1 TITLE PAGE

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3 Original Article

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5 # Fibronectin is a Th1-specific molecule in humans.

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34 ## Abstract

35 Background: Th1 cell mediated immunity is essential for host defense against a variety of
36 intracellular pathogens such as mycobacteria, salmonella and leishmania. A major Th1
37 mediated effector mechanism involves the IFNγ-induced killing of the pathogen by infected
38 macrophages.

39 Objectives: The range of known Th1-specific effector molecules is limited, especially in
40 humans. We sought to identify novel effector molecules that may be involved in Th1-
41 mediated pathogen clearance.

42 Methods: We performed microarray-based analysis of human Th1 and Th2 cells to identify
43 Th1-specific molecules. These analyses identified the extracellular matrix molecule
44 fibronectin as a highly expressed Th1-specific molecule. We examined the expression of
45 fibronectin in a variety of human cell types by real-time RT-PCR, ELISA and Western
46 blotting. We also studied the role of fibronectin in modulating monocyte phenotype by *in*
47 *vitro* culture.

48 Results: We show that human Th1 cells constitutively express and secrete fibronectin after *in*
49 *vitro* differentiation from naïve precursors. Furthermore, we demonstrate that *ex vivo* human
50 Th1 cells selectively express fibronectin when compared to Th2 cells. The predominant
51 isoform of fibronectin expressed by Th1 cells contains additional domains of the protein
52 responsible for α4β1 integrin binding and activation of Toll-like receptor 4. We show that
53 treatment of monocytes with Th1 cell-derived fibronectin induces expression of the pro-
54 inflammatory cytokine IL-6, whilst inhibiting IL-10 expression.

55 Conclusions: Since fibronectin also plays a major role in the attachment and opsonisation of
56 numerous intracellular pathogens we propose that it may be a critical molecule produced by
57 Th1 cells involved in pathogen eradication.

58

59 ## Key Messages

60 - Human Th1 cells selectively express high levels of fibronectin
61 - Th1-cell derived fibronectin induces a pro-inflammatory phenotype in monocytes
62 - Fibronectin may play a important role in pathogen clearance by macrophages

63

64 ## Capsule Summary

65 Th1 cell mediated immunity is essential for host defense but also contributes to the
66 pathogenesis of autoimmune disease. We show here that fibronectin is a pro-inflammatory
67 Th1-specific molecule and may represent a novel therapeutic target.

68

69 Key words

70 Th1 cell, fibronectin, opsonisation, TLR4, integrin, monocyte.

71

72 ## Abbreviations

73 APC: antigen presenting cell

74 EDA: Extra domain A of fibronectin

75 EDB: Extra domain B of fibronectin

76 FN1: fibronectin

77 IFNγ: Interferon-gamma

78 IL-: Interleukin

79 PBMCs: Peripheral blood mononuclear cells

80 PMA: Phorbol myristate acetate

81 STAT: signal transducer and activator of transcription

82 Th: T-helper

83 TLR: Toll-like receptor

84

85 # INTRODUCTION

86 CD4$^+$ T-lymphocytes can be subdivided into at least two functionally distinct subsets, Th1
87 and Th2, based upon the cytokines they secrete upon exposure to antigen[1]. Th1 cells produce
88 IFNγ, TNFα and lymphotoxin and orchestrate phagocyte dependent immune responses to
89 intracellular pathogens[2]. Th2 cells secrete IL-4, IL-5 and IL-13, induce IgE production, and
90 promote eosinophil effector functions in response to extracellular helminth parasites. Both
91 cell types have been implicated in disease when induced inappropriately. Th1 cells often
92 reside at the sites of chronic inflammatory diseases such as rheumatoid arthritis and multiple
93 sclerosis whilst Th2 cells have been implicated in the pathogenesis of allergic diseases and
94 asthma. Recent studies have identified a third subset of CD4$^+$ effector T-cells, termed Th17,
95 that secrete IL-17 and IL-22 and are involved in pro-inflammatory responses to extracellular
96 bacterial and fungal pathogens[3].

97 Several factors influence the development of effector T-cells from naïve precursors
98 including the strength of stimulus, the nature of the accessory cells and the cytokine milieu.
99 The transcription factors that govern Th1/Th2 differentiation have been identified: Th1 cell
100 differentiation is induced by IL-12 from APCs and is controlled by the transcription factor T-
101 bet; whereas Th2 differentiation is induced by IL-4 and controlled by GATA3[4, 5]. Th1-
102 mediated immune responses are critical for the resistance to a variety of infectious pathogens.
103 Genetic defects in the Th1 pathway have demonstrated the key role played by this cell type in
104 protective responses to pathogens. Mutations in the genes encoding IL-12p40, IL-12Rβ1,
105 IFNγR1, IFNγR2 and STAT1 can all cause primary immunodeficiencies in humans resulting
106 in susceptibility to a variety of normally weakly pathogenic bacteria[6].

107 In recent years numerous studies have examined the differentiation of Th1 and Th2
108 cells from naïve precursors and several approaches have been used to identify effector
109 molecules differentially expressed by these subsets[7-9]. The majority of these studies were

110 performed on murine T-cells and have provided considerable insight into the mechanisms
111 that control Th1/Th2 differentiation and function. Despite the considerable body of work
112 investigating Th1 cell mediated disease, very few Th1-specific effector molecules have been
113 identified. We have performed a microarray-based analysis of human Th1/Th2 cells to
114 identify molecules selectively expressed by these cell types. Here, we identify the
115 extracellular matrix molecule fibronectin (FN1) as a gene expressed specifically by Th1 cells.
116 *In vitro* differentiation analyses show that FN1 is selectively induced during Th1
117 differentiation and is constitutively expressed by highly polarized Th1 cells. We demonstrate
118 that *ex vivo* human Th1 cells selectively express FN1 when compared to *ex vivo* Th2 cells.
119 Th1 cells express an unusual isoform of fibronectin which is associated with leukocyte
120 extravasation, bacterial opsonisation and activation of the TLR4 pattern recognition receptor.
121 Furthermore, we show that Th1 cell derived fibronectin modulates the cytokine expression
122 profile of monocytes, inducing a pro-inflammatory phenotype expressing increased levels of
123 IL-6 and reduced levels of IL-10.

124 ## METHODS

125 ### Cell isolation, Th1/Th2 differentiation and flow cytometry

126 *In vitro* differentiation of human Th1/Th2 cells and intracellular staining was performed as
127 previously reported[10] and is described in detail in the Online Repository. *Ex vivo* cell
128 isolations were performed by magnetic selection or flow cytometry and are described in
129 detail in the Online Repository.

130

131 ### RNA isolation, RT-PCR and microarray analyses

132 RNA was isolated and RT-PCR performed as previously reported[11] and is described in detail
133 in the Online Repository. Microarray analyses were performed using Affymetrix U133 plus 2
134 microarrays according to the manufacturers instructions. Details are described in the Online
135 Repository.

136

137 ### Fibronectin ELISA and Western Blotting

138 Fibronectin ELISA on tissue culture supernatants were performed using the QuantiMatrix™
139 Human Fibronectin ELISA kit (Chemicon) or the Human Fibronectin Immunoassay Kit
140 (Biomedical Technologies Inc.) according to the manufacturer's instructions. Since bovine
141 serum contains plasma fibronectin that could cross react with the immunoassay, culture
142 media containing 10% FBS was also examined to determine the background level of
143 fibronectin present.

144 Cytoplasmic protein fractions from Th1/Th2 cells differentiated for 28 days were
145 isolated as previously described using the NE-PER protein isolation kit (Pierce) according to
146 the manufacturer's instructions[11]. Cytoplasmic fractions and tissue culture supernatants were
147 loaded onto NuPAGE 4-12% gels (Invitrogen) according to the manufacturer's instructions.
148 Western blotting and ECL detection were performed as previously described[11]. Primary

149 antibodies used were: rabbit polyclonal anti-Fibronectin (H-300; Santa Cruz Biotechnology),
150 mouse monoclonal anti-GAPDH (6C5; Abcam). Secondary detection antibodies were HRP-
151 conjugated goat anti-mouse IgG (sc-2005; Santa Cruz Biotechnology) and goat anti-rabbit
152 IgG (sc-2004; Santa Cruz Biotechnology).
153

154 Fibronectin purification

155 Th1 cell supernatants were loaded onto a gelatin-sepharose column (GE Healthcare) and flow
156 through was collected. After washing the column with PBS, fibronectin was eluted with 1M
157 arginine. Sequential elutions were collected, combined and concentrated using a Vivaspin
158 microconcentrator with molecular weight cut off of 50 kDa (Vivascience). Excess arginine
159 was removed by sequential washes in PBS. Purity of the fibronectin fractions was assessed
160 by SDS-PAGE, Coomassie staining and Western blotting.
161

162 Monocyte isolation and culture

163 Peripheral blood monocytes were isolated from PBMCs using the MyPure monocyte
164 isolation kit (Invitrogen). Purities were routinely 95% as assessed by FACs. Monocytes were
165 cultured overnight in RPMI 1640 supplemented with 10% FBS, glutamine, penicillin and
166 streptomycin as described above. Where indicated, recombinant human IFNγ (R & D
167 Systems) was added at a final concentration of 50ng/ml and purified Th1 cell derived
168 fibronectin was added at a final concentration of 100 ng/ml.

