

DISSERTATION

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"Identification of novel biomarkers for severe allergic asthma"

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SUMMARY

Asthma is a chronic inflammatory pulmonary disease characterized by recurrent episodes of wheezy, laboured breathing that affects approximately 300 million people worldwide and can be fatal. Current therapies (especially inhaled corticosteroids and ß2-agonists) efficiently control allergic asthma in most of the patients. Development of novel therapies is crucial, however, for disease control in 5-10% of patients with very severe forms of disease that cannot be controlled even with high doses of oral steroids. Additionally, there is still need to improve side effect profile of currently available drugs, especially as they do not cure the disease and have to be continuously administered for a lifetime of a patient.

To guide research of new treatments for asthma, it is crucial to identify and develop novel biomarkers of the disease. Moreover, clinically relevant biomarkers are crucial for understanding of asthma heterogeneity, as the disease differs in severity, natural history, comorbidities, and treatment response between patients. Development of biomarkers for distinct asthma subtypes could ultimately guide research towards development of personalized asthma medications.

The main aim of this work was to detect genomic biomarkers, to develop novel optical imaging techniques and to establish novel treatment modalities for allergic asthma. Therefore, we used 3 novel approaches to (i) provide comprehensive gene expression profile in mice with acute allergic asthma; (ii) visualize allergic inflammation in an experimental mouse model of allergic asthma; and (iii) to treat allergic asthma relapse in mice with a selective muscarinic receptor antagonist drug tiotropium bromide.

To provide a comprehensive gene expression profile in mice with acute allergic asthma, in the manuscript we analyzed 6 publically available expression datasets using a novel and innovative gene- and pathway-driven bioinformatics strategy. We generated and validated an acute asthma signature gene list consisting of asthma-annotated genes as well as of asthma-unrelated genes that constitute an "acute allergic asthma ignorome" in mice. Further, functional and network connectivity analyses indicated that asthma-annotated and -ignorome genes cluster into 4 biologically relevant domains. Finally, within our network we discovered 12 super-connectors genes that link the complex pathophysiological mechanisms underlying asthma and are normalized upon treatment with glucocorticosteroids. Thus, our powerful bioinformatic analyses prioritized asthma-related and -ignorome genes within the clusters and biological domains to provide important insights in asthma pathophysiology.

In publication 1, we developed a novel imaging approach for detection of pulmonary inflammation. Dendritic polyglycerol sulfates selectively bind P- and Lselectins and C3 and C5 complement factors that are upregulated at the site of inflammation. Using fluorescence imaging, we detected specific accumulation of near infrared fluorescence dye labeled dendritic polyglycerol sulfates (dPGS-NIRF) in the lungs of mice with acute allergic asthma. Further, our results show that dPGS-NIRF had a faster uptake compared to other commercially available imaging probes. Altogether, these results suggest that dPGS-NIRF is a potent probe for *in vivo* monitoring of pulmonary inflammation.

Finally, in publication 2, we tested efficacy of selective long-acting muscarinic receptor antagonist tiotropium bromide in acute onset and relapsing mouse models of allergic asthma. Tiotropium bromide is important for the treatment of chronic obstructive pulmonary disease. However, it is not commonly used to treat asthma, even though the mode of action suggests that they may also be effective. Our results demonstrate that tiotropium efficiently suppressed airway hyperreactivity, mucus hypersecretion and airway inflammation in experimental allergic asthma. These data highlighted the anti-inflammatory properties of tiotropium and support its use for the treatment of asthma.

Altogether, the results presented in this thesis provide detailed gene expression profiling in acute allergic asthma, describe a novel imaging technology for visualization of allergic lung inflammation and expand armamentarium of antiasthmatic drugs. While the gene expression data provide multiple novel biomarker and treatment targets, a combination of our imaging approach with the asthma relapse model would provide an elegant experimental setting for their testing.

ZUSAMMENFASUNG

Asthma ist eine chronisch entzündliche Erkrankung der Lunge charakterisiert durch wiederkehrende Episoden pfeifender, schwerfälliger Atmung, von der weltweit etwa 300 Millionen Menschen betroffen sind und tödlich enden kann. Allergisches Asthma kann durch gängige Therapien, vor allem mit inhalativen Kortikosteroiden und ß2-Agonisten, bei den meisten Patienten effizient behandelt werden. Allerdings ist die Entwicklung neuer Therapien für 5-10% der Patienten dringend erforderlich, da diese an sehr schweren Asthma-Formen leiden, welche nicht einmal auf hohe Dosen oral verabreichter Kortikosteroide ansprechen. Außerdem ist es notwendig das Nebenwirkungsprofil erhältlicher Medikamente zu verbessern, da diese nicht zur Heilung führen, sondern lediglich die Symptome lindern und ein Leben lang eingenommen werden müssen.

Zur Erforschung neuer Therapieansätze ist die Identifizierung und Entwicklung neuartiger Asthma-Biomarker essentiell. Zudem sind klinisch relevante Biomarker notwendig, um die Heterogenität des Asthmas zu verstehen, da es sich in Entstehung, Stärke, Verlauf, Komorbiditäten und im Ansprechen auf Therapien je nach PatientIn unterscheidet. Die Entwicklung von Biomarkern für bestimmte Asthma-Subtypen könnte letztendlich zur Etablierung personalisierter Asthma Medikamente führen.

Das Ziel dieser Arbeit war es, genomische Biomarker zu finden, neue Bildgebungstechniken zu entwickeln und neue Therapien für allergisches Asthma zu etablieren. Dafür verwendeten wir 3 neue Ansätze, um (i) ein umfassendes Genexpressions-Profil in Mäusen mit allergischem Asthma zu erstellen; (ii) entzündliches Geschehen in einem experimentellen Maus-Modell für allergisches Asthma zu visualisieren; und um (iii) allergische Asthma-Rückfälle der Mäuse mit dem selektiven Muskarinrezeptor-Antagonisten Tiotropiumbromid zu behandeln.

INTRODUCTION

Zur Erstellung eines umfassenden Genexpressions-Profils in Mäusen mit allergischem Asthma, analysierten wir 6 öffentlich zugängliche Expressions-Datensätze mit einer neuartigen und innovativen Gen- und Signalwegorientierten Bioinformatik-Strategie. Wir erstellten und validierten eine Signatur-Gen Liste für akutes Asthma in Mäusen, bestehend aus Genen, welche dem Asthma zugeordnet sind und Genen, die bisher nicht mit Asthma in Zusammenhang gebracht wurden und als akutes, allergisches "Asthma Ignorome" bezeichnet werden. Funktionelle und Netzwerkkonnektivitäts-Analysen zeigten, dass sich bekannte Asthma Gene und "Asthma Ignorome" Gene in 4 biologisch relevante Domänen gruppieren lassen. Des Weiteren entdeckten wir 12 Super-Konnektor Gene des Asthmas, welche komplexe pathophysiologische Mechanismen verbinden, die mit Glukokortikoid-Behandlung normalisiert werden können. Unsere leistungsstarken bioinformatischen Analysen priorisierten Asthma bezogene und "Asthma Ignorome" Gene in den Clustern und biologischen Domänen, um wichtige Einblicke in die Asthma-Pathophysiologie zu erlangen.

In der ersten Publikation entwickelten wir eine neuartige Bildgebungstechnik, um pulmonale Entzündungen zu detektieren. Dendritische Polyglycerolsulfate binden selektiv P- und L-Selektine und die Komplementfaktoren C3 und C5, welche an Entzündungsstellen hochreguliert sind. Durch Fluoreszenzbildgebung entdeckten wir spezifische Akkumulationen von infrarotnah-fluoreszenz markierten dendritischen Polyglycerolsulfaten (dPGS-NIRF) in den Lungen von Mäusen mit allergischem Asthma. Des Weiteren zeigten unsere Resultate, dass dPGS-NIRF eine schnellere Absorption aufwiesen als andere kommerziell erhältliche Bildgebungs-Sonden. Zusammenfassend konnten wir demonstrieren, dass dPGS-NIRF eine potente Sonde für die *in vivo* Darstellung von Entzündungen ist.

In der zweiten Publikation untersuchten wir die Wirksamkeit des selektiven, lang-wirksamen Muskarinrezeptor-Antagonisten Tiotropiumbromid im akuten und rezidivierenden Maus-Modell für allergische Asthma. Tiotropiumbromid wird zur Behandlung der chronisch obstruktiven Lungenerkrankung eingesetzt, aber üblicherweise nicht in der Asthmatherapie verwendet, obwohl des-

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sen Wirkmechanismus gute Wirksamkeit vermuten lassen. Unsere Resultate zeigen, dass Tiotropiumbromid bei experimentellem allergischem Asthma die Entzündung, Hyperreaktivität und Schleimüberproduktion der Atemwege effizient unterdrücken konnte. Diese Daten demonstrierten die entzündungshemmenden Eigenschaften von Tiotropiumbromid und bekräftigen dessen Einsatz in der Asthmatherapie.

Zusammenfassend zeigen die Resultate dieser Arbeit detaillierte Genexpressions-Profile in akut allergischem Asthma, beschreiben eine neuartige Bildgebungstechnologie zur Visualisierung von allergischen Entzündungen der Lunge und erweitern das Arsenal an anti-asthmatischen Arzneistoffen. Die Genexpressionsdaten liefern zahlreiche neuartige Biomarker und Therapieansätze, während unsere Bildgebungstechnik in Kombination mit dem rezidivierenden Asthma-Modell einen eleganten Versuchsrahmen, bietet diese Biomarker zu testen.

1. INTRODUCTION

1.1. Asthma: definition, epidemiology, etiology, pathogenesis and phenotypes

The first clinical description of asthma (coming from old greek verb αάζειν (aazein), meaning to exhale with open mouth, to pant) is ascribed to Aretaeus of Cappadocia in the first century AD (1). From then on, the definition of asthma has changed to include a heterogenous group of conditions that result in recurrent and reversible airway obstruction (2,3). According to the Global Strategy for Asthma Management and Prevention Report, *"Asthma is a chron-ic inflammatory disorder of the airways in which many cells and cellular ele-ments play a role. The chronic inflammation is associated with airway hyper-responsiveness that leads to recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning.* These episodes are usually associated with widespread, but variable, airflow obstruction within the lung that is often reversible either spontaneously or with treatment." (4).

Asthma is a common disease that affects ~300 million individuals and causes ~250, d000 deaths per year worldwide (2,4). Asthma prevalence increased substantially during second half of 20^{th} century (5). Thereafter, it seems to have plateaued in high income counties, but still increases in many low and middle income countries (4). In Austria, the prevalence of clinical asthma is ~5% (6), and it increased from 1996 to 2002 for 16% in children aged 6-7 years and for 32% in children aged 12-14 years (7).

Multiple genetic and environmental factors are involved in asthma. Twin studies indicate that genetic effects explain 35 to 80% of the variation in the risk for the disease, while the remaining variation is due to non-shared environmental effects (8,9). Numerous genome-wide linkage, candidate gene and genome-wide association studies indicate that it is a polygenic disease with an increasingly large list of genes involved in different biological mechanisms (2,9-11). March et al. (11) categorized asthma-related genes into broad functional groups that include:

- T helper cell type 2 (Th2)-mediated cell response (including transcription factor GATA3, cytokines IL4 and IL13, signaling mediator STAT6 and the beta-chain of the IgE receptor FCERI);
- inflammation (such as cytokine *IL18* and its receptor *IL18R1*);
- environmental sensing and immune detection (genes involved in antigen recognition like Toll-like receptor (TLR) *TLR2, TLR4* and human leukocyte antigen HLA class II molecule);
- airway remodeling (i.e. a disintegrin and metalloproteinase domaincontaining protein 33 (*ADAM33*) that is expressed on smooth muscle cells and lung fibroblasts);
- bronchoconstriction (such as a receptor for acetylcholine CHRNA3/5 and nitric oxide synthase NOS1); and
- epithelial barrier function (including filaggrin (*FLG*), anti-microbial peptide defensin-beta1 (*DEFB1*) and an inhibitor of dendritic cell-mediated Th2-cell differentiation Clara cell 16-kD protein (*CC16*).

In individuals with genetic susceptibility to asthma, environmental factors can either elicit asthma or have protective effects. Factors that contribute to asthma development or can trigger asthma attack include biologic aeroallergens (house dust mites, pollen, animal dander, molds, etc.), air pollutants from tobacco smoke or combustion devices, irritant chemicals and fumes, viruses (especially rhinoviruses), as well as extreme weather conditions and changing climate (2). On the other hand, living on a farm or exposure to day care early in life is associated with decrease risk of asthma later in life (3).

Complexity of the clinical and pathophysiological characteristics that belong to the term "asthma" has recently prompted a concept that asthma consists of multiple phenotypes (3,12-14). The term 'phenotype' is defined as the "observable properties of an organism that are produced by the interactions of the genotype and the environment" (12). According to clinical characteristics, triggers and general inflammatory processes several asthma phenotypes have been proposed (3,12) and there are attempts to describe specific pathogenic mechanisms that might underlie a certain phenotype (13,14). An integrated view of major asthma phenotypes with relevant pathological mechanisms is presented in Table 1.

Table 1. An integrated list of major clinical and molecular asthma pheno-types. Source: Martinez and Vercelli, 2013, (3).

	Natural history	Clinical and physiological features	Pathobiology and biomarkers	Response to therapy				
Th2-high phenotype								
Early-onset allergic	Early onset, mild to severe	Allergic symptoms and other diseases	Thick subepithelial basement membrane, specific IgE, Th2 cytokines	Corticosteroid-responsive Th2-targeted				
Late-onset eosinophilic	Adult onset, often severe	Sinusitis, less allergic	Corticosteroid- refractory, eosinophilia, interleukin 5	Responsive to antibody to interleukin 5 and cysteiny leukotriene modifiers, corticosteroid-refractory				
Th2-low phenotype								
Obesity- related	Adolescent and adult onset	Women mainly affected, very symptomatic, airway hyper-responsiveness less clear	Lack of Th2 biomarkers, oxidative stress	Responsive to weight loss antioxidants, and possibly to hormonal therapy				
Neutrophilic	Adult onset	Low FEV1, more air trapping	Sputum neutrophilia, Th17 pathways, interleukin 8	Possibly responsive to macrolide antibiotics				
Th2=T-helper-type-2 cytokine. FEV1=forced expiratory volume in 1 s.								

In addition to phenotypes, recent progress in methods for molecular phenotying grouped asthma variants into 'endotypes', or "*disease subtypes based on cellular and molecular mechanisms*" (14,15). Asthma endotypes, thus, represent distinct disease entities with consistent pathophysiological mechanisms related to the divergent clinical presentation, epidemiology, and response to different treatments (16). Several studies used gene expression (17,18) and proteomics (19) data to define divergent asthma endotypes. However, there are still no generally accepted criteria that for endotype definition or use in clinics (14,16).

1.2. Allergic asthma pathophysiology

Allergic asthma is an asthma phenotype strongly linked to atopy (a hereditary bias toward development of allergic reactions) and allergy (a type I hypersensitivity reaction to a substance that is present in amounts that do not affect most people). Atopy is a major known predisposing factor for asthma (20), and allergy is involved in ~80% of childhood asthma and in 40-50% of adult asthma (21). Clinically, it can be further subdivided into early-onset (usually during childhood) and late-onset (after 20 years of age) asthma phenotype (22).

Defining features of allergic asthma include an underlying T-helper-type-2 (Th2)-cell- and an IgE-mediated immune response against allergen, a type of antigen that is harmless to the body, but induces an abnormally vigorous immune response. The initial step is allergen sensitization that involves a complex interplay of environmental factors and host immune response ultimately leading to the differentiation of allergen specific Th2 cells (23). Airway epithelium, apart from serving as a physical barrier, is actively involved in the response to environmental insults (e.g., aeorallergens that have proteolytic properties (23)) by secreting inflammatory mediators and antimicrobial peptides, including interleukin (IL)-25, IL-33 and thymic stromal lymphopoientin (TSLP) (24-26). IL-25 and IL-33 activate type 2 innate lymphoid cells (ILC2) to secrete IL-5 and IL-13 that can directly act on lung structural cells (to induce airway hyperresponsiveness (AHR) and mucus oversecretion), as well as on immune system (recruiting eosinophils or inducing alternative activation of macrophages) (27). TSLP, in contrast, induces OX40 ligand expression on dendritic cells, which then prime naïve T cells in secondary lymphatic organs to differentiate into the Th2 cell subtype (28). Th2 cells then secrete IL-4 and IL-13 and induce B-cell isotype switching leading to the generation of allergen-specific IgE and IgG1 (in mice) or IgG_4 (in humans) (29-31). While memory T and B cells usually provide long-term antigen-specific protective immunity to previously encountered pathogens, immunological Th2 memory plays a crucial role in pathophysiology of allergic asthma. Numerous data from animal models and clinical studies support the central role of allergenspecific memory Th2 cells (32-35) and IgE-producing B cells (36,37) in the initiation and maintenance of the key pathophysiological features of the disease.

Re-exposure to the allergen induces, in sensitized individuals, a characteristic biphasic response that includes innate (early-phase) and adaptive (latephase) mechanisms. In the early phase, occurring within minutes upon contact, allergen interacts with IgE bound to high-affinity FcERI receptors on basophils and mast cells, causing their crosslinking and resulting in the release of inflammatory mediators, including histamine, leukotirenes, prostaglandins, chemokines and cytokines (38,39). These mediators, in concert, induce airway smooth muscle contraction, mucus secretion and vasodilatation of blood vessels that mediate the initial symptoms of an asthma attack. Furthermore, as the response progresses to late phase, basophil- and mast cell-derived products enable infiltration of other innate and immune cells, including eosinophils, neutrophils, T and B cells (38,39). Mast cells also secrete several mediators crucial for recruitment of memory Th2 cells and eosinophils, including prostaglandin D2 (PGD2) that binds to chemoattractant receptor homologous molecule expressed on Th2 cells (CRTH2) (40,41). The late phase usually starts a few hours after the early phase response and is mediated mainly by Th2 cells and their cytokines (particularly IL-4, IL-5 and IL-13) (38). IL-4 and IL-13 directly promote multiple features of asthma, including goblet cell metaplasia, airway hyperresponsiveness, smooth muscle remodeling and subepithelial fibrosis, while the main function of IL-5 is the stimulation of eosinophil development, survival and activation (42). Eosinophil accumulation in the lungs, mediated by chemokine CCL11 (43), is one of the defining features of allergic asthma. Their function in the pathology of asthma, however, is still not completely elucidated because of conflicting data obtained with transgenic animals and inhibition experiments (reviewed in (44)). It appears that eosinophils contribute, at least to a part, to various aspects of the disease, including Th2 cell differentiation, airway hyperresponsiveness and remodeling (44). Allergic inflammation induced by allergen re-exposure involves many other leukocytes, including B cells, other helper T cell subtypes (Th17, Th9, Th1, regulatory T cells), neutrophils and basophils (reviewed in (39,45)) that are interconnected in a complex network depicted in Figure 1. It is also important to note that during the inflammatory response to an allergen, leukocytes sometimes show overlapping functions and can compensate for each other.

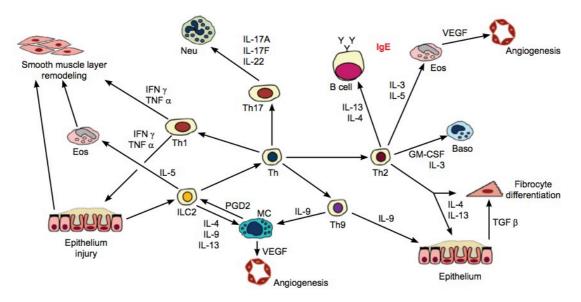


Figure 1. The complexity of inflammatory reactions involved in allergic asthma orchestrated by T helper lymphocytes. Abbreviations: Baso: Basophil, Eos: Eosinophil, GM-CSF: Granulocyte macrophage colonystimulating factor, IFNγ: interferon gamma, IgE: Immunoglobulin E, IL: Interleukin, ILC2: Innate lymphoid cell type 2, MC: Mast cell, Neu: Neutrophil, PGD2: Prostaglandin D2, Th: T helper, TGFβ: Tumor growth factor beta, TNFa: Tumor necrosis factor alpha, VEGF: Vascular endothelial growth factor. Source: Akdis et al 2013 (2).

