Bioinformatic analysis of eosinophil activity and its implications for model and target species

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Abstract
Eosinophils are important immune cells that have been implicated in resistance to gastrointestinal nematode (GIN) infections in both naturally and experimentally infected sheep. Proteins of particular importance appear to be IgA-Fc alpha receptor (FcαRI), C-C chemokine receptor type 3 (CCR3), proteoglycan 3 (PRG3, major basic protein 2) and EPX (eosinophil peroxidase). We used known human nucleotide sequences to search the ruminant genomes, followed by translation to protein and sequence alignments to visualize differences between sequences and species. Where a sequence was retrieved for cow, but not for sheep and goat, this was used additionally as a reference sequence. In this review, we show that eosinophil function varies among host species. Consequently, investigations into the mechanisms of ruminant immune responses to GIN should be conducted using the natural host. Specifically, we address differences in protein sequence and structure for eosinophil proteins.

Introduction
Host immune responses to gastrointestinal nematodes (GINs) are dominated by a Th2 immune response; involving antibodies and immune cells, such as immunoglobulin A (IgA), IgE, mast cells and eosinophils. In particular, ruminants naturally and experimentally infected with GIN demonstrate an increase in blood and tissue eosinophilia, implying that eosinophils may be an important mediator of host immune responses to GIN. However, both phenotypic and bioinformatic evidence suggest that eosinophil activity against GIN may differ between hosts (Urban et al., 1991; Henderson and Stear, 2006). Bioinformatic analyses on eosinophil-associated proteins were used to explore whether differences in resistance to GIN among species were genetic in origin. Specifically, we addressed differences in protein sequence and structure for eosinophil proteins. These proteins included IgA and its receptor, FcαRI, interleukin (IL)-5 and its receptor, IL-5Ra, eotaxin and its receptor, CCR3, major basic protein (MBP, PRG3) and eosinophil peroxidase (EPX). We used known human nucleotide sequences to search the ruminant genomes (Bos taurus, cow; Ovis aries, sheep; Capra hircus, goat), retrieved sequences (Supplementary Table 1), followed by translation to protein and sequence alignments to visualize differences between sequences and species.

Eosinophils and GIN infections
Eosinophils are a sub-type of granulocyte, along with mast cells, neutrophils and basophils. Following proliferation of eosinophil precursors from the bone marrow, eosinophils traffic to sites of infection and are activated. Once activated, eosinophils undergo degranulation, releasing cytotoxic proteins from secondary granules to protect the host against foreign pathogens. Eosinophils are also involved in immune homeostasis and immunity (Rothenberg and Hogan, 2006; Weller and Spencer, 2017). Eosinophils are found in both blood and tissue, however the gastrointestinal tract contains the largest reservoir of eosinophils in the body (Zuo and Hogan, 2006; Weller and Spencer, 2017). The ability of eosinophils to defend the host against parasitic helminths is suggested by the ability of eosinophils to mediate antibody- (or complement-) dependent cellular cytotoxicity (ADCC) in vitro and in vivo (Giacomini et al., 2008; Huang et al., 2015), increased numbers of eosinophils during helminth infections, as well as degranulation in close proximity to helminths in vivo (Rothenberg and Hogan, 2006).

Eosinophils are not only important for GIN infections of humans and mice, but also of ruminants. In particular, GIN infections ravage sheep and goat populations in temperate regions of the world, and in Australia can cost sheep producers up to $500 million per year largely in lost productivity (Lane et al., 2015). In particular, Teladorsagia circumcincta, Haemonchus contortus and Trichostrongylus colubriformis are the dominant GIN-infecting small ruminants (Roerber et al., 2013). The typical immune response to GIN is dominated by Th2 immune responses, namely the production of antibodies such as IgG, IgE and IgA, as well as involvement of mast cells and eosinophils, the details of which have previously been covered by a number of reviews (Maizels and Yazdanbakhsh, 2003; Anthony et al., 2017; Maizels and Yazdanbakhsh, 2003; Anthony et al., 2017; Rothenberg and Hogan, 2006; Weller and Spencer, 2017).

