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ECM1 controls T_H2 cell egress from lymph nodes through re-expression of $S1P_1$

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Type 2 helper T cells (T_H2) are critically involved in allergies and asthma. Here we demonstrate that extracellular matrix protein-1 (ECM1) is highly and selectively expressed in T_H2 cells. ECM1 deficiency caused impaired T_H2 responses and reduced allergic airway inflammation *in vivo*. Functional analysis demonstrated that although the T_H2 polarization of ECM1-deficient cells was unimpaired, these cells had a defect in migration and were retained in peripheral lymphoid organs. This was associated with reduced expression of KLF2 and $S1P_1$. We also found that ECM1 could directly bind the interleukin-2 (IL-2) receptor to inhibit IL-2 signaling and activate $S1P_1$ expression. Our data identify a previously unknown function of ECM1 in regulating T_H2 cell migration through control of KLF2 and $S1P_1$ expression.

$CD4^+$ helper T cells have a crucial role in adaptive immune responses. Naïve $CD4^+$ T cells can differentiate into at least four major subsets, including type 1 and 2 helper T cells (T_H1 and T_H2), inducible regulatory T cells (iT_{reg}) and IL-17-producing helper T cells (T_H17). T_H1 cells, which are induced by IL-12 and produce large quantities of interferon-γ (IFN-γ), are involved in enhancing clearance of certain intracellular pathogens[1]. The differentiation of T_H2 cells, which enhance the clearance of parasites, is coupled to IL-4 production and signaling[2]. In addition, research identifying the T_H17 subset, iT_{reg} cells and, most recently, the IL-9-producing helper T cells (T_H9), which are all induced by transforming growth factor-β in combination with other factors, has added complexity to the known $CD4^+$ effector T cell program[3]. T cell receptor (TCR) and costimulation signaling, as well as polarizing cytokines and lineage-specific transcription factors, are critical for the differentiation of T helper cells[4–6], whereas the array of chemokine receptors expressed by the various T cell subsets directs their migration. Migration of T_H2 cells into inflammatory tissues requires sphingosine-1-phosphate (S1P) interaction with the S1P receptor $S1P_1$ (ref. 7). Although T cell trafficking is crucial for immunological responses, the mechanisms controlling specific migration patterns remain unclear.

Recent studies indicate that emigration of T cells from thymus and secondary lymphoid organs is also regulated by S1P receptors[7–9]. S1P is a natural lysophospholipid found at micromolar concentrations in the plasma. S1P receptors, including $S1P_1$ and $S1P_4$, which are highly expressed on T cells, consist of five known G protein–coupled receptors[10]. An immunosuppressive agent, FTY720, acts as an agonist for S1P receptors. FTY720 binds to $S1P_1$, $S1P_3$, $S1P_4$ and $S1P_5$ and inhibits lymphocyte emigration from lymphoid organs[11,12]. $S1P_1$-deficient T cells in mice fail to exit into blood[13,14]. Conversely, T cells from $S1P_1$-transgenic mice preferentially distribute to the blood rather than to lymphoid organs[15,16], confirming that $S1P_1$ regulates T cell emigration from lymphoid organs. After encountering antigens, T cells downregulate $S1P_1$ and are transiently retained within lymphoid organs. Re-expression of $S1P_1$ 3 d later leads to their exit from lymphoid organs[17]. $S1P_1$ downregulation is initiated by TCR signaling[13]. However, the mechanism(s) controlling $S1P_1$ re-expression remain obscure.

ECM1 was initially identified as an 85-kDa glycoprotein secreted by a mouse osteogenic stromal cell line, MN7 (ref. 18). Its human homolog has been found to regulate endochondral bone formation and to stimulate proliferation of endothelial cells and induce angiogenesis[19]. There are two splice variants in mouse and three splice variants in humans. ECM1 alterations can cause autosomal recessive genodermatosis and lipoid proteinosis[20]. A role has also been reported for ECM1 in angiogenesis, in tumor progression and in tumor metastasis[21,22]. However, a function of ECM1 in immune responses has not yet been identified.

Here we report that ECM1 is preferentially expressed in T_H2 cells and that its induction is particularly pronounced during the late phase of T_H2 differentiation. Moreover, we show that T_H2 cell emigration from lymph nodes into the blood is inhibited in ECM1-knockout mice. We found that this occurs through ECM1-driven regulation of $S1P_1$ re-expression on T_H2 cells 3 d after antigen recognition and that ECM1 can directly bind the IL-2 receptor to inhibit IL-2 signaling and activate $S1P_1$ expression. Our data identify a novel role for ECM1 in controlling T_H2 cell trafficking during an immune response through regulation of $S1P_1$ expression.

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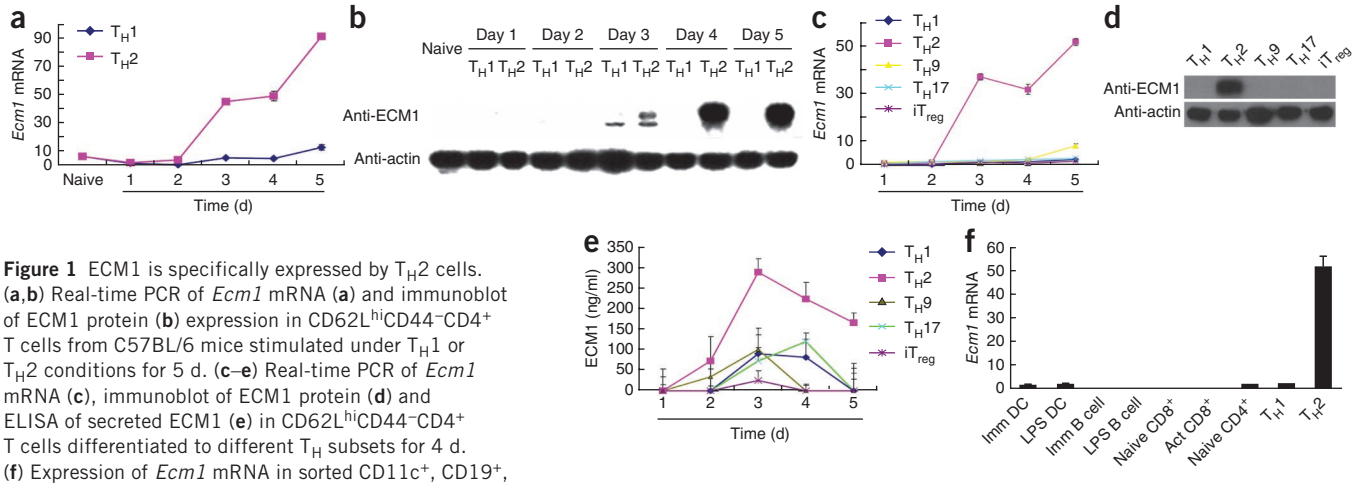

Figure 1 ECM1 is specifically expressed by T_H2 cells. (**a**,**b**) Real-time PCR of *Ecm1* mRNA (**a**) and immunoblot of ECM1 protein (**b**) expression in $CD62L^{hi}CD44^{-}CD4^{+}$ T cells from C57BL/6 mice stimulated under T_H1 or T_H2 conditions for 5 d. (**c**–**e**) Real-time PCR of *Ecm1* mRNA (**c**), immunoblot of ECM1 protein (**d**) and ELISA of secreted ECM1 (**e**) in $CD62L^{hi}CD44^{-}CD4^{+}$ T cells differentiated to different T_H subsets for 4 d. (**f**) Expression of *Ecm1* mRNA in sorted $CD11c^{+}$, $CD19^{+}$, $CD8^{+}$ and $CD4^{+}$ cells stimulated with lipopolysaccharide (dendritic cells (DC) and B cells) or antibodies to CD3 and CD28 (T cells). Graphs show mean ± s.e.m. of three independent experiments. Immunoblot results are representative of two independent experiments.

