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Intercellular Adhesion Molecule-1/LFA-1 Ligation Favors Human Th1 Development

Hermelijn H. Smits,* Esther C. de Jong,* Joost H. N. Schuitemaker,* Theo B. H. Geijtenbeek,† Yvette van Kooyk,* Martien L. Kapsenberg,* and Eddy A. Wierenga**

Th cell polarization toward Th1 or Th2 cells is strongly driven by exogenous cytokines, in particular IL-12 or IL-4, if present during activation by Ag-presenting dendritic cells (DC). However, additional Th cell polarizing mechanisms are induced by the ligation of cell surface molecules on DC and naive Th cells. In the present study, the role of LFA-1/ICAM-1 ligation in human Th cell polarization was investigated. Triggering of LFA-1 on anti-CD3/CD28 stimulated naive Th cells with immobilized Fc-ICAM-1, in the absence of DC and exogenous cytokines, induced a marked shift toward Th1 cell development, accompanied by a dose-dependent decrease in GATA-3 expression and a dose-dependent increase in T-bet expression. Th1 polarization by LFA-1 ligation could be demonstrated only under low cytokine conditions, as it was largely overruled by IL-12 or IL-4. This IL-12-independent Th1-driving mechanism appears to be operated by certain subsets of effector DC. Maturation of DC by poly(I:C), a synthetic dsRNA, used as an in vitro model for viral infections, leads to the generation of Th1-driving effector DC (DC1), which express elevated levels of ICAM-1 but produce only low levels of IL-12p70. Blocking the ICAM-1/LFA-1 interaction in cocultures of these DC with naive Th cells attenuated their Th1-driving capacity. The molecular mechanism by which LFA-1 signaling supports Th1 differentiation is blocked by specific inhibitors of extracellular signal-regulated kinase phosphorylation. The present data indicate the existence of an IL-12-independent, extracellular signal-regulated kinase-mediated mechanism, through which high ICAM-1-expressing DC1 can drive Th1 polarization. This mechanism may be operational during viral infections.


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§ Abbreviations used in this paper: DC, dendritic cell; JNK, Jun NH2-terminal kinase; ERK, extracellular signal-regulated kinase; mDC, mature effector DC; MAPK, mitogen-activated protein kinase; PACE, phosphospecific cell-based ELISA; CD40L, CD40 ligand; β2m, β2 microglobulin; Tim, melting temperature.
be important for driving Th1 cell polarization, as blockage of this interaction leads to increased Th2 cell development from naive Th cells (17–19). In humans the role of ICAM-1/LFA-1 ligation in Th cell polarization has not been evaluated previously.

Therefore, the aim of this study was to investigate the involvement of ICAM-1/LFA-1 ligation in the polarization of human Th1 cells. Experiments were performed in an APC-free and in a DC-dependent system to determine to what extent ICAM-1 expression levels on DC are relevant. Also, the relative contribution of ICAM-1/LFA-1 ligation to the actions of the potent polarizing cytokines IL-4 and IL-12 was studied. Finally, we explored the molecular pathway through which ICAM-1/LFA-1 ligation may drive Th1 responses.

Materials and Methods

Abs, cytokines, and reagents

Human rICAM-1, rICAM-2, and rICAM-3, coupled to human IgG1-Fc, were described before (20). Human IL-4 (20 ng/ml) was obtained from Pharma Biotechnology (Hanover, Germany). Human rIL-12 (sp. act. 1.7 × 10^6 U/mg) was isolated by Sephacryl S200 column chromatography from culture supernatants of J558 cells (J558 cell line expressing mouse CD40L—provided by Dr. Doris Baumann, Genentech, San Francisco, CA). Neutralizing Abs were added in 0.1% Triton X-100 plus 5% BSA and incubated for 2 h before stimulation with anti-CD3/CD28. At day 5, rIL-2 (10 U/ml; Cetus, Emeryville, CA) was added and the cultures were expanded for the next 9 days.

