

Fibronectin is a T_H1-specific molecule in human subjects

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Background: T_H1 cell-mediated immunity is essential for host defense against a variety of intracellular pathogens, such as mycobacteria, salmonella, and *Leishmania* species. A major T_H1-mediated effector mechanism involves the IFN- γ -induced killing of the pathogen by infected macrophages.

Objectives: The range of known T_H1-specific effector molecules is limited, especially in human subjects. We sought to identify novel effector molecules that might be involved in T_H1-mediated pathogen clearance.

Methods: We performed microarray-based analysis of human T_H1 and T_H2 cells to identify T_H1-specific molecules. These analyses identified the extracellular matrix molecule fibronectin as a highly expressed T_H1-specific molecule. We examined the expression of fibronectin in a variety of human cell types by using real-time RT-PCR, ELISA, and Western blotting. We also studied the role of fibronectin in modulating monocyte phenotype using *in vitro* culture.

Results: We show that human T_H1 cells constitutively express and secrete fibronectin after *in vitro* differentiation from naive precursors. Furthermore, we demonstrate that *ex vivo* human T_H1 cells selectively express fibronectin when compared with T_H2 cells. The predominant isoform of fibronectin expressed by T_H1 cells contains additional domains of the protein responsible for $\alpha 4 \beta 1$ integrin binding and activation of Toll-like receptor 4. We show that treatment of monocytes with T_H1 cell-derived fibronectin induces expression of the proinflammatory cytokine IL-6 while inhibiting IL-10 expression.

Conclusions: Because fibronectin also plays a major role in the attachment and opsonization of numerous intracellular pathogens, we propose that it might be a critical molecule produced by T_H1 cells involved in pathogen eradication.

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Key words: T_H1 cell, fibronectin, opsonization, Toll-like receptor 4, integrin, monocyte

CD4⁺ T lymphocytes can be subdivided into at least 2 functionally distinct subsets, T_H1 and T_H2 cells, based on the cytokines they secrete on exposure to antigen.¹ T_H1 cells produce IFN- γ , TNF- α , and lymphotoxin and orchestrate phagocyte-dependent immune responses to intracellular pathogens.² T_H2 cells secrete IL-4, IL-5, and IL-13; induce IgE production; and promote eosinophil effector functions in response to extracellular helminth parasites. Both cell types have been implicated in disease when induced inappropriately. T_H1 cells often reside at the sites of chronic inflammatory diseases, such as rheumatoid arthritis and multiple sclerosis, whereas T_H2 cells have been implicated in the pathogenesis of allergic diseases and asthma. Recent studies have identified a third subset of CD4⁺ effector T cells, termed T_H17 cells, that secrete IL-17 and IL-22 and are involved in proinflammatory responses to extracellular bacterial and fungal pathogens.³

Several factors influence the development of effector T cells from naive precursors, including the strength of the stimulus, the nature of the accessory cells, and the cytokine milieu. The transcription factors that govern T_H1/T_H2 differentiation have been identified: T_H1 cell differentiation is induced by IL-12 from antigen-presenting cells and is controlled by the T-box transcription factor T-bet, and T_H2 differentiation is induced by IL-4 and controlled by GATA3.^{4,5} T_H1-mediated immune responses are critical for resistance to a variety of infectious pathogens. Genetic defects in the T_H1 pathway have demonstrated the key role played by this cell type in protective responses to pathogens. Mutations in the genes encoding IL-12p40, IL-12 receptor $\beta 1$, IFN- γ receptor 1, IFN- γ receptor 2, and signal transducer and activator of transcription 1 can all cause primary immunodeficiencies in human subjects, resulting in susceptibility to a variety of normally weakly pathogenic bacteria.⁶

In recent years, numerous studies have examined the differentiation of T_H1 and T_H2 cells from naive precursors, and several approaches have been used to identify effector molecules differentially expressed by these subsets.⁷⁻⁹ The majority of these studies were performed on murine T cells and have provided considerable insight into the mechanisms that control T_H1/T_H2 differentiation and function. Despite the considerable body of work investigating T_H1 cell-mediated disease, very few T_H1-specific effector molecules have been identified. We have performed a microarray-based analysis of human T_H1/T_H2 cells to identify molecules selectively expressed by these cell types. Here we identify the extracellular matrix molecule fibronectin as a gene (*FN1*) expressed specifically by T_H1 cells. *In vitro* differentiation analyses show that *FN1* is selectively induced during T_H1 differentiation and is constitutively expressed by highly polarized T_H1 cells. We demonstrate that *ex vivo* human T_H1 cells selectively express *FN1* when compared with *ex vivo* T_H2 cells.

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Abbreviations used

EDA: Extra domain A of fibronectin
 EDB: Extra domain B of fibronectin
 FITC: Fluorescein isothiocyanate
FNI: Fibronectin gene
 PE: Phycoerythrin
 PMA: Phorbol 12-myristate 13-acetate
 TLR: Toll-like receptor

T_H1 cells express an unusual isoform of fibronectin that is associated with leukocyte extravasation, bacterial opsonization, and activation of the Toll-like receptor (TLR) 4 pattern-recognition receptor. Furthermore, we show that T_H1 cell-derived fibronectin modulates the cytokine expression profile of monocytes, inducing a proinflammatory phenotype expressing increased levels of IL-6 and reduced levels of IL-10.

METHODS**Cell isolation, T_H1/T_H2 differentiation, and flow cytometry**

In vitro differentiation of human T_H1/T_H2 cells and intracellular staining was performed as previously reported¹⁰ and is described in detail in the Methods section in this article's Online Repository at www.jacionline.org. *Ex vivo* cell isolations were performed by means of magnetic selection or flow cytometry and are described in detail in the Methods section of this article's Online Repository.

RNA isolation, RT-PCR, and microarray analyses

RNA was isolated, and RT-PCR was performed as previously reported,¹¹ which is described in detail in the Methods section of this article's Online Repository. Microarray analyses were performed with Affymetrix U133 plus 2 microarrays (Affymetrix, Santa Clara, Calif), according to the manufacturer's instructions. Details are described in the Methods section in this article's Online Repository.

Fibronectin ELISA and Western blotting

Fibronectin ELISA on tissue culture supernatants was performed by using the QuantiMatrix Human Fibronectin ELISA kit (Chemicon International, Temecula, Calif) or the Human Fibronectin Immunoassay Kit (Biomedical Technologies, Inc, Stoughton, Mass), according to the manufacturer's instructions. Because bovine serum contains plasma fibronectin that could cross-react with the immunoassay, culture media containing 10% FBS was also examined to determine the background level of fibronectin present.

