Focal adhesion kinase-related nonkinase (FRNK) negatively regulates IL-4-mediated inflammation

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Running Title: FRNK blocks IL-4-mediated eosinophil recruitment

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¹ This research was funded by an operating grant from the Canadian Institutes of Health Research. This work was also supported by an equipment and infrastructure grant from the Canadian Foundation for Innovation and the Alberta Science and Research Authority.
Abstract
Focal adhesion kinase (FAK)-related nonkinase (FRNK) is a cytoskeletal regulatory protein recently shown to dampen lung fibrosis, yet its role in inflammation is unknown. Here we show for the first time that expressing FRNK negatively regulates IL-4 inflammation using a human model of eosinophil recruitment. Mechanistically, FRNK blocked 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 IL-4 also increases GATA6 to induce VCAM-1 expression. FRNK blocked IL-4-induced GATA6 transcription, but had little effect on GATA6 protein, and had no effect on STAT6 activation. FRNK can block FAK or Pyk2 signaling, thus we down-regulated these proteins with siRNA to determine if signaling from either protein was involved in regulating VCAM-1 and CCL26. Knocking down FAK, Pyk2 or both had no effect on VCAM-1 or CCL26 expression suggesting 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 for negatively regulating IL-4 inflammation.

Keywords: cell adhesion/endothelial cells/inflammation/leukocyte trafficking
Introduction

Chronic inflammatory diseases are characterized by the inappropriate and unregulated infiltration of leukocytes into the tissue (Ley et al, 2007; Springer, 1994). Cytokines like TNFα and 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 Th2 cytokine that is increased in allergic diseases and asthma and influences the development of allergen specific Th2 cells (Humbert et al, 1996) (Erb & 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 comprised of IL4Rα and the common gamma chain, and the type II receptor comprised of IL4Rα and IL13Rα (Kolaczkowska & Kubes, 2013). In endothelial cells, IL-4 acts solely through the IL-4 type II receptor (Schnyder et al, 1996), leading to increased JAK2 and STAT6 phosphorylation (Palmer-Crocker et al, 1996). Upon phosphorylation, STAT6 translocates into the nucleus where it regulates the expression of over 900 genes including VCAM-1 (Hopkins, 2013), CCL26 (Hoeck & Woisetschlager, 2001) and P-selectin (Adamsson Eryd et al, 2012; 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 & 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 focal adhesion kinase (FAK) related non-kinase (FRNK) in negatively regulating pulmonary fibrosis (Ding et al, 2013). We examined the role of FRNK and the related protein FAK in IL-4-mediated inflammation.

FAK 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 multiple 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.
(Ganguly et al, 2013; Heidkamp et al, 2002; Moreno et al, 2013). For example, the FERM domain also has scaffolding functions independent of kinase activity as shown by its ability to bind MdM2 E3 ligase and p53 leading the ubiquitination and degradation of p53 resulting in protection from apoptosis (Casini et al, 2010). FRNK is autonomously transcribed from the fak gene and contains only the C-terminal FAT domain, and thus lacks kinase activity (Fredj et al, 2006; Moreno et al, 2013). FRNK can act as a dominant negative regulator of FAK kinase activity by displacing FAK at focal adhesions. FRNK can also bind to proteins such as paxillin, p130Cas, GRB2 and p190 RhoGEF, preventing them from binding to FAK (Schaller, 2010).

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

Expressing FRNK in HUVEC blocks IL-4-mediated eosinophil accumulation, firm adhesion and transmigration. Primary human endothelial cells were transduced with Adv-FRNK-GFP. As previously described, greater 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 HUVEC were stimulated with IL-4 for 24 hours and then eosinophil accumulation, rolling, firm adhesion and transmigration under flow conditions were measured. As previously reported, IL-4 alone increased eosinophil recruitment (Cuvelier & Patel, 2001; Moser et al, 1992) (Figure 1 A-C). Transducing cells with GFP prior to IL-4 stimulation had no effect on any of the eosinophil recruitment parameters examined (Figure 1 A-C). Transducing cells with FRNK-GFP prior to IL-4 stimulation blocked eosinophil accumulation (Figure 1A), prevented the transition from rolling to firm adhesion resulting in increased % rolling (Figure 1B) and completely blocked transmigration (Figure 1C).

