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RESEARCH ARTICLE

FRNK negatively regulates IL-4-mediated inflammation

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ABSTRACT

Focal adhesion kinase (FAK)-related nonkinase (PTK2 isoform 6 in humans, hereafter referred to as FRNK) is a cytoskeletal regulatory protein that has recently been shown to dampen lung fibrosis, yet its role in inflammation is unknown. Here, we show for the first time that expression of FRNK negatively regulates IL-4-mediated inflammation in a human model of eosinophil recruitment. Mechanistically, FRNK blocks eosinophil accumulation, firm adhesion and transmigration by preventing transcription and protein expression of VCAM-1 and CCL26. IL-4 activates STAT6 to induce VCAM-1 and CCL26 transcription. We now show that IL-4 also increases GATA6 to induce VCAM-1 expression. FRNK blocks IL-4-induced GATA6 transcription but has little effect on GATA6 protein expression and no effect on STAT6 activation. FRNK can block FAK or Pyk2 signaling and we, thus, downregulated these proteins using siRNA to determine whether signaling from either protein is involved in the regulation of VCAM-1 and CCL26. Knockdown of FAK, Pyk2 or both had no effect on VCAM-1 or CCL26 expression, which suggests that FRNK acts independently of FAK and Pyk2 signaling. Finally, we found that IL-4 induces the late expression of endogenous FRNK. In summary, FRNK represents a novel mechanism to negatively regulate IL-4-mediated inflammation.

KEY WORDS: Cell adhesion, Endothelial cells, Inflammation, Leukocyte trafficking

INTRODUCTION

Chronic inflammatory diseases are characterized by inappropriate and unregulated infiltration of leukocytes into the tissue (Ley et al., 2007; Springer, 1994). Cytokines, such as TNFα and interleukin 4 (IL-4), are typically used in both human and mouse models to simulate the cellular activation that occurs during chronic inflammation (Hickey et al., 1999; Hickey et al., 1997; Moser et al., 1992; Patel, 1998; Patel, 1999). IL-4 is a T-cell subset 2 (Th2) cytokine that is increased in allergic diseases and asthma, and influences the development of allergen-specific Th2 cells (Humbert et al., 1996; Erb and Le Gros, 1996; Ricci et al., 1997; Ying et al., 1997). The mechanisms that initiate IL-4-mediated inflammation are increasingly well understood. IL-4 exerts its effects through two receptors: the type I receptor comprising IL4Rα and the common γ-chain, and the type II receptor comprising IL4Rα and IL13Rα (Luzina et al., 2012). In endothelial cells, IL-4 acts solely through the IL-4 type II receptor (Schnyder et al., 1996), leading to increased phosphorylation of JAK2 and STAT6 (Palmer-Crocker et al., 1996). Upon phosphorylation, STAT6 translocates into the nucleus where it regulates the expression of over 900 genes including those encoding vascular cell adhesion molecule 1 (VCAM-1) (Hopkins, 2013), CCL26 (Tozawa et al., 2011) and P-selectin (Miyazaki et al., 2006; Yao et al., 1996). VCAM-1 and P-selectin then mediate the recruitment of eosinophils, lymphocytes and monocytes (Matsumoto et al., 1997; Patel, 1999), and CCL26 activates eosinophils and supports eosinophil transmigration (Cuvelier and Patel, 2001). Although the mechanisms that initiate IL-4-mediated inflammation are extensively documented, much less is known about how inflammation is resolved. Ding et al. recently identified a novel role for the focal adhesion kinase (FAK)-related nonkinase (PTK2 isoform 6 in humans, hereafter referred to as FRNK) in the negative regulation of pulmonary fibrosis (Ding et al., 2013). We examined the role of FRNK and the related protein FAK in IL-4-mediated inflammation.

FAK, also known as PTK2, is a signaling protein that activates a wide range of cellular responses and is best known for its role in regulating focal adhesion complexes (Schaller, 2010). FAK has several domains, including an N-terminal FERM domain, a kinase domain and a C-terminal FAT domain (Schaller, 2010). Although FAK is primarily known for its kinase activity, the scaffolding activities of the FERM and FAT domains also serve important functions (Nolan et al., 1999; Heidkamp et al., 2002; Zhai et al., 2003). For example, the FERM domain also has scaffolding functions independent of kinase activity as shown by its ability to bind E3 ubiquitin-protein ligase MDM2 and the cellular tumour antigen p53 (p53) leading to the ubiquitylation and degradation of p53, resulting in protection from apoptosis (Lim et al., 2010). FRNK is autonomously transcribed from *PTK2*, but only contains the C-terminal FAT domain of FAK and, thus, lacks the kinase activity (Nolan et al., 1999; Schaller et al., 1993). FRNK acts as a dominant-negative regulator of FAK kinase activity. The mechanisms by which FRNK acts are not well understood though it is known that FRNK can displace FAK at focal adhesions (Heidkamp et al., 2002), as well as bind to proteins such as paxillin, p130Cas, GRB2 and p190 RhoGEF, preventing them from binding to FAK (Schaller, 2010).

We now show that FRNK negatively regulates IL-4-mediated inflammation in a human model of eosinophil recruitment. Exogenous expression of FRNK attenuated recruitment of eosinophils and blocked transmigration by >90%. To understand the mechanism by which FRNK exerted these effects, we examined transcription and translation of VCAM-1 and CCL26. We found that expression of FRNK prevents IL-4-mediated VCAM-1 and CCL26 mRNA expression and protein synthesis. FRNK acted independently of FAK signaling in part by dampening the expression of GATA6, a transcription factor we

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now show is upregulated by IL-4 and, in turn, regulates IL-4-mediated VCAM-1 mRNA and protein expression and recruitment of eosinophils.

RESULTS

Expression of FRNK in HUVECs blocks IL-4-mediated accumulation, firm adhesion and transmigration of eosinophils

Primary human endothelial cells were transduced with N-terminal GFP-tagged FRNK adenoviral vector (Adv-FRNK-GFP). As previously described, more than 85% of cells were positive for FRNK–GFP, endothelial cells were healthy with intact junctions, and transduction alone did not increase the expression of pro-inflammatory mediators such as E-selectin (Parsons et al., 2012). Untreated, GFP-transduced or FRNK–GFP-transduced human umbilical vein endothelial cells (HUVECs) were stimulated with IL-4 for 24 hours, followed by measurements of eosinophil accumulation, rolling, firm adhesion and transmigration under flow conditions. As previously reported, IL-4 alone increased eosinophil recruitment (Cuvelier and Patel, 2001; Moser et al., 1992) (Fig. 1A-C). Transducing cells with GFP prior to IL-4 stimulation had no effect on any of the eosinophil recruitment parameters examined (Fig. 1A-C). Transducing cells with FRNK–GFP prior to IL-4 stimulation blocked eosinophil accumulation (Fig. 1A), prevented the transition from rolling to firm adhesion, resulted in increased rolling (Fig. 1B) and completely blocked transmigration (Fig. 1C).

FRNK blocks IL-4-mediated expression of VCAM-1 and CCL26

To understand the mechanism by which FRNK blocks IL-4-mediated eosinophil recruitment, we first examined gene expression and protein synthesis of VCAM-1 and CCL26. In endothelial cells, IL-4 upregulates VCAM-1 (Palmer-Crocker and Pober, 1995; Schleimer et al., 1992) and CCL26 (Kitaura et al.,