Cytokine production by Th cells

On day 14, the quiescent Th cells were restimulated with PMA (10 ng/ml) and ionomycin (1 μg/ml; Sigma-Aldrich) for 6 h, the last 5 h in the presence of brefeldin A (10 ng/ml; Sigma-Aldrich), and single-cell IL-4 and IFN-γ production was determined by intracellular flow cytometric analysis. Cells were fixed in 2% paraformaldehyde (Merck, Darmstadt, Germany), permeabilized with 0.5% saponin (ICN Biochemicals; Cleveland, OH), and stained with anti-human IFN-γ-FTC and anti-human IL-4-PE (both from BD Biosciences, Franklin Lakes, NJ). In parallel, the cells were stimulated with CD3 and CD28 mAbs to measure total IL-4 and IFN-γ secretion by ELISA in supernatants collected after 24 h.

Real-time quantitative RT-PCR analyses of GATA-3 and T-bet expression

For quantitative analysis of GATA-3 and T-bet mRNA expression, 10^6 naive Th cells were stimulated with anti-CD3/CD28 for 2 days and lysed for total RNA extraction, using a NucleoSpin RNA Isolation kit (Macherey-Nagel, Düren, Germany). First-strand cDNA was synthesized, using a cDNA synthesis kit (MBI Fermentas, St. Leon-Rot, Germany). For quantification of GATA-3, T-bet, and, as a control, β2-microglobulin (β2m) transcripts, real-time quantitative PCR was performed in a LightCycler (Roche Diagnostics, Mannheim, Germany) based on specific primers and general SYBR green master SYBR Green I PCR kit (Roche Diagnostics). A bulk cDNA sample of stimulated human Th cells was used as a standard, and normalization to β2m was performed for each sample.
Clonal rabbit anti-mouse Abs, labeled with HRP (New England Biolabs). After extensive washing the cells were transferred to a 96-well plate (Costar) and the HRP substrate 3,3',5,5'-tetramethylbenzidine (BioSource International, Nivelles, Belgium) was added. The reaction was stopped with 2 M H₂SO₄ after 15 min and the OD₄₅₀/₆₅₀ was used as a measurement of phosphorylation.

Results

ICAM-1/LFA-1 ligation promotes Th1 cell polarization from human naive Th cells

Primary anti-CD3/CD28 stimulation of human naive Th cells in the absence of DC and polarizing factors yields a mixed population of IL-4 and/or IFN-γ producers. This was assessed on day 10 upon subsequent restimulation and was detected both on a per cell basis by intracellular staining (Fig. 1A, left panel) and by measurement of cytokine secretion (Fig. 1B, upper bar). The addition of immobilized Fc-ICAM-1 construct induced a dose-dependent shift toward the Th1 cell phenotype, with gradually increased percentages of IFN-γ producers, decreased percentages of IL-4 producers (Fig. 1A), and an increased IFN-γ:IL-4 secretion ratio (Fig. 1B). Specificity of this Th1-driving effect was demonstrated by the abolishment of Th1 polarization by addition of blocking Abs against either ICAM-1 or LFA-1 (NKI-L15) at the start of the culture (Fig. 1C). Isotype control Ab had no effect (data not shown). Furthermore, mRNA analysis at t = 48 h indicated a dose-dependent drop in the expression of the Th2-specific transcription factor GATA-3 and a dose-dependent increase in the expression of the Th1-specific transcription factor T-bet, with increasing Fc-ICAM-1 concentrations (Fig. 1D), which correlates with the increased IFN-γ and decreased IL-4 production after restimulation on day 10 (Fig. 1, A and B).

In addition to ICAM-1, ICAM-2 and, with lower affinity, ICAM-3 are ligands for LFA-1 (24). When tested in the same culture system, Fc-ICAM-2 and Fc-ICAM-3 induced a similar dose-dependent shift toward Th1 cell differentiation (Fig. 2). As expected from the lower affinity for LFA-1, the Th1-driving effect of ICAM-3 was less pronounced. These results demonstrate a clear Th1-driving effect of ligation of LFA-1.