FRNK blocks IL-4-mediated VCAM-1 and CCL26 expression. To understand the mechanism by which FRNK acted to block IL-4-mediated eosinophil recruitment, we first examined VCAM-1 and CCL26 expression. VCAM-1(Palmer-Crocker & Pober, 1995; Schleimer et al, 1992) and CCL26 (Kitaura et al, 1999; Shinkai et al, 1999) are up-regulated by IL-4 in endothelial cells and our lab showed that together they mediate eosinophil recruitment under flow conditions (Cuvelier & Patel, 2001; Patel, 1998; Patel, 1999). Expressing FRNK blocked IL-4 induced expression of both VCAM-1 and CCL26 as measured by quantitative RT-PCR (Figure 1 D and G), Western blotting (Figure 1 E and F) and ELISA (Figure 1 H). Since eosinophil recruitment in this model is dependent on VCAM-1 and CCL26 (Cuvelier & Patel, 2001; Patel, 1998; Patel, 1999), these data suggest that this is the mechanism by which FRNK blocks eosinophil recruitment. These data also suggest that FRNK is acting early to prevent the transcription of these key mediators. We 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 STAT6 expression, phosphorylation or nuclear translocation. IL-4 activates human endothelial cells by binding to the type II IL-4 receptor (IL-4 receptor α/IL-13 receptor α) (Schnyder et al, 1996), resulting 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, 2002 #2570) where it orchestrates the transcription of target genes including VCAM-1 (Hopkins, 2013) and CCL26 (Hoeck & Woisetschlager, 2001). We found that IL-4 increased STAT6 phosphorylation within 15 minutes of IL-4-stimulation (Figure 2A), as previously reported. Expressing FRNK had no effect on STAT6 phosphorylation or on total STAT6 protein levels (Figure 2A and B). We then examined phospho-STAT6 translocation to the nucleus and found that FRNK had no effect on the ability of phospho-STAT6 to translocate to the nucleus (Figure 2C).

Since STAT6 was unaffected by FRNK, we next examined GATA6 another transcription factor implicated in VCAM-1 regulation. Although GATA6 has been shown to regulate VCAM-1 expression in HUVEC in response to TNFα, a role for GATA6 in IL-4-mediated VCAM-1 expression has not been examined. Because of this, we first examined the ability of IL-4 to regulate GATA6 expression. Under baseline conditions, GATA6 mRNA expression was low but measurable (Figure 2D), as opposed to its family member GATA4, which is not expressed in HUVEC (not detectable by qRT-PCR and (Jenne & Kubes, 2013; Parsons et al, 2013)). IL-4 increased GATA6 mRNA and protein levels (Figure 2D and E).

Effect of GATA6 on IL-4-mediated VCAM-1 and CCL26 expression. siRNA was used to down-regulate GATA6 to determine if GATA6 regulates IL-4-mediated expression of VCAM-1 or CCL26. We also examined the role of GATA6 eosinophil recruitment. siRNA decreased GATA6 expression to baseline levels observed prior to stimulation with IL-4 (Figure 3A and B). Down-regulating GATA6 attenuated VCAM-1 mRNA and protein expression (Figure 3C and D). In contrast, down-regulating GATA6 had no effect on CCL26 expression (Figure 3E and F). Down-regulating GATA6 attenuated eosinophil accumulation and transmigration, but had no effect on the conversion from rolling to firm adhesion (Figure 4 A-C). This is
consistent with decreasing VCAM-1 expression but not CCL26. Indeed, we previously showed that using a mAb directed against VCAM-1 had similar results. Although GATA6 only affects VCAM-1, we next determined if GATA6 mRNA or protein expression was regulated by FRNK. We found that expressing FRNK reduced GATA6 mRNA to control levels (Figure 4D). When GATA6 protein was examined we found that FRNK caused only a modest decrease in GATA6 protein expression (Figure 4E). These data suggest that GATA6 may represent a component of 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 VCAM-1 and CCL26 expression.** FRNK contains a focal adhesion-targeting domain and can displace FAK at focal adhesions. This prevents FAK auto-phosphorylation and blocks downstream signaling. For this reason, FRNK is frequently used as a FAK dominant negative. To determine if FRNK was acting through its actions on FAK signaling, we down-regulated FAK itself. If FRNK acts indirectly through FAK, we would expect that down-regulating FAK would have the same results as expressing FRNK. If FRNK acts independently of FAK, down-regulating FAK would have no effect on VCAM-1 or CCL26 expression. siRNA decreased total FAK expression by >85% and blocked FAK signaling, as measured by auto-phosphorylation (Figure 5A). Down-regulating FAK had no little to no effect on IL-4-induced mRNA expression of either VCAM-1 or CCL26 (Figure 5B and C) and actually increased mRNA for GATA6 (Figure 5G). Similarly, down regulating FAK had no effect on VCAM-1 (Figure 5C and D) or CCL26 protein expression (Figure 5E and F).

