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Signaling Lymphocytic Activation Molecule Is Expressed on CD40 Ligand-Activated Dendritic Cells and Directly Augments Production of Inflammatory Cytokines[1]

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Dendritic cells (DC) comprise a key part of the innate immune system that, upon activation, profoundly influences the nature of the adaptive T cell response. In this study, we present evidence that signaling lymphocytic activation molecule (SLAM), a molecule first identified in activated T and B cells, is strongly up-regulated in DC activated through CD40, as well as in response to inflammatory stimuli, including polyinosinic polycytidylic acid and LPS. mRNA encoding both membrane-bound and soluble secreted isoforms of SLAM was detected in CD40 ligand-activated DC, comprising two of the four known SLAM isoforms. Expression of membrane-bound SLAM protein peaked at 12 h poststimulation with CD40 ligand, gradually returning to baseline levels after 6 days. SLAM up-regulation appears to be a direct result of the induction of DC maturation, as inflammatory cytokines released during this process do not affect SLAM expression. Functionally, engagement of SLAM enhances DC production of IL-12 and IL-8, while having no effect on production of IL-10. Because SLAM is involved in the activation of T cells, the expression of SLAM on DC may provide a bidirectional signaling mechanism in which interacting DC and T cells are simultaneously and synergistically activated to mount proinflammatory Th1 responses. *The Journal of Immunology,* **2001, 167: 3174–3181.**

Dendritic cells (DC)[3] contribute by several mechanisms to the generation of an effective immune response against foreign Ags. As part of the innate immune system, DC express various pattern recognition receptors that can be triggered upon recognition of microbial ligands (1, 2), bacterial CpG DNA (3), and viral RNA (4) to induce the release of proinflammatory cytokines that can influence the nature of the adaptive T cell response (5). Additionally, DC are the most potent of the professional APCs, providing a pathway for the direct activation of specific T cell populations during the adaptive immune response (6, 7).

In order for DC to carry out their duties during an adaptive immune response, they must first be activated to begin the maturation process. When an appropriate maturational cue is received, DC are signaled to undergo rapid morphological and physiological changes that facilitate the initiation and development of immune responses (8). Among these are the up-regulation of molecules involved in Ag presentation; production of proinflammatory cytokines, including IL-12, key to the generation of Th1 responses; and secretion of chemokines that help to drive differentiation, expansion, and migration of surrounding naive Th cells (7, 9, 10). Collectively, these up-regulated molecules facilitate the ability of DC to coordinate the activation and effector function of other surrounding lymphocytes that ultimately provide protection for the host. Although the process of DC maturation is commonly associated with events that lead to the generation of adaptive immunity, many stimuli derived from the innate branch of the immune system are also capable of activating DC to initiate this process. In this manner, DC provide a link between the two branches of the immune response, in which their initial activation during the innate response can influence both the nature and magnitude of the ensuing adaptive response (11).

Several independent pathways have been characterized that induce DC maturation, all of which enhance the ability of DC to initiate and direct the immune response. One such pathway involves the interaction between CD40 on the surface of the DC, and CD40 ligand (CD40L) expressed on activated Th cells (12). The importance of CD40 signaling in the context of the development and establishment of an immune response has been clearly documented. Previous studies using $CD40^{-/-}$ and $CD40L^{-/-}$ mice have demonstrated that these mice are more susceptible to *Leishmania major* infection and have severe defects in the production of inflammatory cytokines, including IL-12, TNF-α, and IFN-γ, compared with wild-type mice (13–17). These mice also appear to have impaired T cell proliferative responses, as well as decreased primary and secondary humoral responses (17, 18). In humans, CD40-activated DC are known to be endowed with an enhanced ability to stimulate $CD4^+$ T cells, primarily by up-regulating Ag presentation and costimulatory molecules and by the release of proinflammatory cytokines (19).

Because of the importance of CD40-CD40L signaling events in the establishment of inflammatory immune responses, we investigated genes that are up-regulated during DC maturation that contribute to their effector function. In this study, we used a subtractive hybridization technique to identify genes induced in CD40L-matured DC. Through this analysis, we have identified signaling lymphocytic activation molecule (SLAM; also known as

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[3] Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; SLAM, signaling lymphocytic activation molecule; TLR, Toll-like receptor.

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CDw150, IPO-3), an unusual costimulatory molecule previously characterized on T and B cells (20, 21). SLAM belongs to the Ig superfamily of receptors, and has been shown to enhance cellular proliferation, production of inflammatory cytokines, and Ig secretion. In T cells, engagement of SLAM was shown to augment production of IFN-γ from cells of the Th1 lineage. Interestingly, committed Th2 cells appeared to be reprogrammed to become Th1/Th0-like following SLAM engagement, down-regulating their production of IL-4 in favor of IFN-γ. In this study, we show that transcripts encoding both membrane-bound and secreted isoforms of SLAM were detected in DC treated with CD40L. SLAM protein was also found to be highly expressed on the surface of DC activated by CD40L, LPS, or poly(I:C), a synthetic dsRNA molecule. SLAM receptor engagement on DC augmented the production of proinflammatory cytokines, including IL-12, pivotal in the differentiation of T cell responses toward the Th1 pattern, but had no effect on production of IL-10, a cytokine involved in the down-regulation of Th1 responses. Together, our data suggest that the expression of SLAM on DC facilitates the generation of proinflammatory Th1 responses.