Review

Cite this article: Jenvey C.J, Alenizi D, Almasi F, Cairns C, Holmes A, Sloan S, Stear MJ (2020). Bioinformatic analysis of eosinophil activity and its implications for model and target species. Parasitology 147, 393–400. https://doi.org/10.1017/S0031182019001768

Received: 31 October 2019
Revised: 8 December 2019
Accepted: 9 December 2019
First published online: 16 December 2019

Key words: Bioinformatics; CCR3; eosinophils; EPX; FcαRI; gastrointestinal nematodes; PRG3; ruminants

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Parasitology
2007; McRae et al., 2015; Motran et al., 2018). Previous research using experimentally and naturally infected sheep have indicated that eosinophils may play an important role in resistance to infection. Investigations into sheep immune responses to T. circumcincta (Gruner et al., 1994; Stear et al., 1995, 2002; Henderson and Stear, 2006; Beraldin et al., 2008), H. contortus (Rainbird et al., 1998; Gill et al., 2000; Balic et al., 2006; Terefe et al., 2007, 2009) and T. colubriformis (Dawkins et al., 1989; Rothwell et al., 1993; Amarante et al., 2007) have all demonstrated increases in eosinophils in resistant animals, resistant breeds and/or in sheep selectively bred for resistance. In addition, differences in numbers of eosinophils and susceptibility to infection have also been observed in goats (Bambou et al., 2013; Basripuzi et al., 2018).

Such findings imply that eosinophils are important cells in ruminant responses to GIN infections. Phenotypic and bioinformatic evidence suggests that there are differences in immune responses to GIN between species. A recent review by Weller and Spencer (2017) discussed a number of unresolved issues when comparing mouse and human eosinophils, namely whether the formation and secretion of eosinophil cytokines is regulated by common mechanisms. In addition, a review by Behm and Ovington (2000) highlighted that IL-5 and eosinophils have different impacts on different helminth infections. Conversely, a review by Meeusen and Balic (2000) suggests that the presence of IL-5 independent eosinophil populations within tissue and peripheral blood may play a role in unnatural nematode–mouse models by increasing resistance to primary infections, and enhancing the development of specific immunity upon subsequent infections. Ultimately, although a number of in vitro studies investigating the mechanisms by which eosinophils cause helminth death have been demonstrated, it is not yet clear whether these same mechanisms also occur in vivo (Motran et al., 2018). Recent updates to the human, mouse, cow, sheep and goat genomes have provided insights into the possible mechanisms of eosinophil function, which can be used to direct functional studies into the mechanisms of eosinophils during GIN infections of ruminants.

**The IgA receptor, FcαRI, may be dysfunctional in goats**

Immunoglobulin A (IgA) is an antibody that plays a crucial role in the immune function of mucus membranes. TGF-β, together with IL-5, is responsible for class-switching of B lymphocytes into IgA-producing plasma cells (Coffman et al., 1989; Sonoda et al., 1989). This local production of IgA is termed secretory IgA and is the predominant form of IgA in mucosal secretions (van Egmond et al., 1989; van Egmond and Stear, 1993; Amarante et al., 2007). As yet, no mouse homologue of FcαRI has been identified (van Egmond et al., 2001; Decot et al., 2005), thus if eosinophils and IgA interact to control nematodes through ADCC, mice may not be appropriate models for studying eosinophil activity against nematodes infecting humans and ruminants.

**Differences in eosinophil responsiveness are not due to IL-5 and IL-5α**

IL-5 is a growth factor and chemoattractant of eosinophils and is involved in the recruitment, activation, degranulation and survival of eosinophils (Lopez et al., 1988; Horie et al., 1996; Fullkerson et al., 2014; Sippel et al., 2018). IL-5 is an important cytokine in the differentiation and activation of anti-parasitic eosinophil responses and has been specifically targeted in mice to demonstrate the in vivo role of eosinophils in helminth infections, as

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discussed in previous reviews (Huang and Appleton, 2016; Meusen and Balic, 2000). However, the reagents and knock-out models used in these rodent studies are generally not available or suitable for large animal experimentation. Several studies have shown that mice vaccinated with recombinant cytokines can induce autoantibodies that specifically cancel out the activity of the native cytokine in vivo (Dalum et al., 1999; Hertz et al., 2001; Richard et al., 2000). This approach may provide an alternative for the in vivo study of immune responses in large animals.

IL-5 acts on target cells by binding to its receptor, IL-5R, which consists of an α and a β subunit, however the α subunit is specific to IL-5R only (McBrien and Menzies-Gow, 2017). IL-5 is a dimeric glycoprotein with a four-helix bundle motif. In complex, IL-5 forms a homodimer which is sandwiched by the IL-5Rα. Binding of IL-5 to the receptor alpha subunit results in recruitment of the β subunit to the receptor (Tavernier et al., 1991; Kusano et al., 2012). The human and mouse cDNA code for proteins of 134 and 133 amino acids in length, respectively, and have 70% amino acid sequence identity (Yamaguchi, 1994).