Another partner to consider in exploring the mechanism by which FRNK is acting is proline-rich tyrosine kinase 2 (Pyk2). Pyk2 is functionally related to FAK, expressed in endothelial cells, and can also be regulated by FRNK (Barlow et al, 2002; Schaller, 2010) (Bhattacharya & Cabral, 2004). For this reason, we examine Pyk2 in this model system. siRNA decreased total Pyk2 expression as measured by quantitative PCR and western blotting (Figure 6B and C). Pyk2 siRNA had no effect on FAK expression nor did FAK siRNA change Pyk2 expression (Figure 6A, B and C). Down-regulating Pyk2 had little to no effect on IL-4-induced mRNA or protein expression of either VCAM-1 or CCL26 (Figure 6C and D).
Similarly, down regulating Pyk2 and FAK together had no effect on VCAM-1 or CCL26 protein expression (Figure 6C and D). When we examined eosinophil recruitment, we found that neither down-regulating either FAK or Pyk2 alone, nor down-regulating both proteins together had an effect on eosinophil accumulation or transmigration (Figure 6E and F). Together these data suggest that FRNK acts independently of FAK and Pyk2 signaling to block VCAM-1 and CCL26 expression.

**IL-4 increases the expression of endogenous FRNK.** We next determined if IL-4 could regulate 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 the expression of FRNK. The primers chosen for quantitative RT-PCR were from the region 5’ of the FRNK start site and are unique to FRNK (Figure 7A). As a control, we also examined FAK expression using primers from the FERM domain, a region not shared with FRNK (Figure 7A). IL-4 increased expression of FRNK mRNA, but had no effect on FAK (Figure 7B). Protein expression was determined by western blot using an antibody that recognizes both FAK and FRNK (Figure 7A). The proteins were differentiated by their molecular weight with FAK running at 125 kD and FRNK running at 45 kD. IL-4 increased FRNK expression at 24 hours with levels remaining elevated at 48 hours (Figure 7C and D).
Discussion

Resolving inflammation is critical for restoring homeostasis following infection or injury. A wealth of data has been published on the mechanisms that positively regulate the adhesion and activation molecules critical for leukocyte recruitment, yet much less is known about the mechanisms that negatively regulate their expression. In this study, we provide data to support a role for FRNK in negatively regulating IL-4-mediated VCAM-1 and CCL26 expression. We previously showed that eosinophil accumulation, firm adhesion and transmigration was dependent on the expression of VCAM-1 and CCL26 (Cuvelier & Patel, 2001; Patel, 1998; Patel, 1999). By preventing the expression of these critical eosinophil adhesion and activation proteins, FRNK blocked eosinophil recruitment to IL-4 stimulated endothelial cells under flow conditions. 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 as a model of Th2 inflammation. IL-4 is an important cytokine in models of allergic disease and asthma (Grunig et al, 1998; Kolaczkowska & Kubes, 2013; Ricci et al, 1997; Wong et al, 2011). In endothelial cells IL-4 exerts its effects through the type II receptor (Palmer-Crocker et al, 1996; Schnyder et al, 1996) resulting in the expression of STAT6-responsive proteins including those involved in eosinophil recruitment (Adamsson Eryd et al, 2012; Cuvelier & Patel, 2001; Hoeck & Woisetschlager, 2001; Hopkins, 2013; Patel, 1998). Although STAT6 is critical for VCAM-1 and CCL26 expression, FRNK had no effect on STAT6 phosphorylation, nuclear localization or total protein expression (Figure 5). This suggested that FRNK was acting independently of STAT6.