169 # RESULTS

170 ## Microarray Analysis Reveals Th1-specific Fibronectin Transcription

171 We have previously reported a method to generate highly polarized human Th1 and Th2 cell
172 populations from naïve $CD4^+$ T-cells after four weeks in culture[10]. Using these culture
173 conditions we produced cells with the characteristic expression patterns of Th1 and Th2
174 cytokines upon activation. Intracellular cytokine staining of the *in vitro* differentiated Th1
175 and Th2 cells demonstrated that prior to stimulation neither population expressed the Th1
176 cytokine IFNγ or the Th2 cytokines IL-4, IL-5 and IL-13 (see Figure E1 in the Online
177 Repository). Upon acute activation with PMA and ionomycin at least 99% of the Th1 cells
178 expressed IFNγ with negligible IFNγ expression by the Th2 cells (Figure E1, upper panel).
179 Approximately 80% of the Th2 cells expressed IL-13 and multicolour analysis demonstrated
180 that there was considerable coexpression of IL-4 and IL-5 (Figure E1, lower panels). The Th1
181 cells did not express Th2 cytokines. These cells were used to perform comparative
182 microarray expression analysis for all known human genes using Affymetrix U133 plus 2
183 microarrays. In these analyses, fibronectin was identified as being highly selectively
184 expressed by Th1 cells (see Table EI in the Online Repository). The mean difference between
185 transcription levels of FN1 in Th1 and Th2 cells is 1,106 fold. Analysis of the microarray
186 data showed that other extracellular matrix molecules, such as collagen, fibrillin, laminin and
187 elastin were either not differentially expressed or not expressed at all (Table E1). This
188 indicates that of the extracellular matrix molecules only fibronectin is selectively transcribed
189 by Th1 cells. Further analysis of the microarray data showed that the gene encoding AICAR
190 Transformylase/IMP Cyclohydrolase (ATIC), immediately adjacent to fibronectin on human
191 chromosome 2, is also not differentially expressed in Th1 versus Th2 cells. This suggests that
192 the Th1-specific transcription of fibronectin is not caused by the adventitious location of the
193 FN1 gene in a Th1-specific chromosomal domain.

194 Th1-specific expression of Fibronectin by *in vitro* differentiated human T-helper cells

195 To further investigate the Th1-specific transcription of the FN1 gene we analyzed highly
196 differentiated Th1 and Th2 cells by quantitative real-time RT-PCR (Figure 1A). As expected,
197 highly Th1-specific and activation responsive transcription of IFNγ was observed in cells
198 cultured for 28 days. Furthermore, Th2-specific transcription of IL-4, IL-5 and IL-13 was
199 found upon Th2 cell activation (Figure 1A). Analysis of these samples demonstrated Th1-
200 specific transcription of the FN1 mRNA (Figure 1A). Interestingly, the FN1 mRNA appears
201 to be constitutively transcribed by Th1 cells prior to activation, in contrast to the cytokine
202 genes. Unstimulated Th1 cells express 1,259 fold more FN1 mRNA than unstimulated Th2
203 cells. Upon activation the FN1 mRNA levels appeared to decrease slightly in Th1 cells with
204 no notable expression in the activated Th2 cells. These data confirm the initial microarray
205 analyses and indicate that human Th1 cells constitutively transcribe the FN1 gene.

206 We then examined the transcription of the FN1 gene during the process of *in vitro*
207 differentiation by real-time RT-PCR on samples from day 14, 21 and 28 of the four-week
208 culture period (Figure 1B). We observed constitutive expression of the FN1 mRNA at each
209 time point of Th1 differentiation with a steady increase in the FN1 mRNA levels as
210 differentiation proceeded. The profile of FN1 mRNA expression during Th1 differentiation
211 parallels the IFNγ mRNA levels with the exception that FN1 is constitutively transcribed.
212 Once again there was negligible FN1 mRNA detected at any time point during Th2
213 differentiation although mRNA for the Th2 cytokine genes steadily increased during the
214 culture period (Figure 1B). The increase in FN1 levels during Th1 differentiation suggests
215 that the ability to transcribe FN1 is a T-helper cell characteristic acquired during Th1
216 differentiation rather than a characteristic lost during Th2 differentiation.

217 To investigate whether fibronectin protein is expressed by the Th1 cells we performed
218 ELISA assays for soluble fibronectin in tissue culture supernatants from the highly polarized

219 Th1 and Th2 cultures (Figure 1C). Soluble fibronectin was readily detectable by ELISA in
220 the Th1 supernatants from cells differentiated for 28 days (478 ng/ml). No detectable
221 fibronectin was identified in the Th2 cell supernatants above the amounts present in the
222 culture media (which contains FBS). Analysis of cell supernatants taken during the *in vitro*
223 differentiation clearly show that the protein levels mirror the mRNA levels, with a steady
224 increase during Th1 differentiation and no detectable fibronectin in the Th2 cultures (Figure
225 1D).

226 Fibronectin is a complex dimeric glycoprotein containing two subunits of
227 approximately 250 kDa each[12, 13]. Alternative splicing produces several different molecules
228 containing variable numbers of Type III FN repeats and also truncated fibronectin molecules.
229 To examine the size of the Th1 cell specific fibronectin protein we performed western blots
230 of cell extracts and tissue culture supernatants (Figure 1E & F). Western blots of cytoplasmic
231 extracts from Th1 and Th2 cells readily detected a single Th1-specific band of over 250 kDa
232 (Figure 1E). Interestingly, the amount of fibronectin in the Th1 cytoplasmic extract decreased
233 upon activation. There was no detectable fibronectin in the Th2 cytoplasmic extracts.
234 Examination of the cell culture supernatants by Western blotting also revealed a Th1-specific
235 band of the same size as the cytoplasmic protein (Figure 1F). A faint band is detectable in the
236 Th2 supernatants but this represents cross-reactivity with the bovine fibronectin in the culture
237 medium from the FBS. These data confirm that the Th1 cells constitutively express full-
238 length fibronectin and suggest that fibronectin is secreted by the Th1 cells upon activation.
239

240 Fibronectin is selectively expressed by *ex vivo* human Th1 cells

241 To determine whether the Th1-specific expression of FN1 that we observe *in vitro* is
242 observed in cells directly isolated from human subjects, we have isolated both Th1 and Th2
243 populations from peripheral blood. *Ex vivo* Th1 cells were isolated by FACS sorting

244 $CD3^+CD4^+IFN\gamma^+$ T-cells and *ex vivo* Th2 cells were isolated by FACS sorting $CD3^+CD4^+IL\text{-}13^+$
245 T-cells (Figure 2A). The relative levels of expression by these cells of IFNγ, IL-13 and
246 FN1 were assessed by real-time RT-PCR (Figure 2B). The data clearly show that FN1 is
247 highly selectively expressed by the $CD4^+IFN\gamma^+$ population in a manner similar to IFNγ,
248 whereas IL-13 is selectively expressed by the $CD4^+$ $IL\text{-}13^+$ cell population. Similar results
249 were obtained using *ex vivo* cells from atopic donors (data not shown). These results show
250 that *ex vivo* Th1 cells selectively express the FN1 gene and that the Th1-specific expression
251 observed after *in vitro* differentiation reflects expression of this gene by *ex vivo* Th1 cells.
252

253 Several alternatively spliced fibronectin isoforms are expressed by Th1 cells

254 The FN1 gene contains at least forty five exons and is subject to several alternative splicing
255 events. These splicing events introduce additional Type III fibronectin repeats into the
256 protein. There are three sites at which alternative splicing occurs, termed extra domain A
257 (EDA), EDB and the variable (V) region (see Figure E2A in the Online Repository). The
258 EDA and EDB regions are absent from most adult tissue but are expressed during embryonic
259 development, tissue injury, inflammation and angiogenesis[13]. The variable region is
260 differentially expressed in a variety of tissues. We have examined the Th1-specific FN1
261 transcripts by RT-PCR and sequencing to ascertain which splicing events occur in Th1 cells.
262 RT-PCR using primers spanning the EDB region identified a single fragment of 296 bp, the
263 size expected for transcripts with the EDB exon absent (see Figure E2B & C in the Online
264 Repository). Cloning and sequencing of the PCR product confirmed that the Th1-specific
265 FN1 transcript does not contain the EDB exon (data not shown).