RESULTS

Specific expression of ECM1 in T_H2 cells

In a gene expression microarray study previously published[23], we found that ECM1 expression is higher in T_H2 than in T_H1 cells. Following this observation, we assessed the mRNA and protein expression of ECM1 in T_H1 and T_H2 cells by real-time PCR and immunoblot analysis. ECM1 was highly expressed in T_H2 cells at the mRNA and protein levels after induction of T_H2 polarization, from day 3 to day 5 (**Fig. 1a**,**b**). To test whether other helper T cell subsets express ECM1, we measured mRNA and protein expression in T_H1, T_H2, T_H9, T_H17 and iT_{reg} cells induced *in vitro* (**Supplementary Fig. 1**). Among these subsets, only T_H2 cells had high ECM1 expression (**Fig. 1c**,**d**).

As ECM1 is a secreted protein, we also tested culture supernatants from the different effector T cell subsets using enzyme-linked immunosorbent assays (ELISAs). T_H2 cells secreted substantially more ECM1 protein into the supernatants than did other T_H subsets (**Fig. 1e**). Real-time PCR analysis of *Ecm1* mRNA in dendritic cells, B cells and nonpolarized $CD8^{+}$ and $CD4^{+}$ cells confirmed that ECM1 expression was restricted to T_H2 $CD4^{+}$ T cells (**Fig. 1f**). Together, these data indicate that ECM1 protein is specifically expressed in T_H2 cells during $CD4^{+}$ T cell differentiation.

ECM1 expression is regulated by STAT6 and GATA-3

As the transcription factors STAT6 and GATA-3 are central regulators in T_H2 cell differentiation[4,6], we tested whether they regulate ECM1 expression. ECM1 protein was not induced in T_H2 cells from STAT6-knockout mice, in comparison to wild-type controls (**Fig. 2a**), and was not induced in T_H1 cells in either STAT6-knockout or wild-type mice. Retroviral transduction of a constitutively active form of STAT6 (STAT6-CA) into either T_H1 or T_H2 cells from STAT6-knockout mice rescued ECM1 expression in T_H2 cells and induced ECM1 expression in T_H1 cells (**Fig. 2b**).

Next, we tested ECM1 expression in T_H2 cells in which GATA-3 expression had been silenced by treatment with small interfering RNA (siRNA). Immunoblot analyses showed that ECM1 protein induction was impaired by knockdown of GATA-3 expression (**Fig. 2c**). Chromatin immunoprecipitation experiments revealed that GATA-3 did not bind to the 5′ regulatory sequences of *Ecm1* on day 2 after T_H2 polarization, but binding to distal GATA-boxes was detectable on day 3, when T_H2 cells started expressing ECM1 (**Fig. 2d**). Thus, ECM1 expression is regulated by the two master regulators of IL-4 signaling, STAT6 and GATA-3, with GATA-3 binding directly to the *Ecm1* promoter to regulate ECM1 transcription.

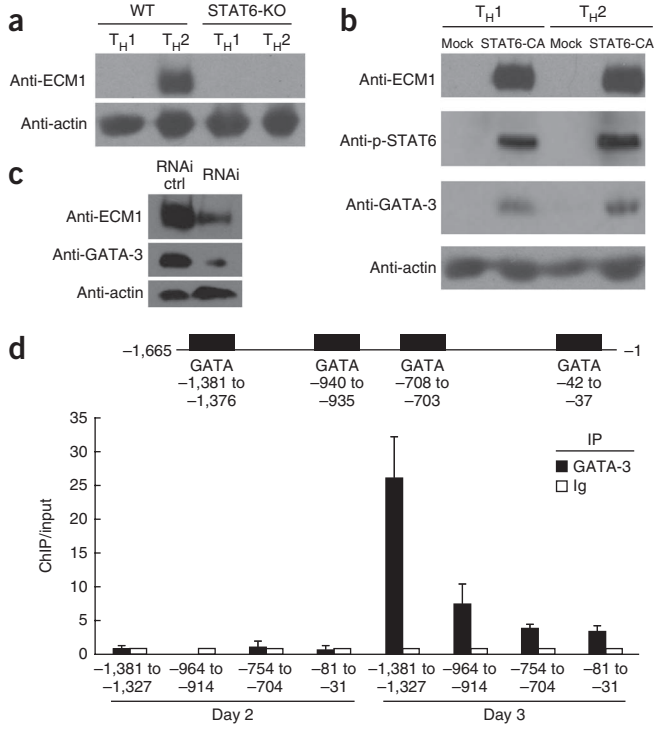

Figure 2 ECM1 expression is regulated by STAT6 and GATA-3. (**a**) Immunoblots from T_H1 and T_H2 cells of wild-type (WT) and STAT6-knockout (KO) mice cultured for 4 d. (**b**) Immunoblots from T cells transfected with retrovirus expressing STAT6-CA plasmid and harvested after sorting and restimulation for 24 h. p-STAT6, phosphorylated STAT6. (**c**) Immunoblots from T_H2 cells transfected with retrovirus expressing GATA-3 siRNA (RNAi). 'RNAi ctrl' denotes a control siRNA. (**d**) Chromatin immunoprecipitation (ChIP) from day 2 and day 3 T_H2 cell samples. Four GATA-boxes are shown on the *Ecm1* 5′ regulatory sequence. Real-time PCR primers were designed for these GATA-box sites. Data in **a**–**d** are representative of three independent experiments; graph shows mean and s.e.m.

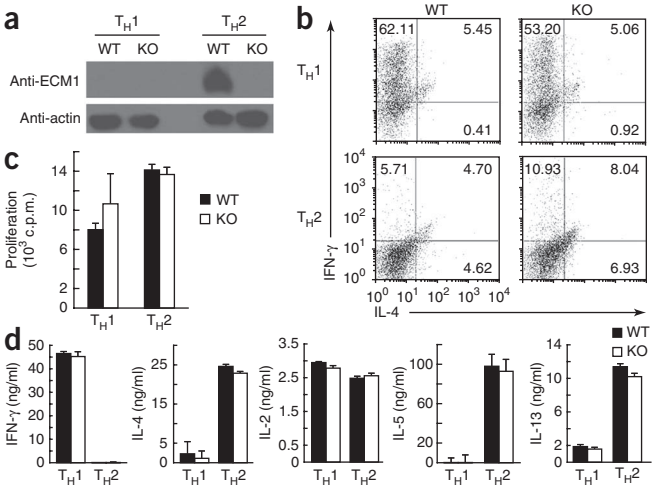

Figure 3 Proliferation and lineage commitment are unaltered in T cells from ECM1-deficient mice. (**a**) Immunoblots from CD4+ T cells of wild-type (WT) and *Ecm1*$^{-/-}$ (knockout, KO) mice activated by anti-CD3 and anti-CD28 under T_H1 or T_H2 conditions. (**b**) Flow cytometry analysis of T cells collected on day 4 after treatment with phorbol 12-myristate 13-acetate and ionomycin for 6 h and brefeldin A for 1 h. Numbers in quadrants represent ratios of cytokine-secreting cells. (**c**) T cell proliferation detected after 24 h restimulation of T_H1 and T_H2 cells. [^{3}H]thymidine was added to the culture 84 h after TCR induction. Samples were collected 12 h later and [^{3}H]thymidine was measured. (**d**) ELISAs from supernatant collected on day 4 under T_H1 or T_H2 conditions. Data in **a**–**d** are representative of three independent experiments; graphs show mean and s.e.m.