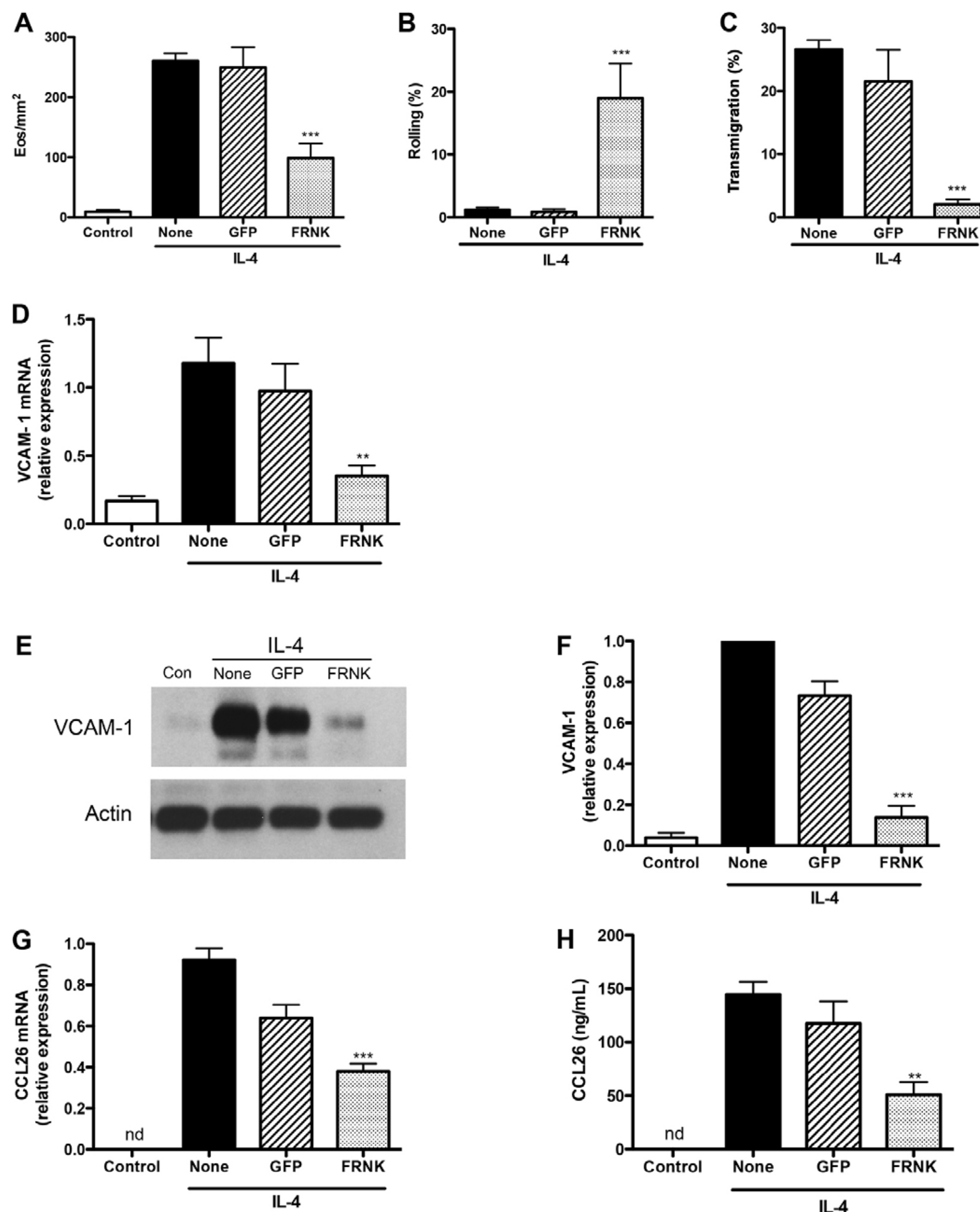

Fig. 1. FRNK blocks eosinophil recruitment and VCAM-1 and CCL26 expression to IL-4-stimulated endothelial cells. HUVECs were transduced with GFP alone or FRNK–GFP prior to stimulation with 20 ng/ml IL-4. After 24 hours, HUVECs were assembled into a parallel-plate flow chamber and eosinophils (0.5×10^6/ml) were perfused across at 1 dyn/cm^2 as described in the Materials and Methods. (A) Total accumulation was determined at 5 minutes, and (B) rolling and (C) transmigration were determined after 10 minutes as described in the Materials and Methods. (D,G) After 24 hours, cells were harvested for mRNA and qRT-PCR was performed to measure mRNA for (D) VCAM-1 or (G) CCL26 and data were normalized based on GAPDH expression as described in the Materials and Methods. Alternatively, cells were lysed for western blots and supernatants were collected for ELISA. (E,F) VCAM-1 expression was determined by western blotting and data were quantified and normalized using actin as a loading control. (H) ELISA for CCL26 was performed as described in the Materials and Methods. Data are mean±s.e.m. of 5 experiments. **P<0.01, ***P<0.001, and nd=not detectible as compared to the GFP control. Representative blots from five experiments are shown in E.

1999; Shinkai et al., 1999), and our lab showed that, together, they mediate eosinophil recruitment under flow conditions (Cuvelier and Patel, 2001; Patel, 1998; Patel, 1999). Expression of FRNK blocked IL-4-induced expression of both VCAM-1 and CCL26, as measured by quantitative real-time PCR (qRT-PCR) (Fig. 1D,G), western blotting (Fig. 1E,F) and ELISA (Fig. 1H). Since eosinophil recruitment in this model depends on VCAM-1 and CCL26 (Cuvelier and Patel, 2001; Patel, 1998; Patel, 1999), our data suggest that this is the mechanism by which FRNK blocks eosinophil recruitment. Our data also suggest that FRNK acts early to prevent the transcription of these key mediators. We, therefore, next examined the effect of FRNK on transcription factors involved in IL-4 activation of endothelial cells.

FRNK has no effect on IL-4-induced expression, phosphorylation or nuclear translocation of STAT6

IL-4 activates human endothelial cells by binding to the type II receptor comprising IL4Ra and IL13Ra, also known as the interleukin 13 receptor, subunit α1 (IL13RA1) (Schnyder et al., 1996), which results in the rapid phosphorylation of JAK2 and STAT6 (Palmer-Crocker et al., 1996). Phosphorylated STAT6 translocates to the nucleus (Khew-Goodall et al., 1999; Schnyder et al., 2002) where it orchestrates the transcription of target genes including those encoding VCAM-1 (Tozawa et al., 2011) and CCL26 (Hoeck and Woisetschläger, 2001). As previously reported, we found that IL-4 increased phosphorylation of STAT6 within 15 minutes of stimulation (Fig. 2A). Expression of FRNK had no effect on STAT6 phosphorylation or on total STAT6 protein levels (Fig. 2A,B). We then examined translocation of phosphorylated STAT6 (P-STAT6) to the nucleus and found that FRNK had no effect on the ability of P-STAT6 to translocate to the nucleus (Fig. 2C).

Because STAT6 was unaffected by FRNK we next examined GATA6, another transcription factor implicated in VCAM-1 gene regulation. Although GATA6 has been shown to regulate VCAM-1 gene expression in HUVECs in response to TNFα, a role for GATA6 in IL-4-mediated VCAM-1 gene expression has not been examined. For this reason, we first examined the ability of IL-4 to regulate GATA6 gene expression. Under baseline conditions, GATA6 mRNA expression was low but measurable (Fig. 2D). As previously reported, its family member GATA4 was not detectable by qRT-PCR (Song et al., 2009; Umetani et al., 2001). We found that IL-4 increased GATA6 mRNA and protein expression (Fig. 2D,E).

