Genetic regulation of the antibody response to the parasite *Ascaris* spp.

Doctoral thesis for obtaining the degree of Doctor in Biomedical Sciences

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Cartagena, Colombia 2017

A mis padres que siempre han creído en mí y me han apoyado en todos los proyectos que he emprendido, gracias por ser mis amigos y motivación durante el doctorado. Los amo con todo mi corazón.

To my parents who have always believed in me and who have supported me in all of the projects that I have undertaken, thank you for being my friends and for being my motivation throughout my Doctorate. I love you with all my heart.

A Luis Caraballo, por ser mi maestro e inspiración para iniciar mis estudios de doctorado, por todas sus enseñanzas, consejos y la disciplina que ha impartido en mí.

To Luis Caraballo, for being my professor and my inspiration to commence my doctorate studies, for all your teachings, advice and discipline which you have imparted to me.

A Nathalie Acevedo, por ser mi modelo a seguir y enseñarme la rigurosidad que merece tener todo aquel que haga ciencia, por su infinita paciencia, por todos sus buenos consejos y enseñanzas de vida.

To Nathalie Acevedo, for being my exemplar and for teaching me the rigorousness that is necessary to possess when one studies sciences, your infinite patience and for all your great advice and life lessons.

ABSTRACT

The nematode *Ascaris* spp. causes ascariasis in humans and pigs. Ascariasis in humans causes anemia, growth retardation and cognitive deficit, being one of the most prevalent helminthiases around the world. The infection in pigs is an ideal model to study immunological aspects of ascariasis in humans. In both hosts, worm loads are aggregated, with few individuals harboring the highest worm burden which may be attributed to genetic factors. The locus 13q33 has been linked as a QTL for *Ascaris* susceptibility and total IgE in humans being *TNFSF13B* (encoding for the cytokine B cell activating factor-BAFF) the major positional candidate gene. A tagSNP in *TNFSF13B* was associated with the levels of IgG to *Ascaris* and IgE to ABA-1 in a Colombian population but causal variants remained to be explored. This thesis aimed to analyze the genetic regulation of the antibody response to *Ascaris* including this genomic region (13q33) and other candidate regions related to type-2 (Th2) immunity, as well as to analyze the relationship between BAFF and the strength of the antibody response to *Ascaris*, in order to get a better understanding of the role of *TNFSF13B*/BAFF in the mechanisms of *Ascaris* susceptibility/resistance using human and pig data under different purposes. In **study I**, the dynamics of antibody levels to *Ascaris* extract and three purified *Ascaris* antigens and their relationship with parasitological indicators of infection intensity in an experimental *A. suum* infection pig model were analyzed. Significant correlations between antibody levels and parasitological phenotypes were found. The levels of IgA to *Ascaris* were higher in the pigs with high worm loads and faecal egg count (FEC). While IgA to ABA-1 levels were higher in pigs with lymphonodular liver white spots, and IgG to GSTA was lower in pigs with the highest FEC, supporting the association between the antibody response and infection intensity. In **study II**, we found inverse relationships between BAFF levels and antibody levels to *Ascaris*. Individuals with high specific-*Ascaris* IgE levels had lower soluble BAFF levels and those with high IgG to *Ascaris* had lower BAFF mRNA expression, in connection with the inverse relationship between BAFF and BAFF-R. In **study III**, we re-sequenced the region 13q33-34 and other candidate genes associated with the Th2 immune response by NGS, and genotyped 101 SNPs in two independent populations. Common and genetic variants with low frequency located in chitinases-related genes and locus 13q33-34 were associated with the strength of IgE to *Ascaris*, ABA-1 and total IgE. We validated the genetic association of *TNFSF13B* with high total IgE in two populations. Two *TNFSF13B* SNPs were associated with both levels of BAFF and specific IgG to *Ascaris*, suggesting that the inverse relationship between BAFF and IgG to *Ascaris* is under genetic regulation. No significant differences were found in the transcriptional expression of *TNFSF13B* in lymphoid tissues of *Ascaris*-infected pigs according infection intensity. In conclusion, this thesis provides genetic evidence supporting the role of *TNFSF13B/BAFF* in the regulation of the specific antibody response to *Ascaris* in humans.

LIST OF SCIENTIFIC PAPERS

Paper I.

Adriana Bornacelly, Per Skallerup, Dilia Mercado, Stig Milan Thamsborg, Nathalie Acevedo, Peter Nejsum and Luis Caraballo. **IgA levels are associated with infection intensity in *Ascaris* infected pigs.** Parasite immunology, submitted.

Paper II.

Adriana Bornacelly, Dilia Mercado, Nathalie Acevedo and Luis Caraballo. **The strength of the antibody response to the nematode *Ascaris lumbricoides* inversely correlates with levels of B-Cell Activating Factor (BAFF).** BMC Immunology 2014; 15:22

Paper III.

Nathalie Acevedo, Adriana Bornacelly, Dilia Mercado, Annika Scheynius, Per Unneberg and Luis Caraballo. **Genetic Variants in *CHIA* and *CHI3L1* Are Associated with the IgE Response to the *Ascaris* Resistance Marker ABA-1 and the Birch Pollen Allergen Bet v 1.** PLoS One 2016; 11(12):e0167453.

Additional papers during doctorate studies not included in this thesis

Acevedo N, Sánchez J, Zakzuk J, **Bornacelly A**, Quiróz C, Alvarez A, Puello M, Mendoza K, Martínez D, Mercado D, Jiménez S, Caraballo L. Particular characteristics of allergic symptoms in tropical environments: follow up to 24 months in the FRAAT birth cohort study. BMC Pulm Med. 2012; 12:13.

Zakzuk J, Acevedo N, Cifuentes L, **Bornacelly A**, Sánchez J, Ahumada V, Ring J, Ollert M, Caraballo L. Early life IgE responses in children living in the tropics: a prospective analysis. Pediatr Allergy Immunol. 2013; 24(8):788-97.

CONTENTS

LIST OF ABBREVIATIONS

ABHD13: AB hydrolase domain containing protein 13
AE: atopic eczema
AID: Activation-induced cytidine deaminase
BAFF: B cell activating factor
BAFF-R: Receptor for the B cell activating factor
BAFF-Tg: BAFF transgenic
Bp: Base pair
cDNA: complementary deoxinucleotide acid
CHIA: Chitinase, Acidic
CHI3L1: Chitinase 3 like 1
CSR: Class switch recombination
DALYs: disability adjusted life years
DNA: deoxyribonucleic acid
ELISA: Enzyme linked immunosorbent assay
epg: eggs per gram
ES: excretory secretory
FEC: Faecal egg count
GALT: Gut-associated lymphoid tissue
GATA3: GATA Binding Protein 3
GST: Gluthathione-S-transferase
GSTA: Gluthathione-S-transferase of *Ascaris*
HDM: house dust mites
Ig: Immunoglobulin
IL: interleukin
IRS2: Insulin receptor substrate 2
kDa: kilodalton
LD: Linkage disequilibrium
LIG4: Ligase 4
L3: third stage larva
L4: fourth stage larva
MAF: Minor allele frequency
MHC: Major Histocompatibility Complex
mRNA: messenger RNA
NGS: Next generation sequencing
PBMC: peripheral blood mononuclear cells
PBS: phosphate buffer saline
PCR: polymerase chain reaction
p.i.: post infection
qPCR: quantitative PCR
QTL: Quantitative trait locus
RNA: ribonucleic acid
SNP: Single nucleotide polymorphism
spp.: species
STHs: soil transmitted helminthes

STAT6: signal transducer and transactivation factor 6
TACI: Transmembrane activator and CAML interactor
tagSNP: Tag single nucleotide polymorphism
Th2: type 2 helper T cells
TNFSF13B: Tumor necrosis factor superfamily member 13b
TF: transcription factor
TXNIP: Thioredoxin-interacting Protein
UTR: untranslated region

CHAPTER ONE

1. Introduction

Infection by *Ascaris* spp. (ascariasis) is one of the most prevalent helminthic diseases with its global prevalence of 14.5%[1] affecting about 800 million people worldwide and conferring 10.5 million of Disability-adjusted life years (DALYs) of the total attributed to neglected tropical diseases[2]. Ascariasis is frequently documented in China, East Asia, sub-Saharan Africa and the Americas[3,4]. The global impact of the infection has remained remarkably constant even through effective anthelminthic campaign programs[5-7]. In Colombia, the average prevalence is 11.3%[8], with high heterogeneities within regions as was reported in the last national survey of parasitism[8]. Rural disperse regions where people live without an adequate sewage system, and with a poor community development, harbor the highest prevalences. The most affected by ascariasis are school-age children and teenagers, with a peak prevalence found in the 5-9 year age[9,10] and a high rate of reinfection up to 12 to 15 years old[11].

The immune response to the infection is characterized by mast cell hyperplasia, eosinophilia, elevated levels of unspecified and specific IgE in circulation, an activation of type-2 cytokine responses including IL-4, IL-5, IL-9, IL-13, goblet cell hyperplasia and mucus hyperproduction and probably involves participation of cells such as tuft, epithelial and other innate lymphoid cells (ILC2) which have been discovered playing essential roles in the initiation of Th2 immunity and worm expulsion especially in *Nippostrongylus brasiliensis* experimental infection models[12-17].

The clinical spectrum of the disease include abdominal distension and pain, nausea, diarrhea, loss of appetite, growth alteration, anemia, cognitive impairment and mental retardation, deficits in cognitive and physical development, intestinal obstruction and asthma-like symptoms such as Loeffler's syndrome[18-23]. It also can affects the immune stimulation after vaccination[24]. Typically *A. lumbricoides* infects humans and *A. suum* pigs, but cross infections have been reported[25-28]. Both species are morphological and antigenic similar,

sharing the same life cycle and host-parasite relationships. However, nowadays there are controversial opinions about whether the two species are just one[29], which seems to be the case according to genetic evidence[30-33].

In pigs, the infection by *Ascaris* has a similar cycle than that observed in humans, passing through intestine, lung and liver; generating lung inflammation, alteration in weight gain and liver condemnation[34]. These similarities and others in some immunological aspects make pigs an ideal model to study the immune response during the course of *Ascaris* infection[35].

In the last three decades, *Ascaris* infection has gained importance due to the association of helminthic diseases with the allergic response, especially with the increase in the prevalences of allergy[36,37] and autoimmune inflammatory diseases worldwide as well as the potential therapeutic applications of helminth products to relieve inflammation[38,39]. Both entities, ascariasis and allergy induce strongly Th2—skewed cytokine responses associated with class-switching to highly produce IgE[40]. But different from allergy, *Ascaris* infection induces a strong immunomodulation, which can modify the intensity of inflammation found in allergic diseases and, as a consequence it can change the course of them[36,41,42]. Indeed, some authors have proposed that the increase in allergies around the world is caused, among other explanations, by the decrease in ascariasis prevalence[43]. However, independently of the epidemiological effects that the reduction of ascariasis in industrialized countries and urban zones of developing countries on allergy[44], understanding the basic mechanisms of the immune responses to both conditions is very important not only in scientific terms but also to explore new approaches for public health interventions[45]. In summary, ascariasis can influence allergy by either stimulating or suppressing the immune allergic response, depending on the severity of the infection, degree of exposure and host genetic susceptibility[46].

There are several ways to analyze the complex relationship between allergy and ascariasis, one of these is analyzing the genetic aspects of the IgE response[36,47]. In this regard, several genes have been associated with levels of total non-specific IgE[48-52] and specific IgE to *Ascaris* antigens[53-55]. At the Institute for Immunological Research of the University of Cartagena, genetic variants of genes *LIG4* and *TNFSF13B* have been associated with specific IgE and IgG response to *Ascaris*, respectively[53]. These genes are in 13q33-34 region which

has been linked as a locus of susceptibility to *Ascaris* in the population of Jirel in Nepal[56]. This locus also influences the levels of total IgE[56]. The causal genetic variants for these associations are unknown; therefore this region has deserved a great interest. This thesis aimed to analyze the genetic regulation of antibody responses to *Ascaris* including this genomic region (13q33-34) and other candidate regions related with the Th2 immunity, as well as to analyze the relationship between BAFF and the strength of the antibody response to *Ascaris* in order to get a better understanding of the role of *TNFSF13B*/BAFF in the mechanisms of *Ascaris* susceptibility/resistance using human and pig data under different purposes.

This chapter is an overview of some general aspects of *Ascaris* spp., such as life cycle, epidemiology, pathology, immune response during infection, the genetic epidemiology of ascariasis, the role of BAFF in the *Ascaris* resistance and antibody production, as well as some considerations about the antibody production between humans and pigs.

1.1. General aspects of *Ascaris* spp.

1.1.1. Morphological description of *Ascaris* and life cycle

The adult worms of *Ascaris*, an intestinal parasite, have a longitude of 15 to 40 cm, with females being larger than males. Its body is reddish yellow, with four longitudinal lines, one mid dorsal, one mid ventral and two laterals. The mouth is at the anterior end of the body and guarded by three lips, one dorsal and two ventrolateral. The posterior end is straight in the female and curved in males. In the posterior end of male, two copulatory spicules and the chitinous penial protrude through the cloacal aperture. Females have a genital pore in the mid-ventral segment of the body. Both male and female reside in the intestine of the host where copulation takes place. Females produce between 200.000 and 1 million eggs per day[57,58].

During the life cycle of *Ascaris* spp. the adult and immature worms live in the small intestine of the host. The eggs produced by adult worms can survive several years in adequate external environmental conditions[59] and are ingested by humans or pigs through contaminated food

or water. Five larval stages have been described, L1 to L5. One-cell egg develops into larva under 2 to 3 weeks and it becomes infective when is ingested via oral route[60]. Before eclosion an embrionated-egg suffers two molts (L1 and L2) and twelve stages of development have been identified during 3 weeks of incubation in *in vitro* studies. These stages include: 1-cell, 2-cell, 3-cell, 4-cell, early-morula (5-10 cells), late-morula (11 or more cells), blastula (a spherical layer of cells surrounding a fluid-filled cavity), gastrula (a layer of cells surrounding the embryo plus a kidney shape invagination in one side of the embryo), pre-larva 1, pre-larva 2, first-stage larva (L1, this larval structure has motility in response to light stimulation but not molt), and second-stage larva (L2, this larval structure has molt and motility in response to stimuli)[60]. The molting from L2 to L3 stages occurs over days 31-38 of culture, and in general after the 4th week of culture all eggs have larval structure with motility[61,62]. Hosts become infected by faecal-oral route.

The infective larva is L3. After infective-embrionated eggs are ingested, the larvae hatches from the eggs into the host intestine, penetrating the caecum mucosa in the proximal region of large intestine (1-2 days post infection, p.i.), and migrating via portal circulation to the liver (3 days p.i.). In the liver, an intense inflammatory reaction with eosinophilia and collagen deposition is generated leading to granulomas formation (lesions known as milk or liver white spots). These lesions will heal unless re-infection occurs, or become fibrotic under persistent reinfections.

The larvae then migrate to the lungs via the circulatory system reaching the alveoli and bronchial tree. Then the larvae reaches the lungs (7 days p.i.), infiltrating alveolar spaces and moving into the pharynx where they are coughed up and swallowed (10 days p.i.), thereby returning to the small intestine where they molt into L4 and adult stages[63,64]. This migration phase also stimulates a potent eosinophilia in the lungs which may cause eosinophilic pneumonia and Loeffler's syndrome. In total, this hepatotracheal migration takes place over a two-week period after the uptake of embrionated eggs by pigs or humans.

Under the days 14 to 21 p.i. around 95% of L4 larvae are expelled[65]. Then, few larvae survive after 28 days p.i. developing into L5 and adults. L5 larvae mature into adult worms residing in the intestinal lumen by 1-2 years unless they become expelled by host immune response

or by de-worming treatment with anthelminthic drugs[4,64]. **Figure 1** shows a schematic life cycle of *A. suum* in pigs.

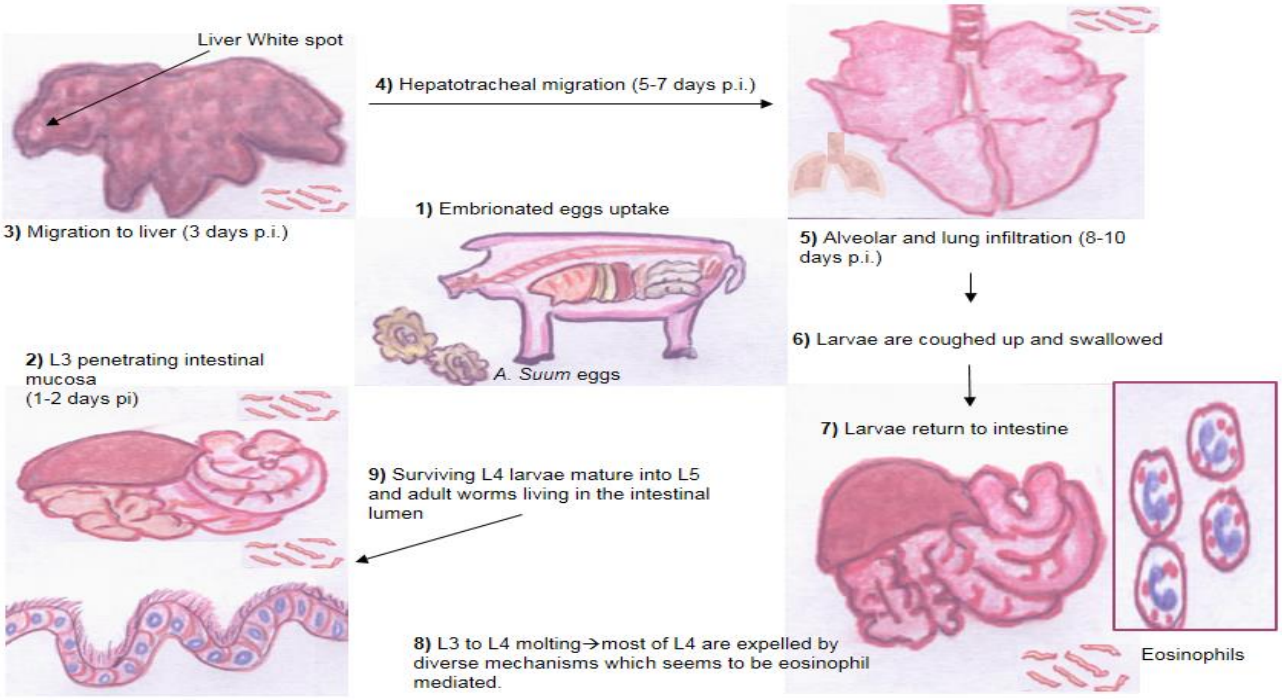

Figure 1. Life cycle of *A. suum* in pigs

1.1.2. Taxonomy of *Ascaris* spp. and cross-infection

Ascaris lumbricoides was described by Linnaeus in 1758 and *Ascaris suum* by Goeze in 1782. *Ascaris* spp. are round worms belonging to the superfamily Ascaridoidea: ubiquitous helminth parasites infecting a wide range of mammals and related to *Parascaris* and *Baylisascaris* branches in the phylogenetic tree of this family[66].

The distinction between *A. lumbricoides* and *A. suum* and the debate about whether they are the same species has not been resolved[29,66] although major genetic evidence (including mitochondrial[30,67,68], ribosomal[32], nuclear[68], microRNA[31] and microsatellite[69] markers) favor the idea of one species[70,71]. More and better molecular tools to explore whether there is gene flow between *Ascaris* populations are required to reach a taxonomic stability to the long and

historically confusing debate about listing names within *Ascaris* taxid, which has great implications in epidemiological and ascariasis management/treatment programs[66]*; e*specially because both species can infect heterologous hosts and cross-infections have been reported[25-28,72,73]. Humans probably have recognized these two ascarids since prehistorical times[29].

1.2. Epidemiology of ascariasis

1.2.1. Prevalence in humans and pigs

Ascaris has a cosmopolitan distribution being found in both temperate and tropical regions. The most recent estimation stated that 800 million of people are infected by *Ascaris* around the world contributing to 10.5 million of DALYs, which correspond to 22% of total DALYs attributable to STH (soil-transmitted helminth) infections[2]. The resting DALYs attributed to STHs were 65% for hookworm and 13% to *T. trichiura*. The vast majority of *Ascaris* infections occur in Asia and Latin America, where poverty, bad sanitary conditions, cultural differences relating to personal and food hygiene, agricultural factors, housing style, social class, gender and in general, different socio-economics conditions promote parasitic exposure early in life and humans become infected by oral contamination with embrionated eggs[2,74].

Within the last two to three decades, reduction in the global prevalence of ascariasis has been reported, which is probably due to large scale deworming programs in China[75], where intestinal helminth infections decreased from 53.6% in 1990 to 19.6% in 2003. In Colombia, the national average prevalence has also decreased from 33.6% in 1980 to 11.3% in 2012-2014[76]. The last national survey for parasitism in Colombia shows that the most affected regions with high prevalences of heavy infection (> 50000 epg) by *Ascaris* are "La Amazonia", "Sierra Nevada de Santa Marta" and "Cinturón árido pericaribeño"[76]. However, high heterogeneities are found within these regions. For example, in a rural area of Loma Arena-Santa Catalina, located near to Cartagena (Colombia), a prevalence of 56% of ascariasis was reported for the year 2004[77], remaining similar to date. As suggested

unpublished data by our group (Caraballo et al. Unpublished data), the prevalence of ascariasis in Cartagena seems to be low but presents itself at early stages in life (for 3 month of age) based on the results of a birth cohort study by our group[78,79]. However, it is worth commenting that the prevalence of ascariasis in Cartagena (Colombia) might be underestimated due to the lack of a prevalence study in the whole population.

Interestingly in some regions of the tropic with high prevalences of ascariasis such as Indonesia, Venezuela, Brazil, Ecuador and Colombia, a perennial co-exposure to high concentrations of house dust mite (HDM) allergens is promoted by temperature and humidity of the environment, conferring particularities to these populations for the study of biological and immunological aspects of the ascariasis-allergy relationship[80].

Ascariasis in pigs is high and it is considered a zoonosis[81]. Its prevalence is variable around the world and even within countries. It depends on the management of farms and it is known that few of them are free of infection[82]. In addition, new indoor production systems may favor helminth transmission[83]. *Ascaris* infection in pigs generates important economic losses, infected pigs have lower food intake, malabsorption and reduced growth rate, impaired lactase activity in the intestinal mucosa, liver condemnation (the loss of the liver as edible offal due to presence of parasitic lesions during post-mortem meat inspection), poor production and quality of meat[84-86]. *A. suum* infections rarely cause specific clinical symptoms in pigs[87,88] except very few cases of coughing and wheezing due to the migration of larvae through lungs. Thus, *Ascaris* infections in pigs seem to be of interest because they have a major economic impact on the pig industry rather than great impact on the health of the pigs. However, keeping in mind cross-infections by *Ascaris* spp. and the very close contact between humans and pigs in some regions of developing countries, more attention should be paid to the control of ascariasis in pigs.

1.2.2. Infection intensity and worm loads distribution

The intensity of *Ascaris* spp. infections in humans and pigs does not follow a normal distribution. Instead, a pattern of aggregation or overdispersion is frequently observed. This

means that most of the worm loads are aggregated in a small proportion of their hosts while the majority of the other individuals infected carry few or no worms **(Figure 2)**[89-92]. In human exposed communities, it is often observed that 10–20% of the population harbor most of the worms[93]. Around 10% of naturally exposed pigs and 21% of experimentally infected pigs have 80% of the worms[94,95], and the majority of infected pigs have only 5-10 adult parasites in their intestines[95]. This aggregation is common in families[96-99]. After deworming this pattern of aggregation is also observed, individuals harboring the highest worm loads before anthelminthic treatment also harbor the highest worm numbers and eggs per gram (epg) of faeces after deworming[95,100,101].

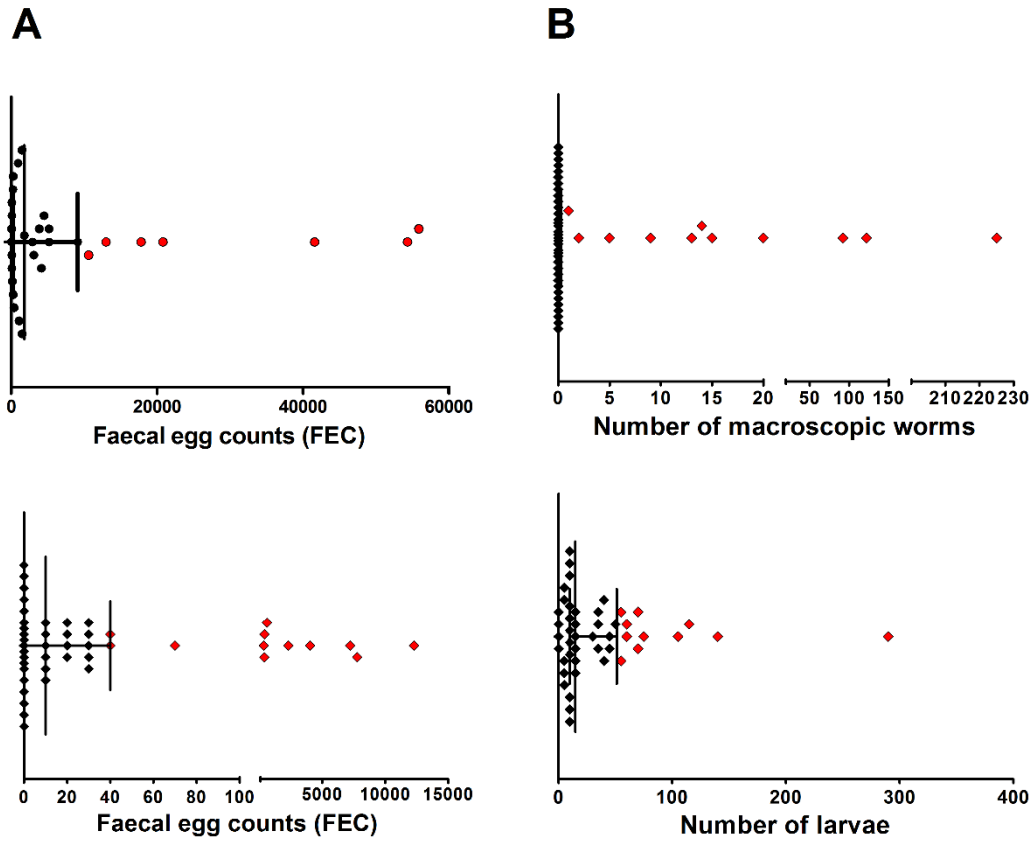

Figure 2. Overdispersion of faecal egg counts (FEC), worms and larvae**. *A.*** *Upper panel:* Distribution of FEC in a subgroup of children between 0-4 years old infected by *A. lumbricoides* belonging to the FRAAT birth cohort study[78], *lower panel*: distribution of FEC in the infected pigs analyzed in paper I. ***B.*** Distribution of intestinal macroscopic worms and larvae in the infected pigs analyzed in paper I. Lines represent median and the interquartile range. The red dots highlight the individuals harboring FEC, macroscopic worms or larvae above 75th percentile.

The individuals harboring large worm burden have a higher risk of morbidity and mortality (for example due to intestinal obstruction); they are significant contributors of infective stages in their communities and are referred to as "wormy persons"[89] for humans, but there can also be "wormy pigs". Due to still unknown mechanisms, in these "wormy" individuals the immunomodulation induced by *Ascaris* allows the parasite survival and evasion of the effector mechanisms leading to its expulsion. This pattern of aggregation is also conditioned by age and sex[90,91]. The most intense infection occurs in children aged 5- 10 years, with a decline in intensity and frequency in adults[96].

The bases of this aggregation/overdispersion and predisposition to reinfection is still not fully clear. Environmental, behavioral and genetic factors are considered contributing to this heterogeneity. In fact, there is strong evidence for genetic components accounting between 30% and 50% of the variation in *Ascaris* worm burden in humans[96]. Genetic factors appear to be also significant in animals. In pigs, heritability of 0.45 for worm count and 0.3 for FEC have been reported[92]. In addition, a QTL on chromosome 4 covering 2.5 Mbp associated with resistance to *A. suum* was recently detected, and two genes (TXNIP and ARNT) within this region were associated with adult worm burden in pigs[102]. Pigs carrying the "susceptible" genotype AB for TXNIP had higher mean macroscopic *A. suum* burden compared with the "resistance" AA genotype[103].

1.3. Immune response in ascariasis

Ascaris has a direct cycle of infection in both, humans and pigs; with a prepatent period between 6 to 8 weeks[64,104]. As other intestinal helminth infections, during ascariasis a Th2 immunity characterized by a polarized cytokine response involving the secretion of IL-4, IL-13 and IL- 5, B-cell isotype switching to IgE, IgG4 (humans), IgG1 (pigs and mice), eosinophilia, basophilia, alternatively activated macrophages, goblet cells and mast cells hyperplasia is observed[40,105,106]. The differentiation of Th2 cells is driven by the binding of type 2-associated cytokines IL-4 and IL-13 to IL-4Rα on the T-cell surface, leading to activation of signalling intermediates such as STAT6 which result in the activation of

GATA3[107]. GATA3 binds to several important sites in the *IL-4* locus including conserved non-coding sequence 1 (CNS1) in the *IL-4/IL-13* intergenic region, conserved noncoding sequence 2 (CNS2) downstream of the *IL-4* coding region, the conserved GATA response element (CRGE) in the *IL-5* promoter and DNase hypersensitivity site II (HSII) in intron 2 of *IL-4*. Although this transcription factor plays a major role in the differentiation toward Th2 response, other important transcription factors are involved. GATA3 regulates the expression of many novel Th2 specific transcription factors whose functions need to be further studied[108]. As mentioned previously, several novel cells such as tuft, epithelial and other innate lymphoid cells (ILC2) have been discovered playing critical roles during the initiation of Th2 immunity against helminths; however, those cells have been evaluated in other nematode experimental models and as a result their specific involvement in ascariasis is unknown.

Little is known about the specific resistance mechanisms in *Ascaris* infection especially in humans. Most of the resistance mechanisms suspected to date have been discovered with other nematode experimental models and henceforth, it can be seen that more studies are required to understand the *Ascaris*-host complex relationships with respect to its susceptibility or resistance; and their implications in other diseases. In the upcoming sections some considerations about resistance mechanisms in *Ascaris*-infection will be described (mainly based on *Ascaris suum* infections in pigs). Among them the initial mucosal immune responses, hepatic barrier, lung and intestinal immune responses for larvae expulsion after hepatotracheal migration and the specific antibody responses.

1.3.1. Intestinal mucosal immune response

The first component of the intestinal mucosal immune response is the contact that the secreted mucus layer makes with the parasite either at larval stage during the early infective process or at adult stages during the reproductive phase of the infection. This layer is composed of a highly ordered hydrated gel formed of mucins, large heavily glycosylated glycoproteins of high molecular weight, secreted from goblet cells that interact with anti-microbial compounds, antibodies and commensal metabolites[109]. It is evident that during a primary intestinal infection that the mucus barrier does not prevent the establishment of

infection. Nonetheless, as infection proceeds a goblet cells hyperplasia is observed[110] and some factors including Muc5a[111], resistin-like molecule RELMβ[112] and indolamine[113] are released by the mucosal epithelium. The exact mechanisms for these molecules expelling parasites are not fully known, but they possibly affect the feeding and development of the parasite[114].

With reference to the cellularity in the mucosa, recently it has been suggested that eosinophils can play an important role in the expulsion of the parasite. At the local cecal mucosa, a marked eosinophilia accompanied by mastocytosis and goblet cell hyperplasia were found. Increased levels of IL-5, IL-13, eosinophil peroxidase and eotaxin, and the fact that eosinophils degranulate in response to contact with infective larvae of *Ascaris* were also found to support the eosinophil influx and its role in the intestinal immunity against infective *A. suum* larvae in pigs[115].

1.3.2. Hepatic barrier

In the liver, a strong neutrophilic and eosinophilic infiltration is induced, together with amiloid deposits in hepatocytes and increased levels of Alanine aminotransferase. During this stage of migration, fibrotic lesions can be observed on the surface of the liver of pigs which are named liver white spots or milk spots and are indicative of a recent infection involved in the hepatic barrier limiting the propagation of the larvae toward the lung[116-118].

The lesions are self-healing and disappear with time. The abscence of milk spots means that it is unlikely that adult worms will be found in the intestine. Two types of milk spots can be found in the liver with those being the granulation-tissue (small and large) and lymphonodular type. However, it is important to highlight that the level of exposure is not reflected in the number of white spots in immune animals[119]. A recent work[120] that intended to get a better understanding of the mechanisms involved in the resistance to *Ascaris* using CBA/ca mice which are a resistant mouse strain[121], found that during infection with *A. suum*, a general stress response is induced with abundant proteins involved in mitochondrial processes including oxidative phosphorilation (OXPHOS) and the trycarboxylic (TCA) cycle

together with proteins related to the innate immune response such as complement component C8a and S100 proteins (S100a8 and S100a9) [120].

1.3.3. Immune responses in lung and intestine

Lung

In addition to the well known Th2-skewed response with high expresion of IL-4, IL-5 and low IFNγ seen in humans and pigs ascariasis[40,106,122], studies comparing the immmunological aspects between *Ascaris*-single and multiple exposed mice have shown that reinfected mice present a reduction in larvae burden associated with a granulocytic pulmonary inflammation and a polarized systemic Th2/Th17 immune response with increasing numbers of circulating cytokines such as IL-4, IL-5, IL-6, IL-10, IL-17A and TNF-α when compared to single-infected animals[123].

Intestine

The migrating larvae L3 reach the small intestine around days 9 - 10 p.i. and shortly after their arrival, the larvae molt to L4 stage. As was previously mentioned, between 14 - 21 days p.i.,there is a phase of gradual larvae expulsion eliminating around 95% of L4 larvae from the small intestine[65,115]. Few surviving larvae will develop into adults 28 days p.i. in the intestine where they have a free movement among the luminal content. It is believed that a protective immunity after a prolonged exposure to *Ascaris* is generated[124].

In *Ascaris*-immune pigs a significant increase in mucosal eosinophils has been reported together with a modest increase in goblet cells and mast cells during the phase of larvae expulsion. Recently it was shown that eosinophils were efficient in killing the larvae in an *in vitro* model when incubated with serum from immune pigs which suggests that *A. suum* specific antibodies are required for efficient elimination of the larvae[115]. In addition to eosinophils, mast cells and histamine released by basophils seem to play a role during this respective phase[115,125]. Another potential factor participating in the larvae expulsion mechanisms is the increased gut movement in the small intestine observed at this time[126].

This protective immunity is supposed to develop at the level of the gut and prevents incoming larvae to penetrate the intestinal tissue and start their hepato-tracheal migration, but little is known about the immunological mechanisms of this protection despite the interest in protective intestinal immunity and a pre-hepatic barrier proposed by Urban et al several years ago[127]. Hence, it is unknown if infected humans develop a pre-hepatic barrier and have this "self-cure"[126]. ,

1.3.4. Antibody responses

In *Ascaris*-infected pigs, the production of antibodies begin early as 5 to 10 days p.i., being maintained until 90-100 days p.i. The maximum peak of the antibody response occurs at 20-40 days p.i.[128]; being IgM, IgA and IgG the main isotypes[129,130]. As was mentioned earlier, a role for antibodies in the self-cure phase of infection (a crucial time-point for resistance) is suspected, but not completely understood[115,126]. The most consistent report shows increased *A. suum*-specific IgM and IgA antibody secreting cells over the course of the expulsion period in the proximal jejujum with elevated *Ascaris*-specific mucosal IgA and systemic IgG1, suggesting that they are important mediators for the expulsion of *A. suum* in infected pigs[131]. Porcine IgE is found in low concentrations in the blood. It is homologous to human and horse IgE and its participation in the context of *Ascaris*-infection has been scarcely studied due to the lack of appropiate reagents. Porcine IgE was purified from serum in 1993 but only in 2008 antibodies potentially useful for immunoassays were obtained[132]. In the context of *Ascaris* infection, one study using an anti-human IgE to detect porcine IgE, found that specific IgE to L4 antigens were detected in serum at 17 days p.i. in comparison to IgA, IgG and IgM levels which were increased since 10 days p.i.[126].

The strength of the antibody response to *Ascaris* is likely determined by age, infection intensity, history of infection and genetic background. Several epidemiological studies have suggested that the specific antibody response to *Ascaris* protects against infection in humans. During human acariasis, antibodies of all isotypes (IgM, IgG, IgA, and IgE) and IgG subclasses (IgG1-IgG4)[133] are produced. High *Ascaris*-specific IgE levels have been associated with resistance to the parasite[134-136]. Higher concentrations of specific IgE to

Ascaris were seen in atopic children having less epg in faeces compared to non-atopic children with similar prevalence of *Ascaris* infection[137].

In a treatment-reinfection study in children from a region of Nigeria where the prevalence of ascariasis in school children is more than 80%, higher levels of IgE to ABA-1 (a lipid-binding protein that is abundant in the pseudocoelomic fluid of the parasites which can be released by the tissue-parasitic larvae and the adult stages) were found in the putative immune children group, harboring no worms in two time-points after anthelminthic treatmet compared to susceptible children[138]. Although ABA-1 is a target of IgE antibody responses in both *Ascaris*-infected humans and rodents, and it has been suggested as an specific-parasite resistance marker is important to remark that only a subset of infected subjects produce antibody to ABA-1, which is probably explained by major histocompatibility complex (MHC)-genetic restriction, as has been demostrated in rodents[139-141]. *Ascaris* FEC in a group of infected young adults were strongly correlated with the levels of *Ascaris*-specific IgG, IgG1 and IgG2[40]. The levels of *Ascaris*-specific IgE are also negatively associated with FEC and the infection intensity[134], whereas the levels of IgG4 were positively associated with infection intensity and the proportion of reinfection in Warao Amerindian children[136]. IgG4 is associated with chronic infection and has been considered as a blocking antibody, limiting the immunological effects driven by IgE-dependent mechanisms[136].

The production of polyclonal IgE may be induced by a combination of direct mitogenic effects on B cells by *Ascaris* antigens[142] and the non-specific induction of IgE secretion in an immune environment associated with the production of large amounts of IL-4. The total IgE levels have been associated with *Ascaris* epg of faeces[56]. In general total IgE is considered to play no protective role during infection. Some authors such as Hagel et al, have hypothetized that total IgE can supress specific IgE responses and also inhibit mast cells activation throught the saturation of their Fcε receptors[143] (**Figure 3.**). That supression may represent a mechanism of parasite evasion as has been proposed for IgG4. Taking this into account, immunity to *Ascaris* may be a balance between the stimulation of immunological effector and blocking mechanisms[134].

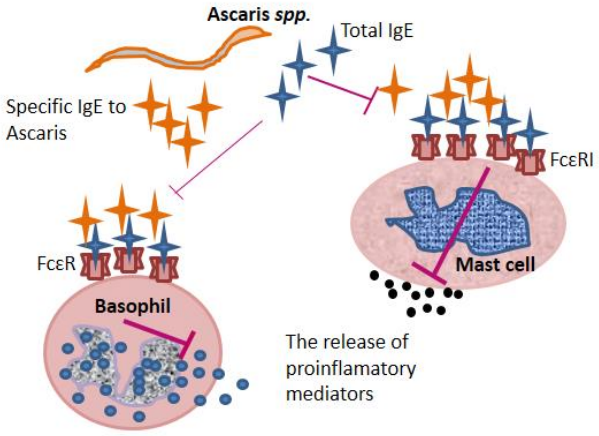

Figure 3. IgE-blocking hypothesis.

Although the protective role of specific antibody responses to *Ascaris* antigens have been recognized[144], it is important to bring to light that their involvement in resistance could be dependent of the type, nature and biological function of the targeted antigen. Immune response to *Ascaris*-antigens has been poorly characterised in comparison to that observed in other nematodes[145]; therefore, the mechanisms of antibodies in resistance to this parasite are not well understood. In fact, contrasting the relatively great number of epidemiological studies, basic experimental work has been very few regarding antibody mediated resistance to *Ascaris* infection, especially in humans.

