Extracellular matrix protein 1 promotes follicular helper T cell differentiation and antibody production

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T-follicular helper (T_{FH}) cells are a subset of $CD4^+$ helper T cells that help germinal center (GC) B-cell differentiation and high-affinity antibody production during germinal center reactions. Whether important extracellular molecules control T_{FH} differentiation is not fully understood. Here, we demonstrate that a secreted protein extracellular matrix protein 1 (ECM1) is critical for T_{FH} differentiation and antibody response. A lack of ECM1 inhibited T_{FH} cell development and impaired GC B-cell reactions and antigen-specific antibody production in an antigen-immunized mouse model. ECM1 was induced by IL-6 and IL-21 in T_{FH} cells, promoting T_{FH} differentiation by down-regulating the level of STAT5 phosphorylation and up-regulating Bcl6 expression. Furthermore, injection of recombinant ECM1 protein into mice infected with PR8 influenza virus promoted protective immune responses effectively, by enhancing T_{FH} differentiation and neutralizing antibody production. Collectively, our data identify ECM1 as a soluble protein to promote T_{FH} cell differentiation and antibody production.

follicular helper T cells | antibody responses | ECM1 | CD4 T cell differentiation | IL-2–pSTAT5 signaling

High-affinity antibodies are critical to immune responses against pathogen infection and are produced by activated B cells and plasma cells. Their production depends on antigen-specific T cells, which transmit activating signals to B cells in GC (1, 2). The specialized T cells located in germinal centers (GCs) are a subset of $CD4^+$ helper T cells called follicular helper (T_{FH}) cells. T_{FH} cells express characteristic genes, including CXCR5 (3–5), PD-1 (6, 7), and ICOS (8) in addition to the transcriptional repressor B-cell lymphoma 6 (Bcl6), which is the master regulator of T_{FH} (9–11).

T_{FH} cell differentiation is generally considered as a multistep and multifactorial process (1). Like other subsets of helper T cells, the cytokine niche is essential for T_{FH} differentiation. In mice, interleukin (IL)-6 and IL-21 play central roles in T_{FH} cell differentiation. IL-6 is expressed in many types of cells, including antigen-presenting cells such as dendritic cells (DCs) and B cells. Mice deficient in IL-6 exhibit impaired initial T_{FH} cell formation (12). During chronic viral infections, late-expressed IL-6 plays a pivotal role in T_{FH} cell maintenance and viral clearance (13). IL-21 is highly expressed in T_{FH} cells and seems to be functionally redundant with IL-6. The loss of either IL-6 or IL-21 alone had a marginal effect on T_{FH} formation during viral infections, but the combined absence of IL-6 and IL-21 led to severely impaired T_{FH} and GC B-cell formation (14).

In contrast, IL-2 is a potent negative regulator for T_{FH} differentiation. A recent study revealed that T cells with low levels of IL-2Rα (CD25) tend to differentiate into T_{FH} cells, suggesting that the IL-2 pathway inhibits T_{FH} differentiation and functions (15). Indeed, IL-2 administration impaired influenza-specific T_{FH} cells and GCs in influenza infections (16). In contrast, IL-2 deprivation or STAT5 deficiency led to enhanced T_{FH} cell formation (17, 18). IL-2 was originally defined as an essential T cell growth factor that promotes the expansion of effector T cells. However, IL-2 signaling does not affect T_{FH} cell survival and proliferation (17). IL-2 and the downstream STAT5 signaling pathway suppress T_{FH} cell differentiation largely by up-regulating Blimp-1 expression and repressing Bcl6 expression. Although the role of the IL-2–STAT5 pathway in T_{FH} differentiation is now clear, the mechanism by which this pathway is regulated during this process remains to be fully investigated.

Extracellular matrix protein 1 (ECM1) is a biologically important protein with roles in multiple tissues and malignant tumors (19). The role of ECM1 was first identified in skin diseases. Mutations in ECM1 have been detected in lipoid proteinosis and lichen sclerosis (20), and these diseases are associated with higher levels of autoantibody production. In addition, ECM1 enhanced angiogenesis and tumor metastasis during cancer development and was identified as a biomarker for cancer (21). In our previous study, we demonstrated that ECM1 regulates $CD4^+$ T cell-mediated immune responses by controlling T_H2 cell migration in an asthma animal model and T_H17 cell differentiation in experimental autoimmune encephalomyelitis (22, 23). However, the function of ECM1 in T_{FH} development remains unknown.

In this study, we found that ECM1 was expressed at high levels in T_{FH} cells. ECM1 deficiency impaired T_{FH} differentiation,

Significance

T_{FH} cell differentiation and antigen-specific antibody production is critical for humoral responses. In this work, we show that extracellular matrix protein 1 (ECM1) is a critical positive regulator in T-follicular helper (T_{FH}) differentiation by repressing IL-2–STAT5–Bcl6 signaling pathway. Importantly, ECM1 effectively enhanced T_{FH} differentiation, germinal center responses, and neutralizing antibody production both in antigen-immunized conditions and influenza infection, which indicate ECM1 may serve as a positive regulator for humoral responses in vivo.

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germinal center formation, and antigen-specific antibody production. In contrast, treatment with recombinant ECM1 protein in wild-type mice enhanced T_{FH} development and GC B-cell responses in vivo. ECM1 inhibited IL-2–STAT5 signaling pathway, down-regulated Blimp-1 expression, and enhanced Bcl6 expression in T_{FH} cells. Importantly, recombinant ECM1 protein administration enhanced T_{FH} differentiation and neutralizing antibody production, which might be helpful for inducing protective immune responses against PR8 influenza virus. Thus, our data demonstrate that ECM1 is a positive regulator of T_{FH} differentiation and antibody production.