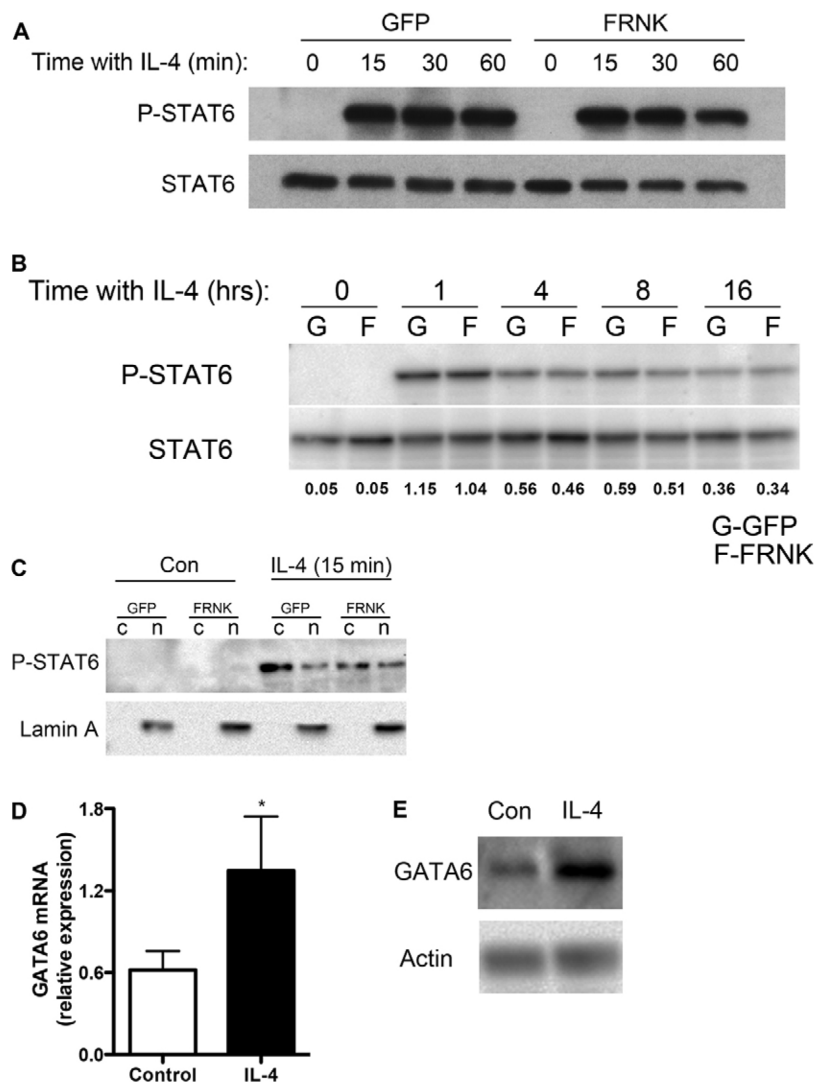

Fig. 2. STAT6 phosphorylation and GATA6 expression. (A-C) HUVECs were transduced with GFP alone or FRNK–GFP prior to stimulation with 20 ng/ml IL-4. After the specified times, cells were either (A,B) lysed and probed for phosphorylated-STAT6 (P-STAT6) then stripped and reprobed for expression of total STAT6, or (C) harvested in cell fractionation media and the cytoplasmic (c) and nuclear (n) fractions were separated, run on SDS-PAGE, and probed for P-STAT6. Lamin A was used to identify the nuclear fraction and demonstrate successful fractionation. (D,E) Alternatively, HUVECs were stimulated with 20 ng/ml IL-4 for 24 hours and cells were either harvested for mRNA or lysed for western blotting. (D) qRT-PCR was performed to measure GATA6 mRNA as described in Materials and Methods. Data were normalized using GAPDH. (E) In parallel experiments, proteins were blotted for GATA6. Con, control. Data in A, C and E are representative of four independent experiments. Time points in B of 0–4 hours are representative of four independent experiments and the data shown were quantified and normalized using total STAT6 as a loading control. Data in D are mean±s.e.m. of ten experiments; *$P<0.05$.

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Effect of GATA6 on IL-4-mediated expression of VCAM-1 and CCL26

Small interfering RNA (siRNA) was used to downregulate GATA6 to determine whether GATA6 regulates IL-4-mediated expression of VCAM-1 or CCL26. We also examined the role of GATA6 in eosinophil recruitment. siRNA decreased GATA6 gene and protein expression to levels observed prior to stimulation with IL-4 (Fig. 3A,B). Downregulation of GATA6 attenuated VCAM-1 mRNA and protein expression (Fig. 3C,D). In contrast, downregulation of GATA6 had no effect on CCL26 mRNA and protein expression (Fig. 3E,F). Downregulation of GATA6 attenuated eosinophil accumulation and transmigration, but had no effect on the conversion from rolling to firm adhesion (Fig. 4A-C). This is consistent with a decrease in VCAM-1 gene and protein expression, and the unchanged expression of CCL26. Indeed, we previously showed that using a mAb directed against VCAM-1 had similar results (Patel, 1998; Cuvelier et al., 2001). Although GATA6 only affects VCAM-1, we next determined whether GATA6 mRNA or protein expression is regulated by FRNK. We found that expression of FRNK reduced GATA6 mRNA to control levels (Fig. 4D). When GATA6 protein was examined we found that FRNK caused only a modest decrease in GATA6 protein expression (Fig. 4E). These data suggest that GATA6 plays a role in the mechanism by which FRNK exerts its effect – yet there are clearly other targets involved.

FRNK acts independently of FAK and Pyk2 signaling to regulate IL-4-mediated expression of VCAM-1 and CCL26

FRNK contains a focal-adhesion-targeting domain and can interfere with FAK signaling at focal adhesions in part by preventing FAK auto-phosphorylation and blocking downstream signals (Shen et al, 1999; Nolan et al, 1999; Heidkamp, 2002). For this reason, FRNK is frequently used as a FAK dominant-negative protein. To determine whether FRNK is acting via its actions on FAK signaling, we downregulated FAK itself. If FRNK acts via FAK signaling, we would expect that downregulation of FAK would yield the same result as expression of FRNK. If FRNK acts independently of FAK signaling, downregulation of FAK would have no effect on expression of VCAM-1 or CCL26. siRNA targeting FAK decreased total expression of FAK by >85% and blocked FAK signaling, as measured by auto-phosphorylation (Fig. 5A). Downregulation of FAK had little to no effect on IL-4-induced mRNA expression of either VCAM-1 or CCL26 (Fig. 5B,C) and increased mRNA for GATA6 (Fig. 5G). Similarly, downregulation of FAK had no effect on protein expression of VCAM-1 (Fig. 5C,D) or CCL26 (Fig. 5E,F).

Protein-tyrosine kinase 2β (PTK2B, hereafter referred to as Pyk2) is another protein to consider when exploring the mechanism by which FRNK is functioning. Pyk2 is functionally related to FAK, expressed in endothelial cells and can also be regulated by FRNK (Govindarajan et al., 2000;

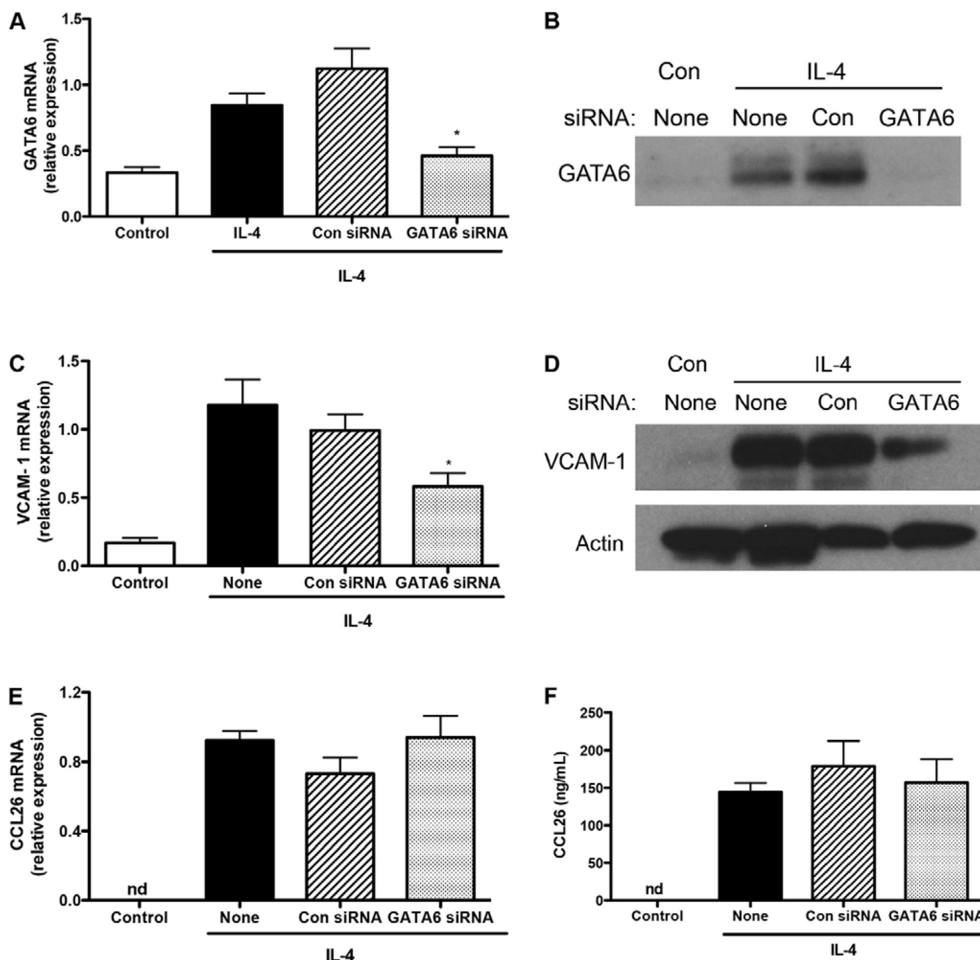

Fig. 3. GATA6 is required for maximal expression of VCAM-1 but is not required for CCL26 expression. HUVECs were pre-treated for 24 hours with control siRNA or siRNA targeting GATA6. (A-E). Following siRNA treatment, cells were stimulated with 20 ng/ml IL-4. After 24 hours, cells were either harvested for mRNA (A,C,E) or lysed for western blotting (B,D) and supernatants were collected for ELISA (F). qRT-PCR was performed to measure mRNA for (A) GATA6, (C) VCAM-1 or (E) CCL26 and the data were normalized using GAPDH as described in the Materials and Methods. In parallel experiments, proteins were blotted for (B) GATA6, (D) VCAM-1 or (F) ELISA was performed on supernatants to measure CCL26 release. Data in A, C, E and F are mean±s.e.m. of five experiments; $^*P<0.05$. (E,F) nd, not detectible as compared to the siRNA control. Blots in B and D are representative of four independent experiments.