Materials and Methods

Preparation, maturation, and stimulation of DC

Peripheral blood taken from healthy donors was enriched for $CD14^+$ cells using the RosetteSep monocyte enrichment kit (StemCell Technologies, Vancouver, British Columbia, Canada). Blood was then centrifuged over a Ficoll gradient (Amersham Pharmacia, Uppsala, Sweden) to isolate PBMC. Adherent cells were isolated by culturing in complete medium (RPMI 1640, 0.1 mM sodium pyruvate, 2 mM penicillin, 50 μg/ml streptomycin; Life Technologies, Grand Island, NY) supplemented with 1% FCS (Omega Scientific, Tarzana, CA) for 2 h. Remaining nonadherent cells were removed by washing with 1× PBS. To generate immature DC, adherent cells were cultured in a CO_2 incubator at 37°C for 7 days in complete medium containing 10% FCS, 800 U/ml GM-CSF (Genetics Institute, Cambridge, MA), and 1000 U/ml IL-4 (Schering-Plough, Madison, NJ) as previously described (22). These cells were nonadherent, displaying typical DC morphology. Purity of the DC was typically >95%, as determined by flow cytometry.

For DC maturation, cells were harvested from flasks after 7 days using PBS-EDTA (1 mM), and washed twice in complete medium. Cells were counted and plated at a concentration of 2.5–5 $\times$ 10^5 DC/ml in either T75 or T25 tissue culture flasks or 96-well tissue culture plates (Corning Glass Works, Corning, NY). DC were treated with 1 μg/ml soluble CD40L trimer (generously provided by Immunex, Seattle, WA) for 24 h to induce maturation. Other maturation stimuli/cytokines used in this study were the following: purified *Salmonella minnesota*-derived LPS (10 ng/ml) and poly(I:C) (20 μg/ml), both purchased from Sigma (St. Louis, MO); recombinant IL-12 (5 ng/ml) and IL-18 (1 ng/ml), both purchased from PeproTech (Rocky Hill, NJ); and TNF-α (50 ng/ml), purchased from Endogen (Woburn, MA).

RNA isolation and purification

DC (5×10^6) cultured in the presence or absence of CD40L for 24 h were harvested, and total RNA was isolated using guanidinium isothiocyanate buffer as previously described (23). RNA was resuspended in RNase-free water, and treated with 10 U DNase I (Promega, Madison, WI) for 1 h at 37°C to remove contaminating genomic DNA. RNA was further purified by standard phenol-chloroform extraction and precipitated overnight in isopropanol at −20°C. RNA pellets were resuspended in RNase-free water containing RNase inhibitor and stored at −80°C.

Subtractive hybridization and identification of differentially expressed cDNA fragments

Subtractive hybridization was performed using the PCR Select Subtractive Hybridization kit (Clontech Laboratories, Palo Alto, CA). This kit contains all necessary reagents for cDNA synthesis, normalization, and subtraction. Total RNA (3 μg) isolated from DC cultured with or without CD40L was used as starting material for this technique, which generated a subtracted population of partial cDNA fragments representing differentially expressed genes enriched in CD40L-stimulated DC.

To identify gene fragments isolated in the subtraction, cDNA taken from our subtracted pool was cloned using the Topo-TA cloning system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ligations were then transformed into DH5α competent cells (Life Technologies) and plated on selective medium containing ampicillin and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) for use in blue-white screening. Colonies containing an insert were grown in selective medium and miniprepped using the Wizard Plus kit (Promega). Cloned cDNA inserts were then sequenced by Applied Biosystems Prism (PerkinElmer, Foster City, CA), and resulting sequences were searched against the BLAST database at the National Center for Biotechnology Information.

Northern blotting and cDNA probe radiolabeling

Total RNA was isolated as described above, and 20 μg of each sample was separated on a 1% agarose gel containing 5% formaldehyde, transferred to nylon membranes (ICN Pharmaceuticals, Irvine, CA) in 10× SSC overnight, and covalently linked to the membrane by UV irradiation using a Stratalinker (Stratagene, La Jolla, CA). To generate probes, cDNA fragments cloned into the Topo-TA vector were amplified using M13 forward and reverse oligonucleotides that flank the cloning site. Resulting PCR products were then gel purified using a Qiaex II kit (Qiagen, Valencia, CA) and radiolabeled by random priming. After incubation with probes, blots were washed and visualized by autoradiography.