Th1 cell differentiation by ICAM-1/LFA-1 ligation is overruled by IL-4 or IL-12

The cytokines IL-4 and IL-12 have powerful capacities to drive Th cell polarization. Therefore, we tested the relative importance of LFA-1-mediated polarization in the presence of these potent cytokines. As shown in Fig. 3A, increasing concentrations of IL-4 dose-dependently abolished the Th1-driving effect of ICAM-1/LFA-1 ligation, indicating that this Th2-driving cytokine overrules

![FIGURE 1. ICAM-1/LFA-1 ligation promotes Th1 differentiation from human naive Th cells. Naive Th cells were stimulated with anti-CD3/CD28 in the absence or presence of an increasing dose of immobilized Fc-ICAM-1. A. After 10 days, IFN-γ and IL-4 production per cell was analyzed by intracellular FACS staining following a 6-h PMA/ionomycin stimulation, the last 5 h in the presence of brefeldin A. B. A parallel stimulation with anti-CD3/CD28 was performed to analyze total IFN-γ and IL-4 secretion by ELISA in the collected 24-h supernatants. C. Naive Th cells were stimulated in the additional presence of neutralizing ICAM-1 or LFA-1 Abs. After 10 days, the single-cell IFN-γ/IL-4 profiles were determined by intracellular FACS analysis, as described in A. D. After 48 h, GATA-3 and T-bet mRNA expression was determined by real-time quantitative RT-PCR analysis, normalized to β₂m transcripts in the same sample, and expressed as the percentage of expression under neutral conditions, set at 100%. The data are presented as mean ± SEM from three independent experiments.](http://www.jimmunol.org/)

![FIGURE 2. Th cell polarization by different ligands for LFA-1. Naive Th cells were stimulated with anti-CD3/CD28 in the absence or presence of increasing concentrations of Fc-ICAM-1, Fc-ICAM-2, or Fc-ICAM-3. After 10 days, IFN-γ and IL-4 production per cell was determined by intracellular FACS analysis as described in Fig. 1A.](http://www.jimmunol.org/)
the effect of LFA-1 ligation. In the presence of moderate to high concentrations of IL-12, ICAM-1/LFA-1 ligation does not add to the Th1-polarizing effect of IL-12 (Fig. 3B). These data indicate that Th1 differentiation by LFA-1 ligation is primarily of importance under low cytokine conditions.

**LFA-1 ligation affects the activation of MAPK**

Recent studies in the mouse have pointed toward a role for MAPK signaling pathways in the control of Th cell responses under low cytokine conditions. These pathways can be induced by TCR triggering, which activates ERK, by CD28 ligation, which activates p38 MAPK, or by the combination of TCR triggering and CD28 ligation, which activates JNK1 and JNK2. JNK1 down-regulates IL-4 production, whereas JNK2 and p38 MAPK increase IFN-γ production, and ERK promotes Th2 responses (reviewed in Ref. 25). However, in human T cells, some of these pathways can have different, even opposing, effects. For example, p38 MAPK was shown to be important for IL-4 production in effector Th2 cells (26, 27), while interruption of the ERK pathway strongly increased, rather than abrogated, IL-4 responses in human Th cells (28).