Normal T cell proliferation and commitment in *Ecm1*$^{-/-}$ mice

To further study the functions of ECM1 in helper T cells, we generated ECM1-knockout mice by deleting exons 2–11 of *Ecm1* (**Supplementary Fig. 2**). PCR of genomic DNA and immunoblot analysis confirmed the lack of ECM1 expression in activated T_H2 cells (**Fig. 3a**). The percentages of T_H1 and T_H2 cell populations, as assessed by intracellular staining for IL-4 and IFN-γ in *in vitro*–induced T_H1 and T_H2 cells, were similar in wild-type and ECM1-knockout mice (**Fig. 3b**). In addition, no substantial difference in cell proliferation was observed between wild-type and ECM1-knockout cells (**Fig. 3c**). ELISA measurements of IL-2, IFN- γ, IL-4, IL-5 and IL-13 secretion showed an essentially unaltered T_H1 and T_H2 cytokine profile in ECM1-knockout cells (**Fig. 3d**). Similar results were obtained after retrovirus infection to overexpress ECM1 or knock down ECM1 in T_H2 cells (**Supplementary Fig. 3**). Together, these data suggest that T_H1 and T_H2 cytokine production and cell proliferation from ECM1-knockout mice are normal.

Defective T_H2 cell migration in ECM1-knockout mice

As ECM1 deficiency did not alter T cell lineage commitment or proliferation *in vitro*, we examined whether ECM1 has a functional role *in vivo*. ECM1-knockout mice showed symptoms of an autoinflammatory disease and did not survive past the age of 6–8 weeks. Therefore, we created ECM1 radiation bone marrow chimeras (wild-type into wild-type and ECM1-knockout into wild-type (referred to as ECM1[BM]-deficient)) to examine the consequences of ECM1 deficiency for immune function *in vivo*. The effect of ECM1 deficiency was explored in a model of experimental allergic airway inflammation induced in ECM1[BM]-deficient mice by two intraperitoneal injections of ovalbumin (OVA) in alum, followed by intranasal challenge with OVA protein for 5 d. Compared to wild-type mice, ECM1[BM]-deficient mice showed substantially fewer eosinophils, macrophages and lymphocytes in their bronchoalveolar lavage (**Fig. 4a**). Moreover, OVA-specific IgE was substantially reduced in the serum of ECM1[BM]-deficient mice (**Fig. 4b**). ECM1[BM]-deficient mice consistently showed less inflammation on histopathologic examination of the lungs (**Fig. 4c**). Thus, ECM1[BM]-deficient mice show suppressed allergic airway inflammation that may be due in part to a reduced *in vivo* T_H2 response.

Evaluation of T cell numbers in the lung-draining lymph node (LLN) and spleen from both wild-type and ECM1[BM]-deficient mice showed there were more CD4+ T cells in peripheral lymphoid organs of ECM1[BM]-deficient mice than in those of wild-type mice (**Fig. 4d**). We further analyzed the different subsets of CD4+ cells (**Fig. 4e**) and found that only T_H2 cells were more numerous in LLNs and spleens from ECM1-knockout mice, whereas CD8+ T cell numbers showed no difference between wild-type and ECM1-knockout mice. These data suggest that in ECM1-knockout mice, T_H2 cells, but not other T cells, have a lower capacity to emigrate from

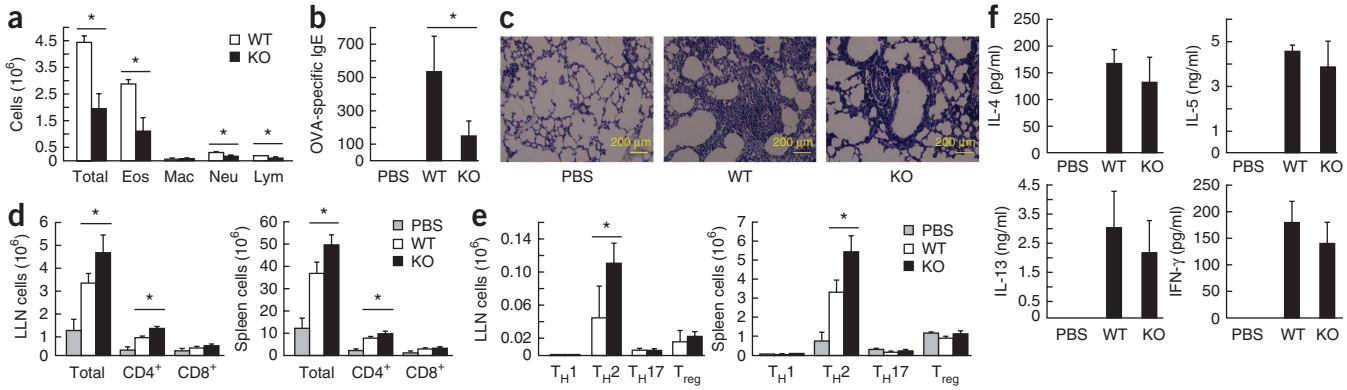

Figure 4 ECM1[BM]-deficient mice show impaired T_H2 function owing to defective T_H2 cell migration *in vivo*. Wild-type (WT) or *Ecm1*$^{-/-}$ (knockout, KO) bone marrow cells (1×10^7) were transferred into irradiated C57BL/6 mice. Two months later, mice were immunized with OVA and alum and challenged with aerosolized OVA as described in Online Methods. Mice immunized with PBS served as a negative control. (**a**) Immune cells counted in bronchoalveolar lavage. (**b**) ELISA showing OVA-specific IgE levels in serum. (**c**) Lung tissue sections stained with hematoxylin and eosin. Scale bar, 200 μm. (**d**,**e**) Numbers of different subsets of cells in spleen and LLN. CD4+, CD8+, T_H1 (IFN-γ+), T_H2 (IL-4+), T_H17 (IL-17A+) and T_{reg} (Foxp3+) cells were measured by flow cytometry. (**f**) ELISA showing cytokine production from supernatants of draining lymph node cells restimulated with OVA for 96 h. *$P < 0.05$. Results in **a**–**f** are representative of three independent experiments; graphs show mean and s.e.m.

