BrMMø. As for the expression of Toll-like receptors (TLRs) in BrMMø, TLR3 was always seen in BrMMø but not in peripheral blood monocytes (PBMo). Also, the expression of TLR3 was slightly enhanced in BrMMø when the cells were treated with interleukin (IL)-4. Moreover, when TLR3 was stimulated with its specific ligand, the double-stranded RNA (dsRNA) poly(I:C), DC-SIGN expression on BrMMø was reduced even in the IL-4-mediated enhanced state. Some reduction may be caused by type I interferons (IFNs), such as IFN-α/β, secreted from BrMMø. Indeed, both IFNs, particularly IFN-β, showed a strong capacity to suppress the enhancement of DC-SIGN expression on IL-4-treated BrMMø and such TLR3-mediated DC-SIGN suppression was partially abrogated by the addition of anti-IFN-α/β-receptor-specific antibodies. As expected, DC-SIGN-mediated HIV-1 transmission to CD4-positive cells by BrMMø was inhibited by either poly(I:C) stimulation or by treatment with type I IFNs. These findings suggest a possible strategy for preventing mother-to-child transmission (MTCT) of HIV-1 via breast-feeding through TLR3 signalling.

Keywords: breast milk macrophages; colostrum/early breast milk; dendritic cell-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN); human immunodeficiency virus type 1 mother-to-child transmission; Toll-like receptor 3; type I interferons

Introduction

Although mother-to-child transmission (MTCT) of human immunodeficiency virus type 1 (HIV-1) has been markedly reduced by antiretroviral treatment and avoidance of breast-feeding,[1] around 400 000 newly infected children have been born, particularly in resource-limited countries (AIDS epidemic update. UNAIDS, http://www.UNAIDS.org accessed 29 July 2008), via vertical transmission during pregnancy, delivery and breast-feeding. Among these three distinct routes, breast-feeding is still a major public health concern in developing countries. The risk of HIV-1 infection of infants via breast-feeding has been found to be influenced by breast milk virus load, which is significantly higher in early/colostrum milk than in mature breast milk.[2]

The majority of cells in colostrum milk have been identified as unique large cells, termed breast milk macrophages (BrMMø), expressing both CD4 and CD14.[3] Importantly, BrMMø also express chemokine receptors such as chemokine (C-X-C motif) receptor 4 (CXCR4) and chemokine (C-C motif) receptor 5 (CCR5), which permit HIV-1 entrance, as well as CD83, a maturation marker of dendritic cells (DCs).[4] Thus, BrMMø have been

identified as DC-lineage HIV-1-vulnerable cells and also express C-type lectin DC-specific intercellular adhesion molecule 3 (ICAM3) grabbing nonintegrin (DC-SIGN),[5] which will tightly capture free HIV-1 virions and transmit them to HIV-1-susceptible infant CD4-positive cells.[3] Moreover, after co-culture with interleukin (IL)-4, BrMMø were found to have enhanced DC-SIGN expression,[4] and became resistant to HIV-1 infection. Therefore, IL-4-treated BrMMø will not be infected by HIV-1 but will rather capture free virus particles via DC-SIGN, and such cell-associated virions would more readily be transmitted to HIV-1-susceptible cells via breast-feeding.

Local production of IL-4 in mastitis may up-regulate the expression of DC-SIGN in BrMMø, which may explain why mastitis is linked to higher HIV load in breast milk and a higher risk of mother-to-infant vertical transmission of the virus.[6] Indeed, it has recently been reported that increased cell-associated HIV-1 but not cell-free virion shedding in breast milk could mediate the association between mastitis and MTCT.[7] In addition, we reported previously that high transmissibility was mediated through HIV-1 virions captured by DC-SIGN but not through cell-free virus particles released from HIV-1-infected cells,[3] although some reports indicate that cell-free HIV-1 in breast milk may contribute to vertical transmission.[8] Therefore, in order to prevent vertical transmission of HIV-1 through breast-feeding, it is necessary to find a way to inhibit the acquisition of free HIV-1 virions via DC-SIGN by suppressing its expression on BrMMø.

