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Microenvironment-Dependent Requirement of STAT4 for the Induction of P-Selectin Ligands and Effector Cytokines on CD4+ T Cells in Healthy and Parasite-Infected Mice

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T effector cells require selectin ligands to migrate into inflamed regions. In vitro, IL-12 promotes induction of these ligands as well as differentiation of CD4+ T cells into IFN-γ-producing Th1 but not Th2 cells. STAT4 is strongly involved in these processes. However, the presence of selectin ligands on various T effector cell subsets in vivo points to more complex regulatory pathways. To clarify the role of the IL-12/STAT4 signaling pathway, we analyzed the impact of STAT4 deficiency on the expression of P-selectin ligands (P-lig) on CD4+ T cells in vitro and in vivo, including conditions of infection. In vitro, we found significant expression of P-lig upon activation not only in the presence, but also in the absence, of IL-12, which was independent of STAT4. TGF-β, an alternative inducer of selectin ligands in human T cells, was not effective in murine CD4+ T cells, suggesting a role of additional signaling pathways. In vivo, a significant impact of STAT4 for the generation of P-lig+ CD4+ T cells was observed for cells from peripheral lymph nodes, but not for those from spleen or lung. However, upon infection with the Th2-inducing parasite Nippostrongylus brasiliensis, P-lig expression became dependent on STAT4 signaling. Interestingly, also the frequency of IL-4-producing cells was greatly diminished in absence of STAT4. These data reveal a hitherto unknown contribution of STAT4 to the generation of Th2 cells in parasite infection and suggest that signals inducing inflammation-seeking properties in vivo vary depending on environmental conditions, such as type of organ and infection. The Journal of Immunology, 2006, 177: 7673–7679.

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appears to be involved in the generation of selectin-binding epitopes in T cells (16).

Whereas these initial in vitro studies suggested a link between IL-12 driven differentiation into Th1 cells and selectin ligand expression, other in vitro studies observed expression of selectin ligands after activation in the absence of IL-12 (7, 17–21). In the human system, also TGF-β has been shown to be a further mediator capable to induce P-lig expression on lymphocytes in a p38-dependent manner (22).

In vivo, the link between Th1 differentiation and selectin ligand expression was much less clear; in fact, selectin ligands were found to be expressed on both IFN-γ and IL-4-producing T cells in men and mice (8, 17, 23). Recent studies emphasized the importance of tissue-dependent factors for the regulation of selectin ligands. Campbell et al. (23) reported a preferential induction on CD4⁺ T cells activated within peripheral lymph nodes, whereas cells activated within mesenteric lymph nodes did not up-regulate P-lig. However, strong inflammatory stimuli were found to induce selectin ligands even in mucosal compartments (24, 25).

These studies left the question open which signaling pathways are involved in induction of selectin ligands on CD4⁺ T cells in vivo, especially under conditions of inflammation or infection. In the present study, we investigated whether STAT4, commonly seen as a signaling pathway associated with Th1 development, might play a broader role under in vivo circumstances, especially in case of Th2-dominated parasite infection. We provide evidence, that STAT4 contributes under homeostatic conditions only to P-lig expression in peripheral lymph nodes but not in the spleen or lung. Surprisingly, upon infection, a major role of STAT4 was not only observed for P-lig expression on CD4⁺ T cells within the inflamed tissues but also for the generation of IL-4-producing effector cells.

Materials and Methods

Mice

Wild-type (WT) BALB/c mice (Bundesinstitut für Risikobewertung) and STAT4⁺/− mice (The Jackson Laboratory) were bred under specific pathogen-free conditions in our animal facility. All animal experiments were performed in accordance with institutional, state and federal guidelines.

Nippostrongyulus infection

WT or STAT4⁺/− mice were subcutaneously infected with 750 L3 larvae of the helminth Nippostrongylus brasiliensis.
Isolation of primary cells for cytometric detection of P-lig expression and cytokine production

For lymphocyte isolation from lymph nodes or spleen, organs were removed and teased through stainless steel meshes. After washing, cells, erythrocytes, and cell debris were removed by density centrifugation (Histopaque-1083; Sigma-Aldrich).