Cytoplasmic protein fractions from T_H1/T_H2 cells differentiated for 28 days were isolated as previously described by using the NE-PER protein isolation kit (Pierce, Cheshire, United Kingdom), according to the manufacturer's instructions.¹¹ Cytoplasmic fractions and tissue culture supernatants were loaded onto NuPAGE 4-12% gels (Invitrogen, Carlsbad, Calif), according to the manufacturer's instructions. Western blotting and enhanced chemiluminescent detection were performed as previously described.¹¹ Primary antibodies used were rabbit polyclonal anti-fibronectin (H-300; Santa Cruz Biotechnology, Santa Cruz, Calif), mouse monoclonal anti-GAPDH (6C5; Abcam, Cambridge, United Kingdom). Secondary detection antibodies were horseradish peroxidase-conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology) and goat anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology).

Fibronectin purification

T_H1 cell supernatants were loaded onto a gelatin-sepharose column (GE Healthcare, Fairfield, Conn), and flow through was collected. After washing the column with PBS, fibronectin was eluted with 1 mol/L arginine. Sequential elutions were collected, combined, and concentrated by using a

Vivaspin microconcentrator with a molecular weight cutoff of 50 kd (Vivascience, Hannover, Germany). Excess arginine was removed by means of sequential washes in PBS. The purity of the fibronectin fractions was assessed by using SDS-PAGE, Coomassie staining, and Western blotting.

Monocyte isolation and culture

Peripheral blood monocytes were isolated from PBMCs by using the MyPure monocyte isolation kit (Invitrogen). Purities were routinely 95%, as assessed with FACS analysis. Monocytes were cultured overnight in RPMI 1640 supplemented with 10% FBS, glutamine, penicillin, and streptomycin, as described above. Where indicated, recombinant human IFN- γ (R&D Systems, Minneapolis, Minn) was added at a final concentration of 50 ng/mL, and purified T_H1 cell-derived fibronectin was added at a final concentration of 100 ng/mL.

RESULTS**Microarray analysis reveals T_H1 -specific fibronectin transcription**

We have previously reported a method to generate highly polarized human T_H1 and T_H2 cell populations from naive $CD4^+$ T cells after 4 weeks in culture.¹⁰ Using these culture conditions, we produced cells with the characteristic expression patterns of T_H1 and T_H2 cytokines on activation. Intracellular cytokine staining of the *in vitro* differentiated T_H1 and T_H2 cells demonstrated that neither population expressed the T_H1 cytokine IFN- γ or the T_H2 cytokines IL-4, IL-5, and IL-13 before stimulation (see Fig E1 in this article's Online Repository at www.jacionline.org). On acute activation with phorbol 12-myristate 13-acetate (PMA) and ionomycin, at least 99% of the T_H1 cells expressed IFN- γ , with negligible IFN- γ expression by the T_H2 cells (see Fig E1, upper panel). Approximately 80% of the T_H2 cells expressed IL-13, and multicolor analysis demonstrated that there was considerable coexpression of IL-4 and IL-5 (see Fig E1, lower panels). The T_H1 cells did not express T_H2 cytokines. These cells were used to perform comparative microarray expression analysis for all known human genes by using Affymetrix U133 plus 2 microarrays. In these analyses fibronectin was identified as being highly selectively expressed by T_H1 cells (see Table E1 in this article's Online Repository at www.jacionline.org). The mean difference between transcription levels of *FNI* in T_H1 and T_H2 cells is 1,106-fold. Analysis of the microarray data showed that other extracellular matrix molecules, such as collagen, fibrillin, laminin, and elastin, were either not differentially expressed or not expressed at all (see Table E1). This indicates that of the extracellular matrix molecules, only fibronectin is selectively transcribed by T_H1 cells. Further analysis of the microarray data showed that the gene encoding AICAR transformylase/IMP cyclohydrolase, which is immediately adjacent to fibronectin on human chromosome 2, is also not differentially expressed in T_H1 versus T_H2 cells. This suggests that the T_H1 -specific transcription of fibronectin is not caused by the adventitious location of the *FNI* gene in a T_H1 -specific chromosomal domain.

 T_H1 -specific expression of fibronectin by *in vitro* differentiated human T_H cells

To further investigate the T_H1 -specific transcription of the *FNI* gene, we analyzed highly differentiated T_H1 and T_H2 cells by means of quantitative real-time RT-PCR (Fig 1, A). As expected, highly T_H1 -specific and activation-responsive transcription of IFN- γ was observed in cells cultured for 28 days. Furthermore, T_H2 -specific transcription of IL-4, IL-5, and IL-13 was found