The GATA family of transcription factors can also regulate VCAM-1 expression, with GATA4 regulating expression in mouse fibroblasts (Lim et al, 2012) and GATA6 acting in human endothelial cells (Wong et al, 2013). 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 HUVEC. In fibroblasts it was the FERM domain acting through GATA4 that was shown to be involved and the FAT domain and FRNK had no effect on GATA4 (Lim et
al, 2012). Furthermore, the FAT domain (FRNK) had no effect on VCAM-1 expression in HUVEC.

GATA4 is not expressed in HUVEC (Jenne & Kubes, 2013; Parsons et al, 2013), suggesting that different mechanisms were at play our model. Since HUVEC express GATA6 (Figure 2 and (Jenne & Kubes, 2013; Parsons et al, 2013)) and TNFα-induced GATA6 can regulate VCAM-1 (Wong et al, 2013), 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. Using siRNA, we showed that GATA6 down-regulation blocked the ability of IL-4 to increase VCAM-1 expression, suggesting that GATA6 was upstream of VCAM-1. This is similar to what was found following TNFα stimulation of HUVEC (Wong et al, 2013). In contrast, GATA6 down-regulation had no effect on CCL26 expression. GATA6 down-regulation had an intermediate effect on eosinophil recruitment, consistent with decreased VCAM-1 expression but normal CCL26 expression. The inability of GATA6 down-regulation to block CCL26 expression may be due to the fact that GATA6 itself is regulated by IL-4. GATA6 levels simply may not be high enough immediately following IL-4 stimulation to have an effect on the induction of CCL26. These data on GATA6 reveal a new mechanism for regulating 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 may be just one component of the mechanisms by which FRNK negatively regulates eosinophil recruitment. These data raise the question about how specifically FRNK blocks VCAM-1 and CCL26 mRNA expression when FRNK has no effect on STAT6 and limited effect on GATA6. Since STAT6 alone is sufficient to drive CCL26 expression, it may be that FRNK affects the ability of P-STAT6 to bind to the promoters of these genes. Alternatively FRNK could act independently of transcription by regulating mRNA stability. Determining that FRNK is not regulating the traditional
transcriptional pathways is an important first step to identifying how FRNK prevents the expression of VCAM-1 and CCL26.

FRNK is typically used to block FAK signaling. FRNK is transcribed from the C-terminus of fak gene (Figure 7A) and can displace FAK from focal adhesions, preventing FAK autophosphorylation and activation (Shen & Schaller, 1999). FRNK can also block FAK signaling by acting as a competitive inhibitor for other FAK binding proteins that influence signaling (Yang et al, 2010). To determine if FRNK was acting through FAK, we down-regulated FAK, which will block all signaling from FAK. Despite blocking >85% of FAK protein expression and completely blocking FAK phosphorylation, down-regulating 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 (Barlow et al, 2002; Schaller, 2010) (Bhattacharya & Cabral, 2004). When we examined the effect of knocking down Pyk2 alone or in conjunction with FAK in this model system had 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 independent of FAK or Pyk2 signaling. Because most studies focus on FRNK’s ability to block FAK’s kinase activity, little is known about the other functions of FRNK. Discrepancies between the phenotype of FAK-KO cells and FRNK overexpressing cells (Heidkamp et al, 2002) have pointed to independent roles for FRNK.