Mononuclear cells from lung cell suspensions were prepared as described previously (26). Briefly, lung tissue was perfused via the right ventricle of the heart with 10–20 ml of PBS. Subsequently, the organs were passed through sieves to obtain single-cell suspensions, and cells were washed. Lung suspensions were subjected to a 40/70% Percoll (Amersham Biosciences) gradient (26) and washed subsequently with PBS containing 0.2% BSA.

Cytometric analysis

P-selectin binding ligands were detected with a P-selectin-human IgG chimeric protein and PE- or cytochrome 5-conjugated anti-human IgG Ab (F(ab')₂) (Dianova) as secondary reagent as described (27). In brief, staining was conducted in HBSS supplemented with Ca²⁺ and Mg²⁺ and with 10 mM HEPES. The optimal concentration of P-selectin-IgG for staining was determined by titration of the chimeric protein for each batch. Background staining was determined by appropriate controls, including staining of FucT-VII-deficient T cells (data not shown) and staining of P-lig⁺ cells in the presence of 5 mM EDTA.

CD4⁺ T cells were stained with anti-CD4-PerCP (BD Pharmingen). Cytometric analysis was performed using FACSCalibur and CellQuest software, both from BD Biosciences.

Detection of P-lig expression and cytokine production of CD4⁺ T cells ex vivo

After preparing single-cell suspensions, cells were stimulated with PMA (10 ng/ml) and ionomycin (500 ng/ml) for 4 h. Brefeldin A was added at 10 μg/ml for the last 2 h of stimulation to prevent secretion of cytokines. After 4 h, cells were stained with anti-CD4 mAb and for P-lig expression as described before. After staining, cells were fixed in HBSS containing Ca²⁺, Mg²⁺, and PFA 2%, permeabilized by 0.5% saponin, and stained with FITC-conjugated anti-IFN-γ (AN18.17.24), anti-IL-4-PE (BD Pharmingen), or anti-IL-10-PE (BD Pharmingen).

Purification of naive CD62L⁺ CD4⁺ cells for in vitro culture

CD4⁺ T cells were purified from pooled peripheral and mesenteric lymph nodes by panning using anti-CD8 (53-672), anti-CD25 (PC/6), anti-Mac-1 (M1/70) and anti-FcR II/III (2.4G2) Abs or by anti-CD4-FITC (GK1.25) and anti-FITC-Multisort Beads (Miltenyi Biotec) to a purity of ≥98%.
Multisort beads were released according to the manufacturer’s suggestion. Naive CD4⁺CD62L⁺ cells were positively selected by high-gradient MACS with anti-CD62L microbeads (Miltenyi Biotec) to a purity of ≥98%. APCs were generated by depletion of spleen cells from CD90⁺ lymphocytes using anti-CD90 microbeads (Miltenyi Biotec).

Cell culture

For effector cell generation, naive T cells were cultured at 1 x 10⁶ cells per ml in complete RPMI 1640 medium, containing 10% FCS and 10 μM 2-ME (Life Technologies) and activated with plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 (1 μg/ml). For culture under Th1 conditions, recombinant murine IL-12 (R&D Systems) at 5 ng/ml, IFN-γ (R&D Systems) at 20 ng/ml, and neutralizing anti-IL-4 Abs at 5 μg/ml (11B11; own production) was added. Th2 cultures contained 30 ng/ml IL-4 (R&D Systems), and 5 μg/ml anti-IFN-γ and 5 μg/ml anti-IL-12 (AN 18.17.24 and C17.8.6; own production). In some experiments, anti-TGF-β Ab (R&D Systems) was added at a concentration of 1 μg/ml. Th0 cultures contained 10 ng/ml IL-2, 5 μg/ml anti-IL-12, 5 μg/ml anti-IFN-γ, and 5 μg/ml anti-IL-4 and were supplemented as indicated with recombinant human TGF-β (R&D Systems) at either 2 or 8 ng/ml.

Statistical analysis

Data are shown as mean and SD. Significance was determined by Mann-Whitney U test. Differences were considered statistically significant with p < 0.05 and highly significant with p < 0.01.