In the present study, careful examination of BrMMø revealed the apparent expression of Toll-like receptor 3 (TLR3) in freshly isolated BrMMø, although we could not detect TLR3 in peripheral blood monocytes (PBMo). However, TLR3 was detected in PBMo when they were stimulated with granulocyte–macrophage colony-stimulating factor (GM-CSF), which is spontaneously produced in BrMMø.[4] Moreover, freshly isolated TLR3-positive BrMMø also expressed DC-SIGN and the expression of TLR3 was slightly enhanced in IL-4-treated BrMMø, in which DC-SIGN expression is significantly enhanced. Thus, we attempted to stimulate TLR3 with one of its ligands, poly(I:C), which is a double-stranded RNA (dsRNA),[9,10] to investigate its effect on DC-SIGN expression, and found a reduction in DC-SIGN expression in both freshly isolated BrMMø and IL-4-treated BrMMø. Also, poly(I:C)-stimulated BrMMø secreted considerable amounts of type I interferons (IFNs), such as IFN-α and IFN-β. As expected, DC-SIGN-mediated HIV-1 transmission to susceptible CD4-positive cells by BrMMø was inhibited by TLR3 signalling with poly(I:C) or treatment of BrMMø with their products, type I IFNs. We discuss our findings in terms of this unique feature of BrMMø and propose a possible strategy for preventing MTCT of HIV-1 via breast-feeding through TLR3 signalling.

Materials and methods

Isolation and culture of BrMMø and PBMo

Breast milk was collected from healthy women within 2–6 days of delivery after informed consent had been obtained under a protocol approved by the Institutional Review Board of Nippon Medical School. Breast milk cells were isolated from freshly obtained breast milk by Ficoll-Hypaque (Amersham-Pharmacia Biotech, Uppsala, Sweden) gradient centrifugation methods as described previously.[4] BrMMø were isolated from freshly collected breast milk cells, and allowed to adhere to polystyrene tissue culture dishes (Corning, New York, NY) for 1–2 hr at 37°. After non-adhering cells had been gently removed, adherent cells were washed with warm RPMI-1640 medium containing 2% fetal calf serum (FCS) (HyClone Laboratories, Logan, UT). The remaining adherent cells were then removed by incubation with 5 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 4° and confirmed to express homogeneous $CD14^+$ cells at approximately 95% using a FACScan (BD Biosciences, Mountain View, CA). To obtain PBMo, $CD14^+$ monocytes were isolated from peripheral blood of healthy volunteers by magnetic depletion using a monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) containing hapten-conjugated antibodies to CD3, CD7, CD16, CD19, CD56, CD123 and Glycopholin A and a magnetic antibody cell sorter (MACS; Miltenyi Biotec) according to the manufacturer's instructions, routinely resulting in > 90% purity of $CD14^+$ cells. The isolated monocytes were cultured in 24-well culture plates (Corning) for 6 days in RPMI-1640-based complete culture medium (CCM)[11] supplemented with 10% FCS (HyClone Laboratories), 20 mM HEPES (Invitrogen, Carlsbad, CA), 50 mM 2-mercaptoethanol (2-ME) (Sigma-Aldrich, St Louis, MO), 2 mM L-glutamine (Sigma-Aldrich) and 100 units of penicillin-streptomycin (Sigma-Aldrich), together with 100 ng/ml GM-CSF (PeproTech, Rocky Hill, NJ) and 20 ng/ml IL-4 (Biosource Intl., Camarillo, CA) to obtain immature DCs (iDCs).

Reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted from 3×10^5 cells of each cell preparation using the commercial RNeasy Kit (Qiagen, Hilden, Germany), and first-strand DNA was synthesized as described previously.[12] Transcripts of TLRs as well as the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by PCR reaction. The primer sets were: GAPDH sense, 5′-GCC TCA AGA TCA TCA GCA ATG C-3′; GAPDH anti-sense, 5′-ATG CCA GTG AGC TTC CCG TTC-3′; TLR1 sense, 5′-CGC ATG GTC CAC ATG CTT T-3′; TLR1 anti-sense, 5′-GCC