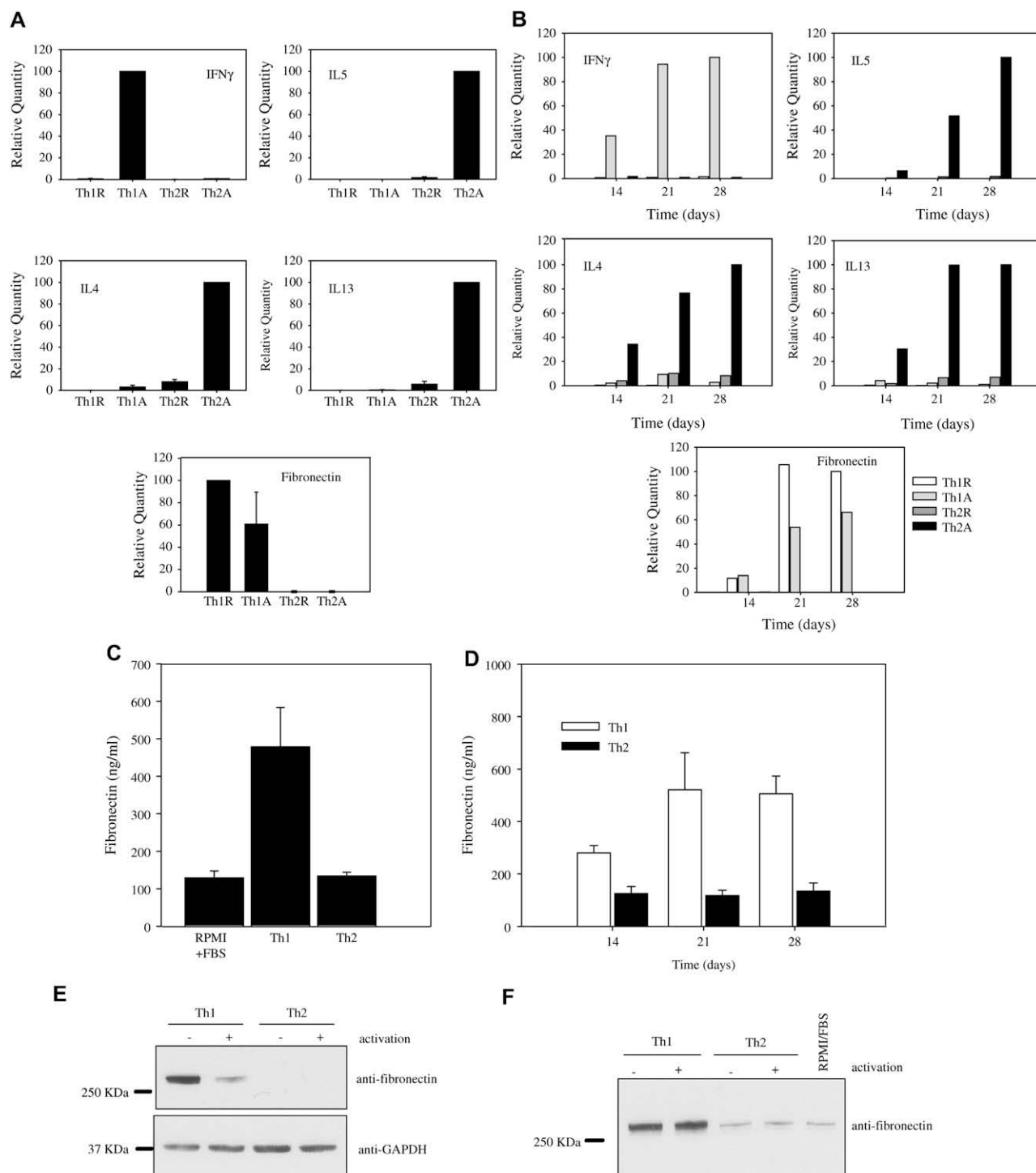


FIG 1. T_H1-specific expression of fibronectin. **A**, *FN1* mRNA is selectively expressed by T_H1 cells. *R*, Resting; *A*, activated. Data are shown as means $\pm$ SEMs ($n = 4$). **B**, *FN1* mRNA is induced during T_H1 differentiation. Data are representative of 2 experiments. **C** and **D**, Fibronectin is secreted by T_H1 cells. Fibronectin ELISA of supernatants is shown (**Fig 1, C**, means $\pm$ SEMs, $n = 4$; **Fig 1, D**, data are representative of 2 experiments). **E** and **F**, Western blotting of cytoplasmic extracts and cell-culture supernatants. $-$, Resting; $+$, activated.

on T_H2 cell activation (**Fig 1, A**). Analysis of these samples demonstrated T_H1-specific transcription of the *FN1* mRNA (**Fig 1, A**). Interestingly, the *FN1* mRNA appears to be constitutively transcribed by T_H1 cells before activation, which is in contrast to the cytokine genes. Unstimulated T_H1 cells express 1,259-fold more *FN1* mRNA than unstimulated T_H2 cells. On activation,

the *FN1* mRNA levels appeared to decrease slightly in T_H1 cells, with no notable expression in the activated T_H2 cells. These data confirm the initial microarray analyses and indicate that human T_H1 cells constitutively transcribe the *FN1* gene.

We then examined the transcription of the *FN1* gene during the process of *in vitro* differentiation by means of real-time RT-PCR

on samples from days 14, 21, and 28 of the 4-week culture period (Fig 1, B). We observed constitutive expression of the *FN1* mRNA at each time point of T_H1 differentiation, with a steady increase in the *FN1* mRNA levels as differentiation proceeded. The profile of *FN1* mRNA expression during T_H1 differentiation parallels the *IFNG* mRNA levels, with the exception that *FN1* is constitutively transcribed. Once again, there was negligible *FN1* mRNA detected at any time point during T_H2 differentiation, although mRNA for the T_H2 cytokine genes steadily increased during the culture period (Fig 1, B). The increase in *FN1* levels during T_H1 differentiation suggests that the ability to transcribe *FN1* is a T_H cell characteristic acquired during T_H1 differentiation rather than a characteristic lost during T_H2 differentiation.

To investigate whether fibronectin protein is expressed by the T_H1 cells, we performed ELISA assays for soluble fibronectin in tissue culture supernatants from the highly polarized T_H1 and T_H2 cultures (Fig 1, C). Soluble fibronectin was readily detectable by means of ELISA in the T_H1 supernatants from cells differentiated for 28 days (478 ng/mL). No detectable fibronectin was identified in the T_H2 cell supernatants above the amounts present in the culture media (which contains FBS). Analysis of cell supernatants taken during the *in vitro* differentiation clearly show that the protein levels mirror the mRNA levels, with a steady increase during T_H1 differentiation and no detectable fibronectin in the T_H2 cultures (Fig 1, D).

Fibronectin is a complex dimeric glycoprotein containing 2 subunits of approximately 250 kd each.^{12,13} Alternative splicing produces several different molecules containing variable numbers of type III fibronectin repeats and also truncated fibronectin molecules. To examine the size of the T_H1 cell-specific fibronectin protein, we performed Western blots of cell extracts and tissue culture supernatants (Fig 1, E and F). Western blots of cytoplasmic extracts from T_H1 and T_H2 cells readily detected a single T_H1 -specific band of greater than 250 kd (Fig 1, E). Interestingly, the amount of fibronectin in the T_H1 cytoplasmic extract decreased on activation. There was no detectable fibronectin in the T_H2 cytoplasmic extracts. Examination of the cell-culture supernatants by means of Western blotting also revealed a T_H1 -specific band of the same size as the cytoplasmic protein (Fig 1, F). A faint band is detectable in the T_H2 supernatants, but this represents cross-reactivity with the bovine fibronectin in the culture medium from the FBS. These data confirm that the T_H1 cells constitutively express full-length fibronectin and suggest that fibronectin is secreted by the T_H1 cells on activation.

***FN1* is selectively expressed by *ex vivo* human T_H1 cells**

To determine whether the T_H1 -specific expression of *FN1* that we observe *in vitro* is observed in cells directly isolated from human subjects, we have isolated both T_H1 and T_H2 populations from peripheral blood. *Ex vivo* T_H1 cells were isolated by means of FACS sorting of $CD3^+CD4^+IFN-\gamma^+$ T cells, and *ex vivo* T_H2 cells were isolated by means of FACS sorting of $CD3^+CD4^+IL-13^+$ T cells (Fig 2, A). The relative levels of expression by these cells of *IFNG*, *IL13*, and *FN1* were assessed by means of real-time RT-PCR (Fig 2, B). The data clearly show that *FN1* is highly selectively expressed by the $CD4^+IFN-\gamma^+$ population in a manner similar to *IFNG*, whereas *IL13* is selectively expressed by the $CD4^+IL-13^+$ cell population. Similar results were obtained by using *ex vivo* cells from atopic donors (data not shown). These