1.4. Role of BAFF in the resistance to *Ascaris* and antibody production

1.4.1. Genetic context

B-cell activating factor BAFF is encoded by a gene of 40244 bp named *TNFSF13B* (TNF superfamily member 13b) which has been reported as an *Ascaris*-susceptibility marker[56,146] and associated with total IgE and specific IgG to *Ascaris* responses[53].

TNFSF13B is located in 13q33, a region with few identified genes; in a 200.000 bp genomic window, there are only two known genes, *ABHD13* and *LIG4*, surrounded by a RNA pseudogene (RNA5SP39) and an uncharacterized LOC105370335 marker. *TNFSF13B* has

seven exons and, several transcripts can be generated by alternative splicing. The first exon encodes for the transmembrane domain of BAFF, the second for the furin like processing site and the others for the tumor necrosis factor (TNF) homology domain (GeneBank, RefSeq Accession code NG_029524). The three most important proteins produced are the functional native transmembrane protein of 285 aa; an exon 3 losing protein called delta BAFF of 266 aa and a soluble form of 164 aa (**Figure 4**.). A little more than 2700 variations have been reported for this gene, most of them being SNPs (approximately 80%). Near to 50 variants have been reported to be pathogenic with a clinical significance (Variation viewer from NCBI, GRCh38).

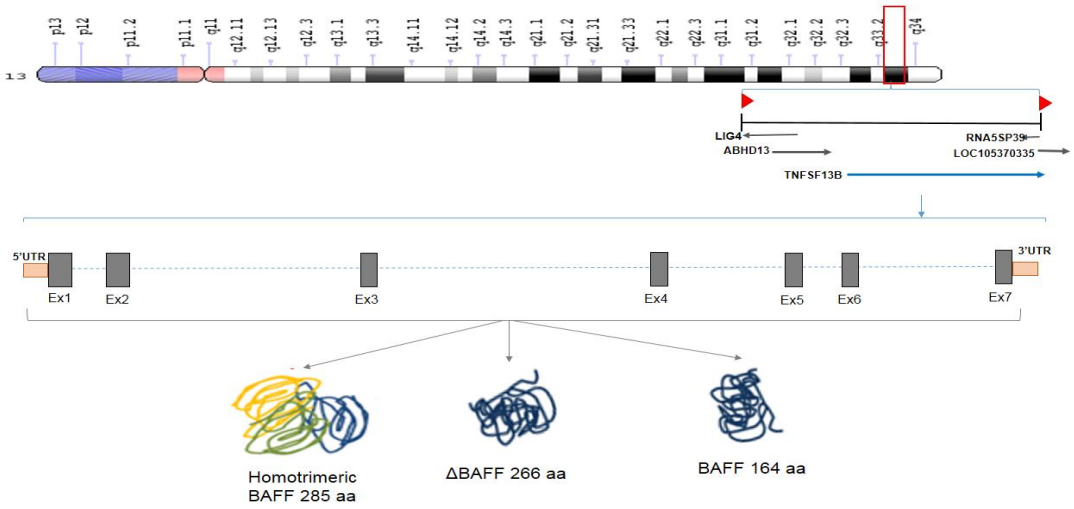

Figure 4. Genomic context and schematic representation of the TNFSF13B gene.

1.4.2. BAFF structure

BAFF is a member of the tumor necrosis factor ligand superfamily of cytokines and it is a major regulator of B cell activation, proliferation, differentiation, survival and immunoglobulin secretion[147-149]. It is mainly expressed by innate immune cells such as neutrophils, macrophages, peripheral blood mononuclear cells (PBMCs) and dendritic cells, although it can be produced by some lymphocytes and non-hematopoietic cells[150,151], B cells in GALT under an intestinal microbiota stimuli[152] and by airway epithelial cells under allergen challenge[153,154]. In its complete form (285 aa) BAFF is expressed as a type II transmembrane protein of a single pass on the plasma membrane where it is cleaved by furine

proteases in its N-terminal end and released into the circulation. Then BAFF adopts a homotrimeric configuration with each monomer folding as a sandwich of two antiparallel β-sheets with a Greek-key topology; the β-strands are connected by loops whose length varies considerably. The core of the protein is mostly hydrophobic but also contains few buried polar residues[155].

1.4.3. BAFF signaling

Soluble BAFF interacts with three receptors: TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor), BCMA (B cell maturation antigen) and BAFF-R (BAFF receptor or BR3). BAFF-R is the most specific receptor and has strong selectivity for BAFF[156]. The other two also exhibit affinity by APRIL (A proliferation-inducing ligand) which is another TNF family ligand member[157]. BAFF-R does not bind APRIL because this requires a specific hydrophobic residue in the conserved β-hairpin structure which is present only in BCMA and TACI, but not in BAFF-R[158].

The binding of BAFF to BAFF-R stimulates a signaling cascade leading to NFκB activation. First, the repressor TRAF3 is degraded in a TRAF2-dependent manner. Then, NIK (MAP 3-NFκB inducing-kinase) and IKK1 (IκB kinase1) are activated, resulting in the NIK stabilization and activation of the alternative NFκB2 signaling that generates the proteolytic processing of NFκB2 (p100) into p52. Finally, one NFκB family transcription factor translocates into the nucleus and regulates the gene expression of pro-survival genes such as Bcl-2 and Bcl-xl[159].

The BAFF/BAFF-R and BCR signaling are coupled and work cooperatively. The BCR and its associated Igα sub-unit may serve as adaptor proteins for BAFF-R signaling[159] and BAFF stimulates the phosphorylation of two important proteins (Igα and Syk) involved in BCR signaling. In addition, both receptors are developmentally regulated at the transitional stages of B cells[160].

1.4.4. Role of BAFF in B and T cells

The role of BAFF in B cell maturation has been studied in BAFF$^{-/-}$ mice, and has shown that maturation of the B cell pool in periphery is impaired with low concentrations of follicular, marginal zone and memory B cells, whereas B cells in bone marrow appeared normal in number and phenotype. Two BAFF deficient mouse strains have been developed by inactivation of this gene; in both, the interruption of B cell development at the stage of transitional B cells has been reported. The defect was localized in the transition from transitional 1 (T1) to transitional 2 (T2) B cells[161].

Other strategies such as BAFF transgenic mice have shown its effect on B and T cell populations. Three different BAFF transgenic mouse strains have been generated, expressing BAFF under one of the following promoters: the liver specific α1 antitrypsin promoter-APOE enhancer [162], the β-actin promoter [163], or VH promoter/Em enhancer [164]. In all these models, the results were remarkably similar: 1) B cell lineage in bone marrow unaffected; 2) T2-B-immature cells and mature B cell in secondary lymphoid organs were expanded, and 3) $CD4^+$ and $CD8^+$ activated T cells increased by twofold. In addition, *in vitro* studies have shown that BAFF is a potent inductor of changes related to the phenotypic and functional maturation of immature B cells, inducing an up-regulation of IgD, CD21 and CD23[165]. BAFF function also has been analyzed in BAFF-R deficient mice. The strain A/WySnJ has a natural deletion in the exon 3 of the BAFF-R gene which encodes the intracellular signaling domain[156,166]. These mice show a phenotype remarkably similar to BAFF deficient mice.

Additionally, BAFF has been recognized as an important co-stimulator of T cell function. Upon TCR ligation, BAFF provides a potent co-stimulatory signal to human T cells enhancing their division and IL-2 production[167]. Human T cells respond to recombinant or endogenous BAFF by secreting IFNγ and IL-2, upregulating CD2, and by proliferating in an IL-2-dependent manner[158].

1.4.5. Role of BAFF in *Ascaris* resistance

Although there is no functional demonstration of the protective role of BAFF in ascariasis or other helminthiases, there is evidence that upon some nematode infections, resistant animals

have increased levels of BAFF. In cattle naturally exposed to *Ostertagia*, *Cooperia* and *Nematodirus* the parasite-resistant animals had increased *tnfsf13b* mRNA levels in the mesenteric lymph nodes[168] and a high *tnfsf13b* mRNA expression have been also detected in the spleen and liver of red grouse infected with *Trichostrongylus tenuis*[169]. In contrast, for the specific case of *Ascaris* infection, a recent analysis of *tnfsf13b* mRNA expression in mesenteric lymph nodes showed no significant differences in the relative expression between pigs with respect to the TXNIP genotypes: "resistance" AA genotype and those with the "susceptible" AB genotype[170].

Additional indirect data supporting a potential role of BAFF in *Ascaris* resistance are from genetic studies. In humans two genome scan studies[56,146] have revealed that BAFF-encoding *TNFSF13B* gene is the major positional marker for the QTL for *Ascaris* susceptibility on chromosome 13 (13q33). In a case-control study, our group found that a *TNFSF13B* intronic tagSNP (rs10508198) was associated with the specific IgG response to *Ascaris*[53]. Adding to this, in the same study lower levels of specific IgE to ABA-1 were found in the asthmatic individuals homozygous for the rs10508198 mutant allele. The IgE response to ABA-1 has been associated with resistance to infection, therefore, this study supports the original finding for this locus as linked with *Ascaris* susceptibility[56]. These genetic reports suggest a potential relationship between *TNFSF13B* and the protective antibody response to *Ascaris* which deserve further investigation. In this thesis we aimed to analyze the genetic variants in *TNFSF13B* and their association with the strength of the antibody response to *Ascaris.*

1.4.6. Role of BAFF in the antibody production

1.4.6.1. General aspects of B cells development and antibody production

B cells differentiate in the bone marrow from stem cells to become mature surface IgM and IgD-expressing cells. The early B cell development includes early proB cells, late proB cells, preB cells, immature B cells, T1 and T2 mature B cells and occurs in the absence of antigen. Mature B cells leave the bone marrow and migrate to secondary lymphoid tissues. T1 B-cells are also emigrant cells from bone marrow to spleen where they constitute the marginal zone[171]. In peripheral lymphoid tissues the B-cells can then mature under the influence of

antigen and T-cell help to undergo isotype switching and affinity maturation by means of somatic mutation. The factors controlling the final differentiation from antibody-secreting B cell to plasma cells and memory cells are not fully characterized, but it is well known that this differentiation requires the participation of the transcription factors Blimp-1(B-lymphocyte-induced maturation protein 1), Xbp1(X-box binding protein 1) and IRF4 (interferon regulatory factor 4)[172].

Antibody repertoire of mature B cells is reached by two antigen-dependent processes that usually occur in the germinal center (GC) of secondary lymphoid organs[173] namely, VDJ gene somatic hyper-mutation and heavy chain class switching (CSR). B-cell activated clones have roughly two ways: one involves the proliferation of B cells and their differentiation into plasma cells, and the other, its entrance into a GC reaction which result in the formation of B memory cells. Memory B cells are formed in lymphoid follicles within the microenvironment of GC. Upon activation of B lymphocytes by antigens in the T cell areas of secondary lymphoid organs, B cells travel through follicle, migrate toward the periphery and then go back to the follicle center where the GC reaction occur thus, leading to B cells proliferation[174].

In the GC, follicular helper T (Tfh) cells, provide critical support for the differentiation of naive B cells into isotype-switched B cells and long-lived memory B cells (LLPC), through their production of cytokines such as IL-4 and IL-21 and co-stimulatory molecules such as CD40L[175]. After providing help to B cells, GC Tfh cells may exit from GC, recirculate into the blood and then return to the GC upon antigen re-exposure[176]. In addition to Tfh cells, the Follicular Dendritic Cells (FDC) present native antigens in the form of immune complexes on the surface of CD21 (CR2) and CD35 (CR1)[177] and switch off the apoptotic machinery contributing to B cell survival due to an up-regulation of c-FLIP which prevents the activation of caspase-8[178], which is a well-known factor involved in the propagation of apoptotic signaling either by direct cleaving and activation of downstream caspases[179,180].

Considering what was previously mentioned with regard to antibody repertoire of mature B cells, these same cells need two signals to initiate CSR to IgA, IgG or IgE, one provided by

a TNF (e.g., CD40L, BAFF, APRIL) or a TLR (e.g., LPS, CpG DNA) ligand, and the other provided by a cytokine (e.g., IL-2, IL-4, IL-6, IL-5, IL-10, IL-21, IFN-γ or TGF-β). For instance, TGF-β causes switching to IgA; T cell–derived IL-10 to IgG1 and IgG3; IFNγ or some other undefined product of Th1 cells appears to induce switching to IgG2; and IL-4 and IL-13 to IgE[172].

Professor Andrea Cerutti, has been a pioneer in the discovery of BAFF properties and its effects on CSR. In 2002, his group in an *in vitro* model, identified that B cells exposed to BAFF and other appropriate cytokines may induce CSR in a CD40L independent way; BAFF together with IL-10 and IL-4 induced CSR to IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE[181]. In another study it was shown that in aerodigestive mucosa, especially in tonsillar crypts, BAFF was produced by DCs promoting the antibody production in a TSLP-dependent manner. Furthermore, these tonsillar crypts were found to be filled with B cells expressing Pax5; a B cell-restricted nuclear protein that regulates immunoglobulin genes[182] and the expression of CD19 and CD20; which are important molecules involved in antigen-independent development as well as the immunoglobulin-induced activation of B cells[183].

1.4.6.2. BAFF and IgA production

A key intestinal strategy to generate immune protection in a non-inflammatory manner is the production of IgA, the most abundant antibody isotype produced in the mucosa of mammals[184]. IgA provides mucosal immune protection as a result of its ability to interact with the polymeric Ig receptor (pIgR), an antibody transporter expressed on the basolateral surface of epithelial cells. After binding to pIgR, IgA dimers secreted by intestinal B cells translocate to the surface of epithelial cells thereby, generating secretory IgA (SIgA) complexes that play multiple protective roles. IgA is also important for the homeostasis between the gut commensal flora and the local immunity[184].

IgA is mainly produced in mucosa and takes place in the GALT[184] and can also be produced in the intestinal lamina propia as was seen in mice lacking Peyer's patches[185]. Most of the work on CSR to IgA showing the participation of BAFF has been done in mice and, evidence in humans is scarce[186]. However, in patients with chronic obstructive pulmonary disease it

has been reported that B cells in bronchial epithelial increase the production of IgA[187]. In this respective tissue, the increased IgA production was promoted by the interaction of two axis IL-6/IL-6R and BAFF/APRIL/TACI[187]. In BAFF overexpressing transgenic mice, elevated concentrations of serum IgA and nephron-mesangial deposits of circulating polymeric IgA can be found exhibiting a similar pathology to the IgA nephropathy[188]. Nonetheless, APRIL seems to be more important than BAFF in the production of IgA since in B cells lacking the expression of TACI a negative regulation of BAFF-induced B cell proliferation and production of IgA and IgG was observed meanwhile, the APRIL-induced production of these antibodies was positively regulated but in collaboration with heparin sulfate proteoglycans[189].

In agreement with other studies, the later mentioned above is supported by the fact that a decreased IgA production in TACI deficient mice was found[190] and that the normal concentrations of IgA in serum, IgA^+ plasma cells in duodenal lamina propia and lower concentrations of IgG and IgM in patients carrying homozygous deletion in the BAFF-R gene were detected[191].

1.4.6.3. BAFF and IgG production

The IgG response plays a very important role in infections and chronic inflammatory diseases and this antibody is most abundantly found in systemic circulation and extravascular fluids in humans[192,193] making it of great serodiagnostic value. Furthermore, the analysis of the IgG profiles to different antigens of a microorganism might reveal different profiles of T helper associated responses that are useful for the design of therapeutic strategies[194].

The effect of BAFF on the IgG production has been long recognized. BAFF-Tg mice display severe hyperglobulinemia associated with the presence of Rheumatoid factor, circulating immune complexes and anti-DNA autoantibodies which result in lupus-like autoimmune manifestations[162]. This protein alone or in conjunction with APRIL can induce CSR to IgG in CD40L deficient mice, and this induction can be mediated by both receptors, BAFF-R and TACI[190]. In fact, in TACI deficient mice a significant defect in the secretion of specific IgG to NP (nitrophenacetyl)-conjugated lipopolysaccharide was found[195]. BAFF together with IL-10 and IL-4 can induce CSR to IgG1, IgG2, IgG3, IgG4, IgA1, IgA2 and IgE in a CD40L

independent way[181]. In the tonsillar crypts of the aerodigestive mucosa, the role played by BAFF on CSR and the TSLP-dependent antibody production has been suggested to occur in a Pax5 regulated manner[182].

1.4.6.4.BAFF and IgE production

To date, there is little evidence showing a direct role of BAFF on IgE production. *In vitro* studies have shown that IgE production is induced after stimulation with IL-4 and anti-CD40, whose signals mimic the T cell help received during the germinal center reaction[196,197]. Although the evidence demonstrating a direct role of BAFF in IgE production is scarce, the fact of this cytokine induces an overexpression of Pax5 creates a potential link, taking into account that Pax5/BSAP has a binding site in the Iε promoter. Pax5 trans-activator of AID expression in GCs[198] which interacts with the recombination-activating gene 1 (RAG1)-RAG2 complex to enhance RAG-mediated V(H) recombination signal sequence cleavage and recombination of a V(H) gene substrate[198].

The fact that BAFF-Tg mice exhibit higher IgE levels as with the other isotypes suggest some BAFF regulation on IgE production[163]. In both humans and mice it has been shown that solely BAFF is unable to induce IgE production; however, when it is administered together with IL-4, immunoglobulin production stimuli is reinforced and synergized. At the molecular level, there are two paths to produce IgE antibodies from IgM; one is the direct switching pathway, in which recombination occurs between Sμ and Sε; the other is the sequential switching pathway, which is a two-step process observed in both mice and humans. In mice, Sμ first recombines with Sγ1, and the hybrid switch region undergoes a second recombination with Sε. In addition, there is indirect evidence suggesting a potential role of BAFF in the local IgE production in nasal mucosa; in patients with chronic rhinosinusitis with nasal polyps, increased concentrations of BAFF has been reported to be associated with the local IgE production and IL-5[199].

1.5. Some considerations about the antibodies in pigs and BAFF

In pigs, the organization of genes encoding for immunoglobulins is similar to chicken, rabbit and rumiants being different to humans and mice[200]. Similar to rabbits, pigs use an unique gene family encoding for heavy chain variable domains (VH3)[201]. They have five antibody isotypes (IgA, IgG, IgM, IgD e IgE) and two light chains (λ, κ). The antibodies begin to be detected early in fetal stages and tend to decrease forward to delivery[202]. Immunoglobulins cannot cross the placenta in pregnant sows hence, neonatal pigs are agammaglobulinemic at birth[203] and passive immunity is conferred via colostrum and milk. In pigs, the antibodies are unavailable to pass across the placenta, probably because their placenta has three layers (endothelial, connective tissue-endometrium and epithelial-endometrial layer) in contrast to humans that only have two layers[203]. Differing from primates, porcine IgA is more abundant in serum and milk than in colostrum while porcine IgG is more abundant in colostrum. Serum IgA is monomeric and colostrum IgA can be monomeric and dimeric. Around the 100% of IgG, 40% of IgA and 85% of IgM found in colostrun all derive from serum[203]. Pigs have several IgG subclasses: IgG1, IgG2a, IgG2b, IgG3, IgG4, IgG5 and IgG6, most of them originated by gene duplication events and exon shuftling[200,201]. All of these subclasses activate the complement cascade, however IgG2 and IgG4 are the major complement activators[204]. Among these subclasses, IgG1 is the most abundant and starts being produced at 50 days of gestation[205]. IgG1 and IgG2 are expressed under Th2 and Th1 cytokine control respectively; the IgG2 production is stimulated using IFNβ and its level is increased in response to IL10[200].

Regarding the role of BAFF in antibody responses in pigs, a recent work[206] shows that the proliferation of porcine B cells treated with BAFF was not affected at least *in vitro*. However, BAFF significantly enhanced cellular viability for both non-activated and CD40L activated B cells compared with cells treated with APRIL throughout the inhibition of the apoptosis of antibody secreting cells (ASC). Furthermore, BAFF appeared to be more effective in enhancing IgM production as compared to APRIL, but there was no difference in IgG secretion[206]. In another study which intended to test the potential of BAFF as a B-cell

targeting genetic adjuvant for a DNA vaccine, it was found that BAFF promotes B-cell survival and that there is a differentiation of foot-and-mouth disease virus (FMDV)-specific memory B cells toward antibody producing cells; porcine BAFF stimulated the FMDV specific and total IgG antibody production supporting the well known potent effect of the cytokine on B cell survival in humans and mice[207].

1.6. Genetic epidemiology of ascariasis in humans

Genetic epidemiology aims to explore the independent role of genetic-environmental determinants, it is the role of genetic variants and their interaction with environmental factors in the occurrence of diseases[208]. As well as to determine the risks related with the allelic variants of candidate genes, to map more accurately regions of the genome for which there is evidence of linkage to disease susceptibility, and to contribute cases to a genome-wide search for susceptibility genes. All these aims of the genetic epidemiology have identified thousands of (mostly common) genetic loci associated with disease traits and biomarkers, with potential use for disease prediction, identification of causal mechanisms and prioritisation of new biological targets for drug discovery[209].

There are two general approaches to the identification of genetic loci: genome scanning and candidate gene studies and there are two methods of data analysis: linkage and association. Linkage analysis involves studying joint inheritance in families. It is less powerful but more robust than association analysis which involves the study of unrelated individuals. In linkage analysis the recombination events are directly observed or inferred in a family pedigree within a limited number of generations, whereas in association analysis the consequences of unobserved recombination events over many past generations within a short interval surrounding a disease locus are exploted[210]. Importantly, the two approaches often leads to the identification of different classes of disease-related genetic variants being most of those common variants. Arguing in favor to the paradigm of that common genetic variants are associated with complex diseases but falling in the explanation of trait heritability[211]. In

addition to common variants, there is extensive evidence for rare variants (MAF < 1%) contributing substantially to complex diseases and the risk of infection[212]. The contribution of low-frequency (MAF 1-5%) and rare variants to human traits, as well as the combination of both, common variants with weak effect and low frequency/rare genetic variants with small to modest effect[209] would not have been possible without the advances in sequencing technology.

In last years, the genomic and genetic research has had several important advances in the field of human genetics of infectious diseases[213]. It is now recognized that host genetics is an important determinant of the intensity of infection and morbidity due to human helminths[214]. Epidemiological studies of a number of parasite species have shown that the intensity of infection (worm burden) is a heritable phenotype with polygenic control[214] and the effects of host genetics are usually considered to be on resistance to infection, through effects on innate or acquired immunity leading to parasite survival, growth or fecundity control[214]. Regarding the genetic epidemiology of parasitic diseases, it is aimed at disentangling the mechanisms underlying immunity and pathogenesis by looking for associations or linkages between loci and susceptibility phenotypes[45]. Therefore, this knowledge is an indispensable approach to understand the molecular basis of common diseases such as ascariasis in exposed populations.

As was mentioned before, *Ascaris* infection exhibits an overdispersed and aggregated ditribution among infected hosts which could be explained by genetic differences among individuals in the infection process or in the immune responses to infection. Two of the major sources of variation in immune responses are variation in antigen recognition which influences the quality of the immune response, and variation in cytokine production which influences the quantity of the immune response. The association between the MHC genes and nematode infections has been suspected but not resistance allele have been confirmed. An analysis of *A. lumbricoides* worm loads in Nigerian children suggested the involvement of the MHCI (A30/31) in determining resistance to infection[215]. In lemurs, the alleles Chme-DRB*8 and Mimu-DRB*28 were positively associated with *Ascaris*-infection[216], and it is has been demostrated that there is a MHC restriction for responses to ABA-1 protein of

Ascaris in rodents[139,217]. In addition, in a recent study[218] in which 87 wild giant pandas were used to investigate MHC variation associated with *Baylisascaris* (a member of the ascaridae family) parasite load. The authors found that Aime-DRB1*10 was significantly associated with parasite infection, but no resistant alleles could be detected[218].

Most of the significant associations with *Ascaris* infection traits have been reported with genes and regions different from MHC. The most informative genetic epidemiology studies of *A. lumbricoides* infection intensity were conducted by Sarah Williams-Blangero et al[56,146] in the Jirel population in Nepal, where the prevalence of ascariasis range between 20-30%. They performed quantitative trait linkage analyses in two 10-cM whole-genome scan studies in a well epidemiological characterized population belonging to a single pedigree. To date, this pedigree represents the most extensive and informative pedigree for the genetic basis study of ascariasis. They identified six potential quantitative trait loci (QTLs) influencing the susceptibility to parasite infection; the most statistically significant were: chromosome 13 (13q33-34, LOD = 3.37), chromosome 11 (11p14, LOD = 3.19), chromosome 8 (8q23, LOD = 3) and chromosome 1 (1p32, LOD = 2.72); the first three QTLs exhibiting genomewide significance. The QTLs on chromosomes 1 and 13 were validated in two genome scans. One limitation of this kind of study is the fact that the resolution of linkage mapping for complex traits is low, so it identifies chromosomal regions, rather than specific genes; therefore, the work of Williams-Blangero et al left the door open for further in-depth mapping studies in each one of the identified QTL regions influencing *Ascaris*-susceptibility.

In chromosome 13 locus the major candidate gene is *TNFSF13B*, which encodes for the B cell activating factor (BAFF) involved in the regulation of B cell activation, maturation and immunoglobulin secretion[149,151]. In a study by our group, two tagSNPs located within 13q33-34 region, on *TNFSF13B* and *LIG4* genes, were associated with the levels of IgG and IgE against *Ascaris,* and IgE to ABA-1 (an *Ascaris* specific resistance marker) in a Colombian sample population[53], suggesting that they could be associated with *Ascaris* susceptibility to infection given that individuals carrying mutant alleles had lower levels of specific antibodies to *Ascaris* or ABA-1.

For the aforementioned reasons the locus 13q33-34 is a very interesting region for a more detailed genetic mapping study in which additional markers within this region are genotyped and the association between specific alleles and *Ascaris*-susceptibility traits are analyzed. This thesis is focused on 13q33-34 region and its association with the strength of antibody response to *Ascaris* taken as an *Ascaris*-susceptibility/resistance trait; this thesis also provides useful knowledge for the characterization of the genetic variants influencing *Ascaris*-susceptibility traits such as antibody responses and parasitological phenotypes as well as the relationship between BAFF and antibody response against *Ascaris*.

In addition to linkage studies, case-control surveys have detected significant associations with *Ascaris* susceptibility for STAT6, IFNγ, IL-13 ADRB2, and IL-10[45], and recently TGFβ[55]. In an endemic *Ascaris* region of China, a common genetic variant in 3'UTR regulatory elements of STAT6 was associated with low worm burden measured as epg and increased resistance to *Ascaris* infection[219]. In this study associations of interactions with IFNγ and IL-13 were also found, however, the variant in STAT6 was the most remarkable association. In children from Venezuela the beta2-adrenoreceptor–*ADRB2* gene was associated with high *A. lumbricoides* FEC[220]. IL-10 SNPs were associated with the *Ascaris* specific IgE and chronic infection in children from Brazil[54] and in the same population SNPs in TGFβ were associated with an increased susceptibility to infection[55].

In Chapter 3 will see how this thesis contributes to the understanding of some of these aspects. In the studies aforementioned there are an indicative that TNFSF13B variants are associated with the levels of antibodies against *Ascaris*, however is not clear how these variants influence the strength of the antibody response. Given that individuals with the higher levels of IgE to ABA-1 for example are the most resistance individuals (as was shown in the study of children in China or Nepal). In turn, to know the genetic variants associated with low specific IgE response are important to understand which variants are associated with the susceptibility to ascariasis and the immunomodulation.

2. Hypothesis and aims

2.1. Hypothesis

The strength of the antibody response to *Ascaris* is partially regulated by common and rare genetic variants in genes located in the genomic region 13q33.

2.2. Aims

The overall aim of this thesis was to identify genetic variants in the locus 13q33-34, and other candidate genomic regions related to type Th2 immune response, influencing the strength of the antibody responses to *Ascaris.* Moreover, to investigate the relationship between BAFF and the antibody response to *Ascaris* and the intensity of the infection.

The specific aims were:

Study I	To analyze the relationship between *Ascaris* antibody response and infection intensity in an experimental *A. suum* infection pig model.
Study II	To investigate the relationship between BAFF and the strength of the antibody response to *Ascaris.*
Study III	To identify common, low frequency and rare variants associated with the strength of antibody response to *Ascaris* in humans.

CHAPTER TWO

3. Materials and methods

This section summarizes the samples and methodologies used that were used to fulfill the specific aims of the thesis. Additional and detailed information can be found in papers I to III.

Experimental *A. suum* infection in pigs

Sixty-two cross-bred Duroc/Danish Landrace/Yorkshire pigs selected based on their genotype at locus SNP TXNIP (AA, AB) associated with susceptibility to *A. suum*[102] (heterozygotes being most susceptible), were allocated into trickle-infected groups (n_{AA}=27; n_{AB}=25) or uninfected groups (n_{AA}=5; n_{AB}=5). Pigs were trickle-infected at 8 weeks of age with *A. suum* eggs (25 eggs/kg/day) twice per week. The pigs were housed in four pens with equal distribution of genotype, litter of origin, sex and weight in each pen. At day 56 pi, the pigs were euthanized and six parasitological phenotypes were evaluated: intestinal number of macroscopic *A. suum* worms (including large juveniles and adults), number of intestinal *A. suum* larvae, total *A. suum* burden (sum of the counts of larvae plus macroscopic adult worms), total number of liver white spots, number of liver white spots of lymphonodular type and FEC. At days 0, 17, 28 and 56 p.i. blood samples were taken for antibody determinations. At day 56 p.i. samples of intestinal mucosa and mesenteric lymph nodes (from jejunum) were taken for gene expression analysis. The samples were immediately snap-frozen in liquid nitrogen and stored at –80°C until RNA extraction.

Study populations and sampling

Colombian CGA study

This case-control study was performed in a population from Cartagena, Colombia. All participants live in an urban, non-industrialized setting, having access to water and electricity and belonging to the lower three (of six) socio-economic strata in the city, where most people

are naturally exposed to *A. lumbricoides*[53]. Eight hundred and ninety-six subjects including 448 asthmatics and 448 non-asthmatics healthy volunteers matched by age and gender were studied in Paper II. Forty eight subjects at the extremes of the distribution of specific IgE levels to *Ascaris* and ABA-1 were selected for the resequencing phase in Paper III. Nine hundred eighty and eight subjects including 391 asthmatics and 597 non-asthmatic controls were selected to analyze the genetic associations in Paper III.

Swedish AE study

The Swedish Atopic Eczema Study comprised 170 atopic-dermatitis patients and 100 healthy controls. We assumed that there is no exposure to *Ascaris* in this cohort, therefore it was not tested for genetic associations with *Ascaris* antigens. This population was included for validation of the genetic associations for total IgE (Paper III).

Experimental procedures

Antigens preparation

The *Ascaris* whole-body extract from adult worms (containing both somatic and ES products) was prepared by the acetone–saccharose precipitation method as described previously[53]. Three recombinant purified antigens (ABA-1, tropomyosin and GSTA) were prepared as follows: ABA-1 was cloned into pGEX-1kT and expressed as a glutathione-S-transferase fusion protein in *Escherichia coli* BL21[53]; the recombinant tropomyosin and GSTA were cloned into pQE-30 UA and expressed in *E. coli* M15 cells as a 6x His-tagged protein as previously described[221,222] (Paper I, II and III).

Targeted resequencing

Targeted resequencing was performed in 14 genes (*CHIA*, *CHI3L1*, *FCER1A*, *IL10*, *TSLP*, *IL5*, *RAD50/IL13*, *IL4*, *IL33*, *STAT6*, *LIG4*, *ABHD13*, *TNFSF13B* and *IRS2*) to evaluate their genetic variations and to select markers for the association study. Genomic DNA was linked to specific adaptor and index sequences, target selected and enriched using biotinylated RNA baits/streptavidin beads. The samples were sequenced with a coverage of 30X in the targeted

regions by next generation sequencing (Ilumina) using a 100 bp sequencing protocol (paired-end) according to manufacturer's instructions (Paper III).

Genotyping

One hundred and one variants were genotyped by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (SEQUENOM®, Inc.). Primer for multiplex PCR and extension reactions were designed by the SpectroDesigner software. PCR and extension reactions were performed according to manufacturer's standard protocols. Concordance analysis with HapMap data was performed (Paper III).

Antibody determinations

CGA study: Specific IgE and IgG to *Ascaris* extract and ABA-1 as well as specific IgE to HDM extracts (*Dermatophagoides pteronyssinus* and *Blomia tropicalis*) and total IgE were detected by ELISA as described previously[53] (Paper II and III).

Swedish AE study: Total IgE and specific IgE to any of the 11 common aeroallergen sources (Phadiatop1) were measured in plasma using Immuno-CAP™. Specific IgE to the purified recombinant allergens Fel d 1 (from cat) and Bet v 1 (birch pollen) were analyzed with the customized MeDALL allergenchip as described previously[223], (Paper III).

Infected pigs: Specific levels of IgA, IgG, IgG_1, IgG_2 and IgE to *A. suum* extract and recombinant proteins (ABA-1, tropomyosin and GSTA) were quantified by ELISA. Total IgE levels were measured by competitive Elisa using a Porcine Immunoglobulin E ELISA Kit as specified by the instructions given by the manufacturer (Paper I).

Quantification of BAFF levels

Soluble BAFF levels in plasma were measured using a quantitative sandwich enzyme immunoassay based on the instructions provided by the manufacturer (Paper II).

Isolation of PBMCs by Ficoll separation

Peripheral blood was diluted 1:2 in PBS and separated by density gradient using Ficoll Histopaque. The layer containing the PBMCs was collected for two purposes: RNA extraction for gene expression analysis, and for BAFF and BAFF-R surface expression by flow cytometry analysis (Paper II).

RNA extraction

RNA was extracted by the phenol-chloroform method using the Trizol® Plus RNA purification kit according to the direction given by the manufacturer (Paper II).

cDNA synthesis

cDNA was synthesized from 1 μg of total RNA using the Superscript III first strand super mix kit following the instructions supplied by the manufacturer. The synthesized cDNA was stored at -20°C until amplification. cDNA was genomic-DNA free, confirmed by an amplification reaction using a non retrotranscriptase enzyme control, (Paper II).

Relative RNA quantification

Relative gene expression was detected by quantitative PCR using Taqman gene expression assays on a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Expression of β2 micro-globulin was used as endogenous control and each sample was tested by duplicate. The C_T value of the β2M gene was subtracted from the C_T values of the target genes. The normalized value was expressed as the delta C_t ($\Delta Ct = Ct_{target\_gene} - Ct_{B2M}$) while the highest ΔC_T value among all samples was subtracted from each sample and the resulting number expressed as the *delta-delt*a C_T. Finally relative expression levels were calculated as $2^{\wedge\text{-}(\Delta\Delta CT)}$ (Paper II).

Cell sorting

Peripheral blood monocytes and B cells were labelled with monoclonal antibodies conjugated to microbeads for negative selection by magnetic associated cell sorting using commercially available kits from Miltenyi Biotec (Paper II).

Flow cytometry

Cell surface expression of BAFF and BAFF-R was analyzed on PBMCs and purified monocytes/B cells by flow cytometry; the expression of cell surface markers was evaluated in a DakoCytomation Cytometer and analyzed by Summit 4.3. Electronic gating was used to evaluate the expression of BAFF and BAFF-R in $CD14^+$ (monocytes) cells and $CD19^+$ B cells, (Paper II).

Microarray for gene expression in pigs

RNA extracted from 48 samples of intestinal mucosa and 24 samples from lymph nodes of infected and non-infected pigs which were used for determining gene expression by Affymetrix Whole Transcript Assays (Gene Titan plate format Gene ST 1.1; Affymetrix, CA, USA).

Bioinformatics and statistical analysis

Resequencing data processing

Sequence reads were aligned to the human genome reference version 19 (hg19) and post-processed for variant calling. Read alignment was done for each sample using BWA software package. Variants were annotated with ANNOVAR and SnpEff and filtered using Variant Filter with respect to several quality scores, such as variant confidence and strand bias. SNPs and indels were processed and filtered seperately (Paper III).

Variant burden analysis

The burden of variants according to specific IgE levels to *Ascaris* and ABA-1 was calculated between groups of high IgE responders and low IgE responders using the Ingenuity Variant Analysis software. The genetic analysis was done for binary and quantitative traits at three levels: gene-gene, variant-variant and gene-variant, including variants that occur in at least 4 high responders but not in low responders. The variants with significant associations were selected as candidates for genotyping (Paper III).

Statistical analysis

The normality of data distribution was explored with Kolmogorov-Smirnov test; statistical analyses were performed using the SPSS and GraphPad Prism softwares (Paper I, II and III). The genetic association analyses between the genotyped variants and the risk of being an antibody high responder and the associations with BAFF levels were done in PLINK. The associations with antibody levels as a continuous variable were modeled by using median regression and quantile regression with the package (quantreg) implemented in R, (Paper III). Microarray gene expression analysis was done using the Transcriptome Analysis Console (TAC 2.0, Affymetrix) and Qlucore softwares.

Pearson and Spearman correlation tests were used to analyze the correlations between antibody levels and parasitological phenotypes, as well as the relationship between antibody levels and soluble BAFF levels and the relationship between BAFF and BAFF-R, according to data distribution (Paper I and II). Mann-Whitney *U* test and *t* test were used to compare antibody levels between infected and non-infected pigs, antibody levels between low and high infection intensity, soluble BAFF levels and BAFF mRNA expression between low and high antibody responders, based on data distribution (Paper I and II).

Antibody levels, BAFF levels according to genotypes and antibody levels among time-points infection were compared using nonparametric tests (Mann Whitney and Kruskall Wallis) according to the number of groups for comparisons (Paper I and III). All regression models were adjusted by age, gender and clinical condition, considering the confounding effects of these covariates in humans and the potential effect of pen, gender and genotype (TXNIP) in the analyses done in pigs. The significance level was set at $p < 0.05$.

Ethical statements

All of the procedures and experiments carried out in this thesis were conducted following the ethical principles for medical research stated in the Declaration of Helsinki. The Ethics Committee of the University of Cartagena (Res. 26/06/ 2009) and the Swedish Regional Ethics Committee (when it was applicable, Drn. 2011/1051-31) approved the study. Written informed consent was obtained from all subjects. Parents/guardians provided informed consent on behalf of all child participants. The pig study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark (Ref. 2010/561-1914). Care and maintenance of all animals were in accordance with applicable Danish and European guidelines.

CHAPTER THREE

4. Results and discussion

This section shows the main findings of the entire body of work of this thesis. Detailed information can be found in papers I-III, although here some additional information to that were presented in papers I-III is discussed.

4.1. Analysis of the relationship between the specific *Ascaris* antibody response and infection intensity (Paper I).

The main goal of this study was to analyze the relationship between the specific *Ascaris* antibody responses and the infection intensity in an *A. suum* experimental controlled trickle-infection pig model. In a total of 46 infected pigs, the specific responses to *Ascaris* whole-body extract and to three recombinant antigens (ABA-1, tropomyosin and GSTA), which are abundant in the body and ES products of *Ascaris*; were evaluated during four time-points of infection (0, 17, 28 and 56 days p.i.), and their relationship with the intensity of six parasitological indicators of infection was analyzed at day 56 p.i..

This study showed significant correlations between specific antibody responses to *Ascaris* and parasitological indicators of infection. Also that high IgA levels to the *Ascaris* and ABA-1 are associated with infection intensity in pigs. Shortly, *Ascaris*-specific IgA levels were significantly increased in pigs with a high number of macroscopic worms and with the highest FEC at day 56 p.i.; the specific IgA to ABA-1 levels were significantly increased in pigs with a high number of lymphonodular liver white spots, while pigs with high FEC have lower levels of specific IgG to GSTA. This supports the idea that antibody responses influence the parasitological outcomes. These results are in agreement with previous studies showing that *A. suum* infection stimulates a dominant IgA response[131,224] and suggesting correlations between antibody response to *Ascaris* and parasitological phenotypes[224-226].