Results

Lacking ECM1 Impairs T_{FH} Differentiation and Antibody Responses. First we examined the role of ECM1 in T_{FH} cell responses. To rule out the possibility of a defect in $CD4^+$ T cell development, we determined the percentage of $CD4^+CD44^{low}CD62L^{high}$ naive T cells and found that there was no difference between wide-type and *Ecm1*$^{-/-}$ mice (*SI Appendix*, Fig. S1*A*). *Ecm1*$^{-/-}$ mice and their wild-type counterparts were immunized using keyhole limpet hemocyanin (KLH) emulsified in complete Freund's adjuvant (CFA), and T_{FH} cell responses were then analyzed on day 7 postimmunization. *Ecm1*$^{-/-}$ mice exhibited normal $CD4^+$ T cell activation, proliferation, and apoptosis (*SI Appendix*, Fig. S1 *B–D*). However, *Ecm1*$^{-/-}$ mice had a significantly lower frequency and total number of T_{FH} cells ($CXCR5^+PD1^+$ or $CXCR5^+Bcl6^+$) than wild-type mice (Fig. 1 *A* and *B*). GC T_{FH} cells are a group of T_{FH} cells that express the highest levels of CXCR5, PD1, and Bcl6, as well as the germinal center marker GL7. These cells were identified as fully polarized T_{FH} cells. We then examined the responses of GC T_{FH} ($CXCR5^+GL7^+$) cells and found that GC T_{FH} differentiation was also substantially reduced in *Ecm1*$^{-/-}$ mice (Fig. 1 *A* and *B*, *Bottom*). We also measured the production of signature cytokines for other $CD4^+$ T cell subsets, such as IFN-γ, IL-4, or IL-17, and found no significant difference between *Ecm1*$^{-/-}$ mice and wild-type mice (*SI Appendix*, Fig. S2). Taken together, these results demonstrate that T_{FH} and GC T_{FH} differentiation were impaired in *Ecm1*$^{-/-}$ mice under immunized conditions.

As T_{FH} cells are the main cognate helpers of B-cell responses, we then examined GC B-cell development and antibody responses in *Ecm1*$^{-/-}$ mice. We observed that GC B-cell development was considerably decreased in *Ecm1*$^{-/-}$ mice compared with their wild-type counterparts (Fig. 1 *C* and *D*). In addition, B cells in *Ecm1*$^{-/-}$ mice exhibited a significantly compromised ability to produce KLH-specific IgG1, IgG2b, IgG2c, and IgG3 at day 7 (Fig. 1*E*) and day 12 (*SI Appendix*, Fig. S3). Additionally, a histologic analysis showed that *Ecm1*$^{-/-}$ mice had fewer and smaller GCs in the draining lymph nodes (Fig. 1*F*). Collectively, our observations suggest that ECM1 exerts a significant impact