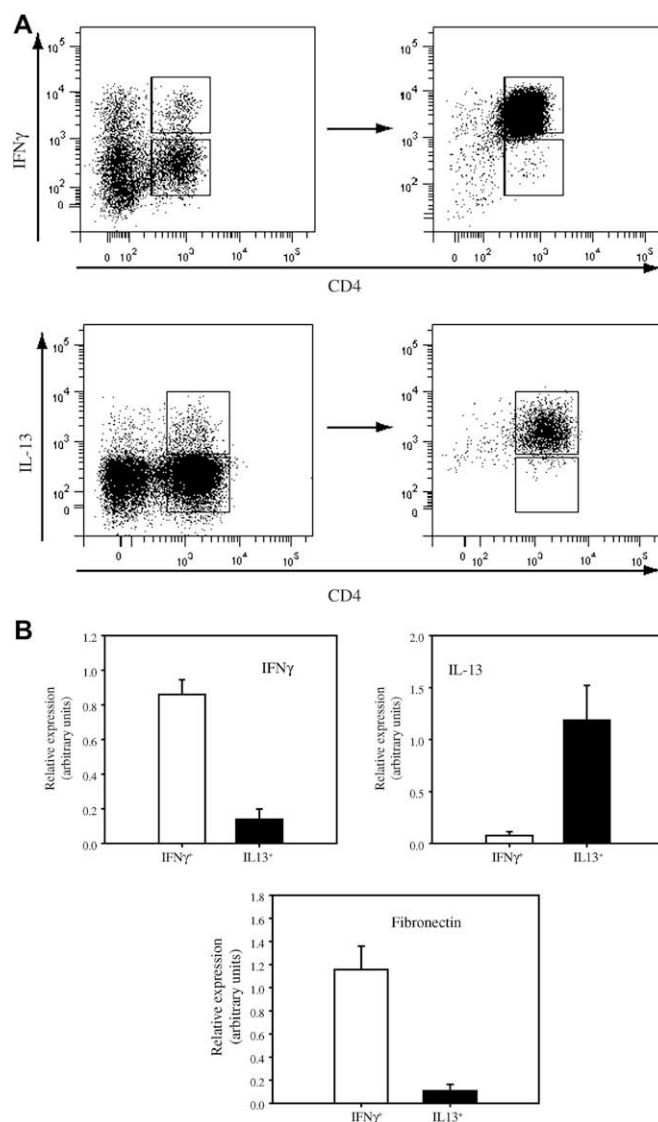


FIG 2. *Ex vivo* T_H1 cells selectively express fibronectin. *Ex vivo* T_H1 and T_H2 cells were isolated based on the expression of CD3, CD4, and IFN- γ or IL-13. **A**, FACS plots show presort and postsort profiles. **B**, RT-PCR on sorted samples (means $\pm$ SEMs, $n = 3$). Gene expression is shown relative to 1 biologic replicate (T_H1 for *IFNG* and *FN1* and T_H2 for *IL13*).

results show that *ex vivo* T_H1 cells selectively express the *FN1* gene and that the T_H1 -specific expression observed after *in vitro* differentiation reflects expression of this gene by *ex vivo* T_H1 cells.

Several alternatively spliced fibronectin isoforms are expressed by T_H1 cells

The *FN1* gene contains at least 45 exons and is subject to several alternative splicing events. These splicing events introduce additional type III fibronectin repeats into the protein. There are 3 sites at which alternative splicing occurs termed extra domain A of fibronectin (EDA), extra domain B of fibronectin (EDB), and the variable region (see Fig E2, A, in this article's Online Repository at www.jacionline.org). The EDA and EDB regions are absent from most adult tissue but are expressed during embryonic development, tissue injury, inflammation, and angiogenesis.¹³

The variable region is differentially expressed in a variety of tissues. We have examined the T_H1 -specific *FN1* transcripts using RT-PCR and sequencing to ascertain which splicing events occur in T_H1 cells. RT-PCR with primers spanning the EDB region identified a single fragment of 296 bp, the size expected for transcripts with the EDB exon absent (see Fig E2, B and C). Cloning and sequencing of the PCR product confirmed that the T_H1 -specific *FN1* transcript does not contain the EDB exon (data not shown).

RT-PCR with primers spanning the EDA and variable regions identified 3 major products of different intensities between 1,000 and 1,700 bp (see Fig E2, B and D). Cloning and sequencing of these PCR products identified a range of different splicing events. The EDA region is absent from some cloned transcripts but is present in 84% of clones identified. Inclusion of the EDA region of fibronectin introduces a binding site for integrins $\alpha9\beta1$ and $\alpha4\beta1$.¹⁴ Germline deletion of the EDA exon in the mouse causes defects in wound healing and also defects in accumulation of extravasated macrophages during acute inflammation.^{15,16} Recent work has shown that the EDA region of *FN1* also acts as an endogenous ligand for the pattern-recognition receptor TLR4 and causes nuclear factor κB activation and secretion of IL-12 and TNF- α by dendritic cells and macrophages.^{17,18}

Sequencing of the variable region reveals a complex pattern of exon use (see Fig E2, E and F). The variable region of fibronectin contains at least 2 recognition sites for the $\alpha4\beta1$ integrin: the LDV and REDV motifs.^{19,20} These motifs are present in the variable region sequences that we have identified (see Fig E2, E and F). The $\alpha4\beta1$ integrin (also known as very late antigen 4) is expressed on lymphoid and myeloid cells, and $\alpha4\beta1$ interactions with fibronectin are important for leukocyte extravasation during acute inflammation.¹²

To further examine the T_H1 -specific expression of the EDA-containing fibronectin isoform in the context of immune cell function, we have screened a large panel of innate and adaptive immune cell types for expression of both total fibronectin and EDA-containing mRNA by means of quantitative real-time RT-PCR (Fig 3). Fig 3, A, clearly shows that of all the cell types examined, only T_H1 cells express high levels of *FN1* mRNA. When specifically examining the expression of the EDA exon, a small amount of expression was detected in $CD4^+CD25^+$ T cells, probably representing the activated T cells in the periphery. A small amount of EDA-specific fibronectin was also detected in natural killer T cells, but the predominant cell type expressing EDA-specific fibronectin was the T_H1 cell (Fig 3, B).

T_H1 cell-derived fibronectin modulates cytokine expression by monocytes

To investigate the potential function of T_H1 cell-derived fibronectin, we purified fibronectin from T_H1 cell-culture supernatants by means of affinity chromatography, taking advantage of the specific binding of fibronectin to gelatin-sepharose.²¹ T_H1 cell supernatants were mixed with a gelatin-sepharose slurry, and the bound fibronectin was washed extensively before elution with arginine (Fig 4, A). We examined the effect of fibronectin on peripheral blood monocytes, which express both TLR4 and $\alpha4\beta1$ integrin, two of the receptors known to bind to fibronectin, including the EDA region. Monocytes were isolated from PBMCs by means of negative magnetic selection and cultured overnight either in culture media or with IFN- γ . After overnight culture, the monocytes were treated with T_H1 cell-derived fibronectin for 4