Although the protective role of IgA levels to specific *Ascaris* antigens in infected pigs is not well defined, there is evidence in other animal models that mucosal IgA is an important mediator for the expulsion of parasites, being most of their protective mechanisms eosinophil mediated[227-229]. In this study, the increase of IgA levels to *Ascaris* antigens (whole-body extract and recombinant proteins) between days 17 and 28 p.i. and the subsequent decrease toward day 56 p.i., was in connection with the decreased infection intensity in most of the pigs at day 56 p.i., and the larvae expulsion that occur in most of the infected pigs during 14 and 21 days p.i.[65,115], suggesting a potential protective role of IgA in the expulsion of larvae. Nevertheless, our results should be extended and confirmed because no larval and worm counts were performed at days 17 and 28 p.i.

Specific immune responses to ABA-1 and GSTA have been associated with the protective immunity to the parasite. The high serum IgA to ABA-1 levels that were detected in the infected pigs with a high number of lymphonodular liver white spots might be the result of the high intestinal IgA production by *Ascaris*-antigens local stimulation (from larvae and adult worms). Still, this possibility cannot be verified in this study because intestinal antibody-producing cells were not evaluated. The relationship found between high FEC and low IgG to GSTA is also consistent with the mechanisms of protection described for GSTs, which involve cytotoxic effects impairing the fecundity and egg viability of the nematodes[230,231], one of the reasons that GSTs are considered vaccine candidates for helminthiases[232-236].

Furthermore, as was revised in section 1.4., the contribution of BAFF in the antibody production mediating CSR to IgA, IgG and IgE is well known but its role in the *Ascaris* resistance is unknown. In agreement with the biological role of BAFF in the production of intestinal IgA, a significant positive correlation between soluble BAFF levels and the specific IgA levels to *Ascaris* whole-body extract in a subgroup of six pigs matched by gender at 28 days p.i. was found (Spearman rho = 0.89, p = 0.03). IgA is the dominant isotype in jejunal mucosa of *A. suum* infected piglets[237], and around 30% of IgA produced in gut-associated lymphoid tissue is transported into systemic circulation contributing to the pool of the

circulating IgA in pigs[238]. The contribution of BAFF in the IgA production has been observed in response to mucosal antigens by activation of follicular B cells through a TI reaction in Peyer's patches, mesenteric lymph nodes and isolated lymphoid follicles[239,240]. The fact that the correlation was found only with the levels of IgA to *Ascaris* whole-body extract might suggest that most of the dominant IgA response in these pigs can be produced in response to *Ascaris* glycosylated antigens.

In order to investigate the relationship between BAFF and *Ascaris* resistance, the RNA expression from 48 samples of intestinal mucosa (jejunum) and 24 of mesenteric lymph nodes at day 56 p.i. was analyzed in a whole-transcript microarray assay. No significant differences in the expression of transcripts for BAFF were found when the comparison between infected and non-infected pigs was done, neither when pigs were grouped based on infection intensity (manuscript in preparation not included in this thesis). This result was in line with a recent report showing that the relative *tnfsf13b* expression was no different between pigs with *TXNIP* AA genotype compared to those AB[170]. The locus *TXNIP* have been associated with *Ascaris* susceptibility[102], pigs of the AA genotype had lower mean macroscopic worm burden than pigs of the AB genotype in a functional study for this marker[103].

BAFF plays crucial roles in gut homeostasis and antibody production; its gene has been linked to *Ascaris* susceptibility in humans, and significant differences in BAFF expression have been found for other nematode infections in animals; supporting the role of BAFF in the mechanisms of *Ascaris* susceptibility and resistance. However, in this thesis no significant differences in BAFF expression were found, showing the biological complexity in the gene regulation mechanisms, which may not always be expressed at transcript level. In addition, in this study the gene expression was evaluated at day 56 p.i., and the differential gene expression at days 17 and 28 p.i. could not be analyzed.

4.2. Relationship between the strength of the antibody response to *Ascaris* and the levels of B-Cell Activating Factor-BAFF (Paper II)

The locus 13q33-34 has been linked to *Ascaris* susceptibility in the Jirel population[56,146], an a tagSNP in *TNFSF13B* (the major candidate gene for these associations) was associated with higher IgG levels to *Ascaris* in a Colombian population[53], suggesting a role for *TNFSF13B* in the specific antibody response to *Ascaris*. However, the relationship between the specific antibody response to *Ascaris* and BAFF has not been evaluated in humans. Based on the results of Paper I, it is evident that the relationship between BAFF and the specific antibody response to *Ascaris* deserves more analysis in a larger dataset. The aim of this study was to analyze the levels of BAFF according to the strength of the antibody response to *Ascaris*, comparing the mRNA expression and the soluble BAFF levels between individuals with low and high *Ascaris* antibody responses in a population naturally exposed to this parasite. In this paper, the BAFF levels were analyzed in a subgroup of 896 individuals classified as 448 asthmatics and 448 controls age and sex matched belonging to the Colombian CGA study.

The results revealed an inverse correlation between levels of BAFF and the intensity of the antibody response to *A. lumbricoides,* thus the soluble BAFF levels being lower in those individuals with specific IgE to *Ascaris* above 75th percentile and BAFF mRNA expression levels lower in those with high specific IgG levels to *Ascaris.* To our knowledge, it is the first study suggesting a relationship between circulating BAFF levels and the human antibody response to a nematode. In contrast to what is generally observed in autoimmune diseases[241-244] and some parasitic infections by protozoans[245], we found an inverse association between soluble BAFF and the strength of the antibody response to *Ascaris*. The fact that the same tendency was observed in both, at protein and mRNA level it is highly suggestive that it represents a biological phenomenon.

Given that most of the specific functions of BAFF might be signaled through BAFF-R; in this study the relation of BAFF and its BAFF-R on mononuclear cells was explored, finding an inverse correlation between the median fluorescence intensity of BAFF-R on the surface

on gated $CD19^+$ B cells and both, the soluble BAFF and mRNA expression levels in agreement with previous reports[246-248]. BAFF is a very important regulator of B cell maturation and proliferation, and, it is has been found inversely correlated with the number of peripheral B cells[247]; furthermore, most of the systemic BAFF is bound to receptors on the surface of B cells and the BAFF binding capacity on follicular B cells is nearly saturated under steady-state conditions *in vivo*[249]. Taking into account the preceding considerations, some small changes in circulating BAFF concentrations may affect the BAFF balance in lymphoid tissues and impair the survival of high-affinity mature B cells clones in the germinal center affecting antibody production. In addition, the inverse relationship between BAFF and BAFF-R provides insights that could explain the previously inverse relationship among BAFF levels and the strength of antibody response to *Ascaris*.

The potential mechanistic link between lower levels of BAFF and the strength of the specific antibodies to *Ascaris* might be related to the inverse relationship between BAFF levels and the BAFF-R expression on B cells, a receptor that was present at the cell surface in all developmental stages of peripheral B cells including switched memory and plasmablasts. Unfortunately in this study, the BAFF surface expression on other different blood cells like neutrophils was not analyzed (beside in monocytes it was not detected), therefore a wide view of the biological regulation of BAFF-BAFF-R axis is very limited.

It is also pertinent to consider that some factors like inflammatory cytokines can affect the BAFF expression and its protein level, and this study was limited to explore the effect of other cytokines in the BAFF levels. Indeed, IL-4 down-regulates the expression of BAFF *in vitro*[250] . It is known that *Ascaris* infections of low intensity are associated with a Th2 immune response rich in IL-4, which it is found at increased levels in asthmatic patients[251,252]. Therefore, in this population, this could be an additional factor introducing variability in the BAFF levels hence making this study limited to rule out the impact of IL-4 on BAFF levels given that IL-4 concentrations were not determined.

In conclusion, this study revealed an inverse relationship between the antibody responses to *Ascaris* and BAFF at the protein and mRNA level although a causal relationship between

them is not demonstrated. Our findings seem to be more related to genetic mechanisms, because firstly, there is previous evidence suggesting that polymorphisms in *TNFSF13B* may influence the *Ascaris* antibody responses[53] and secondly, we also found an association between the IgG responses and the level of mRNA expression for BAFF. Interestingly, neither soluble BAFF nor mRNA expression for BAFF were related to total IgE or IgE sensitization to HDM which is in connection to our previous findings[53].

4.3. Identifying common and genetic variants with low frequency associated with the strength of IgE responses to *Ascaris* in humans (Paper III).

The causal genetic variants for the associations of the locus 13q33-34 with *Ascaris* susceptibility in the Jirel population[56,146] and the association of a tagSNP in *TNFSF13B* with higher IgG levels to *Ascaris* in a Colombian population[53] are unknown. Also, based on the results of Paper II, it is of great interest to analyze the genetic regulation of the strength of the IgE responses to *Ascaris.* Therefore, to obtain a broader view of the regulation of IgE hyper-responsiveness we performed a resequencing of the locus 13q33-34 and other candidate genes associated with the Th2 immune response, with a posterior genotyping and association study including 101 SNPs in 988 individuals belonging to the Colombian CGA study and 270 individuals belonging to the Swedish AE study. The Swedish population was included as a replication (for total IgE) and contrasting population to explore the specificity of the associations.

We found that locus 13q33-34 harbors important genetic variants regulating the IgE hyper-responsiveness and hypo-responsiveness differentially. This being established, SNPs in *TNFSF13B* (rs17565502 and rs8181791) were associated with the strength of total IgE levels in both populations, with rs17565502 also being associated with high IgE to Fel d 1 levels. A SNP in IRS2 (rs12584136) was associated with high total IgE levels and high IgE to *D. Pteronyssinus* levels while *ABHD13* rs3783118 was associated with low levels of specific

IgE to *Ascaris* and *D. pteronyssinus* extracts. Suggesting that those genetic variants are regulating the IgE responsiveness indistinctly of its specificity. These results support the biological role for *TNFSF13B* in the regulation of total IgE levels in humans in agreement with the original discovery for the locus 13q33 as a QTL for total IgE[56]. Since high total IgE levels is a hallmark of both helminth infections and allergic diseases, the conservative role of *TNFSF13B* on this phenotype in both asthmatics and *Ascaris*-exposed Colombian patients, and atopic-eczema non-*Ascaris* exposed Swedish patients supports potential evolutionary links between helminth immunity and allergic responses. The significant association for *IRS2* and the levels of total IgE was also in agreement with a previous study[53]. At our knowledge there are no reported associations for *ABHD13* and IgE responsiveness, therefore this is the first report at this regard. This gene has been hardly studied and gives way for several aspects to be explored in the context of IgE production and *Ascaris* susceptibility. For *ABHD13* rs3783118 a generation of a new binding site for the transcriptional repressor Foxp1 (Fkhd domain) which plays an important role in the differentiation of lung epithelium and it is an essential transcriptional regulator of B-cell development has been predicted[253].

The relative gene expression level was not distinct among genotypes of the genetic variants with significant associations, given that most of the associated genetic variants were located on introns. Other bioinformatic predictions of interest are those for *TNFSF13B* rs17565502 and *IRS2* rs12584136 which generate the loss of the binding site for NFAT5 (Genomatix software). NFAT5 is a member of the nuclear factor of activated T cells (NFAT) family of proteins which play crucial roles in the development and function of the immune system[254]. NFAT proteins bind to the IL-4 promoter region of Th2 cells cooperating in this lineage differentiation[255]. In addition, the synergic action with the proto-oncogene c-Maf[256] and the interferon regulatory factor 4 (IRF4)[257] to potentiate the transcriptional activation of the IL-4 promoter and to initiate endogenous IL-4 production by T cells have been reported. This prediction makes sense biologically taking into account that the protein products of both genes (BAFF and IRS2) have important interactions with IL-4 that lead to the IgE production. BAFF together IL-10 and IL-4 can induce CSR to IgE in a CD40L independent way[181], and *IRS2* is an adaptor molecule which participates in the interleukin IL-4/IL4-Rα signaling pathway which leads to IgE production[258]. Although in this study we did not find significant

associations with genetic variants in IL-4, we did find associations with SNPs in IL-5 and IL-13 and high total IgE levels in agreement with previous reports[259]. Therefore, further studies analyzing the *trans*-acting regulation between genes located in the locus 13q33-34 and those in the Th2-locus could be very interesting for a better understanding of the biological mechanisms regulating IgE responses.

Another remarkable finding was the identification of common and non-frequent variants in chitinases related genes (*CHIA* and *CHI3L1*) and *STAT6* associated with the strength of the specific levels of IgE to ABA-1 in the Colombian CGA study. In connection with the genetic associations of genetic variants in CHIA and the levels of IgE to ABA-1, a recent study showed an impaired expulsion of *N. brasiliensis* in AMCase deficient mice[260], suggesting that *CHIA* might be a potential gene related to *Ascaris* resistance in humans. The association for the SNP in *STAT6* supports the role of this gene as an important gene related with *Ascaris* resistance in compliance with a previous study showing that a 3'UTR SNP is associated with the protection against high *Ascaris* FEC burden in children living in an area of endemic infection in China[219].

This study also showed for first time that the same SNP (*CHI3L1* rs4950928 C/G) was associated with both, the strength of specific IgE to ABA-1 in the Colombian population and the strength of specific IgE to Bet v 1 in the Swedish population, suggesting in part, that IgE responses to helminthes and environmental allergens could be under the same genetic control. This finding was in connection with the recent discovery of that Bet v 1 shares an IgE binding epitope with a Bet v 1 like protein (SmBv1L) from *Schistosoma mansoni*[261]. When we search homology for Bet v 1 and SmBv1L at protein or nucleotide level with *Ascaris* by Blast analyses no matches were found.

In conclusion, this study provides very important evidence for the understanding of the genetic regulation of the strength of IgE responses to *Ascaris* focusing on the genomic region 13q33-34 as of great interest for further fine mapping and association studies with IgE responsiveness. Indeed, recently another region in the chromosome 13 (13q21.31) has been suggested as locus regulating total IgE levels[262]. Given that all three genes, *CHIA, CHI3L1*

and *STAT6* were associated with the strength of IgE to ABA-1 and this response has been related to the natural immunity to *Ascaris*[138], this thesis provides new useful knowledge about the set of genetic variants (common, low- frequency and rare) regulating complex traits like the levels of IgE to ABA-1. However, it is important to remark that in CGA population we have no available information on parasitological phenotypes. Therefore, the associations with the IgE to ABA-1 is not necessarily related to *Ascaris* resistance in spite of elevated specific IgE levels to *Ascaris* and ABA-1 being associated with decreased worm burden as have been reported[134,135,138,263]. The IgE response to ABA-1 has been reported to be restricted to MHC genes[139,141] hence, this work provides valuable information about genes beyond the MHC region influencing this response.

4.4. Identifying genetic variants in *TNFSF13B* regulating the inverse relationship between BAFF and the antibody response to *Ascaris*

The results of the paper II and III in conjunction to previous reports[53,56,146] support a genetic relationship between BAFF and the antibody response to *Ascaris*. In order to get further details about this relationship, we analyzed the association between the *TNFSF13B* genetic variants and the relative expression of BAFF transcripts as well as the soluble BAFF levels in the dataset of paper III. We found that only the intronic SNP rs17499386 was associated with the relative expression of transcripts for BAFF (n = 111; B: 0.787; β: 0.195; 95%IC: 0.024- 1.55; p = 0.024; p_{adj} = 0.04). Interestingly the same SNP was associated with the specific levels of IgG to *Ascaris* (n = 985; B: -0.2; β: -0.1; 95%IC: -0.32,-0.09; p = 0.001; p_{adj} = 0.001) under a dominant linear regression model.

These associations were driven by higher levels of BAFF1 mRNA (p = 0.04) and lower levels of specific IgG to *Ascaris* (p < 0.0001) in carriers of the mutant allele compared to individuals being homozygous for wild type allele. Furthermore, in the subgroup of non-asthmatics controls, the carriers of the mutant allele (C) had lower levels of specific IgG and IgE to ABA-1 **(Figure 6)**. These findings support that the inverse relationship between the strength

of the specific IgG to *Ascaris* and the relative expression of transcripts for BAFF found in paper II is regulated by genetic variants in *TNFSF13B*.

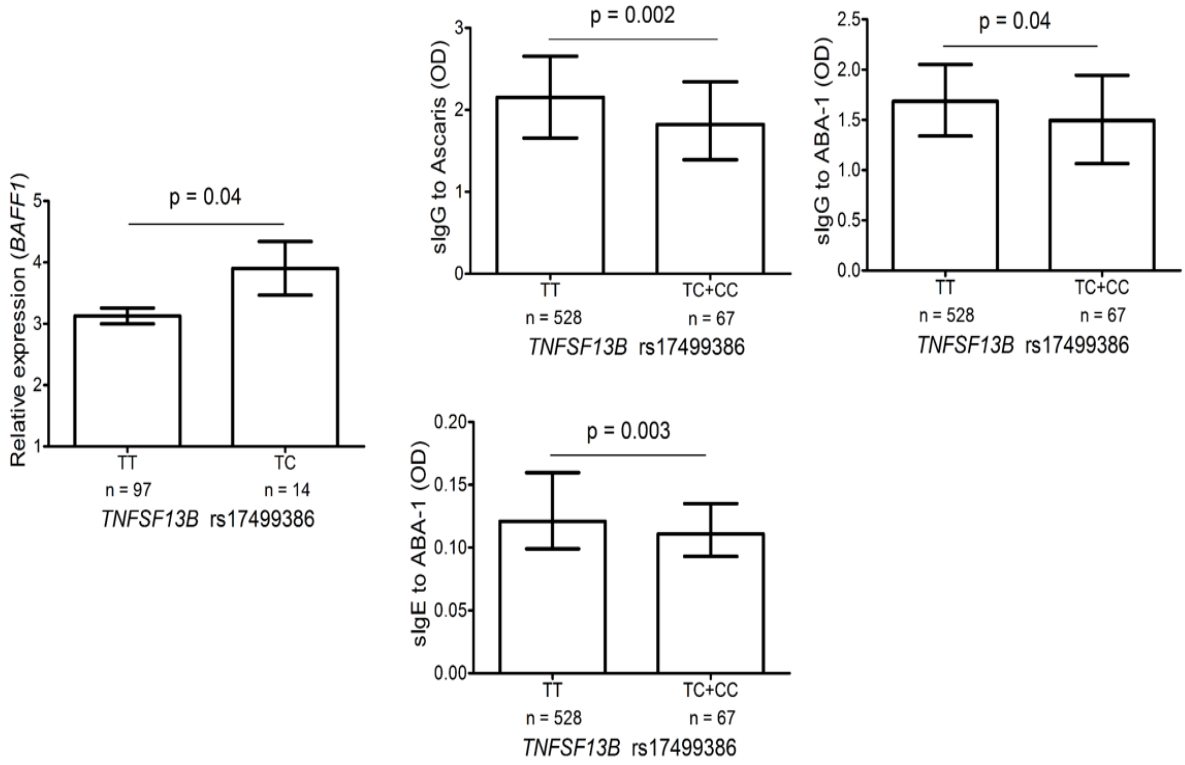

Figure 6. Relative BAFF1 mRNA expression and specific antibody responses to *Ascaris* and ABA-1 according genotypes for *TNFSF13B* rs17499386.

Although few genetic variants regulating the levels of transcripts for BAFF have been reported in humans, our result is in agreement with a previous report showing that a promoter SNP rs9514828 was associated with higher levels of BAFF mRNA in individuals with systemic erythematous lupus[264]. Also, it is in agreement with the previous report by our group stating that an intronic tagSNP (rs10508198, located 5475 bp away) was associated with lower levels of specific IgG to *Ascaris*[53].

Among the genetic variants associated with the soluble BAFF levels, we found that one intronic SNP (rs114688573) with a low frequency distribution in the population (MAF = 0.01) was associated with low soluble BAFF levels (n = 772; B: -238; β: -0.114; 95%IC: -385, -91; p = 0.001; p_{adj} = 0.001), and with high levels of IgG to *Ascaris* (n = 985; B: 0.341; β: 0.086; 95%IC: 0.09 – 0.6; p = 0.007; p_{adj} = 0.007). These associations were driven by

lower soluble BAFF ($p < 0.0001$) and higher IgG to *Ascaris* ($p = 0.005$) in heterozygous individuals compared to homozygous **(Figure 7)**. This finding is also supporting the idea that genetic variants in *TNFSF13B* might regulate or explain the inverse relationship between BAFF levels and specific antibody responses to *Ascaris* reported in paper II. The two potential SNPs (rs17499386 and rs114688573) regulating that inverse relationship are located 2910 bp away each other, making the genomic region around them suitable for further fine mapping studies.

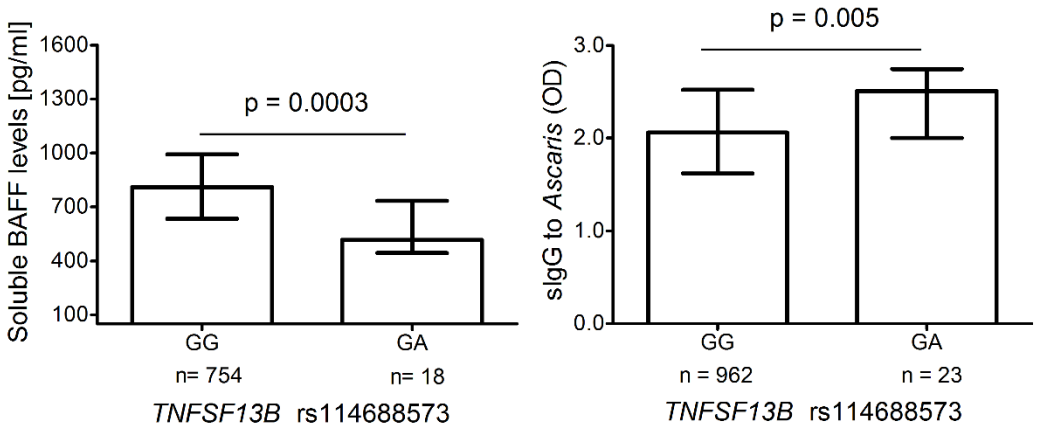

Figure 7. Soluble BAFF levels and specific IgG to *Ascaris* according genotypes for *TNFSF13B* rs114688573.

The results of this study, together with those in papers II and III, suggest that *TNFSF13B* might be related to the susceptibility to *Ascaris* through its effect regulating the *Ascaris*-specific IgG levels more than the specific IgE levels. In connection, a recent study reported that "susceptible" pigs with genotype AB for the locus TXNIP (associated with the *Ascaris*-resistance in pigs) had higher expression of the high-affinity IgG receptor (FCGR1A) as opposed to "resistance" AA pigs in both infected and non-infected animals[170]. Another aspect that requires a deep analysis is the relationship of BAFF with IgG subclasses, being IgG4 of especial interest to be studied due to the fact that it is a sensitive and specific marker for chronic ascariasis, and it is positive correlated to infection intensity[265,266]. In this regard, we found no association between BAFF and the levels of IgG4 to *Ascaris* in the CGA population (data not shown).

5. Concluding remarks and future perspectives

This thesis is based mainly in a genetic association study using a candidate-gene approach (hypothesis-driven) selecting 14 candidate genes on the basis of a priori knowledge of these genes in the immunity type-2 and their relationship with IgE control in humans.

This thesis provides genetic evidence supporting the role of *TNFSF13B/BAFF* in the regulation of the specific antibody response to *Ascaris,* contributing to a better understanding of the role of this gene in the mechanisms of *Ascaris* susceptibility. Here we validated in two populations the role of *TNFSF13B* as a potential gene regulating the total IgE levels in humans which is in agreement with the original discovery of the locus 13q33 as a QTL for total IgE and *Ascaris* susceptibility by Williams-Blangero et al[56]. The differential effects of the *TNFSF13B*-genetic variants in the levels of specific antibody to *Ascaris* are stronger on the IgG response and seem to be mediated by the regulation of the mRNA expression for BAFF and may affect the soluble levels of this cytokine. Additionally, we identify two potential functional variants (rs17499386 and rs114688573) regulating the inverse relationship between BAFF and the strength of the specific IgG to *Ascaris.*

Even though the individual effect of a single polymorphism is expected to be small, in our work individual SNPs appears to be capable of regulating this inverse relationship. Furthermore, fine mapping, replication and functional studies are required. In this context, those studies should be carried out focusing on genotyping at least 50000 bp around the position chr13:108284965 (rs17499386 NCBI, GRCh38).

One of the main limitations to directly relate our findings in the *TNFSF13B, CHIA* and *CHI3L1* genes to the *Ascaris* antibody response with the *Ascaris* susceptibility in humans, is the lack of parasitological data in the population studied in this thesis although in the pig model we found significant correlations between specific antibody responses and parasitological phenotypes. Therefore, further replication studies should be done in endemic populations, conducting a well parasitological, serological and epidemiological

characterization of the population. In this regard, the population living in Loma Arena-Santa Catalina, Colombia, that has been reported to have a high prevalence of parasitism[8] seems to be an affordable population to perform a replication study considering that it shares the same genetic background as the Cartagena population.

Replicative association studies are very important to confirm reported associations. However, more biological functional validating studies are required in order to prove the functional effects of genetic variants which have been investigated and reported in most of the epidemiological genetic studies to date. The lack of functional reports for most of the genetic variants significantly associated in the genetic epidemiological studies difficult the understanding of physiopathology of complex diseases and quantitative traits. Therefore, it is evident that genetic epidemiology should be more integrated with molecular genetics and molecular biology. This body of work provides some candidate variants for functional studies; their effects on transcriptional expression (for those genes expressed at PBMCs and with commercial expression assays available) and the predicting effects affecting binding sites for transcription factors through bioinformatic tools were analyzed. Additional functional approaches such as ChIA-PET (chromatin interaction analysis by paired-end tag sequencing) and chromatin conformational assays can be very useful to study the interactions between the locus 13q33-34 and the Th2 locus.

Other very interesting aspects in which further studies should be focused on are: the disentangling of the BAFF role in the polyclonal B cell activation and generation of specific antibody producing B cells within different lymphoid tissues (i.e. spleen, lymph nodes and bone marrow), liver and intestinal mucosa throughout pig experimental models of ascariasis; and the exploration of *Ascaris* effects on BAFF production given that it is unknown if *Ascaris* can modify the BAFF levels during infection. Experimental *Ascaris* infection models in Pigs are ideal to understand immunological aspects of ascariasis in humans; however, nowadays, some technical limitations concerning the availability of appropriate reagents to experimental porcine work can generate some difficulties in interpreting and extrapolating the results. Therefore, the development of appropriate reagents for the use in pigs is a primary necessity.

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6. Acknowledgements

I would like to express my deepest gratitude to everyone that has contributed to this thesis. In particular to:

Luis Caraballo, my professor and supervisor, for giving me the opportunity to do my PhD in your group and for your invaluable input in all academic discussions about topics covered in this thesis. Thank you very much for the ideas and suggestions that you gave to me that have greatly driven the positive direction in which my thesis work developed. Thanks for your dedication in carefully reading and correcting the manuscripts included in this thesis.

Nathalie Acevedo, my main supervisor, thank you very much for your guidance and support, for your dedication to planning, and doing the experiments included in this thesis work, and also for your thoroughness with respect to the interpretation of the results of our studies. Thank you very much for the ideas and suggestions that you gave to me which a allowed my thesis work to be a piece that was well developed. Thanks for your dedication in carefully reading and correcting the manuscripts included in this thesis. Thanks for your good advice and your hospitality during my research internship in Stockholm-Sweden.

Dilia Mercado, my co-author and friend, thank you for the valuable help in the lab, thank you for performing many of the antibody determinations included in this thesis.

Annika Scheynius and Per Unneberg, co-authors, thank you for your hospitality during my research internship in Stockholm-Sweden and for your dedication in reading and correcting some of the manuscripts included in this thesis. Per Unneberg, thank you for the help in the resequencing data analysis.

Per Skallerup, Peter Nejsum and Stig Millan Thamsborg, co-authors, thank you for performing the *Ascaris*-infection pig model and for your dedication in carefully reading and correcting the paper I included in this thesis.

The University of Cartagena, for the Doctoral Studies scholarship.

Colciencias, Departamento Administrativo de Ciencia, Tecnología e Innovación de Colombia, grant 567-2012 national doctoral studies, thanks for the financial support.

Leonardo Puerta and Niradiz Reyes, doctoral committee members, thanks for your contribution to my education during seminars and presentations.

Mileidis Avila, technical assistant at the Institute for Immunological Research, thank you for the help during blood samples recollection for RNA extraction and flow cytometry analyses included in paper II.

Ana Carolina Mercado, Dalgys Martínez, Eloina Zarate, Velky Ahumada and Stephanie Casadiego, colleagues and friends, thanks for supporting me, for always having a positive attitude and your good advice.

Sandra Coronado, Ronald Regino, Stephanie Casadiego, Emiro Buendía, Marlon Múnera and Neftalí Manotas, colleagues at the Institute for Immunological Research, thanks for your friendly atmosphere in the lab.

Leonardo Machado for being my friend and my emotional support during all these years as a Ph.D. student.

Finally to **my parents** for all your support and love during all these years.

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Study I

IgA levels are associated with infection intensity in *Ascaris* infected pigs

Journal:	*Parasite Immunology*
Manuscript ID	PIM-2017-0059
Manuscript Type:	Original Paper
Date Submitted by the Author:	28-Jun-2017
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Key Words:	Porcine < Host species, Humoral immunity < Immunological terms, Ascaris spp < Parasite, Animal model < Tools and techniques, ELISA/Enzyme-linked immunosorbent assay < Tools and techniques, Serodiagnosis < Tools and techniques

IgA levels are associated with infection intensity in Ascaris infected pigs

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Running title: IgA levels in pigs infected with Ascaris

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Abstract

Aim: To investigate the dynamics of antibody levels to three purified Ascaris antigens in relation to parasitological indicators of infection intensity. **Methods and Results:** Forty-six pigs trickle-infected with A. suum and 9 non-infected controls were studied. Serum IgA and IgG levels against Ascaris whole-body extract and three purified molecules (ABA-1, tropomyosin and glutathione transferase (GSTA)), were measured at 0, 17, 28 and 56 days post first infection (p.i.). IgA levels to Ascaris whole-body extract correlated with the number of macroscopic worms (rho=0.54, p<0.001) and faecal egg counts (FEC) (rho=0.41, p=0.004) at day 56 p.i. IgA levels to Ascaris tropomyosin and ABA-1 correlated with the number of liver white spots of lymphonodular type (rho=0.33, p=0.02 and rho=0.38, p=0.009, respectively). Moreover, IgG antibodies to GSTA at day 28 p.i. and at day 56 p.i. inversely correlated with FEC at day 56 p.i. (rho = -0.30, p=0.04 and rho= -0.37, p=0.01, respectively). Comparisons between groups of pigs with high and low infection intensity confirmed these findings. Conclusion: ascariasis induced high IgA responses against Ascaris antigens and this response was associated with infection intensity. IgG levels to Ascaris GSTA may participate in early response mechanisms towards migration of larvae and/or fecundity of adults.

Keywords

ABA-1, antibody levels, FEC, glutathione transferase, IgG, liver white spots, worm load.

Introduction

Ascariasis continues to be a significant health problem in humans and pigs. Humans are infected with *Ascaris lumbricoides,* affecting approximately 800 million people worldwide with an overall prevalence of 14.5% [1]. Pigs are infected with *Ascaris suum,* which interferes with health and performance, resulting in reduced feed to gain ratios, liver condemnation and compromise vaccine efficacy, incurring in economic losses[2]. Both *Ascaris* species are genetically and evolutionary related and share similar antigenic composition[3-5]. Several molecules of *Ascaris* spp. have been characterized (*e.g*. As12, As14, As16, As24, As37, PAS-1, Asc s 1 (ABA-1), Asc l 3 (tropomyosin) and glutathione S-transferase (GSTA))[6-12]. However, only few studies have evaluated the antibody response against purified antigens of *Ascaris* in pigs[7,13]. To date, most studies in pigs using *Ascaris* adult body fluid (ABF) and excretory-secretory (ES) products from larval stages, have shown that specific antibody responses are detected as early as 7 to 10 days post infection (p.i.) with IgG and IgA as the main isotypes[7,14,15]. Earlier studies using *Ascaris* preparations with somatic antigens have also suggested the presence of immunodominant antigens of ~42 kDa and 97 kDa that may induce potent IgG responses in pigs[16].

ABA-1 is a 14.4 kDa lipid-binding protein so far only found in nematodes[17-19]. The protein is produced as a polyprotein which is post-translationally cleaved to multiple copies of 14 kDa polypeptides[17]. A single unit of ABA-1 is α-helix rich and comprises two subdomains each with a binding site for hydrophobic ligands[18]. The protein is a dominant component of ABF, specially abundant in the pseudocelomic fluid, and also is present in antigen preparations of all stages of *Ascaris*[20]. This protein is also considered as a resistance marker of *Ascaris* infection in humans because there is an association between specific IgE levels to this protein and infection resistance[21,22]. Rodent studies have shown that the host's ability to mount an antibody response to ABA-1 is dependent on certain MHC haplotypes[23,24].

Tropomyosin is a 40 kDa protein with a native structure consisting of two parallel alpha-helical tropomyosin molecules that are wound around each other forming a coiled-coil dimer. This protein belongs to a family of phylogenetically conserved proteins with multiple isoforms found in all eukaryotes. Nematode tropomyosins typically induce strong IgE and

IgG responses upon infection in mammals; indeed, it has been reported that in infections with *Onchocerca volvulus,* anti-tropomyosin antibodies limit microfilaria density and are associated with protective immunity to onchocerciasis[25]. Tropomyosins from shrimp, molluscs, insects and house dust mites have also been decribed as an allergen in humans; with a high degree of cross reactivity among all these sources[26]. In humans, IgE reactivity to *Ascaris* tropomyosin is very frequent in naturally exposed populations[27,28].

GSTA belongs to the family of glutathione S-transferases which are multifunctional enzymes involved in cellular detoxification in eukaryotes by adding reduced glutathione (GSH) to endobiotics, xenobiotics and electrophilic substrates and/or binding to a broad range of molecules including host-immune initiated reactive oxigen species[29,30]. *Ascaris* GSTA has been detected in the nematode intestine and in excretion/secretion products[30], it is know to exist as different isoforms and named as the allergen Asc l 13.0101 in humans[31], however the antigenic strenght of this molecule is still a matter of debate. Human and mice IgG antibodies against GSTs have shown to be protective[32-35] against nematodes by mediating cytotoxic effects[36]. Indeed, GSTs have been targeted as lead candidates for vaccination against several helminths including *Schistosoma* and hookworm[33-35,37].

During the last years we have been studying the antibody response to Ascaris and its purified components ABA-1, tropomyosin and GST; in humans naturally exposed to this nematode in an endemic community in Cartagena (Colombia)[27,31,38], aiming to better understand the host-parasite relationship that influence humoral responses to *Ascaris* and the susceptibility to allergic sensitization and asthma[28,39]. Our studies in infants from the FRAAT birth cohort study revealed that specific IgE antibodies to ABA-1 and tropomyosin are detected early in life in an *Ascaris* endemic region in the tropics[40]. Among *Ascaris* exposed children 75% had detectable IgE antibodies to ABA-1 and 62.5% to *Ascaris* tropomyosin although stool examinations only detected *Ascaris* eggs in 10.5% of children[40]. However, it was needed to evaluate the antibody response to these molecules in a biological system, in which covariates like natural reinfections, use of regular anthelminthic treatments, genotype and environmental co-exposures could be properly controlled. To date, no study has yet evaluated the relationship between antibody levels to these *Ascaris* purified antigens with indicators of

infection intensity. The aims of this study were to analyze the longitudinal changes of antibody levels to *Ascaris* whole-body extract and three recombinant antigens (ABA-1, tropomyosin and GSTA) in an experimental pig model trickle infected with *A. suum* and to analyze the relationship between anti-parasite antibody levels and worm loads at necropsy.

Materials and methods

Experimental animals and parasitological phenotypes

The experimental protocol used to generate *A. suum* infected pigs and details on their phenotypic traits have been described elsewhere[41,42]. Briefly, 62 cross-bred Duroc/Danish Landrace/Yorkshire pigs from 10 litters were purchased from a commercial pathogen-free farm. The pigs were selected based on their genotype (AA, AB) at the single nucleotide polymorphism (SNP) TXNIP associated with susceptibility to *A. suum*[43], with heterozygotes being most susceptible. The piglets were weaned at four weeks of age, then kept in a separate pen (without the sow) until they were seven weeks. The farmer used mixed semen to produce the littermates which were thus full-sibs or half-sibs; males were castrated. Based on genotypes, the pigs were allocated to trickle-infected groups (n_{AA}=27; n_{AB}=25) or uninfected groups (n_{AA}=5; n_{AB}=5).

Pigs were kept at the animal facility at the National Veterinary Institute, Denmark from seven weeks of age until necropsy. The pigs were housed in four pens (three pens for infected pigs, one pen for the uninfected controls), with equal distribution of genotype, litter of origin, sex and weight in each pen. Embryonated eggs used for trickle infections were prepared from female *A. suum* worms collected at a Danish slaughterhouse. At 8 weeks of age the pigs were infected with *A. suum* eggs (25 eggs/kg/day) twice per week. The pens were littered with wood shavings on a daily basis, and water was provided *ad libitum*. The animals were fed a diet consisting of ground barley supplemented with proteins and minerals. The pigs were euthanized using a captive bolt pistol followed by exsanguination at days 55-59 p.i. (for simplicity called day 56 p.i. subsequently). After removing the viscera, the small intestine was opened longitudinally and any macroscopic *A. suum* worms (large juveniles and adults) were removed and counted. Larvae were isolated from the intestinal contents by the agar-gel

method[44]. The total larval burden was calculated by extrapolation from a 20% aliquot count. All worms were stored in 70% ethanol until they were counted.

The liver was carefully examined, and white spots caused by migrating larvae were counted on the diaphragmatic and visceral sides; all livers were examined by the same person who was blinded to pig genotype. Faecal egg counts (FEC) taken at 56 day pi. were determined using a modified McMaster method with an analytical sensitivity of 20 eggs per gram of feces (epg). Samples were processed and counted in duplicate, and the mean of the two measurements was used in the statistical analysis. Six parasitological phenotypes were evaluated: number of macroscopic *A. suum* worms (including large juveniles and adults), number of intestinal *A. suum* larvae, total *A. suum* burden (sum of intestinal larvae and macroscopic worms), total number of liver white spots (visual inspection of surface), number of liver white spots of lymphonodular type and FEC at day 56 p.i.

Blood samples were taken at 0, 17, 28 and 56 days p.i. for antibody determinations. After collection, samples were refrigerated overnight and centrifuged at 1763 *g* for 10 minutes. Serum was then frozen at -20°C until analysis. The study was approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark (Ref. 2010/561-1914). Care and maintenance of all animals were in accordance with applicable Danish and European guidelines.

Antigens

The whole-body extract from adult worms (containing both somatic and ES products) was prepared by the acetone–saccharose precipitation method as described previously[38]. Three recombinant purified antigens (ABA-1, tropomyosin and GSTA) were prepared as follows: cDNA encoding for ABA-1 from *A. suum* (GenBank Accession Number L03211) was cloned into pGEX-1kT, and expressed as a glutathione-S-transferase fusion protein in *Escherichia coli* BL21 (Invitrogen Corporation, Carls-bad, CA, USA). Expression was induced with Isopropyl β-D-thiogalactopyranoside (IPTG, 0.1 mM) during 4 hours at 37°C. The ABA-1 was purified with glutathione–sepharose beads and digested with thrombin; purified protein was dialysed in water and kept at -20°C until use[38]. The recombinant tropomyosin from *A. lumbricoides* (Asc l 3, GenBank Accession Number FJ655903) and the glutathione

transferase from *A. suum*, (GSTA, GenBank accession number X75502.1) were cloned into pQE-30 UA (QIAexpress UA Cloning Kit; Qiagen Inc. Valencia, Calif., USA) and expressed in *E. coli* M15 cells as a 6x His-tagged protein as previously described[27,31].