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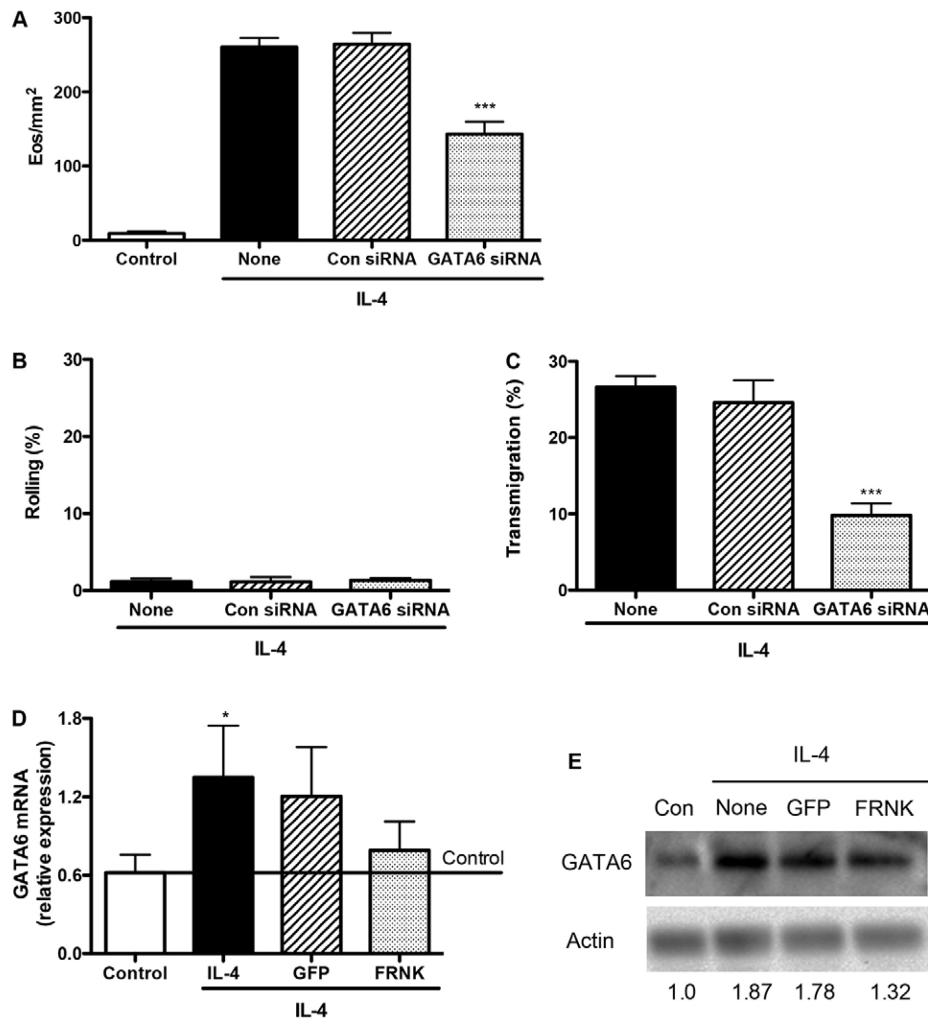

Fig. 4. GATA6 is required for maximal eosinophil accumulation and transmigration. HUVECs were pre-treated for 24 hours with control siRNA or siRNA targeting GATA6 and were then stimulated with 20 ng/ml IL-4. After 24 hours, cells were placed in a parallel-plate flow chamber and eosinophils (0.5×10^6/ml) were perfused across at 1 dyn/cm^2 as described in Fig. 1. (A) Total accumulation, (B) rolling and (C) transmigration were determined as described in Materials and Methods. (D,E) HUVECs were transduced with GFP or FRNK–GFP, stimulated with 20 ng/ml IL-4 for 24 hours and then either harvested for mRNA or lysed for western blotting. (D) qRT-PCR was performed to measure GATA6 mRNA as described in Materials and Methods. Data were normalized against GAPDH. (E) In parallel experiments, proteins were blotted for GATA6; the data were quantified and normalized using actin as a loading control. Data in A–C are mean±s.e.m. of five experiments. Data in D show the mean±s.e.m. of eight experiments. Data in E are representative of four independent experiments and relative expression values are shown. *$P<0.05$ as compared to unstimulated control, ***$P<0.001$ as compared to control siRNA.

Schaller, 2010; Vockel and Vestweber, 2013). For this reason, we examined Pyk2 in our model system. siRNA targeting Pyk2 substantially decreased expression of Pyk2 mRNA and protein following stimulation with IL-4, as measured by qRT-PCR and western blotting (Fig. 6B,C). siRNA targeting Pyk2 had no effect on FAK expression, and siRNA targeting FAK did not change expression of Pyk2 (Fig. 6A-C). Downregulation of Pyk2 had little to no effect on IL-4-induced protein expression of either VCAM-1 or CCL26 (Fig. 6C,D). Similarly, combined downregulation of Pyk2 and FAK had no effect on VCAM-1 or CCL26 protein expression (Fig. 6C,D). When we examined eosinophil recruitment, we found that neither downregulation of FAK or Pyk2 alone, nor downregulation of both proteins together had an effect on eosinophil accumulation or transmigration (Fig. 6E,F). Together, these data suggest that FRNK acts independently of FAK and Pyk2 signaling, in order to block VCAM-1 and CCL26 expression.

IL-4 increases the expression of endogenous FRNK

We next determined whether IL-4 regulates the expression of endogenous FRNK. Endothelial cells were treated with IL-4 for 24, 48 or 72 hours, and mRNA and protein were harvested to determine gene and protein expression of FRNK. The primers chosen for qRT-PCR were from the region 5′ of the FRNK start site that is unique to FRNK (Fig. 7A). As a control, we also examined FAK expression by using primers from the FERM domain, a region not shared with FRNK (Fig. 7A). Stimulation with IL-4 for 24 hours increased expression of FRNK mRNA, but had no effect on FAK mRNA (Fig. 7B). Protein expression was determined by western blotting using an antibody that recognizes both FAK and FRNK (Fig. 7A). The proteins were differentiated by their molecular mass with FAK running at 125 kDa and FRNK running at 45 kDa. Stimulation with IL-4 increased FRNK protein expression at 24 hours with levels remaining elevated at 48 hours (Fig. 7C,D).