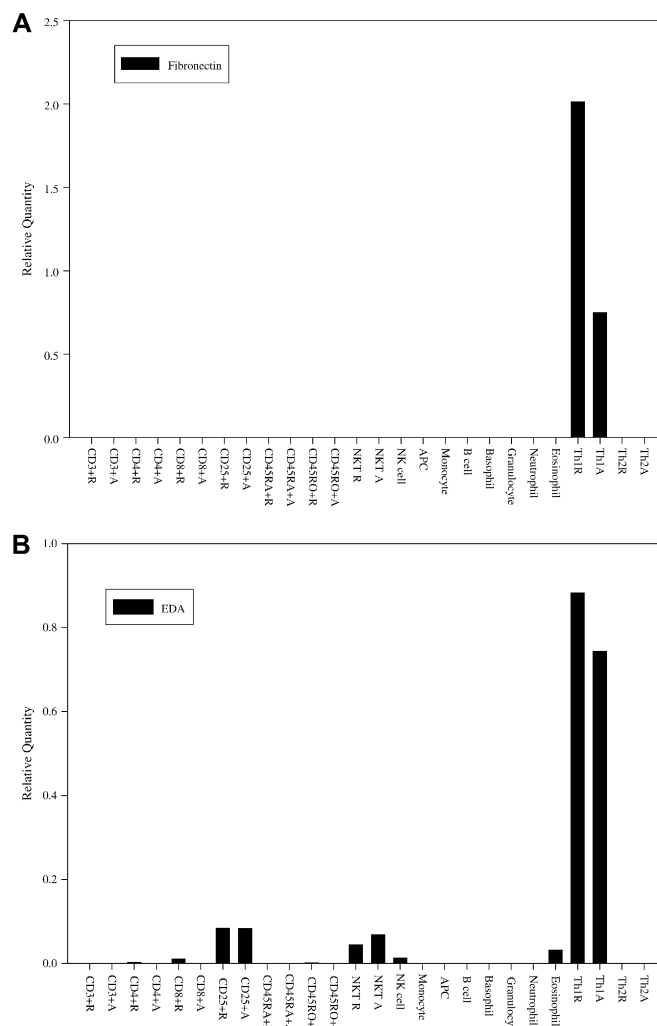


FIG 3. Expression of fibronectin by immune cell types. The results of real-time RT-PCR for total *FN1* (A) and EDA-*FN1* (B) mRNA in a panel of immune cell types are shown. Cell types were isolated by sorting from PBMCs or PMNs, as described in the Methods section, and *in vitro* generated T_H1 and T_H2 cells are also shown. R, Resting cells; A, activated cells (PMA/ionomycin).

hours before harvesting for RNA isolation and quantitative real-time RT-PCR to examine cytokine gene expression. In combination with IFN- γ , fibronectin induced a significant and synergistic upregulation of *IL6* mRNA that was not observed with either fibronectin or IFN- γ alone (Fig 4, B). In contrast, fibronectin treatment alone was sufficient to downregulate *IL10* mRNA expression (Fig 4, C). A similar trend was observed in the presence of IFN- γ , although it did not reach statistical significance (Fig 4, C). These results demonstrate that T_H1 cell-derived fibronectin can modulate monocyte cytokine transcription, promoting the expression of the proinflammatory cytokine IL-6 while downregulating expression of the regulatory cytokine IL-10.

DISCUSSION

Our findings clearly demonstrate that human T_H1 cells generated *in vitro* constitutively express fibronectin at high levels. IFN- γ -secreting $CD4^+$ T_H1 cells isolated directly *ex vivo* also selectively express fibronectin compared with *ex vivo* $IL-13^+$ $CD4^+$

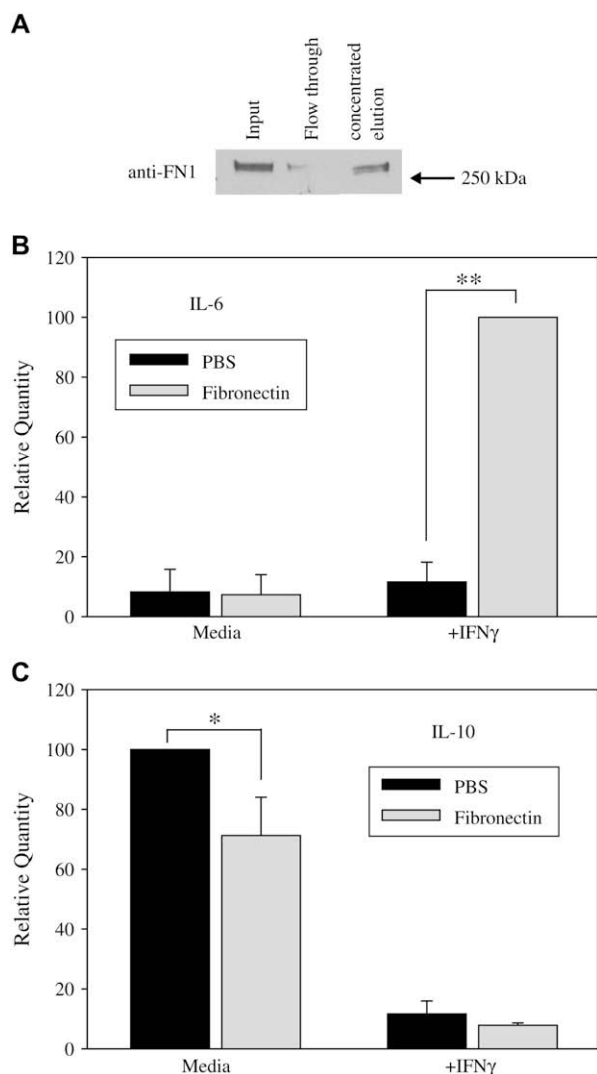


FIG 4. T_H1 cell-derived fibronectin modulates monocyte gene expression. **A**, Monocytes were cultured overnight with or without IFN- γ as indicated. After overnight incubation, fibronectin (shaded bars) or PBS (solid bars) was added for 4 hours. Quantitative real-time RT-PCR was performed for IL-6 (**B**) and IL-10 (**C**). * $P < .05$ and ** $P < .01$, 2-way ANOVA with the Tukey post test.

T_H2 cells. We have also shown that fibronectin expressed by T_H1 cells can promote the expression of the proinflammatory cytokine IL-6 by monocytes while downregulating IL-10 expression. We propose that fibronectin is a new T_H1 -specific effector molecule based on the multiple and complex roles played by fibronectin in immune cell recruitment and function (Fig 5). The interaction between pathogens and fibronectin has long been recognized, and a considerable body of work has shown that many pathogens express specific fibronectin-binding proteins, including mycobacteria, staphylococci, and *Leishmania* species.²²⁻²⁴ Several of these molecules are virulence factors for pathogens and have been proposed to play an important role in bacterial adherence and invasion at the site of injury.^{25,26} By the same mechanism, fibronectin also plays an important role in the opsonization and engulfment of these pathogens by macrophages.²⁷ Therefore expression of fibronectin by T_H1 cells could promote clearance of pathogenic microbes by macrophages, which are also activated to kill phagocytosed bacteria by T_H1 cell-derived IFN- γ .