When considering the mechanisms by which FRNK may act, there may be lessons to 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 (Casini et al, 2010) and GATA4 (Lim et al, 2012). By bringing these proteins together in a complex with Mdm2 and CHIP E3 ligases, respectively, the FERM domain facilitates the poly-ubiquitination and degradation of p53 (Casini et al, 2010) and GATA4 (Lim et al, 2012). It is possible that FRNK can similarly effect the expression of transcription factors important in VCAM-1 and CCL26 expression in HUVEC.
Unlike the FERM domain, FRNK is an autonomously expressed protein that has the potential to be independently regulated. We tested this concept by determining if FRNK could be regulated by IL-4–itself, thus setting up a negative feedback loop to help shut down inflammation. Indeed, we found that IL-4 increased endogenous FRNK expression at 24 and 48 hours.

Based on these data, we propose a model in which FRNK could act to resolve IL-4–induced inflammation (Figure 8). Stimulation of HUVEC with IL-4 increases the expression of GATA6, which in turn increases VCAM-1 expression. IL-4 also increases CCL26. These mediators act together to bind and activate eosinophils. At the same time, IL-4 induces FRNK expression. Once expressed, we speculate that FRNK can act as part of a negative feedback loop to suppress VCAM-1 and CCL26 expression and limit eosinophilic inflammation. Given the lack of a nuclear localization sequence, we think that FRNK prevents the 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. These data are particularly compelling because we also showed that IL-4 increased FRNK expression, suggesting that FRNK may represent an endogenous mechanism to limit IL-4 inflammation. The ability of a naturally occurring variant of FAK to negatively regulate these pro-inflammatory molecules may suggest a role for FRNK in the resolution phase of inflammation.
Materials and Methods

Materials. Medium 199, 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 (HPA) was purchased 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. Anti-FAK, and anti-phospho FAK were purchased from Upstate USA (Charlottesville, VA). C20 anti-FAK antibody that targets the C-terminal region and recognizes FAK and FRNK was from Santa Cruz Biotechnology (Dallas, TX). Anti-phospho STAT6, anti-STAT6, and anti-GATA6 were from New England Biolabs (Whitby, Ontario). Anti-VCAM-1 and a non-immune IgG control were from R&D Systems (Minneapolis, USA). IL-4, CCL26, anti-CCL26 and biotinylated anti-CCL26 were from PeproTech Incorporated (Rocky Hill, NJ). Anti-CD16 and anti-CD3 conjugated to paramagnetic beads were from StemSep (Vancouver, Canada). Fibronectin was from Biomedical Technologies Incorporated (Stoughton, USA). Collagenase was purchased from Worthington Biochemical Corporation (Lakewood, NJ).

Cell isolation and culture. Eosinophils were isolated by density centrifugation through lymphoprep 1077 followed by negative selection using magnetic beads conjugated with anti-CD16 and anti-CD3 as previously described (Cuvelier & 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 and maintained in M199 (Invitrogen, Carlsbad, CA) 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. FRNK-GFP adenovirus was a gift from Dr. Samarel (Chicago, IL) who has used the construct previously in endothelial cells (Tian et al, 2014). 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 were used to assess the efficiency of FRNK transduction or FAK or GATA6 down-regulation, respectively. Maximal levels of down-regulation were achieved 48 hours post-transfection.

Western blotting. Endothelial cells were lysed in hot Laemmli’s buffer. The proteins were separated by SDS-PAGE, transferred to PVDF and probed with the specified antibodies followed by an HRP-conjugated secondary antibody. Bands were visualized using chemiluminescence detection either by film or on a Fluor-S MAX multi-imager (Bio-Rad Laboratories). The membranes were then stripped in IgG elution buffer and reprobed with an antibody directed against either the total protein or actin to determine protein loading. Relative expression was determined by densitometry using NIH ImageJ and data were normalized based on actin expression. Images shown were adjusted for contrast and brightness only, and cropped based on antibody binding and molecular weight (MW) standards. Images from the Fluor-S MAX were also adjusted for size. MW standards are not visible on the blots shown.
Quantitative RT-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, P-selectin, GATA4, GATA, FAK and FRNK mRNA expression was determined using real-time quantitative PCR using TaqMan Universal PCR Master Mix. The probes and primers used were:

VCAM-1
Forward: 5’-CATGGAATTCGAACCAACAACA-3’
Reverse: 5’ GGCTGACCAAGACGGTTGTATC-3’
Probe: [6FAM]CAAACACTTTATGTCAATGTTGCCCCCAG[TAM]

CCL26
Forward: 5’-ACACGTGGGAGTGACATATCCA-3’
Reverse: 5’-GACTTTCTTGCCTTTTTGGTAGTG-3’
Probe: [6FAM]TACAGCCACAAGCCCCTTCCCTGG[TAM]

GATA6
Forward: 5’-AGAAACGCCGAGGGTGAAC-3’
Reverse: 5’-GCACCCCATGGAGTTTCATG-3’

FRNK
Forward: 5’-GTGGCCTGTCTTCTGGACTC-3’
Reverse: 5’-AGGACGAGGGTTTCAAACTG-3’

GATA4 (Hs00171403_m1), FAK (Hs01056460_m1) and Pyk2 (Hs0103403_m1) primers and probe were from Applied Biosystems. GAPDH was used as an endogenous control. Real-time PCR was performed on a StepOnePlus system from Applied Biosystems (Burlington, Ontario, Canada), and data analysis was performed using the associated software. The 2^{ΔΔCt} method was used to analyze the data. Gene expression was normalized to 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 & 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 $\tau_w = \mu \gamma = 6\mu Q/a^2b$. 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 ($0.5 \times 10^6$/mL) were perfused across the monolayer at 1 dyn/cm$^2$. After 4 minutes of perfusion, the inlet line was transferred to HBSS to prevent the binding of new eosinophils for an additional 6 minutes. Interactions between eosinophils and endothelial cells were visualized on a Zeiss Axiovert 100 microscope using either a 10X/0.25NA or 40X/0.60NA phase-contrast objective and recorded via 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 number of rolling, firmly adherent and transmigrated cells were determined at 10 minutes as described (Cuvelier & Patel, 2001; Parsons et al, 2012). 4-10 fields of view were examined for each condition.

Statistics. All experiments were performed at least three times. The data were analyzed using a 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.
Acknowledgements

We thank Ms. Lailey for her technical assistance; Dr. Kubes for critical reading of this manuscript; unit 51 at the Foothills Hospital in Calgary, AB for providing human umbilical cords; and Jennifer Amon of the Live Cell Imaging Facility for assistance with imaging. Dr. K.D. Patel is Alberta Innovates: Health Solutions Medical Scientist.

Author Contributions.

KP wrote the manuscript. RS, PC, HZ and KP conceived and/or designed the experiments. RS, HZ, and KP performed the experiments. All authors edited and provided critical review of the manuscript. All authors discussed the project at all stages.

Conflict of Interest

The authors declare that they have no conflict of interest.
References


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which is expressed in IL-4- stimulated vascular endothelial cells, exhibits potent activity toward eosinophils. *J Immunol* **163**: 1602-1610


Figure Legends

Figure 1. FRNK blocks eosinophil recruitment and VCAM-1 and CCL26 expression to IL-4-stimulated endothelial cells. HUVEC were transduced with GFP alone or FRNK-GFP prior to stimulation with 20 ng/mL IL-4. After 24 hours, HUVEC were assembled into a parallel plate flow chamber and eosinophils (0.5x10^6/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 Material and methods. (D and G) After 24 hours, cells were harvested for mRNA and quantitative RT-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 and 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 ± SEM of 5 experiments. **p<0.01, ***p<0.001, and nd = not detectible as compared to the GFP control. Representative blots from 5 experiments are shown in E.