Without further exposure to allergens, signs of acute disease, such as eosinophilic inflammation, mucus hypersecretion and airway hyperresponsiveness, usually resolve within few weeks (46,47). Mice recovered from acute allergic asthma, however, have inflammatory infiltrates containing memory Th2 cells in the lungs and elevated serum titers of allergen-specific IgG1 and IgE for their lifetime (48). Similarly, lungs of asthmatics contain persistent isolated aggregations of lymphoid cells containing T and B cells (49). Moreover, recent studies suggest that lungs contain a large number of tissue-resident memory T cells (reviewed in (50)). The mechanisms underlying memory CD4⁺ T cell retention in asthmatic lungs are still not fully elucidated and appear to involve CD69 (51) and/or chemokine receptor CCR4 (52).

Allergen re-challenge of recovered mice induces rapid reactivation of resting allergen-specific memory Th2 cells, leading to disease relapse characterized by re-occurrence of airway hyperresponsiveness (AHR), eosinophilic airway and lung inflammation and mucus hypersecretion (48). Clinically, allergic asthmatics have recurrent episodes of asthma attacks associated with allergen exposure (2). Remittent and prolonged exposure to allergens leads to a chronic disease in both experimental animals and humans that is accompanied by healing for which the end result is an altered structure referred to as an airway remodeling (38,53). In addition to other inflammatory features of the disease, changes in the airway wall of asthmatics include epithelial cell shedding, hypertrophy and hyperplasia of airway smooth muscle and increase in mucous glands, causing thickening of airway wall and permanently reducing airway caliber and ultimately leading to permanent decline in lung function (38).

1.3. Treatment of allergic asthma and disease severity

1.3.1. Available treatments for allergic asthma

Allergen avoidance is one of the first described remedies for allergic asthma, dating from the Moses Maimonides in the Middle Ages (54), that remains an important cornerstone in asthma management in modern medicine (2). Besides allergens, asthmatics should also avoid risk factors for allergy and asthma, such as exposure to tobacco smoke, moisture, air pollution and respiratory virus infections (2). Inhalation of allergens or predisposing factors leads to asthma exacerbation, defined as "an acute or subacute episodes of progressively worsening shortness of breath, cough, wheezing, and chest tightness, or some combination of these symptoms, characterized by de-

creases in expiratory airflow and objective measures of lung function (spirometry and peak flow)" (55). If asthma exacerbations occur regularly, unpredictably or are extremely severe, then disease treatment with long-term medications is necessary. The treatment regimes for asthma are internationally agreed upon and guidelines for the management of the disease have been developed (2,4).

The only currently available treatment that can change the course of allergic disease and even lead to immune tolerance to allergens is allergy immunotherapy (56). Controlled administration of allergens in form of subcutaneous immunotherapy (SCIT) and/or sublingual immunotherapy (SLIT) leads to desensitization of mast cells and basophils due to suppression of allergenspecific IgE production by induction of IL-10 producing regulatory T cells (57), tolerance of allergen-specific Th2 cells or induction of Th1 immune response to allergen (58). While allergy immunotherapy is treatment of choice for allergic rhinitis, its widespread use for allergic asthma is currently hindered by lack of efficacy in some patients, high costs and long duration of treatment and possible induction of severe side effects (2,56). Development of novel allergen extracts, adjuvants and treatment regiments for allergy immunotherapy might lead to its more spread use in clinics (56).

Mainstream therapy for allergic asthma includes use of two types of drugs: relievers and controllers (2,4). Relievers are drugs used during acute asthma attack (thus on an as-needed basis) to reverse bronchoconstriction and relieve its accompanying symptoms. The main medication of choice are rapid-acting ß₂-adrenergic receptor agonists, such as salbutamol, terbutalie, fenoterol and reproterol, while alternatives include anticholinergic bronchodilatators, theophylline or systemic glucocorticosteroids (4). In contrast to relievers, controller medications have to be taken daily, long-term (usually lifelong) to keep asthma under control, mainly through their anti-inflammatory effects. The most effective anti-inflammatory controller medication for the treatment of allergic asthma is inhaled glucocorticosteroids (beclomethasone dipropionate, fluticasone propionate, budesonide) that reduce the frequency and severity of exacerbations of asthma, improve lung function and decrease airway hyper-

responsiveness (59-61). Inhaled glucocorticosteroids are usually combined with other medications for full control, including leukotriene modifiers (e.g., cysteinyl-leukotriene 1 (CysLT1) receptor antagonist montelukast or 5-lipoxygenase inhibitor zileuton), long-acting β_2 -adrenergic receptor agonists (e.g., formoterol and salmeterol), theophylline and anti-IgE therapy (e.g., omalizumab) (4). However, all alternative controller treatments have weak anti-inflammatory effects compared to inhaled glucocorticosteroids and they may not be useful as monotherapies (4).

The main goal of asthma treatment is to achieve and maintain symptomatic control of the disease. The Global Initiative for Asthma devised a treatment strategy involving 5 steps, as depicted on Figure 2. Each treatment step provides options for increasing efficacy needed to control the disease in majority of patients (62,63).

Reduce	Treatment Steps			Increase		
Step 1	Step 2	Step 3	Step 4	Step 5		
(If step-up treatment is being		a education. Environmental ntrol, first check inhaler technique		symptoms are due to asthma.		
As needed rapid- acting β_2 -agonist	As needed rapid-acting β_2 -agonist					
	Select one	Select one	To Step 3 treatment, select one or more	To Step 4 treatment, add either		
	Low-dose inhaled ICS*	Low-dose ICS plus long-acting β_2 -agonist	$\begin{array}{c} \text{Medium-or high-dose} \\ \text{ICS plus long-acting} \\ \beta_2\text{-agonist} \end{array}$	Oral glucocorticostero (lowest dose)		
Controller options***	Leukotriene modifier**	Medium-or high-dose ICS	Leukotriene modifier	Anti-IgE treatment		
		Low-dose ICS plus leukotriene modifier	Sustained release theophylline			
		Low-dose ICS plus sustained release theophylline				

* ICS = inhaled glucocorticosteroids **= Receptor antagonist or synthesis inhibitors

*** = Recommended treatment (shaded boxes) based on group mean data. Individual patient needs, preferences, and circumstances (including costs) should be consic

Figure 2. Treatment strategy for allergic asthma. Abbreviations: β_2 -agonist – β_2 -adrenergic receptor agonists, ICS – inhaled corticosteroid. Source: Global Initiative for Asthma 2011 (4).

1.3.2. Disease severity and need for novel therapies

While currently available treatments control symptoms in the majority of asthmatics, 5 to 10% of patients suffer from severe asthma that is associated with poor asthma control, increased risk of frequent severe exacerbations and/or chronic symptoms despite intensive treatment (2). The World Health Organization (WHO) defined severe asthma as: "Uncontrolled asthma which can result in risk of frequent severe exacerbations (or death) and/or adverse reactions to medications and/ or chronic morbidity (including impaired lung function or reduced lung growth in children)." Severe asthma is classified as (i) untreated; (ii) difficult-to-treat; and (iii) treatment-resistant (64). According to the Global Initiative for Asthma, asthma severity is determined on the basis of the treatment intensity needed to control the disease (4). According to this classification, mild asthma may be controlled with low intensity treatment (e.g., low-dose inhaled glucocorticosteroids or leukotriene modifiers), while severe asthma requires a step 4 treatment with oral glucocorticosteroids or the patient remains uncontrolled despite high intensity treatment (4). Importantly, this classification involves both severity of the underlying disease and responsiveness to treatment (65), both of which should be assessed periodically in the patient (66).

Therefore, the development of novel drugs is crucial for disease control in patients with severe, corticosteroid-resistant asthma. Additionally, existing therapies require fewer side effects. Importantly, none of the current asthma controller therapies cure the disease and symptoms usually remerge within few weeks after treatment is terminated (2,67). Furthermore, better understanding of the allergic asthma heterogeneity, consisting of multiple phenotypes and endotypes, will allow optimizing personalized treatment approaches through active case management (68). It is not surprising, thus, that many key molecules and cellular responses defined in basic research are being explored as new targets for testing in the clinics. For example, several novel treatments targeting crucial inflammatory mediators, such as the anti-IL-5 monoclonal antibody, Mepolizuman (69), Dupilumab, the anti-IL-4Ralpha monoclonal antibody that blocks the common receptor for IL-4 and IL-13 (70) and AMG157, anti-TSLP antibody (71), have significantly reduced asthma exacerbations in clinical trials.

1.4. Asthma biomarkers

In medicine, biomarker, or "biological marker", refers to "a broad subcategory of medical signs – that is, objective indications of medical state observed from outside the patient – which can be measured accurately and reproducibly" (72). More precisely, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." (72). A validated biomarker should represent underlying pathological mechanisms of the disease and thus enable diagnosis, indicate treatment responsiveness or allow monitoring of the disease control. The biomarker examples range from blood pressure and pulse, through basic laboratory tests such as leukocyte number in blood to sophisticated laboratory tests, such as measurement of human epidermal growth factor receptor-2 (HER2) expression to predict breast cancer treatment response (73).

Asthma diagnosis and management is based on reported asthma symptoms in combination with lung function tests measuring reversible airway hyperresponsiveness and obstruction. Although these biomarkers provide information on the extent of airflow limitation, they do not monitor the airway and lung inflammation, a central feature of allergic asthma (74-76). Management plan for asthmatic patients should, therefore, include monitoring of airway and/or lung inflammation (74-76).

Bronchoscopy with bronchoalveolar lavage and bronchial biopsy are the gold standards for assessment of extent and type of airway and lung inflammation, respectively (77). However, these invasive techniques are not suitable for routine diagnosis, especially in children (78). Therefore, multiple novel lessinvasive biomarkers are being extensively validated for clinical use in asthma (Figure 3) (68,77,79,80). Among those, sputum induction allows less invasive assessment of airway inflammation and sputum eosinophil counts have been used as biomarkers of airway inflammation and therapy success (81). Despite promising results, the wide use of this biomarker in clinics is disputed due to number of technical limitations and questionable reproducibility (77,79,82).

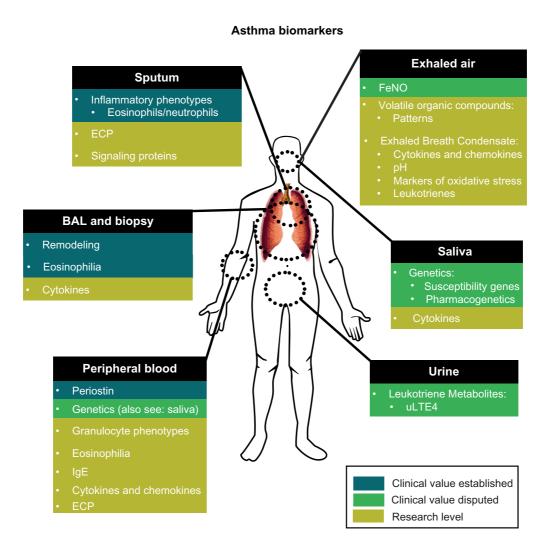


Figure 3. Overview of asthma biomarkers used in clinical practice or currently under research. Abbreviations: BAL, bronchoalveolar lavage; ECP, eosinophil cationic protein; FeNO, fraction of exhaled nitric oxide; IgE, immunoglobulin E; uLTE4, urinary leukotriene E4. Source: Vijverberg et al 2013 (79).

INTRODUCTION

In contrast to sputum collection, more technically feasible biomarker for routine clinical use is measurement of fractional exhaled nitric oxide concentration (FeNO). Initial finding that FeNO is increased in asthmatics compared to healthy controls dates from 1994 (83), and until now its use In asthma management and diagnosis has been extensively studied. However, many factors, such as age, atopy, medication use, therapy adherence or airway infections, influence FeNO levels and preclude its acceptance as a validated biomarker for asthma inflammation (77,79).

Besides sputum induction and FeNO, other asthma biomarkers that are currently being developed include peripheral blood eosinophilia, the measurement of volatile organic compounds in exhaled breath or metabolites in urine and asthma imaging (77,79,80). In addition, unbiased high-throughput screening approaches, including genomics, transcriptomics and proteomics, are powerful techniques that allow biomarker discovery and validation (68,79). Studies using these techniques identified potential endotypes in asthma (18,84), evaluated effects of corticosteroid treatment (85) and identified genes and proteins that could serve as potential novel molecular biomarkers for asthma (17,86,87).

Altogether, combining several biomarkers could provide a more comprehensive approach to monitor the disease than any single biomarker alone (68,79). Recently, George et al. combined clinical biomarker data (including numbers of circulating white blood cells and cardiopulmonary measures) with gene expression analysis from blood to define 3 asthma endotypes (17). In another study, a non-invasive panel of inflammatory biomarkers (*ie*, FeNO, sputum eosinophil count and urinary bromotyrosine level) had better power to predict steroid responsiveness in asthmatics than each of the biomarkers alone (81). Furthermore, transcriptomic approaches provide a great potential for defining divergent asthma phenotypes and endotypes that could ultimately lead to personalized therapy (68,79,88). However, those approaches should be further investigated and eventually incorporated into clinical routine.

1.5. Animal models of allergic asthma

Current understanding of the pathophysiology of allergic asthma and development of novel therapies would be difficult without experimental animal models. There are numerous models of asthma in many different species, including mice, rats, sheep and monkeys (reviewed in (53)). While each animal model has advantages and disadvantages compared to human disease, mice are most widely used, primarily because of the availability of numerous immunological reagents, technologies involving gene manipulation and wellcharacterized inbred strains that allow for the assessment of function of specific factors and cells (53).

There are no standardized experimental protocols for the induction of allergic asthma in mice (39,53,89,90). However, most models use a two step approach that involves initial intraperitoneal sensitization with allergen and the second step is allergen exposure to the respiratory tract with the same allergen administered either in the trachea or nose or in an aerosol. The resulting response depends on the choice of allergen, the inbred mouse strain, the use of adjuvant (e.g., aluminum hydroxide) and the treatment schedule (39,53,89,90). Most models induce elevated levels of allergen-specific IgG1 and IgE, airway and lung allergic inflammation, mucus hypersecretion and airway hyperresponsiveness (53,89-91). These models induce a Th2-mediated late phase response and are successfully used to investigate inflammatory mechanisms and interplay of cells and mediators in asthmatic lungs (53,89,90). However, they do not address all aspects of human disease, including a natural sensitization to airborne allergens that occurs in asthmatics or chronic inflammation of airway wall and airway remodeling (53,89,90).

To mimic exposure of airborne allergens involves airway administration of allergen alone over several days or weeks without adjuvants (53,92). These models also induce acute asthma and allow for the investigation of the mechanisms underlying allergen sensitization via the respiratory mucosa.

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To model structural/remodeling changes in the airways of asthmatics, including subepithelial fibrosis, smooth muscle thickening and increased vascularity, various protocols use an increased number of allergen exposures over many weeks (53,92). In this type of model, inflammation peaks after 2-3 weeks of exposure, while airway hyperresponsiveness and remodeling appear after 5 weeks and remain elevated for up to 9 weeks after cessation of allergen exposure (93,94).

To imitate seasonal allergic asthma, in which allergen exposure is intermittent and recurrent, mice are left to recover from acute exposure to allergen and rechallenged again with the same allergen at later time (48,53). A single secondary allergen challenge induces a robust reappearance of all disease signs (including airway and lung inflammation, airway hyperresponsiveness and mucus hypersecretion). Mice recovered from acute allergic asthma retain local (in the lungs) and systemic (in spleen) Th2 memory that it maintained for their lifetime (48). This model closely resembles patients with intermittent or seasonal asthma and allows for the investigation of Th2 immunological memory.

In summary, none of animal models can be considered perfect surrogates for human asthma, but they should be considered as models of different disease stages and/or phenotypes. Mice as a model species have several advantages over other small animals (such as detailed knowledge on the genome, availability of different reagents and techniques to generate genetically engineered animals), but also several limitations (including differences in lung structure and lack of persistent airway hyperresponsiveness) that should be taken into account when translating results from experimental research to clinical practice. Nevertheless, mouse models of allergic asthma provide an important opportunity to investigate the pathophysiology of asthma and test novel therapies in well-controlled system.

1.6. Allergic asthma imaging

The majority of experimental or clinical allergic asthma studies use end-point analysis of surgical or autopsy samples that preclude the possibility of repeated, longitudinal examination of disease progress. Technical progress over the last decade has lead to the development of novel imaging techniques which allow non-invasive, longitudinal monitoring of dynamic pathological mechanisms involved in allergic asthma in vivo (95). Moreover, advances in imaging of lung of patients with asthma allows for the assessment of airway anatomy and regional lung function to further understand the differences between asthma phenotypes (96). Medical imaging techniques can be divided into 2 major categories: anatomical and functional modalities. Anatomical modalities mainly emphasize tissue structure, and include X-ray, computed tomography (CT), magnetic resonance imaging (MRI) and ultrasound. Functional modalities primary provide information on (patho)physiological activities within the certain tissue and include single photon emission computed tomography (SPECT), position emission tomography (PET) and fluorescence imaging. As functional modalities have limited spatial resolution and anatomical modalities provide limited information on lung function, several modalities can be combined to co-register functional information with anatomical structures.

Computed tomography (CT) images constitutional contrast between air and surrounding lung tissue that allows assessment of airway wall thickness and airway obstruction. However, CT lacks spatial resolution and has to be combined with X-ray to provide anatomical evaluation of the bronchial tree and lung parenchyma. Ct was, either alone or in combination with inhalable contrast agent, successfully used in preclinical animal models to visualize airway inflammation and remodeling (97,98), as well as in clinical studies to determine lung density as a measure of airflow limitation, airway wall thickening and evaluation of therapies in long-term treatment trials (reviewed in (96)). The major drawback of this technique is exposure to high doses of radiation and long scanning times that are needed to achieve high image resolution (<0.05 μ m) (95).

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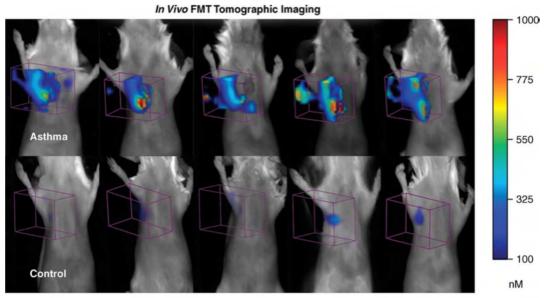
In contrast to CT, magnetic resonance imaging (MRI) is radiation free, which makes this method specially suited for longitudinal studies. Difficulties in MRI lung imaging due to low physical and proton density of the organ can be over-came with the use of safe hyperpolarized gases, such as helium-3 (³He). Hyperpolarized gas lung MRI imaging allows detection of ventilation defects before or after use of bronchonostricting agents (e.g., methacholine) that can be related to asthma severity (99,100). The use of MRI for imaging asthma, however, is limited with low spatial resolution of this technique and the availability and cost of relatively short-lived hyperpolarized gases (96,101). Nevertheless, MRI was successfully used recently to quantify pulmonary inflammation induced by segmental allergen challenge (102).

In contrast to anatomical, functional imaging modalities directly or indirectly monitor cellular or molecular processes in the tissue. Most of these techniques are still under development for allergic asthma and their use is mostly limited to preclinical animal models (96). Positron emission tomography (PET) can detect tiny amounts of radioactively labeled compounds in body tissues, which was used to monitor ventilation in sheep (103) or airway inflammation in rats (104). PET in combination with radioactive-labeled fluorodeoxyglucose was used in two clinical studies on asthmatics to quantify inflammatory response with limited success (105,106). The use of radioactive markers and its low spatial resolution of about 1 cm³, however, precludes the use of PET in small animals and restricts its applicability in humans (95).

Another functional modality that provides a tool for noninvasive repeated examinations of cellular and subcellular activities in an unperturbed environment is *in vivo* fluorescence imaging (Figure 2). Fluorescent dyes that allow tracking of biological processes can be coupled directly to antibodies (107) and small molecules (108), expressed by cells (109), or generated directly in the tissue by administration enzyme-targeted probes that become florescent when activated by different enzymes, such as cathepsins (110) or matrix metalloproteinases (111). The fluorescent signal can be detected by intravital microscopy using fiberoptic bronchoscopy (111), 3D fluorescence molecular tomography (110) and time-domain optical imaging systems (112). Fluores-

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cence imaging allows insight in early pathological processes in experimental models of asthma that cannot be accessed using other imaging methods, while its applicability in humans is still limited by the safety and specificity of fluorescent probes (95). Therefore, there is current need for development of novel specific and safe fluorescent probes that can be used in different areas of asthma research and diagnosis.