Results

Role of IL-12/STAT4 signaling for the induction of selectin ligands in vitro

In vitro data suggested that IL-12 induces P-lig expression on CD4⁺ T cells in a STAT4-dependent manner, whereas IL-4 was rather inhibitory (5, 6). When naive CD4⁺CD62L⁺ T cells from STAT4-deficient or from WT mice were activated by anti-CD3/anti-CD28 under Th1 promoting conditions, i.e., in the presence of IL-12, IFN-γ, and anti-IL-4, induction of P-lig was indeed reduced on STAT4-deficient cells (Fig. 1A); however, the reduction was only partial (50%). Under the conditions used here, T cell activation in the presence of IL-4 and Abs to IL-12 and IFN-γ, i.e., culture conditions promoting Th2 development, also resulted in generation of a varying, but significant number of P-lig-expressing cells if analyzed 4 days after activation. This was found for both WT and STAT4⁻/⁻-deficient cells; induction in the absence of IL-12 was occasionally even higher in STAT4⁻/⁻ cells than in WT CD4⁺ T cells (Fig. 1A). Mean fluorescence intensity (MFI) as a measure of the density of P-lig molecules was comparable among P-lig⁺ Th1 and Th2 cells (Fig. 1A).

Analyzing IFN-γ production by T cells induced under Th1 conditions we found a strong inhibition of IFN-γ-induction cells in STAT4⁻/⁻ T cells, confirming the defect of STAT4⁻/⁻ mice in the generation of a Th1 response (Fig. 1B). Induction of cytokine production appeared to be more impaired than induction of selectin ligands in STAT4⁻/⁻ mice, compared with WT mice.

To look for the kinetics of P-lig induction, prolonged cultures were performed and cells were analyzed on days 4 and 7 after activation. Fig. 1C shows representative histogram plots from T cells cultured for 7 days under Th1 and Th2 conditions from WT and STAT4⁻/⁻ mice. Effector cells from STAT4⁻/⁻ mice induced under Th2-promoting conditions expressed similar levels of P-lig as WT T cells on day 7 after activation, suggesting that STAT4 is not involved in the induction of P-lig under these conditions. In contrast, under Th1 conditions P-lig expression remained lower on STAT4⁻/⁻ T cells than on WT T cells (Fig. 1C).

Comparing P-lig expression on days 4 and 7 after activation we found that, under Th1 conditions, P-lig expression reaches maximal expression already on day 4, whereas under Th2-promoting conditions, P-lig expression further increases up to day 7 after activation (Fig. 1D). These data suggest that an IL-4-dominated environment can be permissive for P-lig induction in vitro.

In the human system, TGF-β-1 was found to induce P-lig expression on CD4⁺ T cells by an alternative pathway (22). We therefore tested whether the presence of TGF-β could contribute to the induction of P-lig observed under Th2 conditions. Neither blockade of TGF-β under conditions promoting Th2 development nor the addition of TGF-β to conditions containing no further cytokine supplement (Th0) had major effects on P-lig induction in WT or STAT4⁻/⁻ cells analyzed on day 4 (Fig. 1D and data not shown) and day 7 after activation (Fig. 1E). This suggests that TGF-β alone does not play a significant role for P-lig induction on murine T cells.

Reduced frequency of P-lig⁺ T cells from lymph nodes of STAT4⁻/⁻ mice

To determine the impact of STAT4-dependent IL-12 signaling on P-lig induction in vivo, we analyzed the frequency of P-lig-expressing cells among CD4⁺ lymphocytes within peripheral lymph...
Reduced induction of P-lig in STAT4−/− mice after infection with the helminth N. brasiliensis

The above in vitro data suggest a partial role of STAT4 for induction of P-lig on Th1, but not on Th2 cells. However, previous data demonstrated a discordance of findings regarding ligand expression in vitro and in vivo; especially expression on ex vivo Th2 effector/memory cells was unexpectedly high and a major impact of immune reactions against infection became evident (8).

We therefore investigated the role of STAT4 signaling under conditions of infection with a Th2-inducing helminth N. brasiliensis. Whereas IFN-γ-producing cells were reduced in uninfected STAT4−/− mice as discussed above, the frequency of IL-4- and IL-10-producing CD4+ cells is unimpaired under homeostatic conditions in the spleen (data not shown).