DISCUSSION

Resolving inflammation is crucial in order to restore homeostasis following infection or injury. A wealth of data has been published on mechanisms that positively regulate the adhesion and activation molecules that are crucial for leukocyte recruitment, yet much less is known about the mechanisms that negatively regulate their gene and protein expression. In this study, we provide data to support a role for FRNK in negatively regulating IL-4-mediated VCAM-1 and CCL26 gene and protein expression. We have previously shown that eosinophil accumulation, firm adhesion and transmigration depend on the expression of VCAM-1 and CCL26 (Cuvelier and Patel, 2001; Patel, 1998; Patel, 1999). By preventing expression of these crucial eosinophil adhesion and activation proteins, FRNK blocked eosinophil recruitment under

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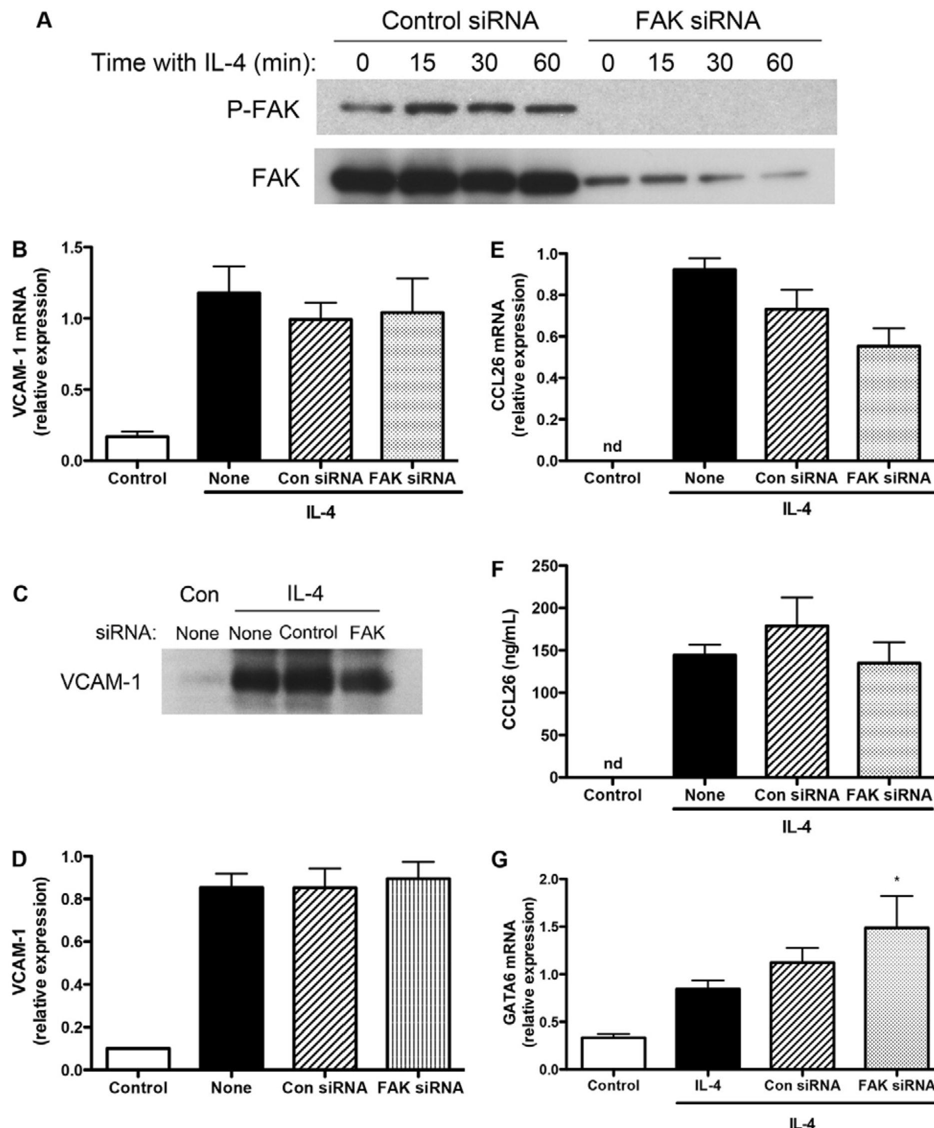

Fig. 5. Downregulation of FAK with siRNA has no effect on expression of VCAM-1 or CCL26. HUVECs were pre-treated for 24 hours with control siRNA or siRNA targeting FAK. (A) Cells were then stimulated for the specified times with 20 ng/ml of IL-4, lysed and blotted for phosphorylated FAK (P-FAK). Blots were stripped and re-probed for total FAK expression. (B-G) Following siRNA treatment, HUVECs were stimulated for 24 hours with 20 ng/ml IL-4, and were either harvested for mRNA or lysed for western blotting and supernatants were collected for ELISA. qRT-PCR was performed to measure mRNA for (B) VCAM-1, (E) CCL26 or (G) GATA6 as described in Materials and Methods. Data were normalized against GAPDH. In parallel experiments, proteins were western blotted for (C and quantified in D) VCAM-1, or (F) ELISA was performed on supernatants to measure CCL26 release. Data are the mean±s.e.m. of five experiments. *$P<0.05$, and nd, not detectible as compared to control siRNA. Representative blots from five experiments are shown in A and C.

flow conditions to endothelial cells stimulated with IL-4. These are the first data showing that FRNK acts as a negative regulator of IL-4-mediated inflammation in human endothelial cells.

We used IL-4 to model Th2 inflammation. IL-4 is an important cytokine in models of allergic disease and asthma (Grünig et al., 1998; Luzina et al., 2012; Pelaia et al., 2012; Ricci et al., 1997). In endothelial cells IL-4 exerts its effects through the IL-4 type II receptor, IL13RA1 (Palmer-Crocker et al., 1996; Schnyder et al., 1996), resulting in the expression of STAT6-responsive proteins including those involved in eosinophil recruitment (Cuvelier & Patel, 2001; Hoeck and Woisetschlager, 2001; Miyazaki et al, 2006; Patel, 1998; Tozawa et al, 2011). Although STAT6 is crucial for VCAM-1 and CCL26 expression, FRNK had no effect on STAT6 phosphorylation, nuclear localization or total protein expression (Fig. 5). This suggests that FRNK acts independently of STAT6.

The GATA family of transcription factors can also regulate VCAM-1 mRNA expression, with GATA4 regulating its expression in mouse fibroblasts (Lim et al., 2012) and GATA6 acting in human endothelial cells (Tsoyi et al., 2010). In both cases, TNFα was used to induce VCAM-1. Intriguingly, FAK was shown to play a role in TNFα-induced VCAM-1 expression in mouse fibroblasts and HUVECs. In fibroblasts, it is the FERM domain acting through GATA4 that was shown to be involved, and the FAT domain had no effect on GATA4 expression (Lim et al., 2012). Furthermore, the FAT domain (FRNK) had no effect on TNFα-induced VCAM-1 expression in HUVECs.

GATA4 is not expressed in HUVECs (Song et al., 2009; Umetani et al., 2001), suggesting that different mechanisms are at play in our model. Because HUVECs express GATA6 (Fig. 2 and (Song et al., 2009; Umetani et al., 2001) and TNFα-induced GATA6 can regulate expression levels of VCAM-1 (Tsoyi et al., 2010), we focused on GATA6. When we examined the ability of IL-4 to induce GATA6, we showed for the first time that stimulating endothelial cells with IL-4 increased GATA6 mRNA and protein expression. Using siRNA, we showed that GATA6 downregulation blocks the ability of IL-4 to increase VCAM-1 mRNA and protein expression, suggesting that GATA6 acts upstream of VCAM-1. This is similar to what has been found following stimulation of HUVECs with TNFα (Tsoyi et al., 2010). In contrast, GATA6 downregulation had no effect on CCL26 expression. GATA6 downregulation had an intermediate effect on eosinophil recruitment, consistent with decreased expression of VCAM-1 but normal expression of CCL26. The inability of