Antibody determinations

Specific levels of IgA, IgG, IgG_1 and IgG_2 to *A. suum* whole-body extract and recombinant proteins were quantified by an indirect enzyme-linked immunosorbent assay (ELISA) in microtiter plates (IMMULON 4HBX, Thermo Fisher Scientific). Optimal dilutions for antigens, serum and conjugate were determined by titration. The plates were coated with *A. suum* extract (1µg/100µl) and recombinant proteins (0.5µg /100 µl) in buffer Carbonate/Bicarbonate (pH 9.6) overnight at 4°C in a humid chamber; then washed 5 times (Automatic plate washer, Thermo Scientific well wash) with 0.05% Tween20-PBS (PBST) and blocked with 200 µl blocking buffer (1% BSA-PBS) for 3 hours. Pre-optimized 2-fold serial dilutions of reference serum samples (serum with the highest optical density, OD values), negative-non-infected controls and study samples were incubated overnight at room temperature (RT) for IgA determination and 1.5 hours at 37°C for IgG, IgG_1 and IgG_2. For measuring IgG and IgG_1 to recombinant proteins, sera were pre-adsorbed with a cell lysate of *E. coli*.

IgA was detected using goat anti pig IgA (Alk. Phos, AA140 AB batch # 130513 AbD Serotec) diluted 1:5000 in 1% BSA 1mM $MgCl_2$ 0.02%-sodium azide Tris buffer (pH 8.0) and incubated 2 hours at RT. The reaction was developed using 4-nitrophenyl phosphate disodium salt hexahidrate (pNPP, Sigma-Aldrich 2640) in 15 ml of 10% diethanolamine, 0.5 mM $MgCl_2$.

For IgG detection a rabbit anti pig IgG peroxidase conjugate (A5670, Sigma-Aldrich, USA) diluted 1:10,000 in PBST was used and plates were incubated for 1 hour at 37°C; the reaction was developed using *O*-phenylenediamine dihidrocloride, OPD (Sigma-Aldrich P8287) in phosphate–citrate 0.05 M sodium perborate buffer (Sigma P4922) adding 200 µl per well and incubating for 30 minutes in dark. The reaction was stopped with 50 µl of 3M H_2SO_4 and read at 492 nm.

IgG_1 and IgG_2 were detected with mouse monoclonal antibodies specific for porcine IgG_1 (Clone K139 3C8- Serotec) or IgG_2 (Clone K68-Serotec) in a pre-optimized dilution (1:400) in PBST and incubated at 37°C for 1.5 hour. Bound antibodies were visualized by adding a goat anti-mouse IgG (whole molecule Alk. Phosphatase conjugated A3562 Sigma-Aldrich) diluted 1:10,000 in PBST and incubated at 37°C for 1 hour. The reaction was developed with 4-nitrophenyl phosphate disodium salt hexahidrate (pNPP, Sigma-Aldrich 2640) in 15 ml of 10% diethanolamine, 0.5 mM $MgCl_2$. For IgA, IgG_1 and IgG_2 the reaction was stopped with 100 µl of 3N NaOH and the absorbance was measured at 405 nm using a spectrophotometer (Spectra Max 250; Molecular Devices, Sunnyvale, CA, USA).

Total IgE levels were measured by competitive ELISA using Porcine Immunoglobulin E ELISA Kit (Cusabio cat number CSB-E06803p) and following the technical Instructions of the manufacturer. Detection range of this assay was 0.05ng/ml-12.5 ng/ml.

Statistical analysis

The normality of the data was explored with the Kolmogorov-Smirnov test. We compared antibody levels between infected and non-infected pigs using the Mann-Whitney U test. Differences in antibody levels among days 0, 17, 28 and 56 p.i. were analyzed using the Friedman test with Dunn's comparison of all pairs of columns. Correlations between antibody levels and parasitological phenotypes were calculated using the Spearman correlation test. Pigs were classified in groups of low infection or high infection intensity based on the 75th percentile of each parasitological phenotype as a cut-off and antibody levels were compared between low and high infection groups by Mann-Whitney U test. The potential effect of pen, gender and genotype (TXNIP) was analyzed by logistic regression. Statistical analyses were performed using the Statistical Package for the Social Sciences software (SPSS version 20, Armonk, NY, USA IBM Corp) and GraphPad Prism version 7 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Parasitological phenotypes

One pig of each genotype was sacrificed on day 13 post first infection to test the infectivity of the *A. suum* egg batch and these two animals were excluded from the analyses. Two of the infected pigs (both genotype AA) died during the experiment for reasons not related to the study treatment. Serology could not be determined for two infected pigs (both genotype AB) and one control pig (genotype AB). The final dataset hence comprised 46 trickle-infected pigs (n_{AA}=24; n_{AB}=22) and 9 uninfected pigs (n_{AA}=5; n_{AB}=4).

Descriptive statistics (mean ± s.e.m) of parasitological phenotypes at day 56 p.i. in the infected pigs (n = 46) were as follows: macroscopic A. suum burden (11.2 ± 5.7), number of intestinal A. suum larvae (36.4 ± 7.3), total number of liver white spots (392.7 ± 18.8), number of liver white spots of lymphonodular type (9.07 ± 1.1) and FEC (779.57 ± 352.63 epg). The sample distribution for each parasitological phenotype is presented in **Figure 1**. From these results we identified groups of pigs at the extreme of the distribution (≥ 75th percentile) that were regarded as harboring high intensity of infection and compared with the low infection group (<75th percentile) in additional analyses. Non-infected pigs (n = 9) were negative for all parasitological phenotypes.

Dynamic of antibody response to Ascaris whole-body extract

Ascaris-specific IgA levels increased from day 17 p.i., reached its maximum levels at day 28 p.i., and decreased by day 56 p.i. Ascaris-specific IgG levels showed a sustained increase from day 17 p.i. until day 56 p.i. (**Figure 2A**). Ascaris-specific IgG1 increased from day 28 p.i. to 56 p.i., while Ascaris-specific IgG2 levels were similar across all the time-points (**Figure 2A**). Of these four isotypes, the IgA isotype was the most prominent
267 (**Supplementary Fig. 1A and Fig. 1B**) and IgG levels were mainly IgG1 (**Supplementary Fig.1C**). The levels of all the aforementioned isotypes were significantly higher at all time points compared to baseline (day 0) and to the levels observed in non-infected pigs (**Supplementary Fig. 1**). Total IgE levels were higher in infected pigs after day 0; however no significant differences were observed overtime (**Supplementary Fig. 1D**).

Dynamic of antibody responses to recombinant antigens

The IgA levels to ABA-1, tropomyosin and GSTA had a similar dynamic, increasing from day 17 p.i., reaching its maximum at day 28 p.i., and decreasing at day 56 p.i., (**Figure 2B**). Compared to day 0 (baseline), tropomyosin-specific IgA levels were significantly higher at days 17 p.i. ($p = 0.004$) and 28 p.i. ($p = 0.01$), while for ABA-1 ($p = 0.008$) and GSTA ($p = 0.03$), the increase in specific IgA antibodies were only significant at day 28 p.i.

We found higher levels of IgG to all the recombinant tested at day 0 than at other time points (data not shown); suggesting a passive immunity via colostrum and/or milk transmission[45], therefore we here did not conduct further analysis on the relationship between IgG_1 and IgG_2 levels to purified molecules and infection intensity.

Relationship between antibody response and infection intensity

We next sought to investigate the correlation between antibody levels and infection intensity using six different phenotypes. We found significant correlations between antibody levels to *Ascaris* whole-body extract at day 56 p.i., and worm and egg count phenotypes (**Figure 3**). Hence, Ascaris-specific IgA levels correlated with the number of macroscopic *Ascaris* (rho = 0.54, $p < 0.001$), total *A. suum* burden (rho=0.42, p=0.003) and FEC (rho = 0.41, p = 0.004). Ascaris-specific IgG correlated with the number of macroscopic Ascaris (rho = 0.30, p = 0.03) and the total A. suum burden (rho=0.32, p=0.02). *Ascaris*-specific IgG2 correlated with the number of larvae (rho = 0.33, p = 0.02) and the total *A. suum* burden (rho=0.35, p=0.01). Regarding the antibody response to purified antigens, we found significant positive correlations between the number of lymphonodular liver white spots with the IgA levels to tropomyosin (rho = 0.33, p = 0.024) and with IgA levels to ABA-1 (rho = 0.38, p = 0.009) (**Figure 3**).

Moreover, antibody levels to GSTA were significantly correlated with FEC and that relationship was detected earlier than with any other molecule: GSTA-specific IgA levels at day 17 p.i. significantly correlated with FEC (rho=0.35, p=0.01) while GSTA-specific IgG levels at day 28 p.i. and at day 56 p.i. inversely correlated with this phenotype (rho = -0.30, p=0.04; rho= -0.37, p=0.01, respectively) suggesting that production of antibodies to this

molecule may have a negative impact on larvae migration and/or adult fecundity later in the course of infection.

We then studied the antibody levels to *Ascaris* whole-body extract 303 according to the infection intensity, which by grouping the individuals at the extreme of the distribution increases the power to define a wormy pig. Ascaris-specific IgA levels were significantly increased in pigs with high number of macroscopic worms at day 56 p.i. (Mann Whitney U test p value < 0.0001, **Figure 4A**) and with the highest FEC (p=0.001, **Figure 4B**). IgA levels to the purified proteins did not differ according to the infection intensity of macroscopic worms, number of larvae or FEC.

Based on the significant correlations between IgA levels to tropomyosin and ABA-1 and the number of lymphonodular white spots (**Figure 3**) and considering that this type of lesion can be regarded as a marker of host immunity in recently infected animals[46], we analyzed the relationship between specific IgA levels to tropomyosin, ABA-1 and GSTA in pigs classified as having high or low numbers of lymphonodular white spots based on being above or within the 75th percentile of the population (**Figure 1E**). We found significant increased levels of IgA to ABA-1 in pigs with high number of lymphonodular white spots (Median, IQR: 0.29 (0.22 – 0.35) compared to those with low number of lymphonodular white spots (Median, IQR: 0.20 (0.17 – 0.26)), Mann Whitney U test p value = 0.027 (**Figure 4C**). For this phenotype, IgA to tropomyosin or GSTA did not differ between groups. Total number of white spots (including both granulation-type and lymphonodular-type) was not related with antibody levels.

As described above, we found a significant negative correlation between specific IgG levels against GSTA and FEC (**Figure 3**). In agreement with those findings the comparison based on the 75th percentile of the FEC confirmed that pigs with high FECs had lower levels of specific IgG to GSTA (Mann Whitney U test p value = 0.01, **Figure 5**). We did not find significant differences between the SNP TXNIP genotype and antibody levels to whole-body extract or to any of the purified proteins tested. Moreover, none of the antibody levels associated with parasitological phenotypes was affected by pig gender or pen.

Discussion

In this study we analyzed the longitudinal changes in antibody levels to *Ascaris* whole-body extract and three purified *Ascaris* antigens not previously tested in a pig model trickle-infected with *A. suum*. To our knowledge this is the first report showing that high IgA levels to the *Ascaris* extract and ABA-1 are associated with infection outcomes in pigs including infection intensity and the number of lymphonodular liver white spots. Our results are in agreement with previous studies showing that *A. suum* infection stimulates a dominant IgA response and suggesting correlations between antibody response to *Ascaris* and parasitological phenotypes[15,47,48]. The time trends in IgA levels observed in this study also reproduce those reported for the *Ascaris* adult body fluid (ABF) and the excretory-secretory (ES) products from larval stages, which are produced early as 10 to 14 days p.i., and decrease after 4 weeks[14,47].

IgA is the dominant isotype in jejunal mucosa of *A. suum* infected piglets[49]. Most of the IgA is synthesized by plasma cells in the gut-associated lymphoid tissue and about 30% of this gut IgA is transported to the general circulation through the lymphatic system, contributing to the pool of the circulating IgA in pigs[50]. The high serum IgA levels to the extract that we detected in the pigs infected with the highest worm loads may be the result of the migration of larvae and/or intestinal antigen loads inducing high levels of local IgA; a possibility we cannot verify because we did not evaluated the presence of antibody-producing cells in the intestine.

The protective role of IgA levels to specific *Ascaris* antigens in infected pigs is not well defined. However, other animal models have shown that mucosal IgA is an important mediator for the expulsion of parasites: in sheep infected by *Teladorsagia circumcincta*, IgA mediates suppression of worm growth and fecundity, possibly in conjunction with eosinophils[51]. In cattle, a similar effect has been reported for the resistance to *Ostertagia ostertagi*[52]. In rats, the killing of schistosomes by eosinophils has been shown to be IgA mediated[53]. Furthermore, in humans a highly glycosylated isoform of FcαRI is found on eosinophils and it is known that sIgA (secretory IgA) strongly stimulate the degranulation of these cells[54]. Recent studies have shown a predominant role for cellular mechanisms (specifically those eosinophils-mediated) in the intestinal immunity against *Ascaris*[55]. Our

study support a significant association between IgA levels to the *Ascaris* whole-body extract and infection markers like the number of macroscopic worms and FEC. IgA levels may protect from infection because after their increase between 17 to 28 days p.i., most of the pigs showed decreased IgA levels by day 56 p.i. **(Figure 2)** in connection with low levels of infection **(Figure 1)**. In the small group of susceptible pigs with a high infection intensity, IgA levels remains relatively high, suggesting that the constant stimulation by larvae and adult worms sustain an increased synthesis of IgA.

We detected IgA antibodies to ABA-1 early during infection (day 17 p.i.). Previous studies in mice and humans have supported the role of ABA-1 as a resistance marker of *Ascaris* infection[21,22]. In this study, IgA levels to ABA-1 at day 56 p.i. significantly correlated with the number of lymphonodular liver white spots **(Figure 4C)**. Those lesions are a fibrotic inflammatory response composed of leucocyte infiltrations involved in the hepatic barrier limiting the propagation of the larvae towards the lung[56-58]. Interestingly, in pigs with high infection intensity, IgA levels to ABA-1 followed a different dynamic than those to tropomyosin and GSTA and remained in plateau at day 56 p.i. (data not shown). More experimental studies are needed to elucidate the relationship between specific IgA levels to ABA-1 with the infection burden and the number of lymphonodular liver white spots.

The increased levels of IgG1 together with no changes in the IgG2 levels is consistent with a Th2 polarization and were similar to previous reports describing that IgG2 seems to be a poor indicator for *A. suum* infection/exposure in pigs[15,59]. We here also found that the IgG1/IgG2 ratio was high in infected pigs at days 28 and 56 p.i. (**Supplementary Fig.1C**) as previously described[15].

As expected, total serum IgE levels were increased in infected pigs compared to non-infected **(Supplementary Figure 1)**, however there were no significant associations between this antibody and infection intensity (data not shown). A limitation of our study is that we measured specific IgE responses with a cross-reactive α-human IgE monoclonal antibody because the appropriate reagents for measuring IgE in pigs were not available; still, we did not find any significant relationship between specific IgE to *Ascaris* antigens and parasitological phenotypes but this relationship remains to be properly investigated.

Moreover, since the design of this model only collected information on most parasitological phenotypes at day 56 p.i., the relationship between antibody levels to *Ascaris* with histopathological features and parasitological phenotypes at days 17 p.i. and 28 p.i. remains to be formally studied; especially, because we here obtained suggestive evidence that IgG antibodies to GSTA as early as day 28 p.i. could predict FEC at day 56 p.i. The mechanisms behind these findings may involve antibody-mediated inhibition of enzymatic functions[36] and alterations in larvae metabolism or fecundity[30,60,61]. These results also suggest a potential use of GSTA as an antigen in serological screenings.

In summary, this is the first longitudinal study evaluating the antibody response to the *Ascaris*-purified antigens ABA-1, tropomyosin and GSTA in trickle-infected pigs. The results show that an Ascaris infection stimulates a strong IgA response within the first four weeks post infection and that this IgA response may play a role in the host's ability to eliminate worms. Specific IgA antibodies to the nematode polyprotein ABA-1 were associated with the number of lymphonodular liver white spots at necropsy. Moreover, the results revealed an inverse relationship between IgG levels against *Ascaris* GSTA and faecal egg excretion suggesting a role of this family of enzymes in *Ascaris* fecundity and metabolism.

Competing interests

The authors declare that no competing interest.

Funding

This work was funded by the Administrative Department of Science, Technology and Innovation (Colciencias, Colombia, Grant 680-2009); by the University of Cartagena, Colombia and by the Danish Agency for Science, Technology and Innovation (grant awarded to P.N.). AB was supported by a PhD study grant from Colciencias-Colombia (Grant 567-2012). PS was supported by a University of Copenhagen (Denmark) PhD grant. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Authors' contributions

AB and DM performed the antibody measurements. PS, SMT, PN originally conceived and conducted the pig infections and provided the samples for antibody measurements. PS, SMT and PN measured the infection phenotypes. AB, NA prepared the recombinant *Ascaris* purified proteins. AB, PS, NA analyzed the data. AB, NA conducted the statistical analyses. SMT, NA, PN, LC conceived the investigation and supervised the experimental work and data analysis. AB, NA, and LC wrote the original draft. AB, PS, DM, SMT, NA, PN and LC wrote, revised, edited and approved the final version of the manuscript.

Acknowledgements

We thank Dr. Malcolm Kennedy of University of Glasgow for providing the clone of ABA-1 and Dr. Ana Maria Presas of National Autonomous University of Mexico for providing the *A. suum* whole-body extract. This work was supported by the Administrative Department of Science, Technology and Innovation (Colciencias, Colombia, Grant number 680-2009 to LC), by the University of Cartagena, Colombia and by the Danish Agency for Science, Technology and Innovation (Grant number 271-09-0162, awarded to P.N.). AB was supported by a PhD study grant from Colciencias-Colombia (Grant number 567-2012). PS was supported by a University of Copenhagen 33 (Denmark) PhD grant. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing interests

The authors declare that no competing interest**.**

Figure legends

Figure 1. Distribution of six parasitological phenotypes in 46 pigs trickle-infected with *A. suum* and necropsied at day 56 post infection (p.i.). **A.** Number of macroscopic *A. suum* worms (including large juveniles and adults). **B.** Number of intestinal larvae. **C.** Total *A. suum* burden (combined number of larvae and macroscopic worms). **D.** Total number of liver white spots including granulation-type and lymphonodular-type. **E.** Number of lymphonodular white spots. **F.** Faecal egg counts (FEC). Each pig is represented by a dot; red dots indicate individuals with observed values equal or above $\geq 75^{th}$ percentile of the distribution. Lines indicate median ± interquartile range.

Figure 2. Dynamics of antibody levels to *A. suum* whole-body extract and three purified *Ascaris* antigens in pigs trickle-infected with *A. suum* as determined at 0, 17, 28 and 56 days p.i. **A.** Antibody levels of IgA, IgG, IgG_1 and IgG_2 to *Ascaris* extract. **B.** Specific IgA levels to recombinant proteins of Ascaris: ABA-1, tropomyosin and GSTA. Lines represent median OD values.

Figure 3. Correlations between antibody levels as determined at day 56 p.i. and parasitological phenotypes. The spearman correlation coefficient is presented inside the square. Non-significant correlations are indicated by a dot. Asterisks indicate the level of significance *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$.

Figure 4. IgA levels to *A. suum* whole-body extract, tropomyosin and ABA-1 according to the infection intensity. **A.** IgA levels to *Ascaris* extract between pigs with low and high number of macroscopic worms at day 56 p.i. **B.** IgA levels to *Ascaris* whole-body extract between pigs with low and high FEC at day 56 p.i.. **C.** IgA levels to ABA-1 between pigs with low and high numbers of lymphonodular liver white spots at day 56 p.i. Each dot represents an individual. Lines represent median and interquartile ranges.OD: optical density.

Figure 5. IgG levels to GSTA according to the infection intensity defined as low or high faecal egg counts (FEC) at day 56 p.i. Each dot represents an individual. Lines represent median and interquartile ranges. OD: optical density.

Supplementary data

Supplementary figure 1.

Antibody response to Ascaris suum whole-body extract in infected and non-infected pigs. **A.** IgA levels. **B.** IgG levels. **C.** IgG1/IgG2 ratio. **D.** Total IgE levels. Mean and s.e.m. (standard error of mean) are shown. Asterisks indicate the level of significance from the comparison of infected vs. non-infected pigs at each time point *** $p < 0.0001$, ** $p < 0.001$, * $p < 0.05$.

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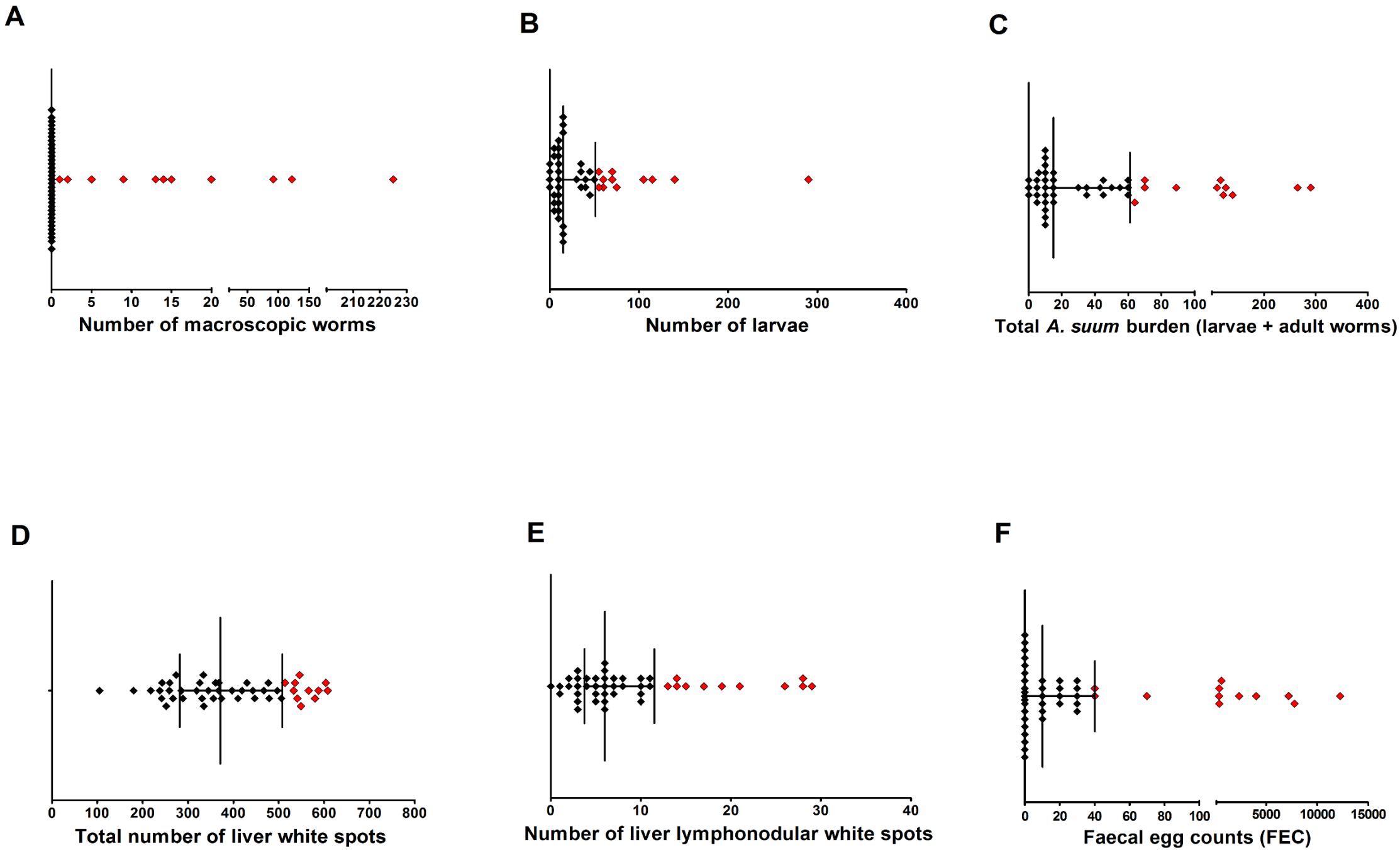

A
0 5 10 15 20 50 100 150 210 220 230
Number of macroscopic worms
B
0 100 200 300 400
Number of larvae
C
0 20 40 60 80 100 200 300 400
Total A. suum burden (larvae + adult worms)
D
0 100 200 300 400 500 600 700 800
Total number of liver white spots
E
0 10 20 30 40
Number of liver lymphonodular white spots
F
0 20 40 60 80 100 5000 10000 15000
Faecal egg counts (FEC)

Figure 1

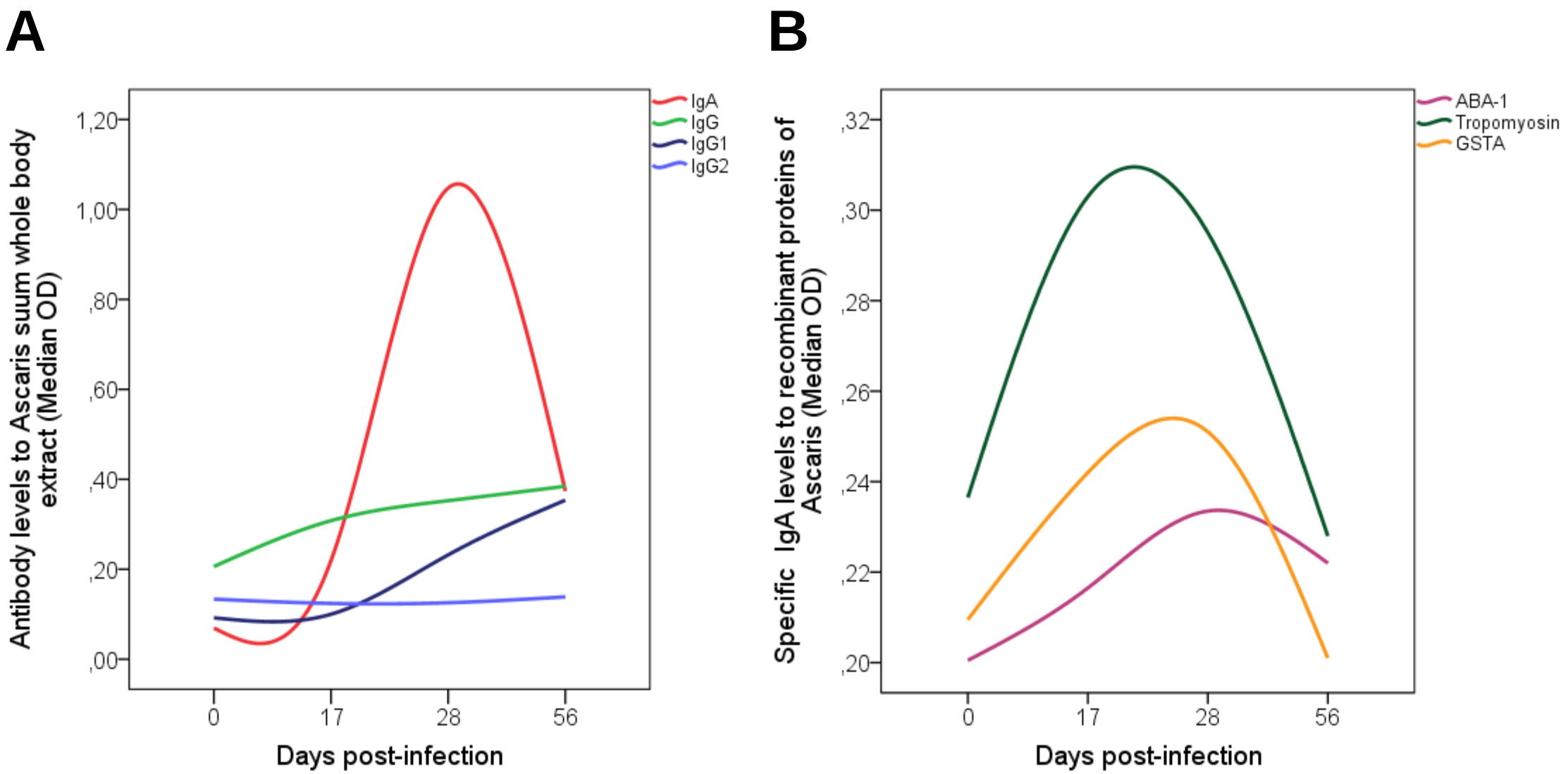

A
Antibody levels to Ascaris suum whole body extract (Median OD)
1,20
1,00
,80
,60
,40
,20
,00
0
17
28
56
Days post-infection
IgA
IgG
IgG1
IgG2
B
Specific IgA levels to recombinant proteins of Ascaris (Median OD)
,32
,30
,28
,26
,24
,22
,20
0
17
28
56
Days post-infection
ABA-1
Tropomyosin
GSTA

Figure 2

Day 56 p.i.	*Ascaris* whole body extract				ABA-1		Tropomyosin		GSTA	
	IgA	IgG	IgG_1	IgG_2	IgA	IgG	IgA	IgG	IgA	IgG
Number of macroscopic worms	0.54 ***	0.30*								
Number of larvae				0.33*						
Number of *Ascaris* (total)	0.42**	0.32*		0.35*						
FEC	0.41**									-0.37*
Number of liver white spots										
Number of liver white spots (lymphonodular)					0.38**		0.33*			

Figure 3

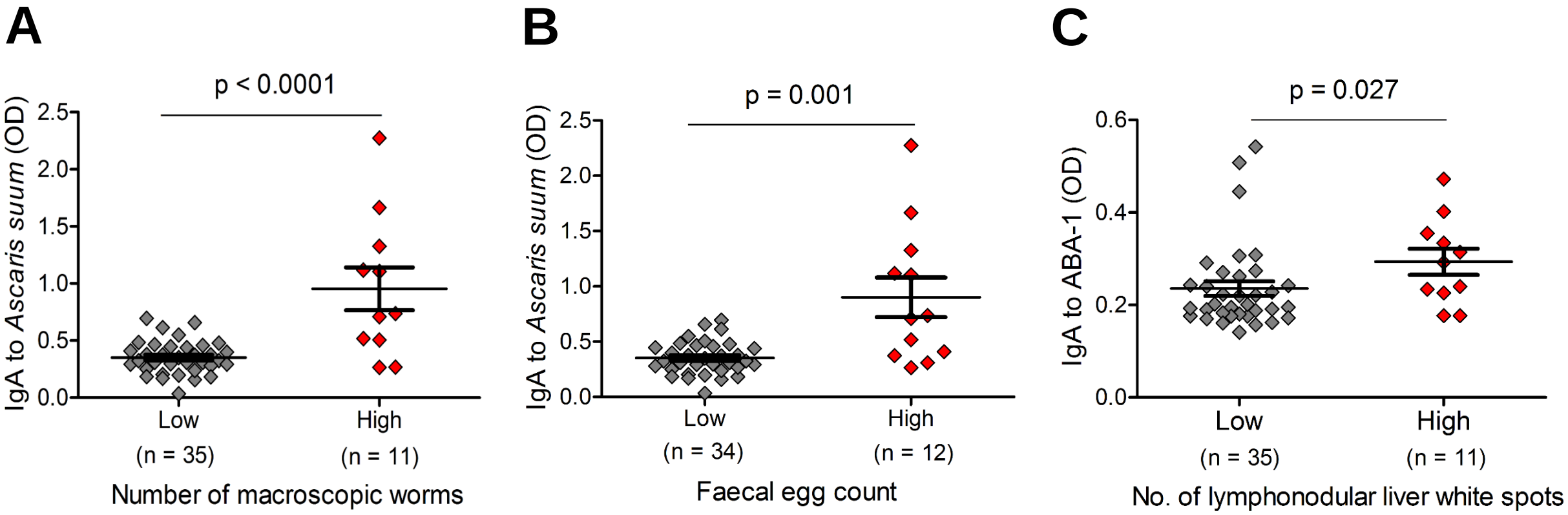

A
p < 0.0001
IgA to Ascaris suum (OD)
2.5
2.0
1.5
1.0
0.5
0.0
Low
(n = 35)
High
(n = 11)
Number of macroscopic worms
B
p = 0.001
IgA to Ascaris suum (OD)
2.5
2.0
1.5
1.0
0.5
0.0
Low
(n = 34)
High
(n = 12)
Faecal egg count
C
p = 0.027
IgA to ABA-1 (OD)
0.6
0.4
0.2
0.0
Low
(n = 35)
High
(n = 11)
No. of lymphonodular liver white spots

Figure 4

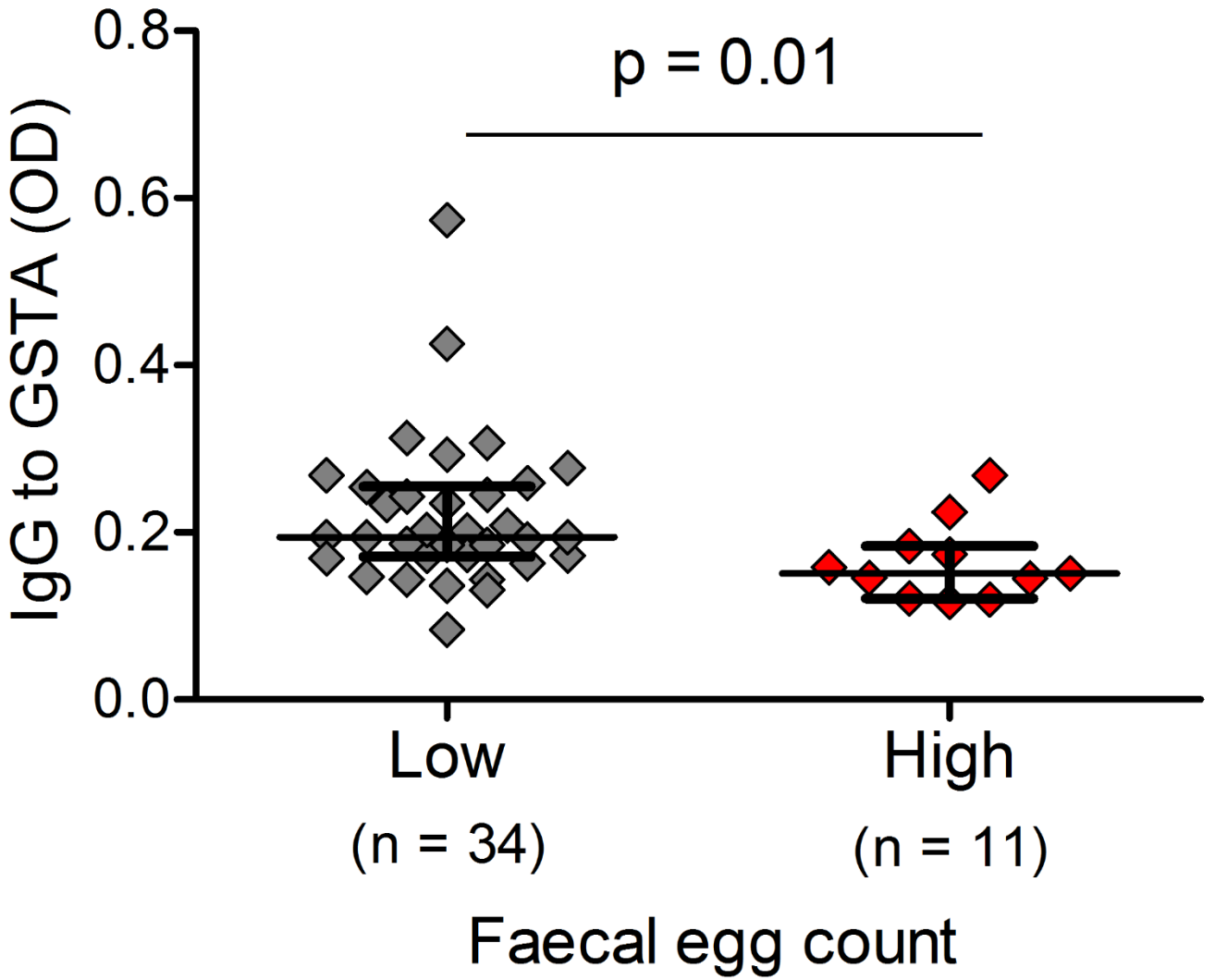
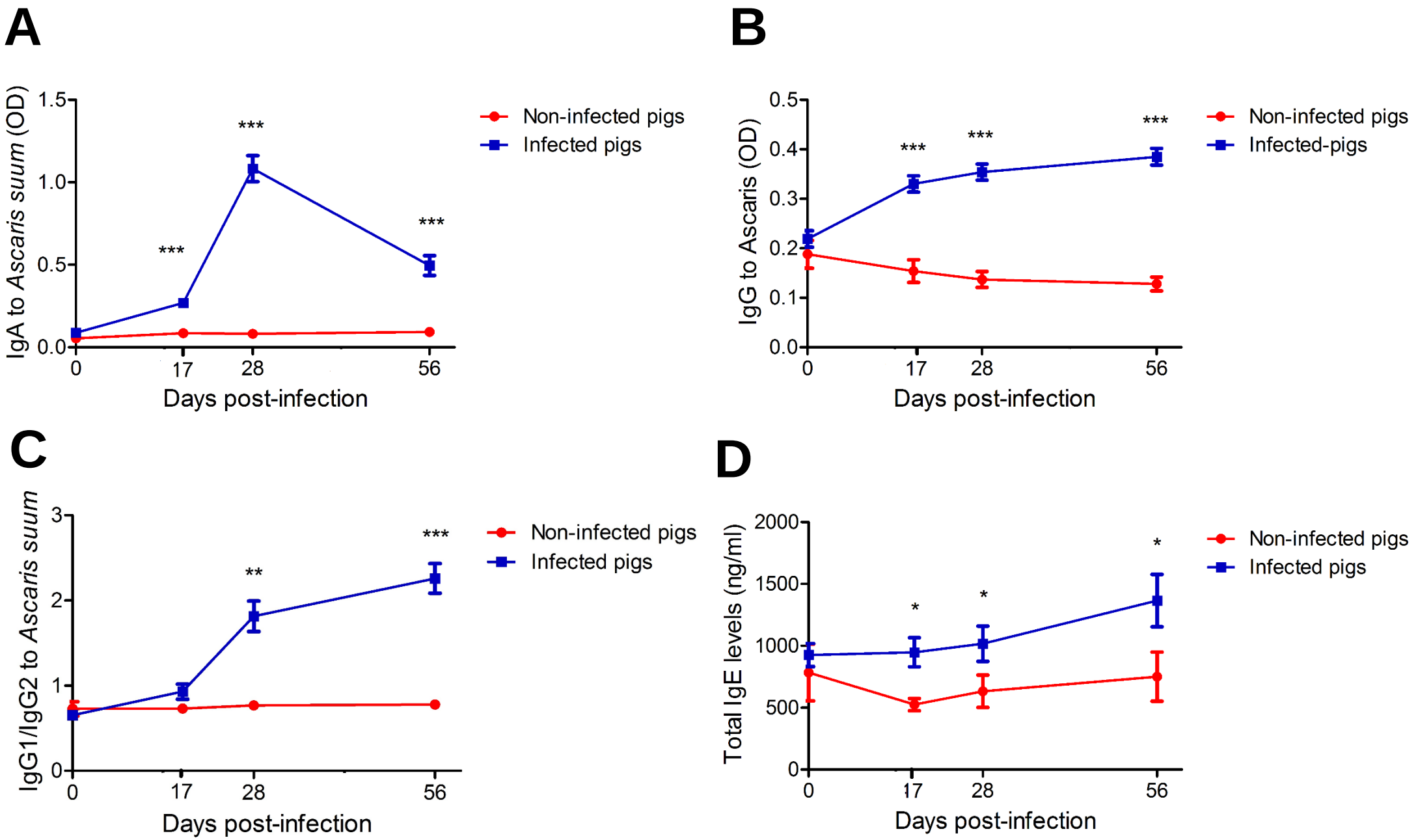

p = 0.01
IgG to GSTA (OD)
0.8
0.6
0.4
0.2
0.0
Low
(n = 34)
High
(n = 11)
Faecal egg count

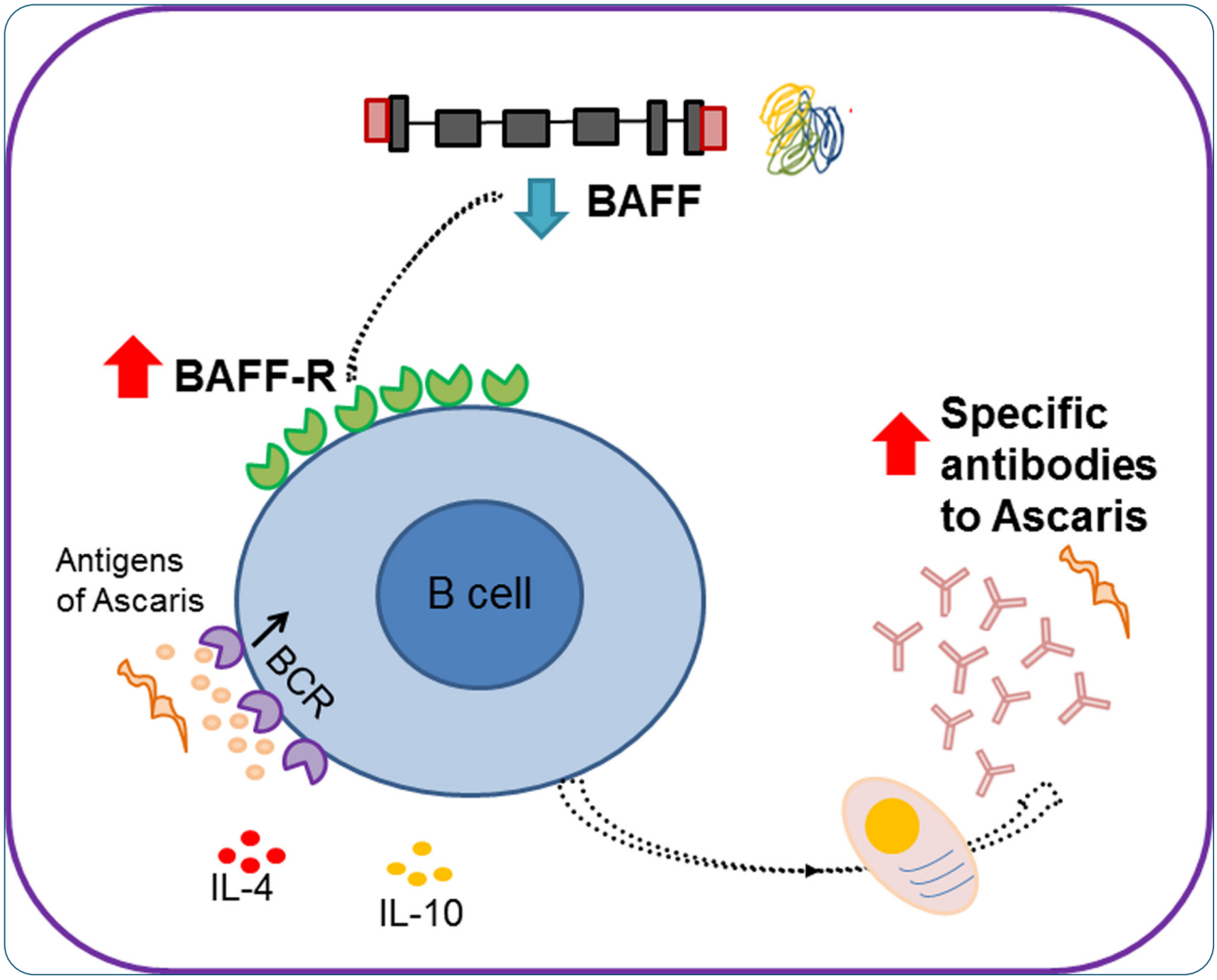
Figure 5



Supplementary Fig 1

Study II

BMC Immunology



The strength of the antibody response to the nematode *Ascaris lumbricoides* inversely correlates with levels of B-Cell Activating Factor (BAFF)

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BMC Immunology

RESEARCH ARTICLE Open Access

The strength of the antibody response to the nematode *Ascaris lumbricoides* inversely correlates with levels of B-Cell Activating Factor (BAFF)

Adriana Bornacelly[1,2], Dilia Mercado[1], Nathalie Acevedo[1,2] and Luis Caraballo[1,2*]

Abstract

Background: B-Cell Activating Factor (BAFF) is a cytokine regulating antibody production. Polymorphisms in the gene encoding BAFF were associated with the antibody response to *Ascaris* but not to mite allergens. In the present study we evaluated the relationship between BAFF and specific antibodies against *Ascaris* and mites in 448 controls and 448 asthmatics. Soluble BAFF was measured by ELISA and *BAFF* mRNA by qPCR. Surface expression of BAFF and its receptor (BAFF-R) was analyzed by flow cytometry.