Figure 2. STAT6 phosphorylation and GATA6 expression. HUVEC 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 and B) lysed and probed for phospho-STAT6 then stripped and reprobed for total STAT6 expression, or (C) harvested in cell fractionation media and the cytoplasmic (c) and nuclear (n) fractions were separated, run on SDS-PAGE, and probed for phospho-STAT6. Lamin A was used to identify the nuclear fraction and demonstrate successful fractionation. (D and E) Alternatively, HUVEC were stimulated with 20 ng/mL IL-4 for 24 hours and cells were either harvested for mRNA, or lysed for western blotting. (D) Quantitative RT-PCR was performed to measure GATA6 mRNA as described in the methods. Data were normalized using GAPDH. (E) In parallel experiments, proteins were blotted for GATA6. Data in A, C and E are representative of 4 independent experiments. Time points in B of 0 to 4 hours are representative of 4 independent experiments and the data shown were quantified and normalized using total STAT6 as a loading control. Data in D are mean ± SEM of 10 experiments and *p<0.05.
**Figure 3. GATA6 is required for maximal expression of VCAM-1 but is not required for CCL26 expression.** HUVEC were pretreated for 24 hours with control siRNA or GATA siRNA. (A-E) Following siRNA treatment, HUVEC were stimulated with 20 ng/mL IL-4. After 24 hours, cells were either harvested for mRNA, or lysed for western blotting and supernatants were collected for ELISA. Quantitative RT-PCR was performed to measure mRNA for (A) GATA6, (C) VCAM-1 or (E) CCL26 and the data were normalized using GAPDH. 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 ± SEM of 5 experiments. *p<0.05 and nd = not detectible as compared to the siRNA control. Blots in B and D are representative of 4 independent experiments.

**Figure 4. GATA6 is required for maximal eosinophil accumulation and transmigration.** HUVEC were pretreated for 24 hours with control siRNA or GATA siRNA. HUVEC were then stimulated with 20 ng/mL IL-4 and after 24 hours, HUVEC were placed in a parallel plate flow chamber and eosinophils (0.5x10^6/mL) were perfused across at 1 dyn/cm^2 as described in figure 1. (A) Total accumulation, (B) rolling and (C) transmigration were determined as described in the Material and methods. (D and E) HUVEC were transduced with GFP or FRNK-GFP, stimulated with 20 ng/mL IL-4 for 24 hours and then cells were either harvested for mRNA, or lysed for western blotting. (D) Quantitative RT-PCR was performed to measure GATA6 mRNA as described in the methods. Data were normalized using GAPDH. (E) In parallel experiments, proteins were blotted for GATA6 and the data were quantified and normalized using actin as a loading control. Data in A-C are mean ± SEM of 5 experiments. Data in D are mean ± SEM of 8 experiments. Data in E are representative of 4 independent experiments and relative expression values are shown. *p<0.05 as compared to the unstimulated control and ***p<0.001 as compared to control siRNA.

**Figure 5. Down-regulating FAK with siRNA has no effect on VCAM-1 expression or CCL26 expression.** HUVEC were pretreated for 24 hours with control siRNA or FAK siRNA. (A) Cells were then stimulated for the specified times with 20 ng/mL of IL-4, lysed and
blotted for phospho-FAK. Blots were stripped and re-probed for total FAK expression. (B-G) Following siRNA treatment, HUVEC 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. Quantitative RT-PCR was performed to measure mRNA for (B) VCAM-1, (E) CCL26 or (G) GATA6 as described in the methods. Data were normalized using 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 mean ± SEM of 5 experiments. *p<0.05, and nd = not detectible as compared to control siRNA. Representative blots from 5 experiments are shown in A and C.

**Figure 6. Down-regulating FAK and Pyk2 together has no effect on VCAM-1 expression, CCL26 expression or eosinophil recruitment.** HUVEC were pretreated for 24 hours with control siRNA, or siRNA directed against FAK, Pyk2 or both FAK and Pyk2 together (FAK/Pyk2). Following siRNA treatment, HUVEC 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. Quantitative RT-PCR was performed to measure mRNA for (A) FAK or (B) Pyk2 and the data were normalized using GAPDH. (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 and F) After IL-4 stimulation, HUVEC were placed in a parallel plate flow chamber and eosinophils (0.5x10⁶/mL) were perfused across at 1 dyn/cm² as described in figure 1. (E) Total accumulation, and (F) transmigration were determined as described in the Material and methods. Data are expressed as mean ± SEM of between 3 and 6 experiments. *p<0.05, and nd = not detectible as compared to control siRNA. Blots in C are representative of 6 independent experiments.