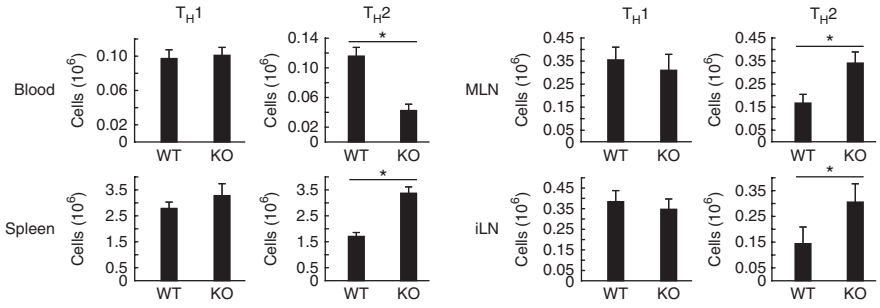

Figure 5 ECM1 functions in control of T_H2 cell migration. Shown are numbers of $CFSE^+$ T_H1 cells or $CFSE^+$ T_H2 cells in spleen, blood, inguinal lymph nodes (iLN) and mesenteric lymph node (MLN), 24 h after T_H1 and T_H2 cells were cultured for 4 d, labeled with CFSE and injected intravenously into C57BL/6 mice. WT, wild-type mice; KO, ECM1-knockout mice. $*P < 0.05$. Graphs show mean and s.e.m. from three independent experiments.

draining lymph nodes to lung tissue. However, upon *ex vivo* recall with OVA protein, LLN cells from wild-type and ECM1-knockout mice produced similar amounts of T_H1 and T_H2 cytokines (**Fig. 4f**), consistent with the *in vitro* observation (**Fig. 3d**). These results are compatible with the idea that the impairment of the T_H2 response in ECM1[BM]-deficient mice *in vivo* is associated with $CD4^+$ T cell retention in peripheral lymphoid organs.

ECM1 controls T_H2 cell migration

As $CD4^+$ cell exit from lymph nodes and airway inflammation were reduced in ECM1[BM]-deficient mice, we hypothesized that ECM1 controls T_H2 migration. To directly address this question, we compared the T_H1 and T_H2 cell trafficking of wild-type and ECM1-knockout T cells *in vivo*. T_H1 or T_H2 cells from wild-type or ECM1-knockout donors were polarized for 4 d *in vitro* (to reach high expression of ECM1), labeled with the cytosolic dye CFSE and injected intravenously into wild-type C57BL/6 mice. The T_H2 cells, but not the T_H1 cells, were retained in peripheral lymphoid organs of C57BL/6 recipients (**Fig. 5**). In contrast, there were considerably fewer T_H2 cells found in peripheral blood. These results support the idea that ECM1 deficiency lowers the ability of T_H2 cells to migrate from peripheral lymphoid organs to an inflammatory site.

ECM1 is not required for T_H1 and T_H17 response *in vivo*

To further analyze the function of ECM1 in induction of T_H1 and T_H17 responses *in vivo*, we immunized wild-type and ECM1[BM]-deficient bone marrow chimeras with OVA protein in complete Freund's adjuvant. These immunizing conditions drive a T_H1- and T_H17-dominated response[24]. Lymphocytes from draining lymph nodes were isolated on day 5 after immunization and restimulated *ex vivo* with OVA for 4 d. Cell numbers in the draining lymph nodes indicated that $CD4^+$ and $CD8^+$ T cell emigration was not affected by ECM1 deficiency under T_H1 and T_H17 polarizing conditions (**Supplementary Fig. 4a**). ELISAs of supernatants from OVA-restimulated lymphocytes showed no difference in IL-4, IL-17A and IFN-γ production between wild-type and ECM1[BM]-deficient mice (**Supplementary Fig. 4b**). These data support the idea that T_H1 and T_H17 function is not affected by ECM1 deficiency.

ECM1 deficiency reduces $S1P_1$ expression in T_H2 cells

As ECM1 is secreted by T_H2 cells and our data suggest that it specifically regulates T_H2 cell migration, we explored the mechanism by which ECM1 accomplishes this regulation. Because T cell trafficking is directed by responses to chemokines, we applied mRNA microarray technology to identify genes with altered expression in ECM1-knockout T_H2 cells. Notably, the chemokine receptors $S1P_1$ and CCR4 were downregulated in ECM1-knockout T_H2 cells (**Fig. 6a**). In contrast, expression of $S1P_1$ and CCR4 was normal in ECM1-knockout T_H1 cells. $S1P_1$ expression is regulated by KLF2 and Foxo1 transcription factors and is important in T cell migration[25,26]. Real-time PCR of *Klf2* and *Foxo1* mRNA expression in T_H1 and T_H2 cells showed that KFL2 but not Foxo1 was downregulated in ECM1-knockout T_H2 cells (**Fig. 6b**). In addition, time-course analysis of ECM1 and $S1P_1$ re-expression (**Supplementary Fig. 5**) suggested that ECM1 contributed to $S1P_1$ re-expression 3 d after TCR engagement. These findings suggest that ECM1 regulates expression of $S1P_1$ by controlling its transcription factor, KLF2.

As ECM1 deficiency in T_H2 cells was associated with the downregulation of $S1P_1$, we asked whether ECM1 replacement would be sufficient to restore $S1P_1$ expression. An *Ecm1*-encoding retrovirus restored *Ecm1* mRNA expression in T_H2 cells from ECM1-knockout mice (**Fig. 6c**) as well as expression of the mRNAs *S1pr1* (encoding $S1P_1$) and *Klf2*, whereas expression of *S1pr4* and *Foxo1* mRNA were unaffected (**Fig. 6c**). In addition, ECM1 re-expression restored $S1P_1$-dependent chemotaxis of ECM1-knockout T_H2 cells to levels comparable to those of wild-type T_H2 cells in a standard *in vitro* transmigration assay (**Fig. 6d**). To demonstrate that these effects were specific to $S1P_1$, we tested the chemotactic response to the chemokines CXCL12 and CCL21, which might recruit cells expressing the adhesion marker CXCR4 and the lymph node–homing receptor CCR7 after restoration of ECM1 expression. ECM1 deficiency did not affect the CXCL12 and CCL21 chemotaxis effect (**Supplementary Fig. 6a**). Moreover, we did not see an effect of ECM1 deficiency or restoration in T_H1 cells (**Supplementary Fig. 6b,c**).

We further confirmed these observations *in vivo* using adoptive transfer of ECM1-knockout T_H2 cells forced to express ECM1, KLF2 or $S1P_1$. To exclude confounding effects of T cell ingress into lymphoid organs, L-selectin antibody, which is only efficient at blocking entry into peripheral LNs, was injected intravenously into the recipient mice 24 h after adoptive transfer of transduced cells. ECM1, KLF2 or $S1P_1$ expression rescued ECM1-knockout T_H2 cell migration *in vivo*, resulting in levels comparable with those in wild-type T_H2 cells (**Fig. 6e**). Together, these data indicate that ECM1 has an essential role in upregulating KLF2 and $S1P_1$ expression in T_H2 cells.

We next investigated whether ECM1 carries out its functions through endocrine or exocrine pathways. ECM1-knockout T cells polarized for 3 d under T_H2-inducing conditions were labeled with CFSE and cultured for 24 h with unlabeled T_H2 polarized wild-type cells (**Fig. 7a**), followed by separation by sorting. The $S1P_1$-induced chemotactic response from ECM1-knockout ($CFSE^+$) T_H2 cells was rescued by culturing with wild-type ($CFSE^-$) T_H2 cells (**Fig. 7b**). Consistent with this observation, *S1pr1* and *Klf2* mRNA expression were also substantially greater in $CFSE^+$ ECM1-knockout T_H2 cells, whereas $S1P_4$ and Foxo1 expression were unaffected (**Fig. 7c**). To further confirm that ECM1 effected migration extrinsically, we used adoptive transfer *in vivo*. Wild-type and ECM1-knockout T_H2 cells cultured for 3 d were labeled with different concentrations of