266 RT-PCR using primers spanning the EDA and V regions identified three major
267 products of different intensities between 1000 bp and 1700 bp (see Figure E2B & D in the
268 Online Repository). Cloning and sequencing of these PCR products identified a range of

269 different splicing events. The EDA region is absent from some cloned transcripts but is
270 present in 84% of clones identified. Inclusion of the EDA region of fibronectin introduces a
271 binding site for integrins $\alpha9\beta1$ and $\alpha4\beta1$[14]. Germline deletion of the EDA exon in the mouse
272 causes defects in wound healing and also defects in accumulation of extravasated
273 macrophages during acute inflammation[15, 16]. Recent work has shown that the EDA region of
274 FN1 also acts as an endogenous ligand for the pattern recognition receptor TLR4 and causes
275 NF-κB activation and secretion of IL-12 and TNFα by dendritic cells and macrophages[17, 18].
276 Sequencing of the V region reveals a complex pattern of exon usage (see Figure E2E
277 & F in the Online Repository). The V region of fibronectin contains at least two recognition
278 sites for the $\alpha4\beta1$ integrin, the LDV and REDV motifs[19, 20]. These motifs are present in the V
279 region sequences that we have identified (see Figure E2E & F in the Online Repository). The
280 $\alpha4\beta1$ integrin (also known as VLA-4) is expressed on lymphoid and myeloid cells and $\alpha4\beta1$
281 interactions with fibronectin are important for leukocyte extravasation during acute
282 inflammation (reviewed in Ref [12]).
283 To further examine the Th1-specific expression of the EDA-containing fibronectin
284 isoform in the context of immune cell function we have screened a large panel of innate and
285 adaptive immune cell types for expression of both total fibronectin and EDA containing
286 mRNA by quantitative real-time RT-PCR (Figure 3). Figure 3A clearly shows that of all the
287 cell types examined only Th1 cells express high levels of fibronectin mRNA. When
288 specifically examining the expression of the EDA exon a small amount of expression was
289 detected in CD4+CD25+ T-cells, probably representing the activated T-cells in the periphery.
290 A small amount of EDA specific fibronectin was also detected in NKT-cells but the
291 predominant cell type expressing EDA specific fibronectin was the Th1 cell (Figure 3B).
292
293

294

295

296 Th1-cell derived fibronectin modulates cytokine expression by monocytes

297 To investigate the potential function of Th1 cell derived fibronectin we purified fibronectin
298 from Th1 cell culture supernatants by affinity chromatography taking advantage of the
299 specific binding of fibronectin to gelatin-sepharose[21]. Th1 cell supernatants were mixed with
300 a gelatin-sepharose slurry, and the bound fibronectin washed extensively, before elution with
301 arginine (Figure 4A). We examined the effect of fibronectin on peripheral blood monocytes
302 which express both TLR4 and α4β1 integrin, two of the receptors known to bind to
303 fibronectin including the EDA region. Monocytes were isolated from PBMCs by negative
304 magnetic selection and cultured overnight either in culture media or with IFNγ. After
305 overnight culture the monocytes were treated with Th1 cell derived fibronectin for 4 hours
306 before harvesting for RNA isolation and quantitative real-time RT-PCR to examine cytokine
307 gene expression. In combination with IFNγ, fibronectin induced a significant and synergistic
308 upregulation of IL-6 mRNA that was not observed with either fibronectin or IFNγ alone
309 (Figure 4B). In contrast fibronectin treatment alone was sufficient to downregulate IL-10
310 mRNA expression (Figure 4C). A similar trend was observed in the presence of IFNγ
311 although it did not reach statistical significance (Figure 4C). These results demonstrate that
312 Th1 cell derived fibronectin can modulate monocyte cytokine transcription promoting the
313 expression of the pro-inflammatory cytokine IL-6 whilst downregulating expression of the
314 regulatory cytokine IL-10.

315 ## DISCUSSION

316 Our findings clearly demonstrate that human Th1 cells generated *in vitro* constitutively
317 express fibronectin at high levels. IFNγ-secreting $CD4^+$ Th1 cells isolated directly *ex vivo*
318 also selectively express fibronectin compared to *ex vivo* $IL\text{-}13^+CD4^+$ Th2 cells. We have also
319 shown that fibronectin expressed by Th1 cells can promote the expression of the pro-
320 inflammatory cytokine IL-6 by monocytes whilst downregulating IL-10 expression. We
321 propose that fibronectin is a new Th1-specific effector molecule based upon the multiple and
322 complex roles played by fibronectin in immune cell recruitment and function (Figure 6). The
323 interaction between pathogens and fibronectin has long been recognized and a considerable
324 body of work has shown that many pathogens express specific fibronectin binding proteins
325 including mycobacteria, staphylococcae and leishmania[22-24]. Several of these molecules are
326 virulence factors for pathogens and have been proposed to play an important role in bacterial
327 adherence and invasion at the site of injury[25, 26]. By the same mechanism, fibronectin also
328 plays an important role in the opsonisation and engulfment of these pathogens by
329 macrophages[27]. Therefore expression of fibronectin by Th1 cells could promote clearance of
330 pathogenic microbes by macrophages which are also activated to kill phagocytosed bacteria
331 by Th1-cell derived IFNγ.

332 Fibronectin also plays an important role in leucocyte extravasation during an
333 inflammatory response via binding to integrin α4β1 on the surface of both lymphoid and
334 myeloid cells[12]. Therefore Th1-specific expression of fibronectin may also recruit other cell
335 types including macrophages and also more Th1 cells (Figure 5). It is interesting that Th2
336 cells do not bind to fibronectin, further emphasizing the Th1-specific nature of fibronectin[28].
337 It is also important to note that the Th1-specific isoforms of fibronectin containing EDA
338 harbour several additional binding sites for integrin α4β1 and α9β1 and thus may promote
339 extravasation more effectively[14]. The EDA-containing isoforms of fibronectin are not

340 normally expressed in adult tissue but have been identified in response to tissue injury,
341 wound healing[29, 30], fibrosis[31], and allograft transplantation[32]. EDA-fibronectin has also been
342 observed in the synovial fluid in rheumatoid arthritis[33, 34], in multiple sclerosis[35, 36] and
343 psoriasis[37] all diseases with a significant Th1-mediated pathology. In these disease settings
344 the source of EDA-fibronectin has not been entirely clear with some evidence for
345 macrophage or fibroblast involvement. Our work suggests that one source of EDA-
346 fibronectin is likely to be the Th1 cell. Recent work using mutant mouse strains in which the
347 EDA exon was deleted has demonstrated that this region plays a role in wound healing and
348 also in macrophage accumulation during inflammation[15, 16]. It would be interesting to
349 examine the effect of deletion of the EDA region in Th1 mediated infectious disease mouse
350 models and autoimmune pathologies such as experimental autoimmune encephalomyelitis.
351 Recent work by two groups has shown that the EDA region of fibronectin expressed
352 by Th1 cells is an endogenous ligand for TLR4[17, 18]. Ligation of TLR4 classically induces
353 NF-κB signaling and promotes Th1 immunity via stimulation of antigen presenting cells to
354 produce pro-inflammatory cytokines such as IL-6 and also IL-12[38]. We have shown here that
355 the Th1-derived fibronectin containing the EDA region can induce IL-6 gene expression from
356 monocytes and also suppress IL-10 gene expression. The role of EDA-fibronectin as an
357 endogenous ligand for TLR4 and for integrin binding is particularly interesting in the light of
358 recent studies showing that the TLR4 signaling pathway is dependant upon integrin signaling
359 to localize the adaptor molecule TIRAP to the plasma membrane in macrophages[39]. It is
360 therefore possible that EDA-fibronectin can perform both roles in macrophage activation.
361 Elucidation of the the relative roles of integrin and TLR4 signaling by Th1 derived
362 fibronectin in monocyte/macrophage activation will require further studies beyond the scope
363 of this report. Experiments to dissect the role of the different domains and their effect on
364 TLR4 and integrins will be technically challenging since fibronectin is a very large molecule