**Figure 7. IL-4 selectively increases endogenous FRNK in HUVEC.** (A) Diagrams showing a comparison of FAK and FRNK cDNA and protein are shown. HUVEC were stimulated with 20 ng/mL IL-4. (B) After 24 hours, cells were harvested for mRNA and quantitative RT-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 the *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 figure 7A and as described in the *Materials and methods*. (D) The data were normalized using actin as a loading control and densitometry was performed as described in the *Materials and methods*. Data in B and D are mean ± SEM of 5 or 4 experiments, respectively. **p<0.01 and ***p<0.001. A representative blot is shown in C.

**Figure 8. A model for resolving IL-4-mediated eosinophil recruitment.** (1.) IL-4 binds to the type II receptor on endothelial cells leading to increased STAT6 phosphorylation. P-STAT6 dimerizes and translocates to the nucleus where it induces the transcription of VCAM-1 and CCL26. IL-4 also induces expression of GATA6 that in turn enhances expression of VCAM-1. (2.) VCAM-1 and CCL26, together with P-selectin, mediate eosinophil accumulation, firm adhesion and transmigration (Patel, 1998) (Cuvelier & Patel, 2001). (3.) IL-4 increases the expression of FRNK. (4.) FRNK acts independently of FAK to block the transcription of VCAM-1 and CCL26. In the absence of VCAM-1 and CCL26, eosinophils are no longer recruited.
Figure 1

A. Eos/mm²

B. Rolling (%)

C. Transmigration (%)

D. VCAM-1 mRNA (relative expression)

E. VCAM-1

F. VCAM-1 (relative expression)

G. CCL26 mRNA (relative expression)

H. CCL26 (ng/mL)

IL-4

None  GFP  FRNK

nd

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*
Figure 2

A. Time with IL-4 (min):

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GATA6 mRNA (relative expression)

* 

B. Time with IL-4 (hrs):

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P-STAT6

STAT6

C. Con vs IL-4 (15 min)

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D.

![Graph showing GATA6 mRNA expression](image)

E. Con vs IL-4

GATA6

Actin
**Figure 3**

A. Control

B. Con siRNA

C. GATA6 siRNA

D. None

E. Con

F. None

---

**GATA6 mRNA** (relative expression)

- Control
- IL-4
- Con siRNA
- GATA6 siRNA

**VCAM-1 mRNA** (relative expression)

- Control
- None
- Con siRNA
- GATA6 siRNA

**CCL26 mRNA** (relative expression)

- Control
- None
- Con siRNA
- GATA6 siRNA

---

**CCL26 (ng/mL)**

- Control
- None
- Con siRNA
- GATA6 siRNA
Figure 4

A. Eosinophils/mm²

B. Rolling (%)

C. Transmigration (%)

D. GATA6 mRNA (relative expression)

E. Western Blot

- GATA6
- Actin

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<th>FRNK</th>
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* indicates statistical significance.
Figure 6

A.

FAK (Relative expression)

B.

Pyk2 (Relative expression)

C.

IL-4

D.

CCL26 (ng/mL)

E.

Eos/mm

F.

Transmigration (%)

siRNA: None Con FAK Pyk2 F+P

FAK

Actin

Pyk2

Actin

VCAM-1

Actin

IL-4

IL-4

IL-4

IL-4

IL-4

ns

ns

ns

ns

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Figure 7

A.

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Unique leader

B.

- FAK cDNA
  - ATG
  - Unique leader

- FRNK cDNA
  - ATG

C.

<table>
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D.

<table>
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IL-4 Receptor Type II (IL4Rα/IL13Rα)

1. IL-4

2. VCAM-1

3. FRNK

gata6

4. FRNK

P-STAT6

CCR3

α4β1

Eosinophil

CCL26

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