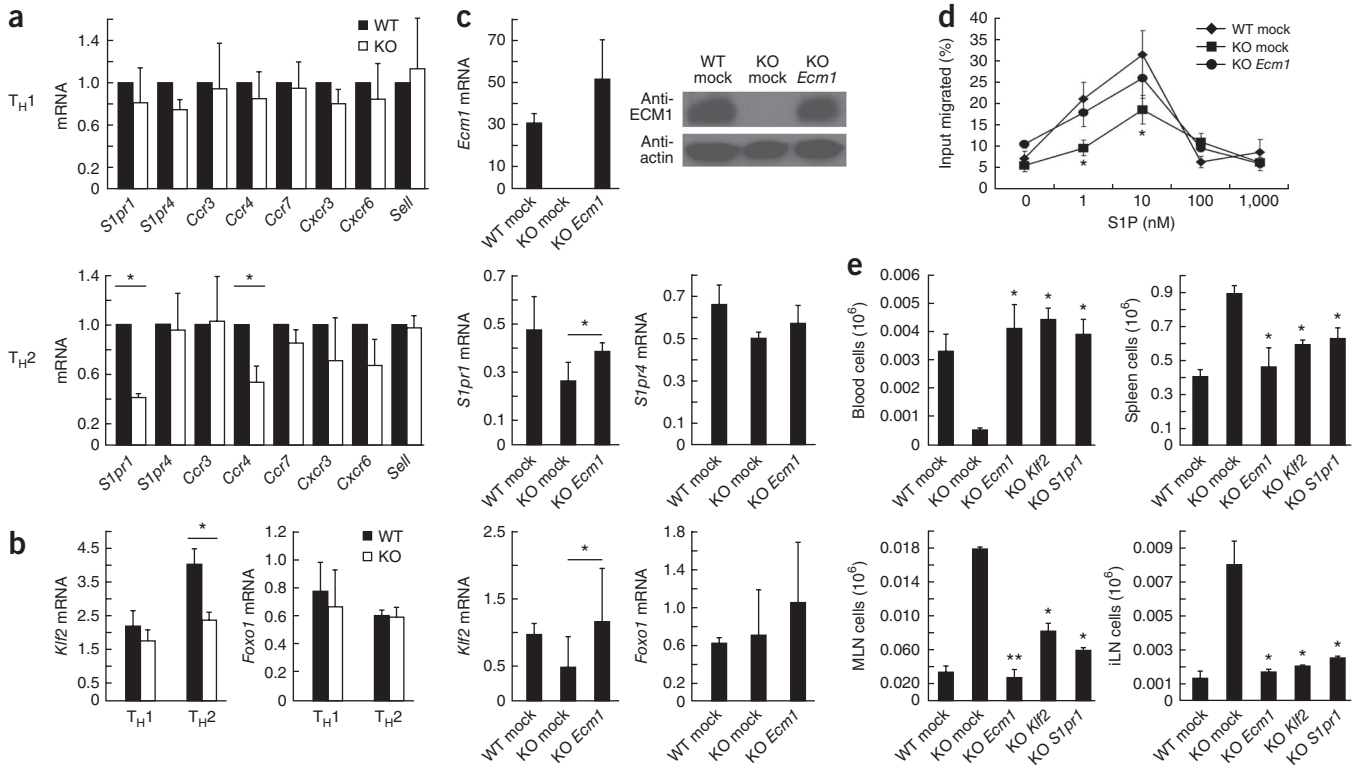

Figure 6 ECM1 deficiency reduces expression of $S1P_1$ in T_H2 cells. (**a**) mRNA levels determined by real-time PCR of T_H1 and T_H2 cells from wild-type (WT) and ECM1-knockout (KO) mice cultured for 4 d. *Sell* encodes L-selectin. (**b**) mRNA levels for the transcription factors KLF2 and Foxo1 detected by real-time PCR. (**c**) mRNA levels and immunoblots from WT and KO T_H2 cells infected by ECM1-expressing retrovirus as described in Online Methods; data were obtained 4 d after TCR signaling treatment. (**d**) Chemotactic response of T_H2 cells infected with a control retrovirus vector or one encoding *Ecm1* from WT and KO mice. Shown is the percentage of the input cell population that responded to each concentration of S1P. (**e**) Numbers of $CFSE^+$ WT T_H2 cells or $CFSE^+$ KO T_H2 cells in spleen, blood, inguinal lymph nodes (iLN) and mesenteric lymph node (MLN) of injected mice. WT or KO T_H2 cells were transfected with *Ecm1*, *Klf2*, *S1pr1* or control plasmid, cultured for 4 d and injected intravenously into C57BL/6 mice; 24 h later, 100 μg antibody to L-selectin was injected intravenously, and $CFSE^+$ cell numbers were measured 1 d later. $^*P < 0.05$; $^{**}P < 0.01$. Data in **a**–**e** are representative of three independent experiments; graphs show mean ± s.e.m.

CFSE and co-cultured for 24 h. The mixed cells were then adoptively transferred to wild-type C57BL/6 recipients. Exposure to extracellular ECM1 restored the ability of ECM1-knockout T_H2 cells to migrate *in vivo* (**Fig. 7d**). These data suggest that ECM1 secreted by wild-type T_H2 cells induces $S1P_1$ expression via KLF2 and restores migration of ECM1-knockout T_H2 cells.

ECM1 inhibits IL-2 signaling

Finally, we wanted to explore the mechanism by which ECM1 regulates $S1P_1$ expression in T_H2 cells. KLF2 and $S1P_1$ expression in T cells is controlled by signals from the kinases PI(3)K and mTOR, which in turn are activated by IL-2–IL-2 receptor interactions[27]. To determine whether PI(3)K and mTOR were involved in ECM1 function, we activated T cells for 3 d and then treated them with PI(3)K- and mTOR-specific inhibitors (LY294002 and rapamycin, respectively) for 24 h. Inhibition of PI(3)K and mTOR signaling enhanced *Klf2* and *S1pr1* mRNA expression in wild-type, but not in ECM1-knockout T_H2 cells (**Fig. 8a**). This suggests that the regulation of KLF2 and $S1P_1$ expression by ECM1 is not dependent on PI(3)K and mTOR signaling.

We next tested whether STAT5 phosphorylation, which is also downstream of IL-2R signaling, is involved in ECM1-mediated $S1P_1$ regulation. We used recombinant human IL-2 to enhance IL-2 signaling and mouse IL-2 neutralizing antibody to block IL-2 signaling. The basal level of STAT5 phosphorylation in ECM1-knockout T_H2 cells was higher than in wild-type T_H2 cells (**Fig. 8b**), whereas *Klf2* and *S1pr1* mRNA expression were lower (**Fig. 8c**). Blocking IL-2 signaling reduced STAT5 phosphorylation, but increased *Klf2* and *S1pr1* mRNA expression. Conversely, adding exogenous IL-2 increased STAT5 phosphorylation and reduced *Klf2* and *S1pr1* mRNA expression (**Fig. 8b**,**c**). However, we did not find this pattern in T_H1 cells (**Supplementary Fig. 7**). These data identify STAT5 as another negative regulator of *Klf2* and *S1pr1* mRNA expression in T_H2 cells and implicate IL-2 signaling in ECM1-mediated regulation of *S1pr1* mRNA expression.