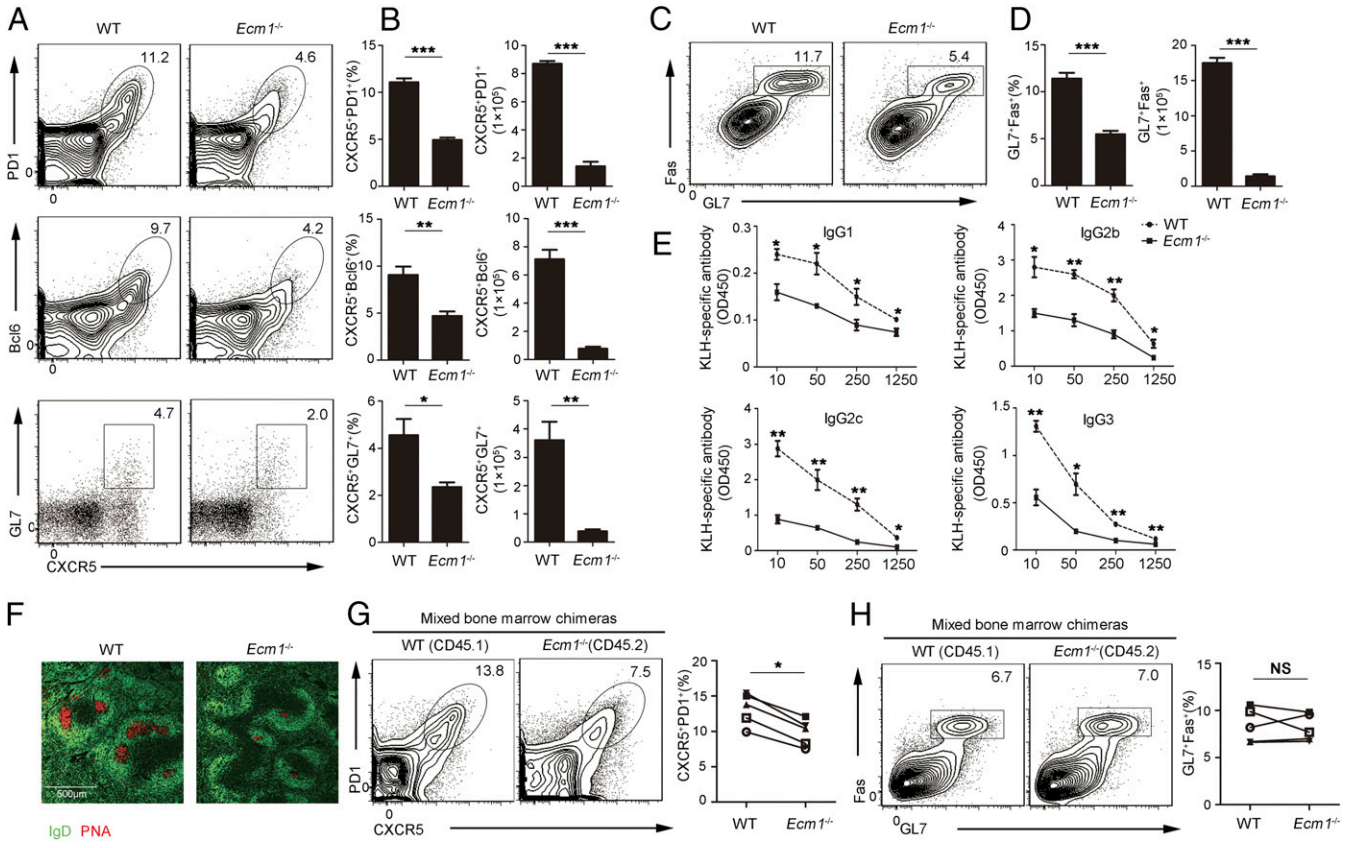

Fig. 1. Lacking ECM1 impairs T_{FH} differentiation and antibody responses. (*A–F*) Wild-type (WT) or *Ecm1*$^{-/-}$ mice were immunized with KLH emulsified in CFA. (*A*) Flow cytometry of $CD4^+$ T cells obtained from inguinal lymph nodes (iLNs) at day 7 postimmunization. Numbers adjacent to outlined areas indicate the percentage of $CXCR5^+PD1^+$ (*Top* row), or $CXCR5^+Bcl6^+$ T_{FH} cells (*Middle* row), or $CXCR5^+GL7^+$ GC T_{FH} cells (*Bottom* row) among all $CD4^+$ T cells ($n = 5$ per group). (*B*) The frequency (among $CD4^+$ T cells) and total number of T_{FH} or GC T_{FH} cells ($n = 5$ per group) are shown. (*C*) Flow cytometry of $GL7^+Fas^+$ cells among $B220^+$ B cells. (*D*) The frequency (among $B220^+$ cells) and total number of GC B cells are shown. (*E*) ELISA for KLH-specific IgG1, IgG2b, IgG2c, and IgG3 in sera. (*F*) Confocal microscopy of B-cell follicles (IgD^+) and GCs (PNA^+) of LNs from wild-type and *Ecm1*$^{-/-}$ mice. (Scale bars, 500 μm.) (*G* and *H*) Mixed bone marrow chimera mice were subjected to KLH plus CFA immunization ($n = 5$ per group). (*G*) Flow cytometry of CD45.1 (wild type) and CD45.2 (*Ecm1*$^{-/-}$) $CD4^+$ T cells. The percentage of $CXCR5^+PD1^+$ cells among total $CD4^+$ T cells is shown. (*H*) Flow cytometry of CD45.1 and CD45.2 $B220^+$ B cells. The percentage of $GL7^+Fas^+$ cells among total $B220^+$ B cells is shown. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, NS, not significant.

on T_{FH} and GC B-cell differentiation and antigen-specific antibody production.

To determine whether ECM1 promotes T_{FH} differentiation through an autocrine manner, we reconstituted lethally irradiated *Rag1*$^{-/-}$ mice using mixed CD45.2 *Ecm1*$^{-/-}$ and CD45.1 wild-type bone marrow cells at a ratio of 1:1 to generate chimeric mice. Eight weeks after reconstitution, both wild-type and *Ecm1*$^{-/-}$ T or B cells developed normally. The mice were immunized with KLH emulsified in CFA, and T_{FH} cell and GC B-cell responses were analyzed 7 d postimmunization. We found that there was no difference in GC B-cell development between wild-type and *Ecm1*$^{-/-}$ B cells in the mixed bone marrow chimeras (Fig. 1*H*). However, T cells derived from *Ecm1*$^{-/-}$ bone marrow still exhibited impaired T_{FH} differentiation compared with those derived from wild-type mice (Fig. 1*G*), suggesting autocrine ECM1 by T cells plays more important roles in the regulation of T_{FH} cell differentiation than paracrine or endocrine ECM1 under the physiological condition. Of note, *Ecm1*$^{-/-}$ CD4$^+$ T cells in mixed bone marrow (Fig. 1*G*) had a slightly less severe defect in T_{FH} cell differentiation than CD4$^+$ T cells in *Ecm1*$^{-/-}$ mice (Fig. 1*A*), which might be due to the effect of paracrine or endocrine ECM1 from wild-type cells. Collectively, our results suggest that ECM1 acts on CD4$^+$ T cells partially through an autocrine manner during T_{FH} cell development.

Exogenous ECM1 Treatment Promotes T_{FH} Differentiation in Vivo. Considering ECM1 is a secreted protein and can be expressed and purified in vitro (23), we tested whether treatment with exogenous ECM1 would manipulate T_{FH} development in vivo. A purified mouse recombinant ECM1–human Fc fusion protein or control IgG protein was expressed using the Bac-to-Bac baculovirus expression system and injected into wild-type mice together with KLH emulsified in CFA. The data showed that treatment with the exogenous recombinant ECM1 protein resulted in more T_{FH} and GC B-cell development than that observed in mice treated with the IgG protein (Fig. 2 *A–D*). Consistent with these results, administering ECM1 increased the production of antigen-specific high-affinity IgG1 and IgG2c antibodies (Fig. 2*E*). These data showed that treatment with exogenous ECM1 was sufficient to promote T_{FH} generation and GC responses in vivo.