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Figure 4. Imaging of allergic airway inflammation with a fluorescence imaging. Mice with acute allergic asthma show a consistent lung fluorescence 24 hours after administration of a near-infrared fluorescent probe activated by cathepsin (ProSense 680), while control mice show minimal signal in the lungs. Signals were detected using VisEn FMT 2500 in vivo imaging system. Source: Jannasch et al 2009 (95).

Altogether, each imaging modality has a unique combination of strengths and weaknesses that are summarized in Table 2. The choice of imaging method depends on the aspects of particular study. In clinics, quantitative CT and hyperpolarized gas MRI allow investigation of structural differences present in the lungs of asthmatics (96). In preclinical research, on the other hand, fluorescence imaging allows insights in inflammatory processes that cannot be assessed using other methods (95). For a detailed understanding of asthmatics

pathophysiology, the optimal approach would consist of a combination of different techniques that allows for simultaneous detection of structural and functional information.

Table 2. Summary table comparing different imaging techniques for al-lergic asthma. Source: Jannasch et al 2009 (95).

	ст	PET/SPECT	MRI	Fluorescence imaging
Pros	No limit in depth	No limit in depth	No radiation	No radiation
	High spatial resolution		No limit in depth	High sensitivity
			High spatial resolution	
Cons	Radiation	Low spatial resolution	Low sensitivity	Limited depth
		Radiation	Long scanning times	Low spatial resolution
Best use	Morphological imaging	In combination with CT	Morphological and functional imaging	Functional imaging
		Translational research		

2. AIMS OF THE THESIS

The main aim of this thesis was to detect genomic biomarkers, to develop novel optical imaging techniques and to establish novel treatment modalities for allergic asthma. Detailed molecular genetic characterization of pathophysiological responses involved in allergic asthma and development of novel imaging technologies to monitor disease progression *in vivo* will provide opportunities for new treatments. There is one manuscript and 2 published papers reported in this thesis.

In the manuscript, we show the results of a meta-analysis of 6 publically available gene expression experiments from in vivo mouse allergic asthma models. We created an acute asthma signature gene list consisting of 933 genes of which 493 are unrelated to asthma and constitute an "acute allergic asthma ignorome" in mice. Asthma-annotated and ignorome genes were confirmed in an independent mouse model, thus verifying the generalizability of these genes expressed in multiple experimental protocols. We ascertained through functional and network connectivity analyses that asthma-annotated and -ignorome genes clustered into 4 biologically relevant domains. Within these networks, we discovered 12 super-connectors genes that link the complex pathophysiological mechanisms underlying asthma and are normalized upon treatment with glucocorticosteroids in an independent mouse model of allergic asthma and have a strong disease phenotype. Overall, our powerful bioinformatic analyses prioritized asthma-related and -ignorome genes within the clusters and biological domains to provide important insights in asthma pathophysiology.

The published paper 1 describes a non-invasive *in vivo* imaging approach to visualize allergic inflammation in an experimental mouse model of allergic asthma. We used dendritic polyglycerol sulfates, which bind to selectins and complement factors, labeled with near infrared fluorescence dye (dPGS-NIRF) in combination with time-domain optical imager. The dye accumulated in the lungs of mice with allergic inflammation that resulted in greater fluores-

cence intensity in the thoracic area of asthmatic in comparison to healthy mice, representing a novel approach to monitor lung inflammation.

In published paper 2, we investigated the effects of tiotropium bromide, a selective long-acting muscarinic receptor antagonist, in mouse model of allergic asthma relapse. Tiotropium bromide is used for the treatment of chronic obstructive pulmonary disease, but is currently not approved for use in asthma and pre-clinical and clinical studies have to demonstrate its efficacy in this disease. We showed that tiotropium bromide efficiently suppresses airway hyperreactivity, mucus hypersecretion and airway inflammation in experimental allergic asthma. These data highlight the anti-inflammatory properties of tiotropium bromide and support its use for the treatment of asthma.

3. PUBLICATIONS

3.1. Manuscript

Revealing the acute asthma ignorome: characterization and validation of uninvestigated gene networks

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Revealing the acute asthma ignorome: characterization and validation of uninvestigated gene networks

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Author contributions:

M.R. performed multi-dataset bioinformatics analysis, network analysis and contributed to manuscript preparation; J.M.G.M. performed multi-dataset bioinformatics, network analysis and contributed to manuscript preparation; B.B. designed and performed in vivo experiments, analyzed the samples and contributed to manuscript preparation; D.C. performed and supervised computational analysis; P.M. contributed to in vivo experiments and sample analyses; C.L. participated in quantitative PCR analyses; M.M.E. designed in vivo experiments, contributed to manuscript preparation; E.S. designed and supervised computational analysis, contributed to manuscript preparation. All authors read and approved final version of the manuscript.

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Running title: Microarray analysis reveals asthma ignorome

Descriptor number that best classifies the subject of your manuscript: *1.10 Asthma Mediators*

Total word count: 3336

At a Glance Commentary: A novel multi-dataset bioinformatics strategy enabled us to unambiguously define acute asthma signature genes. A literature search indicated that 53% of those genes belong to the "acute asthma ignorome", which are defined as genes without previous publications related to allergic asthma. An experimental validation and functional annotation of these genes indicates novel areas of biology to be pursued further and provides a novel perspective for future basic and translational asthma research.

This article has an online data supplement, which is accessible from this issue's table of content online at www.atsjournals.org

Abstract

Rationale: Although multiple pathways involved in allergic asthma are known, work in other disease areas indicates that there is still a vast "ignorome" to be discovered, and that this can shed new insights on disease pathogenesis.

Objectives: Integrating systems biology and experimental validation to identify and validate novel asthma gene networks.

Methods: A systems biology approach on existing data provided a gene signature, evaluated by network analysis and literature mining and validated in a mouse model, with and without steroid treatment.

Measurements and Main results: We generated a signature of 933 genes and experimentally validated their deregulation in our experiment (Pearson r=0.86-0.88). The genes grouped into 7 clusters and 4 biological domains related to inflammation, lung-specific insult response, stem cell proliferation and circadian rhythm. Literature mining showed that 13 genes account for over one third of asthma publications, and 50% of the genes are novel, making up the "acute asthma ignorome". Two domains containing the highest proportion of ignorome genes were related to cell proliferation in CD34⁺, CD71⁺, CD105⁺ cells and circadian rhythm. Network topology identified 1) novel hubs (*e.g.* IGSF6, PLEK, SLC15A3, and GSTA3) and 2) super-connector genes with robust small expression changes that linked multiple biological domains. Systemic treatment with dexamethasone targeted the super-connector, glucocorticoid receptor, *NR3C1*, reverted the majority of the inflammation-related, and partially reverted lung-specific insult response super-connectors.

Conclusions: Our approach characterizes and validates gene networks underlying acute asthma, shedding light on the asthma ignorome and key gene networks not yet investigated in the disease.

Abstract word count: 249

Key words: acute allergic asthma, ignorome, multi-dataset bioinformatics analysis, systems biology, microarray

Introduction

Allergen exposure causes a complex interaction of cellular and molecular networks leading to allergic asthma in susceptible individuals. Experimental mouse models of allergic asthma are widely used to understand disease pathogenesis and elucidate mechanisms underlying the initiation of allergic asthma (1). For example, gene profiling of lung tissue from experimental mice during the initiation of allergic asthma in experiments with different protocols (2-7) validated well-known genes and identified new genes with roles in disease pathogenesis such as *C5* (3), *ARG1* (8), *ADAM8* (9), and *PON1* (7), and dissected pathways activated by *IL13* (10) and *STAT6* (11). Despite these datasets revealing large numbers of genes expressed in lungs during the onset of allergic asthma, there is a skewed distribution of research and literature coverage on a small number of genes, as has been noted in other fields (12-14).

To fully understand the genes and pathways in the lung that are involved in the initiation of allergic asthma, we reviewed all 23 published gene expression experiments performed on lungs from mice with allergic asthma to select those of sufficient robustness to be utilized for a multi-dataset bioinformatics analysis. We focused on 6 studies that share a common microarray platform, have a robust disease phenotype and were derived from experiments using different mouse strains and experimental protocols (2-7). We used a novel bioinformatics strategy combining gene-centric and pathway-centric approaches (see methods) to generate a stringent asthma signature list. This list included a large set of protein-coding genes that had no published function or literature connecting them to allergic asthma either at the level of the whole organism or for individual organs, tissues or cells, genes for which the term "ignorome" was recently coined (15). Here, we present a gene signature list from mice consisting of 933 genes with 440 asthma-annotated and 493 "acute allergic asthma ignorome" genes.

Materials and methods

Data selection

From the 23 experiments identified in the literature, we selected 6 datasets based on studies using the Affymetrix Mouse Genome 430 2.0 or Murine Genome U74 Version 2 Arrays microarray platform and mouse models of ovalbumin (OVA) and house dust mite induced acute asthma characterized by airway eosinophilia, airway hyperresponsiveness (AHR) and mucus hypersecretion. Within these independent datasets, we extracted in total 10 comparisons of asthmatic vs. control samples (see Table 1).

Multi-dataset bioinformatics analysis for selection of acute asthma signature list

We retrieved raw data from the GEO database (16) and analyzed them using a common pipeline for signal normalization and extraction of differentially expressed genes (see supplementary methods and Figure 1). We extracted differentially regulated genes for each experiment, which were then utilized for our multi-dataset bioinformatics analysis combining two approaches: a topdown, biological process enrichment-driven approach, from now on called 'pathway-driven', and a bottom-up, 'gene-driven' approach (see supplementary methods). The two approaches were merged to generate a final union list.

Gene annotation for acute asthma ignorome detection and its functional annotation

To assess the extent of the acute asthma ignorome, we performed an unambiguous literature search based on Entrez Gene and PubMed databases (see online supplementary methods). To annotate the genes from the acute asthma signature list (including the asthma ignorome), we compiled a list of human orthologs, mapped it to the STRING interaction network, and performed a topological analysis for clusters of densely connected genes ($n \ge 5$; details in supplementary methods). For each cluster, we identified three sets of genes: (i) hub genes (the top 10% of the genes in the list ranked by betweennes centrality, defined as the ratio of shortest paths passing through a node, being a common measure of centrality of a node parameter in descending order); (ii) peripheral genes (1-degree nodes connected to a clique, *i.e.* a set of nodes completely connected to each other); and (iii) super-connectors (genes connecting more than 5 clusters). Each cluster was then further characterized in terms of functional enrichment, tissue specific expression and literature representation (see supplementary methods).

Biological validation experiments

We used 8-10 week old female BALB/c mice (Charles River, Sulzfeld, Germany). All experimental protocols complied with the requirements of the Animal Care Committee of the Austrian Ministry of Science. All mice were immunized with OVA (Sigma Chemical Co., St. Louis, MO) dissolved in PBS intraperitoneally on days 0 and 21 and intranasally on day 32 with OVA or PBS. In treatment experiments, we administered vehicle or dexamethasone (Sigma) intraperitoneally at 1mg/kg 30min before and 24 and 48h after challenge. Details on animal treatment and measurements of airway hyperresponsiveness, airway and lung inflammation, mucus hypersecretion and serum OVA-specific antibody titers are reported in supplementary methods. Separate groups of mice were used for lung mRNA extraction at 24 or 72h after intranasal challenge with PBS or OVA. Methods for RNA extraction, reverse-transcription, quantitative polymerase chain reaction and statistical analysis are also reported in supplementary information.

Results

Acute allergic asthma gene signature list

To select the datasets for analysis, we analyzed 23 microarray studies of lung RNA from mice at the initiation of allergic asthma [(16), GEO database (http://www.ncbi.nlm.nih.gov/geo/)]. Our selection criteria included the pheno-type elicited and data robustness (see methods). Six microarray datasets [(2, 10, 17, 18), GSE1301 and GSE3184] from 5 different mouse strains, 2 allergens, 6 experimental protocols were subdivided into 10 comparisons of control *vs.* asthmatic mice (A-J) (Table 1 and Figure 1).

To create an acute asthma signature list from these 6 microarray datasets, we used a multi-dataset bioinformatics analysis strategy including a pathway-

driven and gene-driven approach (see methods and online supplementary methods; Figure 1) to ensure that both enriched pathways as well as robust genes not annotated to pathways were maintained in the final signature (see methods). The pathway-enrichment-driven approach yielded 493 genes, the gene-driven approach 602 genes, giving a final acute asthma signature list of 933 genes.

We validated the robustness of the signature by measuring 59 genes through qPCR in an independent experimental model of allergic asthma (Supplementary figure E1). Despite the difference in experimental setup and technology platform, we observed a significant positive correlation of 0.8660 and 0.8819 (both p < 0.00001) between the two datasets at 24 h and 72h post-challenge, respectively (Figure 2, Supplementary figure E2). These data indicate that stringent and multi-dataset bioinformatics analysis across independent experiments can yield robust gene signatures, which are found to be applicable regardless of experimental context and are representative of general features of the disease.

Allergic asthma ignorome

To determine the already known association of the 933 genes with allergic asthma, we enumerated publications associated with asthma, as well as asthma annotation in other sources (CTD and Malacards, see supplementary methods). Figure 3A illustrates the number of genes associated with asthma using these methods. There is an inverse correlation between these data sources and the number of genes, i.e. a large number of publications are associated with relatively few genes: 13 genes (*Ccl11, Ccl5, Cd14, Cysltr1, Gstm1, Ifng, Il10, Il13, Il33, Il4, Il4ra, Tlr2,* and *Tlr4*) account for approximately 1/3 of the articles published on asthma for these 933 genes (Figure 3). In our dataset, there are 493 genes (Figure 3B) that are as yet unrelated to asthma and belong to the acute allergic asthma "ignorome". These data, combined with the robustness of the gene signature shown above, substantiate that the scientific community has so far focused only on a small subset of genes which are likely to play a role in asthma and that there is a substantial set of genes which warrant further investigation.

Network connectivity analysis

To define the allergic asthma gene network, we analyzed the connections of 897 human orthologs from 933 acute asthma mouse genes (see methods). We detected a network of 779 densely connected genes in which there are 7 topological clusters containing 763 genes (Figure 4A), whereas 118 genes were outside of the network (Supplementary Table E2). In the 7 topological clusters, 409 genes are associated with asthma publications (Figure 4B) and 645 are related to "inflammation or immunity" (Figure 4C) in at least one relevant publication.

The 7 topological clusters were annotated for functional enrichments (see methods) (Table 2, Supplementary figures E3-E7). The clusters fall into 4 major biological domains, of which two are well studied, and two novel: 1) inflammation, including clusters 1A-B: cytokine-cytokine receptor signaling, leucocyte endothelial migration (353 genes, 66.86% published in asthma) 2) circadian rhythm, cluster 2, including genes expressed in the lung as well as CD4⁺, CD8⁺, CD56⁺ and CD71⁺ cells (15 genes, 86.7% as yet unpublished in asthma), 3) lung-specific insult response including clusters 3A-C: extracellular matrix remodeling, adherens and tight junctions, and mucus secretion (309 genes, of which 48.5% are published in connection to asthma), and 4) stem cell proliferation in CD34⁺, CD71⁺, CD105⁺ cells (82 genes, of which 85.4% unpublished in asthma) (Table 2, Figure 4A).

Investigating, in greater depth, the connectivity between the genes contained in these domains (see supplemental methods) enabled the characterization of the connectivity hierarchy amongst them (Figure 5). As expected, clusters belonging to the inflammation domain have the strongest connectivity between themselves, as well as lung to inflammation domains. Interestingly, the genes within the 2 novel domains identified (stem cell proliferation and circadian rhythm) are strongly connected to a specific cluster within the lung-specific insult response, *i.e.* extracellular matrix remodeling. The same domains are also interconnected by a single gene, $ROR\alpha$ that belongs to the circadian rhythm domain and couples the stem cell proliferation and the

extracellular matrix remodeling cluster in the lung-specific insult response domain (Figure 5).

When these novel clusters are annotated for publications in inflammation or immunity, they reveal a substantially larger body of literature (73.3% of circadian rhythm genes and 56.1% of the stem cell genes are associated), indicating that their role in the broader field of immunology is already being investigated, although not yet associated specifically to asthma.

The reliability of the identified functional gene clusters is corroborated by manual inspection of well established key genes in asthma, e.g. the inflammation cluster contains lung allergic immune response genes (e.g. cluster 1A: II4, II4r, II13, II33, CCL11, CCL17 and CCL22; and cluster 1B: Cysltr1, Alox5ap, Arg1, Adam8 and Fcer1g), the lung-specific insult response clusters with known extracellular matrix remodeling, tight junctions, and mucus production genes (e.g. Gstm1, Vegfa, Chi3l1, Pcna, in cluster 3B, e.g. Pard3, Prkci, and in cluster 3C, e.g. muc4, muc5B and muc5AC). In contrast, stem cell proliferation clusters contain genes that are essential for DNA replication and cell proliferation, which were not previously attributed to asthma. Enrichment analysis indicated that these genes are expressed mainly in cells that mediate leukocyte proliferation [CD34⁺ and CD71⁺ cells; (19-21)] and tissue regeneration [CD105⁺ cells; (22)] in allergic asthma. Cluster 2 contains well-known circadian rhythm genes e.g. NR1D1 [or Rev-Erba], NR1D2, PER2, PER3. Most of them have not been related to asthma yet, but growing evidence suggests they play a role in the immune system (23, 24). This evidence suggests that our approach is able to identify valid functional clusters within the overall acute asthma gene signature and to identify genes that are less explored in the context of asthma.

We utilized the network connectivity information to prioritize specific genes that are likely to play an important role in the above domains. We concentrated on two categories of genes: hub genes, *i.e.* genes that are central, given the network structure and peripheral genes, *i.e.* genes which, despite not having a large number of connections themselves, are connected to a set of genes having full mutual interplay (*cliques*, see methods). The assumption is that hub genes play an important role in the overall network, while peripheral genes have reciprocal influence on specific *cliques*. This approach led to the identification of 34 hub genes and 8 peripheral genes in the inflammation domain, and 29 hub genes and 11 peripheral genes in the lung-specific insult response domain. The majority of the peripheral genes (14/22) have not yet been associated with asthma, but most of them have been associated with inflammation (17/22). In contrast, only 11/61 hub genes have not been associated with asthma, and 4/61 have not been associated with inflammation. Interestingly, some well-studied genes are hubs, e.g. as IFNG, TLR4 and PTPRC, but several others are not, e.g. IL13, IL4 and IL4R. The ignorome hubs and peripheral genes are summarized in Table 3. They include IGSF6 and SLC15A3, which are expressed on antigen presenting cells and involved in antigen recognition (25, 26), as well as the peripheral gene CLEC5A (also known as MDL-1), which is involved in the innate immune response to microorganisms (27-29). Within the lung-specific insult response domain, a hub gene TGFBI and a peripheral gene BAMBI belong to the TGFB signaling pathway, important for lung remodeling (30).

Super-connector genes link functional domains

We then searched for "super-connectors", *i.e.* genes that link at least 5 clusters, and thus, identified 12 genes (Figure 6). There are 9 super-connectors in the inflammation domain that are cytokines *IL6* and *IL1B*, cell surface receptors *CD4* and *CD44*, signaling molecules *STAT1*, *TRAF6* and *RELA*, as well as *CADM1*, a receptor that mediates mast cell adhesion to lung structural cells (31) and the glucocorticoid receptor *NR3C1*. The other 3 of 12 molecules belong to cluster 3 (extracellular matrix remodeling) and include *PRKCD*, a protein kinase that plays an important role in tissue remodeling (32), an antihemophilic factor *VWF* with multiple pro-inflammatory roles (33) and *ERBB2*, an orphan tyrosine kinase that plays an important role in cancer (34)).

The super-connector fold change fluctuated considerably, albeit in limited ranges, as indicated by the median fold changes that ranged from -0.01 and 0.32 within each microarray comparison (Figure 7). This finding is consistent with a previous analysis (35), indicating that genes with high connectivity have

minimal fold change in gene expression. Despite this, we found that the expression change determined by qPCR was significant for 9/12 genes and concordant with the microarray data for 8 of 12 (67%) super-connector genes (Figure 7). Moreover, we observed a significant positive correlation of 0.69 (p = 0.0132) between the two datasets at 72h post-challenge (Supplementary figure E8). These data indicate that genes, which are likely to play an important role, would often escape attention in a single experiment analysis due to their minimal fold-change, but can emerge from an approach that combines computational dataset analysis and gene network connectivity investigation.