RT-PCR

Total RNA was isolated as described above and reverse transcribed using Superscript II RT (Life Technologies) to generate cDNA for use in RT-PCR. Reactions were conducted for a total of 35 cycles, consisting of a denaturation at 94°C for 30 s and annealing/extension at 65°C for 1 min. RT-PCR typically contained 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2 U *Taq* polymerase, and 20 pM 5′ and 3′ oligonuclotide primers (Life Technologies). The sequences of the primer pairs used, 5′ and 3′, were the following: mcvSLAM, sSLAM, vSLAM, and cSLAM, as described (24); IL-12 (p40), CCCTGACATTCTGCGTTCAGGTCC and TGGGTCTATTCCGT TGTGTC; β-actin, GGACGACATGGAGAAGATCTGG and ATAGTA ATGTCACGCACGATTTCC; M13 forward, GTTTTCCCAGTCACGACG; and M13 reverse, CAGGAAACAGCTATGAC. Reactions were run on agarose gels and visualized by ethidium bromide staining.

Flow cytometry

To assess the cell surface expression of SLAM, standard flow cytometric analysis was performed. Cells were harvested and blocked with human serum for 1 min at 25°C to reduce nonspecific FcR binding. Cells were then stained with an unconjugated anti-SLAM mAb (IPO-3, IgG2a; Kamiya Biomedical, Seattle, WA). All other Abs used were purchased from Caltag Laboratories (South San Francisco, CA), PE CD1a (VIT 6b, IgG1), CD3 (S4.1, IgG2a); or BD PharMingen (San Diego, CA), PE CD80 (L307.4, IgG1), PE CD86 (2331, IgG1), PE HLA-DR (G46-6, IgG2a). Appropriate isotype control Abs (mouse PE IgG1, PE IgG2a, or unconjugated IgG2a) were used in all experiments. A FITC-conjugated goat anti-mouse IgG secondary was used to detect all unconjugated mAb used. After staining, cells were washed and fixed in 1% paraformaldehyde, and analyzed on a BD Biosciences (Mountain View, CA) FACScan flow cytometer. For all data acquisition, live cells were gated on and 5000 gated events were collected from each sample. All data analysis was conducted using WinMDI 2.8 (J. Trotter, The Scripps Research Institute, San Diego, CA).

In vitro stimulation of DC

DC were harvested on day 7 and plated in six-well tissue culture plates in 2 ml of complete medium. All cells were stimulated with CD40L trimer (1 μg/ml) for 24 h to induce SLAM expression. Cells were then washed thoroughly with complete medium, and fresh medium containing a sterile anti-SLAM mAb (IPO-3), an isotype-matched control, or LPS (10 ng/ml) were added. Abs used for tissue culture did not contain azide and contained <1 ng/ml LPS contamination, as determined by a *Limulus*-amoebocyte assay (BioWhittaker, Walkersville, MD). Following cell stimulation, supernatants were harvested at various time points and analyzed for the presence of IL-12, IL-10, and IL-8 cytokines using a standard sandwich ELISA. Abs and protein standards used for IL-12 and IL-10 ELISA were purchased from BioSource International (Camarillo, CA). IL-8 ELISA reagents were purchased from BD PharMingen.

Results

Identification of CD40L-inducible genes in DC by subtractive hybridization

It has previously been shown that CD40L is one of many stimuli capable of inducing DC maturation and subsequent expression of genes involved in directing the adaptive T cell response toward the Th1 lineage (10, 25). To better understand the role of DC in inflammatory responses, we used subtractive hybridization to identify genes up-regulated by CD40L in DC. To this end, DC from healthy donors were cultured in the presence or absence of soluble CD40L trimer, and total RNA was isolated for use in subtractive hybridization. Activation of the DC by CD40L was confirmed by assaying for up-regulation of IL-12 p40 by RT-PCR and ELISA (data not shown).

We performed three separate subtractive hybridization experiments and isolated a total of 2300 cDNA fragments from the CD40L-stimulated DC. A portion of these cDNA fragments was sequenced and identified using the BLASTN database at the National Center for Biotechnology Information. A partial list of the genes identified is shown in Table I, organized into five general categories based on either cellular location or proposed function. Some of the genes identified, such as IL-1β, IL-12, and macrophage-derived chemokine, are known to be up-regulated in mature DC (26, 27), providing an internal control for the reliability of this technique. Of particular interest to us were known or novel genes having immunological relevance, specifically those that may participate in regulating Th1 responses. One such gene was SLAM, which encodes a protein that has been previously characterized in activated T and B cells, but not DC (20, 21). Therefore, we decided to investigate the potential relevance of SLAM expression on DC function.

SLAM up-regulation in CD40L-activated DC

To confirm SLAM mRNA was up-regulated in DC stimulated with CD40L, we performed Northern blot analysis on CD40L-treated and untreated DC. Cells were stimulated in the presence or absence of soluble CD40L trimer, and total RNA was isolated. RNA was transferred to nylon membranes and hybridized with radiolabeled gene-specific probes. Fig. 1 shows that SLAM mRNA is significantly up-regulated in CD40L-stimulated DC. Similar results were obtained for the characteristic Th1 cytokine IL-12 and for the chemokines, macrophage-derived chemokine and monokine induced by IFN-γ, used as positive controls. All blots were normalized to β-actin to ensure equal RNA loading. These data corroborate the results obtained by subtractive hybridization, indicating the up-regulation of SLAM in CD40L-activated DC.