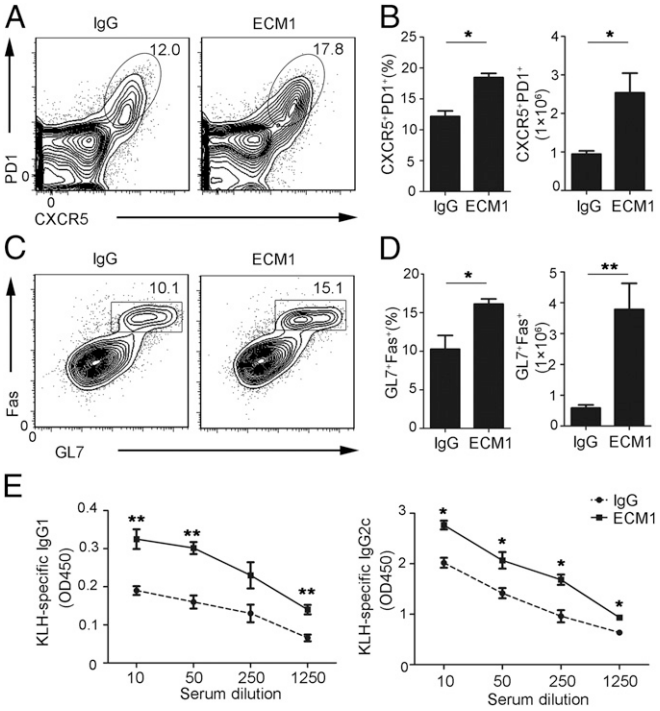

Fig. 2. Exogenous ECM1 treatment promotes T_{FH} differentiation in vivo. Wild-type C57BL/6 mice were immunized with KLH and injected with 200 μg IgG or recombinant ECM1 protein on day 0, 2, 4, or 6 after immunization and analyzed on day 7. (*A*) Flow cytometry of CXCR5$^+$PD1$^+$ cells among CD4$^+$ T cells (n = 5 per group). (*B*) The frequency (among CD4$^+$ T cells) and total number of T_{FH} cells. (*C*) Flow cytometry of GL7$^+$Fas$^+$ GC B cells among B220$^+$ B cells. (*D*) The frequency (among B220$^+$ cells) and total number of GC B cells. (*E*) ELISA for KLH-specific IgG1 and IgG2c in serum obtained from immunized mice. Data are presented as OD_{450} values. $^*P < 0.05$, $^{**}P < 0.01$.

The IL-6/IL-21/STAT3 Pathway Induces ECM1 Expression in T_{FH} Cells. Next we detected ECM1 expression of CD4$^+$ T cells in immunized mice in vivo. CD4$^+$CD44$^+$CXCR5$^-$PD1$^-$ (non-T_{FH}) and CD4$^+$CD44$^+$CXCR5$^+$PD1$^+$ (T_{FH}) cells from wild-type C57BL/6 mice that were immunized using KLH emulsified in CFA were sorted. Like T_{FH} signature genes, such as *Bcl6*, *Cxcr5*, and *Pdcd-1*, *Ecm1* mRNA expression levels were increased in T_{FH} cells compared with non-T_{FH} cells (Fig. 3*A*). Then we sought to figure out the mechanism by which ECM1 is regulated in T_{FH} cells. We cultured naive CD4$^+$ T cells and stimulated them with different cytokines and anti-CD3 and anti-CD28 antibodies in vitro and then analyzed ECM1 expression levels on day 4. The results showed that IL-6 and IL-21 strongly induced both ECM1 mRNA and protein expression (Fig. 3 *B* and *C*) and seemed to have synergistic roles (Fig. 3*D*). In contrast, ICOS signaling had no obvious effect on the expression of ECM1 (Fig. 3 *B* and *C*). Both IL-6 and IL-21 transduced downstream signaling primarily by activating the transcriptional factor STAT3, and we therefore wondered whether the up-regulation of ECM1 was STAT3 dependent. A bioinformatics analysis identified four STAT3-binding sites in the promoter and the first intron region of the *Ecm1* locus. A chromatin immunoprecipitation (ChIP) analysis of STAT3 in wild-type T_{FH}-like cells showed that STAT3 bound specifically to these regions and especially to the promoter region, suggesting that ECM1 is a direct target of STAT3 (Fig. 3*E*). To further address whether the induction of ECM1 by IL-6 and IL-21 is STAT3 dependent, we used niclosamide, a STAT3 inhibitor, in the culture of T_{FH}-like cells and found ECM1 expression was dramatically repressed (Fig. 3*F*). Moreover, ECM1 was hardly expressed in *Stat3*-deficient CD4$^+$ T cells (Fig. 3*H*). Taken together, our data suggest that ECM1 could be strongly induced by IL-6 and IL-21 in T_{FH} cells in a STAT3-dependent manner.

ECM1 Promotes T_{FH} Development by Antagonizing the IL-2–STAT5 Signaling Pathway. We next sought to determine the mechanism by which ECM1 enhances T_{FH} differentiation. In a previous report, we found that ECM1 binds to IL-2Rβ (CD122) and blocks the interaction between IL-2 and IL-2R, thereby negatively regulating the IL-2–STAT5 signaling pathway (22). Because the activation of STAT5 has been reported to inhibit T_{FH} development (17, 18), we hypothesized that ECM1 promotes T_{FH} differentiation via the disruption of the IL-2–IL-2R–STAT5 signaling pathway. In vitro differentiated T_{FH}-like cells were cultured with ECM1 recombinant protein, and STAT5 phosphorylation was detected on days 1 and 2. As expected, STAT5 phosphorylation was decreased upon the treatment of ECM1 recombinant protein (Fig. 4*A*). STAT5 activation has been reported to negatively regulate Bcl6 expression (17). Consistent with these results, we found that exogenous ECM1 enhanced Bcl6 expression in a dose-dependent manner (Fig. 4 *B* and *C*). We next differentiated naive CD4$^+$ T cells that were exposed to excess IL-2 with or without the recombinant ECM1 protein. The expression of the *Bcl6* mRNA was inhibited by IL-2, in agreement with a previous report (16). Interestingly, ECM1 significantly rescued the expression of Bcl6 in cells treated with IL-2 (Fig. 4*D*).

Next, we investigated the expression of typical T_{FH} genes in *Ecm1*$^{-/-}$ T_{FH}-like cells. In accordance with the results described