Results: Individuals with specific IgE levels to *Ascaris* >75th percentile had lower levels of soluble BAFF; those with specific IgG levels to *Ascaris* >75th percentile had reduced *BAFF* mRNA. Total IgE and specific IgE to mites were not related to BAFF levels. There were no differences in soluble BAFF or mRNA levels between asthmatics and controls. There was an inverse relationship between the cell-surface expression of BAFF-R on $CD19^+$ B cells and BAFF levels at the transcriptional and protein level.

Conclusions: These findings suggest that differences in BAFF levels are related to the strength of the antibody response to *Ascaris*.

Keywords: *Ascaris*, Antibodies, Asthma, BAFF, BAFF-R, IgE, IgG, Immune response, Nematode, Parasite

Background

Infection by *Ascaris lumbricoides* (ascariasis) is one of the most prevalent helminthic diseases, affecting about 1.5 billion people worldwide. The immune response to this nematode has as hallmarks the induction of anti-*Ascaris* IgE/IgG antibodies and a strong Th2-driven inflammation [1]. Specific antibodies to *Ascaris* have been associated to parasite resistance [2-5] and both the immunoglobulin repertoire as well as the strength of the antibody responses are genetically regulated [6]. In mice, the production of IgE to the *Ascaris* resistance marker ABA-1 is HLA restricted [7]. In humans, two linkage scans identified a QTL (Quantitative Trait Loci) on chromosome 13q33.3 associated with the susceptibility to *A. lumbricoides* [8,9]. This region harbors the gene *TNFSF13B* (Tumor Necrosis Factor Ligand Superfamily, member 13b) encoding for the cytokine B-Cell Activating Factor, BAFF. The wild type allele of an intronic polymorphism in this gene (rs10508198) was found associated with higher IgG levels against *Ascaris* while the mutant allele was associated with less IgE to ABA-1 in asthmatics patients [10] suggesting a role of *TNFSF13B* in the antibody response to *Ascaris*.

BAFF (also known as BlyS, CD257), is a member of the tumor necrosis factor ligand superfamily of cytokines and is a major regulator of B cell activation, proliferation, differentiation, survival and immunoglobulin class-switching [11-13]. It is mainly expressed in innate immune cells such as neutrophils, macrophages and dendritic cells [14,15] but is also produced by non-hematopoietic cells [16,17]. This molecule may exist as cell surface-bound or soluble forms, the latter secreted after a furin cleavage [18,19]. Soluble BAFF interacts with three receptors: TACI (transmembrane activator and calcium-modulating cyclophilin ligand interactor), BCMA (B cell maturation antigen) and BAFF-R (BAFF receptor or BR3), the latter expressed on peripheral B cells [20,21]. *In vitro* experiments have demonstrated the critical role of BAFF on the production of

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IgA and IgG antibodies and the synergic effect with IL-4 on the transcription of IgE [22]. In these processes the BAFF-R also plays an important role [23,24].

Changes in BAFF levels are detectable in plasma during different immune related conditions [16]. In addition, there is evidence that upon nematode infection, resistant animals have increased levels of BAFF. In cattle naturally exposed to *Ostertagia*, *Cooperia* and *Nematodirus* the parasite-resistant animals had increased *BAFF* mRNA levels in the mesenteric lymph nodes [25]. High *BAFF* mRNA expression have been also detected in the spleen and liver of red grouse infected with *Trichostrongylus tenuis* [26]. Further studies in protozoan infections found that BAFF might be associated to variations in the strength of specific antibodies; for instance, administration of an antibody blocking BAFF signaling in mice infected with *Trypanosoma cruzi* induced a significant reduction of serum specific-IgM to the parasite [27]. Besides, there is evidence that *Plasmodium* parasites can modulate the BAFF pathway in the host, compromising protective antibody memory [28]. Although these investigations suggest that *TNFSF13B* may be a candidate gene underlying phenotypic variation in response to parasite infections, few have been done in humans. There are no studies evaluating the role of BAFF on the strength of *Ascaris* specific antibody production or total IgE in humans naturally exposed to this parasite. Since it has been observed that asthmatics have a higher antibody response to nematodes [29], these individuals are an interesting group for analyzing these traits. The aims of this study were (a) to investigate the relationship between BAFF and specific antibody levels to *Ascaris*, (b) to evaluate the relationship between BAFF and total IgE and specific IgE to non-parasitic allergens in asthmatic patients and non-asthmatic controls and (c) to evaluate the relationship between BAFF levels and the cell-surface expression of its receptor (BAFF-R) on $CD19^+$ B cells.

Results and discussion

BAFF and the antibody response to *Ascaris*

The demographical characteristics and antibody responses of the studied population are presented in Table 1. In the entire dataset (n = 896), BAFF values in plasma were non-normally distributed with median 792.1 pg/ml (interquartile range 594.2–978.7 pg/ml) Figure 1A. There were no differences in soluble BAFF levels according to age, gender or disease status. IgE to *Ascaris* was higher in asthmatic patients while IgG to *Ascaris* was higher in controls and total serum IgE was higher in asthmatics (Table 1).

The relationship between levels of soluble BAFF and antibodies to *Ascaris* was first evaluated using bivariate correlations stratified by healthy and asthmatics. Because soluble BAFF and the specific IgE levels to *Ascaris* were non-normally distributed, the non-parametric Spearman test was used. We found a significant inverse correlation between soluble BAFF and specific IgE to *Ascaris* (r = -0.10, p = 0.03) in the group of healthy individuals (n = 448). A similar tendency was observed when comparing soluble BAFF levels between subjects with positive IgE sensitization to *Ascaris* (OD ≥ 0.113, n = 392) and those non-sensitized (n = 425): soluble BAFF was lower in *Ascaris* sensitized

Table 1 Descriptive of the study population

Variables	Asthmatic patients (n = 448)	Non-asthmatic controls (n = 448)	p-value
Age, years (Mean ± SD)	34.4 ± 18.2	36.7 ± 18.6	0.06
Gender, female, n (%)	256 (57.1)	249 (55.6)	0.6
Soluble BAFF levels [pg/ml][a]	768 (554.2–999.1)	804.3 (628.5–964.3)	0.16[b]
mRNA levels *BAFF1*[c] (Mean ± SD)	3.21 ± 1.39	3.12 ± 1.14	0.6[d]
mRNA levels *BAFF2*[c] (Mean ± SD)	2.79 ± 1.04	2.70 ± 0.94	0.5[d]
Total IgE, UI/ml[a,e]	699.7 (236.3–1065.8)	148.8 (58.3–408.1)	<0.001[b]
Ig levels to parasite (OD)[a,e]			
sIgE to *Ascaris*	0.117 (0.100–0.150)	0.105 (0.091–0.134)	<0.001[b]
sIgG to *Ascaris*	1.99 (1.60–2.34)	2.17 (1.73–2.69)	<0.001[b]
sIgE to ABA-1	0.125 (0.098–0.187)	0.119 (0.099–0.154)	0.18[b]
sIgG to ABA-1	1.49 (1.09–1.83)	1.70 (1.36–2.11)	<0.001[b]
Ig levels to HDM (OD)[a,e]			
IgE to *D. pteronyssinus*	0.191 (0.115–0.520)	0.099 (0.089–0.122)	<0.001[b]
IgE to *B. tropicalis*	0.231 (0.110–1.06)	0.098 (0.089–0.122)	<0.001[b]

SD: Standard deviation.
[a]Median (Inter Quartile Range).
[b]Mann-Whitney U test.
[c]Measured in PBMCs (n = 131, including 71 asthmatics and 60 controls).
[d]Independent samples t-test.
[e]Data available only for 369 asthmatics.

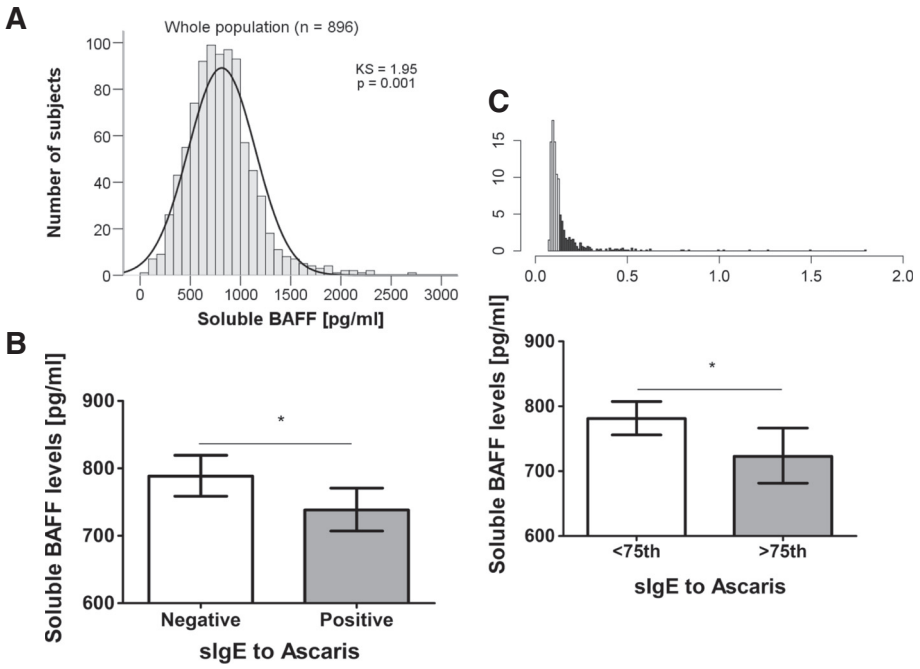

Figure 1 BAFF levels according to the IgE antibody response to *Ascaris*. **A**. Distribution of soluble BAFF levels in the population; KS: Kolmogorov-Smirnov **B**. Soluble BAFF levels in individuals with negative (n = 425) or positive (n = 392) IgE to *Ascaris* **C**. Distribution of specific IgE to Ascaris; density (y-axis), optical density units (x-axis), shaded bars represent individuals with levels >75th percentile (upper panel). Soluble BAFF levels between individuals with specific IgE levels to *Ascaris* below (n = 583) and above (n = 234) 75th percentile (lower panel). All lines indicate geometric mean (95%CI), *p < 0.05.

(median 786.3 pg/ml; IQR 595.1–973.6) than in non-sensitized (829.6 pg/ml; IQR 653.3–1013, Mann-Whitney U test, p = 0.03), Figure 1B.

Considering that parasite loads are over-dispersed within populations [30] and that individuals at the extreme of the distribution might carry biological traits influencing parasite susceptibility, we analyzed the relationship between soluble BAFF and the strength of the antibody response by stratifying the participants according their antibody levels, above 75th percentile (>75th) and below 75th percentile (<75th), Figure 1C (upper panel). We found that those with high specific IgE levels to the *Ascaris* extract (n = 234) had less concentrations of soluble BAFF in plasma (median 786.3 pg/ml; IQR 573.8–985) than those with IgE levels <75th percentile (n = 583, median 819.7 pg/ml; IQR 653.6–991.3; Mann-Whitney U test p = 0.04), Figure 1C. This association remained significant in the linear regression model using the square-root transformed soluble BAFF levels, and after adjustment by age, gender and disease status (p = 0.028). To explore if this relationship was reflected at the gene expression level, we analyzed a subgroup of subjects (n = 131) with mRNA data in peripheral blood mononuclear cells (PBMCs), but we found no association between the relative expression of *BAFF* mRNA and specific IgE levels to *Ascaris* (data not shown).

Besides, there was no significant correlation between soluble BAFF levels and IgG levels to Ascaris using non-parametric tests. Since IgG levels were normally distributed, we tested a more powerful parametric test (Pearson's r) using square-root-transformed soluble BAFF levels, with similar results. Interestingly, there was a significant inverse correlation between *BAFF* mRNA levels and IgG to *Ascaris* (r = - 0.22, p = 0.01), Figure 2A. This relationship was also observed when the comparison was done according to the strength of the antibody response: individuals with specific IgG levels to *Ascaris* >75th percentile had lower *BAFF1* mRNA levels (n = 40, mean 2.68 ± 1.0 SD) than those < 75th percentile (n = 91, mean 3.38 ± 1.3 SD, p = 0.003), Figure 2B.

The relationship between BAFF and serum antibody levels has been extensively analyzed in the context of autoimmune diseases, where increased levels of soluble BAFF have been associated with increased titers of auto-antibodies of the IgG isotype [11,31-34]. However, the effects of BAFF on specific antibody levels may differ depending on the context, for instance, inverse relationships between *BAFF* mRNA levels and the risk of developing donor-specific antibodies have been observed after transplantation [35]. To our knowledge this is the first study suggesting a relationship between circulating BAFF

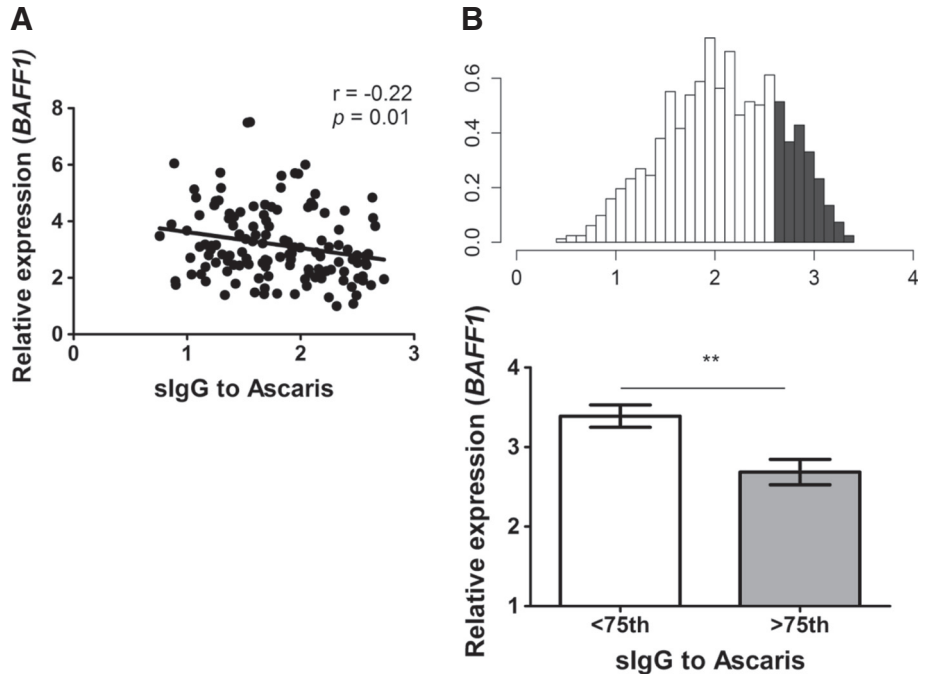

Figure 2 *BAFF* mRNA levels according to the specific IgG to *Ascaris*. **A**. Correlation between the mRNA expression for *BAFF1* and the levels of specific IgG to *Ascaris*. The numbers indicate the Pearson correlation coefficient (r) and the p-value (two-tailed); each dot represents an individual **B**. Distribution of specific IgG levels to Ascaris; density (y-axis), optical density units (x-axis), shaded bars represent individuals with IgG levels >75th percentile (upper panel); *BAFF1* mRNA levels between individuals with IgG to *Ascaris* below (n = 91) or above (n = 40) 75th percentile (lower panel), **p < 0.005.

levels and the human antibody response to a nematode. In contrast to what is generally observed in autoimmune diseases and some parasitic infections, we found an inverse association between soluble BAFF and the strength of the antibody response to *Ascaris*. The fact that the same tendency was observed both at protein and mRNA level is highly suggestive that it represents a biological phenomenon. The mechanisms underlying this relationship are unknown.

The mean difference in soluble BAFF levels between subjects with high specific IgE to *Ascaris* and the rest of the population was 52 pg/ml. The biological significance of this finding need to be further investigated but there are several studies suggesting that it could be relevant. It has been shown that most of the systemic BAFF is bound to receptors on B cell surfaces and that BAFF binding capacity on follicular B cells is nearly saturated under steady-state conditions in vivo [36]. In this scenery a small change in circulating BAFF concentrations may affect the BAFF balance in lymphoid tissues and impair the survival of high-affinity B cells clones in the germinal center. In addition, it is known that for some cytokines the biological impact is in the order of few pg/ml; for example, concentrations of IFNγ between healthy and malaria infected children differ in 8.1 pg/ml, and this small change correlated with soluble BAFF levels and concentration of antibodies [37]. Moreover, differences in cytokine levels upon stimulation of PBMCs with phytohemagglutinin or *Ascaris* were small but the impact on antibody production was remarkable [38]. Differences in levels of pro-inflammatory cytokines between healthy individuals and patients with autoimmune conditions have been also described in this range [39].

Previous studies showed that *TNFSF13B* gene is a QTL for *Ascaris* susceptibility [8,9], harboring genetic variants associated with IgE response to *Ascaris* and worm burden [10,40]. Now we have data suggesting that, in addition to genetic evidence, there is a relationship between the antibody responses to *Ascaris* and BAFF at the protein and mRNA level; although a causal relationship between them has yet to be demonstrated. Our findings seem to be more related to genetic mechanisms because first, there is previous evidence suggesting that polymorphisms around *TNFSF13B* may influence the antibody responses and second, we also found an association between the IgG responses and the level of mRNA expression. It is also pertinent to consider that *A. lumbricoides* can modify BAFF levels during infection, as has been reported for malaria parasite in acutely infected children [37]. However, in our study it is not possible to assess a direct effect of the parasite on BAFF production because it was designed to evaluate the antibody responses to *Ascaris* and not the acute infection. Besides, we should keep in mind that low BAFF

levels and high antibody responses might be independent consequences of the *Ascaris* infection without any causal relationship. Indeed, IL-4 has shown to down-regulate the expression of BAFF *in vitro* [41]. It is known that *Ascaris* infections of low intensity are associated with a Th2 immune response rich in IL-4 and in this population this could be an additional factor introducing variability in BAFF levels.

Soluble BAFF, total IgE, specific IgE and asthma

The relationship between BAFF and total plasma IgE is still controversial [42-45]. In this study we found no association between soluble BAFF and total IgE when analyzed as continuous variables. Total IgE values were transformed to a categorical variable based on the 75th percentile in non-asthmatic controls and asthmatic patients as a cut-off to define high total IgE levels but there were no significant differences (Figure 3). In addition, BAFF mRNA levels were not associated with total IgE. Our findings are in agreement with previous studies showing no relationship between BAFF levels and total IgE [43]. Regarding specific IgE to non-parasite/environmental allergens, we found that BAFF levels were not related to specific IgE to *D. pteronyssinus* and *B. tropicalis*, the main sensitizers in this population. There are contradictory published results around this point: it has been reported a significant increase in BAFF levels after allergen exposure in the bronchoalveolar lavage fluid of allergic patients [46] but no relationship was found between soluble BAFF and specific IgE reactivity to fungal allergens [42]. Our results support previous findings detecting association between BAFF variants and specific IgE to *Ascaris* but not to mites [10]. Some studies have suggested a relationship between BAFF and asthma in humans [44,47]. However, in agreement with Lei *et al.* [48], we found no differences in BAFF between asthmatic patients and controls neither at protein nor at the mRNA levels (Table 1). It is worth mentioning that our results refer to circulating form of the cytokine and its expression in peripheral blood leukocytes. Although several covariates might influence BAFF synthesis, the levels we found in healthy controls were comparable to those observed in other populations [49-51].

BAFF mRNA levels inversely correlate with the cell surface expression of BAFF receptor (BAFF-R) on B cells

Since there were previous reports showing an inverse relationship between BAFF and BAFF receptor [50,52,53], the expression of membrane-bound BAFF and BAFF-R was evaluated on PBMCs (n = 113). We did not detect cell surface expression of BAFF in gated $CD14^+$ cells (monocytes), lymphocytes or gated $CD19^+$ cells (B cells); and, as previously described [54], the BAFF-R was highly expressed in peripheral B cells but not in monocytes (Figure 4A). Similar findings were obtained when cell-surface expression of BAFF and BAFF-R were analyzed in sorted monocytes (Figure 4B) and B cells (Figure 4C) from six non-asthmatic controls. As expected [54] we found that plasmablasts ($CD27^{high}$, $CD38^{high}$), switched-memory B cells, mature naïve B cells and transitional B cells expressed BAFF receptor (Additional file 1). The relationship between the cell-surface expression of BAFF-receptor (BAFF-R) on $CD19^+$ B cells (median fluorescence intensity, MFI) with the soluble BAFF levels and the BAFF mRNA levels in PBMCs was evaluated in the subgroup of 113 individuals with flow cytometry data. These observations had a normal distribution in this dataset and therefore parametric tests (Pearson's correlation) were used (Additional file 2). We found a significant inverse correlation between *BAFF1* mRNA and the cell surface expression of BAFF-R in $CD19^+$ B cells (r = - 0.23, p = 0.01), Figure 5A. This finding was

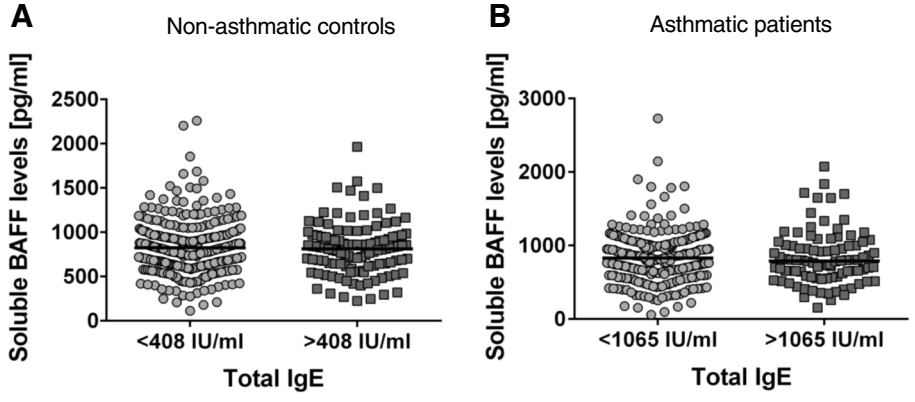

Figure 3 Soluble BAFF levels according to total IgE levels. A. Soluble BAFF levels in non-asthmatic controls according to total IgE below (n = 335) or above (n = 113) 75th percentile **B**. Soluble BAFF levels in asthmatics according to total IgE below (n = 277) or above (n = 92) 75th percentile.

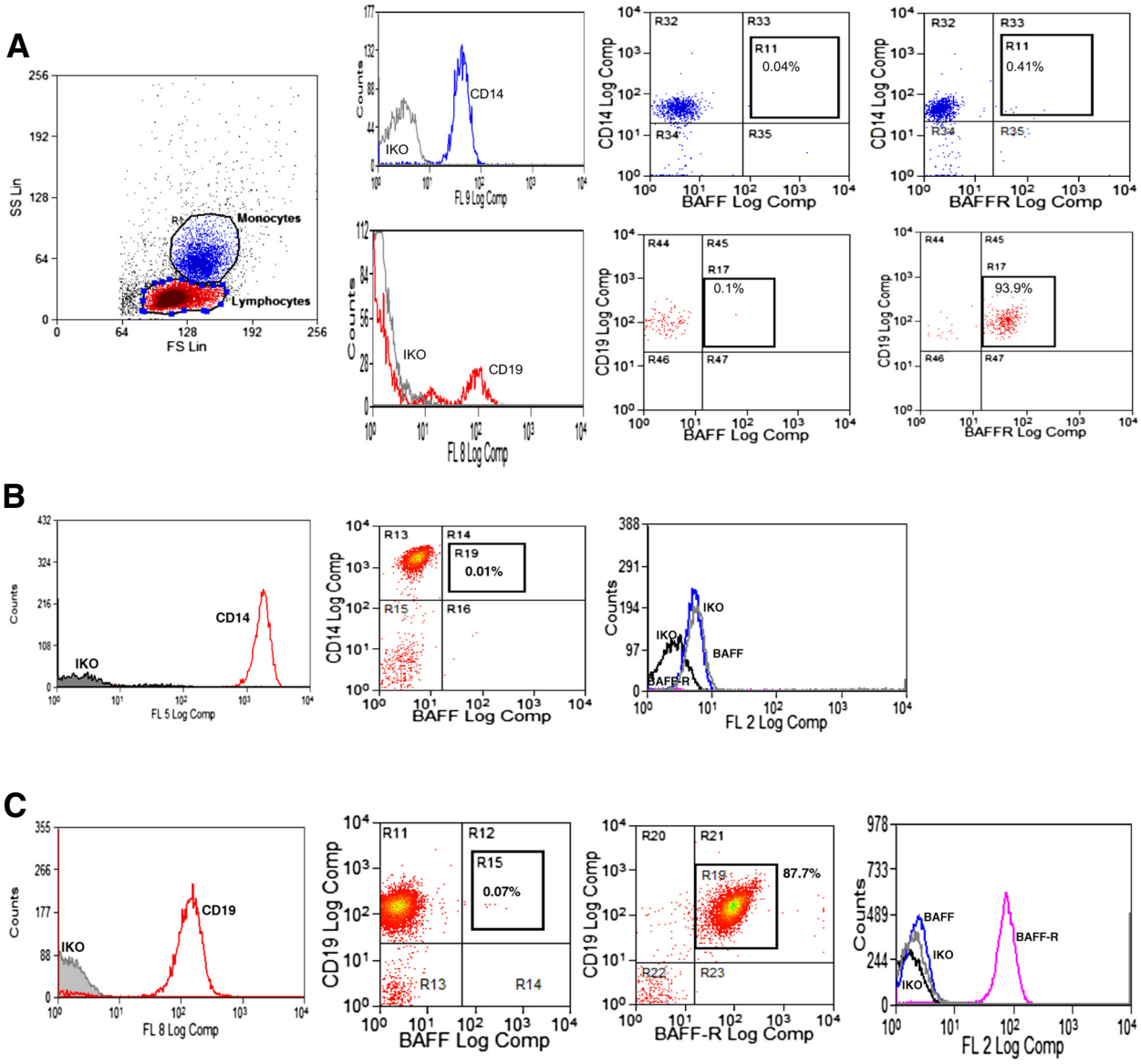

Figure 4 Cell surface expression of BAFF and BAFF-R in PBMC, monocytes and B cells. A. Cell surface expression of BAFF and BAFF-R on gated monocytes ($CD14^+$) and B cells ($CD19^+$). A representative example out of 113 PBMCs samples tested. IKO: isotype control **B**. Cell surface expression of BAFF and BAFF-R in purified monocytes **C**. Cell surface expression of BAFF and BAFF-R in purified B cells.

independent of age, gender, disease status and the proportion of monocytes in the sample as tested in a linear regression model. However, no correlation was found with *BAFF2* mRNA levels (r = - 0.13, p = 0.1). Regarding protein levels, we found an inverse correlation between soluble BAFF levels and the cell surface expression of BAFF-R in $CD19^+$ B cells (r = -0.27, p = 0.003) Figure 5B. These observations were significant after adjustment by age, gender, disease status and the proportion of monocytes in the sample. The inverse relation between soluble BAFF levels and the cell surface expression of BAFF-R has been previously reported in mice and patients with deficiency of BAFF-R [50], patients with systemic lupus erythematous and Sjogren's syndrome [52] hyper-IgE syndrome [53] and acute malaria [37]. In this model, the 8.2% of the variance in BAFF-R expression can be explained by the soluble BAFF levels. In the context of immune responses to *Ascaris*, it remains to be elucidated how this BAFF/BAFF-R axis is related to the synthesis of specific antibody levels.

The relationship between circulating soluble BAFF levels and *BAFF* mRNA levels in blood mononuclear cells

Another question to be addressed was the relationship between soluble BAFF and BAFF mRNA levels, and whether those can serve as proxies for mechanisms contributing to the variation in specific antibody levels. Immune responses

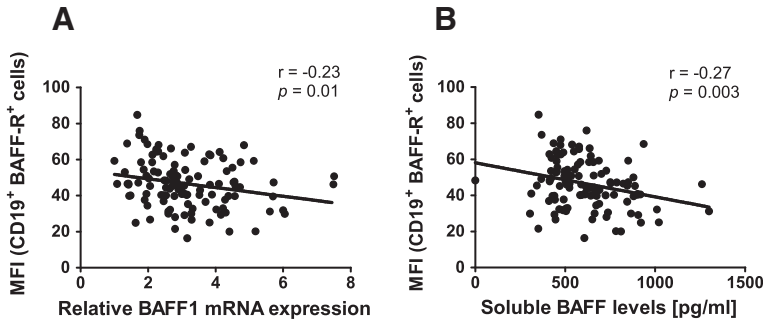

Figure 5 Relationship between cell surface expression of BAFF-R and BAFF levels. A. Correlation between the median fluorescence intensity (MFI) of BAFF-R on gated $CD19^+$ B cells and the mRNA levels for *BAFF1* in PBMCs **B**. Correlation between the MFI of BAFF-R on gated $CD19^+$ B cells and soluble BAFF levels in plasma. The numbers indicate the Pearson correlation coefficient (r) and the p-value (two-tailed). Each dot represents an individual.

to antigens including helminths take place in lymphoid tissues and in infiltrated organs. There, BAFF is produced by many cells including lymphoid tissue stroma cells and resident monocytes [55,56]. Levels of circulating soluble BAFF has shown to correlate with local BAFF activity at the germinal center [36]. In this study we found a significant positive correlation ($r = 0.68$, $p < 0.001$), between the mRNA levels for *BAFF1* (exon 3-4) and *BAFF2* (exon 4-6), however, soluble BAFF levels were only significantly correlated to *BAFF1* mRNA levels (Figure 6A) and not to *BAFF2* mRNA (Figure 6B). From the transcripts to soluble BAFF there are several steps: as a type II transmembrane protein of 285 amino acids (aa) in the plasma membrane, BAFF is cleaved by furine proteases in its N-terminal end (TNF-homology domain) and released in the circulation. There it forms homotrimers of 152 aa amenable to be detected by ELISA; homotrimers can also form other assemblies of less abundance (i.e. BAFF 60 mer) [16,57]. There is another isoform expressed at low levels and called ΔBAFF (266 aa), that can form heteromultimers with BAFF (285 aa) and negatively regulate the secretion of the latter [58]. The relation between soluble BAFF levels and *BAFF1* mRNA levels could be explained because this transcript contains exon 3, which is informative for both BAFF and ΔBAFF. More studies are needed to elucidate if differential expression of BAFF isoforms contribute to the strength of specific antibody levels.

Conclusions

Our findings show that soluble BAFF levels are lower in subjects with high specific IgE to *Ascaris*, suggesting that this cytokine plays a role in the strength of the antibody responses to this nematode and supporting previous molecular genetics studies. Interestingly, soluble BAFF levels are not related with total IgE or IgE sensitization to house dust mites, also in agreement with our previous findings. In addition, we found no differences in BAFF levels between asthmatic patients and controls. As mRNA, BAFF was expressed in mononuclear cells but, as a protein it was not detected in cell-surface including monocytes and B cells. The potential mechanistic link between lower levels of BAFF and the strength of the specific antibodies to *Ascaris* might be related with the inverse relationship between BAFF levels and the BAFF-R expression on B cells, a receptor that was present at the cell surface in all developmental stages of peripheral B cells including

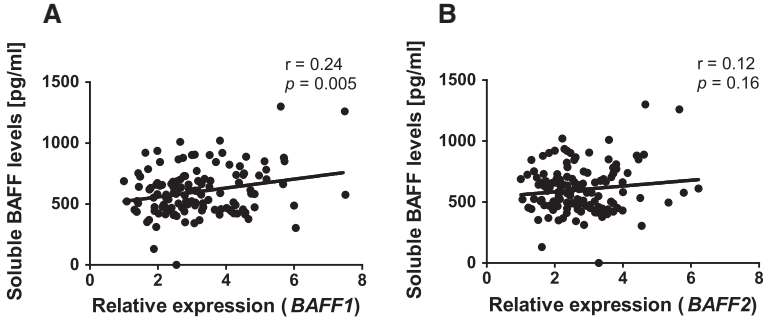

Figure 6 The relationship between soluble BAFF levels and *BAFF* mRNA levels in blood mononuclear cells. A. Correlation between soluble BAFF levels and *BAFF1* mRNA expression **B**. Correlation between soluble BAFF levels and *BAFF2* mRNA expression. The numbers indicate the Pearson correlation coefficient (r) and the p-value (two-tailed). Each dot represents an individual.

switched memory and plasmablasts. The role of BAFF on the susceptibility to *Ascaris* infection and its clinical implications deserve further confirmatory and mechanistic studies in larger cohorts.

Methods

Study design and population

This is a case-control study performed in Cartagena, Colombia. Eight hundred ninety six subjects including 448 asthmatics and 448 non-related healthy volunteers were selected from a well-characterized dataset [10] and matched by age and gender (Table 1). The matching process was done randomly, without knowledge of anti-*Ascaris* IgE or IgG levels. All participants live in an urban, non-industrialized setting, having access to water and electricity and belonging to the lower three (of six) socio-economic strata in the city, where most people are naturally exposed to *A. lumbricoides*. Measurements of soluble BAFF (n = 896) were done in non-previously thawed plasma samples from the repository in which anti-*Ascaris* antibody determinations were done previously [10]. A random subgroup of these subjects (n = 131, 71 asthmatics and 60 controls) were visited by a physician of the research staff who obtained 20 mL blood samples for gene expression, flow cytometry analyses, soluble BAFF, total IgE and specific antibodies to *Ascaris* and ABA-1. Asthma diagnosis was confirmed according GINA guidelines, including spirometry. Allergic sensitization was defined as a positive IgE result to the mites *Dermatophagoides pteronyssinus* and/or *Blomia tropicalis*. The study was approved by the Ethics Committee of the University of Cartagena. A full verbal explanation of the investigation was given and written informed consent was obtained from all participants.

Antibody measurements

Total IgE was determined by duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (RIDASCREEN; R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Specific IgE and IgG to *Ascaris* extract and ABA-1 as well as specific IgE to mite extracts were detected by ELISA as described previously [10,59]. ABA-1 is a nematode specific allergen from *Ascaris* (Asc s 1) that is useful for avoiding cross reactivity with mite allergens [59]. Also, antibody responses to ABA-1 have been associated with resistance to ascariasis [3,5]. The responses to *Ascaris* and ABA-1 were analyzed as dichotomous variables, using two cut-off points: the first was the optical density (OD) value of 0.113 (mean OD of six negative, non-allergic, non-parasitized controls + 3 standard deviations) which defined sensitized (positive) and non-sensitized (negative) individuals. The second was the 75th percentile of antibody levels in non-asthmatics individuals (i.e., 0.134 OD units for IgE to *Ascaris*; 0.154 OD units for IgE to ABA-1; 2.68 OD units for IgG to *Ascaris* and 2.11 OD units for IgG to ABA-1). To analyze the strength of total IgE levels the 75th percentile was used as cut-off to define high total IgE phenotype. This percentile was calculated separately in healthy controls (>75th = 408.1 IU/mL) and asthmatics (>75th = 1065.8 IU/mL).