To date, four different SLAM mRNA splice variants have been identified in T and B cells (20, 24). In addition to the membrane-bound isoform of SLAM, a secreted SLAM isoform lacking the 30-aa transmembrane domain, a variable SLAM isoform with a truncated cytoplasmic domain, and a cytoplasmic SLAM isoform lacking the leader sequence have also been described. Although both the membrane-bound and secreted isoforms of SLAM have been demonstrated to have physiological effects on studies using T and B cells, no function has been assigned to the variable or cytoplasmic isoforms of SLAM. To determine which isoforms of SLAM mRNA were induced in DC by CD40L, we performed RT-PCR using SLAM isoform-specific oligonucleotide primers. The cDNA template was diluted up to 50-fold to ensure results were not due to nonlinear amplification, and all reactions were normalized to β-actin mRNA levels. As shown in Fig. 2, mRNA encoding both the membrane-bound and secreted isoforms of SLAM was strongly up-regulated by DC in response to CD40L. No message encoding the variable or cytoplasmic SLAM isoforms was detected (data not shown).

To determine whether SLAM protein was expressed on CD40L-activated DC, cells were labeled with anti-SLAM mAbs and analyzed by flow cytometry. Fig. 3*A* shows that CD40L induced the expression of SLAM protein, as well as the expression of CD1a, CD80, CD86, and HLA-DR molecules, known to be up-regulated

Table I. *Differentially expressed genes in CD40L-activated DC*

Cytokines/chemokines	Cell growth/apoptosis
IL-1β	G9 (sialidase)
IL-12 (p40) (NKSF, CTLMF)	Glutamate transporter-1
Macrophage-derived chemokine	EF-1α
Macrophage-inflammatory protein 3α	Cholesterol acetyl transferase
Monokine induced by IFN-γ	Cyclin G2
	Ferritin L chain
Membrane receptors	Manganese superoxide dismutase
SLAM (CDw 150, IPO-3)	Spermine synthase
β_2-microglobulin	JKTBP
Fas (Apo-1)	MIHC-I (TNFR/TRAF associated protein)
Leptin-related receptor	cIAP-2
IL-10R	CCR7 (EBI-1)
CD83 (BL11)	
TMP-21	Novel genes/unknown function
	Centaurin β2 (KIAA0042)
Cytoplasmic/structural	RB-1
Fibroblast tropomyosin	RBP-1
α-Centractin	My028
TM30	Beige protein homolog (CHS)
Vimentin	KIAA0005
ARC21	SAMSN1
GBP-2	IPF 53
RACK1	PSCDBP (Cytohesin binding protein HE)
Secretory granule core peptide	Hlark
Monocyte/neutrophil elastase inhibitor	PCCX1
71-kDa heat shock protein	
90-kDa heat shock protein	
Synexin (annexin VII)	
LAMP	

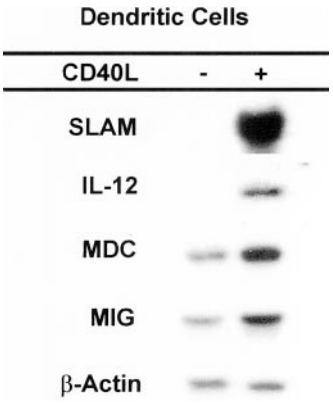

FIGURE 1. Confirmation of subtractive hybridization results by Northern blotting analysis. Total RNA (20 μg) isolated from monocyte-derived DC cultured in the presence or absence of soluble CD40L trimer (1 μg/ml) was transferred to nylon membranes and probed with radiolabeled gene-specific probes against genes identified by subtractive hybridization. All blots were normalized to β-actin levels to ensure equal RNA loading.

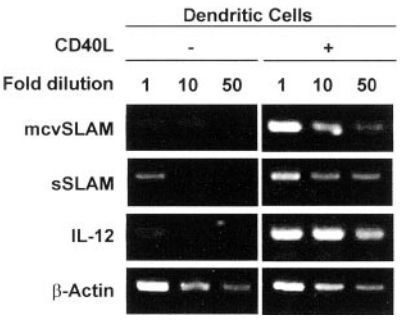

FIGURE 2. RT-PCR for SLAM isoforms in CD40L-activated DC. cDNA derived from the total RNA taken from DC cultures was analyzed for the presence of transcripts encoding the four known isoforms of SLAM. Isoform-specific oligonucleotides designed for the amplification of membrane-bound, cytoplasmic, and variable SLAM (mcvSLAM), or soluble SLAM (sSLAM) products are shown. No message was ever detected using similar oligonucleotides designed to amplify the cytoplasmic or variable SLAM isoforms. cDNA templates were diluted up to 50-fold to safeguard against nonlinear amplification. All reactions were normalized to β-actin levels to ensure equivalent amounts of template were used.