365 containing numerous domains with overlapping functions making the use of function
366 blocking antibodies against each domain extremely difficult to interpret.
367 We have shown that human Th1 cells selectively express fibronectin at high levels
368 both *in vitro* and *ex vivo* when compared to Th2 cells and other immune cell types. It will be
369 interesting to examine pro-inflammatory Th17 cells for fibronectin expression once the
370 methods for efficient generation of human Th17 cells have been characterized. The Th1-
371 specific expression of fibronectin at first seems to contrast with data supporting a role for
372 fibronectin in Th2-mediated disease pathologies such as asthma, however it is likely that
373 other cell types contribute to the fibronectin deposition seen in Th2 disease. In fact, although
374 it is clear that there is increased extracellular matrix deposition in the subepithelial reticular
375 basement membrane in asthma, there is little evidence of increased total or EDA-containing
376 fibronectin in seasonal or chronic asthma[40]. Collectively our data suggest that fibronectin can
377 function as a Th1-specific effector molecule. Germline deletion of fibronectin is lethal in
378 mice, which has hampered study of the function of this molecule *in vivo*. It would be
379 intriguing to examine the role of EDA-containing fibronectin in Th1 disease models in which
380 the EDA exon has been deleted.
381
382
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387

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496
497
498

499 **Figure legends**

500 **Figure 1. Th1-specific expression of fibronectin.** (A) FN1 mRNA is selectively expressed
501 by Th1 cells. (R) resting (A) activated. Data shown are the mean ± SEM, n=4. (B) FN1
502 mRNA is induced during Th1 differentiation. Representative of two experiments. (C & D)
503 Fibronectin is secreted by Th1 cells. Fibronectin ELISA of supernatants. (C) mean ± SEM,
504 n=4. (D) Representative of two experiments. (E & F) Western blotting of cytoplasmic
505 extracts and cell culture supernatants, (-) resting, (+) activated.
506
507 **Figure 2. *Ex vivo* Th1 cells selectively express fibronectin.** *Ex vivo* Th1 and Th2 cells were
508 isolated based on the expression of CD3, CD4 and IFNγ or IL-13. (A) FACS plots show pre
509 and post sort profiles. (B) RT-PCR on sorted samples, mean± SEM, n=3. Gene expression is
510 shown relative to one biological replicate (Th1 for IFNγ and FN1 and Th2 for IL-13).
511
512 **Figure 3. Expression of fibronectin by immune cell types.** Real-time RT-PCR for total
513 fibronectin (A) and EDA-fibronectin (B) mRNA in a panel of immune cell types. Cell types
514 were isolated by sorting from PBMCs or PMNs as described in methods, *in vitro* generated
515 Th1 and Th2 cells are also shown. R, resting cells; A, activated cells (PMA/Ionomycin).
516
517 **Figure 4. Th1 cell derived fibronectin modulates monocyte gene expression.** (A).
518 Monocytes were cultured overnight with or without IFNγ as indicated. After overnight
519 incubation fibronectin (light bars) or PBS (dark bars) was added for 4 hrs. Quantitative real-
520 time RT-PCR was performed for IL-6 (B) and IL-10 (C). Statistical analysis: 2 way ANOVA
521 with Tukey post test *$p<0.05$ **$p<0.01$.
522

523 **Figure 5. Model for the role of fibronectin in Th1-cell functions.** FnBP, fibronectin

524 binding protein; TLR4, toll-like receptor 4; α4β1, integrin α4β1; MØ, macrophage.

525

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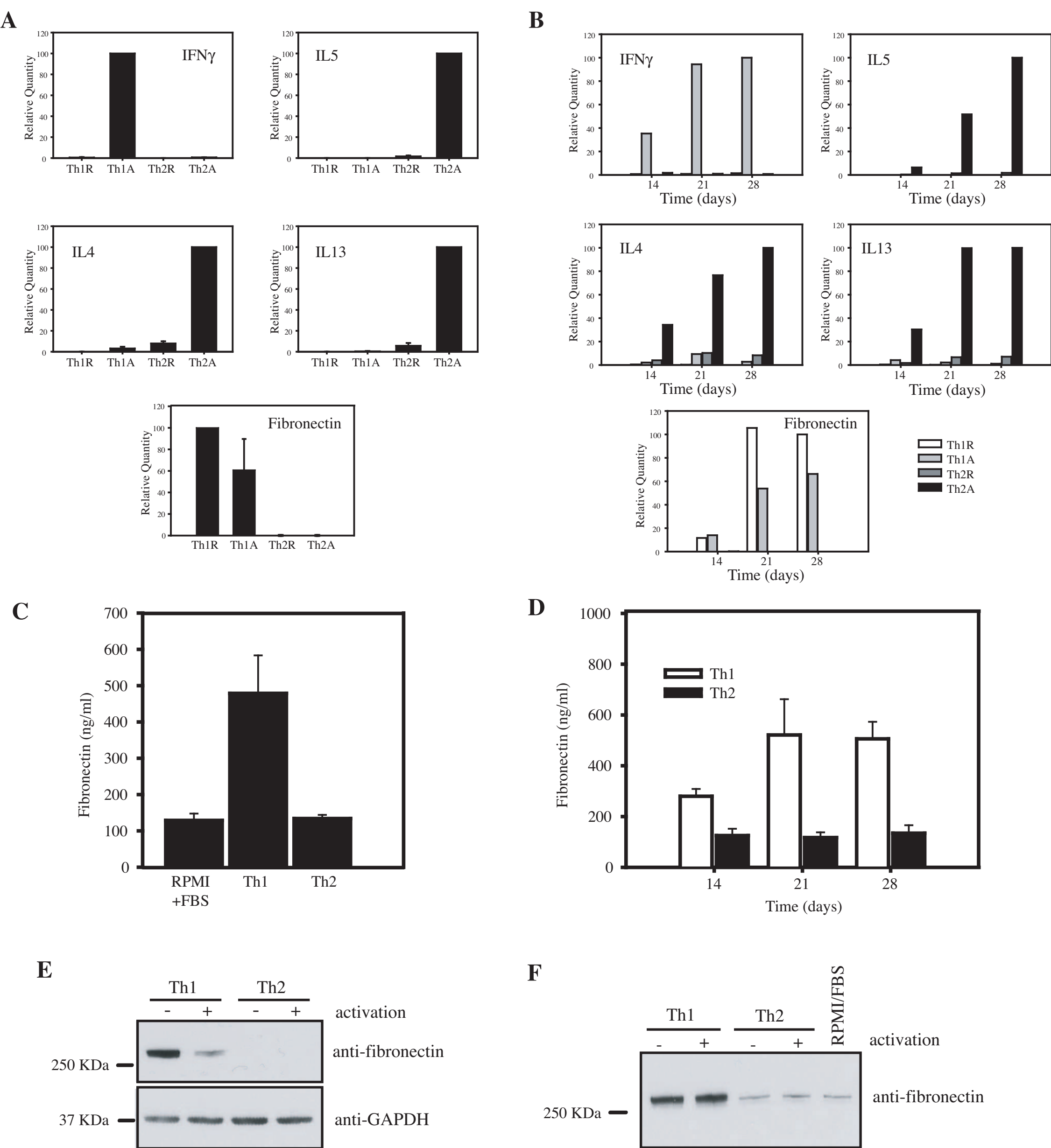

Figure 1
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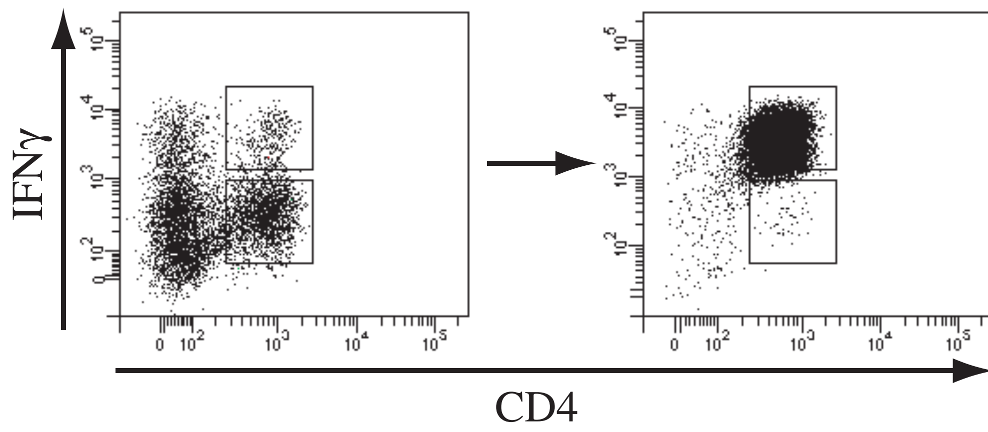