Quantification of BAFF levels in plasma

Soluble BAFF in plasma was measured using a quantitative sandwich enzyme immunoassay according to the manufacturer's instructions (Quantikine Human BAFF/BLyS kit, R&D Systems Cat. SBLYS0). A standard curve with four parameter logistic (4-PL) curve-fit was used to extrapolate the sample concentration value. Intra assay variation coefficients were lower than 10%. The quantification range of the assay was between 62.5 and 4000 pg/ml.

Isolation of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was diluted 1:2 in 1× PBS and separated by density gradient using Ficoll Histopaque (Sigma Aldrich, Catalog 1077-1). Tubes were centrifuged during 30 minutes at 400 *g* at room temperature and without brake. The layer containing the PBMCs was transferred into a new tube, washed twice with 50 mL of 1× PBS and resuspended in 1 mL of 1× PBS. A 10μl aliquot was stained with 0.4% Trypan Blue and counted in a hemocytometer. For gene expression analysis, 1×10^7 cells were placed in a new tube, centrifuged at 300 *g*, 4°C during 5 minutes. The supernatant was discarded and the cell pellet homogenized in 750 μl of Trizol® (Life Technologies) and stored at -70°C until use. The remaining cells were used for flow cytometry analysis (see below).

RNA extraction

Cell homogenates were thawed during 15 minutes at room temperature (protected from light) and 150 μl of chloroform were added to each tube, vigorously mixed by hand and centrifuged at 12 000 *g* during 15 minutes at 4°C. The aqueous upper phase was transferred to a fresh tube, diluted in 400 μl of 70% ethanol and pipetted into a spin column (Trizol® Plus RNA purification kit, Life Technologies, Catalog: 12183555). Each sample was centrifuged at 12000 *g* during 15 seconds at room temperature, the flow-through was discarded and the column containing the RNA washed three times. The spin column was centrifuged at 12 000 *g* during 1 minute and 50 μl of RNAse free water were added for RNA elution (12 000 *g* during 2 minutes at room temperature). The RNA yield was between 8.1 μg and 25 μg. The A260/A280 ratio ranged from 1.92 to 2.03 and for the ratio A260/A230 from 0.71 to 2.36. Integrity of RNA was also verified by electrophoresis as two intact bands of 28S and 18S ribosomal RNA.

cDNA synthesis

cDNA was synthesized from 1 μg of total RNA using the Superscript III first strand super mix kit (Invitrogen, Catalog 11752050) following manufacturer's instructions. RT-PCR reactions included retrotranscriptase, reaction mix (oligodT 22.5 μM, random hexamers 2.5 ng/μl, 10 mM $MgCl_2$ and dNTPs), total RNA and RNAse-free water (Ambion Cat. AM9937). The synthesized cDNA was diluted with 80 μl of water and stored at -20°C until amplification. cDNA was genomic-DNA free, confirmed by an amplification reaction using a non retrotranscriptase enzyme control (RT-minus control).

Quantitative PCR

BAFF mRNA was detected by quantitative PCR (qPCR) using Taqman gene expression assays on a 7300 Real-Time Polymerase Chain Reaction (PCR) system (Applied Biosystems, Foster City, CA, USA). To detect differential splicing, the mRNA was measured in two separated assays: assay 1 targeting the exons 3 and 4 (Cat. Hs00198106, 84 bp amplicon) and assay 2 targeting the boundaries of exons 4–5 and 5–6 (Cat. Hs00902574, 73 bp amplicon). The two isoforms were named *BAFF1* and *BAFF2* (Additional file 3). Expression of β2 micro-globulin was used as endogenous control (Cat. Hs00984230, 81bp amplicon). Each sample was tested by duplicate and the average C_T value exported from the SDS Software (Applied Biosystems). The C_T value of the β2M gene (Mean C_T 18.8 ± 0.40 SD) was subtracted from the C_T values of the target genes (Mean C_T 25.7 ± 0.60 SD for *BAFF1* and Mean C_T 27.1 ± 0.67 SD for *BAFF2*). The normalized value was expressed as the delta C_t ($\Delta Ct = Ct_{BAFF} - Ct_{B2M}$). The highest ΔC_T value among all samples was subtracted from each sample and the resulting number expressed as the *delta-delt*a C_T. Relative expression levels were calculated as $2^{\wedge\text{-}(\Delta\Delta CT)}$ [60]. The probes for BAFF were FAM-labeled and for β2 micro-globulin were VIC-labeled, all assays included NFQ (non- fluorescent quencher).

Flow cytometry on PBMCs

Cell surface expression of BAFF and BAFF-R was analyzed on PBMCs by flow cytometry (n = 113). Cells were resuspended in MACS buffer (0.5% BSA in PBS) at a final concentration of 10^5 cells/100 μl per tube. Fcγ receptors were blocked with 1 μl of normal mouse serum (e-Bioscience, Catalog 24-5544) during 10 minutes at 4°C. A panel of fluorochrome-conjugated monoclonal antibodies (APCe-Fluor α-human CD14, Catalog: 47-0149; APC α-human CD19, Catalog 17-0199; PE α-human BAFF/BLyS, Catalog 12-9017 and FITC α-human BR3/BAFF-R, Catalog 11-9117, all from e-Bioscence) were added to the cells and incubated 30 minutes at 4°C protected from light. Each assay included the unstained sample and a panel of appropriate isotype controls to set the gates of positive and negative populations. After staining, cells were washed twice with PBS and resuspended in 500 μl of PBS. Data on 20000 events per sample were acquired using a Dako Cytomation Cytometer (Beckman Coulter, Inc. CA, USA) and analyzed by Summit 4.3 (Beckman Coulter, Inc. CA, USA). Electronic gating was used to evaluate the expression of BAFF and BAFF-R in $CD14^+$ cells and $CD19^+$ B cells.

Flow cytometry in purified monocytes and B cells

Peripheral blood monocytes and B cells were purified by magnetic associated cell sorting (MACS) using negative selection protocols (Monocyte Isolation kit catalog 130-091-153 and B cells isolation kit catalog 130-091-151, Miltenyi Biotec). Briefly, 30×10^6 PBMCs were resuspended in MACS buffer containing 0.5% BSA - PBS pH 7.2. Then, biotin-conjugated antibody cocktails were added and the mixture incubated by 10 minutes at 4°C. Non target cells were magnetically labeled with a cocktail of monoclonal antibodies targeting CD2, CD14, CD16, CD36, CD43 and CD235a for the B cell separation protocol and CD3, CD7, CD16, CD19, CD56, CD123 and CD235a for the monocyte separation protocol (Miltenyi Biotec). Anti-biotin microbeads were added and the cells incubated by 15 minutes at 4°C. After washing, cells were resuspended in MACS buffer at a final volume of 500 μl and passed through the column. The flow-through was collected and centrifuged at 300 *g* during 5 minutes at 4°C. The supernatant was discarded, and the cells reconstituted in 500 μl of MACS buffer and counted. Purified cells were stained with specific antibodies to detect BAFF and BAFF-R in different subpopulations (Additional file 4). The purity of monocytes and B cells was 91% and 90% respectively.

Statistics

Statistical analyses were performed using the statistical package for the social sciences software (SPSS version 17 for Windows; SPSS Inc., Chicago, IL, USA) and GraphPad Prism software v.5. Bivariate correlations were used to analyze the relationship between soluble BAFF and the antibody levels as continuous variables; since soluble BAFF was non-normally distributed, the non-parametric Spearman test was used. IgG levels were normally distributed therefore soluble BAFF levels were transformed to their square root to achieve normality and the Pearson correlation test was applied for this isotype. The comparisons of soluble BAFF between the groups of antibody responders (<75th and >75th) were done using Mann-Whitney *U* test (p-values presented after 10000 permutations). Linear regression was then used to model the effect of antibody grouping on transformed soluble BAFF levels adjusting by age, gender and disease status (asthma yes/no). Since *BAFF* mRNA values in PBMCs were normally distributed (Additional file 2), the comparison between groups of antibody responders (<75th and >75th) were done by

independent samples *t* test and further verified by adjusted linear regression models. MFI of BAFF-R was also normally distributed and its relationship with soluble BAFF and transcript levels were explored using Pearson correlation test. Since BAFF transcripts were measured in unfractionated mononuclear cells, the percentage of monocytes per sample was included as covariate in adjusted linear models. The relationship between soluble BAFF levels and the mRNA levels of the transcripts *BAFF1* and *BAFF2* was analyzed using Pearson correlation test. A p value ≤0.05 (two-tailed) was considered statistically significant.

Additional files

Additional file 1: Cell surface expression of BAFF-R in subpopulations of purified B cells.

Additional file 2: Distribution of the median intensity levels of BAFF-R in B cells, soluble BAFF levels and mRNA levels in PBMCs for the subgroup of 113 individuals.

Additional file 3: Schematic representation of the gene encoding BAFF and the exons covered by the gene expression assays.

Additional file 4: Antibodies used for flow cytometry in PBMCs, monocytes and B cells.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
AB, NA performed experiments and statistical analyses; DM performed antibody measurements; NA participated in the study design and supervised data analysis. LC conceived the investigation and supervised the general aspects of the work and data analyses. AB, NA, LC wrote the manuscript. All authors read and approved the final version of the manuscript.

Acknowledgements
We thank all the patients and controls for their voluntary participation in the study; also to our scientific collaborators at the Genetics and Molecular Biology Group (University of Cartagena, Colombia) for the infrastructure support to perform the cell isolations. We also thank Dr. Malcolm Kennedy (University of Glasgow) for providing the ABA-1. This work was funded by the Administrative Department of Science, Technology and Innovation (Colciencias, Colombia) Grant 680-2009; and by the University of Cartagena, Colombia. AB received funds from Fundemeb. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Received: 7 January 2014 Accepted: 2 June 2014
Published: 7 June 2014

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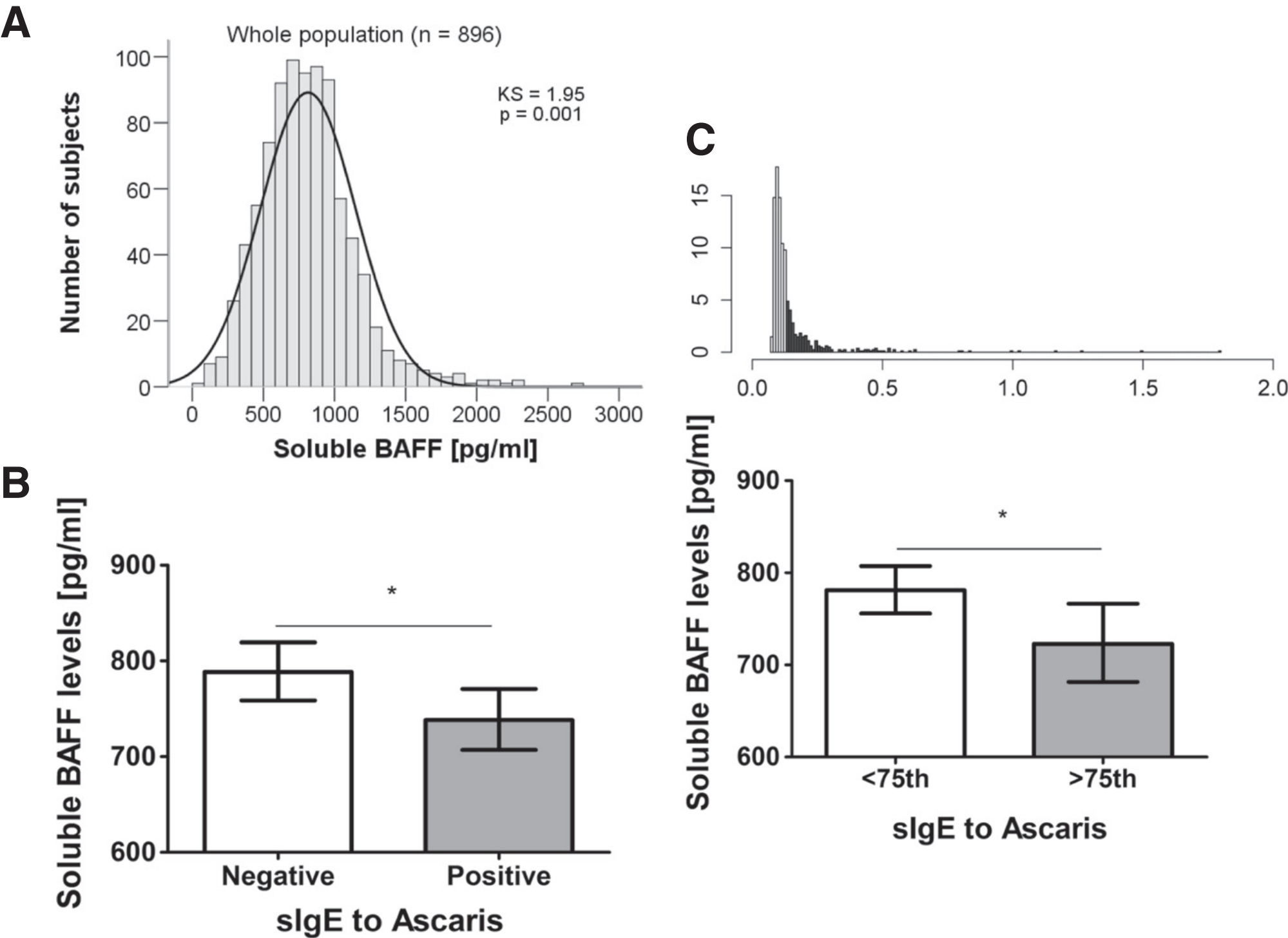
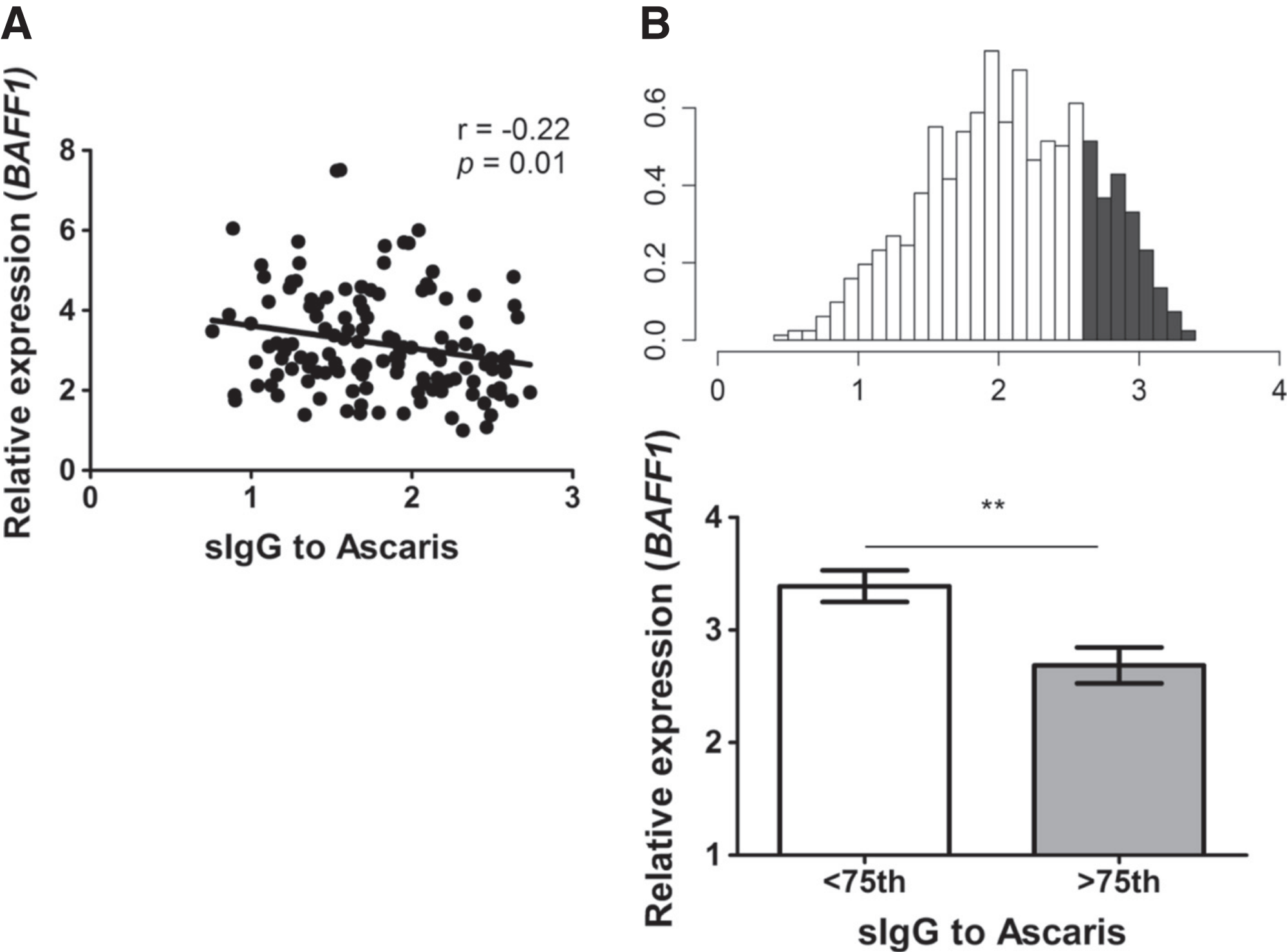
doi:10.1186/1471-2172-15-22
Cite this article as: Bornacelly *et al.*: The strength of the antibody response to the nematode *Ascaris lumbricoides* inversely correlates with levels of B-Cell Activating Factor (BAFF). *BMC Immunology* 2014 **15**:22.


A
Whole population (n = 896)
KS = 1.95
p = 0.001
Number of subjects
Soluble BAFF [pg/ml]
B
Soluble BAFF levels [pg/ml]
Negative
Positive
sIgE to Ascaris
C
Soluble BAFF levels [pg/ml]
<75th
>75th
sIgE to Ascaris


A
Relative expression (BAFF1)
r = -0.22
p = 0.01
sIgG to Ascaris
B
0.0
0.2
0.4
0.6
0
1
2
3
4
**
Relative expression (BAFF1)
<75th
>75th
sIgG to Ascaris

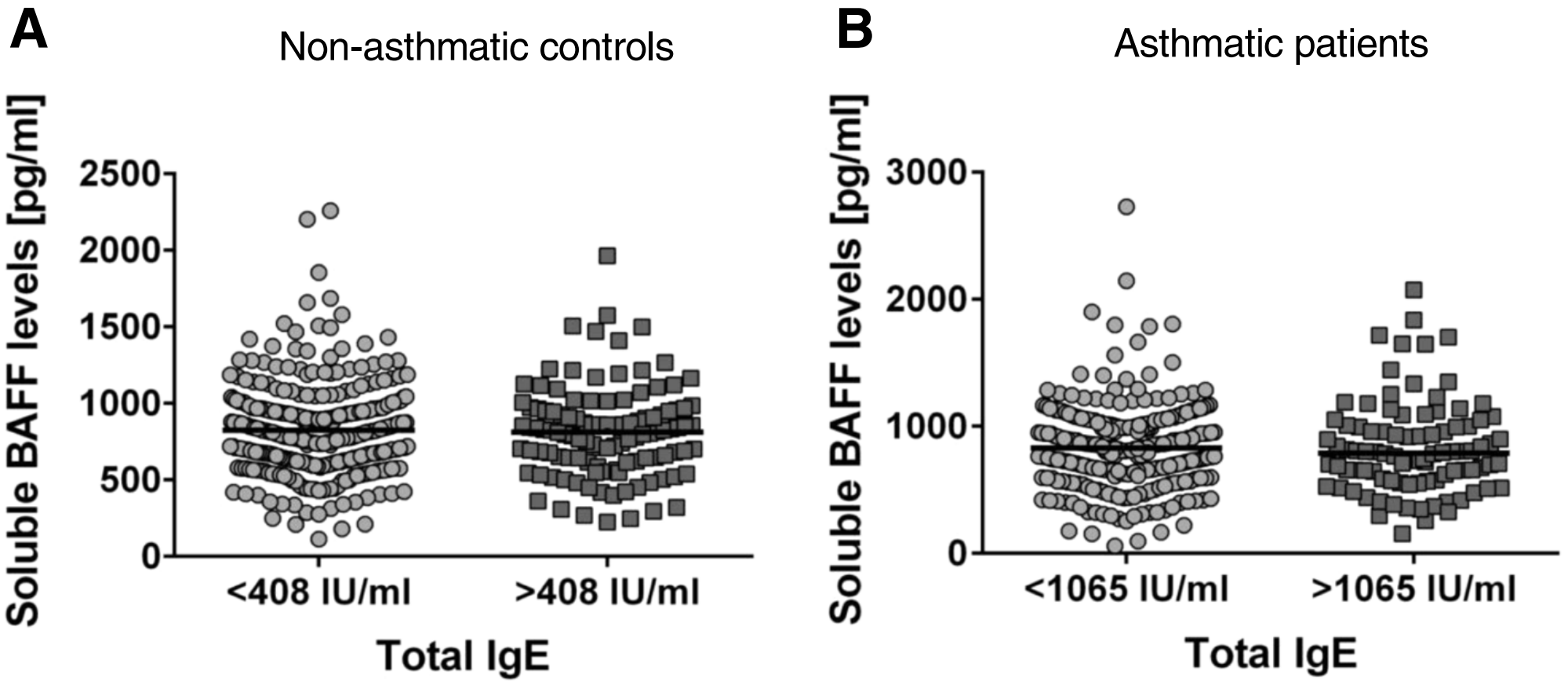
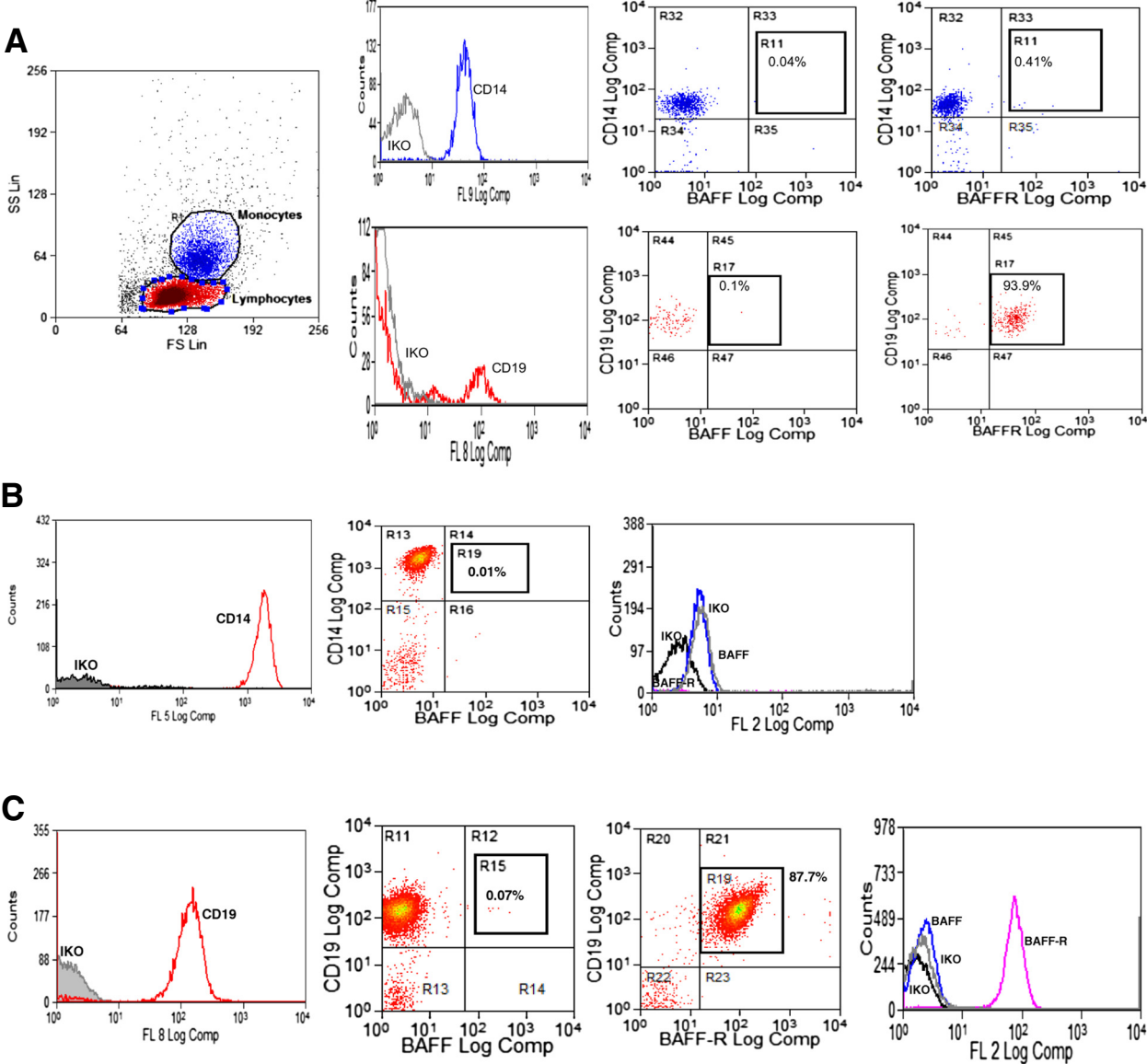
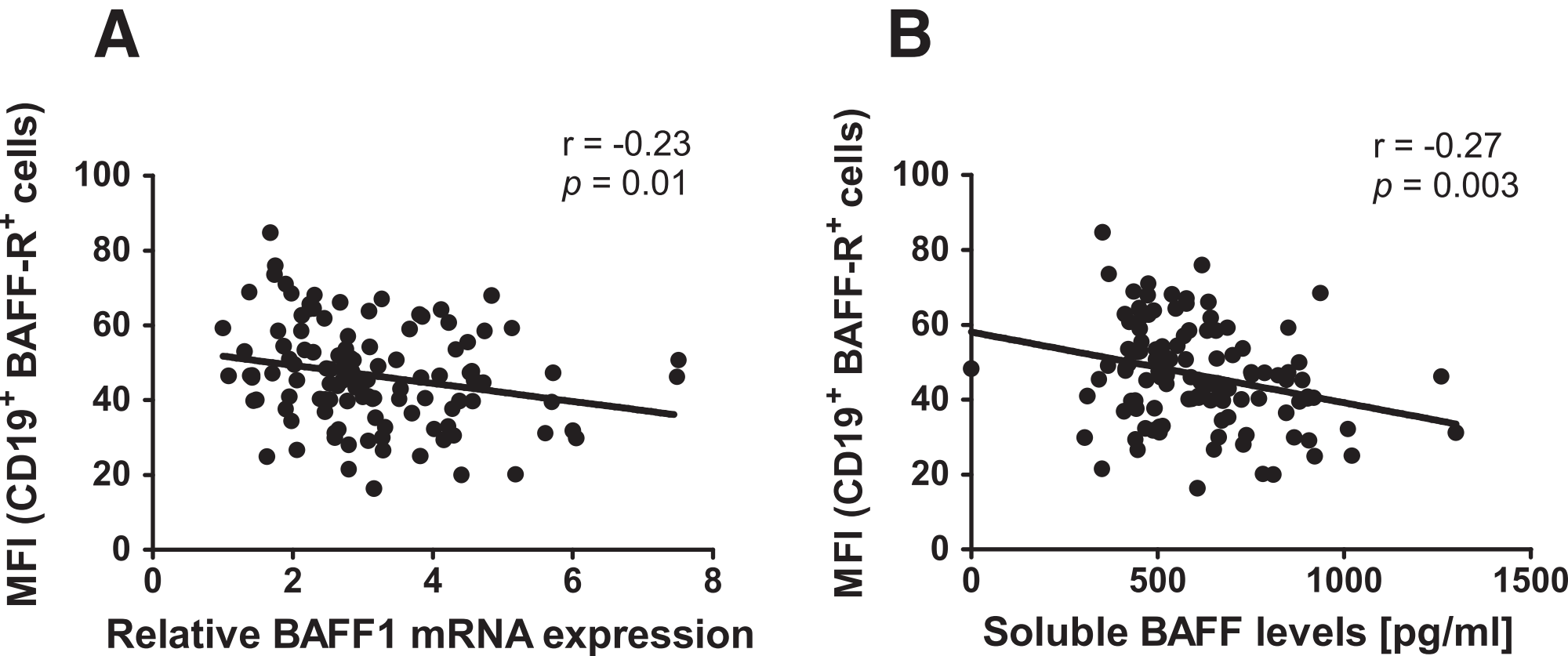
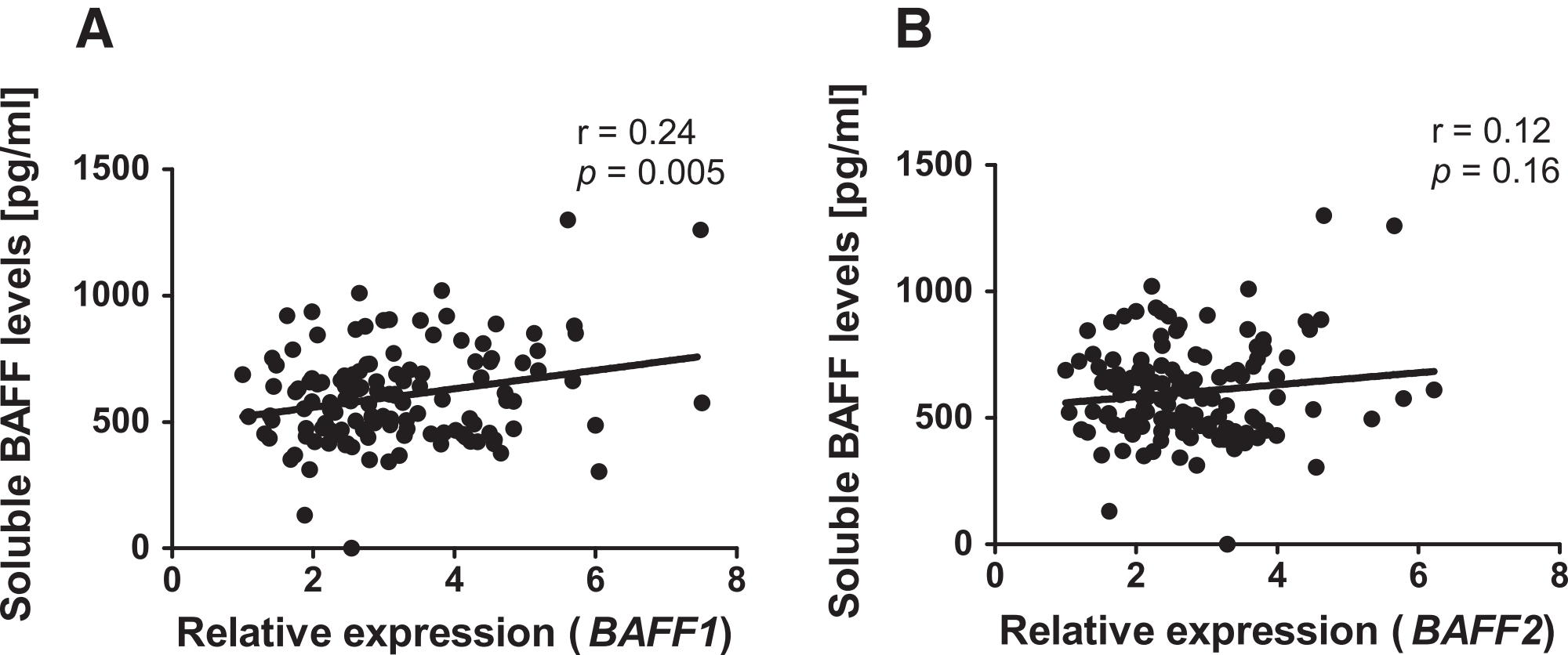

A
Non-asthmatic controls
Soluble BAFF levels [pg/ml]
2500
2000
1500
1000
500
0
<408 IU/ml
>408 IU/ml
Total IgE
B
Asthmatic patients
Soluble BAFF levels [pg/ml]
3000
2000
1000
0
<1065 IU/ml
>1065 IU/ml
Total IgE

Figure 3

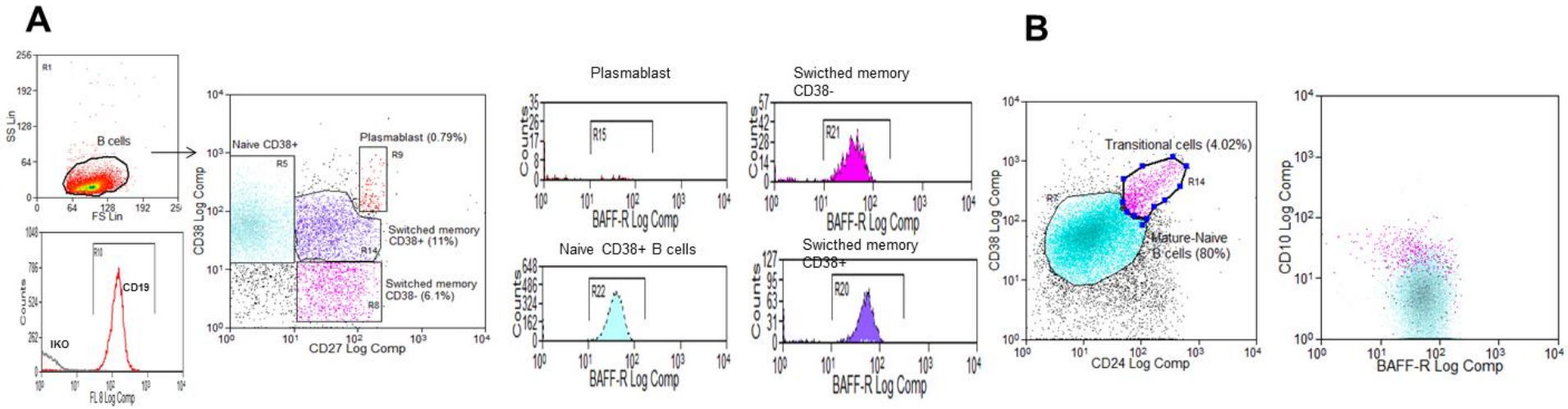

A
SS Lin
FS Lin
Monocytes
Lymphocytes
CD14
IKO
Counts
FL 9 Log Comp
CD14 Log Comp
BAFF Log Comp
R11
0.04%
BAFFR Log Comp
R11
0.41%
CD19
FL 8 Log Comp
CD19 Log Comp
R17
0.1%
R17
93.9%
B
FL 5 Log Comp
R19
0.01%
BAFF
BAFF-R
FL 2 Log Comp
C
R15
0.07%
R19
87.7%
BAFF-R Log Comp


A
r = -0.23
p = 0.01
MFI (CD19+ BAFF-R+ cells)
100
80
60
40
20
0
0
2
4
6
8
Relative BAFF1 mRNA expression
B
r = -0.27
p = 0.003
MFI (CD19+ BAFF-R+ cells)
100
80
60
40
20
0
0
500
1000
1500
Soluble BAFF levels [pg/ml]


A
r = 0.24
p = 0.005
Soluble BAFF levels [pg/ml]
1500
1000
500
0
0
2
4
6
8
Relative expression (BAFF1)
B
r = 0.12
p = 0.16
Soluble BAFF levels [pg/ml]
1500
1000
500
0
0
2
4
6
8
Relative expression (BAFF2)

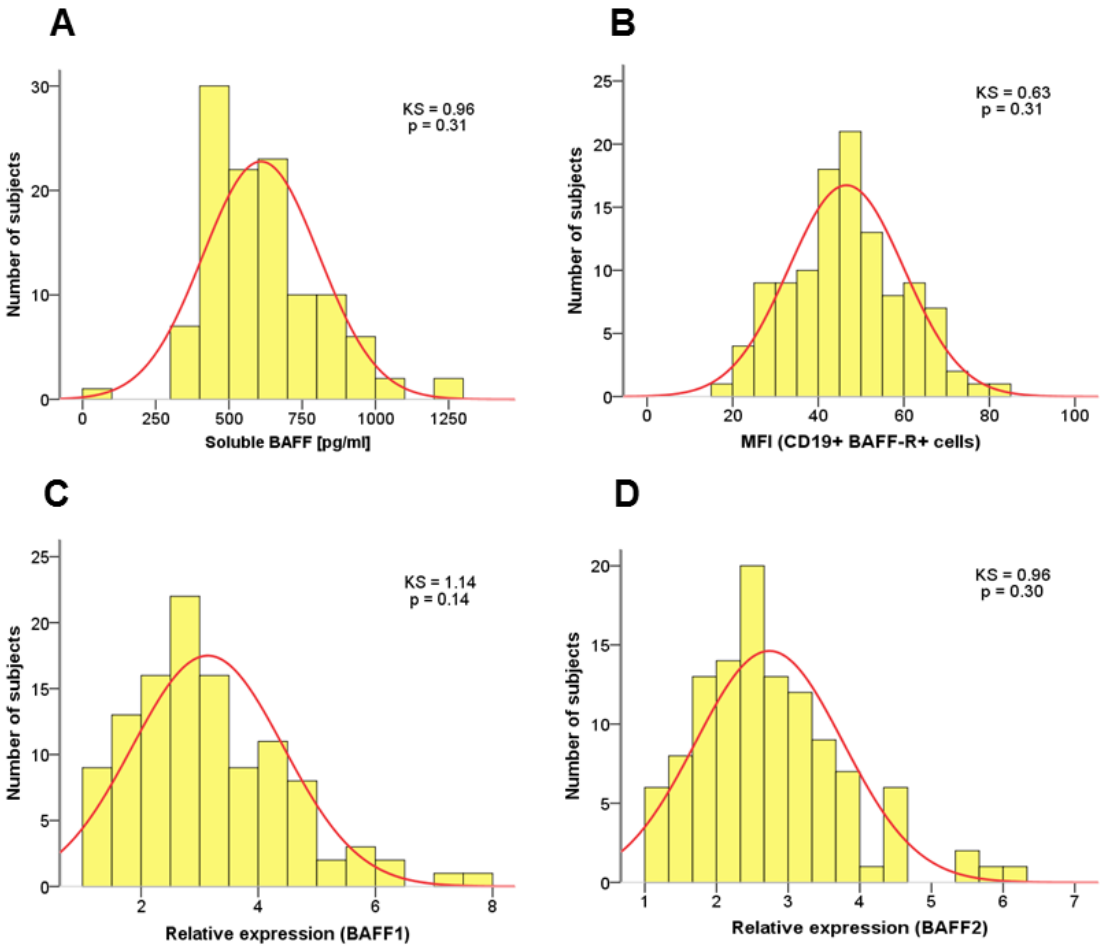
Additional file 1: Figure S1

Cell surface expression of BAFF-R in sub-populations of purified B cells



A. Cell surface expression of BAFF-R in plasmablasts, switched memory CD38^{+} and CD38^{-} and naïve B cells **B.** Cell surface expression of BAFF-R in transitional and mature naïve B cells.

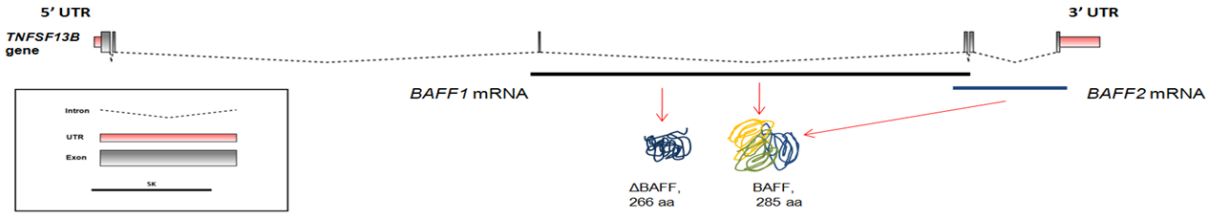
Additional file 2: Figure S2



Distribution of variables in a subgroup of individuals with mRNA and flow cytometry data (n = 113) **A.** Soluble BAFF levels in plasma **B.** Median fluorescence intensity of BAFF-R on gated $CD19^{+}$ B cells **C** *BAFF1* mRNA expression, and **D** *BAFF2* mRNA expression.