in mature DC. To confirm that SLAM was expressed on DC and not a contaminating subpopulation, cells were labeled with anti-SLAM and anti-CD1a mAbs conjugated with either FITC or PE dye and analyzed by two-color flow cytometry. As shown in Fig. 3*B*, 74% of the $CD1a^+$ DC cell population was also positive for SLAM expression following maturation with CD40L. These levels are relatively high when compared with the expression levels observed in previous studies using both activated T and B cells, which seem to be between 25 and 50% positive (20, 21). Fetal thymocytes, however, express SLAM at levels comparable with DC, approaching 80% positive. Finally, to ensure that the SLAM protein detected was not the result of a DC/T cell aggregate, cells were then labeled with mAbs to CD1a and CD3 conjugated with either FITC or PE dye. Only 7.4% of the $CD1a^+$ population was also $CD3^+$, suggesting that SLAM expression on DC was not a result of T cell contamination.

To examine the kinetics of SLAM induction on DC, cells were treated with CD40L and assayed for cell surface expression at various time points. As shown in Fig. 3*C*, SLAM is first detectable on the surface of DC as early as 12 h poststimulation with CD40L. SLAM expression observed at this time appears to approach maximal levels, as no significant increase in expression could be detected even after 2 days. Four days after the initial stimulation with CD40L, SLAM expression begins to diminish and ultimately reaches baseline levels on day 6. These kinetics appear to closely mirror those observed in B cells, in which SLAM expression is observed as early as 6 h and reaches maximal levels 24–48 h following stimulation (21, 24). Thus, SLAM expression appears to be rapidly up-regulated on DC during the maturation process.

SLAM expression is up-regulated in response to other known DC maturation stimuli

In addition to CD40L, other stimuli have been shown to have the ability to drive DC activation and maturation. Several microbial ligands are believed to be potent mediators of DC maturation, LPS being one of the best characterized. Recently, it has also been shown that the 19-kDa lipoprotein from *Mycobacterium tuberculosis* mediates DC maturation via Toll-like receptor 2 (TLR) (28), a determination made based on changes in cell surface protein expression, uptake and presentation of Ag, and secretion of proinflammatory cytokines such as IL-12 that characterize this process. Another stimulus having the ability to drive DC maturation is poly(I:C) (29), a synthetic molecule originally designed to induce production of type I IFNs important for the control of viral infections (30, 31). Much like CD40L and LPS, poly(I:C) is also capable of inducing DC to secrete high levels of proinflammatory cytokines such as IL-12; however, its receptor has not yet been identified.

To determine whether these ligands also induce SLAM expression in DC, immature DC were cultured in the presence of different maturation stimuli and assayed for cell surface expression of SLAM by flow cytometry. Fig. 4*A* shows that both CD40L and poly(I:C) up-regulated SLAM expression on DC to similar levels. Of the microbial ligands we tested, only LPS was capable of inducing expression of SLAM on DC, whereas the 19-kDa lipoprotein from *M. tuberculosis* (19 kDa), the OspA lipoprotein from *Borrelia burgdorferi* (OspA), and the 47-kDa lipoprotein from *Treponema pallidum* (Tp47) had no effect. However, all the microbial stimuli added to immature DC cultures were able to induce high levels of IL-12 production (Fig. 4*B*), suggesting that the lack of SLAM expression observed under some conditions was not due to DC unresponsiveness. Additionally, cytokines typically secreted by DC during maturation, such as IL-12 and IL-18, had no effect on SLAM expression when added alone or in combination. Minimal up-regulation of SLAM was observed on DC in response to TNF-α; however, this effect was inconsistent and never equivalent to the levels observed following stimulation with CD40L, LPS, or poly(I:C). Collectively, our data suggest that SLAM is up-regulated by DC during their maturation with CD40L, poly(I:C), or LPS, and is not the result of secondary events, such as cytokine release, that occur during the maturation process.

Engagement of SLAM on DC augments secretion of Th1-inducing, but not Th2-inducing cytokines in vitro

Previous studies describing SLAM expression on activated T and B cells have shown that this molecule exhibits a number of costimulatory properties, including the ability to enhance cellular proliferation and inflammatory cytokine release (20, 21, 24, 32). Evidence also exists to suggest that SLAM serves as its own ligand, exhibiting weak homophilic binding properties in vitro (33). It is therefore possible that interacting DC and T cells expressing SLAM could be activated simultaneously through this molecule.