Molecular modelling indicates that it is unlikely that IL-5 and its receptor are responsible for differences in eosinophil responses between sheep and goats. For IL-5, the predicted protein sequences differed in 6 out of the 432 amino acids. All of these differences occurred in the first 42 amino acids of the protein, which included within the 15–34 amino acids that aligned with the signal peptide for human IL-5Rα, as well as within a region lacking secondary structure. This indicates that the observed sequence differences are unlikely to result in a dysfunctional IL-5 and IL-5R.

### The eotaxin receptor, CCR3, contains a frameshift mutation in goats

Eotaxin is a chemoattractant cytokine which is important for promoting eosinophil recruitment and degranulation (Garcia-Zepeda et al., 1996; Davoine and Lacy, 2014), and has been shown to be important for eosinophil recruitment during helminth infections (Rothenberg et al., 1997; Mochizuki et al., 1998; Ruth et al., 1998; Culley et al., 2000; Simons et al., 2005). Eotaxin belongs to the CC chemokine family, which is distinguished by two cysteines immediately adjacent to the N terminus. Eotaxin has been determined to be in equilibrium between a monomer and a dimer at near physiological pH, however, functional eotaxin is present as a monomer (Crump et al., 1998). It exhibits a chemokine-like fold consisting of three anti-parallel β-strands with an overlying α-helix (Crump et al., 1998). There are three molecules of Eotaxin, CCL11 (Eotaxin-1), CCL24 (Eotaxin-2) and CCL26 (Eotaxin-3); however, Eotaxin-1 is the dominant isoform. The highest levels of Eotaxin-1 are found in the GI system and can be produced by a variety of cells (Kitaura et al., 1996; Ying et al., 1999). Eotaxin-1 is important for the release of eosinophil precursors from the bone marrow (Palframan et al., 1998) and is activated by Th2 cytokines (Mochizuki et al., 1998) and inhibited by Th1 cytokines (Miyamasu et al., 1999; Fukuda et al., 2002). Eotaxin-2 is synthesized and released by mucosal epithelial cells and macrophages, while Eotaxin-3 is produced by endothelial and epithelial cells (Kitaura et al., 1999; Shinkai et al., 1999; Dulkys et al., 2001). Eotaxin-2 and Eotaxin-3 can also recruit eosinophils, but at later stages of infection (>24 h) (Ying et al., 1999; Rosenwasser et al., 2003; Kalomenidis et al., 2005; Schratt et al., 2006). All Eotaxin isoforms are associated with a single receptor, CCR3, however the receptor binds to the different isoforms with different affinities (Kitaura et al., 1999). CCR3 is very abundant in eosinophils (approximately 40–400 × 10^3 receptors per cell), but is also expressed at lower levels in basophils, mast cells and a subset of Th2 lymphocytes (Sallusto et al., 1997; Ugccioni et al., 1997; Romagnani et al., 1999). CCR3 also binds to other non-eosinophil selective CC chemokines, but with lower affinity compared to Eotaxin-1 (Porath et al., 1996; Baggiolini et al., 1997; Sabroe et al., 1999). CCR3 is a G-protein-coupled receptor of 335 amino acids in length and shares 63 and 51% sequence homology with CCR1 and CCR2, respectively (Daugherty et al., 1996). The CCR3 gene codes for four cysteine residues, one in each of the extracellular domains, and a serine/threonine-rich cytoplasmic tail, all of which are highly conserved features of chemokine receptors (Porath et al., 1996). Uniquely, CCR3 contains a cluster of negatively charged amino acids distal to the transmembrane helix IV in the second extracellular loop (Daugherty et al., 1996).