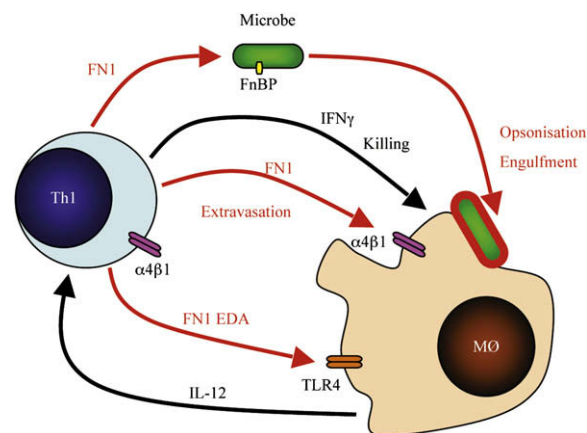


FIG 5. Model for the role of fibronectin in T_H1 cell functions. *FcBP*, Fibronectin-binding protein; $\alpha4\beta1$, integrin $\alpha4\beta1$; *MO*, macrophage.

Fibronectin also plays an important role in leucocyte extravasation during an inflammatory response through binding to integrin $\alpha4\beta1$ on the surface of both lymphoid and myeloid cells.¹² Therefore T_H1 -specific expression of fibronectin might also recruit other cell types, including macrophages, and also more T_H1 cells (Fig 5). It is interesting that T_H2 cells do not bind to fibronectin, further emphasizing the T_H1 -specific nature of fibronectin.²⁸ It is also important to note that the T_H1 -specific isoforms of fibronectin containing EDA harbor several additional binding sites for integrin $\alpha4\beta1$ and $\alpha9\beta1$ and thus might promote extravasation more effectively.¹⁴ The EDA-containing isoforms of fibronectin are not normally expressed in adult tissue but have been identified in response to tissue injury, wound healing,^{29,30} fibrosis,³¹ and allograft transplantation.³² EDA-fibronectin has also been observed in the synovial fluid in patients with rheumatoid arthritis,^{33,34} multiple sclerosis,^{35,36} and psoriasis,³⁷ all diseases with a significant T_H1 -mediated pathology. In these disease settings the source of EDA-fibronectin has not been entirely clear, with some evidence for macrophage or fibroblast involvement. Our work suggests that one source of EDA-fibronectin is likely to be the T_H1 cell. Recent work with mutant mouse strains in which the EDA exon was deleted has demonstrated that this region plays a role in wound healing and also in macrophage accumulation during inflammation.^{15,16} It would be interesting to examine the effect of deletion of the EDA region in T_H1 -mediated infectious disease murine models and autoimmune pathologies, such as experimental autoimmune encephalomyelitis.

Recent work by two groups has shown that the EDA region of fibronectin expressed by T_H1 cells is an endogenous ligand for TLR4.^{17,18} Ligation of TLR4 classically induces nuclear factor κB signaling and promotes T_H1 immunity through stimulation of antigen-presenting cells to produce proinflammatory cytokines, such as IL-6 and IL-12.³⁸ We have shown here that the T_H1 -derived fibronectin containing the EDA region can induce *IL6* gene expression from monocytes and also suppress *IL10* gene expression. The role of EDA-fibronectin as an endogenous ligand for TLR4 and for integrin binding is particularly interesting in the light of recent studies showing that the TLR4 signaling pathway is dependant on integrin signaling to localize the adaptor molecule TIR domain-containing adaptor protein (TIRAP) to the plasma membrane in macrophages.³⁹ It is therefore possible that EDA-fibronectin can perform both roles in macrophage activation. Elucidation of the relative roles of integrin and TLR4

signaling by T_H1-derived fibronectin in monocyte/macrophage activation will require further studies beyond the scope of this report. Experiments to dissect the role of the different domains and their effect on TLR4 and integrins will be technically challenging because fibronectin is a very large molecule containing numerous domains with overlapping functions, making the use of function-blocking antibodies against each domain extremely difficult to interpret.

We have shown that human T_H1 cells selectively express fibronectin at high levels both *in vitro* and *ex vivo* when compared with T_H2 cells and other immune cell types. It will be interesting to examine proinflammatory T_H17 cells for fibronectin expression once the methods for efficient generation of human T_H17 cells have been characterized. The T_H1-specific expression of fibronectin at first seems to contrast with data supporting a role for fibronectin in T_H2-mediated disease pathologies, such as asthma; however, it is likely that other cell types contribute to the fibronectin deposition seen in T_H2 disease. In fact, although it is clear that there is increased extracellular matrix deposition in the sub-epithelial reticular basement membrane in patients with asthma, there is little evidence of increased total or EDA-containing fibronectin in patients with seasonal or chronic asthma.⁴⁰ Collectively, our data suggest that fibronectin can function as a T_H1-specific effector molecule. Germline deletion of fibronectin is lethal in mice, which has hampered study of the function of this molecule *in vivo*. It would be intriguing to examine the role of EDA-containing fibronectin in T_H1 disease models in which the EDA exon has been deleted.

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Key messages

- Human T_H1 cells selectively express high levels of fibronectin.
- T_H1 cell-derived fibronectin induces a proinflammatory phenotype in monocytes.
- Fibronectin might play an important role in pathogen clearance by macrophages.

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METHODS

T_H1/T_H2 cell differentiation

Venous blood was taken from nonatopic healthy human volunteers by using heparin as an anticoagulant. Ethical approval for the use of human volunteers in this study was obtained from the institutional ethical review committee. PBMCs were isolated with Lymphoprep (Nycomed, Zurich, Switzerland), according to the manufacturer's instructions. $CD4^+$ T cells were isolated from PBMCs by using a CD4 Positive Isolation Kit (Invitrogen), according to the manufacturer's instructions. Naive $CD45RA^+$ cells were purified from $CD4^+$ cells by means of depletion of $CD45RO^+$ cells with mouse anti-human $CD45RO$ antibody (UCHL1; BD Biosciences, San Jose, Calif; $0.5 \mu\text{g}/1 \times 10^6$ cells) and rat anti-mouse IgG Dynabeads (Invitrogen), according to the manufacturer's instructions. The purity of naive cell populations was assessed by means of FACS analysis, as previously described,^{E1} and was at least 99% $CD4^+CR45RA^+$.

Purified $CD45RA^+$ cells (1×10^6 cells/mL) were cultured in RPMI 1640 supplemented with 10% FCS, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 $\mu\text{g}/\text{mL}$ streptomycin (all from Invitrogen). Cells were stimulated with plate-bound anti-CD3 (1 $\mu\text{g}/\text{mL}$, clone OKT3) and anti-CD28 (2 $\mu\text{g}/\text{mL}$, clone 15E8; Sanquin, Amsterdam, The Netherlands), rIL-2 (50 U/mL; Eurocet, Amsterdam, The Netherlands), and anti-IL-10 (5 $\mu\text{g}/\text{mL}$, clone JES3-9D7; BD Biosciences), rIL-12 (2.5 ng/mL; R&D Systems) and anti-IL-4 (5 $\mu\text{g}/\text{mL}$, clone MP4-25D2; BD Biosciences) were added to direct T_H1 differentiation. For T_H2 differentiation, rIL-4 (12.5 ng/mL; R&D Systems) and anti-IFN- γ (5 $\mu\text{g}/\text{mL}$, clone B-B1; Biosource International, Camarillo, Calif) were added. After 4 days, the cells were expanded under the same conditions in the absence of anti-CD3 or anti-CD28. Cells were then restimulated every 7 days. When required, cells were activated with PMA (5 ng/mL; Sigma, St Louis, Mo) and ionomycin (500 ng/mL; Calbiochem, Nottingham, United Kingdom) for 4 hours.