We next addressed whether ECM1 binds to IL-2 receptor to affect STAT5 phosphorylation. ECM1 and hemagglutinin-tagged IL-2 receptor subunits (CD25, CD122 and CD132) were transfected into HEK293T cells. ECM1 immunoprecipitated with the IL-2 receptor β-subunit (CD122), but not with CD25 or CD132 (**Fig. 8d**). However, ECM1 deficiency did not affect the levels of IL-2 receptor expression (**Fig. 8e**). Notably, STAT5 phosphorylation can also be induced by IL-7 and IL-15 (ref. 28). We examined whether downstream signaling through IL-7R and IL-15R was affected by ECM1. Although IL-7 and IL-15 promoted STAT5 phosphorylation and inhibited $S1P_1$ expression when added to the T_H2 polarizing cultures, ECM1 deficiency did not affect IL-7- or IL-15-mediated STAT5 phosphorylation (**Supplementary Fig. 8**). This indicates that ECM1 affects only IL-2 signaling, and not IL-7 or IL-15 signaling, to regulate $S1P_1$ expression. ECM1 can interact with perlecan[19], and perlecan in serum can

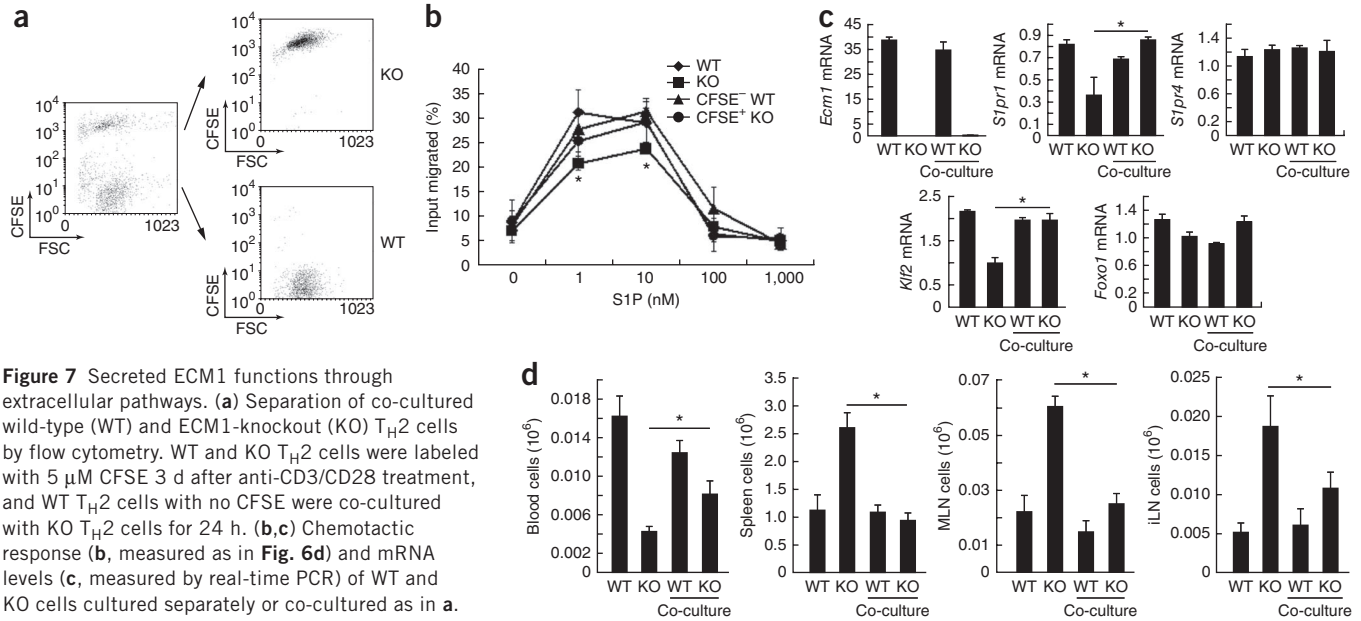

Figure 7 Secreted ECM1 functions through extracellular pathways. (**a**) Separation of co-cultured wild-type (WT) and ECM1-knockout (KO) T_H2 cells by flow cytometry. WT and KO T_H2 cells were labeled with 5 μM CFSE 3 d after anti-CD3/CD28 treatment, and WT T_H2 cells with no CFSE were co-cultured with KO T_H2 cells for 24 h. (**b**,**c**) Chemotactic response (**b**, measured as in **Fig. 6d**) and mRNA levels (**c**, measured by real-time PCR) of WT and KO cells cultured separately or co-cultured as in **a**. (**d**) Numbers of $CFSE^+$ cells in spleen, blood, iLN and MLN of mice injected with separately cultured or co-cultured cells. Three days after TCR engagement, WT or KO T_H2 cells were labeled with different concentrations of CFSE (WT, 0.5 μM; KO, 5 μM) and co-cultured for 24 h. Cells were injected intravenously into C57BL/6 mice. After 24 h, 100 μg anti-L-selectin was injected intravenously. $CFSE^+$ cell numbers were measured 1 d later. $^*P < 0.05$. Data in **a**–**d** are representative of three independent experiments; graphs show mean ± s.e.m.

regulate IL-2R signaling[29]. However, ECM1 inhibited STAT5 phosphorylation and promoted $S1P_1$ expression under serum-free conditions, in the absence of perlecan (**Supplementary Fig. 9**). Together, these data indicate that ECM1 binds to the IL-2Rβ subunit to inhibit IL-2 signaling.

To examine whether ECM1 could alter IL-2 signaling and thus regulate $S1P_1$ expression indirectly, we used the co-culture method described above and added ECM1-neutralizing antibody to change the ECM1 concentration available to the wild-type and ECM1-knockout T_H2 cells. ECM1 rescue in ECM1-knockout T_H2 cells inhibited STAT5 phosphorylation and promoted *S1pr1* mRNA expression, whereas neutralization of ECM1 in wild-type T_H2 cells promoted STAT5 phosphorylation and inhibited *S1pr1* mRNA expression (**Fig. 8f**,**g**). Together, these findings confirm that ECM1 can directly bind to the IL-2 receptor to inhibit IL-2 signaling and promote $S1P_1$ expression in T_H2 cells (**Supplementary Fig. 10**).