We addressed the question of whether Th1 polarization by LFA-1 ligation is mediated via the activation of one or more of these MAPK pathways. Naive Th cells stimulated with anti-CD3/CD28 in the presence of 500 ng/ml Fc-ICAM-1 displayed a shift toward Th1 cell development. Addition of a chemical inhibitor of ERK phosphorylation (PD90859) to anti-CD3/CD28-activated naive Th cells resulted in strong dose-dependent induction of IL-4 production and inhibition of IFN-γ production, while in the ICAM-1-stimulated naive Th cells this effect was reduced and required higher concentrations of PD90859 to yield equal percentages of IL-4-producing Th cells upon restimulation (Fig. 4A). These data suggest that ERK activity is increased in ICAM-1-stimulated naive Th cells. This was indeed confirmed by the analysis of phosphorylated (active) ERK in 48-h-stimulated naive Th cells by PACE (23) (Fig. 4C). p38 MAPK phosphorylation was detectable only after 3 days but did not differ between cells stimulated with and without ICAM-1 (Fig. 4C; day 3 data not shown). Accordingly, addition of the chemical p38 MAPK inhibitor, SB203580, did not differentially affect the Th cell cytokine profile in cells stimulated with or without ICAM-1 (Fig. 4B), suggesting that p38 MAPK is not involved in ICAM-1-induced Th1 polarization. Although elevated levels of phosphorylated JNK1/2 were demonstrated in the ICAM-1-stimulated Th cells at day 2 (Fig. 4C), the functional consequences for ICAM-1-driven Th1 polarization cannot be studied in this experimental setup, as no specific JNK inhibitor is available yet.
Low IL-12-producing poly(I:C)-matured DC drive Th1 polarization through elevated expression of ICAM-1

Next, we addressed the question of whether LFA-1-mediated signaling indeed contributes to Th1 polarization under more physiological conditions, i.e., during the interaction of naive Th cells with DC. We generated different Th1-driving DC1 subsets with different ICAM-1 expression levels. To this end, maturation was induced in immature monocyte-derived DC either with the synthetic dsRNA poly(I:C) or by the combination of LPS plus IFN-γ. As a comparison, maturation was induced by LPS alone, yielding non-polarizing DC0. In addition to ICAM-1, expression of the other ligands for LFA-1, ICAM-2, and ICAM-3 were analyzed on both DC1 subsets and the DC0 subset. As shown in Fig. 5A, ICAM-2 was hardly expressed on either DC subset and was expressed on ICAM-3 only moderately, but was elevated on the poly(I:C) DC1. ICAM-1 was more abundantly expressed and particularly high on the poly(I:C) DC1. In combination with this high ICAM-1 expression, poly(I:C) DC1 produced only limited quantities of IL-12p70, as compared with the LPS/IFN-γ-matured DC1 (Fig. 5B). In fact, IL-12p70 production by the poly(I:C) DC1 hardly differed from the levels produced by the nonpolarizing LPS-matured DC0. As the impact of LFA-1 triggering on Th1 polarization is most evident under such low cytokine conditions (Fig. 3B), we tested to what extent the Th1-driving capacity of poly(I:C) DC1 (Fig. 5C) was mediated by ICAM-1 and IL-12p70. Coculture experiments with naive Th cells demonstrated that blocking the ICAM-1/LFA-1 interaction, either with anti-ICAM-1 or with anti-LFA-1, clearly reduced the Th1-driving potential (Fig. 5C). Likewise, neutralization of IL-12p70 only partially blocked Th1 polarization. Addition of anti-ICAM-3 instead did not substantially reduce Th1 polarization in any DC subset (Fig. 5C). Full abrogation of Th1 polarization (to the basal level induced by LPS-matured DC0; Fig. 5C, upper bar) was obtained only by combining IL-12p70 neutralization and blocking the ICAM-1/LFA-1 interaction, demonstrating the contribution of ICAM-1 in the Th1-driving mechanism of poly(I:C) DC1 (Fig. 5C). In contrast, the Th1-polarizing effect of LPS/IFN-γ DC1 is fully IL-12 dependent (Fig. 5C).

**Discussion**

In this study we show that LFA-1 ligation on activated human naive Th cells leads to a shift toward Th1 cell differentiation, accompanied by a rapidly increased T-bet:GATA-3 mRNA expression ratio. The Th1-driving effect of the ICAM-1/LFA-1 interaction is primarily of importance under low cytokine conditions, as the polarizing cytokines IL-4 and IL-12 dose-dependently overrule the Th1-driving effect. Indeed, experiments with the poor IL-12-producing poly(I:C) DC1 expressing high levels of ICAM-1 indicated that ICAM-1 actively contributes to their Th1-driving capacity. Exploring the molecular mechanism by which LFA-1 ligation supports Th1 cell differentiation, we have found evidence that the MAPKs ERK and JNK may be involved.