Dexamethasone treatment reverts super-connector gene expression

The glucocorticoid receptor NR3C1 is the only super-connector that linked all 4 major domains and robustly changed expression across microarray comparisons and our gPCR. Given existing knowledge of the role of NR3C1 in allergic asthma, we tested the effect of dexamethasone, a steroid that binds to NR3C1 (36), in our animal model on both the disease phenotype and superconnector gene expression. As expected, dexamethasone significantly suppressed airway and lung inflammation, mucus hypersecretion and lung Th2 cytokine gene expression (Supplementary figure E9). Out of the 9 superconnector genes that were significantly deregulated in our model, dexamethasone partially or completely reverted 7 genes towards control values (Figure 8). Although STAT1 was not deregulated in our model, it was downregulated significantly after dexamethasone treatment, which is in agreement with a previous study (37). TRAF6 and VWF were the only 2 superconnectors significantly down-regulated in our model that were unaffected by dexamethasone treatment. Out of the 9 super-connectors belonging to the inflammation domain, 6 were partially or completely reverted by dexamethasone treatment, whereas of the 3 super-connectors belonging to extracellular matrix remodeling, ERBB2 is partially reverted by dexamethasone, *PKRCD* is not deregulated, and *VWF* is not reverted by the treatment. In conclusion, systemic treatment with glucocorticoids modulated the majority of the super-connectors belonging to the inflammation domain, but only partially reverts one gene belonging to the extracellular matrix remodeling domain.

Discussion

Here, we implement a novel computational approach used to analyze multiple microarray datasets derived from lungs of mice with acute onset allergic asthma and integrate it with independent experiments to unravel the asthma ignorome and dissect it into domains, clusters, hubs and peripheral genes.

Technical limitations reduce the power of microarrays in detection of potentially important genes with limited variation in expression levels, especially when the sample size is small (38, 39). Gene signals from small specific cell populations in complex tissues, such as lung, are hence diluted to low levels that are not accurately detected by microarray analyses (38, 39). Using our approach, we reduced biological noise and detected genes with significant < 1.5-fold changes that were excluded from previous individual microarray studies (2, 10, 17, 18) and meta-analysis (40), which generated a reliable, robust gene expression signature for acute allergic asthma in mice.

Our signature gene list consists of 41% asthma-annotated genes and 59% of genes not previously associated with disease, defined as the ignorome (15). Of the asthma-annotated genes, 13 genes associated with Th2 and inflammatory responses account for 1/3 of all asthma related publications and the remaining genes play roles in inflammation. Strikingly, there are a large number of ignorome genes involved in inflammatory pathways, DNA replication, cell cycle and wound healing, which may include important disease targets. Taken together, these data support the notion that biomedical research is skewed towards a relatively small number of 'successful' genes (12, 13, 15, 41). We argue that novel insights into the pathogenesis of allergic asthma should be achieved irrespective of the current literature bias. The main ignorome clusters relate to stem cell proliferation and circadian rhythm domains. Both functional domains are well established in asthma (19, 20, 22, 42) and yet the individual genes identified in this study have as yet not been associated to it, thus providing interesting candidates to pursue these functional areas in the disease.

Understanding hubs (predominantly asthma-annotated) and peripheral genes (mostly ignorome) is essential for establishing functional interplay within the clusters and determining why key Th2 genes e.g. *IL13, IL4* and *IL4R* are neither hub nor peripheral genes (43, 44). These data support the finding that there is no correlation between a gene's degree of interaction, importance in the pathogenesis, and frequency of occurrence in the scientific literature (14).

Notably, all super-connectors are asthma-annotated and belong to either inflammation or lung-specific insult response domains. In our dexamethasone experiment, we found that only *TRAF6* and *VWF* had not changed expression levels. However, it is possible that they might have changed, if higher steroid doses were used, especially because steroids are known to increase *VWF* (45, 46) and *TRAF6* (47) expression. Although we focused on these superconnectors, there is evidence that 41% (314) of genes interconnect 3 or more clusters, 1/3 of them being an ignorome, further demonstrating a large constellation of interrelated clusters across tissues and biological domains.

There are caveats regarding the signature gene list and analyses presented here. Firstly, these analyses are based on the early events at the initiation of acute allergic asthma in the lungs of mice and may not reflect the same multiplicity of clusters and biological domains that would appear when testing chronic disease or disease relapse. Secondly, according to the current paradigm, genes in the inflammatory and immune response are expected, however, the remodeling response appears earlier than expected and these genes may reflect an early response of the lung to inflammatory signals and may represent the activation of healing mechanisms. Thirdly, it is not known whether human gene expression profiles from patient lungs would produce a similar gene list and functional clusters. Asthma is a heterogeneous disease that includes several distinct disease phenotypes and endotypes (48) and is not usually studied at acute onset, but rather during ongoing disease. Multiple dataset analyses including more gene expression profiles from mouse and man and at different stages of disease and in distinct patient subgroups may result in the prioritization of essential genes and networks at the core of disease and simultaneously highlight molecular differences between phenotypes.

41

In conclusion, the integration of prior literature knowledge and unbiased multiple dataset analysis paradigms through the lens of systems biology provides important insights into asthma pathogenesis, allowing to bring novel biology and existing knowledge on a balanced plate, highlighting connections between the two and providing novel avenues to combat the disease.

Acknowledgments

The authors acknowledge excellent technical help of Drs. Rui-Yun Lee, Gordana Mokrovic and Daniela Reiner in various stages of experimental work.

Tables

		S		Study		•	Antigen		Sampling
No.	GSE	GPL	PMID	Comparison	Affimerix Mouse Array	Mice	(Mice number)	No. of challenges	time (hr after last challenge)
1	13032	1261	19491150	А	Genome	A/J	PBS (3) OVA (3)	3	3
1				В 430 2.0	B6	PBS (3) OVA (3)	3	3	
2	6858	1261	17437023	С	Genome 430 2.0	BALB/c	PBS (4) OVA (4)	7	24
3	9465	1261	19057703	D	Genome 430 2.0	A/J	PBS (3) OVA (3)	1	96
4	1301	339-340	-	Е	U74 Version 2	BALB/c	PBS (3) HDM (3)	2	72
	3184	339	-	F G	U74 Version 2	СЗН	PBS (5) OVA (5)	1	6
_						СЗН	PBS (5) OVA (5)	1	24
5				Н		Version 2	A/J	PBS (5) OVA (5)	1
				I		A/J	PBS (5) OVA (5)	1	24
6	18010	1261	19770271	J	Genome 430 2.0	B6.Il4raQ576/Q576 B6.Il4raQ576/Q576/IL-13tg	- (5) IL-13 (8)	7	24

Table 1. Gene expression studies of asthma used for the bioinformatics analysis.

Six GEO Datasets (http://www.ncbi.nlm.nih.gov/gds/) studies were selected for meta-analysis on the basis of the microarray platform and *in vivo* experimental protocol. From those 6 studies, 10 asthmatic vs. healthy control comparisons (A to J) have been extracted and further analyzed.

17 tissues potentially found i lenes, as for Sept 2014) nness Centrality

Maximum of 5 tissues with the first overlap for As Top 5 hub genes ordered Peripheral genes defined Peripheral genes defined

Table 2. Biological processes, pathways and tissue expression enrichment analysis of main clusters in asthma-signature genes list, examples of hubs, peripheral genes, super-connectors and genes present in Malacards, a genedisease association database.

				_		Selected genes		Percentage of g	Percentage of genes in PubMed related to
Cluster	N Gene Ontology Biological Processes	KEGG Pathways	Human Athlas Enriched Tissues	Hubs	Peripheral St	uper-connectors	Peripheral Super-connectors Malacards Asthma	"asthma"	"inflammation OR immunity"
₹	239	ISAAGGO TONINE CANARE RELEPTOR INTERACTION AsaAGGO HEMANOPELTIC CELLI LIKAGE ISAAGGO TOLL LIKE RECEPTOR SIGNAL NO PATHWAY HEMAGES OLL ANTERESON MICENEL ANTERSON MICENES HEMAGES OLAK STAT SIGNALING PATHWAY	C 105- Merida C 105- Boldereg _sell) Whorefelood of SmothMuscle	6	HLA-D082 C HLA-D082 082 C C C C C S S R R A C	MPN MPA MPA HLADOB2 CR4 RFAF6 RFAF6 RFAF6 RFAF6 CADM1 CD44 LL5	L13 COL11 COL11 LLRR LLR RMSE6 LLR RMSE3 LL COL17 COL15 FOER2	*	%L 98
8	Immune strain protess (20.00.2376) defense response (00.000685) Immune response (00.000685) 114 Immäe immune response (00.0046087) respirationy bust (00.0046730)	HSAuger DELKOYCTF REAVENDOTHEM MARKENDINEM MARKENDINEM SALAGBAF CFESLINUNR I SIGNALING SPATHAWY HSAAQRAF CFESLINUNR I SIGNALING SPATHAWY HSAAGBAS NHTUBAL HILLER CELL MEDINTED CYTOTOXICTY HSAAGBAS NHTUBAL HULER CELL MEDINTED CYTOTOXICTY HSAAGBAS INOSITOL PHOSPHAFE METBABOLISM	CD14+ Monocytes CD34+ Myeloid WholeBlood Lung	TYROBP PLEK TIGB2 C10A SLC15A3	COTL1 PRICKLE1 SIGLEC8 SDPR SPON2 CLEC5A	1	PLA2G7 ALOXSAP CYSLTR1 ADAM8	48.2%	84.2%
7	regulation of the Arrentation process (CO305122) regulation of transaction, DNA-dependent (CO305025) to Reme busynthes process (CO300575) 15 equation of transaction from RNA polymerase II promoter (CO3005415) regulation of transaction (CO3005416)	ISBARTO FOR CAUNN HYPT, THE CONNEL MET ABOL USM ISBARDO GLYCHKE SERIKE AND THRECONNEL MET ABOL USM ISBARDOS GLYCHKE SKND PROLINEL MET ABOL ISM ISBARDOS PORPHYRIN AND CHLOR OPHYLL MET ABOL ISM	CD71+_EarlyErythroid (2) Lung (1) CD66- MCCalis (1) CD8+_Telles (1) CD4+_Teells (1)	ALAS1 DBP GATM ALAS2 NR1D1		I	I	13.3%	73.3%
3А	regulation of the proference (IO CO04256) regulation of apportens (CO04366) regulation of apportens (CO04366) regulation of cell migration (CO003034) regulation of cell migration (CO003034) regulation of cell midlon (CO0051270)	IBSAGE TEA RECERTION NIERACTION BRAGE IPCOAL ADRESION BRAGE IPCOAL ADRESION BRAGE IPCOAR EMBARY BRAGE IPCOARE ARTHANK BRAGE IPCOARE ARTHANK BRAGE IPCOARE ARTHANK BRAGE IPCOARE ARTHANK	SmoothAusole Lung	NEGFA FN1 ERBB2 IT GB1 CAV1	TFPI2 PF AQP5 V1 AQP5 V1 MYO1B MYO1B EFHD2 TSPAN7 MMP19 MYC MMP19 MYC TMEM173 TMEM173 BEX1 BEX1	PRCKD VWF ERBB2	CHIA ALOXI2 ALOXI5 CAT CAT	48.6%	84.8%
38	establishment or maintenance of cell potarty (GO:0007163) 19	HSA04530 TIGHT JUNCTION	BronchialEpithelialCells (3) CD105+ Endothelial (1) UNDeBbood (1) CD71+_EarlyErythroid (1)	PRKCI SQSTM1 PARD3 PLN GRIA1	NA	I	I	42.1%	63.2%
ĸ	Immonsaccinate metabolis, bross (CO 2005966) enluar actorial metabolis, process (CO 2006066) 8 ventricular cardiac muscle morphogenesis (GO 2005010) cardiac muscle contraction (CO 2006046) cardiorhydrate metabolis process (CO 2006176)	A constance recent Nu Liver Te constructions of the constance of the constance of the constant	Trachea (2) CD71+ Ean/Erythroid (1) CD54NKCells (1) CD14+_Monocytes (1) Lung (1)	4	TFF2 SLC26A4 CHST2	I	I	62.5%	100.0%
4	orgamelle organization (CO:008196) chromesone organization (CO:0081526) 82 regulation of cell cycle (CO:0051726) DNA replication (CO:000529) DNA replication (CO:000259)	NAGATIO ELL CYCIE ISAO0230 PURINE METABOLISM HSA00240 PYRIMIDNE METABOLISM	721.B_tymphobiasts CD105+_Endothelial CD71+_EartyErythroid CD34+ cells	MAD2L1 NME1 PTTG1 TK1 RRM2	ADAM19 CD14	I	I	14.6%	56.1%
Maximur. Maximur	Maximum of 5 enriched categories ordeed by Enricht Combined score showing an adjusted Puelue < 0.05. Naximum of 5 fissues within a tist of 17 tissues potentially found in the whole lung tissue samples in dusters 1 to 7 Naximum of 5 fissues within a tist of 17 tissues potentially found in the whole lung tissue samples in dusters 1 to 7	jjusted P-value < 0.05. ue samples in datasets under study (See Supplementary Figures 2-8 for	details on Human Atlas Gene expression for s	all genes in cluster	rs 1 to 7				

Table 3. List of hub and peripheral genes related to acute asthma ignorome.

Gene Name	Network role	Functional Domain	Published in
			inflammation&immunity
C1QA	Hub	Inflammation	Yes
IFITM	Hub	Inflammation	Yes
HCLS1	Hub	Inflammation	Yes
IGSF6	Hub	Inflammation	No
PLEK	Hub	Inflammation	No
SLC15A3	Hub	Inflammation	No
CLEC5A	Peripheral	Inflammation	Yes
COTL1	Peripheral	Inflammation	Yes
MTPN	Peripheral	Inflammation	No
PRICKLE1	Peripheral	Inflammation	No
SDPR	Peripheral	Inflammation	No
GSTA3	Hub	Lung-specific insult response	No
CYP2B6	Hub	Lung-specific insult response	Yes
TGFBI	Hub	Lung-specific insult response	Yes
COL1A2	Hub	Lung-specific insult response	Yes
FGF1	Hub	Lung-specific insult response	Yes
BEX1	Peripheral	Lung-specific insult response	No
EFHD2	Peripheral	Lung-specific insult response	No
BAMBI	Peripheral	Lung-specific insult response	Yes
CHST2	Peripheral	Lung-specific insult response	Yes
ITGB1BP2	Peripheral	Lung-specific insult response	Yes
MYO1B	Peripheral	Lung-specific insult response	Yes
TFPI2	Peripheral	Lung-specific insult response	Yes
TMEM173	Peripheral	Lung-specific insult response	Yes
TSPAN7	Peripheral	Lung-specific insult response	Yes

Figures

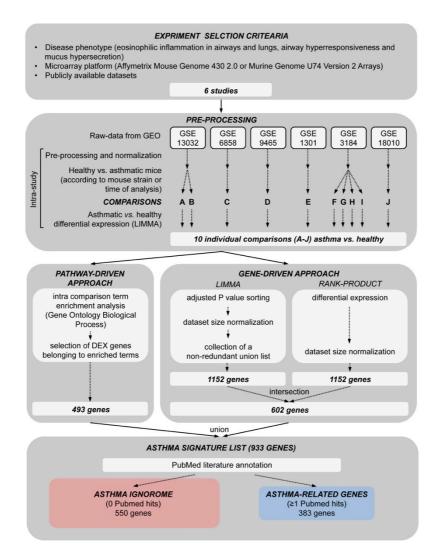


Figure 1. Schematic overview of analysis pipeline. Using experiment selection criteria, we selected six studies that were further subdivided into 10 comparisons of control and asthmatic mice according to mouse strain and time of analysis. After initial analysis with liner models to obtain differential gene expression, data from each comparison were re-analyzed using pathway- and gene-driven approaches (for details please refer to online supplementary methods). Lists of differentially regulated genes generated in the 2 approaches were merged into a final asthma signature list of 933 genes, which was used for literature-coverage searches in PubMed.

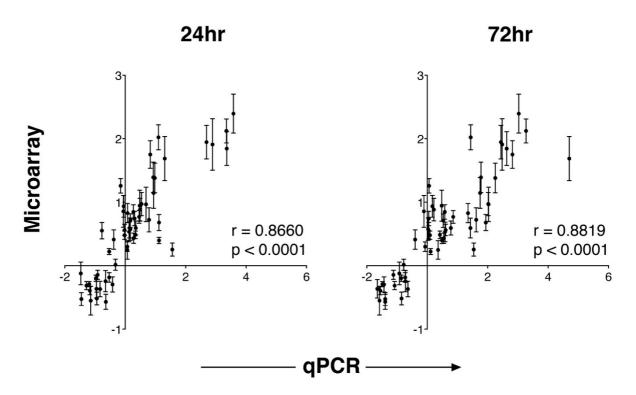
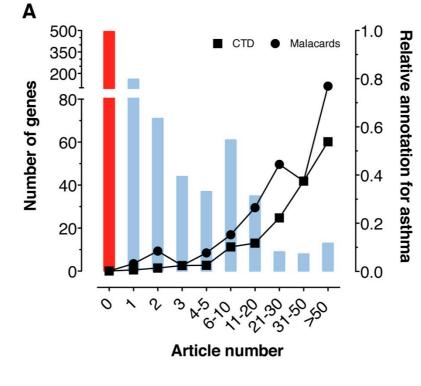


Figure 2. Microarray data for 59 randomly selected genes from 10 comparisons of control and asthmatic mice correlate to quantitative PCR data from our independent mouse asthma model. For qPCR, ovalbumin (OVA)-sensitized BALB/c mice received PBS (controls) or OVA challenge (A) 24h or (B) 72h before extraction of total lung RNA. Pearson r coefficients and p values for each correlation are indicated.