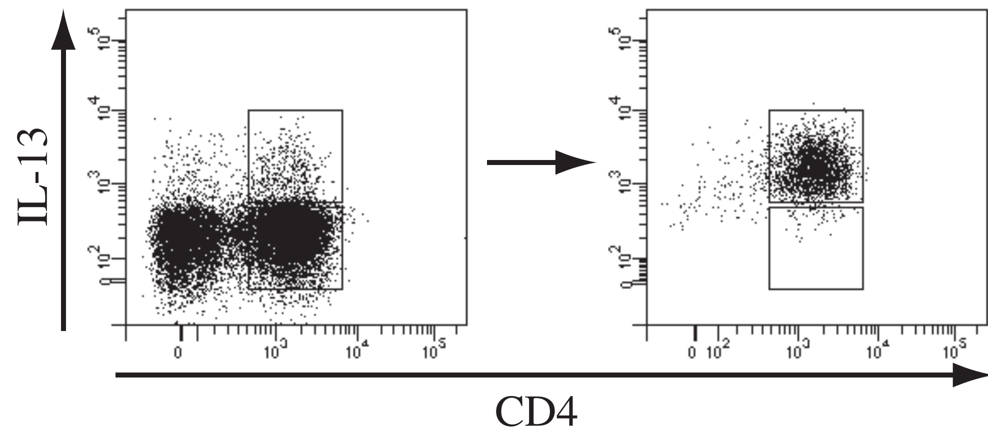

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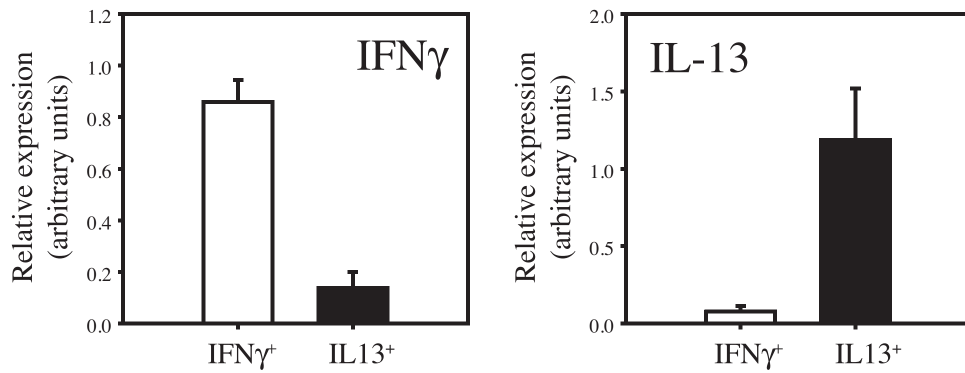

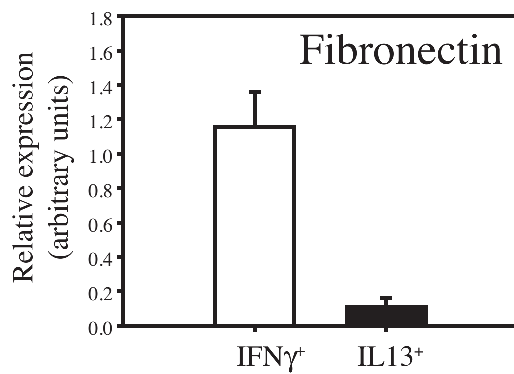

Figure 2
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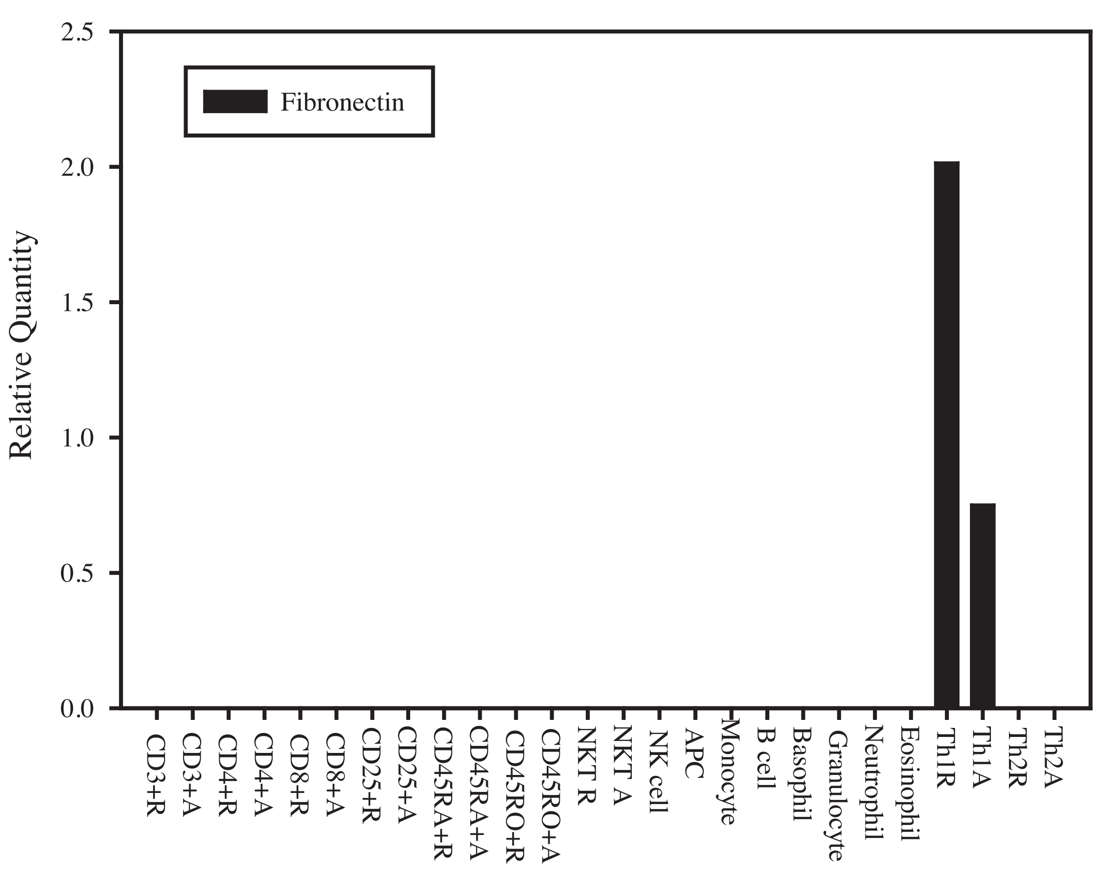
A



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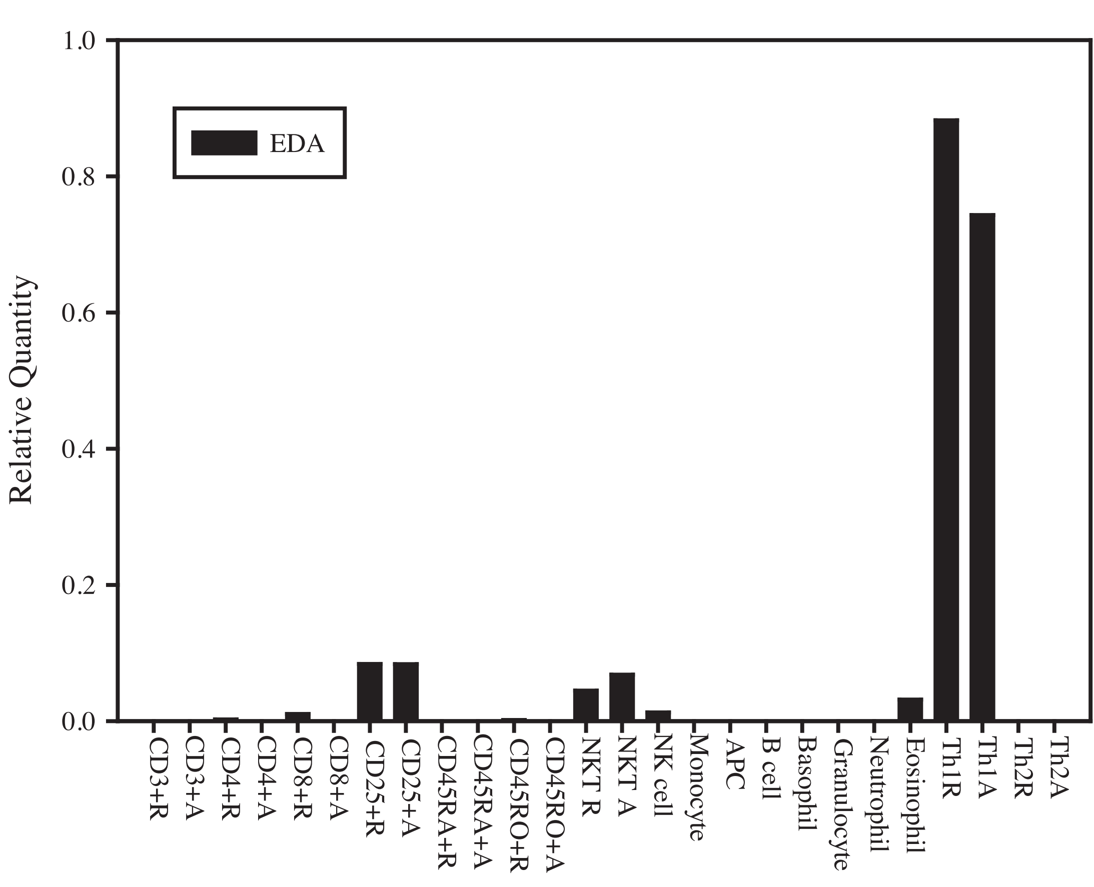