Additional file 3: Figure S3

Schematic representation of the gene encoding BAFF and the exons covered by the gene expression assays



To take into account differential splicing, the mRNA levels for BAFF were measured by two separated assays: Assay 1 (*BAFF1*) targeted the exons 3 and 4 (Cat. Hs00198106, 84 bp amplicon) and Assay 2 (*BAFF2*) targeted the boundaries of exons 4-5 and 5-6 (Cat. Hs00902574, 73 bp amplicon). Both transcripts were correlated ($r = 0.68$, $p < 0.001$).

Additional file 4: Table S1

Antibodies used for flow cytometry in PBMCs, monocytes and B cells

Specificity	Isotype	Catalog number	Clone	Manufacturer
APC-F780 anti-human CD14	IgG1k	47-0149	61D3	eBioscence
PECy7 anti-human CD14	IgG2a	A22331	RMO52	Beckman Coulter
APC anti-human CD19	IgG1k	17-0199	HIB19	eBioscence
FITC anti-human BAFF	IgG1k	11-9017	1D6	eBioscence
PE anti-human BAFF	IgG1k	12-9017	1D6	eBioscence
FITC anti-human BAFF-R	IgG2a k	11-9117	8A7	eBioscence
PE anti-human BAFF-R	IgG2a k	12-9117	8A7	eBioscence
FITC anti-human CD24	IgG2a k	555427	ML5	BD
PE-Cy7 anti-human CD38	IgG1k	25-0389	HIT2	eBioscence
PE-Cy5 anti-human CD10	IgG2b	15-0106	eBioCB-CALLA	eBioscence
APC anti-human CD27	IgG1k	17-0279	O323	eBioscence
PE-Cy7 anti-human CD27	IgG1k	25-0279	0323	eBioscence
FITC anti-human IgG	IgG1	MCA647F	MK1A6	AbD serotec

Study III

PLOS ONE

RESEARCH ARTICLE

Genetic Variants in *CHIA* and *CHI3L1* Are Associated with the IgE Response to the *Ascaris* Resistance Marker ABA-1 and the Birch Pollen Allergen Bet v 1

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Citation: Acevedo N, Bornacelly A, Mercado D, Unneberg P, Mittermann I, Valenta R, et al. (2016) Genetic Variants in *CHIA* and *CHI3L1* Are Associated with the IgE Response to the *Ascaris* Resistance Marker ABA-1 and the Birch Pollen Allergen Bet v 1. PLoS ONE 11(12): e0167453. doi:10.1371/journal.pone.0167453

Editor: Esaki M. Shankar, Central University of Tamil Nadu, INDIA

Received: August 22, 2016

Accepted: November 14, 2016

Published: December 15, 2016

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This work was funded by the Administrative Department of Science, Technology and Innovation (Colciencias, Colombia) Grant 680-2009, the University of Cartagena, Colombia; grants to AS from the Swedish Research Council, the Cancer and Allergy Research Foundation, and through the regional agreement on medical training and clinical research (ALF) between Stockholm

Abstract

Helminth infections and allergic diseases are associated with IgE hyperresponsiveness but the genetics of this phenotype remain to be defined. Susceptibility to *Ascaris lumbricoides* infection and antibody levels to this helminth are associated with polymorphisms in locus 13q33-34. We aimed to explore this and other genomic regions to identify genetic variants associated with the IgE responsiveness in humans. Forty-eight subjects from Cartagena, Colombia, with extreme values of specific IgE to *Ascaris* and ABA-1, a resistance marker of this nematode, were selected for targeted resequencing. Burden analyses were done comparing extreme groups for IgE values. One-hundred one SNPs were genotyped in 1258 individuals of two well-characterized populations from Colombia and Sweden. Two low-frequency coding variants in the gene encoding the Acidic Mammalian Chitinase (*CHIA* rs79500525, rs139812869, tagged by rs10494133) were found enriched in high IgE responders to ABA-1 and confirmed by genetic association analyses. The SNP rs4950928 in the Chitinase 3 Like 1 gene (*CHI3L1*) was associated with high IgE to ABA-1 in Colombians and with high IgE to Bet v 1 in the Swedish population. *CHIA* rs10494133 and *ABDH13* rs3783118 were associated with IgE responses to *Ascaris*. SNPs in the Tumor Necrosis Factor Superfamily Member 13b gene (*TNFSF13B*) encoding the cytokine B cell activating Factor were associated with high levels of total IgE in both populations. This is the first report on the association between low-frequency and common variants in the chitinases-related genes *CHIA* and *CHI3L1* with the intensity of specific IgE to ABA-1 in a population naturally exposed to *Ascaris* and with Bet v 1 in a Swedish population. Our results add new information about the genetic influences of human IgE responsiveness; since the genes encode for enzymes involved in the immune response to parasitic infections, they could be helpful for understanding helminth immunity and allergic responses. We also

County Council and the Karolinska Institutet and the Karolinska University Hospital, and by grant numbers 048488, 042679, and WT083625MA to MK from the Wellcome Trust, UK. RV research was founded in part by grant F4605 of the Austrian Science Fund (FWF).

Competing Interests: RV has received research grants from the European Union, by Biomay AG, Vienna, Austria, Thermofisher, Uppsala, Sweden and Fresenius Medical Care, Bad Homburg, Germany. He serves as a consultant for Biomay AG, Vienna, Austria, Thermofisher, Uppsala, Sweden and Fresenius Medical Care, Bad Homburg, Germany. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

confirmed that *TNFSF13B* has an important and conserved role in the regulation of total IgE levels, which supports potential evolutionary links between helminth immunity and allergic response.

Introduction

Upon infection with helminths humans synthesize specific IgE antibodies to parasite components as well as high levels of total IgE. The intensity of this response differs among exposed individuals [1–4], which seems to be determined by environment and their genetic backgrounds. Studies in animals [5–7] suggest that the specificity of the IgE to helminth components is determined by alleles of the major histocompatibility complex (MHC). However, the complete set of genes regulating this response and to common allergens is not defined.

The intestinal helminth *Ascaris lumbricoides* infects about 0.9 billion people worldwide [8], inducing specific IgE against its proteins (e.g. the polyprotein allergen ABA-1, tropomyosin, glutathione-S-transferase) [9–11], high levels of total IgE and, in general, a strong Th2 response [12]. Therefore, the increase in IgE elicited by *Ascaris* infection (ascariasis) is a good model for analyzing the genetics of IgE responsiveness. In addition, ABA-1 (also designated Asc s 1) is considered a resistance marker for *Ascaris* and also an *Ascaris*-specific component since it has no cross-reactivity with house dust mite (HDM) allergens [13, 14]. ABA-1 is therefore an excellent tool with which to investigate the genetics of the IgE response to *Ascaris*.

Genetic studies on the susceptibility to *Ascaris* have identified several associated loci including the signal transducer and activator of transcription 6 (*STAT6*) [15, 16], β2-adrenoreceptor (*ADRB2*) [17], tumor necrosis factor superfamily member 13B (*TNFSF13B*) [18–20], and ligase 4 (*LIG4*) [20]. Still, very little is known about the genetic influences on the IgE response to *Ascaris* and *Ascaris*-specific components such as ABA-1 in humans. Also, it remains to be defined whether the IgE responses to helminth and environmental allergens are under the same genetic control, which is of evolutionary significance given the features that anti-helminth immunity and allergic inflammation have in common [21, 22]. This question has been addressed by genetic epidemiology studies [15, 16, 23] and bioinformatics approaches [24] that evaluate each phenotype in separate populations, but studying the problem in populations naturally exposed to both helminths and allergens could be more informative.

We previously observed a great inter-individual variation in the IgE antibody response to *Ascaris* [1, 4, 25] that allowed the grouping of individuals into “high" and "low" IgE response phenotypes. In preliminary studies using few tag-SNPs, we detected associations between polymorphisms in chromosome 13q33 and IgE levels to *Ascaris* [20]. In the present work we fine-mapped these signals and explored other genes that might be of relevance, under the hypothesis that total and specific IgE levels are complex traits influenced by combinations of common and rare variants. To explore if these variants may also affect the IgE responses to non-parasite allergens we included a sample set of Swedish allergic patients. The aims of this study were (1) to perform targeted resequencing of promoters, untranslated regions (UTR), exons, and introns of 14 genomic regions to identify genetic variants associated with IgE responsiveness to *Ascaris*, (2) from these variants to select a panel for genotyping two populations with different genetic and environmental backgrounds and (3) to investigate whether variants influencing IgE response to Ascaris are associated with the IgE response to non-parasite allergens. We here detected significant associations between polymorphisms in chitinase related genes and the intensity of the specific IgE response to the *Ascaris* resistance marker ABA-1 supporting

that genetic factors play an important role in host responses to this parasite. We also add evidence suggesting that genes at 13q.33 locus are involved in the regulation of total and specific IgE response in humans.

Materials & Methods

Ethics statement

This study was conducted following the ethical principles for medical research stated in the Declaration of Helsinki. The Bioethics Committee of the University of Cartagena (Res. 26/06/2009) and the Swedish Regional Ethics Committee (Drn. 2011/1051-31) approved the study. Written informed consent was obtained from all subjects. Parents/guardians provided informed consent on behalf of all child participants.

Population characteristics and samples

For resequencing phase, forty eight subjects from Cartagena, Colombia (CGA cohort, see below) at the extremes of the distribution ($\leq 25^{th}$ and $\geq 75^{th}$ percentiles) of specific IgE levels to *Ascaris* and ABA-1 were included (**Table 1**). For the genotyping phase, samples from 1258 individuals from two independent cohorts (Colombia and Sweden) were selected to analyze genetic associations with total serum IgE, specific IgE to *Ascaris*, ABA-1 and other non-parasitic allergenic sources.

In both cohorts, individuals with allergic diseases (i.e. asthma, eczema) were included to provide more subjects with specific allergen IgE sensitization and to model the effect of genotypes on total IgE levels.

The Colombian cohort (Candidate Genes for Asthma, CGA) consists of 988 subjects; 597 non-asthmatic controls and 391 asthmatics (**Table 2**). Asthma was defined according to the Global Initiative for Asthma criteria using a standardized questionnaire previously tested in patients with a history of physician-diagnosed asthma. A physician belonging to the research staff confirmed the diagnosis. Subjects meeting the following criteria were recruited: current asthma, ≥ 8 years old and a history of ≥ 2 years of asthma, ≥ 3 episodes of asthma symptoms (wheezing, chest tightness and dyspnea) in the last 12 months or absence of symptoms due to

Table 1. Descriptive of individuals selected for targeted re-sequencing from the Candidate Genes for Asthma (CGA) cohort

Variables	low IgE (n = 20)	high IgE (n = 28)	*p* value[e]
Age, years (mean ±SD)	46.9 ± 17.1	34.8 ± 20.6	0.03
Gender, female (%)	9 (45)	16 (57)	0.4
Asthma, n (%)[a]	10 (50)	14 (50)	1
Total IgE (IU/ml)[b]	239 (145–826)	807 (447–1145)	0.01
IgE to Ascaris (OD)[b]	0.08 (0.07–0.08)	0.27 (0.23–0.52)	<0.001
IgE to ABA-1 (OD)[b, c]	0.08 (0.08–0.08)	0.48 (0.34–0.72)	<0.001
IgG to Ascaris (OD)[b, d]	2.04 (1.31–2.58)	2.77 (2.31–3.03)	0.003

[a] Asthmatic patients are a representative group for analyzing IgE response to *Ascaris* since it has been described that asthmatics have a higher antibody response to nematodes [1, 20].

[b]Median (interquartile range).

[c]ABA-1 is a fatty acid binding protein of 14.6 kD, very abundant in the pseudocelomic fluid of adult parasites and considered a resistance marker to *Ascaris* infection [13, 14].

[d] Levels of IgG to *Ascaris* extract denote that individuals in both groups have been exposed to the parasite.

[e] Comparisons of continuous variables calculated by *t*-test (age) and Mann Whitney U test (IgE variables); and by chi square for categorical variables. IU, international units; OD, optical density units.

doi:10.1371/journal.pone.0167453.t001

Table 2. Descriptive of the two populations analyzed in genetic association tests

Candidate Genes for Asthma cohort (CGA, n = 988)			
Variables	**Non-asthmatics controls (n = 597)**	**Asthmatic patients (n = 391)**	***p* value[c]**
Age years (mean ± SD)	35.6 ±18	36.1 ±18.1	0.6
Gender, female (%)	339 (56.8)	246 (63)	0.06
Total IgE (IU/ml)[a,b]	125.4 (46.9–297.8)	714.8 (250–1074.5)	<0.001
Ig levels to parasite (OD)[a]			
IgE to Ascaris	0.105 (0.091–0.132)	0.118 (0.101–0.154)	<0.001
IgE to ABA-1	0.119 (0.099–0.157)	0.122 (0.098–0.187)	0.1
IgG to Ascaris	2.11 (1.63–2.62)	2.01 (1.63–2.39)	0.02
IgE levels to HDM (OD)[a,b]			
IgE to *D. pteronyssinus*	0.097 (0.088–0.119)	0.209 (0.117–0.605)	<0.001
IgE to *B. tropicalis*	0.098 (0.088–0.120)	0.279 (0.114–1.30)	<0.001
Swedish Eczema Study cohort (n = 270)			
Variables	**Healthy controls (n = 100)**	**Eczema patients (n = 170)**	***p* value[c]**
Age years (mean ±SD)	37.6 ± 14.3	33 ± 13.6	0.008
Gender, female (%)	61 (61)	102 (60)	0.8
Objective SCORAD index	0	33 (27–41)	-
Asthma and/or rhinitis, n (%)	0	133 (78.2)	-
Phadiatop positive, n (%)	10 (10)	129 (75.8)	<0.001
Total serum IgE (kU/l)[a]	21.5 (13–46.5)	160 (51.2–852.5)	<0.001
Fel d 1 IgE (ISU)[a]	nd	0.30 (0–4.5)	-
Bet v 1 IgE (ISU)[a]	nd	0.30 (0–10.3)	-

[a] Median (interquartile range)

[b] Data for 389 asthmatics and 593 non-asthmatics controls

[c] Comparisons of continuous variables calculated by *t*-test (age) and Mann Whitney U test (antibody variables); and by chi-square for gender.

IU, international units; OD, optical density units; HDM: house dust mites

ISU: ISAC standardized units; SCORAD (SCORing Atopic Dermatitis) index: A clinical tool used to assess the extent and severity of eczema; nd = not determined.

doi:10.1371/journal.pone.0167453.t002

the use of antiasthmatic medications. Children under 8 years of age were excluded to avoid asthma misdiagnosis due to the high prevalence of transitory wheezing in this age range. Unrelated control subjects without a history of asthma, allergy or other diseases were recruited randomly from the same neighborhoods as the patients, using a questionnaire. All participants lived in an urban, non -industrialized setting, belonging to the lower three (out of six) socioeconomic strata in the city, where most people are naturally exposed to HDM [26] and *A. lumbricoides* and receive periodically anthelminthic treatment. The genetic background of this population resulted from racial admixture between Native Americans, Spaniards, and an important proportion (37.9%) of African ancestry [27, 28]. The DNA samples (from peripheral blood) were obtained from a well-characterized repository at the Institute for Immunological Research in Cartagena, Colombia; they were extracted between 2002 and 2004 and have been kept at -80˚C [1, 20]. Each DNA sample used for sequencing was evaluated for DNA integrity by visualization in 1% agarose gel and had A_{260}/A_{280} ratio between 1.8 and 2.09 (mean, sd, 1.93±0.05). Both cases and controls had total IgE and specific IgE to Ascaris and HDM and statistical analyses were adjusted by disease status.

The Swedish cohort (Swedish Eczema Study) comprised 170 atopic-dermatitis (AD) patients and 100 healthy controls. They were recruited from the Stockholm area and examined by a dermatologist at the Dermatology and Venereology Unit, Karolinska University Hospital

in Stockholm, Sweden, during September until May to avoid the summer season as previously described [29, 30]. Inclusion criteria for AD patients were: diagnosis according to the UK working party, moderate to severe eczema, and skin lesions not only restricted to the hands. The severity of the eczema was assessed using the objective SCORAD index. The healthy controls were subjects who did not have clinical symptoms or history of allergy or skin disease and were genotyped to serve as controls in the estimation of allele frequencies in this population. DNA samples were extracted from peripheral blood using the QIAamp DNA Blood Mini Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). The demographical characteristics of this population are presented in **Table 2.** We assumed that there is no exposure to *Ascaris* in this cohort, therefore it was not tested for *Ascaris* allergens and the analyses on the IgE responses to *Ascaris* and ABA-1 were done in the CGA cohort.

Allergens and IgE determinations

In the CGA cohort, total IgE was determined in duplicate using an enzyme-linked immunosorbent assay (ELISA) kit (RIDASCREEN; R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions. Specific IgE to *Ascaris* extract and ABA-1 (bacterial recombinant type 1A unit of the As-NPA array of the polyprotein [31, 32] as well as specific IgE to HDM extracts (*Blomia tropicalis* and *Dermatophagoides pteronyssinus*) were detected by ELISA as described previously [20]. In the Swedish cohort, total IgE and specific IgE to any of 11 common aeroallergen sources (Phadiatop®) were measured in plasma using ImmunoCAP™ (Phadia AB, Uppsala, Sweden). Specific IgE to the purified recombinant allergens Fel d 1 (from cat) and Bet v 1 (birch pollen) were analyzed with the customized MeDALL allergen-chip (Phadia Multiplexing, Thermo Fisher Scientific, Vienna, Austria) as described by Lupinek *et al* [33]. In brief, sera samples were tested undiluted, and after washing and rinsing, arrays were scanned by a confocal laser scanner and evaluated by the Microarray Image Analyzer v3..1.2 software (Phadia AB). ISAC standardized units (ISU) of IgE reactivity to Bet v 1 and Fel d 1 were used for quantitative trait analyses because these allergens were the most frequent sensitizers in this population [30] and the distribution of IgE levels allowed modeling the effect of genetic variants.

Targeted resequencing

Targeted resequencing was performed in 14 genes (*CHIA*, *CHI3L1*, *FCER1A*, *IL10*, *TSLP*, *IL5*, *RAD50/IL13*, *IL4*, *IL33*, *STAT6*, *LIG4*, *ABHD13*, *TNFSF13B* and *IRS2*) to evaluate their genetic variation and select markers of IgE hyper-responsiveness for further association studies.

Genomic coordinates of the coding and non-coding regions of the 14 genes included in the study were extracted via the UCSC browser. A library of RNA baits (120 mer) was designed using e-Array and produced by chemical synthesis (Agilent Technologies). For targeted enrichment, 3 μg genomic DNA from each individual was fragmented by sonication (Covaris S2 instrument) and then linked with specific adaptors and indexes. The samples were incubated overnight with the biotinylated RNA baits (SureSelect, Agilent). After targeted selection using magnetic streptavidin beads, the enriched regions were eluted according to the manufacturer's instructions. After amplification, the samples were sequenced using a 100 bp sequencing protocol (paired-end). The sequencing runs were performed according to manufacturer's instructions (Illumina) with a setup aiming for a minimum coverage of 30X in the targeted regions. The production of the libraries and the sequencing procedures were done at Science for Life Laboratory in Stockholm, Sweden. The flow chart for data analysis is presented in **S1 Fig.**

Data processing

Sequence reads passing Ilumina's chastity filter were aligned to human genome reference version 19 (hg19) and post-processed for variant calling. Read alignment was done for each sample with BWA version 0.6.2 [34] and sample-specific bam files were generated (Sequence Alignment Map-format in binary format). We used Picard tools version 1.126 to sort the bam files, mark duplicates, and calculate alignment, insertion and hybrid selection metrics. Genome Analysis Tool Kit (GATK) [35] version 3.3–0 base quality recalibration (BQR) and local realignment were applied around indels. Variant calling, filtering, and variant quality score recalibration (VQSR) was done following best practice guidelines [36]. Alignments and variants were visualized with the Integrative Genome Viewer (IGV) version 2.3.19 [37]. Variants were annotated with ANNOVAR [38] and SnpEff [39]. Phasing information for each gene was obtained by running GATK Read Backed Phasing on reads mapping to target gene regions including 50 kb flanking regions. Finally, 2423 raw variants that passed filtering criteria were uploaded and analyzed into Ingenuity Variant Analysis software (www.qiagen.com/ingenuity) from QIAGEN Redwood City.

Burden analysis

The.vcf files containing all the variants per individual were uploaded to the Ingenuity Variant Analysis software. The burden of variants according to specific IgE levels to *Ascaris* and ABA-1 was calculated between groups of high IgE responders (HR, levels $\geq$ percentile 75th) and low IgE responders (LR, levels $\leq$ percentile 25th). Ingenuity Variant Analysis software identifies genes that exhibit significant differences in variants of low frequency (MAF < 0.05) between groups. The statistical test is based on an extension of the Optimal unified Sequence Kernel Association Test (SKAT-O) and can be used to find variants associated with dichotomous and quantitative traits [40]. Starting from 2423 variants, we used the filter "confidence" to select those with a high call quality (CQ) and read depth (RD), and this resulted in 1955 variants for analysis (CQ = 100 and RD = 30). To identify variants with putative functional effects, the predicted deleterious filter was also applied. After this filter 338 variants remained in the analysis, with a predicted effect or association with a phenotype according to the American College of Medical Genetics and Genomics guidelines; and/or association with gain or loss function of a gene. The genetic analysis was done at three levels: gene-gene, variant-variant and gene-variant, including variants that occur in at least 4 high responders but not in low responders. The statistical association analysis was done for binary (HR, LR) and the specific IgE levels to *Ascaris* and ABA-1. Variants with a p value less than 0.05 after Bonferroni corrections and with an odds ratio greater than or equal to 1.5 between high and low responders were considered statistically significant. In addition, for quantitative traits the analysis was corrected by age, gender and asthma status.

Selection of variants and genotyping

One hundred-one SNPs were selected for genotyping by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry (SEQUENOM®, Inc.). A detailed list of SNPs genotyped is presented in **S1 Table**. The variants for genotyping were selected based on the following criteria: (1) A statistically significant association in the variants burden analysis with specific IgE levels to *Ascaris* and ABA-1; (2) the most informative TagSNPs around the regions associated in the burden analysis (3) SNPs with a clinical or functional association with total IgE levels in PubMed and dbSNP (NCBI); (4) SNPs predicted to affect transcription factor-binding sites by different publicly available bioinformatics tools (i.e. F-SNP and Genomatix software suite v 3.1) and/or (5) related to significant promoter or enhancer chromatin

state annotations based on ENCODE data (explored using Haploreg2). Ninety SNPs passed the quality criteria and were further analyzed in both populations. Primer for multiplex PCR and extension reactions were designed by the SpectroDesigner software (Sequenom GmbH, San Diego, CA, USA, available on request). PCR and extension reactions were performed according to manufacturer's standard protocols. Concordance analysis with HapMap data was performed. Ten novel variants that were found enriched in high IgE responders in the burden analysis were included in the multiplex PCR reactions (of which seven were successful). The required 50 bp upstream and downstream flanking regions were extracted from the UCSC Genome Browser using Galaxy utilities (http://galaxyproject.org/).

Statistical analysis

The genetic association analyses between the genotyped variants (n = 91) with the risk of being a high responder (HR) ($\geq$ percentile 75th) were done in PLINK v 1.09 (http://pngu.mgh.harvard.edu/~purcell/plink/). IgE levels according to genotypes were compared using non-parametric tests (Mann Whitney and Kruskall Wallis). The associations with IgE levels as a continuous variable were modeled by using median regression and quantile regression with the package (quantreg) implemented in R. The regression models were adjusted by age, gender and clinical condition (asthma in CGA and atopic eczema in Swedish cohort) considering the confounding effect of these covariates on IgE levels (p_{adj}). The significant level was set at $p < 0.05$.

Results

Differential distribution of variants in targeted genes

Targeted resequencing in 48 individuals from the CGA cohort resulted in 2423 variants (1851 located on the targeted genes). Of these, 1290 were already known (dbSNP137) and 561 novel (without a reference SNP ID number in dbSNP build 137) including 1663 single nucleotide substitutions and 188 indels. A summary of targeted resequencing metrics by gene region is presented in **S2 Table**. The highest numbers of variants were found in *IRS2* (n = 341) and *IL-33* (n = 311) and the lowest in *IL-5* (n = 17). There were remarkable differences in the distribution of the 75 coding variants among the loci studied, suggesting different degrees of conservation. For instance, the gene *CHIA* encoding for the acidic mammalian chitinase (AMCase) contained the highest number of exonic variants with 14 non-synonymous, 6 synonymous, 1 stop-gain and 1 frameshift, while the genes encoding the cytokines *IL-10* and *TSLP* had no coding variants. Based on the ratio of the number of observed variants to those expected from the gene size, the most polymorphic genes were *LIG4*, *IRS2*, *IL13* and *CHI3L1* (**Table 3**).

Burden analysis of variants

We analyzed 1955 variants (out of 2423) with call quality of 100 in the Phred Scale and a read depth of 30x for their enrichment according to the intensity of the IgE levels (burden analysis). Seventy variants, distributed among 8 genes (*CHIA*, *CHI3L1*, *TSLP*, *IL13*, *LIG4*, *ABHD13*, *IRS2 and STAT6*) were enriched in high IgE responders to whole *Ascaris* antigen and ABA-1 (IgE level $\geq$ 75th percentile) and absent in low responders. These included 65 single nucleotide variations (SNVs), three insertions and two deletions, 70% of them being non-coding variants. Coding variants included 11 missense; 8 synonymous, 1 stop gain and 1 causing a frameshift change. The distribution of variants enriched in high IgE responders to *Ascaris* is shown in **Fig 1**.

Table 3. Distribution of variants in the genes analyzed by targeted resequencing (CGA cohort)

locus	Gene symbol	Gene name	# Variants (total)	# Novel variants	# Coding variants	Gene size (kb)	# Variants F/E (1kb)*
1p:13.2	*CHIA*	Acidic mammalian chitinase	196	27	22	29.7	6.5
1p:22.2	*IL10*	Interleukin 10	33	5	0	4.8	6.7
1q:23.2	*FCER1A*	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	107	30	2	18.5	5.7
1q:31.1	*CHI3L1*	Chitinase 3–like 1	80	11	8	7.8	10.1
5q:22.1	*TSLP*	Thymic stromal lymphopoietin	43	10	0	6.3	6.7
5q:31.1	*IL5*	Interleukin 5	17	0	2	2.0	8.1
5q:31.1	*IL13*	Interleukin 13	30	4	1	2.9	10.2
5q:31.1	*IL4*	Interleukin 4	84	27	3	8.6	9.6
9p:24.1	*IL33*	Interleukin 33	311	98	3	42.1	7.3
12q:13.3	*STAT6*	Signal transducer and activator of transcription 6	132	71	3	16.0	8.2
13q33.3	*LIG4*	Ligase 4	179	97	9	10.9	16.3
13q33.3	*ABHD13*	AB hydrolase domain containing protein 13	87	15	1	15.8	5.4
13q33.3	*TNFSF13B*	B-cell activating factor	211	67	4	38.8	5.4
13q34	*IRS2*	Insulin receptor substrate 2	341	99	17	32.7	10.4
			1851	561	75	-	-

*The ratios of the number of variants found/expected (F/E) were calculated based on the frequency of SNP throughout the human genome, one in every 1000 base pairs.

doi:10.1371/journal.pone.0167453.t003

Variants associated with the IgE responses to *Ascaris* and ABA-1

Table 4 shows all the genetic associations found in this study. The burden analysis revealed two coding variants in *CHIA* (rs79500525, G/A, and rs139812869, G/A) that were enriched in high IgE responders to *Ascaris* extract and ABA-1. They were located 508 base pairs apart and in strong linkage disequilibrium (D' = 0.99). These variants were tagged by an intronic SNP (rs10494133, T/C), observed in 14% of the individuals in the CGA dataset and associated with IgE levels to ABA-1 above percentile 75th under additive (aOR: 1.39, 95%CI 1.04–1.85, p_{adj} = 0.02) and dominant models (aOR: 1.39, 95%CI 1.01–1.93, p_{adj} = 0.04), Fig 2A.

Haplotype analysis revealed a significant increased risk of high IgE levels to ABA-1 (OR: 2.32, 95%CI 1.03–5.23, p = 0.04) in carriers of the minor allele A in the two coding variants and the minor allele C in rs10494133 (global haplotype p value = 0.04 (Table 5).

We then implemented quantile regression analyses to model the effect of these genetic variants on IgE levels to ABA-1 as a continuous variable. The tagSNP rs10494133 (T/C) was associated with increased IgE levels to ABA-1 independently of age, gender or the presence of asthma (p_{adj} = 0.04). Since only one individual was homozygous for the A/A genotype in rs79500525 and rs139812869, dominant code for quantile regression (GG vs. GA + AA) was used, confirming that the allele A was significantly associated with increased IgE levels to ABA-1 (p = 0.03). The tagSNP *CHIA* rs10494133 T/C was also associated to high IgE responses to *Ascaris* under additive (aOR = 1.35, 95%CI 1.02–1.79, p_{adj} = 0.04) and dominant models (aOR = 1.43, 95%CI 1.04–1.97, p_{adj} = 0.03), driven by a higher frequency of the minor allele C in subjects with IgE levels to *Ascaris* above percentile 75th (Fig 2A). This was confirmed by quantile regression analysis (p = 0.01) but was not significant after adjusting by age, gender and the presence of asthma (p_{adj} = 0.2). There was also association between high IgE response to ABA-1 and *STAT6* rs73118440 (G/T) under additive model and quantile regression analysis,

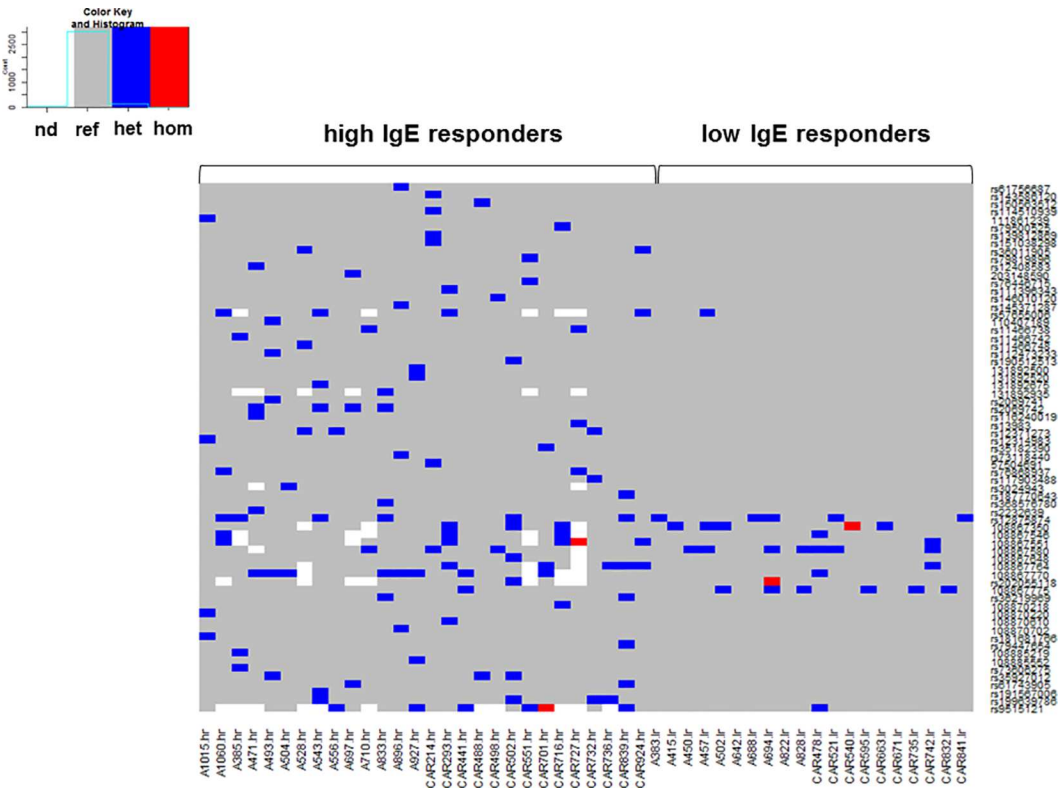

Fig 1. Burden of 70 single nucleotide variants with differential enrichment between high (≥75th percentile) and low (≤25th percentile) IgE responders to Ascaris and ABA-1. Each column corresponds to the pattern of one individual. The color scale indicates the reference genotype (grey) or the presence of single nucleotide variants in heterozygous (blue) or homozygous (red).

doi:10.1371/journal.pone.0167453.g001

Fig 2B. Neither *CHIA* nor *STAT6* variants were associated with IgE responses to HDM or other common allergens.

Variants associated with the IgE responses to common allergens

To address whether loci influencing IgE levels to *Ascaris* or ABA-1 were involved in the response to non-parasite allergens we explored associations with common allergens. In the CGA cohort *IRS2* rs12584136 C/A was associated with high IgE response to *D. pteronyssinus* (and not to parasite allergens) under additive model, dominant model and quantile regression analyses (Table 4). In patients from the Swedish cohort (n = 170) the SNP rs17565502 (A/C) located in the gene encoding B cell activating factor (*TNFSF13B*) was associated with an increased risk of high IgE responses to the cat allergen Fel d 1(>4.52 ISU). This association was significant under the additive model and quantile regression analysis.

Variants associated with IgE levels to both Ascaris and common allergens

Variants in two genes were associated with both Ascaris and common allergens (Table 4). In the CGA cohort *CHI3L1* (rs4950928, C/G) was associated with high IgE response to ABA-1 under recessive model, which was confirmed by quantile regression analysis, Fig 3A. Also, in patients from the Swedish cohort two variants in the *CHI3L1* gene (rs4950928 C/G and rs880633 C/T) were associated with high IgE response (≥75th percentile) to the birch pollen

Table 4. Genetic variants associated with the strength of the IgE response in Colombian and Swedish populations

Gene	SNP	Allele	Phenotype	Population	aOR (95%CI)	model	P_{adj}	P_{adj} (quantreg)[a]
CHIA	rs10494133	T/C	high IgE to ABA-1	Colombia	1.39 (1.04–1.85)	additive	0.02	0.04
					1.39 (1.01–1.93)	dominant	0.04	
CHIA	rs10494133	T/C	high IgE to *Ascaris*	Colombia	1.35 (1.02–1.79)	additive	0.02	0.2
					1.43 (1.04–1.97)	dominant	0.03	
STAT6	rs73118440	G/T	high IgE to ABA-1	Colombia	2.49 (1.06–5.83)	additive	0.03	0.006
IRS2	rs12584136	C/A	high IgE to *D. pteronyssinus*	Colombia	2.14 (1.24–3.67)	additive	0.007	0.03
TNFSF13B	rs17565502	A/C	high IgE to Fel d 1	Sweden	1.87 (1.07–3.29)	additive	0.02	0.003
CHI3L1	rs4950928	C/G	high IgE to ABA-1	Colombia	1.77 (1.02–3.09)	recessive	0.04	0.003
CHI3L1	rs4950928	C/G	high IgE to Bet v 1	Sweden	2.52 (1.21–5.25)	dominant	0.01	0.02
CHI3L1	rs880633	C/G	high IgE to Bet v 1	Sweden	1.82 (1.07–3.10)	additive	0.02	0.004
					2.44 (1.07–5.57)	recessive	0.03	
ABHD13	rs3783118	A/C	IgE to *Ascaris* below 75th percentile	Colombia	0.53 (0.31–0.89)	additive	0.01	0.00001
					0.53 (0.31–0.90)	dominant	0.01	
ABHD13	rs3783118	A/C	IgE to *D. pteronyssinus* below 75th percentile	Colombia	0.49 (0.28–0.87)	additive	0.01	0.13
					0.50 (0.28–0.89)	dominant	0.01	
TNFSF13B	rs17565502	A/C	high total IgE	Colombia	1.78 (1.16–2.75)	additive	0.009	0.03[b]
					1.75 (1.09–2.83)	dominant	0.02	
TNFSF13B	rs8181791	A/G	high total IgE	Sweden	1.97 (1.14–3.41)	additive	0.01	0.0006
					4.81 (1.72–13.4)	recessive	0.002	
IRS2	rs12584136	C/A	high total IgE	Colombia	2.71 (1.25–5.90)	allelic	0.01	0.00007[c]
IL5	rs2069816	A/C	high total IgE	Colombia	2.33 (1.08–5.0)	additive	0.03	0.02[b]
					2.64 (1.18–5.91)	dominant	0.02	
IL13	rs20541	G/A	high total IgE	Sweden	1.74 (1.0–3.0)	additive	0.05	0.008

[a] The model fits quantile 75th ($\tau = 0.75$) and computed the standard errors by using the Powell kernel version of the covariance matrix estimate (se ="ker").

[b] Estimated model on quantile 50th ($\tau = 0.50$).

[c] For the case of this variant the standard error was computed by the method "nid" which presumes local (in tau) linearity of the conditional quantile functions and computes a Huber sandwich estimate using a local estimate of the sparsity.

doi:10.1371/journal.pone.0167453.t004

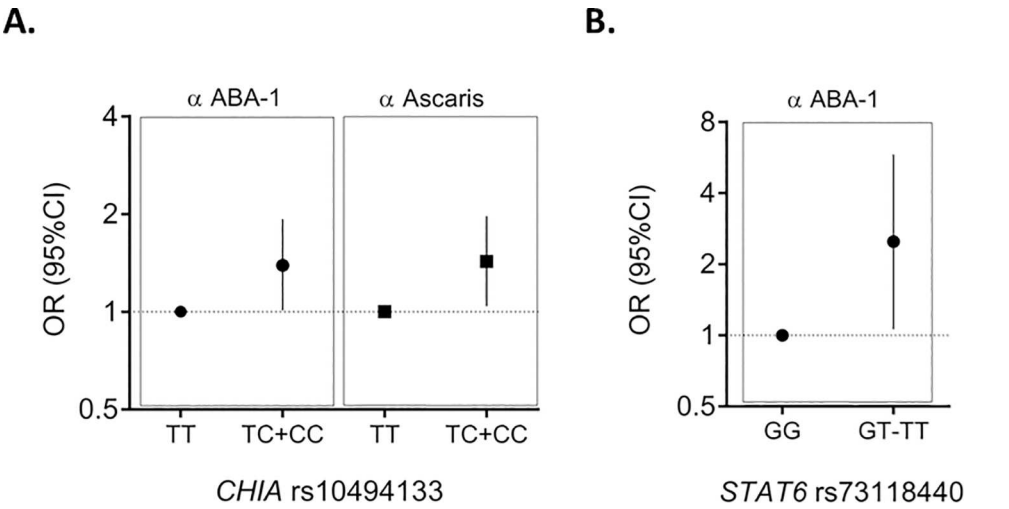

Fig 2. Genetic loci associated with the risk of having high IgE response (≥75th percentile) to ABA-1 and *Ascaris* extract in the CGA dataset. A) Effects of the tagSNP *CHIA* rs10494133 on the risk of high IgE response to ABA-1 (filled circle) and to the *Ascaris* extract (filled square) under dominant model. B) Effects of *STAT6* rs73118440 on the risk of high IgE response to ABA-1. OR: Odds ratio; CI: confidence interval.

doi:10.1371/journal.pone.0167453.g002

Table 5. Haplotype association between genetic variants in *CHIA* and IgE response to ABA-1 in the CGA cohort (n = 988)

rs79500525	rs139812869	rs10494133	IgE to ABA-1 <75th percentile (n = 736)	IgE to ABA-1 >75th percentile (n = 252)	OR (95% CI)	p-value
G	G	T	0.87	0.82	1.0	-
G	G	C	0.11	0.15	1.36 (1.01–1.83)	0.04
A	A	C	0.008	0.02	2.32 (1.03–5.23)	0.04
A	A	T	0	0	-	-

Adjusted global haplotype association p-value = 0.04

doi:10.1371/journal.pone.0167453.t005

allergen Bet v 1 (>10.3 ISU) (Fig 3A). It is worth mentioning that these SNPs are 3081 base pairs apart, in strong LD (D' = 0.94).