Article, nr	Gene
0	1100001G20Rik, 1190002H23Rik, 1190002N15Rik, 2610524H06Rik, 2810417H13Rik, A930001N09Rik, Abca3, Abca8a, Acp5, Acsi4, Acvr2b, Acvr11, Adam19, Adam19, Adam52, Adoy4, Adoy6, Adoy7, Adoy6, Ahcyl2, Ahsp, Alf1, Alas2, Alht18a1, Alas1, Anta, Ampd2, Ankrd44, Anxa8 ⁺ , Apex1, Apinr, App2, Ar, Af8b, Amt2, Appc1b, Apl22, AW112010, Azgp1, B4gaint1, Bambi, Basp1, Bcap29, Bc10, Bc3, Beaz, Bhihe40, Bink, Bmi1, Bmp2R, Bmp6. Bmx, Bnij3, Bnijbl, Brit, Cria, Cita, Cita, Cita, Cita, Cita, Cardo, Cadot18b, Codeba, Codeb8b, Codeba, Codeb8, Codeb4, Codeb4, Codec4, Clecc4e, Clecc5a, Clic1, Clic3, Clip4, Clin6, Cotm8, Code20, C
1	Abcb1a, Abi3bp, Acaa1b, Aglg1, Alpl, Arhgdib, Batt3, Bid, Birc3, C4b, Ccl4, Ccl8, Ccr9, Cd163, Cd177, Cd22, Cd300a, Cd52, Cd53, Cd68, Cd72, Cdk1, Cdkn1a, Ch25h, Chi3l4, Cish, Cidn10, Clec10a, Cp, Cr2, Cs12ra, Cst3r, Cstb, Ctse, Cxc113, Cxc116, Cxcl9, Cyp22, Cyr61, Dabz, Dag1, Dokt, Ddit3, Dic1, Dok2, Egr2, EliS, Emr1, Epb4 114b, Ephx2, Erang1, Etx5, F10, F13a1, F5, Fga, Fg7, Fp1Z, Foxp1, Frmd6, Fscn1, Fyb, Gad445g, Gbp1, God2, Girx, Gyrasp1, Gyr2, Griat, H2-DMa, H2-Oa, Hpgd, Hspa1a, Hspb1, Hvcn1, Id3, Ifi30, Igfrir, Igtp, Irf5, Igm1, Iria1, Itga7, Iltga7, Junb, Kil2, Kil4, Lag3, Lama3, Lck, Lcp1, Lgals1b, Llith3, Lpin2, Lib, Lyst, Map2K3, Marco, Mgl2, Me4Aeb, Msr1, Myocd, Nc4f, Nry1, Cosm, Part3, Per1, Pgr, Fm1, Pin, Polrók, Prixch, Prax, Pin22, Pyhin1, Rac2, Raged3, Rasa2, Reck, Rg4, Saa3, Scnnt, Sdc4, Sema3, Sema4d, Strp1, Sic2a1, Slit2, Sox17, Spon2, Srgn, Sit8ia2, Swap70, Syne2, Tapbp, Txx2, TfH, Thbd, Tihbs1, Timp3, Tirap, Tinfs19, Tnis19,
2	Adrb1. B2m, Batf, Bcl6, Birc5, Bpifa1, Card10, Ccl12, Ccl3, Cd74, Chi33, Citta, Clec4n, Ccl3a1, Ctgf, Cxcl2, Cxcl5, Cxcr4, Cybb, Erbb2, F3, Fabp1, Fgr, Fpr2, Hck, Hexb, Hspa1b, Igf1, Igfbp3, Irf7, Itgal, Itln1, Kl1f5, Lbp, Lcn2, Ly86, Merk, Mmp19, Mmp8, Myc, Ncl2, Ndst1, Ntrk2, Olr1, Park7, Per2, Pt4, Pglyrp1, Psmb8, Ptch1, Piges, Ptpn2, Rbp4, Relb, Ripk2, Sema4a, Stpi1, Sic11a1, Sipi, Socs2, Sox9, Sox9, Tap2, Tbxas1, Tek, Thy1, Tnfrs9, Trxb, Trp63, Uch11, Vidir
3	Acvr1, Adam17, Adcy9, Adh1, Alox12, Cadm1, Colö, Ccl9, Cd48, Cfh, Csf1, Clss, Cxcl1, Cyp2d22, Dll4, Earl, Epas1, Fabp4, Fn1, Gsto1, Hey1, Ifngr2, Inpp5d, Itgax, Kdr, Lat, Mki67, Muc4, Ncf1, Nikb2, Notch1, Npy, Ppara, Ptpn1, Pycard, S100a9, Selpig, Serpina3c, Serpina3c, Serpina3m, Serpi
4-5	2210018M11Rik, Angpt1, Casp3, Cc11, Ccl22, Ccr1, Ccr7, Cd44, Cd83, Cd8a, Cklf, Clec7a, Col1a1, Cx3cl1, Darc, Dusp1, Ephx1, Fcgr1, Gpx1, H2-Q7, Hix, II7r, Jak2, Lgais3, Ltf, Mrc1, Nikbia, Pla2g7, Pon1, Ppp1r14a, Prkcd, Rbpj, S100a8, Sod2, Splnk1, Stat5b, Tnfsf13b
6-10	Agr2, Arva5, Apoe, Aqp5, Areg, Arg2, Calca, Cat, Cat/1, Cl220, Cd7, Cd274, Cd28, Cd38, Cd40, Csf2rb2, Cxcl11, Cxcl12, Cyba, Cyp1a1, Fas, Fogr2b, Fogr3, H2-Aa, H2-Ab1, H2-M3, Hdc, Ido1, Ighg, Ighm, II16, II18r1, II1a, II2rg, Irf4, Itga4, Itgam, Itgb1, Itgb2, Kitt, Mif, Pdcd11g2, Piger4, Pigs1, Pipn6, Retnia, Rora, Selp, Siglec5, Sic26a4, Socs1, Socs3, Stat5a, Tff2, Tgfb2, Timp1, Tir1, Tir6, Tnc, Tnfrsf1b
11-20	Ace, Adam8, Adora2b, Adra2a, Alox15, Alox5ap, Arg1, C3, C3ar1, C5ar1, Cc17, Cc124, Ccr2, Ccr5, Cd4, Clp, Chia, Clca3, Cxc110, Fcer1g, Fcer2a, Hc, Hmox1, II1r1, II1m, Il2ra, Mmp12, Muc5b, Pde4d, Spp1, Stat1, Sykb, Tir7, Vcam1, Vdr
21-30	Chi3l1, Csf2, Clla4, Ear2, Icam1, II1b, Myd88, Nr3c1, Pona
31-50	Ccl2, Cd2, II18, II1r11, II6, Muc5ac, TH2, Vegfa

>50 Ccl11, Ccl5, Cd14, Cysltr1, Gstm1, Ifng, II10, II13, II33, II4, II4ra, Ptprc, Tlr4

В

Figure 3. An acute asthma ignorome. (A) PubMed literature coverage of 933 genes from acute asthma signature list related to "asthma". The *x*-axis represents the number of asthma-annotated literature for each gene in Pub-Med in November 2014. The left *y*-axis shows the gene number (shown as bars), while the right *y*-axis indicates the relative annotation to gene-disease association databases (Comparative Toxicogenomics Database (CTD) or Ma-

lacards; shown as lines). (B) List of MGI gene symbols for 933 asthma signature genes according to their number of asthma-annotated literature in Pub-Med according to the method described in online supplementary methods and exact number of publications is listed in Supplementary table E2.

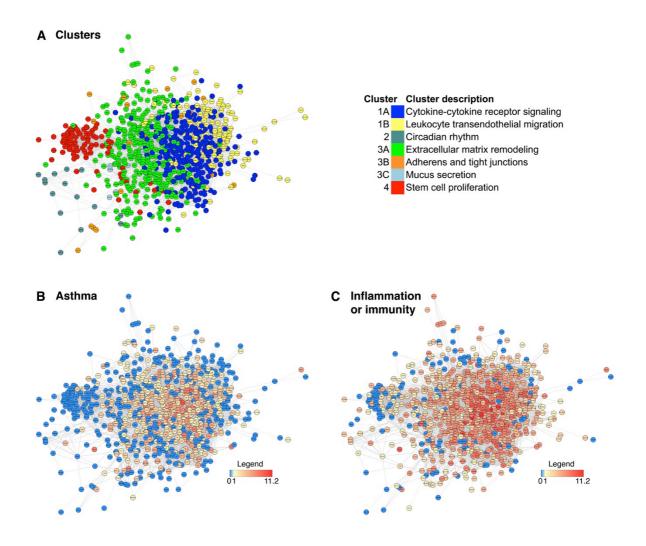


Figure 4. Network representation of 759 human ortholog genes in 7 clusters from our asthma-signature list. (A) Within the network we have detected 7 main clusters using STRINGdb library in Bioconductor that were further functionally annotated to reveal biological functions with online tool EnrichR (for details please refer to Online supplementary material). Genes are colored according to literature number associating each gene to (A) "asthma" or (B) "inflammation OR immunity". For each gene, we have retrieved number of publications in PubMed in November 2014

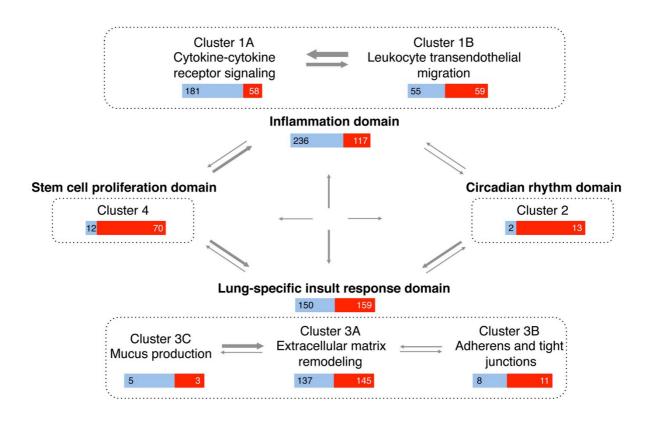


Figure 5. Schematic diagram depicting connectivity between 4 domains and individual clusters in acute asthma signature list. Bars indicate number of asthma-related (blue) and –ignorome (red) genes in each cluster or domain. Arrows indicating connections between clusters or domains are scaled according to relative connection strength between clusters and domains (see supplementary methods for details).

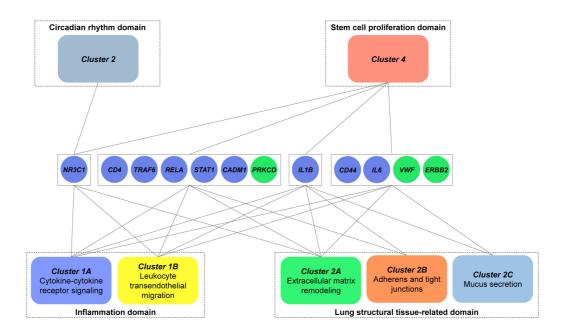


Figure 6. Schematic diagram depicting 12 super-connector genes in the asthma-signature gene list. Gene circle color indicates the cluster of origin and connected to the pertaining topological cluster. Topological clusters are grouped into 4 biological domains by dotted rectangles.

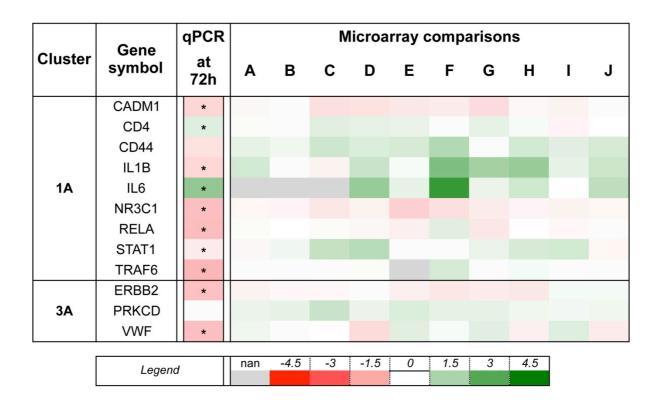


Figure 7. Expression profiles of 8 from 12 super-connectors were confirmed with quantitative PCR. Data are presented as mean log_2 fold changes of gene expression by quantitative real-time PCR and microarray relative to control mice. Quantitative real-time PCR data were determined in whole lung extracts and are pooled from 2 independent experiments (n=6). Microarray data are from 6 publicly available datasets broken down into 10 direct comparisons of asthmatic and control mice (please refer to Supplementary data and Figure 1 for details). * p < 0.05 compared with PBS challenged mice (unpaired *t*-test).

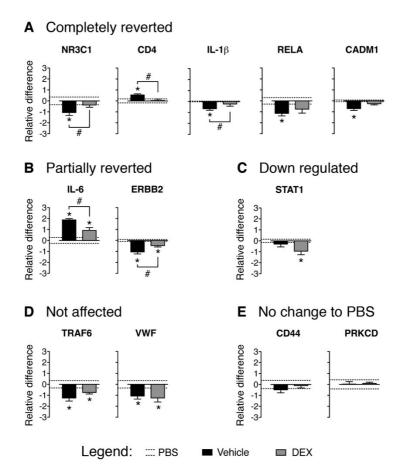


Figure 8. Expression of super-connectors in acute allergic asthma after dexamthasone (DEX) treatment. Total lung RNA was extracted from mice intranasally challenged with PBS (controls) and mice that received vehicle or dexamethasone before and after ovalbumin challenge to induce allergic asthma. Samples were collected at 72h after allergen challenge and super-connector expression was determined with quantitative PCR. Super-connectors are grouped according to their change to dexamethasone treatment into (A) completely reverted, (B) partially reverted, (C) further down regulated, (D) not affected with dexamethasone treatment, and (E) not changed in comparison to PBS controls. Data are presented as mean log₂ fold changes of gene expression relative to control (PBS) mice and are pooled from 2 independent experiments (n=6). * p < 0.05 compared with PBS challenged mice; # p < 0.05 compared with vehicle-treated group (unpaired *t*-test).

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Revealing the acute asthma ignorome: characterization and validation of uninvestigated gene networks

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Online Data Supplement

Supplementary materials and methods Supplementary tables Supplementary figures Supplementalry references

Supplementary materials and methods

Multi-dataset bioinformatics analysis

To overcome the limitations of direct comparison of the results obtained by other laboratories with their own differing analysis pipelines, we retrieved the raw data from the GEO database (E1) and re-analyzed them using a common pipeline for signal normalization and extraction of differentially expressed genes with limma and Affy Bioconductor libraries (E2). Raw data were normalized using robust multi-array average [RMA; (E3)]. In each experiment, genes with expression values lower than the first quartile of the entire distribution of values were considered as non-expressed and hence removed from the analysis. We applied parametric moderated *t*-statistics, implemented in limma library, to identify differentially expressed genes between asthmatic and control samples. Each differential expression analysis was conducted separately for each experiment. Significantly up and down regulated genes were selected using an adjusted *p*-value threshold of 0.05 (Benjamini-Hochberg correction (E4)).

The resulting gene lists were then utilized for our multi-dataset bioinformatics analysis that combines two approaches: a top-down, biological process enrichment driven approach, from now on called 'pathway-driven', and a bottomup, 'gene-driven' approach. The former strategy is based on the selection of common asthma-specific pathways from a combination of enriched biological terms found across individual studies. In the latter strategy, a list of differentially expressed genes has been created across all individual datasets, normalizing the size of the list to balance the different statistical power of each experimental design. Results from each single approach have been merged in a comprehensive list of genes. Human orthologs of such genes were used to query a network of interactions and investigated in terms of topological structure using STRINGdb Bioconductor library (E5) and Cytoscape (E6). The details of each strategy are described below.

Pathway-driven strategy

We performed GO.BP (Gene Ontology Biological Process) term enrichment analysis using the lists of differentially expressed genes derived from each microarray comparison, selected at a stringency of adjusted *p*-value < 0.05, using NCBI DAVID online tool (E7). 76 GO.BP were selected according to the following criteria: ease score < 0.01, presence in at least 2 studies, fold enrichment > 2; categories which were also enriched in the background of mouse transcriptome alone were filtered out. We calculated sematic similarity among enriched terms using GO.SemSim Bioconductor library (E8). Similarities were used to build clusters of semantically related terms, including regulation of morphogenesis, T-cell selection, antigen processing and presentation, response to external stimuli, immune signaling, and leukocyte-mediated response (Supplementary figure E10). Differentially expressed genes referring to enriched GO.BPs were retained to form a non-redundant list of interesting targets (*n*=493), namely "pathway-driven" list.

Gene-driven strategy

To obtain a list of differentially expressed genes (DGEs) based on a 'genedriven approach', we performed a two-step analysis: normalized union of DGEs and Rank Product of DGEs.

Normalized Union of DGEs

The genes obtained from the limma differential expression analysis were ranked from highest to lowest significance for all 10 comparisons performed (A to J). The comparison which produced the smallest list of genes was used to define a cut-off for the remaining comparisons performed to produce 10 lists of genes of the same size, all containing highly significant genes (adjusted *p*value < 0.05). This approach was used to ensure, on the one hand a stringent approach for the inclusion of genes, and on the other hand to obtain a balanced contribution of each study for the downstream analysis. We then performed a non-redundant merge of these 10 lists.

Rank Product of DGEs

We used a non-parametric approach to analyze the 10 studies together based on the Rank Product using the RankProd Bioconductor library (E9). This approach makes it possible for datasets generated at different laboratories or from different environments to be combined for study and it has been previously used with success for meta-analyses of gene expression data (E10). RankProd uses the rank product non-parametric method to identify up- or down-regulated genes in one condition against another condition, based on consistent fold change across experiments, rather than t statistics. In our comparison of asthmatic vs. control samples, Rank Product detected genes that were consistently highly ranked in a number of lists, *i.e.*, genes that were consistently found among the most strongly up- or down-regulated genes in a number of replicate experiments [see (E9) for further details]. The RankProd Bioconductor package was used to calculate rank product statistics on data coming from the 10 different lists in this study. We derived a list of upregulated and a list of down-regulated genes. We then ranked these based on their meta-analysis Rank Product FDR (*pfp*) and identified a *pfp* cutoff which would allow us to retain the same number of genes obtained in the normalized union approach, with the same up- and down-regulated proportions, to balance the contribution of both approaches. We combined the list of genes from the normalized union step (*t*-statistics) with the genes coming from the Rank Product step (non-parametric) and produced an intersection containing genes that were identified by both statistical approaches, leading to a highly stringent and robust set of differentially expressed genes which ranked highly both in terms of *t*-statistics and fold-change rank product.

Gene Annotation

For each gene in our list, we performed a literature search in PubMed to identify publications associated to asthma as keyword in the title or abstract. We used NCBI Entrez Gene search engine [(E11); http://www.ncbi.nlm.nih.gov/gene/] on EntrezId terms for either mouse genes or their human orthologs (see below). We used a ruby script to retrieve NCBI publication numbers using either "Asthma" or "Immunity OR Inflammation" in the Title/Abstract of the papers and the number of resulting publications was

PUBLICATIONS

used to annotate each gene (date of publication number retrieval, 28th of November 2014; see Supplementary Table E2). To validate 550 genes that had 0 publications for "asthma", we performed additional searches in PubMed using the gene abbreviation as a keyword. For 444 genes, we confirmed 0 publications records for "asthma" and for the remaining 106 genes, the publication number was manually curated to retrieve a final list of 493 genes, whose function was not related to "asthma". An overlap of our asthma signature with known as asthma-related annotated genes was calculated using the Comparative Toxicogenomics Database (CTD) via the enrichment ToppGene Suite (E12) and with Malacards (E13) through GeneCards [(E14), http://www.genecards.org/]. GeneCards allergic asthma-related genes were retrieved through the advanced search "allergic asthma" as keyword in February 2014.

Network analysis and network cluster annotation

Pathway-driven and gene-driven lists were merged into a final union list. We compiled a list of corresponding human orthologs: First, BioMart was used to automatically map the human orthologs from the gene symbols (<u>www.biomart.org</u>). Second, for those genes without any annotated ortholog using BioMart, a combined search using EnsEMBL and MGI databases was undertaken (<u>www.ensembl.org</u> and <u>http://www.informatics.jax.org/</u>). For those mouse genes that showed more than one potential human ortholog, all genes were retrieved. We used a full list of human orthologs to query STRING interaction network using STRINGdb library in Bioconductor. We performed a topological analysis using STRINGdb library again, searching for clusters of densely connected genes: a group of nodes having denser relations with each other compared with the rest of the network. STRINGdb integrated igraph (E15) functions for this purpose. Clusters with at least 5 genes were entitled for further analysis.

Functional annotation of genes within 7 clusters

For each cluster, we identified three sets of genes: hub genes, peripheral genes and super-connectors. Hubs were defined as the top 10% of the genes in the each cluster ranked by betweenness centrality parameter in descending order. Peripheral nodes were defined as 1-degree nodes connected to a clique (a maximal complete subgraph where all vertices are connected). Superconnectors were defined as genes connecting more than 5 clusters.

Each cluster was further analyzed using the online tool EnrichR (E16). We analyzed enrichments for the human orthologs in the following gene sets: Gene Ontology Biological Process (GO.BP), KEGG Pathways and Human Atlas. Gene sets with p < 0.05 were considered significant. We also extracted expression values for each gene from the BioGPS (E17) dataset within 17 different that could tissues be represented in asthmatic lungs (http://biogps.org/downloads/): 12 related to leukocytes and 5 related to lung tissues. We used pheatmap R library [R package version 0.7.7. http://CRAN.R-project.org/package=pheatmap] to produce clustered heatmaps for genes in each cluster characterized in the network analysis (Supplementary Figures E3-E7).

Calculation of relative connection strength between clusters and domains

To assess the connectivity between different clusters as well as between biological domains, we processed the raw counts of genes and connections in each cluster and each domain. Briefly, we obtained a "connectivity ratio" for each cluster by dividing the number of connections found in each cluster by the square of the number of genes present in that cluster. We then used this "connectivity ratio" to normalize the number of connections of a cluster with another specific cluster by the connectivity ratio of the originating cluster. Finally, to obtain biological domain connectivity scores, we used the average of the connectivity scores of all clusters found within the biological domain.

Acute allergic asthma model

In all experiments, we used 8-10 week old female BALB/c mice (Charles River, Sulzfeld, Germany) provided with OVA-free food (SSNIFF, Soest, Germany) and autoclaved water *ad libitum*. All experimental protocols complied with the requirements of the Animal Care Committee of the Austrian Ministry of Science. All mice were immunized with 10 µg of ovalbumin (Sigma Chemical Co., St. Louis, MO) in 200 µl of PBS phosphate buffered saline (PBS) int-

raperitoneally (i.p.) on days 0 and 21 and then intranasally challenged on day 32 with (i) 100 μ g of ovalbumin in 50 μ l of PBS or (ii) 50 μ l of PBS (Supplementary Figure 8A).