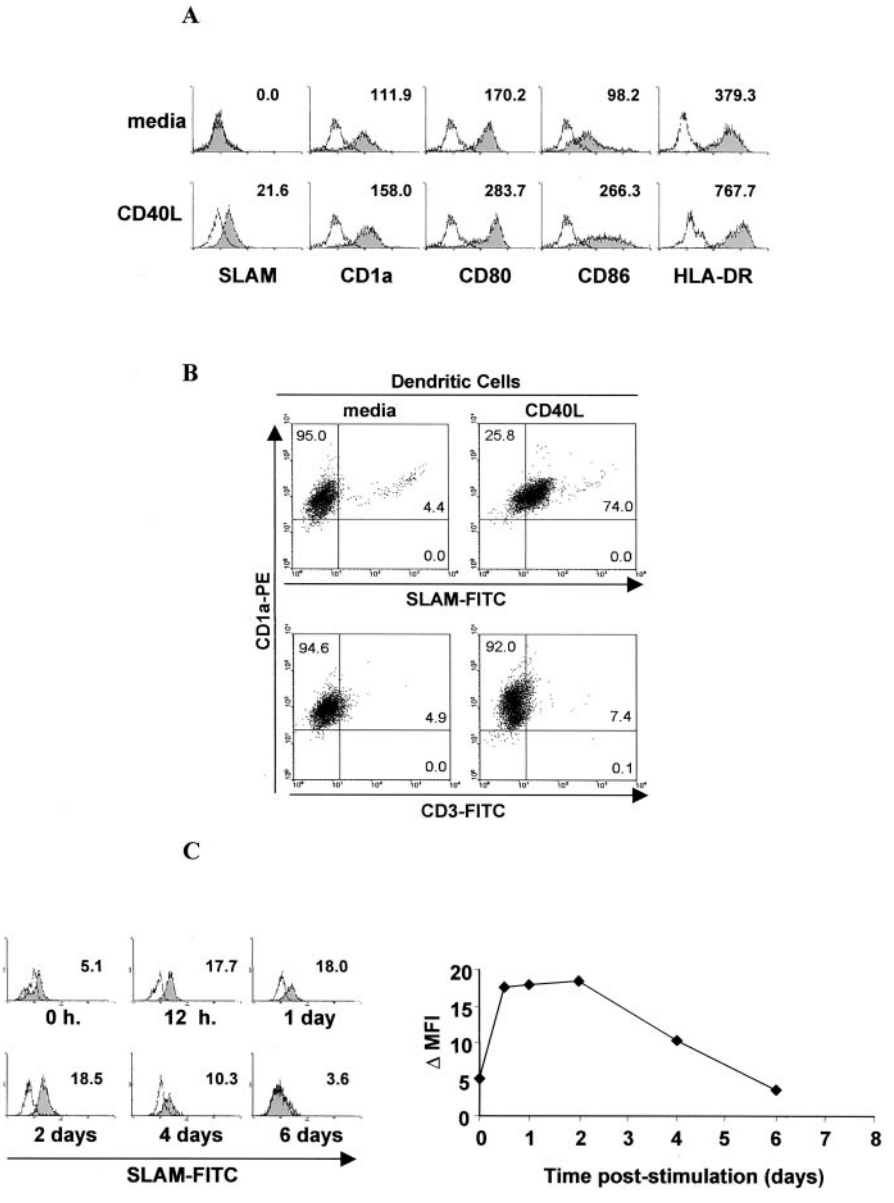

FIGURE 3. *A*, SLAM protein expression on CD40L-matured DC. DC were stimulated for 24 h in the presence or absence of CD40L trimer (1 μg/ml) and analyzed for expression of SLAM, CD1a, CD80, CD86, and HLA-DR by flow cytometry using isotype control mAbs (open histograms), or mAb recognizing these molecules (filled histograms). Numbers in the *upper corner* of each histogram represent the mean fluorescence intensities. *B*, Two-color flow cytometric analysis of DC for surface expression of SLAM. DC were stimulated as in *A* and labeled with either FITC- or PE-conjugated isotype controls or mAb recognizing CD1a, CD3, or SLAM. *C*, Time course analysis of SLAM expression on DC. DC stimulated with CD40L trimer were harvested at various time points over the course of 6 days and analyzed for cell surface expression of SLAM by flow cytometry using isotype control Abs (open histograms) or mAb recognizing SLAM (filled histograms). Numbers on each histogram represent the change in mean fluorescence intensity (Δ MFI) relative to the negative control for each time point. These data are also shown graphically.

Given the ability of SLAM engagement to affect T cell function, we wanted to determine whether SLAM receptor ligation had an effect on DC function. To this end, DC were treated with soluble CD40L trimer to induce expression of SLAM and thoroughly washed before restimulation with agonistic anti-SLAM mAbs. As a positive control for the ability to restimulate DC after their initial exposure to CD40L, LPS was added to some samples. Supernatants from these cultures were collected and analyzed for cytokine release by ELISA. As shown in Fig. 5, retreatment of SLAM-expressing DC with anti-SLAM mAbs, but not an isotype control mAb, augmented the amount of IL-12 produced by these cells. Similar results were obtained for IL-8 production, albeit to a lesser degree. The increase in IL-12 produced in response to SLAM engagement appeared to peak at 12 h, tapering off at the 24-h point. The kinetics of IL-8 production were somewhat different, with levels still increasing after 24 h and beginning to diminish 48–72 h poststimulation (data not shown). These changes in cytokine production were dose dependent, as lower concentrations of the anti-SLAM Ab had more modest, yet still significant, effects. The amount of IL-10 produced by these cells was not affected by the anti-SLAM or isotype control mAbs, although LPS was able to induce low levels of IL-10. These results suggest that the engagement of SLAM enhances the ability of DC to secrete proinflammatory cytokines and chemokines that contribute to the development of the Th1 response.