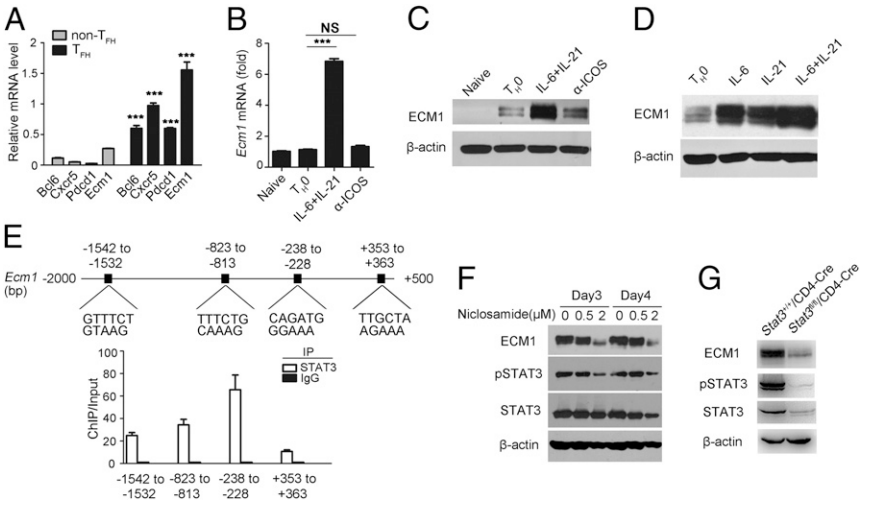

Fig. 3. The IL-6/IL-21/STAT3 pathway induces ECM1 expression in T_{FH} cells. (*A*) *Bcl6*, *Cxcr5*, *Pdcd1*, and *Ecm1* mRNA expression was measured. (*B* and *C*) Naive $CD4^+$ T cells were stimulated with IL-2 (T_H0), or IL-6 and IL-21, or anti-ICOS signaling. The mRNA expression levels (*B*) and protein expression levels (*C*) of ECM1 were detected. (*D*) Naive $CD4^+$ T cells were stimulated with IL-6 or IL-21, or IL-6 and IL-21. ECM1 protein level was detected. (*E*) Naive $CD4^+$ T cells were stimulated with IL-6, IL-21, anti–IL-4, anti–IFN-γ, and anti–TGF-β (T_{FH}-like cells). A ChIP analysis of T_{FH}-like cells was performed. Four predicted STAT3-binding sites are shown in black boxes. IgG, negative control. (*F*) Immunoblot analysis of ECM1 and pSTAT3 levels in T_{FH}-like cells cultured with or without niclosamide. (*G*) $CD4^+$ T cells from *Stat3*$^{fl/fl}$/CD4-Cre mice or littermates were cultured under IL-6 and IL-21 stimulation, and immunoblot analysis of ECM1 and pSTAT3 levels were detected on day 4. ***$P < 0.001$, NS, not significant.

above, we found that *Bcl6* expression was lower and *prdm1* expression was higher in *Ecm1*$^{-/-}$ cells than in wild-type cells (Fig. 5*E*). However, the expression levels of other T_{FH} cell-related genes, including *Cxcr5*, *Ccr7*, *Icos*, *Sh2d1a*, *Batf*, and *Maf*, were unaltered, suggesting that ECM1 mainly promoted T_{FH} differentiation by regulating Bcl6 and Blimp-1 (Fig. 4*E*). Previously, our data showed that ECM1 regulates T_H2 cell migration by regulating *Klf2* and *S1pr1* expression in T_H2 cells (22). However, we observed no significant difference of *Klf2* or *S1pr1* mRNA level in ECM1-deficient T_{FH}-like cells compared with that in wild-type T_{FH}-like cells (Fig. 5*E*). Next, we added exogenous ECM1 protein to T_{FH}-like cell culture media and found that exogenous ECM1 rescued *Bcl6* expression and suppressed *prdm1* expression, although to a lesser extent (Fig. 4*F*). Our data suggest that ECM1 regulates the T_{FH} differentiation signaling network via the IL-2–STAT5 pathway.

Inhibiting IL-2 Signaling in ECM1-Deficient Mice Rescues T_{FH} Cell Development in Vivo. We next tested the function of the ECM1–IL-2–STAT5–Bcl6 axis in vivo. We hypothesized that the deficiency in T_{FH} cell differentiation that was observed in *Ecm1*$^{-/-}$ mice was mainly caused by overwhelming IL-2 signaling and that a blockade against IL-2 or IL-2R would rescue or partially rescue this deficit. *Ecm1*$^{-/-}$ and wild-type mice were immunized with KLH and then intraperitoneally treated with PBS or anti–IL-2 (α–IL-2) plus anti-CD122 (α-CD122) antibodies. After 7 d, $CD4^+$ T cells and $B220^+$ B cells obtained from inguinal lymph nodes (iLNs) were analyzed. Indeed, the treatment with anti–IL-2 plus anti-CD122 antibodies substantially restored the deficiency in T_{FH} and GC B-cell development that was observed in *Ecm1*$^{-/-}$ mice (Fig. 5). Together, these results further demonstrate that ECM1 enhanced T_{FH} differentiation by antagonizing IL-2 signaling.

ECM1 Enhances T_{FH} Differentiation and Neutralizing Antibody Production During Influenza Infection. Influenza virus infection induces a robust protective immune response that is accompanied by effective T_{FH} differentiation and GC responses (16, 24). To increase our understanding of the role of ECM1 in pathological conditions, we infected C57BL/6 mice with the A/Puerto Rico/8/34 (PR8, H1N1) influenza virus and then injected control human IgG or recombinant ECM1 protein on day 1, 3, 5, or 7. We measured immune responses on day 10, and found that the treatment of recombinant ECM1 protein resulted in enhanced T_{FH} differentiation (Fig. 6 *A* and *B*). To determine whether ECM1 promotes the development of influenza-specific $CD4^+$ T cell responses, we used a fluorochrome-labeled $IA^bNP_{311–325}$ MHC class II tetramer to identify influenza