After s.c. infection with infective third-stage larvae of N. brasiliensis, parasitic larvae migrate via the blood stream into the lung where they are coughed up and swallowed within 2 days. As shown in Fig. 3A, a strong Th2-biased immune response occurs within the lung associated with the appearance of large numbers of IL-4- and IL-10-producing cells. Furthermore, a strong increase in the frequency of P-lig-expressing cells is observed within the lung (Fig. 3B). The frequency of P-lig+ cells increased among all subsets of effector cells present within the lung and reached up to 60% among IL-4- and among IL-10-producing cells (Fig. 3C).

In STAT4−/− mice, infection with N. brasiliensis also resulted in an increase in the frequency of P-lig-expressing CD4+ T cells within the lung, compared with uninfected mice; however, the increase was much lower than in WT mice (Fig. 4, A and B). Surprisingly, reduced frequencies were also found for IL-4-producing Th2 cells (Fig. 4B), unraveling an hitherto unknown role of STAT4 signaling in the induction of Th2 cells in vivo.

Although the frequency of P-lig+ CD4+ T cells was strongly reduced, MFI on the remaining P-lig+ cells was comparable among WT and STAT4−/− cells (Fig. 4C). The frequency of P-lig-expressing cells among individual populations of cytokine-producing cells was not significantly reduced (data not shown), and also the total number of mononuclear cells recovered from the lung did not significantly differ among WT and STAT4−/− mice (data not shown).

Discussion

Studies on the regulation of selectin ligand expression on CD4+ effector cells have yield contradictory results: On one site, different in vitro studies observed a strongly biased expression of selectin ligands on in vitro generated Th1 effector cells but not on Th2 cells (4, 19) while in vivo, no restriction of P-lig expression to Th1 cells was seen (8, 28).

Furthermore, recent data showed a tissue-dependent induction in vivo (23). This raised the question which factors regulate P-lig expression in vivo and what role the IL-12/STAT4 signaling pathway plays, which was shown to up-regulate FucT-VII and C2GlcNAT-I in vitro (5, 29, 30). In a Th1-dominated transfer model using transgenic CD4+ T cells and i.p. application of specific Ag in CFA, a reduced induction of Ag-specific P-lig+ T cells
was observed after IL-12 blockade (24). Similarly, in a the Th1-dominat Leishmania major infection model, pretreatment of mice with anti-IL-12 also reduced the frequency of E- and P-lig CD4+ T cells and suppressed induction of FucT-VII (31), suggesting a major role of the IL-12/STAT4-pathway for the induction of selectin ligands in vivo.

The present study focuses on the question whether STAT4 is indeed a dominant regulator of the synthesis of selectin ligand epitopes and what role distinct conditions in vivo such as organ environment or activation in the course of an infection do play. Especially the role of STAT4 for the differentiation of Th2 cells had not been addressed so far.

The data of this study confirm a major, although not indispensable, function for the in vitro generation of P-lig+ effector cells under Th1-inducing conditions, i.e., in the presence of IL-12. In vivo, P-lig expression only on T cells from peripheral lymph nodes was (partly) dependent on STAT4, whereas spleen cells were not significantly affected. Thus, the contribution of the STAT4 signaling pathway to selectin ligand induction is less dominant or even lacking in some compartments, in contrast with its more prominent role in cutaneous reactions. As mentioned above, evidence for early IL-12 activity in Th2 reactions has been provided. If this time window is short enough, no stable cytokine imprinting can occur and a later IL-4-dominated phase is able to suppress IL-12R expression and IFN-γ production via up-regulation of GATA-3 (36). In our hands, IL-4, at least in the mouse, does not significantly down-regulate the expression of selectin ligands (our unpublished data). Accordingly, early induction of P-lig by IL-12 might last throughout a later conversion of the inflammation into a Th2 milieu and lead to the appearance of P-lig+ Th2 cells.

In conclusion, this study unravels an unforeseen complexity in the regulation of selectin ligands in vivo, and a major role of the STAT4 pathway in generation of both IL-4-producing T cells and of P-lig-expressing cells in a classical Th2-dominated infection model.

Disclosures
The authors have no financial conflict of interest.

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