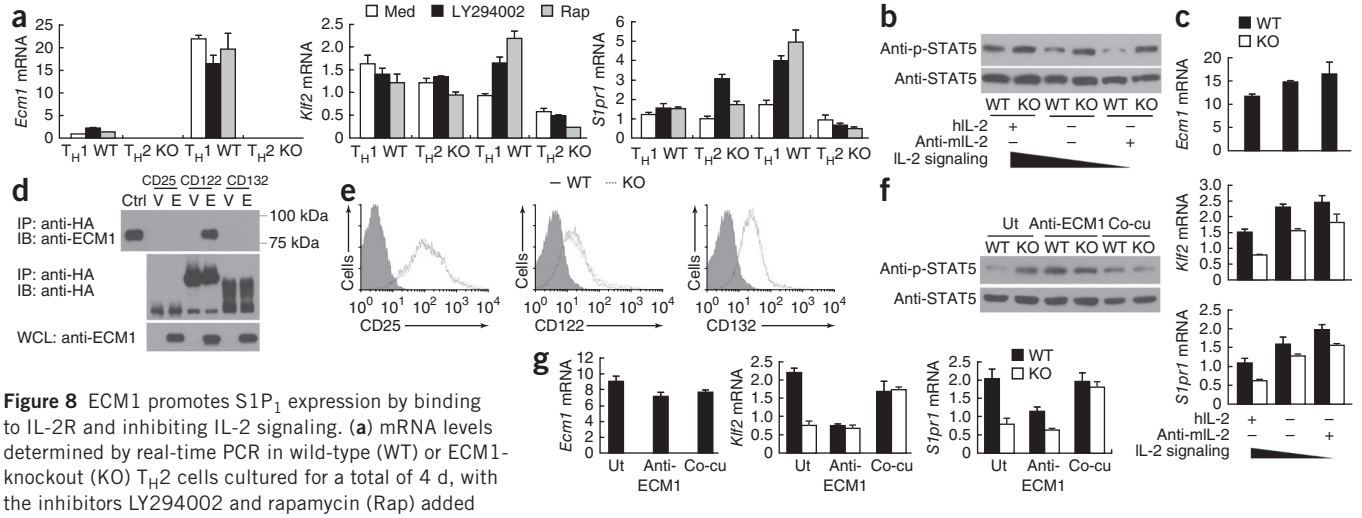

Figure 8 ECM1 promotes $S1P_1$ expression by binding to IL-2R and inhibiting IL-2 signaling. (**a**) mRNA levels determined by real-time PCR in wild-type (WT) or ECM1-knockout (KO) T_H2 cells cultured for a total of 4 d, with the inhibitors LY294002 and rapamycin (Rap) added 24 h before termination of culture. Med, medium alone (negative control). (**b**,**c**) Immunoblots (**b**) and mRNA levels measured by real-time PCR (**c**) from WT or KO T_H2 cells cultured for 4 d with mouse IL-2 (mIL-2) neutralizing antibody added after 2 d to block human IL-2 (hIL-2) signaling. (**d**) Immunoprecipitation (IP) and immunoblot (IB) analysis of lysates of HEK293T cells expressing ECM1 and various IL-2 receptor units. Ctrl, positive control; V, vector; E, ECM1; WCL, whole-cell lysis. (**e**) CD25, CD122 and CD132 expression determined by flow cytometry of T_H2 cells. (**f**,**g**) Immunoblots (**f**) and mRNA levels measured by real-time PCR (**g**) from T_H2 cells prepared as in **b**,**c** and subjected to ECM1 co-culture as in **Figure 7**. ECM1 neutralizing antibody was used for blocking secreted ECM1 protein. Ut, untreated; Co-cu, co-culture. $^*P < 0.05$. Data in **a**–**g** are representative of three experiments; graphs show mean and s.e.m.

DISCUSSION

Here we report that ECM1, a molecule previously found to have a role in angiogenesis and tumor growth, is also involved in immunity. We show that ECM1 is selectively expressed in T_H2 cells and is crucial in T_H2 cell trafficking, in particular in the egress of newly primed T_H2 cells from the lymph node. In an OVA-induced T_H2-mediated allergic airway inflammation model, ECM1 deficiency reduced airway inflammation and IgE responses. Instead of being trafficked to the lung, the OVA-specific $CD4^+$ T cells were retained within spleen and lymph nodes in the ECM1-knockout mice, which is consistent with the altered migration patterns observed in C57BL/6 mice. These data suggest that ECM1-knockout effector T_H2 cells fail to migrate to inflammatory sites.

Many extracellular proteins are secreted and then feed back to regulate signaling in $CD4^+$ T cells[30]. In this study, we have demonstrated that ECM1 expressed by T_H2 cells behaves in this way. ECM1 was not induced until 48 h after T cell receptor engagement, and its expression was enhanced during the late phase of T_H2 differentiation. This expression pattern implies that ECM1 may have a function during the later phase of T_H2 cell development. The fact that co-culture of wild-type and ECM1-knockout T_H2 cells restored the migration of ECM1-knockout T_H2 cells toward S1P shows that secreted ECM1 signals to T_H2 cells. Nevertheless, the data do not exclude the possibility that ECM1 can act directly on the producing cells, without first being secreted.

Both $S1P_1$ and CCR4 were initially identified as potential ECM1 targets; however, here we have concentrated on the role of $S1P_1$ in ECM1-mediated T_H2 cell migration. Our data do not exclude a role for CCR4, the chemokine receptor classically associated with T_H2 cells[31], in T_H2 responses. We confirmed that CCR4 expression was reduced by ECM1 deficiency and was rescued by ECM1 retrovirus transduction, suggesting that CCR4 is another pathway in which ECM1 may be involved (data not shown). It is also important to mention that although there was a difference in the CD4/CD8 ratio between wild-type and ECM1-knockout thymi, radiation chimeras reconstituted with wild-type and ECM1-knockout bone marrow did not show such a difference, suggesting that T cell development is not substantially changed in ECM1-knockout bone marrow chimeras (data not shown).

Antigen presence causes a transient retention of antigen-reactive cells in the lymphoid organs[10], ensuring that T cells receive adequate interaction with antigen-presenting cells before T cells exit[10]. *In vitro* and *in vivo* data suggest this retention is due to $S1P_1$ downregulation on antigen-activated T cells, causing loss of chemotactic responsiveness[13]. However, after 3 d, when activated effector T cells began to appear in circulation, T cells' *S1pr1* mRNA expression was high and cells had regained S1P responsiveness. Although it has been suggested that $S1P_1$ downregulation is controlled by TCR engagement[13], the mechanism of its re-expression is unknown. Our current data indicate that ECM1 assists KLF2 and $S1P_1$ re-expression when T_H2 cells are ready for emigration from lymphoid organs.

It has been proposed that L-selectin is regulated by KLF2 in T cells[25]. However, our mRNA microarray data and real-time PCR showed no substantial difference in L-selectin expression between wild-type and ECM1-knockout T_H2 cells. Other regulatory influences may counteract the effect of KLF2 on L-selectin expression in ECM1-knockout cells. IRF1 and Mzf1, which have been reported to promote L-selectin expression[32], were upregulated in ECM1-knockout T_H2 cells (data not shown), which may explain the normal L-selectin levels in ECM1-knockout T_H2 cells.

Although both STAT5 phosphorylation and PI(3)K-mTOR activation are downstream of IL-2 receptor, PI(3)K-mTOR signaling seems not to be involved in ECM1-mediated $S1P_1$ regulation. STAT5 phosphorylation is thought to be important in proliferation[33], but ECM1 deficiency did not affect T_H2 cell proliferation. The timing of ECM1 expression could be important in this case. After antigen engagement, activated T cells begin to proliferate, and IL-2 signaling is crucial for this process. At this early stage, STAT5 phosphorylation, together with TCR activation, may be involved in inhibiting $S1P_1$ expression. Subsequently, however, strong IL-2 signaling may inhibit expression of migration-related molecules. Inhibition of STAT5 expression at later stages, when ECM1 is expressed, would primarily affect migration, rather than proliferation.

In summary, we have demonstrated that ECM1 is expressed and secreted by T_H2 cells from day 2 after antigen engagement and feeds back to regulate transcription of KLF2 and $S1P_1$ and thus control T_H2 cell migration from lymph nodes to sites of inflammation. These findings shed new light on the regulation of T cell migration in immune-mediated disease and may suggest future approaches to control T_H2 cell trafficking in diseases such as allergy and asthma.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Accession codes. GEO: microarray data, GSE19707 (series), and GSM491757 and GSM491758 (specific array files).