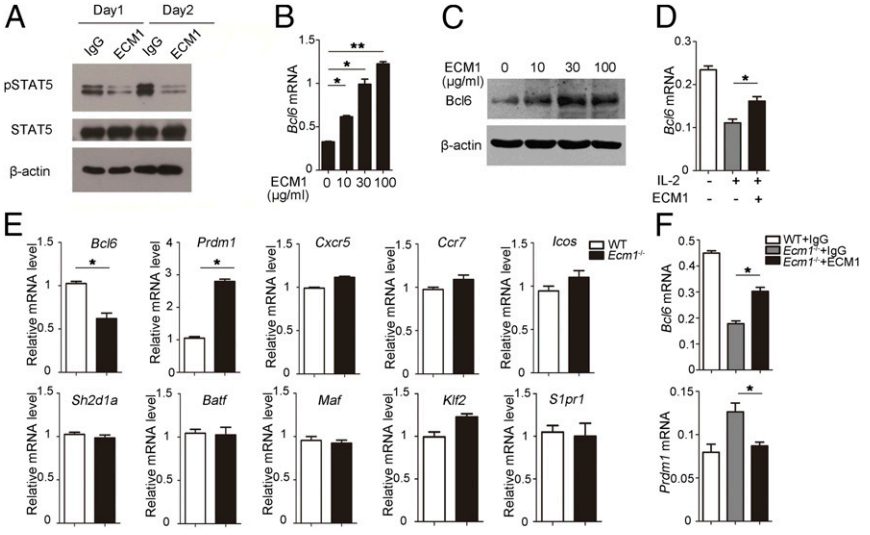

Fig. 4. ECM1 promotes T_{FH} development by antagonizing the IL-2–STAT5 signaling pathway. (*A*) Immunoblot analysis of pSTAT5 in T_{FH}-like cells cultured for 1 or 2 d with 100 μg/mL recombinant ECM1 or control IgG proteins. (*B* and *C*) T_{FH}-like cells were cultured with different doses of recombinant ECM1 protein, and *Bcl6* mRNA and protein expression levels were detected. (*D*) Naive $CD4^+$ T cells were cultured in excess IL-2 with or without recombinant ECM1 protein, and *Bcl6* mRNA expression levels were detected. (*E*) The expression of *Prdm1*, *Cxcr5*, *Ccr7*, *Icos*, *Sh2d1a*, *Batf*, *Maf*, *Klf2*, and *S1pr1* was detected in *Ecm1*$^{-/-}$ T_{FH}-like cells or wild-type cells. (*F*) *Bcl6* or *Prdm1* expression levels were assessed in wild-type or *Ecm1*$^{-/-}$ T_{FH}-like cells cultured in the presence or absence of 100 μg/mL recombinant ECM1 protein. *$P < 0.05$, **$P < 0.01$.

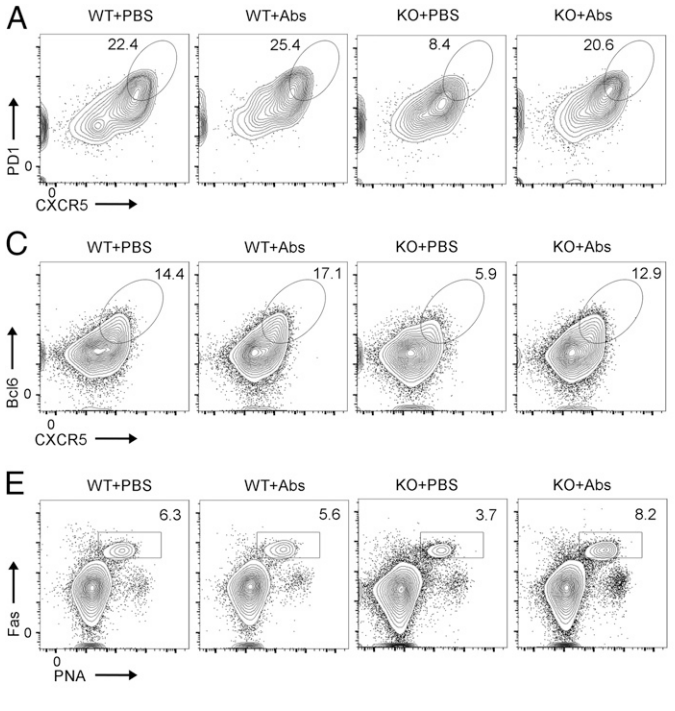

Fig. 5. Inhibiting IL-2 signaling in ECM1-deficient mice rescues T_{FH} cell development in vivo. *Ecm1*$^{-/-}$ or wild-type mice were immunized with KLH and intraperitoneally injected with PBS or anti–IL-2 (α-IL2) plus anti-CD122 (α-CD122). (*A*) Flow cytometry of CD4$^+$ T cells obtained from iLNs of immunized mice. Numbers adjacent to outlined areas indicate the percentage of CXCR5$^+$PD1$^+$ T_{FH} cells among total CD4$^+$ T cells (n = 3~5 per group). (*B*) The frequency (among CD4$^+$ T cells) of T_{FH} cells is shown as in *A*. (*C*) Flow cytometry of CD4$^+$ T cells in iLNs obtained from immunized mice. The numbers adjacent to outlined areas indicate the percent of CXCR5$^+$Bcl6$^+$ T_{FH} cells among total CD4$^+$ T cells. (*D*) The frequency (among CD4$^+$ T cells) of CXCR5$^+$Bcl6$^+$ T_{FH} cells is shown as in *C*. The data are representative of two independent experiments. (*E*) Flow cytometry of B220$^+$ T cells in iLNs obtained from immunized mice. Numbers adjacent to outlined areas indicate the percent of PNA$^+$Fas$^+$ GC B cells among total B220$^+$ B cells. (*F*) The frequency (among B220$^+$ B cells) of GC B cells is shown as in *E*. The data are representative of two independent experiments. Small horizontal lines indicate the mean ± SD. *P < 0.05, **P < 0.01 (two-tailed Student's t test).

nucleoprotein (NP)-specific T cells (Fig. 6*C*). There was no significant difference in the percentage of NP-specific CD4$^+$ T cells between mice treated with IgG or ECM1, indicating that ECM1 did not affect influenza-specific CD4$^+$ T cell expansion (Fig. 6*C*). However, ECM1 dramatically increased the frequencies and total numbers of NP-specific T_{FH} and GC T_{FH} cells (Fig. 6 *D*–*G*). Consistent with these results, we found that the development of GL7$^+$Fas$^+$ GC B cells and IgD$^-$CD138$^+$ plasma cells was also promoted by treatment with ECM1 (Fig. 6 *H*–*K*). Notably, treatment with ECM1 increased both influenza-specific IgG antibodies