Mouse studies (17–18) with ICAM-1-deficient APC or blocking Abs to ICAM-1 already indicated a profound inhibitory effect of ICAM-1 on IL-4 production by activated naive Th cells, suggesting at least prevention of Th2 development as a consequence of LFA-1/ICAM-1 interaction. The mouse experiments did not indicate an active induction of IFN-γ production or Th1 development. In contrast, previous studies with human memory CD4+ Th cells showed that repeated costimulation by ICAM-1 did lead to increased secretion of IFN-γ, but not IL-4 or IL-5 (29, 30). These results are confirmed in our present study and are further extended by showing that initial polarization of human naive Th cells upon LFA-1 costimulation by ICAM-1 gives rise to memory Th cells that produce high amounts of IFN-γ but hardly any IL-4. The question remains what precise mechanisms are involved in this shift in Th cell polarization. It is well known that the ligation of LFA-1 by ICAM-1 helps to stabilize the physical APC-Th cell interaction, allowing for a higher TCR occupancy, and thereby to enhance or prolong TCR-dependent signals (31), such as inositol phospholipid hydrolysis, increased intracellular Ca2+ levels (32, 33), and phosphorylation of phospholipase Cγ1 (34). Indeed, it has been reported that LFA-1/ICAM-1 interaction can lower the required Ag dose for Th cell activation by 10- to 100-fold (35, 36). Several studies support the “strength of signal” hypothesis as an additional factor influencing Th cell polarization. According to this
hypothesis, triggering of high numbers of TCR will lead to IFN-γ-producing Th cells, whereas low number TCR triggering will favor the development of IL-4-producing Th cells (37). The contribution of LFA-1/ICAM-1 ligation to this model was elegantly demonstrated by a study of Ruedl et al. (38) in which LFA-1 triggering by ICAM-1 lowered the required Ag dose for CD40L up-regulation on the Th cell, which is essential for induction of IL-12 production, by the Ag-presenting DC, which, in turn, can promote Th1 responses. Nevertheless, this model can only in part explain our present data, because 1) poly(I:C) DC1 produce only low levels of IL-12p70 upon CD40L stimulation, which does not fully account for the Th1 responses induced by these high ICAM-1-expressing DC, and 2) naive Th cells stimulated with anti-CD3/CD28, in the absence of APC, also showed a dose-dependent increase in Th1 cell polarization with increasing concentrations of immobilized ICAM-1-Fc. Other studies also suggest that the “strength of signal” hypothesis is incomplete. For example, Abraham et al. (39) demonstrated that high-density TCR ligand expression could not compensate for the lack of LFA-1/ICAM-1 interaction in Th cell activation and that only TCR engagement together with costimulation through either LFA-1 or CD28 was sufficient to induce detectable levels of IL-2 mRNA (40), albeit by qualitatively different signaling routes (41). Furthermore, it has been described that LFA-1 ligation on human CD8+ T cells can up-regulate the activity of phosphatidylinositol 3-kinase, sphingomyelinase, and JNK, all similar to pathways induced by CD28 ligation, but not identical, as LFA-1 and CD28 ligation display a different profile of sensitivity to inhibitors of phosphatidylinositol 3-kinase (33). Therefore, a likely explanation would be that the shift in Th1 polarization induced by LFA-1 ligation is the combined result of several factors, involving modifications of TCR-dependent signals and perhaps the induction of unique signaling pathways. In the present study, increased levels of activated ERK in ICAM-1-costimulated Th cells were demonstrated, both by experiments with a specific ERK inhibitor and by PACE. As ERK phosphorylation is induced upon TCR triggering, part of our results may be explained by LFA-1-induced modifications of TCR-dependent signals, which may lead to a reduction of IL-4-producing Th cells. In the mouse, JNK is involved in the induction of IFN-γ production in Th cells and, although there are no reports on this effect in human Th cells, it is tempting to speculate that the elevation of activated JNK observed in ICAM-1-costimulated Th cells by PACE at day 2 promotes the development of IFN-γ-producing Th cells and together with increased ERK activity leads to enhanced Th1 polarization. Because JNK is not activated by TCR triggering alone but requires costimulation via CD28 or LFA-1, we hypothesize that the Th1 shift observed in this study is accomplished in part by modifications of TCR-dependent signaling, e.g., via ERK activation, but may additionally be the result of specific ICAM-1-induced signaling, like the activation of JNK.