Dexamethasone treatment protocol

For treatment, we administered dexamethasone (Sigma) at the dose of 1 mg/kg, 30 minutes before and 24 and 48h after ovalbumin challenge. Dexamethasone was first dissolved in DMSO (Sigma) and then diluted with PBS to a final concentration of 0.1 mg/ml (final concentration of DMSO was 5%). Control mice received intraperitoneal administration of PBS in the same treatment schedule. All solutions were administered to the mice in 10 ml/kg.

Airway hyperresponsiveness

At 24h after OVA challenge, we measured airway resistance and dynamic compliance in anesthetized and ventilated animals by Resistance and Compliance System (Buxco Electronics Ltd., Troy, NY, USA). Briefly, PBS, followed by increasing concentrations of methacholine (Sigma), was nebulized for 3 min and lung function was recorded and calculated using FinePoint software (Buxco Electronics Ltd.).

Airway inflammation

Seventy-two hours after intranasal OVA challenge, the lungs were washed with total volume of 1 ml of PBS to collect bronchoalveolar lavage fluid (BAL). The total number of cells in BAL was enumerated in an improved Neubauer hemocytometer and cytocentrifuged preparations (Cytospin-4, Thermo Shandon Corporation, Pittsburg, PA, USA) were stained with the Kwik-Diff (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA) to determine the percentage of inflammatory cells by morphological examination of at least 300 cells.

Lung inflammation and mucus hypersecretion

Lungs were dissected after bronchoalveolar lavage and fixed in paraformaldehyde. Paraplast-embedded lung sections of 3 µm were stained with hematoxylin and eosin (H&E) for morphological evaluation and with periodic acid-Schiff stain (PAS) for enumeration of mucopolysaccharide positive cells. Intensity of inflammation on the H&E stained slides was graded according to our semi-quantitative scoring system as described previously (E18). The number of mucus-containing cells per millimeter of basement membrane was determined with ProgRes CapturePro Software (Jenoptik, Jena, Germany).

Serum OVA-specific immunoglobulin

The measurement of OVA-specific IgG1 and IgE titers in sera was done by inhouse ELISA as described before (E18).

mRNA extraction and quantitative PCR

Separate groups of mice were used for lung mRNA extraction at 24 or 72h after intranasal challenge with PBS or OVA. Briefly, total RNA was isolated using RNeasy Mini Kit (Qiagen, Germantown, MD, USA) and the integrity of isolated RNA was confirmed using an Agilent 2100 Bioanalyzer and Agilent RNA 6000 Nano Assay Kit (Agilent Technologies, Pao Alto, CA, USA). After reverse-transcription with the Superscript III first-strand cDNA synthesis supermix kit (Invitrogen, Carlsbad, CA, USA), gene expression was analyzed using the SYBR green method for quantitative polymerase chain reaction (qPCR) on StepOnePlus real time PCR system (Applied Biosystems, Foster City, CA, USA). Collected data were analyzed the $\Delta\Delta$ CT method for which the geometric mean of threshold cycle (CT) values for hypoxanthine-guanine phosphoribosyltransferase (HPRT), β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control and mean of CT values from recovered mice was used as a reference sample. The list of primers (Invitrogen or Microsynth, Balgach, Switzerland) can be found in Supplementary table E1.

Correlation of microarray and qPCR data

Data from selected 59 genes with divergent fold changes were analyzed with qPCR and correlated to average microarray expression across 10 divergent microarray comparisons (A-J) used in this study. To include gene into analysis, its expression had to be detectable in control and asthmatic mice with qPCR; i.e. genes with Ct value of 40 in any group were excluded from analysis.

Statistical analysis

To compare airway hyperresponsiveness data, area under curve was calculated for each experimental animal on the graph of resistance or compliance (y axis) vs. the methacholine concentration (mg/ml; x axis). Before analysis, the values for the calculated area under curve for resistance and compliance, the airway inflammation data, as well as the mucus-positive cell counts in the lungs were log-transformed to equalize variances. Those data were analyzed with Student's *t*-test (for experiments with control and asthmatic mice) or with one-way ANOVA followed by Tukey's multiple comparison test (in experiments with dexamethasone treatment). Scores for lung inflammation were analyzed with Chi-squared test for trend. Pearson r correlation was used to analyze microarray and qPCR data correlation. Unpaired *t*-test was used to compare differences in gene expression determined with qPCR between control and asthmatic mice as well as vehicle-treated and dexamethasone-treated OVA-challenged mice. All analyses were done using GraphPad Instat v.5.0 (GraphPad Software Inc., San Diego, CA, USA) and p-values were considered significant at 0.05 threshold.

Supplementary tables

Supplementary table E1. List of primers (5' -> 3') used for chemokine expression analysis. Primer sequences were mainly selected from PrimerBank (<u>http://pga.mgh.harvard.edu/primerbank/</u>).

Gene	NCBI Gene ID	Forward primer	Reverse primer
HPRT	15452	TTGCTCGAGATGTGATGAAGGA	AAAGTTGAGAGATCATCTCCACCAA
β-Actin	11461	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC
GAPDH	14433	ACCCAGAAGACTGTGGATGG	CACATTGGGGGTAGGAACAC
ALAS1	11655	TCGCCGATGCCCATTCTTATC	GGCCCCAACTTCCATCATCT
ALPL	11647	GTGACTACCACTCGGGTGAAC	CTCTGGTGGCATCTCGTTATC
BAMBI	68010	CATTGCTGGCGGACTGATCTT	CTTGCCCCTTCTTGGAATGGT
CADM1	54725	GAACCAGCAGTTCACGATTCT	AGCAAGCATAGCATGGCAAAC
CD44	12505	TCTGCCATCTAGCACTAAGAGC	GTCTGGGTATTGAAAGGTGTAGC
CLEC5A	23845	TTATTGGTTTGGTACGTCAGCC	TGGTCCTGATTGGTAACATTGC
CTSS	13040	TAGAGGCAGACGCTTCCTATC	CGGGAGCTGAATGTACCTTGA
CXCL10	15945	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
CXCL9	17329	GGAGTTCGAGGAACCCTAGTG	GGGATTTGTAGTGGATCGTGC
DCK	13178	TGGCAAGCTCAAAGATGCAGA	AGAAGCGAAAATGTACCTGTCAC
ENPP1	18605	GAGTGTCCAGCAGAGTTTGAAT	CACCCCAGGTGTGCAAATACT
ERBB2	13866	GAGACAGAGCTAAGGAAGCTGA	ACGGGGATTTTCACGTTCTCC
HCLS1	15163	GGCCACGAGTATGTTGCTGAT	CCCTCTCAACTCCATATTTGCC
IFNγ	15978	ATGAACGCTACACACTGCATC	CCATCCTTTTGCCAGTTCCTC
IGSF6	80719	TAGAAGTGGACTACGGTTCT-	GTCTGCCTCATTTCTGCATCC
IL13	16163	GGATATTGCATGGCCTCTGTAAC	AACAGTTGCTTTGTGTAGCTGA
IL1β	16176	TTCAGGCAGGCAGTATCACTC	GAAGGTCCACGGGAAAGACAC
IL4	16189	ACTTGAGAGAGATCATCGGCA	AGCTCCATGAGAACACTAGAGTT
IL5	16191	CTCTGTTGACAAGCAAT-	TCTTCAGTATGTCTAGCCCCTG
IL6	16193	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
KLF4	16600	ATCCTTTCCAACTCGCTAACCC	CGGATCGGATAGCTGAAGCTG
LCK	16818	AACTTCGTGGCGAAAGCAAAC	CTGACCGACAGGGAAAAGGAC
MAD2L1	56150	GTGGGAAGAATCGGGACCG	CAGTCATTGACAGGGGTTTTGT
MUC5AC	17833	GTGGTTTGACACTGACTTCCC	CTCCTCTCGGTGACAGAGTCT
MUC5B	74180	AAGCTGCCCTACAGTCGTG	GACTAGGCGGATGCTGATTTT
NME1	18102	AGGAGCACTACACTGACCTGA	GGTTGGTCTCTCCAAGCATCA
NR1D1	217166	GCTCAGCGTCATAATGAAGCG	GGGCCGAATATACGTGGGT
NR3C1	14815	CCGGGTCCCCAGGTAAAGA	TGTCCGGTAAAATAAGAGGCTTG
PARD3	93742	ACATGCTGATACCGGATTGGA	CACTGAAAGGCACTACATGGATT
PER2	18627	CAGGTTGAGGGCATTACCTCC	AGGCGTCCTTCTTACAGTGAA
PER3	18628	TCAAGACGTGAGGGCGTTCTA	CATTCATACTGCGAGGCTCTTT

PLEK	56193	TTCAGAGGGGTTATCATCAAGCA	CCCAGCAGGATCATAGTAGTGC
PPARA	19013	TACTGCCGTTTTCACAAGTGC	AGGTCGTGTTCACAGGTAAGA
PRKCD	18753	TGGGGGTGACCTGATGTTC	CCAGCACCAACAATACCTGTAA
PRKCI	18759	CCACACTTTTCAAGCCAAACG	TGCACTTGTATCCTTGTCGTC
RELA	19697	AGCGCGGGGACTATGACTT	GCCCGGTTATCAAAAATCGGAT
RORA	19883	GATGACCTCAGCACCTATATG-	CGGGTTTGATCCCATTGATGTC
RRM2	20135	GGAAGCTCTGAAACCCGATGA	ACTTCTTGGCTAAATCGCTCC
SIGLEC5	233186	TGCACAGAAAATCGACACAGG	TCAGAGCTATCACAAGCACAGA
STAT1	20846	GCTGCCTATGATGTCTCGTTT	TGCTTTTCCGTATGTTGTGCT
TFF2	21785	CCTTGGTGTTTCCACCCACTT	AGCAGCAGTTTCGACTGGC
TGFBI	21810	TGTCCTGGATAT-	GGTGGTCGATCCCACAACTC
TIMP1	21857	GCAACTCGGACCTGGTCATAA	CGGCCCGTGATGAGAAACT
TK1	21877	AAGCTGCCTACACGAAGAGG	CGGCACACGGAGTGATACTT
TMEM17	72512	CTACATTGGGTACTTGCGGTT	GCACCACTGAGCATGTTGTTATG
TRAF6	22034	AAAGCGAGAGATTCTTTCCCTG	ACTGGGGACAATTCACTAGAGC
TYROBP	22177	GAGTGACACTTTCCCAAGATGC	CCTTGACCTCGGGAGACCA
VWF	22371	CTTCTGTACGCCTCAGCTATG	GCCGTTGTAATTCCCACACAAG

Supplementary table E2. Gene expression values and network properties of mouse genes in asthma-signature list and their human orthologs.

Supplementary Table 2. Table of mouse-human orthologs gene expression and network p