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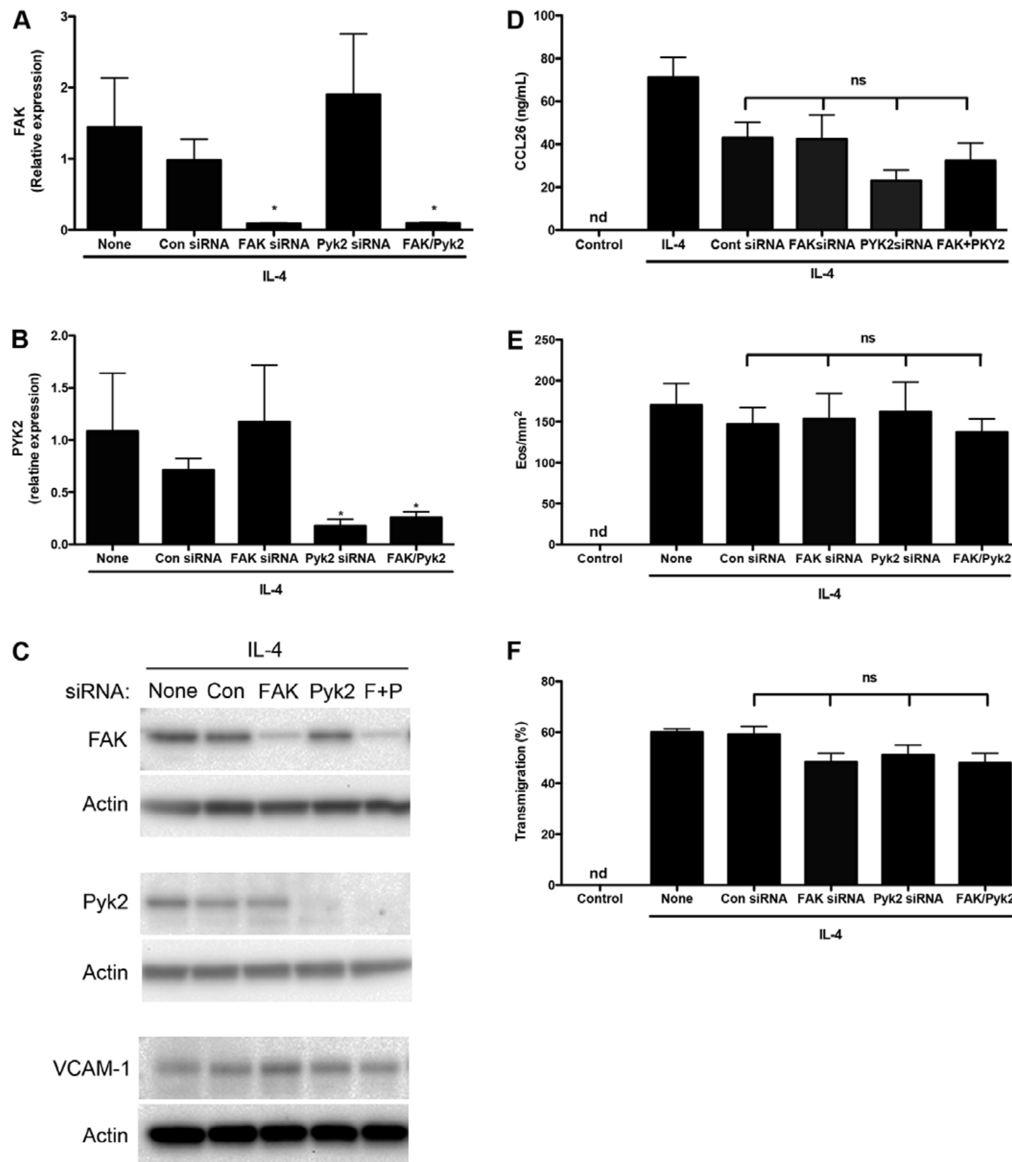

Fig. 6. Combined downregulation of FAK and Pyk2 has no effect on VCAM-1 expression, CCL26 expression or eosinophil recruitment. HUVECs were pre-treated for 24 hours with control siRNA, or siRNA directed against FAK, Pyk2 or both FAK and Pyk2 together (FAK/Pyk2). Following siRNA treatment, HUVECs were stimulated for 24 hours with 20 ng/ml IL-4 and cells were either harvested for mRNA or lysed for western blotting and supernatants were collected for ELISA. qRT-PCR was performed to measure mRNA for (A) FAK or (B) Pyk2 and the data were normalized using GAPDH as described in the Materials and Methods. (C) In parallel experiments, proteins were blotted for FAK, Pyk2, or VCAM-1, and (D) ELISA was performed on supernatants to measure CCL26 release. (E,F) After stimulation with IL-4 (20 ng/ml), HUVECs were placed in a parallel-plate flow chamber and eosinophils (0.5×10^{6}/ml) were perfused across at 1 dyn/cm^2 as described for Fig. 1. (E) Total accumulation, and (F) transmigration were determined as described in Materials and Methods. Data are shown as the mean±s.e.m. of three to six experiments. *$P<0.05$; nd, not detectable; ns, not significant as compared to control siRNA. Blots in C are representative of six independent experiments.

GATA6 downregulation to block CCL26 expression might be due to the fact that GATA6 itself is regulated by IL-4. GATA6 levels simply might not be high enough immediately after stimulation with IL-4 to have an effect on the induction of CCL26. Our data on GATA6 reveal a new mechanism to regulate VCAM-1 and eosinophil recruitment in IL-4-stimulated endothelial cells.

FRNK attenuated the IL-4-induced increase in GATA6 expression. FRNK had a significant effect on GATA6 mRNA expression, yet the effect on protein expression at 24 hours was limited. These data, combined with the fact that GATA6 had no effect on CCL26, suggest that FRNK-mediated regulation of GATA6 is just one component of the mechanisms by which FRNK negatively regulates eosinophil recruitment. Our data raise the question of how exactly FRNK blocks VCAM-1 and CCL26 mRNA expression, when FRNK has no effect on STAT6 and only a limited effect on GATA6. Since STAT6 alone is sufficient to drive CCL26 transcription, it might be that FRNK affects the ability of P-STAT6 to bind to the promoter of these genes. Alternatively FRNK might act independently on transcription by regulating mRNA stability. Whether FRNK does or does not regulate the traditional transcriptional pathways is an important first step towards finding out how FRNK prevents the gene expression of VCAM-1 and CCL26.

FRNK is typically used to block FAK signaling. FRNK is transcribed from the C-terminus of the *Ptk2* gene (also encoding FAK) (Fig. 7A) and can displace FAK from focal adhesions (Heidkamp et al., 2002), prevent FAK autophosphorylation and block signaling (Shen and Schaller, 1999). FRNK can also block FAK signaling by acting as a competitive inhibitor for other FAK-binding proteins that influence signaling (Neff et al., 2003; Shen and Schaller, 1999; Schaller, 2010). To determine whether FRNK acts by blocking FAK signaling, we downregulated FAK, which will block all signaling from FAK. Despite blocking >85% of FAK protein expression and completely blocking FAK phosphorylation, downregulation of FAK had no effect on GATA6, VCAM-1 or CCL26 expression. Pyk2 is functionally related to FAK, expressed in endothelial cells, and can also be regulated by FRNK (Govindarajan et al., 2000; Schaller, 2010; Vockel and Vestweber, 2013). When we examined the effect of Pyk2 knockdown alone or in conjunction with FAK in this model

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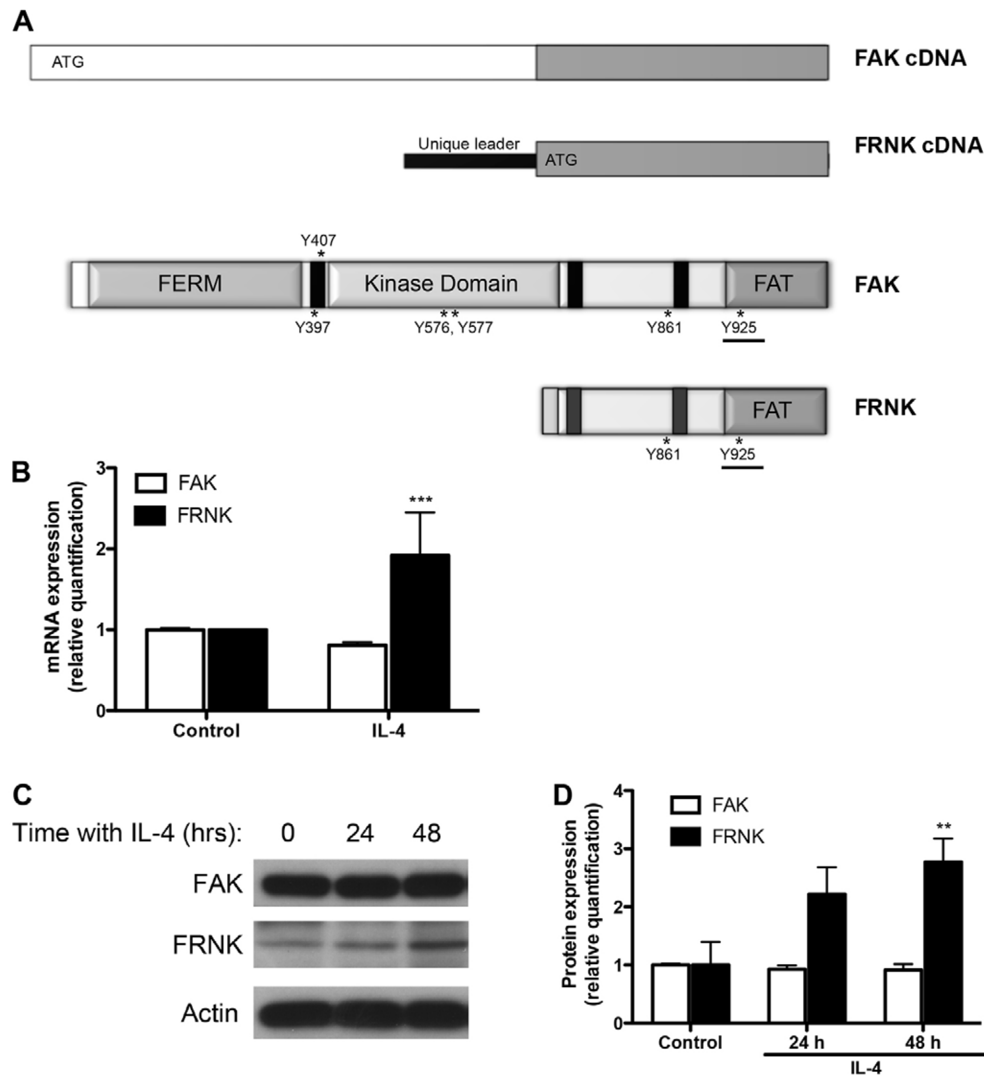