Discussion

The activation and maturation of DC provide a key pathway by which the innate immune response influences the nature and magnitude of the adaptive immune response. A number of receptors on the surface of DC are known to mediate their maturation, including CD40 (19), the receptor for CD40L expressed on T cells. To better understand the role of mature DC in the establishment of adaptive immune responses, we investigated genes that are up-regulated in CD40L-activated DC. Using subtractive hybridization, we identified SLAM, a molecule previously identified on activated T and B cells and shown to have costimulatory properties for Th1 cytokine responses (20). Flow cytometry confirmed that SLAM was expressed on CD40L-matured DC. Other stimuli known to induce the maturation of DC, including LPS and poly(I:C), were also able to induce SLAM expression, but not microbial lipoproteins such as the *M. tuberculosis* 19-kDa lipoprotein. The induction of SLAM

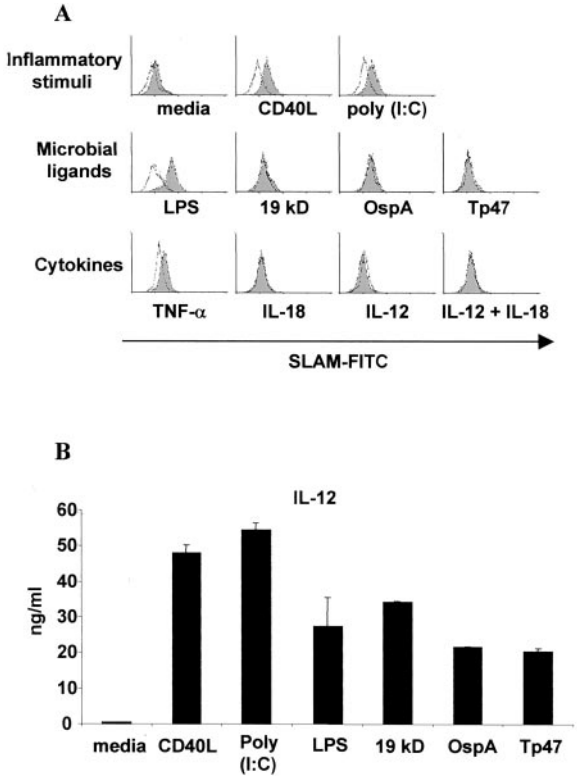

FIGURE 4. *A*, SLAM expression on DC in response to various maturation stimuli. DC (5×10^5/ml) were treated for 24 h in the presence of various different stimuli and analyzed for cell surface expression of SLAM by flow cytometry using an isotype control (open histograms) or mAb recognizing SLAM (filled histograms), followed with an appropriate FITC-conjugated secondary Ab. *B*, IL-12 production by DC in response to maturation stimuli. Cell-free supernatants were taken from DC cultures used for flow cytometry and analyzed for IL-12 p40 by ELISA. Error bars represent ±SEM.

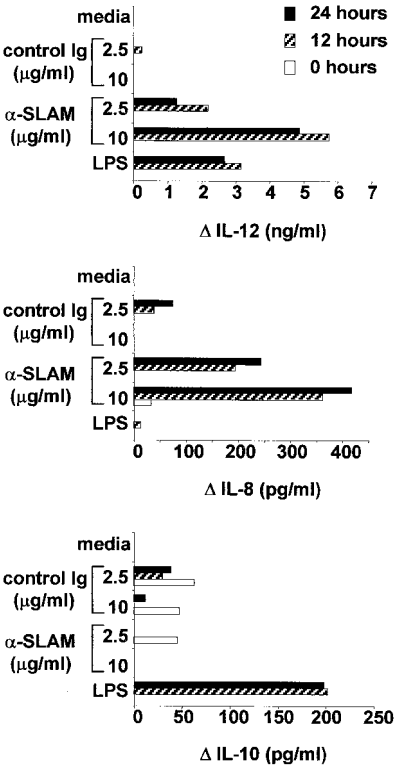

FIGURE 5. Inflammatory cytokine production in mature DC in response to SLAM receptor engagement. DC (5×10^5/ml) were treated with soluble CD40L trimer (1 μg/ml) for 24 h to induce maturation. Cells were thoroughly washed and treated with an isotype control or agonist anti-SLAM mAbs at the concentrations indicated or LPS (10 ng/ml) for an additional 24 h. Cell-free supernatants were collected at various intervals and analyzed for IL-12 p40, IL-8, and IL-10 release by ELISA. Values shown represent the average change in cytokine production (Δ cytokine) for triplicate samples above background levels observed for medium controls at each time point. Background levels for the cytokines assayed at 24 h were ~2.5 ng/ml (IL-12), 300 pg/ml (IL-8), and 100 pg/ml (IL-10). All data are representative of three independent experiments.

appears to be a direct effect of the maturation stimuli, as cytokines such as IL-12, IL-18, and TNF-α that are secreted during DC activation and maturation had no effect on SLAM expression. Functionally, SLAM receptor engagement on DC augmented production of IL-12 and IL-8, but not IL-10. These data suggest that SLAM expression on mature DC may play a role in facilitating the ability of DC to initiate inflammatory immune responses by increasing local cytokine concentrations that impact the nature and magnitude of the adaptive T cell response.