Figure 3
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Figure No. 4- Unmarked

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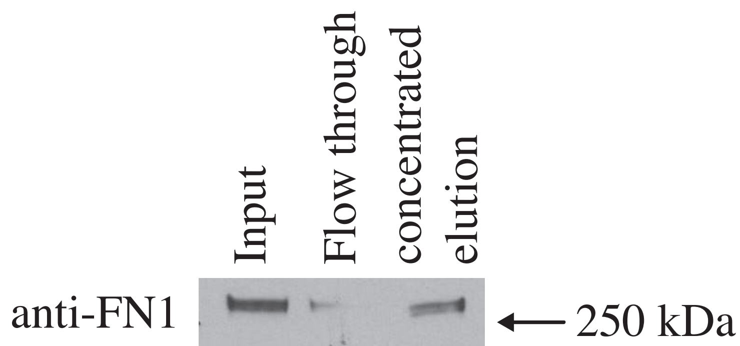

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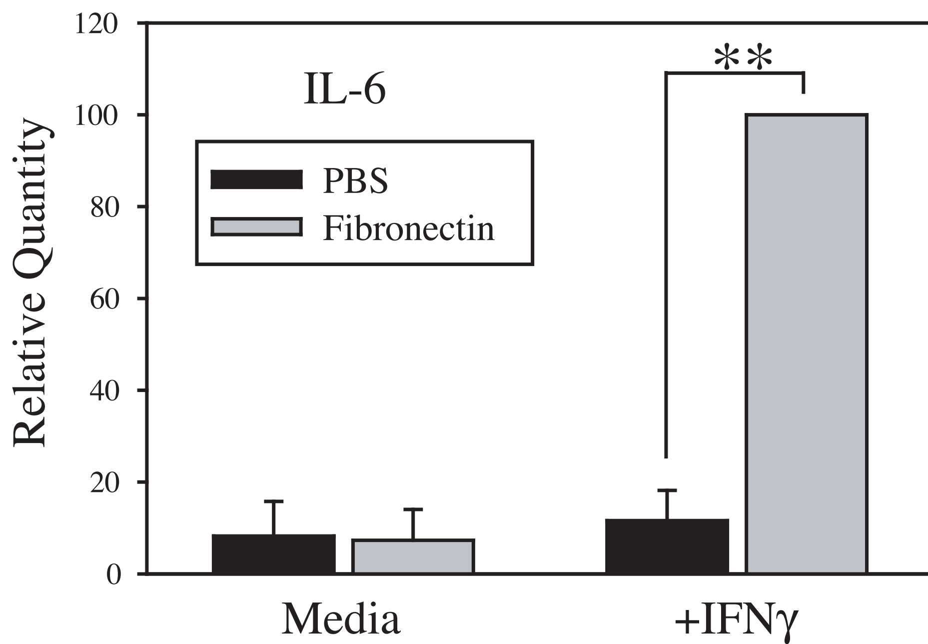

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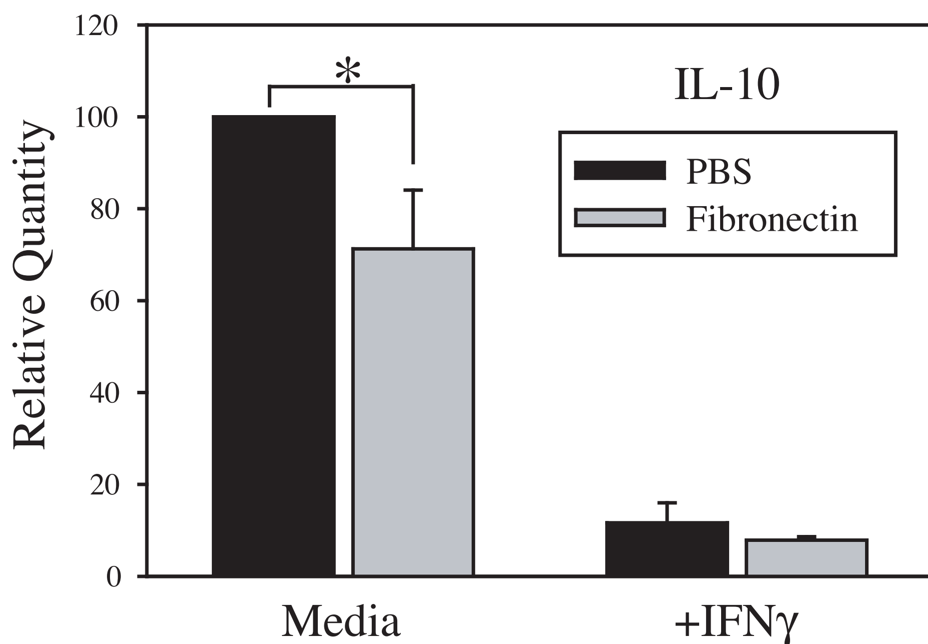

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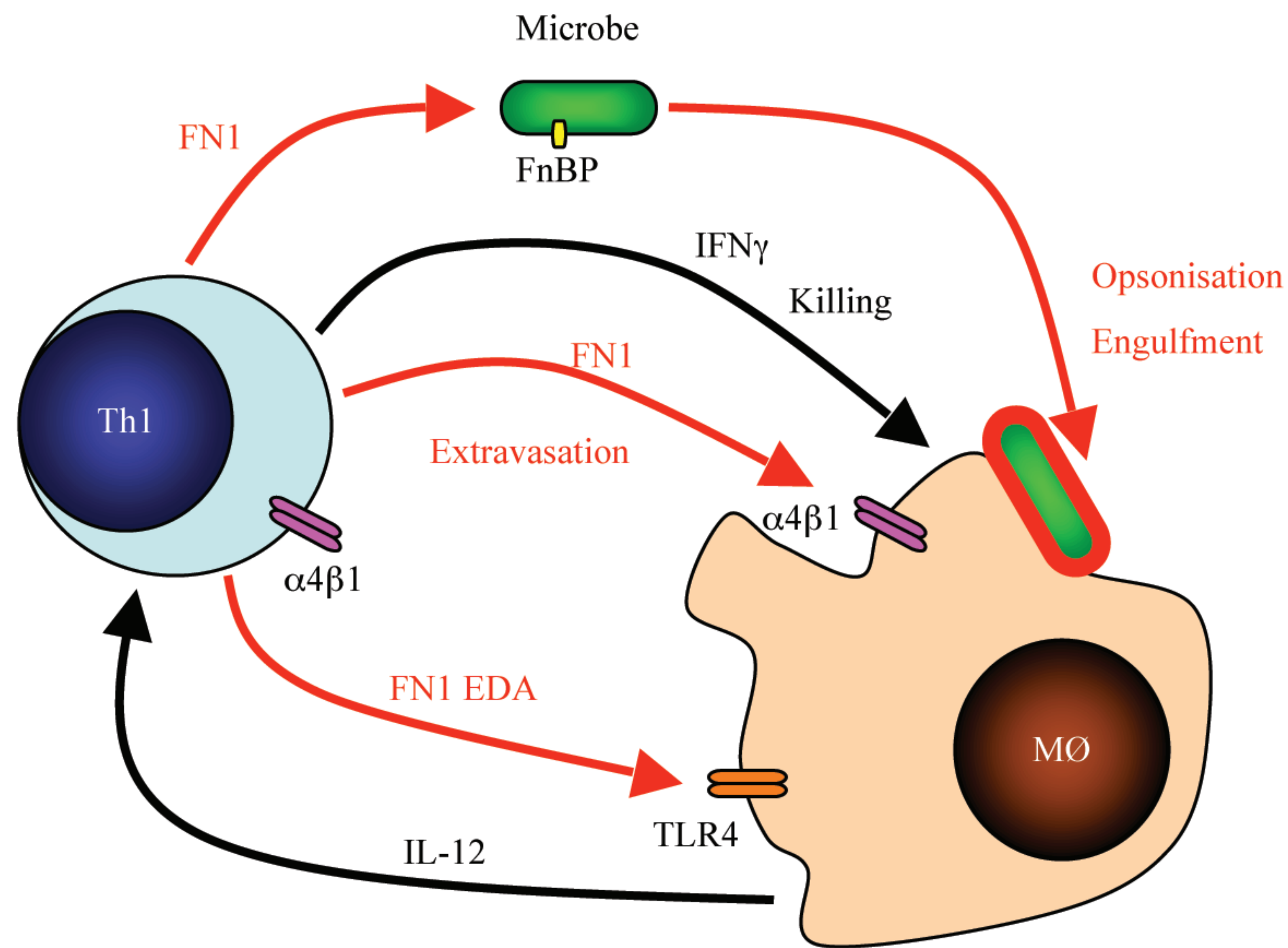