Fig. 7. IL-4 selectively increases endogenous FRNK in HUVECs. (A) Diagrams showing a comparison of FAK and FRNK cDNA and protein are shown. HUVECs were stimulated with 20 ng/ml IL-4. (B) After 24 hours, cells were harvested for mRNA and qRT-PCR was performed to measure mRNA for FAK and FRNK. Primers unique for FAK were based on sequences in the FERM domain and primers unique for FRNK were based on sequences in the unique leader sequence of FRNK. Data were normalized based on GAPDH expression as described in Materials and Methods. (C) Alternatively, cells were stimulated for 24 or 48 hours then lysed for western blots and FAK and FRNK expression was determined by western blotting using an antibody that recognizes a peptide sequence in the FAT domain as indicated in Fig. 7A and as described in Materials and Methods. (D) The data were normalized using actin as a loading control and densitometry was performed as described in Materials and Methods. Data in B and D are the mean±s.e.m. of five or four experiments, respectively. $^{**}P<0.01$, $^{***}P<0.001$. A representative blot is shown in C.

system, we saw no effect on VCAM-1 or CCL26 expression and no effect on eosinophil recruitment. These are the first data to show a role for FRNK in repressing the effect of pro-inflammatory mediators independently of FAK or Pyk2 signaling. Because most studies focus on the ability of FRNK to block the kinase activity of FAK, little is known about the other functions of FRNK. Discrepancies between the phenotypes of cells expressing no FAK, kinase dead FAK, auto-phosphorylation-deficient FAK and FRNK (Heidkamp et al., 2002; Richardson et al., 1997; Nolan et al., 1999; Schaller et al., 1993) have pointed to independent roles for FRNK.

When considering the mechanisms by which FRNK might act, lessons can be learned by closely examining the other scaffolding domain of FAK. Like FRNK, the FERM domain serves as a scaffold, binding to proteins such as p53 (Lim et al., 2010) and GATA4 (Lim et al., 2012). By bringing these proteins together in a complex with MDM2 and the E3 ubiquitin-protein ligase CHIP, respectively, the FERM domain facilitates poly-ubiquitylation and degradation of p53 (Lim et al., 2010) and GATA4 (Lim et al., 2012). It is possible that FRNK similarly affects the expression of transcription factors that are important for VCAM-1 and CCL26 gene expression in HUVECs.

Unlike the FERM domain of FAK, FRNK is autonomously expressed and has the potential to be independently regulated. We tested this hypothesis by determining whether FRNK is regulated by IL-4 itself, thus setting up a negative feedback loop to help shut down inflammation. Indeed, we found that stimulation with IL-4 increased expression of endogenous FRNK at 24 and 48 hours.

Based on these data, we propose a model in which FRNK acts to resolve IL-4-induced inflammation (Fig. 8). Stimulation of HUVECs with IL-4 increases protein expression of GATA6, which in turn increases VCAM-1 mRNA and protein. IL-4 also increases CCL26 mRNA and protein. These mediators act together to bind and activate eosinophils. At the same time, IL-4 induces FRNK expression. Once the protein is expressed, we speculate that FRNK acts as part of a negative feedback loop to suppress VCAM-1 and CCL26 protein expression, and limit eosinophilic inflammation. Given the lack of a nuclear localization sequence, we think that FRNK prevents transcription of VCAM-1, CCL26 and GATA6 through an as yet, unknown intermediary.

This study has revealed a novel role for FRNK in the negative regulation of VCAM-1, CCL26 and – to a lesser extent – GATA6 in IL-4-stimulated endothelial cells, resulting in decreased eosinophil recruitment. Our data are particularly compelling because we also show that stimulation with IL-4 increased FRNK

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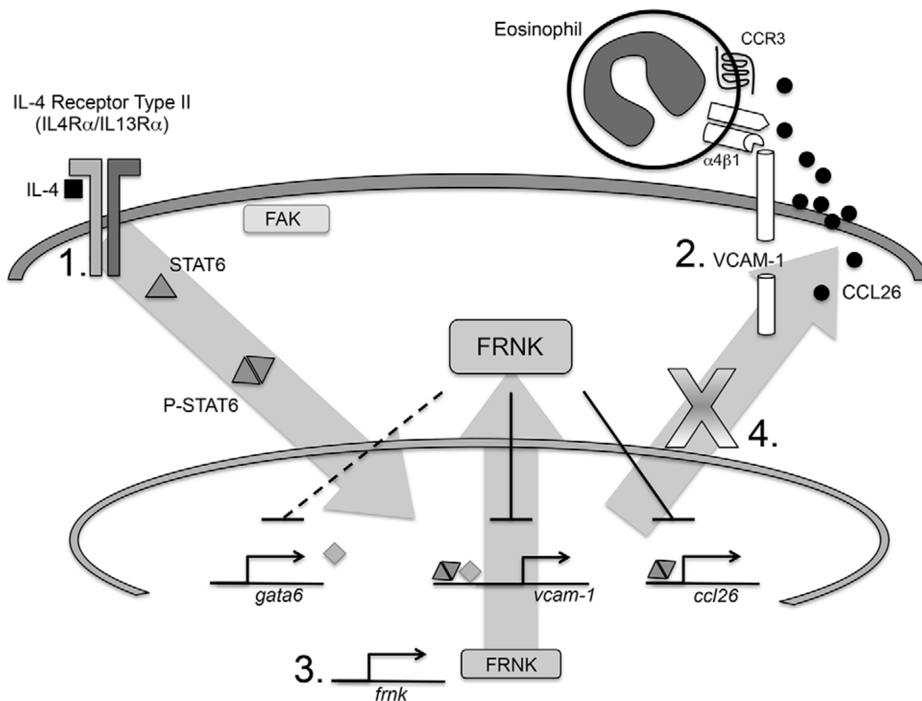

Fig. 8. Model of IL-4-mediated recruitment eosinophils. (1) IL-4 binds to the IL-4 Receptor Type II (IL13RA1) on endothelial cells leading to increased phosphorylation of STAT6. Phosphorylated STAT6 (P-STAT6) dimerizes and translocates to the nucleus where it induces the transcription of the genes encoding VCAM-1 and CCL26. IL-4 also induces transcription and protein expression of GATA6 that in turn enhances transcription and protein expression of VCAM-1. (2) VCAM-1 and CCL26, together with P-selectin, mediate eosinophil accumulation, firm adhesion and transmigration (Patel, 1998) (Cuvelier and Patel, 2001). (3) IL-4 increases the transcription and protein expression of FRNK. (4) FRNK acts independently of FAK signaling to block transcription and subsequent protein expression of VCAM-1 and CCL26. In the absence of VCAM-1 and CCL26, eosinophils are no longer recruited.

expression, suggesting that FRNK might represent an endogenous mechanism to limit IL-4-mediated inflammation. The ability of a naturally occurring variant of FAK to negatively regulate these pro-inflammatory molecules suggests a role for FRNK in the resolution phase of inflammation.