Intracellular cytokine staining

Resting cells were activated with PMA (5 ng/mL) and ionomycin (500 ng/mL) for 4 hours, and monensin (2 $\mu\text{mol}/\text{L}$; Sigma) was added for the final 2 hours of activation. Cells were harvested in FACS tubes and placed on ice, 7-amino-actinomycin D (4 $\mu\text{g}/\text{mL}$; Sigma) was added, and cells were incubated for 10 minutes on ice. Cells were then washed with FACSFlow (BD Biosciences) and processed for intracellular cytokine staining with a Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions. Antibodies used were as follows (BD Biosciences unless stated otherwise): phycoerythrin (PE)-labeled anti-IL-4 (Fastimmune), allophycocyanin-labeled anti-IL-5 (TRFK5), PE-labeled anti-IL-13 (JES10-5A2), and fluorescein isothiocyanate (FITC)-labeled anti-IFN- γ (B27). Samples were analyzed on a FACSCalibur (BD Biosciences). Live cells were analyzed for cytokine expression based on forward and side scatter and exclusion of 7-amino-actinomycin D. At least 10,000 live cells were analyzed for each sample. Quadrant markers were set based on background staining of matched control antibodies (also from BD Biosciences) and on resting unactivated cells treated in parallel.

Ex vivo cell isolation

As a marker of *ex vivo* T_H1 cells, $CD3^+CD4^+IFN-\gamma^+$ cells were isolated from PBMCs by using the IFN- γ -FITC secretion assay detection kit (Miltenyi Biotec, Bergisch Gladbach, Germany) in combination with anti-CD3-PE (BD Biosciences) and anti-CD4-allophycocyanin (Miltenyi Biotec), according to the manufacturer's instructions. *Ex vivo* $CD3^+CD4^+IL-13^+T_H2$ cells were isolated from PBMCs by using the IL-13-PE secretion assay detection kit (Miltenyi Biotec), anti-CD3-FITC (BD Biosciences), and anti-CD4-

allophycocyanin (BD Biosciences). PBMCs were activated for 3 hours with PMA and ionomycin before cell isolation, according to the manufacturer's instructions, to detect cytokine-positive cells. Other *ex vivo* cell populations were isolated from blood by means of magnetic or FACS sorting with lineage-specific markers: $CD3^+$ ($CD3^+$ cells); $CD4^+$ ($CD3^+CD4^+$ cells); $CD8^+$ ($CD3^+CD8^+$ cells); $CD25^+$ ($CD4^+CD25^+$ cells); $CD45RA^+$ ($CD4^+CD45RA^+$ cells), $CD45RO^+$ ($CD4^+CD45RO^+$); natural killer T ($CD3^+CD56^+$ cells); natural killer ($CD3^-CD56^+$ cells); monocyte ($CD14^+$ cells); antigen-presenting cell ($CD3^-CD19^-CD14^-CD56^-HLA-DR^+$); B cell ($CD19^+$ cells); basophil ($CD203c^+$); granulocyte (Histopaque PMN pellet with RBC lysis); neutrophil ($CCR3^-$ granulocytes); and eosinophil ($CCR3^+$ granulocytes). Cells were FACS sorted on a FACS Aria (BD Biosciences), and purity after sorting was greater than 85% in all experiments. Granulocyte purities and morphologies were assessed by means of May-Grunwald-Giemsa staining.

RNA isolation and RT-PCR

Isolation of total cellular RNA was performed with the RNeasy mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions, and reverse transcription was performed as previously described.^{E2} Quantitative real-time RT-PCR was performed with Taqman MGB Gene expression assays (Applied Biosystems, Foster City, Calif). Probe sets used were as follows: *IL4*, Hs00929862_m1; *IL5*, Hs00174200_m1; *IL6*, Hs00174131_m1; *IL10*, Hs00174086_m1; *IL13*, Hs00174379_m1; *IFNG*, Hs00174143_m1; *FN1*, Hs00277509_m1; *FN1* EDA region, Hs01549959_m1; and 18s rRNA, Hs99999901_s1. Conventional RT-PCR was performed as previously described by using Amplitaq Gold Taq polymerase (Applied Biosystems). Primers for amplifying the alternatively spliced regions of *FN1* were designed with MacVector and ordered from MWG Biotech. Primer sequences were as follows: EDB region, TACCACAACCCCTACAAACGGC and TTTTCA CAGGTGAGTAACGCACC; EDA and variable region, TCCCAGATG CAAGTGACCGATG and GGTAACAACCTCTTCCCGAACC. PCR products were cloned into pCR 2.1 TOPO (Invitrogen), according to the manufacturer's instructions, and 40 individual clones of the *FN1* regions were sequenced by Geneservice. Sequences have been deposited in GenBank with the following accession numbers: EF550130, EF550131, EF550132, EF550133, EF550134, and EF550135.

Microarray analysis

cRNA samples were prepared for microarray hybridization to GeneChip U133 plus 2 arrays, according to the manufacturer's instructions (Affymetrix). Fragmented cRNA was hybridized to GeneChip arrays at 45°C for 18 hours. Arrays were washed and stained with streptavidin-PE, according to the manufacturer's instructions, on the GeneChip fluidics station 450 (Affymetrix). Fluorescent signals were detected with the GeneChip scanner 3000. Images were analyzed with the GeneChip operating software (Affymetrix) to generate raw data as .cel files. Further analysis was performed with GeneChip operating software to identify differentially expressed genes. Data shown in Table E1 are representative of 3 independent biologic replicates for each condition.

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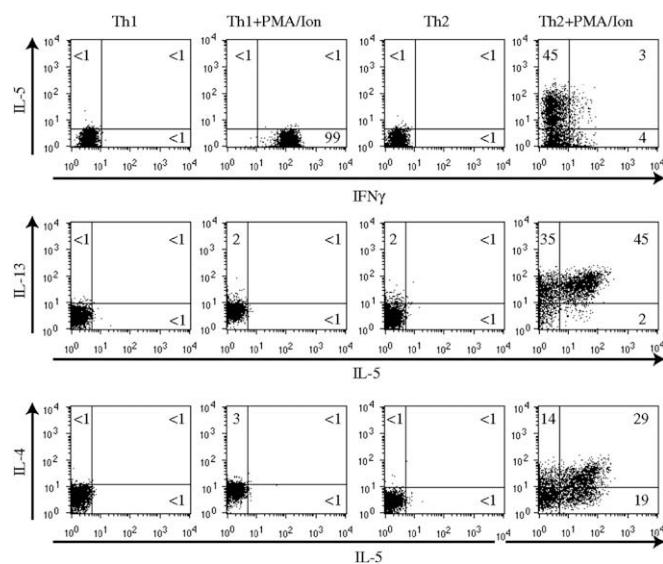


FIG E1. *In vitro* differentiation of human T_H1 and T_H2 cells. Naive CD4⁺ T cells were isolated from PBMCs and cultured for 28 days under T_H1- or T_H2-polarizing conditions. Where indicated, cells were activated for 4 hours with PMA (5 ng/mL) and ionomycin (Ion; 500 ng/mL), and intracellular cytokine staining was performed. Data are representative of 6 independent experiments from different healthy donors.