* Genes which human or ** Genes not in the first NiN: Not in Network Pumbed search was mad	thologs were not found in co-er 7 clusters with n<5 le using the formula: GENE AND r, stating that a logarithm fold ci o clusters 3 and 4 since human Human ortholog(*) #N/A	xpression matrix	s stract] or GEI	NE AND Immu	nity OR Inflame	hation[Title/	Abstract] (28t	th November 2	2014)										
nan means not a number Ear1 and Ear2 were set to	r, stating that a logarithm fold c o clusters 3 and 4 since human	hange of express orthologs of tho los	ion was not p le genes belo 21Fold-Chane	ossible to be o nged to differ e) of expressio	calculated ent clusters (RN on in Asthmatic	ASE3 in clust vs Control fo	ter 4 and RNA or individual n	GE2 in cluster	3) nparisons as	in Figure 1				,	Network properties		Total number of PUBMED hits fo	er search terms in Title/	Abstract for mouse and human genes
MGI Symbol 1100001G208ik	Human ortholog(*) #N/A	A B	0.14	0.384	E 1.285 nan	1	0.974	H 1.154	1.104	1 212		n Number NiN	HL N	bs Pe	NO	SuperConnector NO	"ASTHMA"	"IMMUP	NITY OR INFLAMMATION"
1190002H23Rk 1190002N15Rk 2210018M11Rik 2610524H06Rik	RGCC C3orf58 C11orf30*	-0.1815 -0.2295 0.0505 0.095	0.088 -0.1305 0.1855 0.055	-0.727 -0.7495 -0.206 0.515 1.6785	-0.782 -0.3397 -0.1257 0.541	-1.117 -1.072 0.028 0.173 1.126	-0.611 -1.098 -0.514 0.026 0.166	0.023 -0.584 -0.174 0.165	1.104 -0.623 -0.452 -0.128 0.102 0.141	-0.191 -0.246 0.17 0.254 0.856	0.028 ** -0.2267 ** -0.0877 0.07		4 N		NO NO NO	ND ND ND		0	1
	KIAA0101			0.515		0.173 1.126	0.026	0.189	0.102 0.141	0.254 0.856		NN	-4 N	0	NO	NO		0	0
A930001N09Rik Abca3	CREBRF ABCA3	-0.2073 -0.4517	-0.064	-0.3043	-0.394 -0.3513	-0.4415	-1.28	-0.314 -0.376	-0.415	-0.136	-0.262 ** -0.2647 1A		N		NO NO	NO NO		0	1
Abca8a Abcb1a Abi3bp	ABCB1 ABI38P ACAA1	-0.274 -0.1885 -0.3525 -1.356	-0.064 -0.2683 -0.299 -0.4315 -0.2305 -0.798	-1.001 -0.881 -1.4955 -1.844 -0.4355 2.172	-0.758 -0.65 -0.6925 -1.358	-0.4415 -0.521 -0.897 -0.83 -1.955 -1.013	-1.28 -0.072 -0.598 -0.018 -0.166 -1.344	-1.146 -0.269 -1.45 -1.617	-0.627 -0.363 -0.383 -1.051	-0.136 -0.016 -0.392 -0.291 -0.648 -0.627	-0.286 -0.3505 3A -0.6045 ** -1.398 3A	NIN	N N N	5	NO NO NO	ND ND ND		1	25
Abi3bp Acaa1b Ace Acp5	ACAA1 ACE ACP5	-1.356 -0.171 0.549	-0.798 -0.0585 0.147	-1.844	-1.358 -0.5775 1.426	-1.013 -0.765 1.715	-1.344 -0.539 0.715	-1.617 -0.213 0.864	-1.051 -0.335 0.754	-0.627 -0.52 0.592	-1.398 3A 0.237 3A 1.599 1B		N N N		ND ND	NO NO NO		1	4
Acsi4 Acyr1	ACSL4	0.5373	0.249	0.377	0.234	0.452	0.386	0.557	0.56	0.375			N	2	NO NO	NO NO		0	0
Acwr2b Acwr11 Adam17	ACVR28 ACVR11 ADAM17	-0.2285 -0.449 0.0837	0.249 -0.2095 -0.149 -0.159 0.063	2.172 0.377 -0.3335 -0.0925 -0.8055 0.5233 0.733 2.349	-0.412 -0.7105 0.1843	0.452 -0.601 -0.274 -0.9415 0.217	0.386 -0.171 -0.919 -0.236 0.499	0.557 -0.24 -0.397 -0.744 0.264	0.56 -0.39 -0.427 -0.447 -0.086	-0.034 -0.2 0.18	0.0783 3A -0.162 3A -0.07 3A 0.1423 3A		N	5	ND ND	NO NO NO		0	1
Adam17 Adam19 Adam8	ADAM17 ADAM19 ADAM8	0.0725	0.063 0.1185 0.86	0.5233 0.733 2.349	0.171	0.18	-0.114	0.264 0.49 1.238	-0.086 -0.108 1.412	0.18 0.1 0.397	0.4115		4 N N		YES NO	NO NO NO		3 0 16	64 7 19
Adamts2 Adcy4	ADAMTS2	0.2095 -0.331 -0.148 0.0827 -0.139	0.005	0.838 -0.63 -0.185 0.6313 -0.9	0.302	0.301 nan -0.557 -0.287 0.002 -0.568	-0.059	nan nan	nar	0.022	0.024 3A -0.209 1A -0.077 1A -0.173 1A -0.637 1A		N	2	NO NO	NO NO		0	6
Adcy6 Adcy7 Adcy8	ADCY6 ADCY7 ADCY8	-0.148 0.0827	-0.031 -0.033 -0.051 0.0103	-0.185 0.6313	-0.233 0.195 -0.613	-0.287 0.002	-0.558 -0.294 -0.151	-0.235 0.286 -0.243	-0.417 -0.661 -0.576	-0.037 0.035 -0.033	-0.077 1A -0.173 1A		N N N		NO NO NO	ND ND		0	2
Adcy9 Adh1	ADCY9 ADH1C	-0.2037		-0.550	-0.5688	-0.4535	-0.863	-0.327	-0.619	0.032	-0.074 3A		N		NO NO	NO NO		3	0
Adora2b Adra2a	ADORA2B ADRA2A	0.3125	-0.188 0.3095 1.6005 -0.079 0.0086 3.274 0.191 1.522	-0.439 0.3695 0.486 -0.405 -0.2612 1.743 -0.1857 -0.517	-0.139 0.4403 0.302 -0.54 -0.1388 2.255 -0.0713 -0.0713 -0.294	-1.021 0.514 0.983 nan -0.573 -1.263 2.133 0.13	0.699 nar	0.272 nan	0.398 nar	0.024	0.5837 1A 1.1045 1A		N		NO NO	NO NO		12 15	50 3
Adrb1 Agfg1 Agr2	ADRB1 AGFG1 AGR2 AHCYL2	-0.098 -0.0224 3.371 0.4577 1.432	-0.079 0.0086 3.274	-0.405 -0.2612 1.743	-0.54 -0.1388 2.255	-0.573 -1.263 2.133	-1.055 -1.015 1.464 1.097 0.222	-0.331 -0.331 2.319 0.718 0.085	-0.364 -0.057 0.702 0.818 0.098	0.11 0.057 2.097 0.473 0.659	-0.348 1A -0.344 ** 1.455 3A 0.5597 **		N	5	NO NO NO	NO NO		1	10 0
Agr2 Ahcyl2 Ahsp		0.4577 1.432	0.191 1.522	-0.1857 -0.517				0.718	0.818	0.473 0.659		NIN	N N N	0	NO	NO NO NO		0	2
Alf1 Ak2 Alatio	AIF1 AK2 AKTR	0.329	1.522 0.553 0.2495 -0.13 -0.152 -0.138 0.465 -1.313 -0.032	1.0465	0.510	0.743	0.642	0.65 0.32 -0.62 -0.672 -0.233 0.46	0.335 0.533 -0.291 -0.831 -0.543 0.259	0.715 0.464 -0.081 -0.043 0.006 0.479	0.783 18 0.8055		4 N	5	NO NO	NO NO		0	13 0
Alas1 Alas2 Aldh18a1	AKTIP ALAS1 ALAS2 ALDH18A1	-0.099 -0.2895 -0.378 0.548	-0.152	-0.761 0.029 -1.854 0.6523	-0.641 -0.199 -0.272 0.661	-0.322 -0.964 -1.157 1.096	-0.654 -0.683 -0.652 -0.758	-0.672	-0.831	-0.043	0.8055 0.039 ** 0.105 0.712 0.426 -0.125 3A 0.178 3A		2 Yi 2 N 4 N	s	NO NO NO	NO NO		0	1
Aldh18a1 Aldh3a1 Alox12	ALDH18A1 ALDH3A1 ALOK12	0.548 0.04 -0.409	0.465	0.6523 -1.596 -0.815	0.661 0.305 -0.028	1.096 -0.803 -0.381 nan	-0.758 -0.67				0.426 -0.125 3A		4 N N	5	ND ND	ND ND ND		0	0
Alox12 Alox5ap	ALOX15	1.474	0.995	-0.815 2.594 0.693 -0.059 -0.371 0.4275 -0.935 -0.935 -0.3025	0.154	2.166 0.691	0.328	0.697 0.584	-0.008	0.245	0.178 3A 0.423 3A 0.323 18 0.298 3A -0.156 38 0.0925 -0.0295 3A -0.5607 **		N	2	NO NO	NO NO		3 17 18	30 71 24
Alpi	ALPL AMOT AMPD2	-0.029 -0.212 0.237	0.995 0.248 0.47 -0.02 0.223	-0.059 -0.371	-0.349 -0.1183 0.177	2.166 0.691 -0.34 -0.168 0.371	0.322 -0.252 0.878	-0.034 -0.29 0.64	-0.008 0.258 0.051 -0.216 0.272	0.245 0.526 -0.182 -0.282 0.475	0.298 3A -0.156 3B		1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1	s	ND ND	NO		1	4
Amot Ampd2 Angp11 Ankrd44	AMPD2 ANGPT1 ANKRD44	0.237 -0.494 -0.1905	0.223 -0.2345 -0.0585	0.4275	0.177 -0.5285 -0.361	0.371 -0.804 -0.4495	0.878 -0.332 -0.34	0.64 -0.094 -0.144	0.272 -0.387 -0.591	0.475 -0.153 -0.157	0.0925 -0.0295 3A		4 N N		ND ND	NO NO NO		0 4	1 32
Ama5 Ama8*	ANKAS	-0.069	-0.009	-0.15 0.172	-0.118 0.7875	-0.4405 -0.447 0.251	-0.282	-0.249	-0.181	-0.067 0.403	0.139 34		2 N	2	NO NO	NO NO		7	13
Apex1 Apinr	APEXI APINR APINR APIP2	0.2853 -0.433 -0.1722	0.238 -0.3885 -0.1976	-0.3025 -0.15 0.172 0.565 -0.629 -0.4192	-0.118 0.7875 0.5443 -0.482 -0.287	-0.447 0.251 0.179 -0.48 -0.796	0.247 -0.929 0.079	0.233 -0.829 -0.831	0.343 -0.341 -0.307	-0.067 0.403 0.107 -0.239 -0.149	0.282 3A -0.2615 1A 0.2004 3A		N	5	NO NO NO	ND ND		0	8 2
Apip2 Apoe Aqp5	APOE AQP5	-0.1722 -0.515 -0.223	-0.1976 -0.505 -0.05	0.228	0.153	0.287	0.356	-0.831 -0.205 -0.855	-0.307 -0.205 -0.789	-0.149 -0.06 -0.237	0.2004 3A 0.022 3A -0.036 3A		N YI N	s	ND ND YES	NO NO NO		6	1 410
Ar Areg	AR ABS/C*	-0.171	0.051	-0.677 0.59	-0.397 0.695	-0.2105 nan -0.423	-0.877 nar 0.276			0.741	-0.5867 **		N	2	NO NO	NO NO		0 7	10
Arg1 Arg2 Adoptib	ARG1 ARG2 ARIGDIB ARISB	2.254 0.261 0.345 0.091	1.924 0.408 0.19 0.0287	-0.677 0.59 5.352 1.065 0.972 0.339	-0.397 0.695 0.172 1.021 0.781 0.2273	-0.2105 nan -0.423 5.222 1.225 0.72 -0.003	2.703 1.605 0.894 0.037	4.262 0.685 0.844 0.128	-0.348 1.222 0.906 0.382 0.176	2.784 0.2 0.768 0.169	4.087 1B 0.838 ** 0.467 1B 0.3917 **		N N N	2	NO NO NO	NO NO		18 8	42 20
Arhgdib Arl8b Arnt2						u.72 -0.003 0.254 nor	0.037	0.128 0.128	0.382 0.176 nar		0.3917 ** 0.366 3A		N	5	ND ND	NO NO		0 0	3 0 2
Arpc1b Ato1a2	ARPC1B ATP1A2	-0.0235	0.3335	0.573	0.3905	0.254 nan 0.883 -0.209	.0 547	0.638 -1.009		0.644	0.366 3A 0.4525 3A -0.0452 **		N N N	2	ND ND	NO NO NO		0	0 2
Atp2b2 AW112010	ATP282 #N/A	0.5595 0.233 0.159 -0.09	0.276	0.064	-0.413 -0.3317 1.153 -0.195 nan 0.297 0.393	0.216 0.574	0.484 0.641	-0.038 0.832	0.027	0.198 0.73	0.4757 0.621 -0.18 3A 0.684 1A	NN	4 N N N		NO NO NO NO	NO NO		0	3
Azgp1 82m 84gaint1	AZGP1 B2M B4GALNT1 BAMBI	0.373	-0.19 0.276 0.239 -0.326 0.01 0.219 -0.065	0.064 1.23 -0.694 0.538 1.161 -1.148	-0.195 man 0.297 0.393	0.337	0.204 0.726	0.37 0.016	0.19 0.318	0.22	0.684 1A 0.4745 3A		N	5	NO NO	NO NO		2	3 108 5
Bambi Basp1	BAMBI BASP1 BATF	-0.339 0.774 0.571	-0.065 0.685	-1.148 1.218	0.393 -0.634 1.763 0.664	0.67 -0.637 1.278	0.726 -0.805 1.341	0.016 -0.388 0.889	0.318 -0.681 0.843	0.265 -0.229 0.437	0.4745 3A -0.322 3A 1.336 ** 0.488 3A		N N N	5	ND YES ND ND	ND ND ND		0	2
Batt3 Brow 30	BATF BATF3 BCAP29 BCL10	0.571 1.375 0.772 0.1485	0.685 0.179 1.115 0.358 0.092	0.347	0.66 0.66 0.292 0.188	0.454	1.092	0.524	-0.681 0.843 0.839 1.145 0.563 0.192	0.281	0.488 3A 0.873 1A		N N N	5	NO NO NO	NO		1	10
Bcap29 Bcl10 Bcl3	BCL10 BCL3 BCL6	0.1485 1.052 0.2145	0.092 0.7 0.3	-1.148 1.218 0.347 1.804 0.949 0.2218 0.383 -0.001 - 1.92	0.188 0.802	1.278 0.454 0.941 0.559 0.1715 0.406 0.05	-0.805 1.341 1.092 1.427 0.636 0.857 1.759 0.451	-0.588 0.889 0.524 0.723 0.903 -0.002 0.648 -0.083	0.192 0.906 0.273	-0.229 0.437 0.281 0.61 0.572 0.151 0.282 0.141	0.488 3A 0.873 1A 0.801 ** 0.2475 1A 0.896 1A -0.244 1A		N	5	ND ND	NO NO NO		0	30 14
Bcló Bex2 Bhlhe40	BEX1 BHI HEAD	0.2145	-0.418	-0.001 -1.428	-0.599	-1.093	-0.372	-0.969	0.273	0.141	-1.274 3A		. N	2	ND YES ND	NO NO		2	40 0
Bid Birc3	BID BIRC3 BIRC5	-1.168 0.671 0.299 0.1995 2.022	-0.046 0.276 1.708	0.656	0.5305 0.388 2.281	0.796 0.542	0.806	0.807 0.644 0.311	-0.315 0.71 0.887 0.969 0.261	-0.344 0.36 0.466 0.482 0.9	0.336 3A 0.1095 1A 0.991		2 N N 4 N	2	NO NO NO	ND ND ND		1	15 30
Birc5 Birk	BIRCS BLNK COMMD3-BMI1	2.022 0.49 -0.1215		-0.001 -1.428 1.279 0.656 0.695 2.21 2.289 -0.3535	2.281 0.901 -0.226	-1.093 0.752 0.796 0.542 1.69 1.596 -0.853	0.806 1.638 0.446 0.714 -0.659	0.64	0.261 0.455 -0.321	0.9 0.83 -0.141	0.49 1A		4 N N	2	ND ND	NO NO		2	17
Bmi1 Bmp2k Bmp6	BMP2K BMP5	0.2643	-0.0945 0.1127 _0.23	-0.3535 0.5643	-0.226 0.343 -0.877	-0.853 0.419 -1.004	0.97	-0.1 0.615 -0.416 -0.267 -0.263 -1.94 0.249 -0.143	0.604	-0.141 0.551 -0.236	-0.359 ** -0.394 3A -0.239 3A -0.057 3A -0.1767 3A -2.122 ** 1.675 ** -0.1353 1A		N	2	NO NO	NO NO		0	2 0
Brrix Brrip3	BMX BNIP3 BNIP3L BPIFA1	-0.376 -0.225 -0.2513 -0.368	0.1127 -0.23 -0.381 -0.18 -0.1613 0.044	-0.3535 0.5643 -1.316 -0.855 -0.54 -0.3103 -2.167 1.372 -0.1525	0.343 -0.877 -0.644 -0.25 -0.23 0.335	0.419 -1.004 -0.719 -0.248 -0.504 -1.539	-0.441 0.061 -0.233 -0.493 -1.195 0.859 -0.399	-0.4 -0.267	0.604 -0.514 -0.453 -0.16 -0.177 -0.279	0.551 -0.236 -0.278 -0.062 0.031 0.58 1.584 -0.031	-0.239 3A -0.057 3A		N N	5	NO NO NO	NO NO		0	6 2
Bnip3l Bpifa1 Bpifb1	BNIP3L BPIFA1 RDIFR1	-0.2513 -0.368	-0.1613 0.044	-0.3103 -2.167	-0.23 0.335	-0.504 -1.539	-0.493 -1.195	-0.263 -1.94	-0.177 -0.279 1.201	0.031 0.58	-0.1767 3A -2.122 **		N		NO NO	NO NO		2	1114
Btrc Clqa	BPIFB1 BTRC C1QA	0.845 -0.1017 0.657	0.962 -0.028 0.561	-0.1525 2.682	1.2635 -0.1214 1.192	1.412 -0.171 1.928	-0.399		1.201 -0.157 0.208	-0.031 0.778			N N YI	s	ND ND	NO NO		0	10
C1qb C1qc	C108 C10C C18 C15	0.657 0.805 0.939 0.203 0.106	0.561 0.7667 0.739 0.126 0.067	2.682 2.9423 3.036 0.802 0.415	1.192 1.224 1.345 0.818 0.402	1.928 2.139 1.984 0.124 0.275	0.852 1.278 0.993 0.386 0.325	1.78 1.252 0.588 0.556	0.208 0.431 0.29 0.07 0.002	0.778 1.079 0.785 0.591 0.258	1.356 18 1.454 18 0.635 3A 0.094 3A		N N N	5	NO NO NO NO	NO NO		0	6
Ctra Cts	C18 C15 C3	0.203 0.106 0.918	0.126 0.067 0.612	0.802 0.415 1.381	0.818 0.402 0.599	0.124 0.275 1.68	0.386 0.325 0.826	0.588 0.556 1.057	0.002	0.591 0.258 1.081	0.635 3A 0.094 3A 1.131 1A		N		NO NO	NO NO		0	9 200
C3ar1 C4b	C3 C3AR1 C4B C5AR1	0.741 0.155 -0.469	0.612 0.6827 0.336 -0.184	1.381 2.9583 0.64 -0.221	0.599 1.9973 0.438 -0.103	1.68 2.254 0.433	0.826 0.784 0.225	1.057 1.115 0.41	0.638 0.627 -0.151	1.081 0.648 -0.126	1.131 1A 1.1583 1A 0.664 3A -0.002 1A		N N N	5	ND ND ND	ND ND ND		14	46 36
CSar1 Cadm1 Calca	CSAR1 CADM1 CALCA CAPG	-0.469 -0.0827 nan na	-0.0195	-0.221 -0.534	-0.103 -0.4698 -0.303 1.1205	0.433 -0.125 -0.362 -0.061 1.021	0.784 0.225 1.024 -0.312 0.226 0.403 -1.199 0.158	1.115 0.41 0.164 -0.56 0.542 0.612 -0.528 0.067	-0.151 0.184 -0.101 0.024 0.283	0.648 -0.126 -0.101 -0.21 -0.435 0.888 -0.351 0.294	-0.002 1A -0.033 1A		N	2	NO NO NO	YES		14 3	91 4
Capg Card10 Casp3	CAPS CARD10 CASP3	0.758	0.478 -0.129 0.1065	1.788 -0.454 0.8925	-0.303 1.1205 -0.459 0.3483	-0.061 1.021 -0.756 0.403	0.403	0.612	0.283	0.888	-0.002 1A -0.033 1A -0.0565 1A 1.302 -0.465 1A 0.121 3A		4 N 4 N Y	5	NO NO NO	NO NO NO		0	51 0 5
Casp3 Casp4 Cat	CASP3 CASP4 CAT	0.193 0.591 -0.4345	0.1065	0.83	0.3483 1.172 -0.4095	0.403	0.158 1.429 -0.384	0.067 0.549 -0.695	-0.704 0.01 1.194 -0.176 -0.194 -0.331 0.002 0.248	0.294 0.367 -0.217	0.467 1A		YI N YI	5	ND ND	NO NO		5	65 25
Cav1 Cav2	CAT CAV1 CAV2	-0.4345 -0.116 -0.1285	0.458 -0.299 -0.054 0.0035	-0.567 -0.551 -0.328	-0.4095 -0.409 -0.3325	0.579 -0.81 -0.575 -1.136 0.632 0.768	-0.384 -0.391 -0.513	-0.595 -0.238 -0.266	-0.176 -0.194 -0.331	-0.217 -0.212 -0.358	-0.492 3A -0.247 3A -0.245 3A		Y	5	NO NO	NO NO		9	19 51 0
Ccdc109b Ccdc80	CAV1 CAV2 CCDC1098 CCDC60	0.568	-0.1135	-0.567 -0.551 -0.328 1.196 0.664 -1.0795	-0.409 -0.3325 0.375 0.5655	0.632 0.768	-0.391 -0.513 0.171 0.378	-0.238 -0.266 0.622 0.776	0.002 0.248	-0.212 -0.358 0.418 0.377	-0.492 3A -0.247 3A -0.245 3A 0.417 ** 0.2565 3A -0.3635 **		N	5	NO NO NO	NO NO		0	0
Codc85a Codc99 Col1	SPDL1	-0.219 0.54 0.839	-0.105 0.291 0.874	-1.0795 0.898	-0.7165 0.952	-0.9 0.302 0.247 nan	-0.232 -0.152	-0.18 0.095	-0.021 0.157 nar	-0.285 0.017	0.361	NIN	N		ND ND	NO NO		0	0
Cd11 Cd12	CCL11 CCL8* CCL17 CCL2	3.343 1.964 2.538 1.788	2.318 1.605 1.85 1.314	0.898 0.537 2.14 1.913 0.765 0.975 0.798	0.38 nan 2.336 0.504 1.878		3.231 1.81 1.316 1.873	2.843 2.419 0.406 1.159	nar 2.42 0.688 1.798 1.464 0.434 0.539	2.307 1.516 0.633 0.557 -0.318 0.037	1.364 1A 1.263 1A 2.129 1A 1.013 1A		N	5	NO NO	ND NO		57 2	77 29
Cd17 Cd2	CCL17 CCL2	2.538 1.788	1.85 1.314	1.913	0.504	1.73 0.815 0.606	1.316 1.873	0.406	1.798 1.464	0.633 0.557	2.129 1A 1.013 1A		N	5	NO NO	NO NO		17 32	35 437
Cri20 Cri22 Cri24	CCL20 CCL22 CCL24	-0.121 1.359 2.584	0.166 0.548 1.985	0.798	0.414 1.013 0.127	0.54 0.856 1.411	1.143 0.31 2.043	0.206 0.322 1.966	0.539		0.423 1A 1.007 1A 1.478 1A		YI N	2	NO NO	NO NO		4	44 23 22
Cd3 Cd4	CC1313 CC14 CC15 CC15*	nan na 0.232	n 0.259 0.027 0.269	0.798 2.13 1.213 0.612 1.354 1.563	2.96 2.129 0.903 0.75	1.411 0.309 0.279 0.198 2.093	2.323 2.34 1.033 0.437	0.746 0.547 0.688 0.833	2.229 1.303 0.587 0.03 0.17	0.152 0.07 0.333 0.665 0.697 2.305	2.355 1A 0.397 1A 0.098 1A 2.3945 1A		N N YI	5	NO NO NO NO	ND ND ND ND		2	22 70 44 172 15
Cd5 Cd5 Cd7	CCL15* CCL15* CCL7;COL15-CCL14	-0.2 0.895 2.035	0.269	1.354 1.563 0.921	0.903 0.75 1.521	0.198 2.093 0.555	1.033 0.437 2.218	0.688	0.03 0.17 1.624	0.333 0.665 0.697	0.098 1A 2.3945 1A 0.477 1A		N N	5	NO NO	NO NO		51 3 8	172 15 42
Cc18 Cc19	CCL8*;CCL23 CCL15*;CCL15-CCL14;CCL23* CCNE1	2.484	1.628 2.388 1.2775 0.22	0.921 4.946 2.8215 0.3965	1.521 3.004 1.772 0.639	0.555 4.127 3.226 0.3165 nam	2.218 2.381 1.291	1.603 3.461 2.493	1.624 1.664 1.243		0.477 1A 2.732 1A 2.766 1A 0.157		N N 4 N	2	NO NO NO	ND ND ND		1 3	22 12
Cone1 Cor1	0081	1 011	0.731	1.4605	0.947	1.337	2.634	2.009	1.433	0.998	0.868.14			0	NO	NO		4	1 63
Cor1 Cor2 Cor5 Cor7 Cor9	CCR2 CCR2 CCR2 CCR7 CCR9	0.316 0.83 0.275	0.433 0.9665 0.082	1.0685 2.0477 0.803	0.295	0.3965 1.05 0.132	0.821 0.987 0.352	0.975 1.271 0.165	0.339 0.806 0.254	0.865 0.657 0.687	0.2425 1A 0.94 1A 0.364 1A 0.56 1A		N N YI	s	ND ND	NO NO NO		16 11 4	244 344 343 32 32 32 32 34 34 34 35 35 35 35 35 35 35 35 35 35 35 35 35
Cd14	CD14	nan na 0.551	n ni 0.565		1.731	0.4225 nan 0.831	nar 2.409			0.713	1.773		4 N 4 N	2	ND YES	NO NO NO NO NO NO NO NO NO NO NO NO NO N		1 67 1	24 207
Cd177 Cd2	CD103 CD177* CD2	0.238 0.427	1.088	1.201 0.803	3.046	0.381	2.229	1.404	1.576	1.105 0.721	1.922 1A 0.027 **		N	2	NO NO	NO NO		1 40	29
Cd177 Cd2 Cd209a Cd22	CD177* CD2 CLEC4M CD22	0.238 0.427 0.492 -0.1125	1.088 0.15 0.307 -0.2325	0.893 0.694 1.201 0.803 -0.148 1.195 0.681	3.046 0.463 -0.525 0.851	0.381 0.505 0.557 0.953 0.24	0.745 2.229 0.886 0.828 0.606 0.367	nan 0.582 3.094 1.404 0.462 0.291 0.506 0.253	nar 1.618 0.806 1.576 0.139 1.431 0.452 0.452	1.052 1.105 0.721 0.433 0.469 0.348	1.922 1A 0.027 ** 0.462 1A 0.0295 1A		N N N N	2	NO NO	NO NO		0	16 13
Cd247 Cd24a	CD247 CD24 CD274	-0.048 0.216 0.137	-0.093 0.152 0.129	0.0603		0.24 0.185	0.367	0.253 0.123 0.607	0.221 0.682	0.348			NNN		NO NO	NO NO		0	18 5
Cd28 Cd300a Cd300f Cd37	CD28 CD300A CD300LF CD37			1.347 0.9995 1.193 0.958 0.9535 -0.3325 1.561	0.431 0.1933 1.677 0.854 0.32 1.173 0.5995	0.185 0.377 0.197 0.4885 0.628 0.951	0.679 0.981 0.457 0.253 1.335 0.514 0.626 0.925	0.123 0.607 0.693 0.319 0.951 0.343 0.436 0.786	0.682 1.01 0.145 0.021 0.485 0.238	0.078 0.363 0.371 0.245 0.376 0.4 0.01 0.622	0.328 1A 0.558 1A -0.1385 ** 0.571 1B 0.804 1B -0.1065 1B		N N N	5	NO NO	NO NO		10	99 2
Cd300ff Cd37	CD300LF CD37	0.037 0.405 0.1415	0.119 0.465 -0.0525	0.958	1.173	0.628	1.335	0.951 0.343	0.485	0.376	0.804 18		N		NO NO	NO NO		0	57
Cd3d Cd3d	CD38 CD3D	0.0295	-0.1135	-0.3525 1.561 0.525	0.942	0.009	0.625 0.925 0.069	0.435 0.785 0.39	0.348	0.01 0.622 -0.137	0.305 1A		N N YI	s	NC NC NC NC NC NC NC NC NC NC	NO NO YES		7 0 11	142
Ci40 Ci44 Ci48 Ci52	CD40 CD44 CD48	0.3947 0.448 0.436 0.501	-0.025 0.2497 0.2603 0.329 0.42	0.525 0.682 0.8303 1.775 1.873	0.4957 0.6017 0.968 1.425	0.354 0.432 0.744 1.358 1.706	0.925 0.069 0.71 1.308 0.518 1.155 1.867 0.646	0.39 0.582 0.101 1.003 1.25 1.399 1.055	0.137 0.613 0.771 0.334 0.753	-0.137 0.523 0.479 0.64 1.023 0.924 0.997	0.02 1A 0.7317 1A 0.838 1A 0.87		11 11 11 11 11 11 11 11 11 11 11 11 11	s	NO NO	YES NO YES NO NO		8 5	186 119 8 1
Cd52 Cd52		0.436	0.329 0.42	1.775 1.873 1.395	0.968	1.358 1.706 1.295	0.518 1.155 1.867	1.003 1.25 1.999	0.334 0.753	0.64	0.838 1A 0.87 0.5245 18	NN	N	5	ND ND	NO NO		3 1	8
Cd53 Cd68 Cd72 Cd74	CD53 CD68 CD72 CD74	0.363 0.884 0.003 0.001	0.098 0.356 0.529	1.235	1.112 1.771 1.628 0.305	1.235 1.819 0.611	0.646	1.055 0.513	1.366 0.218 0.11	0.997	0.5745 18 2.234 1A 0.123 1A 1.148 1A		N N N	5	ND ND	NO NO NO NO NO		1	5 12 1
Cd74 Cd79b	CD74 CD798 CD89	0.001	0.356 0.529 0.08 -0.176 0.556 0.489	1.109	0.305	0.611 0.322 1.305 1.792 1.407	0.243	0.513 0.146 0.699 0.849 0.736	0.279	0.366	1.148 1A 0.047 1A		N	2	ND ND	NO NO		2	1 39 0 10
Cd79b Cd83 Cd84 Cd8a	CD798 CD83 CD84 CD84	-0.241 0.938 0.556 -0.076	0.556 0.489 0.024	2.114 1.529 1.109 1.196 1.899 1.429 0.6633 -0.123	0.634 0.979 0.659 0.6458	1.792 1.407 0.215	0.546 0.27 0.243 1.435 2.799 0.504 0.051 -0.856	0.849 0.736 0.27	0.218 0.11 0.279 0.74 2.123 0.348 0.008 -0.75	0.401 0.366 1.205 1.117 0.53 0.049 0.101	0.047 1A 0.957 1A 0.89 1B -0.0382 1A		N N N N		ND ND	ND ND ND		0 4	10 4 104
Cd8a Cd97 Cdc20 Cdc42ep3	CD8A CD97 CDC20 CDC42EP3	-0.076 -0.292 0.5767 -0.2135	0.024 -0.092 0.6163	1.3937	-0.482 1.435 -0.046	0.215 -0.531 0.782 -0.451	-0.029	0.27 -0.253 0.216 -0.44	-0.75 0.19 -0.393	0.101 0.433 -0.157	-0.0382 1A -0.144 ** 0.3117 -0.263 **		4 N			NO NO		0	8
Cato42ep3 Cato45 Catoa5	CDC42EP3 CDC45 CDC45	-0.2135 0.564 1.163	-0.0435 0.54 1.1025	-0.1395 0.639 1.1315	-0.046 0.893 1.7675	-0.451 0.314 nan 1.091	-1.135 0.108	-0.44 n nan 0.011	-0.393 nar 0.122		-0.263 ** 0.185 0.703		4 N 4 N		NO NO	NO NO		0	1
Cdo45 Cdo45 Cdo45 Cdb2 Cdb2 Cdb5	CDC45 CDC45 CDC45 CDH2 CDH5	0.564 1.163 -0.005 -0.2305	0.6163 -0.0435 0.54 1.1025 -0.132 -0.133	-0.1395 0.639 1.1315 -0.518 -0.6215 2.167	0.893 1.7675 -0.0035 -0.7095 2.245	-0.451 0.314 nan 1.091 -0.284 -0.73 0.959	0.108 0.067 0.079	-0.44 nan 0.001 -0.634 -0.34 -0.029	-0.189 -0.117	0.72 0.144 -0.994 0.399	0.185 0.703 -0.5165 3A 0.2625 3A		N	5	ND ND	NO NO		0	3
	CDK1 CDKN1A	1.565 0.219 0.7105	1.432 -0.0335 0.4475	2.167	2.245	0.959	0.057	-0.029 0.264 0.147	0.1 0.644 0.075	0.399 0.05 0.652	0.0535 3A		4 N 1	s	NO NO	NO NO		1	236
Cdkn1a Cdk1 Cenpe Cenpf Cep55 Cfd	CDT1 CENPE CENPF CEP55 CFD			1.142 1.268 1.655	-0.096 1.0545 1.473 1.082 1.7625 0.12	0.002 0.803 0.478 nan 0.753 nan 1.023 -0.38	0.415 nar	n nan	nar		0.479 0.285 0.17 0.792 0.443 38		- N 4 N 4 N	-	ND ND	NO NO		0 0	0
Cep55 Cfd Cfb	CEP55 CFD CFH	0.654 1.829 -0.753 -0.0425	0.912 1.374 -0.961 0.0075	-0.371 1.142 1.268 1.655 2.756 -1.671 -0.0515 0.946 -1.201	1.7625 0.12 -0.252 nan		-0.047 -0.425 0.207 0.06	0.027 -1.36 0.461 0.215	0.194 0.187 0.206 0.396	0.529 -0.523 -0.077 0.455	0.792 0.443 3B		4 N 4 N 4 N 4 N 4 N N N N		NO NO NO NO NO NO NO NO NO NO NO NO NO N	NC NC NC NC NC NC NC NC NC NC NC NC NC N		0	1
Cfi	CFH CFI CFP	0.745	0.0075 0.346 0.672		0.422 0.635	0.833	0.06		0.396	0.455	-0.281 3A 1.577 3A 0.676 1B		N	5	NO	NO NO		0 12	78 4 8 3
ch25h Chad	CH25H CHAD	2.74 0.997	2.411 0.841	1.355 0.675	2.226 0.826	1.039 2.038 0.152	2.523 0.894	2.178 0.267	0.502 2.077 0.493	0.554 1.631 0.464	0.676 18 2.502 ** 0.5 3A		N	2	ND ND	NO NO		1	3