Molecular modelling of the Eotaxin receptor, CCR3, indicates that it may be dysfunctional in goats. The sheep and goat CCR3 protein sequences differed by only two amino acids. The two sheep protein sequences were identical except for two substitutions. The two goat sequences were derived from the goat genome sequence and the other from mRNA extracted from the liver of an Osmanabadi goat. The goat genome sequence contained a frameshift deletion on chromosome 22 at 52,155,650, which corresponded to amino acid 330 of the CCR3 protein (Fig. 2). This deletion was not present in the mRNA; therefore, it is possible that this deletion is merely a
MBP-2 is the only MBP molecule present in ruminants

MBP-1 is an abundant granule protein of human eosinophils. Its homologue, MBP-2, is unique to eosinophils (Acharya and Ackerman, 2014). MBP is localized within the crystalline core of the eosinophil and is an important mediator of eosinophil function (Gleich and Adolphson, 1986; Kita, 2011). MBP is highly basic, which results in the binding of MBP to cell membranes. Cytotoxic mechanisms of MBP involve surface interchange to increase cell membrane permeability and interrupting tissue enzyme activity (Ackerman et al., 1985; Gleich and Adolphson, 1986; Swaminathan et al., 2001). MBP has been demonstrated to be toxic against Schistosoma mansoni by disrupting the cell membrane via the binding of heparin (Butterworth et al., 1979), as well as being important in the control of Litomosoides sigmodontis in mice (Specht et al., 2006). Structurally, MBP is most like C-type lectins, except that it lacks a calcium binding site, and instead binds selectively to heparin and heparin sulphate, glycosaminoglycans and chondroitin sulphate B (Swaminathan et al., 2005; Wagner et al., 2007). Human MBP-1 is 222 amino acids long, consisting of a signalling peptide, pro-peptide and two chains (Swaminathan et al., 2001). It has been suggested that the pro-peptide protects the eosinophil from MBP during transport from the Golgi apparatus to the crystalline core by masking the mature domain, as well as by blocking glycosylated binding sites to inactivate the protein (Swaminathan et al., 2001). The mature domain is highly basic and is the region where carbohydrate recognition occurs. There is a 66% amino acid sequence identity between MBP-1 and MBP-2, with MBP-2 being less basic. MBP-2 contains 10 cysteine residues, eight of which are conserved in MBP-1, including those cysteines that are involved in disulphide bridges. The conservation of these disulphide bridges is consistent with other C-type lectins and is thought to be important for tertiary structure and function (Wagner et al., 2007).

Sequence searches of MBP indicate that only MBP-2 is detectable in ruminant genomes; it may function similarly to MBP-1. A total of three sequences for sheep (XM_027979083.1, XM_027979084.1 and XM_027979084.1) and two sequences for cows (NM_001098471.1 and NM_001098471.1) were retrieved. Of the two human MBP sequences, the ruminant sequences were most similar to MBP-2 (55–58% homology). Homology between the sheep sequences was between 72 and 79%, while homology between the goat sequences was 75%. The highest homology was observed between sheep sequence XM_027979084.1 and goat sequence XM_018058941.1, with 95%, which implies a recent divergence of the C-type lectin domain, as well as by blocking glycosylated binding sites to inactivate the protein (Swaminathan et al., 2001). The mature domain is highly basic and is the region where carbohydrate recognition occurs. There is a 66% amino acid sequence identity between MBP-1 and MBP-2, with MBP-2 being less basic. MBP-2 contains 10 cysteine residues, eight of which are conserved in MBP-1, including those cysteines that are involved in disulphide bridges. The conservation of these disulphide bridges is consistent with other C-type lectins and is thought to be important for tertiary structure and function (Wagner et al., 2007).

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MBP-1, ruminant MBP-2 may function as MBP-1, being more cytotoxic and abundant. Functional assays are required to determine if this hypothesis is in fact the case.

**Goat EPX lacks a nitrosylated tyrosine**

Similar to MBP-2, eosinophil peroxidase (EPX) is unique to eosinophils and is the most abundant cationic protein within the matrix of the specific granule of the eosinophil (Acharya and Ackerman, 2014). Human EPX is 715 AA long, located on chromosome 17. It is structurally similar to myeloperoxidase (MPO), which is present in neutrophil-specific granules (Loughran et al., 2008). EPX uses hydrogen peroxide to produce toxic reactive oxygen species, such as hypohalous acids, and is capable of killing parasites, including S. mansoni (Auriault et al., 1982), Toxoplasma gondii (Locksley et al., 1982) and L. sigmodontis (Specht et al., 2006). In addition to eosinophils, mast cells also play a role in parasitic infections. High concentrations of EPX have been demonstrated to result in mast cell lysis. Mast cell lysis is followed by the binding of EPX to mast cell granules to form a complex, which results in the retention of secretory activity on mast cells (Henderson et al., 1980). In addition, a study by Metzler et al. (2011) demonstrated donors that were deficient in MPO failed to form neutrophil extracellular traps (NET), indicating that MPO is essential for NET formation. Based on this evidence, it is possible that EPX could be involved in eosinophil extracellular trap (EET) formation, which would implicate eosinophil proteins in the direct control of parasitic infections.