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

Z. Li designed (with help from Z. Liu) and performed mouse experiments and *in vitro* cell culture experiments, analyzed data and wrote the manuscript; Y. Zhang did all western blotting and real-time PCR experiments and helped in mouse experiments and analyzing data; X.W., K.M. and Y. Zheng helped in mouse experiments; Z.T. bred mice and confirmed the *Ecm1*$^{-/-}$ genotype; J.W. helped with immunoprecipitation experiments; G.L., L.T. and Y.J. made two ECM1 antibodies; M.Q. supplied reagents; S.S. performed flow cytometry; X.Z. helped design experiments and edit the manuscript; and B.S. conceived of the research, directed the study and edited the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 and STAT6-knockout mice were obtained from the Jackson Laboratory and held under specific pathogen-free conditions at the Animal Care Facility of Chinese Academy of Sciences. Mice 6–8 weeks old were used in cell culture. Animal care and use were in compliance with Institute of Biochemistry and Cell Biology's guidelines. ECM1-knockout mice were generated by knocking out exons 2–11 of the mouse *Ecm1* gene. The ECM1-knockout effect was confirmed by real-time PCR and immunoblot. Mice were initially created on the 129 X C57BL/6 background, then extensively back-crossed to C57BL/6.

Cell purification and differentiation *in vitro*. Naive $CD4^+$ ($CD4^+CD44^{low}CD62L^{hi}$) T cells, $CD4^+$ T cells, $CD8^+$T cells, $CD19^+$ B cells and retrovirally transduced GFP^+ cells were purified by FACS Aria (BD Biosciences), routinely to more than 95% purity. The method for generation of bone-marrow dendritic cells has been described[34]. The method of $CD4^+$ T cell differentiation *in vitro* has been described[23]. Mouse IL-7 and mouse IL-15 were obtained from R&D. Functional grade mouse IL-2 antibody (JES6-1A12) was from eBioscience. T cell serum-free medium was from Celprogen.

Retroviral transduction. The cDNA of *Ecm1* and STAT6-CA were cloned into mouse stem cell retrovirus vector. To construct retrovirus-mediated *Ecm1* and GATA-3 siRNA vector, mouse *Ecm1* and GATA-3 RNA interference target sequence was designed and the complementary oligonucleotides were synthesized and cloned into multiple cloning sites of the retrovirus vector. The retroviral transduction protocol has been described[23].

Real-time PCR. Total RNA was extracted from cultured cells with TRIzol (Invitrogen) according to the manufacturer's instructions. The methods for cDNA generation and real-time PCR have been described[23].

Flow cytometry and sorting. For analysis of intracellular cytokine production, method has been described previously[23]. For flow cytometry analysis, allophycocyanin anti-mouse IFN-g (XMG1.2) was obtained from eBioscience. Phycoerythrin anti-mouse IL-4 (BVD4-1D11), phycoerythrin anti-mouse CD8α (53-6.7), fluorescein isothiocyanate anti-mouse CD4 (GK1.5), allophycocyanin anti-mouse CD25 (PC61.5), allophycocyanin anti-mouse CD122 (TM-b1) and phycoerythrin anti-mouse CD132 (TUGm2) were purchased from BD Pharmingen. Naïve T cells were sorted by $CD4^+CD44^{low}CD62L^{hi}$. $CD4^+$ T cells, $CD8^+$ T cells, $CD19^+$ B cells, retrovirally transduced GFP^+ cells and CFSE-labeled cells were purified by FACS Aria (BD Biosciences) to routinely more than 95% purity.

Enzyme-linked immunosorbent assay. For analysis of cytokine production in the supernatant, IL-4 IL-2, IL-17A, IL-5, IL-13 and IFN-γ ELISA Duoset kits were purchased from R&D Systems and used according to the manufacturer's protocol. We measured OVA-specific IgE by coating 100 μg/ml OVA and detecting IgE with horseradish peroxidase (HRP) anti-IgE (R35-72) from BD Pharmingen. We prepared ECM1 N- and C-terminal antibodies for ECM1 ELISA assay. The N-terminal antibody was used as the capture antibody, and the HRP-labeling C-terminal antibody was used as the detection antibody to detect ECM1 in supernatant.

Bone marrow transfer and adoptive transfer. Bone marrow cells from C57BL/6 and ECM1-knockout mice were depleted of T cells by CD4(L3T4) microbeads (MACS Miltenyi Biotec). Cells were transferred intravenously into the lethally irradiated (950 rad) C57BL/6 mice within 24 h. The reconstitution effect was detected 6–14 weeks after bone marrow cells were transferred. Before transfer, T cells were labeled with CFSE. T cells (1×10^7) were injected intravenously into C57BL/6 mice. Total cell numbers were determined in spleen, blood, inguinal lymph node and mesenteric lymph node. $CFSE^+$ ratio was determined by flow cytometry and numbers of $CFSE^+$ cells were calculated. Functional-grade anti-L-selectin (MEL-14) was purchased from eBioscience.

OVA-induced allergic airway inflammation. The allergic airway inflammation induction has been described[35]. Measurements of serum OVA-specific IgE production and histological analysis were carried out as described[35].

OVA and complete Freund's adjuvant immunization. Mice (6–8 weeks old, five mice per group) were immunized with OVA (0.5 mg/ml) emulsified in complete Freund's adjuvant (0.5 mg/ml) at the base of the tail (100 μl for each mouse) for 5 d. Splenocytes from the immunized mice were restimulated with OVA protein for 3–4 d and cytokine expression was analyzed by ELISA.

Immunoprecipitation and immunoblot analysis. Immunoprecipitation and immunoblot analysis was performed using standard protocols. For immunoprecipitation analysis, protein A/G plus-agarose immunoprecipitation reagent was purchased from Santa Cruz, protease inhibitor cocktail was obtained from Roche and anti-hemagglutinin (HA.11; 16B12; CO-MMS-101R) was obtained from Covance. For immunoblot analysis, antibodies against phosphorylated (p)-STAT6 (Tyr 641-R), total STAT6 (S-20) and GATA-3 (HG3-31) were obtained from Santa Cruz. Antibodies against p-STAT5 (Tyr694) and total STAT5 (3H7) were from Cell Signaling. The inhibitors of PI(3)K and mTOR signaling, LY294002 and rapamycin, were obtained from Sigma-Aldrich. The N-terminal and C-terminal ECM1 rabbit polyclonal antibodies were raised against recombinant bacterially produced mouse ECM1 protein fragments encompassing N- and C-terminal peptides. Anti-actin was purchased from Sigma.

^{3}H proliferation assay. Purified $CD4^+$ T cells were cultured at about 0.5×10^6 to 1×10^6 cells/ml in 200 μl of medium and were stimulated for 72 h on plates precoated with anti-CD3 (clone 145-2C11, BD Pharmingen). For costimulation of T cells, soluble anti-CD28 (clone 37.51, BD Pharmingen) was also added at 1 μg/ml. Proliferation was assessed by the incorporation of [^{3}H] thymidine added at 1 μCi/well during the last 16 h of culture in triplicate wells. Cells were collected using a cell harvester and [^{3}H]thymidine was quantified by scintillation counting.

Transwell assay. The chemotaxis assay has been described[36]. $CD4^+$ T cells were cultured until use. T cells were washed and resuspended in 100 μl serum-free RPMI 1640 medium, and 5×10^5 T cells from each group were placed in 24-well Transwell plates (5-μm pore size, Corning). The bottom chambers were filled with various concentrations of S1P (Sigma) suspended in serum-free medium. Plates were cultured for 3 h at 37 °C, at which point migrated T cells were collected from the lower chamber and cells were counted in a blood cell counting chamber under light microscopy.

Statistics. Results represent the mean ± s.e.m. where applicable. Student's *t*-test was used to compare two independent groups. For all tests, values of $P < 0.05$ were considered statistically significant. A value of $P < 0.01$ was considered highly statistically significant.

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