MATERIALS AND METHODS

Materials

Medium199, penicillin/streptomycin/glutamine, Hank's buffered saline solution (HBSS), trypsin-EDTA, lymphoprep 1077, OPTI-MEM, TRIzol and Bisbenzimide H33258 were purchased from Invitrogen Life Technologies (Burlington, Ontario). Dextran was from Sigma (Oakville, Canada). Human plasma albumin was from Gemini Bio-Products (Sacramento, CA). High-capacity cDNA Reverse Transcription kit, TaqMan Gene Expression Master Mix, and SYBR Green PCR Master Mix were purchased from Applied Biosystems (Burlington, Ontario, Canada). siRNA duplexes, HiPerFect, and RNeasy Lipid Tissue Mini kit were from Qiagen Incorporated (Mississauga, Ontario). Triton X-100 was purchased from Calbiochem-Novabiochem Corporation (San Diego, CA). The parallel-plate flow chambers used were the µ-slide VI from ibidi (Munich, Germany) and the circular flow model from GlycoTech (Rockville, Maryland). All other chemicals were from BDH Incorporated (Toronto, Canada). Plasticware was from VWR International (West Chester, PA) and Becton Dickinson (Franklin Lakes, NJ).

Antibodies and proteins

Antibodies against FAK and phosphorylated FAK were purchased from Upstate USA (Charlottesville, VA). C20 anti-FAK antibody targets the C-terminal region and recognizes FAK; FRNK was from Santa Cruz Biotechnology (Dallas, TX). Antibodies against P-STAT6, STAT6 and GATA6 were from New England Biolabs (Whitby, Ontario). The antibody against VCAM-1 as well as a non-immune IgG control were from R&D Systems (Minneapolis, MN). IL-4, CCL26, and antibodies against CCL26 and biotinylated CCL26 were from PeproTech Incorporated (Rocky Hill, NJ). Antibodies against CD16 and CD3 conjugated to paramagnetic beads were from StemSep (Vancouver, Canada). Fibronectin was from Biomedical Technologies Incorporated (Stoughton, MA). Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ).

Cell isolation and culture

Human eosinophils were isolated by density centrifugation through lymphoprep 1077, followed by negative selection using magnetic beads conjugated to anti-CD16 and anti-CD3 antibodies as previously described (Cuvelier and Patel, 2001). Eosinophils were typically >95% pure, with lymphocytes as the main contaminating cell type. Endothelial cells were isolated from human umbilical cords (Foothills Hospital, Calgary, Alberta, Canada) as previously described, HUVECs were maintained in M199 medium (Invitrogen, Carlsbad, CA) supplemented with 20% human serum (Ganguly et al., 2012). Cells were used at first passage. The University of Calgary Conjoint Health Research Ethics Board approved all procedures requiring human subjects.

Adenoviral transduction and siRNA transfection of endothelial cells

N-terminal GFP-tagged FRNK adenoviral vector (Adv-FRNK-GFP) was a gift from Samarel (Layola University, Chicago, IL) who had previously used the construct in endothelial cells (Holinstat et al., 2006). Adenoviral GFP was from the Libin Gene Therapy Unit (University of Calgary, Calgary, AB). Endothelial cells that were between 80%–95% confluence were infected with Adv-FRNK-GFP or Adv-GFP. Endothelial cells were used 24–48 hours post infection as described previously (Parsons et al., 2012). The siRNA transfection protocol was performed using HiPerFect according to the manufacturer's instructions as described previously (Parsons et al., 2012). Live-cell imaging and western blotting, respectively, were used to assess the efficiency of FRNK transduction and FAK or GATA6 downregulation. Maximal levels of downregulation were achieved 48 hours post-transfection.

Western blotting

Endothelial cells were lysed in hot Laemmli buffer. The proteins were separated by SDS-PAGE, transferred to a PVDF membrane and probed with the specified antibodies followed by an HRP-conjugated secondary antibody. Protein bands were visualized using chemiluminescence detection either on film or on a Fluor-S MAX multi-imager (Bio-Rad Laboratories). The membranes were then stripped in IgG elution buffer and re-probed with an antibody directed either against the total protein or actin to determine protein loading. Relative expression was determined by densitometry using NIH ImageJ, and data were normalized against

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actin expression. Images shown were adjusted for contrast and brightness only, and cropped based on antibody binding and molecular mass (MW) standards. Images from the Fluor-S MAX were also adjusted for size. MW standards are not visible on the blots shown.

Quantitative real-time PCR

Treated endothelial cells were lysed using TRIzol reagent and RNA was isolated using RNeasy Lipid Tissue Mini kit. cDNA was generated using the High-capacity cDNA Reverse Transcription kit according to the manufacturer's instructions. VCAM-1, CCL26, GATA4, GATA6, FAK and FRNK mRNA expression was determined conducting quantitative real-time PCR (qRT-PCR) using TaqMan Universal PCR Master Mix. The probes and primers used were VCAM-1: forward, 5′-CATGGAATTCGAACCCAAACA-3′, reverse 5′ GGCTGACCAAGA-CGGTTGTATC-3′, probe: [6FAM]CAAACACTTTATGTCAATGTT-GCCCCCAG[TAM]; CCL26: forwar 5′-ACACGTGGGAGTGACATA-TCCA-3′, reverse 5′-GACTTTCTTGCCTCTTTTGGTAGTG-3′, probe: [6FAM]TACAGCCACAAGCCCCTTCCCTGG[TAM]; GATA6: forward 5′-AGAAACGCCGAGGGTGAAC-3′, reverse 5′-GCACCCCATGGA-GTTTCATG-3′; FRNK: forward 5′-GTGGCCTGTCTTCTGGACTC-3′, reverse 5′-AGGACGAGGGTTTCAAACTG-3′. GATA4 (Hs00171403_m1), FAK (Hs01050460_m1) and Pyk2 (Hs00168444_m1) primers and probes were from Applied Biosystems. GAPDH was used as an endogenous control. qRT-PCR was performed on a StepOnePlus system from Applied Biosystems (Burlington, Ontario, Canada), and data analysis was performed using the associated software. The $2^{\Delta\Delta Ct}$ method was used to analyze the data. Gene expression was normalized agaisnt GAPDH for each sample and expressed as fold change.

Eosinophil recruitment under flow conditions

Interactions between endothelial cells and freshly isolated human eosinophils were examined under flow conditions as described previously (Cuvelier and Patel, 2001). A parallel plate flow chamber was used to mimic the hydrodynamic conditions found in post-capillary venules *in vivo*. Fluid shear stress in this system is calculated using the equation $\tau_w = \mu\gamma = 6\mu Q/a^2 b$ as described (Lawrence et al., 1990). Eosinophil accumulation, rolling, firm adhesion and transmigration were determined as previously described. Confluent endothelial cells were stimulated for 24 hours with 20 ng/ml of IL-4. In some experiments, endothelial cells were first pre-treated with siRNA, transduced with Adv-FRNK or Adv-GFP or treated with FAK inhibitors. DMSO alone was used as a vehicle control when appropriate. After stimulation, a parallel-plate flow chamber was assembled and eosinophils (0.5×10^6/ml) were perfused across the monolayer at 1 dyn/cm^2. After 4 minutes of perfusion, the inlet line was transferred to HBSS for an additional 6 minutes, in order to prevent the binding of new eosinophils. Interactions between eosinophils and endothelial cells were visualized on a Zeiss Axiovert 100 microscope using either a 10×/0.25 NA or 40×/0.60 NA phase-contrast objective and recorded by using a charge-coupled device camera (KP-M1U; Hitachi Denshi, Ltd). The total number of cells accumulated on the monolayer was determined at 5 minutes of perfusion, and the numbers of rolling, firmly adherent and transmigrated cells were determined at 10 minutes as described (Cuvelier and Patel, 2001; Parsons et al., 2012). For each condition, four to ten fields of view were examined.

Statistics

All experiments were performed at least three times. The data were analyzed by *t*-test when comparing two groups and analysis of variance with the appropriate post-tests when comparing more than two groups. Nonparametric tests were used when required. $P<0.05$ was considered significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

K.D.P. wrote the manuscript. R.S., P.C., H.Z. and K.D.P. conceived and/or designed the experiments. R.S., H.Z. and K.D.P. performed the experiments. All authors edited and provided a critical review of the manuscript. All authors discussed the project at all stages.

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