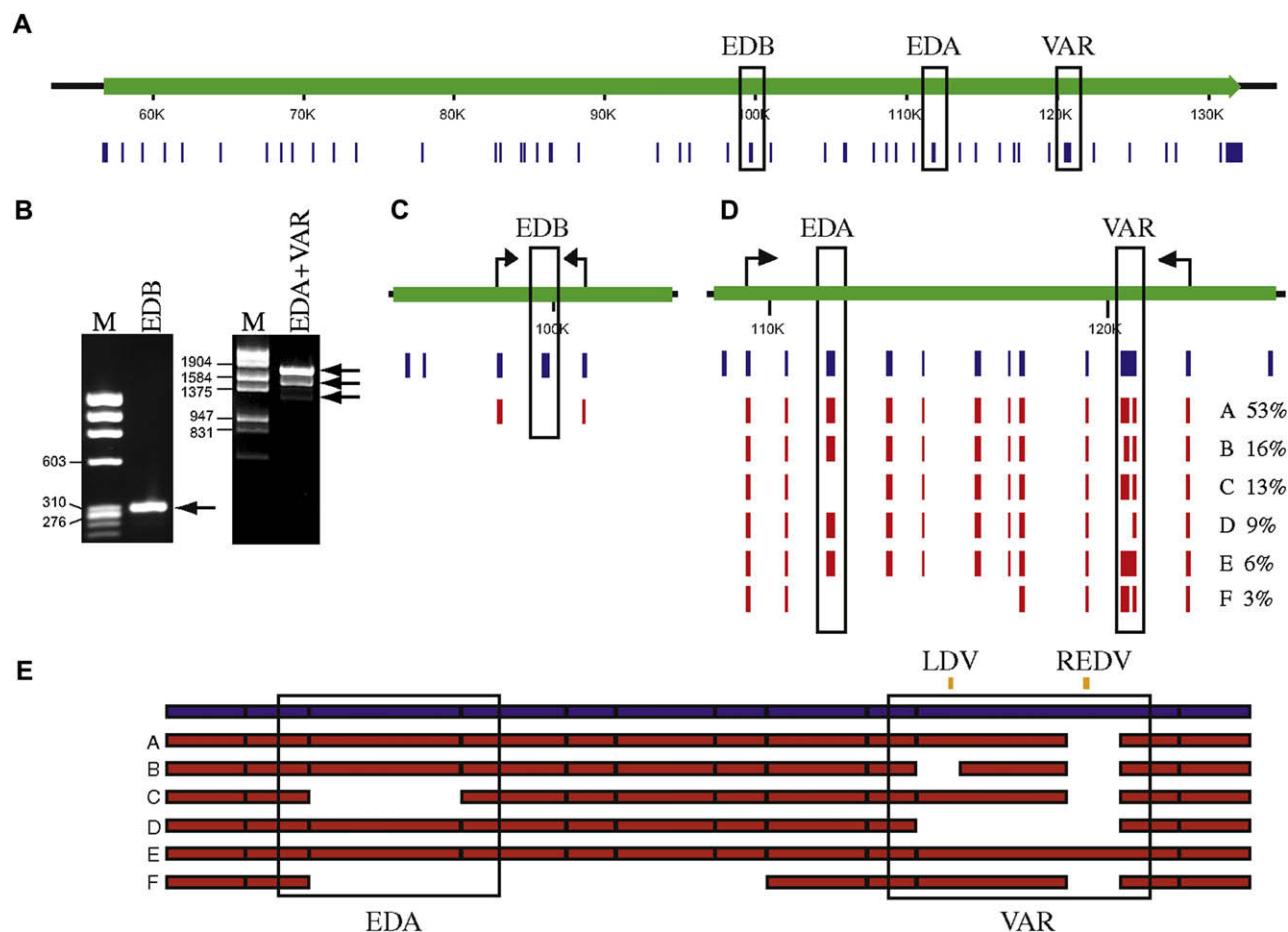


FIG E2. Variable splicing of T_H1 cell fibronectin. **A**, Human fibronectin gene. *Blue boxes* represent exons. *Black boxes* indicate regions of variable splicing. *VAR*, Variable region. **B**, RT-PCR for EDB, EDA, and variable regions using primers indicated in Fig E2, **C** and **D**. **C** and **D**, Variably spliced regions. *Red boxes* represent exons identified by sequencing. Frequency of exon splice variants (Fig E2, **A-F**) is shown. **E**, Coding sequence of T_H1 cell fibronectin isoforms around the EDA and variable regions (*black boxes*). **F**, Alignments of the variable regions and LDV and REDV motifs are highlighted.

[illegible]

FIG E2. (Continued)

TABLE E1. Affymetrix GeneChip analysis of human T_H1/T_H2 cells

Gene name	Affymetrix probe set	T _H 1 resting	T _H 2 resting	Fold difference	T _H 1 activated	T _H 2 activated	Fold difference
<i>FN1</i>	211719_x_at	2,349.1	1.5	1,566	1,627.3	1.1	1,479
<i>FN1</i>	212464_s_at	2,052	1.7	1,207	1,184	1.7	696
<i>FN1</i>	216442_x_at	2,186.1	2.6	840.8	1,442.7	1.7	848.6
<i>COL13A1</i>	208535_x_at	7	22.5	−3.21	1.5	1.8	−1.2
<i>COL6A3</i>	201438_at	15.9	1	15.9	92.2	12.2	7.5
<i>FBN1</i>	202766_s_at	17.6	24.9	−1.41	9.6	1.8	5.3
<i>FBN2</i>	203184_at	1.7	3.7	−2.17	8.1	13.1	−1.61
<i>LAMA1</i>	227048_at	9	0.4	22.5	8.7	5	1.74
<i>LAMC1</i>	200771_at	13.3	6.3	2.1	62.7	6.9	9.09
<i>ELN</i>	212670_at	3.4	2.2	1.55	3.2	1.9	1.68
<i>ATIC</i>	208758_at	669.5	521.7	1.28	1,242.3	1,184	1.05
<i>IL4</i>	207539_s_at	15.4	352.2	−22.8	177.8	5,197.8	−29.2
<i>IL5</i>	207952_at	3.3	1,219.5	−369.5	3.5	11,774.4	−3,364
<i>IL13</i>	207844_at	58.1	1,504.4	−25.9	409.3	10,536.3	−25.7
<i>IFNG</i>	210354_at	5,905.7	70.9	83.3	12,481.4	7,906.1	1.58