The *ABHD13* rs3783118 A/C, located in the *Ascaris* susceptibility locus (Cr. 13q33.3), was under-represented in the group of high responders to *Ascaris* under the dominant model. Quantile regression confirmed this finding showing significant association with lower IgE levels (p_{adj} = 0.00001). In the CGA dataset we found that, as occurred with the *Ascaris* extract, *ABHD13* rs3783118 was associated with lower levels of IgE to *D. pteronyssinus* under dominant model. This effect was driven by one or two copies of the minor allele C (p_{adj} = 0.03), Fig 3B.

Variants associated with total IgE levels

We investigate associations with high total IgE levels (≥ 75th percentile) in allergic patients from the CGA dataset and the Swedish cohort. In the CGA cohort *TNFSF13B* rs17565502 A/C was associated with high total IgE levels (≥ 1074.5 IU/ml) under the additive model. In the Swedish cohort *TNFSF13B* rs8181791 A/G was associated with high total IgE levels (≥852.5 kU/l) under additive and recessive models (Fig 4). Both associations were also observed when

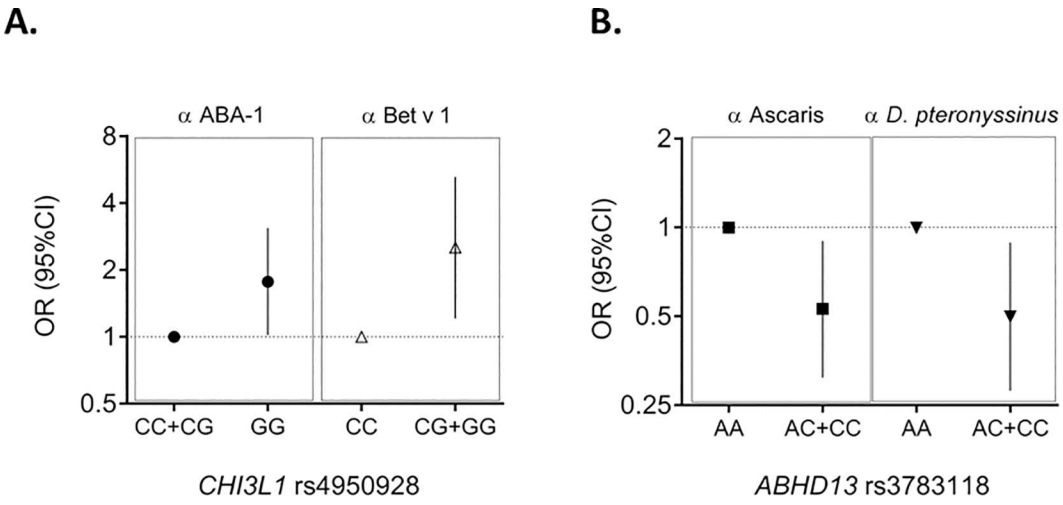

Fig 3. Genetic loci associated with the risk of having high IgE response to both Ascaris and common allergens. A) Effects of *CHI3L1* rs4950928 on the risk of high IgE response to ABA-1 (filled circle) and to the pollen allergen Bet v 1 (white triangle). The association with ABA-1 was detected in the CGA cohort and the association with Bet v 1 in the Swedish Eczema Cohort. **B)** Effect of *ABDH13* rs3783118 on the risk of high IgE response (≥75th percentile) to the extracts of *Ascaris* and HDM in the CGA dataset. Risk of IgE response to the *Ascaris* extract (filled square); Risk of IgE response to the *D. pteronyssinus* extract (filled triangle). OR: Odds ratio; CI: confidence interval.

doi:10.1371/journal.pone.0167453.g003

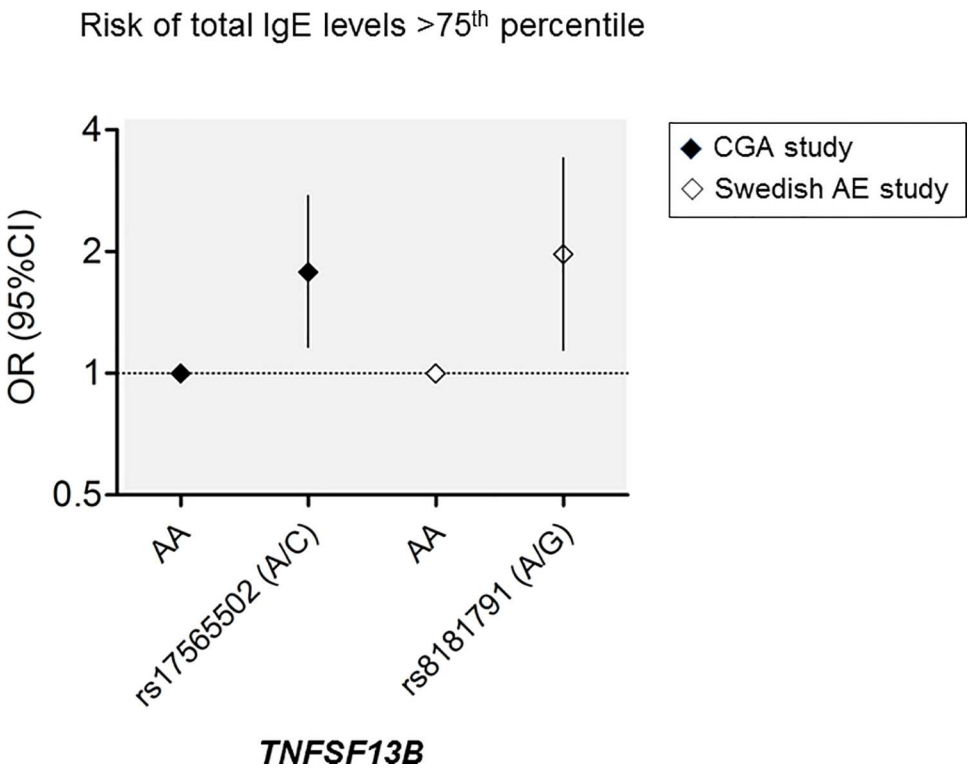

Fig 4. Effect *TNFSF13B* SNPs on the risk of high total serum IgE levels. Asthmatic patients from the CGA dataset (n = 391) (filled rhomboid) and AE patients from the Swedish eczema study (n = 170) (white rhomboid). OR: Odds ratio; CI: confidence interval.

doi:10.1371/journal.pone.0167453.g004

total IgE levels were modeled as a continuous variable by quantile regression (**Table 4**). Other variants associated with high total IgE were the coding SNP *IL13* rs20541 G/A; *IRS2* rs12584136 C/A and the *IL5* rs2069816 A/C. **Fig 5** summarizes the main genetic associations found in the study.

Despite some genes evaluated in this study have been associated with asthma in particular populations, we did not find any variant significantly associated with this condition (data not shown), however, this study was designed to study QTL for total and specific IgE and is underpowered to address associations with disease phenotypes.

Discussion

Tropical settings provide several advantages for investigating the molecular genetics of the IgE responses in helminthiases and allergies. First, due to the high prevalence of these conditions [8, 41, 42], studies analyzing the influences of genetic variants on the IgE response to allergens from both sources can be performed simultaneously in the same population; and second, the perennial exposure to allergenic components allows selecting extreme phenotypes of IgE responses. Also, it allows the comparisons with populations living in temperate climates and industrialized settings, which could be very informative. Using deep sequencing and a powerful design of extreme phenotypes case-control study [43], we discovered genetic variants in genes *CHIA* and *CHI3L1* (chromosome 1) overrepresented in high IgE responders to *Ascaris* and ABA-1 (here a purified recombinant IgE binding protein of *Ascaris*).

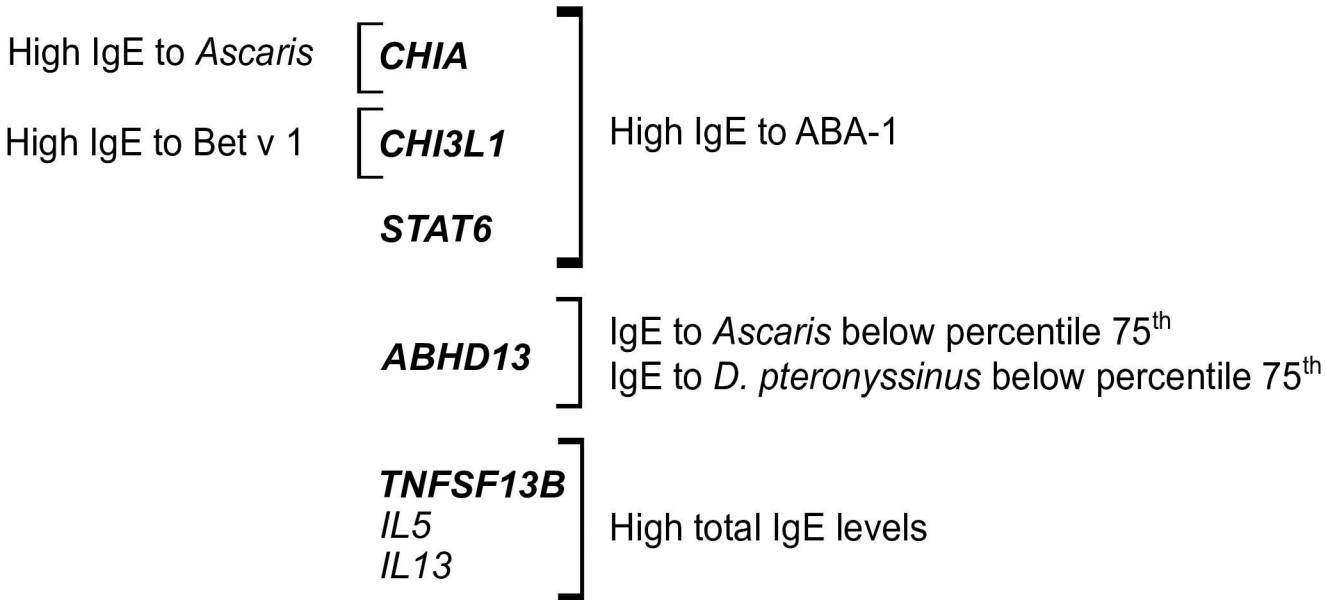

Fig 5. Summary of the genes influencing IgE levels in this study. Associations with those in bold are described for the first time. *TNFSF13B* was associated with total IgE levels in both Colombian and Swedish populations.

doi:10.1371/journal.pone.0167453.g005

There is evidence for considering ABA-1 as a resistance marker for ascariasis [13], but among infected individuals some respond strongly and others not at all, despite having attested infection and immune responses to other components of the parasite [6, 13, 25]. This also occurs in animals, in association with MHC polymorphisms [7], suggesting an important genetic influence on the regulation of this response. There are no previous studies about the influence of non-MHC genes on the overall immune response to ABA-1, but we now find that genes beyond the MHC region are influential.

This is the first report of association between chitinase related genes and the IgE responsiveness to a nematode allergen (ABA-1). *CHIA* has 13 exons spanning around 29 kb and encoding AMCase, a protein with functional catalytic and chitin-binding domains in humans. This enzyme is produced by epithelial cells and alveolar macrophages, and Th2 cells are potent stimulators of its expression at both mRNA and protein level [44]. *CHI3L1* is a gene of 8 Kb, which encodes YKL40, a 40 kDa heparin and chitin-binding glycoprotein. Chitinases and chitinase-like proteins may act directly as chemotactic agents or by inducing other chemokines that attract eosinophils and T cells to the sites of parasitic infection. AMCase activity is required for the increased expression of chemokines involved in the recruitment of monocytes, macrophages, eosinophils and neutrophils. Also, it reduces the expression of the Th1 chemokines interferon gamma-inducible protein 10 (IP-10) and interferon-inducible T-cell alpha chemoattractant (I-TAC), thus contributing to a stronger Th2 response [45]. Furthermore, in mice, Ym1 (a chitinase-like protein) has been reported as a potent chemotactic agent for eosinophils and $CD4^+$ T cells [46]. These functions are likely to be related to the resistance to helminths [47]; pertinently, *CHIT1* deficient individuals from South India were more susceptible to *Wuchereria bancrofti* infection [48], although such protective effect has not been replicated in other studies [49, 50]. Our study was not designed to directly investigate the genetics of susceptibility to ascariasis, but, considering the biological role of chitinases and chitinase-like proteins in the context of Th2-mediated inflammation and the predicted functional effect of the detected variants (*e.g.* rs880633 Arg145Gly), our findings suggest that they

are relevant in the regulation of the intensity of the specific IgE response to ABA-1, which is potentially important information for understanding the genetic susceptibility to ascariasis. It is worth adding that *CHIA* rs10494133 was also associated with the intensity of the IgE response to the *Ascaris* extract, which can be explained because ABA-1 is abundant in this extract. However, the underlying mechanisms of these associations remain unknown and require functional studies.

Our results also support previous findings suggesting that *STAT6* is involved in the susceptibility to Ascaris. Gao et al [15] showed that variants of this gene were associated with low parasite infestation in humans. Therefore, our findings support indirectly the protective role of the IgE response to ABA-1. Interestingly, these genes have been associated with asthma in other populations and our results suggest that these associations could be related to their effects on IgE production, a known risk factor for asthma.

The association of *ABHD13* with the IgE responsiveness to *Ascaris* supports previous findings linking the 13q33 locus with the susceptibility to *Ascaris*-infection and the regulation of IgE responses to the parasite [18–20]. In our previous study we did not explore the effect of *ABHD13* but we now found that the rs3783118 variant was associated with lower levels of specific IgE to *Ascaris* and *D. pteronyssinus*. This variant generates a new binding site for the transcriptional repressor Foxp1 (Fkhd domain) that plays an important role in the differentiation of lung epithelium and is an essential transcriptional regulator of B-cell development [51]. Since there is cross reactivity between *Ascaris* and HDM extracts [14] the associations between *ABHD13* and the IgE responses to both sources may involve cross reactive components. Interestingly the SNP was not associated with the IgE response to ABA-1, which does not cross-reactive immunologically with HDM [14].

We did not include parasitological data in this study; therefore, the associations with IgE levels are not necessarily related to resistance to Ascaris infection. However, immunity to helminthiases involves a wide spectrum of effector mechanisms including the specific IgE response [52–54]. The relative importance of these antibodies has not been defined [55], although several studies have shown that elevated specific IgE levels to *Ascaris* or the purified allergen ABA-1 are associated with resistance to this nematode and decreased worm burden [13, 53, 54, 56, 57]. Since the original linkage study performed by Williams-Blangero et al. [18] identified 13q33 as a quantitative trait locus for resistance to ascariasis (as detected by parasite egg loads) and in this study we detected significant associations between genes underlying the 13q33 locus and specific IgE levels to *Ascaris*, it is feasible that genetic regulation of antibody production may play a role on *Ascaris* susceptibility.

Another aspect of the 13q33 locus is its influence on total IgE levels. In this study it was driven by variants in *TNFSF13B* present in both populations, Swedish [58] and Colombian [27]. This suggests a biological role for this gene on the regulation of total IgE levels in humans. Since high total IgE levels is a hallmark of both, helminth infections and allergic diseases, the conservative role of *TNFSF13B* on this phenotype supports potential evolutionary links between helminth immunity and allergic responses. *TNFSF13B* encodes B cell-activating factor (BAFF), a well-known major regulator of B cells development that has a critical role on the production of IgA and IgG and the synergic effect with IL-4 on the class-switching to IgE [59]. In addition, changes in BAFF levels are detectable in plasma during different immune-related conditions and nematode infections [60], and in exploring the relationship between circulating BAFF levels and antibody response in humans, we found an inverse correlation between levels of BAFF and the intensity of the antibody response to *A. lumbricoides* [1]. Although total IgE levels are markedly influenced by environment (mainly helminthiases), heritability of this trait was high ($h^2 = 0.53$) when analyzing a large pedigree from the Jirels population in Nepal [18], which is highly exposed to *A. lumbricoides*. In addition, the fact that the association was also

found in subjects from Sweden, where intestinal parasite infections are not endemic, rule out the potential confounding effect of helminthiases. Genomic region 13q33 is evidently of interest for further fine mapping and association studies in larger populations. Recently, another region in the chromosome 13 (13q21.31) has been suggested as locus regulating total IgE levels [61].

Another variant influencing total IgE levels in the Swedish population was *IL13* rs20541, which has been associated with allergic phenotypes and helminth susceptibility in parasite exposed populations [62–66] suggesting that this is a regulatory locus common to both allergy and parasite responses. We also found significant associations between *IL5* and total IgE levels in the Colombian population replicating previous findings on the influence of this gene on IgE production [67, 68].

Excepting *ABHD13* rs3783118, genetic variants associated with specific IgE reactivity to *Ascaris* or ABA-1 were not associated with the IgE response to HDM, suggesting that in the CGA dataset specific IgE responses to parasite and HDM allergens are controlled by different genes, which seems to contradict the idea that allergic response is a side effect of helminth immunity; however, in the Swedish cohort the IgE response against two common inhaled allergens (Bet v 1 and Fel d 1) were associated with *CHI3L1* and *TNFSF13B* respectively, which could reflect differences in the regulation of gene expression by environment. Recently, using bioinformatics tools, it was found that Bet v 1 shares an IgE binding epitope with a Bet v 1 like protein (SmBv1L) from *Schistosoma mansoni* [69]. The authors of that work confirmed the IgE binding using sera of infected individuals from Uganda, under the hypothesis that current IgE responses to common allergens is a remnant from originally protective immunity to metazoan parasites. Our findings on *CHI3L1* suggest that chitinase related genes may have evolved in the context of more primitive immune responses than those elicited by non-parasite allergens.

In summary, we have uncovered genetic variants strongly associated with the IgE response to the nematode *Ascaris* and its resistance marker ABA-1. Most associations were restricted to the response to this parasite and not to other allergens such as HDM. We also confirm previous associations, especially the relevant role of locus 13q.33 in modulating total and specific IgE levels.

Supporting Information

S1 Fig. Flow chart summarizing the research questions and phases of the study. Samples from the Colombian Dataset are indicated by the acronym CGA; Individuals were classified as high IgE responders (HR) or low IgE responders (LR) based on the percentile corresponding to their IgE levels; SKAT-O: Optimal unified Sequence Kernel Association Test; HDM: House Dust Mites; AE: atopic eczema. *Adjusted by age, gender and disease status. **For this trait only data from patients was analyzed.
(PDF)

S1 Table. List of variants genotyped for the association study in CGA and the Swedish Eczema Cohort. Success rate for genotyping (%), the exact p-value for the calculations on Hardy-Weinberg equilibrium in controls (HWE) and the minor allele frequencies in each population are indicated in columns. The variants without rs number correspond to novel single nucleotide substitutions as detected in CGA.
(XLSX)

S2 Table. Descriptive of sequencing metrics by gene region in the CGA cohort
(DOCX)

Acknowledgments

We thank all the patients and controls for their voluntary participation in the study. Also we thank Dr. Lena Lundeberg and PhD Catharina Johansson for their support with Swedish cohort recruitment. We acknowledge Colciencias for financial support (Contract 680–2009), the Science for Life Laboratory (SciLifeLab) and the National Genomics Infrastructure (NGI) for sequencing service and the Mutation Analysis Facility (MAF) at Karolinska Institutet for genotyping service.

Author Contributions

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Formal analysis: NA AB PU IM.

Funding acquisition: NA LC.

Investigation: NA AB DM PU IM MK.

Methodology: NA AB DM PU LC.

Project administration: LC.

Resources: NA RV MK AS LC.

Software: NA AB PU.

Supervision: NA LC.

Validation: NA AB DM PU IM RV AS LC.

Visualization: NA AB LC.

Writing – original draft: NA AB LC.

Writing – review & editing: NA AB DM PU IM RV MK AS LC.

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1. RESEQUENCING PHASE			
Genetic variation in 14 genes		CGA dataset (n = 48)	Sequencing, read mapping (hg19), variant annotation, QC + filtering → 2423 variants
2. BURDEN ANALYSIS PHASE			
Are there genetic variants enriched in high IgE responders to Ascaris and ABA-1?		CGA dataset (n = 48) HR = 28 (>75th) LR = 20 (< 25th)	*Ingenuity variant analysis (IVA)* Confidence filter → 1955 variants Predicted deletereous filter → 338 variants Genetic and statistical associations in IVA SKAT-O model for binary (HR vs. LR) and quantitative trait (IgE level to Ascaris and ABA-1)
3. ASSOCIATION STUDY PHASE			
Are there variants associated with IgE response to Ascaris/ABA-1? and Are they related with the IgE response to common allergens and total IgE levels?	IgE response to Ascaris, ABA-1 and HDM	CGA dataset (n=988)	*Logistic regression** Association with IgE levels (above and below percentile 75th) *Quantile regression** Association with IgE levels as a continuous variable *Mann Whitney and Kruskall Wallis tests*
	IgE response to common allergens in AE patients	Swedish AE dataset (n=170)	
	total IgE in allergic patients**	CGA dataset (n = 391) Swedish AE dataset (n = 170)	

S2 Table. Descriptive of sequencing metrics by gene region in the CGA cohort

Chrom	Gene	# Reads	% Aligned	% duplicated	Insert size	%Reads on target	Mean coverage	% 10X cov	% 30X cov	Total variations	In dbSNP	Ts/Tv all	Ts/Tv (dbSNP)	Ts/Tv (novel)
1	*CHIA*	421858	41.80	32.06	256	89.9	736	100	99.5	145	138	2.54	2.53	3
1	*IL10*	308778	33.01	31.66	256	89.0	906	100	99.7	27	23	1.27	1.33	1
1	*FCER1A*	449056	43.20	32.00	259	83.2	636	100	98.5	75	69	1.80	1.78	2
1	*CHI3L1*	430195	38.73	31.35	258	88.0	871	100	99.3	67	58	2.14	2.05	3
5	*TSLP*	316097	36	31.4	258	89.3	687	100	98.1	42	32	1.16	1.29	0.8
5	*IL13*	279437	30.6	31.2	259	90.1	719	99	96.5	29	24	2.00	2.29	1
5	*IL4*	334026	35	31.7	257	86.9	811	99	98.7	46	42	2.15	1.92	3
5	*IL5*	272047	32.6	31.2	259	78.4	639	100	98.1	53	44	2.00	1.92	2.5
5	*IL33*	824546	56.6	31.9	254	85.1	626	99.8	97.8	219	192	1.41	1.41	1.5
9	*STAT6*	583424	44.2	31.6	257	89.2	787	100	98.6	62	56	4.18	4.89	1
12	*LIG4*	351454	38.4	31.6	256	91.2	607	98	95.6	101	76	1.87	1.95	1.63
13	*ABHD13*	390925	42.0	31.8	258	88.4	558	99.4	95.3	71	60	1.68	1.55	3
13	*TNFSF13B*	709635	55	31.9	256	82.8	547	99.7	96.1	205	172	1.68	1.68	1.67
13	*IRS2*	918602	57.4	31.6	259	91.7	628	98.5	94.8	204	181	2.07	2.24	1.11

Here is shown % for coverage 10X and 30X. Ts/Tv = Transition transversion for all and separating by included in dbSNP. Ts/Tv (novel) = not included in dbSNP

Assay_ID	Alleles	SuccessRate% (CGA)	SuccessRate% (MALF)	HWE_Exact_p-value_control (CGA)	HWE_Exact_p-value_control (MALF)	MAF (CGA)	MAF (MALF)
c13LIG4_21	C/A	0	0	NA	NA	0	0
c1CHIA_23	T/C	0	0	NA	NA	0	0
rs112583572	A/T	0	0	NA	NA	0	0
rs13983	T/C	0	98,3	NA	monomorphic	0	0
rs145021969	C/T	0	0	NA	NA	0	0
rs146319305	T/C	0	99,4	NA	monomorphic	0	1
rs147640309	T/C	99,7	99,4	monomorphic	monomorphic	0	0
rs3024496	G/A	0	0	NA	NA	0	0
rs35927012	T/C	0	0	NA	NA	0	0
rs76446715	C/G	0	98,9	NA	monomorphic	0	0
rs76724577	G/A	0	0	NA	NA	0	0
rs111396343	G/T	98,2	99,7	monomorphic	monomorphic	0,001	0
c1CHI3L1_59	C/T	99,6	99,4	1	monomorphic	0,002	0
c5TSLP_60	C/T	99,7	99,7	1	monomorphic	0,002	0
rs146010120	C/T	98,0	99,7	1	1,000	0,002	0,006
c12STAT6_69	G/A	99,7	99,7	1	monomorphic	0,003	0
c5TSLP_18	G/C	99,6	99,4	1	monomorphic	0,003	0
c5TSLP_83	T/C	98,2	99,7	1	monomorphic	0,003	0
c5TSLP_10	C/G	99,7	99,7	1	monomorphic	0,004	0
rs187770643	C/T	99,5	99,4	1	monomorphic	0,004	0
rs117903488	T/C	99,4	100	1	monomorphic	0,005	0,004
rs56154409	C/T	97,8	99,7	1	monomorphic	0,005	0
c9IL33_54	A/G	99,4	100	1	monomorphic	0,006	0
rs144519557	T/C	97,9	99,7	1	monomorphic	0,006	0
rs145443280	T/C	99,7	99,4	1	1,000	0,006	0,002
rs147988352	G/A	99,4	100	1	monomorphic	0,006	1
rs180851865	C/T	99,6	99,4	1	monomorphic	0,006	0
rs2243249	T/C	99,1	99,4	1	monomorphic	0,006	0
rs73606275	A/T	99,6	99,7	1	monomorphic	0,006	0
rs77666548	T/C	99,6	99,4	1	monomorphic	0,006	0
c13ABHD13	G/C	47,6	20,8	1	monomorphic	0,007	0
rs116764155	G/A	97,9	99,2	1	monomorphic	0,007	0
rs11466748	G/C	99,3	100	1	monomorphic	0,008	0
rs182211245	T/C	98,1	99,7	1	monomorphic	0,008	0
rs11466742	G/C	99,3	100	1	1,000	0,011	0,028
rs73118440	G/T	99,4	99,2	1	1,000	0,011	0,048
rs114688573	G/A	99,6	99,7	1	monomorphic	0,012	0
rs139812869	G/A	99,3	100	1	monomorphic	0,012	0
rs79500525	A/G	99,2	100	1	monomorphic	0,012	0
rs78060317	G/A	98,7	98,3	1	monomorphic	0,013	0
rs79660931	G/A	98,0	99,7	1	monomorphic	0,015	0,002
rs73396600	G/C	97,2	99,7	1	monomorphic	0,017	0
rs36219969	T/A	99,6	99,7	1	monomorphic	0,018	0

rs77007163	G/C	99,4	100	1	monomorphic	0,018	0,002
rs11466737	C/G	98,9	99,4	1	monomorphic	0,019	0
rs11466738	G/C	98,1	99,7	1	monomorphic	0,02	0,002
rs61752461	C/T	99,3	99,7	1	1,000	0,021	0,002
rs2069742	G/A	99,2	99,4	1	monomorphic	0,024	0
rs928550	G/C	97,7	99,7	1	monomorphic	0,027	0
rs11466743	G/A	99,7	99,4	0,875	monomorphic	0,028	0
rs3093767	T/C	97,7	99,7	0,749	monomorphic	0,029	0
rs6697497	T/C	98,1	98,6	1	monomorphic	0,029	0
rs74399908	T/C	99,2	99,2	1	1,000	0,029	0,041
rs9670655	G/T	99,6	99,4	1	1,000	0,029	0,006
rs2069816	C/A	99,6	99,4	0,876	1,000	0,033	0,006
rs12584136	C/A	99,0	100	0,155	0,952	0,04	0,089
rs2071580	C/T	97,2	99,7	1	1,000	0,042	0,017
rs2069817	C/T	99,1	100	1	monomorphic	0,045	0
rs2232642	C/T	98,8	100	1,000	monomorphic	0,053	0
rs3783118	C/A	99,1	100	0,730	1,000	0,058	0,046
rs17499386	C/T	98,9	100	0,829	0,113	0,059	0,141
rs55726619	T/C	95,4	99,7	1	1,000	0,059	0,037
rs3024950	G/A	99,4	99,4	0,986	monomorphic	0,069	0
rs11466744	G/T	99,5	99,4	0,813	monomorphic	0,072	0
rs1059513	T/C	98,9	100	0,463	1,000	0,078	0,106
rs3093740	G/T	99,2	99,2	0,676	monomorphic	0,08	0,004
rs2069750	C/G	96,9	98,1	0,208	monomorphic	0,085	0,002
rs3818822	A/G	99,0	99,7	0,785	0,946	0,092	0,117
rs76754615	A/T	98,8	100	1,000	0,296	0,093	0,141
rs17564816	G/A	98	99,2	0,286	1,000	0,096	0,187
rs2099435	C/T	98,9	100	0,452	0,930	0,101	0,137
rs16841997	C/T	99,2	99,4	0,044	0,176	0,102	0,097
rs2251746	C/T	97,5	99,7	0,939	1,000	0,107	0,257
rs7320328	C/A	96,8	99,7	0,879	monomorphic	0,116	0,002
rs9520829	C/G	98,1	99,2	0,541	0,698	0,118	0,33
rs16972207	C/G	98,5	100	0,297	1,000	0,12	0,189
rs10494133	C/T	99,0	99,7	1	0,504	0,137	0,126
rs3759465	T/C	98,5	100	0,353	0,030	0,141	0,006
rs16972197	G/C	98,1	100	0,482	0,030	0,144	0,006
rs3858811	G/C	98,1	100	0,075	1,000	0,144	0,231
rs16972194	G/A	98	99,7	0,816	0,030	0,15	0,006
rs11102248	C/T	97,7	99,7	0,672	0,498	0,155	0,298
rs1224141	G/T	98,6	99,4	1,000	0,560	0,156	0,257
rs1224145	C/G	98,6	99,4	0,408	0,833	0,157	0,266
rs1224149	G/T	98,6	100	0,484	0,431	0,161	0,298
rs17565502	C/A	98	97,2	0,941	1,000	0,182	0,355
rs9514828	T/C	98,1	100	0,959	0,554	0,199	0,465

rs11619378	G/T	98,8	100	0,884	0,296	0,204	0,141
rs4950928	C/G	97,7	99,7	0,344	1,000	0,243	0,222
rs1295687	C/G	95,6	99,4	0,363	1,000	0,262	0,071
rs4771646	T/C	97,0	99,7	0,793	0,974	0,266	0,306
rs880633	C/T	98,9	99,7	0,065	0,834	0,271	0,446
rs11069727	A/G	98,1	100	1,000	0,152	0,298	0,235
rs4771648	A/G	96,1	98,9	0,039	0,439	0,304	0,358
rs20541	G/A	97,2	99,7	0,195	0,948	0,318	0,256
rs2243267	G/C	97,0	99,7	0,769	0,028	0,347	0,231
rs8181791	A/G	98,6	100	0,844	0,677	0,363	0,343
rs1518111	C/T	99,2	99,4	0,365	0,213	0,394	0,214
rs2582869	G/A	97,5	100	0,607	0,853	0,444	0,404
rs2275353	G/A	97,5	99,7	0,217	0,795	0,472	0,117
rs190777329	G/T	98,1	99,7	monomorphic	monomorphic	1	0

S1 Table. List of genotyped variants for genetic association study in CGA and the Swedish Eczema Cohort

Assay_ID	Gene	Alleles	SuccessRate% (CGA)	SuccessRate% (Swedish AE study)	HWE_Exact_p-value_control (CGA)	HWE_Exact_p-value_control (Swedish AE study)	MAF (CGA)	MAF (Swedish AE study)
rs147640309	LIG4	T/C	99,7	99,4	monomorphic	monomorphic	0	0
rs111396343	CHI3L1	G/T	98,2	99,7	monomorphic	monomorphic	0,001	0
c1CHI3L1_59	CHI3L1	C/T	99,6	99,4	1	monomorphic	0,002	0
c5TSLP_60	TSLP	C/T	99,7	99,7	1	monomorphic	0,002	0
rs146010120	CHI3L1	C/T	98,0	99,7	1	1,000	0,002	0,006
c12STAT6_69	STAT6	G/A	99,7	99,7	1	monomorphic	0,003	0
c5TSLP_18	TSLP	G/C	99,6	99,4	1	monomorphic	0,003	0
c5TSLP_83	TSLP	T/C	98,2	99,7	1	monomorphic	0,003	0
c5TSLP_10	TSLP	C/G	99,7	99,7	1	monomorphic	0,004	0
rs187770643	LIG4	C/T	99,5	99,4	1	monomorphic	0,004	0
rs117903488	STAT6	T/C	99,4	100	1	monomorphic	0,005	0,004
rs56154409	IL4	C/T	97,8	99,7	1	monomorphic	0,005	0
c9IL33_54	IL33	A/G	99,4	100	1	monomorphic	0,006	0
rs144519557	IL33	T/C	97,9	99,7	1	monomorphic	0,006	0
rs145443280	FCER1A	T/C	99,7	99,4	1	1,000	0,006	0,002
rs147988352	IL33	G/A	99,4	100	1	monomorphic	0,006	1
rs180851865	TNFSF13B	C/T	99,6	99,4	1	monomorphic	0,006	0
rs2243249	IL4	T/C	99,1	99,4	1	monomorphic	0,006	0
rs73606275	IRS2	A/T	99,6	99,7	1	monomorphic	0,006	0
rs77666548	LIG4	T/C	99,6	99,4	1	monomorphic	0,006	0
rs116764155	ABHD13	G/A	97,9	99,2	1	monomorphic	0,007	0
rs11466748	TSLP	G/C	99,3	100	1	monomorphic	0,008	0
rs182211245	IL33	T/C	98,1	99,7	1	monomorphic	0,008	0
rs11466742	TSLP	G/C	99,3	100	1	1,000	0,011	0,028
rs73118440	STAT6	G/T	99,4	99,2	1	1,000	0,011	0,048
rs114688573	TNFSF13B	G/A	99,6	99,7	1	monomorphic	0,012	0
rs139812869	CHIA	G/A	99,3	100	1	monomorphic	0,012	0
rs79500525	CHIA	A/G	99,2	100	1	monomorphic	0,012	0
rs78060317	TNFSF13B	G/A	98,7	98,3	1	monomorphic	0,013	0
rs79660931	IL33	G/A	98,0	99,7	1	monomorphic	0,015	0,002
rs73396600	IL33	G/C	97,2	99,7	1	monomorphic	0,017	0
rs36219969	LIG4	T/A	99,6	99,7	1	monomorphic	0,018	0
rs77007163	IL33	G/C	99,4	100	1	monomorphic	0,018	0,002
rs11466737	TSLP	C/G	98,9	99,4	1	monomorphic	0,019	0
rs11466738	TSLP	G/C	98,1	99,7	1	monomorphic	0,02	0,002
rs61752461	CHIA	C/T	99,3	99,7	1	1,000	0,021	0,002
rs2069742	IL13	G/A	99,2	99,4	1	monomorphic	0,024	0
rs928550	ABHD13	G/C	97,7	99,7	1	monomorphic	0,027	0
rs11466743	TSLP	G/A	99,7	99,4	0,875	monomorphic	0,028	0
rs3093767	LIG4	T/C	97,7	99,7	0,749	monomorphic	0,029	0
rs6697497	IL10	T/C	98,1	98,6	1	monomorphic	0,029	0
rs74399908	TNFSF13B	T/C	99,2	99,2	1	1,000	0,029	0,041
rs9670655	ABHD13	G/T	99,6	99,4	1	1,000	0,029	0,006
rs2069816	IL5	C/A	99,6	99,4	0,876	1,000	0,033	0,006
rs12584136	IRS2	C/A	99,0	100	0,155	0,952	0,04	0,089
rs2071580	CHI3L1	C/T	97,2	99,7	1	1,000	0,042	0,017
rs2069817	IL5	C/T	99,1	100	1	monomorphic	0,045	0
rs2232642	LIG4	C/T	98,8	100	1,000	monomorphic	0,053	0
rs3783118	ABHD13	C/A	99,1	100	0,730	1,000	0,058	0,046
rs17499386	TNFSF13B	C/T	98,9	100	0,829	0,113	0,059	0,141
rs55726619	IL33	T/C	95,4	99,7	1	1,000	0,059	0,037
rs3024950	STAT6	G/A	99,4	99,4	0,986	monomorphic	0,069	0
rs11466744	TSLP	G/T	99,5	99,4	0,813	monomorphic	0,072	0
rs1059513	STAT6	T/C	98,9	100	0,463	1,000	0,078	0,106
rs3093740	LIG4	G/T	99,2	99,2	0,676	monomorphic	0,08	0,004
rs2069750	IL13	C/G	96,9	98,1	0,208	monomorphic	0,085	0,002
rs3818822	CHIA	A/G	99,0	99,7	0,785	0,946	0,092	0,117
rs76754615	TNFSF13B	A/T	98,8	100	1,000	0,296	0,093	0,141
rs17564816	TNFSF13B	G/A	98	99,2	0,286	1,000	0,096	0,187
rs2099435	IRS2	C/T	98,9	100	0,452	0,930	0,101	0,137

rs16841997*	FCER1A	C/T	99,2	99,4	0,044	0,176	0,102	0,097
rs2251746	FCER1A	C/T	97,5	99,7	0,939	1,000	0,107	0,257
rs7320328	IRS2	C/A	96,8	99,7	0,879	monomorphic	0,116	0,002
rs9520829	TNFSF13B	C/G	98,1	99,2	0,541	0,698	0,118	0,33
rs16972207	TNFSF13B	C/G	98,5	100	0,297	1,000	0,12	0,189
rs10494133	CHIA	C/T	99,0	99,7	1	0,504	0,137	0,126
rs3759465	TNFSF13B	T/C	98,5	100	0,353	0,030	0,141	0,006
rs16972197	TNFSF13B	G/C	98,1	100	0,482	0,030	0,144	0,006
rs3858811	TNFSF13B	G/C	98,1	100	0,075	1,000	0,144	0,231
rs16972194	TNFSF13B	G/A	98	99,7	0,816	0,030	0,15	0,006
rs11102248	CHIA	C/T	97,7	99,7	0,672	0,498	0,155	0,298
rs1224141	TNFSF13B	G/T	98,6	99,4	1,000	0,560	0,156	0,257
rs1224145	TNFSF13B	C/G	98,6	99,4	0,408	0,833	0,157	0,266
rs1224149	TNFSF13B	G/T	98,6	100	0,484	0,431	0,161	0,298
rs17565502	TNFSF13B	C/A	98	97,2	0,941	1,000	0,182	0,355
rs9514828	TNFSF13B	T/C	98,1	100	0,959	0,554	0,199	0,465
rs11619378	ABHD13	G/T	98,8	100	0,884	0,296	0,204	0,141
rs4950928	CHI3L1	C/G	97,7	99,7	0,344	1,000	0,243	0,222
rs1295687	IL13	C/G	95,6	99,4	0,363	1,000	0,262	0,071
rs4771646	IRS2	T/C	97,0	99,7	0,793	0,974	0,266	0,306
rs880633	CHI3L1	C/T	98,9	99,7	0,065	0,834	0,271	0,446
rs11069727	TNFSF13B	A/G	98,1	100	1,000	0,152	0,298	0,235
rs4771648*	IRS2	A/G	96,1	98,9	0,039	0,439	0,304	0,358
rs20541	IL13	G/A	97,2	99,7	0,195	0,948	0,318	0,256
rs2243267	IL4	G/C	97,0	99,7	0,769	0,028	0,347	0,231
rs8181791	TNFSF13B	A/G	98,6	100	0,844	0,677	0,363	0,343
rs1518111	IL10	C/T	99,2	99,4	0,365	0,213	0,394	0,214
rs2582869	TNFSF13B	G/A	97,5	100	0,607	0,853	0,444	0,404
rs2275353	CHI3L1	G/A	97,5	99,7	0,217	0,795	0,472	0,117
rs190777329	IL33	G/T	98,1	99,7	monomorphic	monomorphic	1	0