During the course of maturation, DC are transformed into highly specialized immune cells with an enhanced ability to affect the activation and differentiation of surrounding lymphocytes (6, 7, 10). This ability of mature DC is largely a result of their increased expression of costimulatory molecules and cytokines that facilitate the activation of T cells. We investigated the process of DC maturation by activating immature DC with CD40L and characterizing the types of genes that are induced in these cells once maturation is triggered. Using subtractive hybridization, we observed the up-regulation of a number of genes encoding cytokines (e.g., IL-12), chemokines, and cell surface receptors (e.g., CD80 and CD86) that are known to be associated with DC maturation (17, 19). We also discovered that mature DC expressed SLAM, a molecule previously believed to be restricted to activated T and B cells (20, 21). These data implicate SLAM as a new marker of CD40L-mediated DC maturation.

In addition to their ability to receive signals from activated T cells during the adaptive immune response, DC are also integrally involved in innate immunity to microbial pathogens. We found that two microbial ligands that have been shown to drive DC maturation, namely the TLR4 ligand LPS and poly(I:C), a synthetic dsRNA molecule used to mimic viral RNA, up-regulated SLAM expression on DC. Interestingly, microbial lipoproteins, TLR2 ligands, were unable to induce SLAM expression in DC, despite the fact that 19 kDa has recently been shown to drive DC maturation (28). Given our current understanding that LPS mediates signaling via TLR4 (34) and lipoproteins via TLR2 (35), it is tempting to speculate that TLR4, but not TLR2, selectively mediates the up-regulation of SLAM expression on DC during maturation. Alternatively, it is possible that LPS-mediated induction of SLAM expression on DC occurs through a TLR-independent pathway (36, 37). Collectively, these data suggest that SLAM may also be expressed on DC that undergo maturation in response to bacterial and viral pathogens during the innate immune response.

One of the most influential roles of DC in establishing protective immunity is to help drive the expansion and commitment of surrounding T cells toward either the Th1 or Th2 lineage (7). The mechanisms surrounding these events are believed to be partially dependent on the pattern of cytokines secreted by DC into the local

microenvironment (5). Activation of SLAM on DC using an agonist mAb augmented the release of the Th1 cytokine IL-12, but not IL-10, a Th2-inducing cytokine. SLAM engagement also enhanced DC production of IL-8, a chemotactic cytokine for neutrophils and T cells (38). The effects resulting from SLAM receptor engagement on DC may contribute to their ability to help initiate Th1 responses.

Previous studies have described that SLAM engagement on activated T cells leads to the generation of a Th1 cytokine response. In one study, SLAM was shown to augment the production of IFN-γ from cells of the Th1 lineage (20). Given that homophilic SLAM-SLAM interactions have been shown to occur in binding assays (33), it is tempting to speculate that DC and T cells, or any other pair of SLAM-expressing cells, may interact via SLAM-SLAM interactions. An interaction of this sort could lead to the bidirectional activation of these cells, synergizing in the generation of a Th1 response. We suspect that SLAM is one part of a matrix of receptors expressed on activated DC that can be engaged upon interaction with a T cell to aid in potentiating immune responses. Alternatively, the existence of a secreted isoform of SLAM suggests that receptor engagement does not necessarily involve direct cell-to-cell contact, and may be mediated instead through an indirect mechanism (24, 39).

Developing our overall understanding of SLAM function may also have clinical benefits, in light of several studies that have correlated its expression with human disease. First, in patients affected with acute multiple sclerosis, a neurological disorder believed to result in part from the unchecked activation of cell-mediated immunity, SLAM expression on $CD4^+$ T cells was higher compared with patients with stable multiple sclerosis and healthy controls (40). Second, a recently discovered gene that maps to the same chromosome locus for X-linked lymphoproliferative disease has been identified as SLAM-associated protein, a protein shown to negatively regulate signaling events through SLAM (41). Mutations in the gene encoding SLAM-associated protein were found in three X-linked lymphoproliferative disease patients, and it is believed that the inability of these patients to control B cell proliferation is partially due to a lack of regulation of SLAM-mediated signaling events. Finally, SLAM has been implicated as a receptor for the measles virus, known to cause severe immunosuppression (42), possibly due to the impairment of receptor function upon binding (43). Given that both expression and function of SLAM have been correlated with the pathogenesis of these diseases, further investigation of SLAM expression profiles and function is warranted. The knowledge obtained from these and future studies may yield clinical benefits, in which activation or blockade of SLAM on immune cells may provide useful therapeutic strategies for treatment of these and other human diseases.

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