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5 Original Article

6

7 Fibronectin is a Th1-specific molecule in humans.

8

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26 # METHODS

27 ## Th1/Th2 cell differentiation

28 Venous blood was taken from non-atopic healthy human volunteers using heparin as an
29 anticoagulant. Ethical approval for the use of human volunteers in this study was obtained
30 from the institutional ethical review committee. PBMCs were isolated using Lymphoprep®
31 (Nycomed) according to the manufacturer's instructions. $CD4^+$ T-cells were isolated from
32 PBMCs using a CD4 Positive Isolation Kit (Invitrogen) according to the manufacturer's
33 instructions. Naïve $CD45RA^+$ cells were purified from $CD4^+$ cells by depletion of $CD45RO^+$
34 cells using mouse anti-human CD45RO antibody (UCHL1; BDBiosciences; 0.5μg/1x10^6
35 cells) and rat anti-mouse IgG Dynabeads® (Invitrogen) according to the manufacturer's
36 instructions. The purity of naïve cell populations were assessed by FACs as previously
37 described[E1] and were at least 99% $CD4^+CR45RA^+$.

38 Purified $CD45RA^+$ cells (1x10^6/ml) were cultured in RPMI 1640 (Invitrogen)
39 supplemented with 10% FCS, 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin
40 (Invitrogen), and 100 μg/ml streptomycin (Invitrogen). Cells were stimulated with plate-
41 bound anti-CD3 (1μg/ml; clone OKT3) and anti-CD28 (2μg/ml; clone 15E8; Sanquin), rIL-2
42 (50U/ml; Eurocetus) and anti-IL-10 (5μg/ml; clone JES3-9D7; BDBiosciences). To direct
43 Th1 differentiation, rIL-12 (2.5ng/ml; R&D Systems) and anti-IL-4 (5μg/ml; clone MP4-
44 25D2; BDBiosciences) were added. For Th2 differentiation, rIL-4 (12.5ng/ml; R&D
45 Systems) and anti-IFNγ (5μg/ml; clone B-B1; Biosource) were added. After four days, the
46 cells were expanded under the same conditions in the absence of anti-CD3 or anti-CD28.
47 Cells were then re-stimulated every seven days. When required, cells were activated with
48 PMA (5ng/ml; Sigma) and ionomycin (500ng/ml; Calbiochem) for 4 hours.

49

50

51

52 ## Intracellular cytokine staining

53 Resting cells were activated with PMA (5ng/ml) and ionomycin (500ng/ml) for 4 hours,
54 monensin (2 µM; Sigma) was added for the final 2 hrs of activation. Cells were harvested in
55 FACs tubes and placed on ice, 7-amino-actinomycin D (4 µg/ml; Sigma) was added and cells
56 were incubated for 10mins on ice. Cells were then washed with FACSFlow (BD Biosciences)
57 and processed for intracellular cytokine staining with Cytofix/Cytoperm kit (BD Biosciences)
58 according to the manufacturer's instructions. Antibodies used (BD Biosciences unless stated
59 otherwise): PE anti IL-4 (Fastimmune), Allophycocyanin anti-IL-5 (TRFK5), PE anti-IL-13
60 (JES10-5A2), FITC anti-IFNγ (B27). Samples were analysed on a FACSCalibur® (BD
61 Biosciences). Live cells were analysed for cytokine expression based upon forward and side
62 scatter and exclusion of 7-amino-actinomycin D. At least 10,000 live cells were analysed for
63 each sample. Quadrant markers were set based upon background staining of matched control
64 antibodies (also from BDBiosciences) and on resting, unactivated cells treated in parallel.

65

66 ## *Ex vivo* cell isolation

67 As a marker of *ex vivo* Th1 cells, $CD3^{+}CD4^{+}IFN\gamma^{+}$ cells were isolated from PBMCs using the
68 IFNγ-FITC secretion assay detection kit (Miltenyi) in combination with anti-CD3 PE
69 (BDBiosciences) anti-CD4-APC (Miltenyi) according to the manufacturer's instructions. *Ex*
70 *vivo* $CD3^{+}CD4^{+}IL\text{-}13^{+}$ Th2 cells were isolated from PBMCs using the IL-13-PE secretion
71 assay detection kit (Miltenyi), anti-CD3-FITC (BDBiosciences) and anti-CD4-APC
72 (BDBiosciences). To detect cytokine positive cells, PBMCs were activated for 3 hours using
73 PMA and Ionomycin prior to cell isolation according to the manufacturer's instructions.
74 Other *ex vivo* cell populations were isolated from blood by magnetic or FACs sorting using
75 lineage specific markers. CD3+ (CD3+ cells); CD4+ (CD3+CD4+ cells); CD8+ (CD3+CD8+

76 cells); CD25+ (CD4+CD25+ cells); CD45RA+ (CD4+CD45RA+ cells), CD45RO+
77 (CD4+CD45RO+); NKT (CD3+CD56+ cells); NK (CD3-CD56+ cells); Monocyte (CD14+
78 cells); APCs (CD3-CD19-CD14-CD56-HLA-DR+); B-cell (CD19+ cells); Basophil
79 (CD203c+); Granulocyte (Histopaque PMN pellet with RBC lysis); Neutrophil (CCR3-
80 granulocytes); Eosinophil (CCR3+granulocytes). Cells were FACS sorted on a FACS Aria
81 (BDBiosciences), purity post sort was greater than 85% in all experiments. Granulocyte
82 purities and morphologies were assessed by May-Grunwald Giemsa staining.
83
84
85 ## RNA isolation and RT-PCR
86 Isolation of total cellular RNA was performed using the RNeasy mini kit (Qiagen) according
87 to the manufacturer's instructions and reverse transcription was performed as previously
88 described[E2]. Quantitative real-time RT-PCR was performed using Taqman® MGB Gene
89 expression assays (Applied Biosystems). Probe sets used were: IL-4, Hs00929862_m1; IL-5,
90 Hs00174200_m1; IL-6, Hs00174131_m1; IL-10, Hs00174086_m1; IL-13, Hs00174379_m1;
91 IFNγ, Hs00174143_m1, FN1, Hs00277509_m1; FN1 EDA region, Hs01549959_m1; 18s
92 rRNA, Hs99999901_s1. Conventional RT-PCR was performed as previously described using
93 Amplitaq Gold Taq polymerase [1]. Primers for amplifying the alternatively spliced regions of
94 FN1 were designed using MacVector and ordered from MWG Biotech. Primer sequences
95 were: EDB region, TACCACAACCCCTACAAACGGC and
96 TTTTCACAGGTGAGTAACGCACC; EDA and V region,
97 TCCCAGATGCAAGTGACCGATG and GGTAACAACCTCTTCCCGAACC. PCR
98 products were cloned into pCR 2.1 TOPO (Invitrogen) according to the manufacturer's
99 instructions and 40 individual clones of the FN1 regions were sequenced by Geneservice.