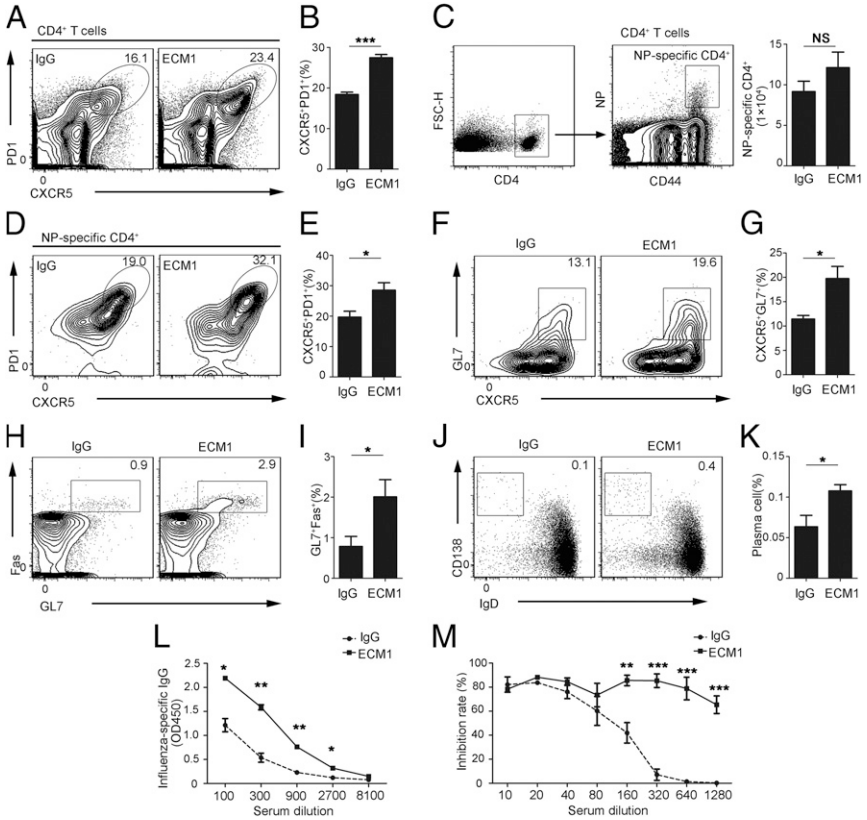

Fig. 6. ECM1 enhances T_{FH} differentiation and neutralizing antibody production during influenza infection. (*A*–*K*) Mediastinal lymph nodes were obtained from mice and analyzed on day 10 (n = 6 per group). (*A*) Flow cytometry of CXCR5$^+$PD1$^+$ cells among CD4$^+$ T cells. (*B*) The frequency (among CD4$^+$ T cells) of T_{FH} cells is shown as in *A*. (*C*) Activated (CD44$^+$) NP-specific CD4$^+$ T cells are shown, and the number of NP-specific CD4$^+$ T cells was calculated. (*D*) Flow cytometry of CXCR5$^+$PD1$^+$ cells among NP-specific CD4$^+$ T cells. (*E*) The frequency (among NP-specific CD4$^+$CD44$^+$ T cells) of NP-specific T_{FH} cells was assessed. (*F*) Flow cytometry of CXCR5$^+$GL7$^+$ cells among NP-specific CD4$^+$CD44$^+$ T cells. (*G*) The frequency (among NP-specific CD4$^+$CD44$^+$ T cells) of NP-specific GC T_{FH} cells is shown. (*H*) Flow cytometry of GL7$^+$Fas$^+$ cells among B220$^+$ B cells. (*I*) The frequency (among B220$^+$ cells) of GC B cells is shown. (*J*) Flow cytometry of IgD$^-$CD138$^+$ plasma cells among B220$^+$ B cells. (*K*) The frequency (among B220$^+$ cells) of plasma cells is shown. (*L*) ELISA of influenza-specific IgG in serum obtained from infected mice. (*M*) In vitro microneutralization against PR8 influenza virus in serum obtained from infected mice. Data are presented as the percentage inhibition. All data are representative of three independent experiments. Small horizontal lines indicate the mean ± SD. *P < 0.05, **P < 0.01, ***P < 0.001, NS, not significant (two-tailed Student's t test).

and neutralizing antibodies according to ELISA and microneutralizing experiments, respectively (Fig. 6 *L* and *M*). All of these results indicate that ECM1 enhanced the T_{FH} cell response and the production of neutralizing antibodies against influenza infection, both of which are critical to protective humoral immunity against influenza virus infection.

Discussion

In this study, we found that ECM1 is induced by IL-6 and IL-21 in $CD4^+$ T cells and performs a critical function during T_{FH} differentiation by antagonizing IL-2 signaling. Mice deficient in ECM1 have lower levels of Bcl6, which impairs T_{FH} cell development, GC B-cell reactions, and antigen-specific antibody production, whereas ECM1 administration increased T_{FH} differentiation and GC responses in vivo, both in antigen immunization and influenza virus infection conditions. Mechanically, ECM1 inhibited IL-2–STAT5 signaling, down-regulated Blimp1 expression, and promoted Bcl6 expression in T_{FH} cells. Our data demonstrate that ECM1 is a positive regulator of both T_{FH} differentiation and humoral immunity.

Our data reveal a mechanism by which different cytokines and soluble factors work together to regulate T_{FH} development. IL-6 and IL-21 induce ECM1 expression in T_{FH}, and are subsequently secreted into the extracellular space, where they act as a potent blocker of IL-2 signaling. Several groups have demonstrated that IL-2 strongly inhibits T_{FH} differentiation. Thus, IL-6 and IL-21 promote T_{FH} development by inducing ECM1 to inhibit the negative effect of IL-2. Therefore, ECM1, an extracellular soluble factor, participates in cytokine networks that regulate T_{FH} differentiation and thereby contributes to the formation of a microenvironment that is beneficial for T_{FH} differentiation. It would be interesting to determine whether other soluble factors, in addition to ECM1, play roles in regulating T_{FH} differentiation.

In a previous study, we found that ECM1 down-regulated KLF2, which blocked IL-2 signaling and thereby promoted the reexpression of the chemokine receptor S1PR1 (22). Recent studies have shown that KLF2 and S1PR1 are also important during T_{FH} development (25, 26). However, in our study, we found that ECM1 did not regulate the KLF2–S1PR1 pathway in T_{FH} cells. Alternatively, ECM1 down-regulated the expression of Blimp-1 and enhanced the T_{FH} master regulator Bcl6 expression and thereby promoted T_{FH} differentiation. Therefore, ECM1 blocked IL-2–STAT5 signaling to promote cell differentiation in T_{FH} cells, and to facilitate cell migration in T_H2 cells, however, the downstream signaling was mediated through Blimp-1–Bcl6 or KLF2–S1PR1, respectively. These results indicate that ECM1 regulates different $CD4^+$ subsets via diverse mechanisms and controls $CD4^+$ T cell differentiation and function in a sophisticated manner.