EPX is structurally distinct from the other granule proteins, being a two-chain (55-kDa heavy chain and 12.5-kDa light chain) haemoprotein, although EPX is highly cationic, much like MBP-1 and eosinophil cationic protein. A feature of EPX is that it post-translationally modifies itself via the nitrosylation of a specific tyrosine residue (Tyr-488) during synthesis and packaging of the granule proteins into the developing eosinophil (Ulrich et al., 2008). Tyr-488 has also been shown to be surface exposed, which may assist in the production of reactive oxygen species by EPX. Other granule proteins are also nitrosylated by EPX, but it is unclear whether this is important in protection of the host against helminths.

Molecular modelling of EPX indicates that this protein may be dysfunctional in goats. Based on searches using the human (NM_000502.6) and bovine (XM_024980582.1) EPX sequences, no annotated sequences were retrieved from either the sheep or goat genomes. However, short sequences that matched the reference, but not annotated with an associated gene, were retrieved and re-aligned to the reference. The sheep sequence contained one single-nucleotide polymorphism (SNP), while the goat sequence contained two SNP, one of which was in the same location as in the sheep sequence. The sheep and goat sequences were 90.3% homologous, with homology of sheep and goat to the cow sequence being 84.9 and 82.9%, respectively. The cow, sheep and goat sequences all contained MPO-like protein domains, all of which were conserved except for single-residue substitutions within each domain. In addition to the MPO-like domains, the cow and sheep sequences also contained a tryptic peptide fragment, which was conserved in all ruminant sequences except for a single substitution (alignment to human sequence; Arg to His). Of note, the nitrosylated tyrosine was not conserved in the goat sequence and was substituted for a cysteine (Fig. 4). The nitrosylated tyrosine is important for EPX-mediated activities, including the post-translational nitration of eosinophil secondary granule proteins, which in turn, may influence inflammatory responses. The importance of a nitrosylated tyrosine in GIN infections of ruminants has not yet been established; however, the absence of this residue may be responsible for the relative susceptibility of goats to GIN infection. Functional studies are required to determine whether the SNP identified in the sheep and goat sequences may be sequencing error rather than true SNP, as well as to confirm the importance of the nitrosylated tyrosine in resistance and susceptibility to GIN infection.

**Directions for future research**

Phenotypic and bioinformatic analyses, in combination, are valuable in assessing differences in immune responses between species in order to better direct the design of functional studies. Some eosinophil proteins may be at least partly responsible for the susceptibility of certain ruminant species to GINs. In particular, sequence variation in the IgA receptor, FcεRI, the Eotaxin receptor, CCR3 and EPX may contribute to the susceptibility of goats to GIN infection. The different conformation of goat FcεRI, as compared to sheep and human, indicates that it is unlikely that it would be fully effective in antigen-dependent cellular cytotoxicity processes, which have been shown to be important for some helminth infections (Huang et al., 2015). In addition, goat CCR3 may contain a frameshift mutation, which would in turn affect the role of Eotaxin-1 in eosinophil recruitment and degranulation. Goat EPX lacks a nitrosylated tyrosine. In humans, the nitrosylated tyrosine in EPX is important for the post-translational modification of EDGPs during eosinophil maturation. Goat EPX may not be involved in this process. Finally, although MBP-1 is the most abundant and important cationic protein in human eosinophils, it has no orthologue in ruminant genomes. MBP-2 appears to be the only MBP molecule present in ruminants and it may function similarly to human MBP-1. Future research should focus on functional studies to confirm these findings and should involve the extraction of these proteins from the host of interest, followed by sequencing, crystallography...
and in vitro assays to assess the overall importance of these proteins to the susceptibility of ruminants to GIN.

Perhaps the clearest finding of this review is that bioinformatic analyses indicate that the function of specific eosinophil proteins may vary among host species. Therefore, functional studies of eosinophil activity should be performed in the target species. Extrapolations from one species to another need to be interpreted with great care.

**Supplementary material.** The supplementary material for this article can be found at https://doi.org/10.1017/S0031182019001768

**Conflict of interest.** None.

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