100 Sequences have been deposited in GenBank, accession numbers: EF550130, EF550131,
101 EF550132, EF550133, EF550134, EF550135.
102

103 ### Microarray analysis

104 cRNA samples were prepared for microarray hybridisation to GeneChip U133 plus 2 arrays
105 according to the manufacturer's instructions (Affymetrix). Fragmented cRNA was hybridised
106 to GeneChip arrays at 45°C for 18 hours. Arrays were washed and stained with streptavidin-
107 PE according to the manufacturer's instructions on the GeneChip fluidics station 450
108 (Affymetrix). Fluorescent signals were detected using the GeneChip scanner 3000. Images
109 were analysed using the GeneChip operating software (GCOS, Affymetrix) to generate raw
110 data as .cel files. Further analysis was performed in GCOS to identify differentially expressed
111 genes. Data shown in Table 1 are representative of three independent biological replicates for
112 each condition.
113

114 ## References

115

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121 120:56-63.

122
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124

125 Table

126 Table EI. Affymetrix GeneChip analysis of human Th1/Th2 cells

Gene Name	Affymetrix Probeset	Th1 Resting	Th2 Resting	Fold Difference	Th1 Activated	Th2 Activated	Fold Difference
FN1	211719_x_at	2349.1	1.5	1566	1627.3	1.1	1479
FN1	212464_s_at	2052	1.7	1207	1184	1.7	696
FN1	216442_x_at	2186.1	2.6	840.8	1442.7	1.7	848.6
COL13A1	208535_x_at	7	22.5	-3.21	1.5	1.8	-1.2
COL6A3	201438_at	15.9	1	15.9	92.2	12.2	7.5
FBN1	202766_s_at	17.6	24.9	-1.41	9.6	1.8	5.3
FBN2	203184_at	1.7	3.7	-2.17	8.1	13.1	-1.61
LAMA1	227048_at	9	0.4	22.5	8.7	5	1.74
LAMC1	200771_at	13.3	6.3	2.1	62.7	6.9	9.09
ELN	212670_at	3.4	2.2	1.55	3.2	1.9	1.68
ATIC	208758_at	669.5	521.7	1.28	1242.3	1184	1.05
IL4	207539_s_at	15.4	352.2	-22.8	177.8	5197.8	-29.2
IL5	207952_at	3.3	1219.5	-369.5	3.5	11774.4	-3364
IL13	207844_at	58.1	1504.4	-25.9	409.3	10536.3	-25.7
IFNγ	210354_at	5905.7	70.9	83.3	12481.4	7906.1	1.58

127

128

129 Figure Legend

130 Figure E1. *In vitro* differentiation of human Th1 and Th2 cells. Naive $CD4^+$ T-cells were

131 isolated from PBMCs and cultured for 28 days under Th1 or Th2 polarizing conditions.

132 Where indicated, cells were activated for 4 hours with PMA (5ng/ml) and ionomycin

133 (500ng/ml) and intracellular cytokine staining performed. Data are representative of six
134 independent experiments from different healthy donors.
135
136 Figure E2. Variable splicing of Th1 cell fibronectin. (A) Human fibronectin gene. Blue
137 boxes represent exons. Black boxes indicate regions of variable splicing; EDA, extra domain
138 A; EDB, extra domain B; VAR, variable region. (B) RT-PCR for EDB, EDA and variable
139 regions using primers indicated in C & D panels. (C & D) Variably spliced regions, red boxes
140 represent exons identified by sequencing. Frequency of exon splice variants (A-F) is shown.
141 (E) Coding sequence of Th1 cell fibronectin isoforms around the EDA and variable regions
142 (black boxes). (F) Alignments of the variable regions, LDV and REDV motifs are
143 highlighted.
144

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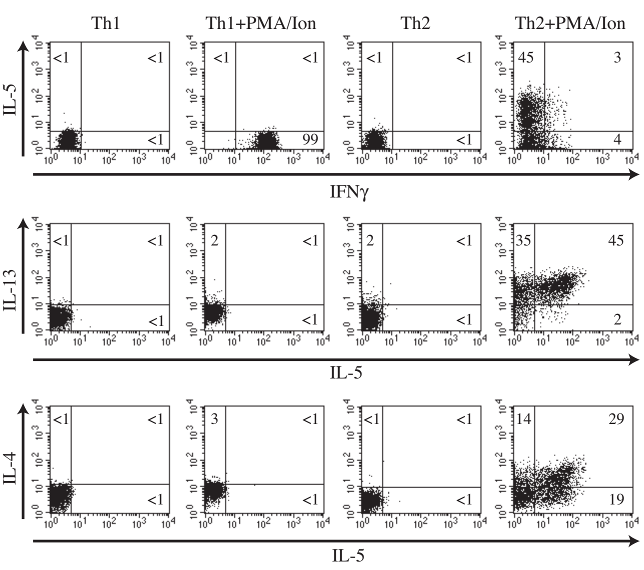

Figure E1
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Repository - Unmarked E Figure No.2

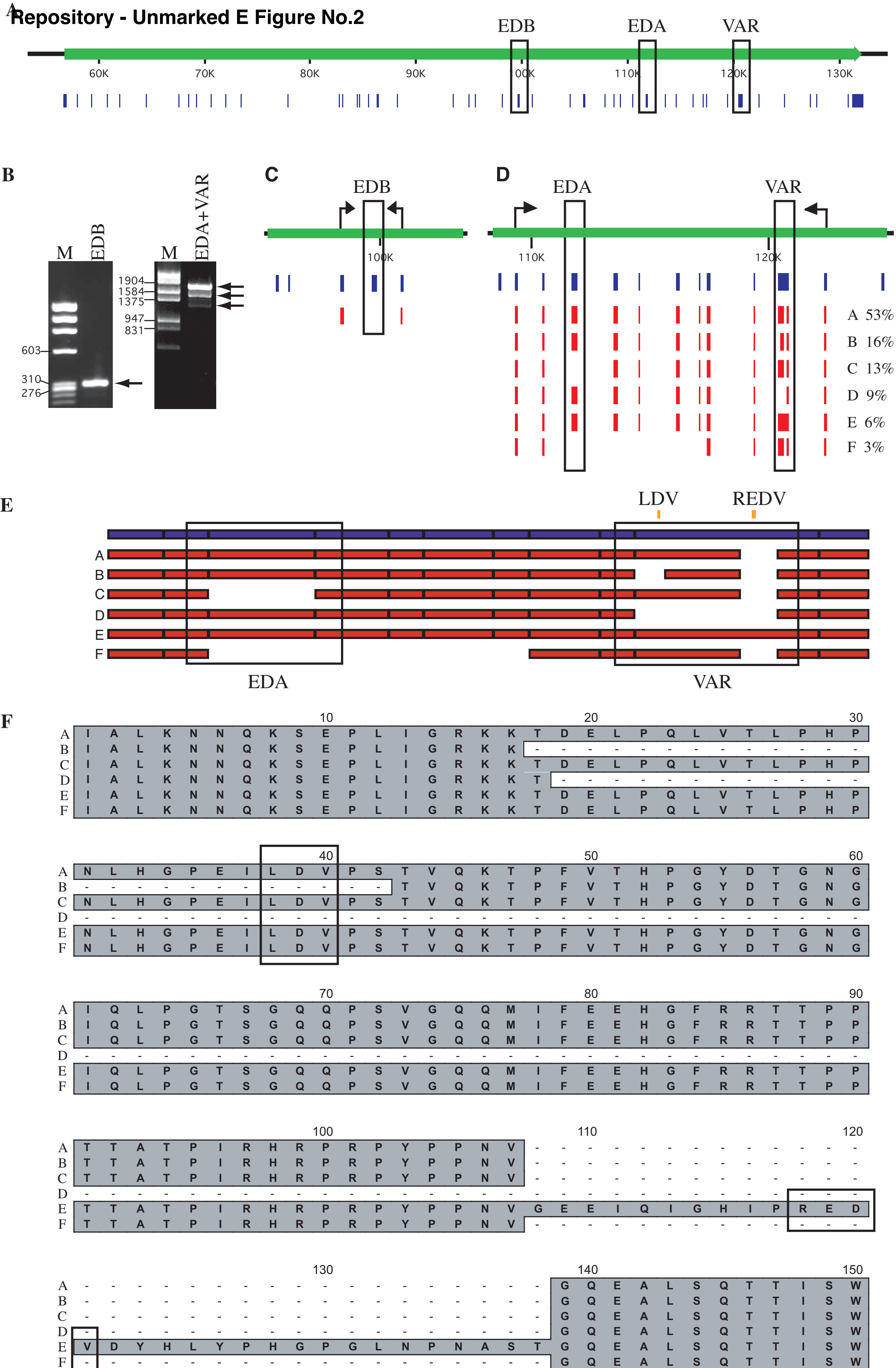

Figure E2
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