Materials and Methods

Detailed methods can be found in *SI Appendix*.

Mice. Mice were housed in specific pathogen-free animal facilities at the Animal Care Facility of the Chinese Academy of Sciences and used according to protocols approved by the Institutional Animal Care and Use Committee. C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center. *Ecm1*$^{-/-}$ mice were previously described (22). CD45.1 mice were obtained from Y. Zhang, Institut Pasteur of Shanghai, Chinese Academy of Sciences. *Rag1*$^{-/-}$ mice were provided by X. Liu, Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. *Ecm1*$^{-/-}$ mice and their littermate controls were used when they were 5 to 6 wk old. In all other cases, 6- to 8-wk-old mice were used.

Immunization and Infection. The mice were immunized with 100 μg KLH (Sigma) emulsified in 0.5 mg/mL CFA (Sigma) at the base of the tail (100 μL per mouse). For blockade of IL-2 signaling in vivo, each mouse was treated with 0.5 mg anti–IL-2 (JES6-1A12, BioXcell) and 0.5 mg anti-CD122 (5H4, BioXcell) or PBS by i.p. injection on days −1, 1, 3, and 5 (immunization on day 0). To induce infection with influenza, the mice were infected intranasally with mouse-adapted influenza virus strain A/Puerto Rico/8/34 (PR8, H1N1) at a dose of 450 $TCID_{50}$ (the half maximal tissue culture infectious dose) per 30 μL. Mice were injected with 200 μg IgG or recombinant ECM1 protein on day 1, 3, 5, or 7 after virus infection.

Statistics. Comparisons between two different groups were performed using unpaired two-tailed Student's *t* test. Values of $P < 0.05$ were considered statistically significant. Statistical analyses were performed using Prism 5 software (GraphPad).

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1. Crotty S (2011) Follicular helper CD4 T cells (TFH). *Annu Rev Immunol* 29:621–663.
2. Vinuesa CG, Linterman MA, Yu D, MacLennan IC (2016) Follicular helper T cells. *Annu Rev Immunol* 34:335–368.
3. Schaerli P, et al. (2000) CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J Exp Med* 192:1553–1562.
4. Kim CH, et al. (2001) Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. *J Exp Med* 193:1373–1381.
5. Breitfeld D, et al. (2000) Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J Exp Med* 192:1545–1552.
6. Keir ME, Butte MJ, Freeman GJ, Sharpe AH (2008) PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 26:677–704.
7. Good-Jacobson KL, et al. (2010) PD-1 regulates germinal center B cell survival and the formation and affinity of long-lived plasma cells. *Nat Immunol* 11:535–542.
8. Akiba H, et al. (2005) The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. *J Immunol* 175:2340–2348.
9. Johnston RJ, et al. (2009) Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation. *Science* 325:1006–1010.
10. Nurieva RI, et al. (2009) Bcl6 mediates the development of T follicular helper cells. *Science* 325:1001–1005.
11. Yu D, et al. (2009) The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment. *Immunity* 31:457–468.
12. Choi YS, Eto D, Yang JA, Lao C, Crotty S (2013) Cutting edge: STAT1 is required for IL-6-mediated Bcl6 induction for early follicular helper cell differentiation. *J Immunol* 190:3049–3053.
13. Harker JA, Lewis GM, Mack L, Zuniga EI (2011) Late interleukin-6 escalates T follicular helper cell responses and controls a chronic viral infection. *Science* 334:825–829.
14. Karnowski A, et al. (2012) B and T cells collaborate in antiviral responses via IL-6, IL-21, and transcriptional activator and coactivator, Oct2 and OBF-1. *J Exp Med* 209:2049–2064.
15. Choi YS, et al. (2011) ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. *Immunity* 34: 932–946.
16. Ballesteros-Tato A, et al. (2012) Interleukin-2 inhibits germinal center formation by limiting T follicular helper cell differentiation. *Immunity* 36:847–856.
17. Johnston RJ, Choi YS, Diamond JA, Yang JA, Crotty S (2012) STAT5 is a potent negative regulator of TFH cell differentiation. *J Exp Med* 209:243–250.
18. Nurieva RI, et al. (2012) STAT5 protein negatively regulates T follicular helper (Tfh) cell generation and function. *J Biol Chem* 287:11234–11239.
19. Hameed A, Nasir M, Ajmal M, Latif L (2009) Novel human pathological mutations. Gene symbol: ECM1. Disease: Lipoid proteinosis. *Hum Genet* 126:336.
20. Chan I (2004) The role of extracellular matrix protein 1 in human skin. *Clin Exp Dermatol* 29:52–56.
21. Lee KM, et al. (2015) ECM1 regulates tumor metastasis and CSC-like property through stabilization of β-catenin. *Oncogene* 34:6055–6065.
22. Li Z, et al. (2011) ECM1 controls T(H)2 cell egress from lymph nodes through reexpression of S1P(1). *Nat Immunol* 12:178–185.
23. Su P, et al. (2016) Novel function of extracellular matrix protein 1 in suppressing Th17 cell development in experimental autoimmune encephalomyelitis. *J Immunol* 197: 1054–1064.
24. León B, Bradley JE, Lund FE, Randall TD, Ballesteros-Tato A (2014) FoxP3+ regulatory T cells promote influenza-specific Tfh responses by controlling IL-2 availability. *Nat Commun* 5:3495.
25. Weber JP, et al. (2015) ICOS maintains the T follicular helper cell phenotype by down-regulating Krüppel-like factor 2. *J Exp Med* 212:217–233.
26. Lee JY, et al. (2015) The transcription factor KLF2 restrains $CD4^+$ T follicular helper cell differentiation. *Immunity* 42:252–264.