In this study, we identified a novel IL-12-independent Th1-driving mechanism, actively operated by poly(I:C) DC1. These cells, obtained by maturation of monocyte-derived immature DC in the presence of poly(I:C), are characterized by elevated expression of ICAM-1 and low levels of IL-12, both equally contributing to the Th1 polarization. Poly(I:C) is a synthetic dsRNA molecule and may represent a model for viral infections. In this respect, the poly(I:C)-induced up-regulation of ICAM-1 on DC and subsequent in vitro Th1 cell polarization is nicely corresponding to numerous in vivo studies that showed that viral infections lead to the induction of Th1 responses and increased levels of ICAM-1 expression (reviewed in Ref. 42). Furthermore, studies with IL-12p40−/− and IL-12p70−/− mice (43) and patients with a functional mutation in the IL-12R (44) demonstrated that virus-specific Th1 responses could still be mounted, indicating also that in vivo alternative, IL-12-independent, Th1-driving mechanisms are operational, especially during viral infections. Based on our present findings, it is tempting to speculate that the LFA-1/ICAM-1 interaction is relevant in this respect. In vivo yet other mechanisms may be involved, because during viral infections high levels of type I IFNs are produced that, in humans, can induce Th1 responses as well (45). In our experiments, only minimal expression of IFN-α was detected in the poly(I:C) DC, and blockage did not result in an inhibition of Th1 polarization.

In addition to viral infections, under low cytokine conditions in vivo LFA-1/ICAM-1 interaction may drive Th1 responses. Those conditions may occur in newborns, which are characterized by an immature immune system, e.g., indicated by the deficient IL-12p70 production by monocyte-derived cord blood DC (46). Consequently, during primary immune responses the polyclonal contribution of LFA-1/ICAM-1 ligation may be relatively high.

Animal models for Th1-mediated responses or diseases also provide evidence that ICAM-1-LFA-1 interaction may be involved in Th1 polarization in vivo. For example, blocking Abs to LFA-1 and ICAM-1 were shown to prevent allograft rejection, associated with a shift from a Th1 to a Th2 cell cytokine profile (47–49). Similarly, ICAM-1 deficiency in nonobese diabetic mice blocked accelerated diabetes, in part explained by affecting the generation and/or expansion of islet-specific Th1 cells (50). Also, in an experimental autoimmune encephalomyelitis disease model, ICAM-1-deficient mice showed reduced T cell proliferation and reduced Th1 cytokine production in response to myelin Ag (51). The latter two studies suggest the involvement of ICAM-1 in autoreactive Th1 cell development, although it was not specified which cell type, e.g., DC, was essential in this respect. Furthermore, studies with ICAM-1 knockout mice indicated that ICAM-1 is required for rapid activation of Th1 cells to control early acute phase genital chlamydial infection, suggesting a role for ICAM-1 in the control of infectious diseases (52). Therefore, evidence from both in vitro and in vivo models points toward a role for ICAM-1-driven Th1 development. Blockage of ICAM-1-LFA-1 interaction may therefore provide potent therapeutic possibilities for the treatment of autoimmune diseases or prevention of graft rejection by transplantation, whereas therapeutic triggering may be beneficial to reduce Th2-mediated disease.

References