Chaf1b	CHAF18	0.327 0.606 0.691 1.076 0.609 1.41	0.4155 0.167 nan nan	nan nan	0.257 1.499 3A	4	NO	NO	NO	0
Chaf1b Chi311 Chi313 Chi314	CHI3L1 #N/A #N/A	1.076 0.609 1.41 2.293 1.154 1.247 6.065 5.905 6.687	0.968 1.077 0.712 0.441 2.89 2.178	0.971 0.597 1.147 2.321 1.108 1.356 3.885 1.057 2.673	1.499 3A 1.199 Ni 7.699 Ni	N	NO NO NO	ND ND ND	NO NO NO	25 2 1
Chia Chit2	CHIA CHST2	2.738 2.22 2.391 -0.081 0.089 -0.111	-0.665 3.031 1.777	2.1 1.24 2.583	3.445 3A		NO NO	NO YES	NO NO	20 0
Cita Cish Cit	CIITA CISH CIT	-0.008 0.197 1.059 1.236 0.523 -0.144 0.236 0.035 0.262		0.137 0.31 0.459 0.581 0.966 0.32 nan nan	0.1435 1A 0.422 1A 0.0025	4	NO NO NO	ND NO	NO NO NO	2 1 0
Ckap4 Ckif Chun11	CKAP4 CKLF CKMT1A-CKMT18*	0.3558 0.34 0.608 0.2346 0.2052 0.4485	0.4677 0.644 0.303 0.456 0.194 -0.127 0.6105 1.017 0.838	0.442 0.342 0.38 0.263 -0.042 0.26 -0.033 -0.148 0.324	0.1263 **	2	NO NO	NO NO	NO NO	0 4
Cks1b Cks2	CKS1B* CKS2	1.0565 0.897 1.5795 0.904 0.869 1.7595	1.3525 1.606 0.168 1.618 1.338 0.48 .3388 6.819 6.718 .0.084 -0.115 0.286	0.617 0.356 0.964 0.004 0.27 0.378 6.414 4.778 5.875 0.028 0.185 0.522	1.2765 0.7695 0.4405 4.429 3C 0.416 1B 0.576 1B	4	NO NO NO	NO NO NO	NO NO	0
Cica3 Cidn10 Cidn18	CLCA1 CLDN10 CLDN18	-0.214 -0.021 -0.28	-0.489 -0.655 -0.271 -	6.414 4.778 5.875 0.028 0.185 0.522 0.891 -0.225 -0.093			NO	NO	NO NO	15 1 0
Cldn22 Clec10a Clec14a	CLDN24 CLEC10A CLEC14A	0.692 0.457 0.323 1.4 0.769 0.963 -0.477 -0.2025 -0.9555	0.303 0.264 nan nan 0.792 1.041 0.634 -0.667 -0.773 -1.226	nan nan 1.023 0.25 0.488 0.362 -0.561 -0.053	0.397 ** 0.546 3A -0.5195 1B		NO NO	NO NO	NO NO	0 1 0
Clec14a Clec4a2 Clec4d Clec4e	CLECI4A CLECI4A CLEC4D CLEC4E	-0.477 -0.2025 -0.555 0.419 0.3235 1.462 -0.036 0.106 0.16 0.94 -0.321 -0.422	0.792 1.041 0.634 -0.667 -0.773 -1.226 0.8485 1.048 0.177 2.548 -0.162 2.048 3.1725 nan 2.072	0.362 -0.561 -0.053 0.845 0.495 0.417 1.163 1.026 0.17 1.324 1.464 0.386	-0.5195 18 0.357 18 0.754 18 0.3275 18		NO NO NO	NO NO NO	NO NO NO	0
Clec4n Clec5a Clec7a	CLECGA* CLECSA CLEC7A	0.4635 -0.196 1.738 nan nan 1.171 0.543 0.61 2.632	2.4235 -0.143 0.839	0.685 0.497 0.427 0.515 0.731 0.154 0.835 0.862 0.661	0.2165 1B 1.436 1B 1.67 1B		NO NO NO	ND ND YES ND	NO NO NO	2
Clic1 Clic3 Clip4	CLEC7A CLIC1 CLIC3 CLIP4	0.0635 0 0.226	1.884 1.937 1.373 0.176 nan 0.411 -0.327 -1.164 -0.599 - 0.339 0.081 0.553 -	0.307 0.221 0.334	1.67 18 0.14 3A 0.535 ** 0.6157 **		NO NO NO	ND NO NO	NO NO NO	0
Clip4 Clin6 Cmtm6	CLIP4 CLN6 CMTM6	-0.138 0.044 -0.32 0.1195 0.2623 0.7357 -0.042 0.036 0.144 0.021 0.1225 -0.0005	-0.327 -1.164 -0.599 - 0.339 0.081 0.553 - 0.188 nan -0.085 - 0.048 0.211 0.325 -	-0.605 -0.665 0.078 -0.082 0.385 0.274 -0.111 -0.32 -0.026 -0.156 0.488 0.29	0.253	4	NO NO NO	NO NO	NO NO	0
Cmtm7 Cmtm8 Colla1	CMEMT	0.107 0.179 1.069			0.0565 1A -0.060 ** 0.5295 3A 0.1177 3A 0.111 3A		NO	NO	NO	0
Colla2 Colla1	CMTMB COLIA1 COLIA2 COLIA2	0.975 0.2575 1.376	-0.152 -0.212 -0.215 - 1.583 0.486 0.041 0.6893 0.817 0.239 1.3085 0.886 0.824	0.263 0.008 0.338 0.263 0.195 0.014 0.228 0.161 0.046 0.706 0.096 0.223 1.104 0.033 0.439	0.1177 3A 0.111 3A		NO NO YES NO	ND ND ND	NO NO NO	0 2
CoHa3 Col5a2 Col5a2	COL4A3 COL5A2 COL6A2	-0.2145 -0.1275 -0.4475 0.645 0.2105 0.974 1.0895 0.4795 0.61	0.872 0.495 0.27	0.279 -0.382 -0.461 0.885 -0.104 0.361 0.924 -0.086 0.415	-0.013 3A		NO NO	ND ND	NO NO	0
Col8a1 Coro1a Coro2b	COLBA1 CORD1A CORD2B	-0.147 -0.309 0.0023 0.2155 0.2525 0.9563 -0.164 -0.0325 -0.606	-0.136 0.372 -0.1 0.578 0.829 1.091 -0.454 -0.376 -0.3	0.609 -0.016 0.114 1.008 0.499 1.34 0.189 -0.155 -0.254	0.0837 3A -0.005 18 0.0605 **		NO NO	ND ND ND	NO NO NO	0
Cotl1 Cox7a1	COTL1 COX7A1	0.5186 0.3794 1.347 -0.015 -0.347 -1.148	-0.205 0.129 -0.018 -	0.857 0.319 0.888 -1.656 -0.161 0.479	0.4782 18 0.337 3A		NO NO	YES NO	NO NO	0
Cp Cpem1 Cr2	CPXM1 CR2	0.728 0.232 1.777 -0.749 -0.064 0.534	1.222 0.020 0.660	0.682 0.995 0.672 1.146 0.256 0.485 0.054 0.182 0.367 0.211 -0.279 0.086 0.538 0.445 0.211	0.5753 3A 0.73 ** -0.382 1A		NO NO	ND ND	NO NO	0
Creb3 Creb311 Creld2	CREB3 CREB3L1 CRELD2	0.663 0.242 0.669	-0.0565 -0.287 -0.309 - 0.435 0.366 0.33 0.218 1.31 0.11	0.772 0.147 0.513	-0.382 1A -0.0165 3A 0.465 ** 0.369 **		NO NO NO NO	ND ND ND ND ND	NO NO NO NO	0
Csf1 Csf2 Csf2ra	CSF1 CSF2 CSF2RA	0.1555 0.0735 0.2115 0.071 -0.067 0.417 0.488 0.253 1.293			0.361 1A		NO YES NO	NO	NO	3 26 1
Csf2ra Csf2rb Csf2rb2 Csf3rb2	CSF2RA CSF2RB CSF2RB CSF3R	0.483 0.253 1.293 0.8855 0.354 1.403 1.073 0.321 1.654 -0.308 0.157 -0.361	0.677 1.246 0.747 1.537 1.1865 0.93 1.739 1.134 0.863 0.073 0.312 0.987	0.392 0.008 0.062 1.91 1.007 0.693 1.272 1.086 0.863 0.934 0.537 0.052	0.4505 1A 1.284 1A 0.979 1A 0.342 1A		YES NO NO NO	ND ND ND	NO NO NO	6
Cstb Ctgf Ctla4	CSTB CTGF CTLA4	0.4385 0.244 1.5635 0.378 0.203 -0.178	1.0645 1.052 0.092	0.46 0.234 0.29 0.511 -0.985 0.177 0.4 0.281 0.057	0.9715 18 -0.336 3A 0.239 1A		NO NO NO	ND ND ND	NO NO NO	1 2 22
Ctsa4 Ctnnd1 Ctsa Ctsb	CTNND1 CTSA CTSB	nan nan 1244 -0.1313 -0.0513 -0.4323 0.197 0.1375 0.413 0.2392 0.064 1.1026	1.4 nan 1.507 -0.474 -0.46 -0.577 -0.338 0.4145 0.33 -0.5244 0.8015 0.598	0.4 0.281 0.057 -0.166 -0.192 -0.32 0.333 0.03 0.555 0.595 0.234 0.376	0.239 1A -0.0037 3A 0.418 3A 0.8488 3A		NO NO NO	ND NO NO	NO NO NO	22 0 0
Ctsb Ctse Ctsk	CTSB CTSE CTSK	0.2392 0.064 1.1026 -0.273 0.125 -0.275 1.596 0.547 2.681	0.208 0.773 0.885	0.611 0.191 -0.172	0.8488 3A -0.054 3A 2.805 3A		NO NO	NO NO NO	NO NO	0
Ctss	CTSS CTSZ CTTNBP2NL	0.603 0.346 1.62	1.017 1.708 0.616 1.1973 1.761 1.012 -0.5237 -0.744 -0.054	0.719 0.415 0.487 1.12 0.516 0.853			YES	NO NO	NO NO NO	3
Cttnbp2nl Cx3cl1 Cxcl1	C/3011 C/012	-0.09 0.1315 -0.434 0.9933 0.151 0.57	1.017 1.708 0.616 1.017 1.708 0.616 1.1973 1.761 1.012 -0.5237 -0.744 -0.054 - -0.202 -0.2345 0.004 - 1.4653 0.9115 3.442	0.719 0.415 0.487 1.12 0.516 0.853 0.186 -0.469 -0.112 0.434 0.045 -0.269 1.74 2.729 0.848	0.1057 1A -0.0677 ** 0.1055 1A 1.7617 1A		NO NO NO	ND ND ND	NO	4
Cxc110 Cxc111 Cxc112	CXCL10 CXCL11 CXCL12	2.19 1.766 2.312 nan nan nan 0.216 0.1063 0.4657	1.038 nan 0.21	0.786 2.666 1.114 0.11 0.802 0.343 0.149 -0.205 0.185	0.455 1A 0.12 1A 0.496 1A		YES NO NO	NO NO	NO NO	17 6 6
Cxc113 Cxc116 Cxc12	OK113 OK116 OK13 OK16	0.103 -0.2205 0.921 0.1545 0.257 1.422 nan nan nan	1.523 1.665 0.581 0.5525 0.728 0.132 4.528 nin 3.502 3.398 0.791 4.333	1.251 0.968 0.576 0.277 0.283 0.304 0.677 2.218 0.412 2.478 3.512 0.735	0.552 1A 0.4695 1A 2.657 1A 4.201 1A		NO NO NO	ND ND ND	N0 N0 N0	1 1 2
Card Card Card	CKC16 CKC19 CXC19	nan nan 0.694 3.072 2.339 2.7315		1.657 2.119 1.656			YES	NO	NO	2
Cxcr4 Cyba Cybb Cybrd1	CYBA CYBB CYBRD1	-0.48 0.073 0.675 0.355 0.222 1.27 0.403 0.1323 1.9503 0.205 0.089 0.3265 0.223 -1.649 -1.26	0.057 0.097 0.531	0.257 -0.912 -0.099 0.503 0.151 0.712 0.465 0.608 0.541	0.21 1A 1.188 3A 1.083 1B		NO NO	ND ND	NO NO	6
Cyp1a1 Cyp2b10	CYP1A1 CYP286		0.337 0.3415 0.51 1.8997 0.3115 0.5 -0.0155 0.78 nan nan -0.825 -0.205 -0.529 - -0.784 -1.06 -0.858 -0.7305 -0.919 -0.34	-0.222 -0.288 0.83 -0.64 -0.601 -0.173 -0.51 -0.301 -0.191	1.083 18 0.536 3A -0.16 3A -0.1113 3A		NO NO YES NO	ND ND ND ND ND	NO NO NO NO	7
Cyp2d22 Cyp2f2 Cyp4b1 Cyp4f18	CYP2D6*;CYP2D7P CYP2F1 CYP481	-0.3565 -0.2875 -0.8945 -0.372 -0.341 -1.023 -0.314 -0.082 -1.235	-0.376 -1.011 -0.391 -	0.444 -0.329 -0.047	-0.2475 3A -0.378 3A -0.498 3A		NO NO	NO NO	NO NO	3 1 0
Cyp4f18 Cyp7b1 Cyr61	CYP4F2 CYP7B1 CYR61	0.665 0.263 2.172 0.9005 0.6295 1.245 -0.0893 -0.547 -0.7838	0.684 1.489 1.404 1.0155 0.259 0.431 -0.4095 -0.4685 1.153	0.537 0.415 0.275 1.339 0.63 0.63 0.397 0.963 0.139	0.11 3A 0.7085 ** 0.1685 3A		NO NO NO	ND ND ND	NO NO NO	0
Cysltr1 Cyth3 D17H6S56E-5	CYSLTR1 CYTH3	0.364 0.438 0.2045 -0.2185 -0.214 -0.603 0.7915 0.7895 1.521	-0.425 0.46 nan nan -0.5725 -0.3875 -0.448 -	nan nan -0.469 -0.332 -0.026 -0.131 0.587 0.252	0.057 1B -0.545 ** 1.4505 Ni		NO NO NO	NO NO NO	NO NO NO	53 0
D17H6556E-5 Dab2 Dag1 Darc	0.00	0.7915 0.7895 1.521 0.1595 0.1575 0.2092 -0.24 -0.1653 -0.2687 0.129 0.412 0.558 -0.201 -0.248 1.705	1.5625 1.568 0.922 - 0.2485 0.131 1.016 -0.271 -0.566 -0.428 - 0.471 0.168 0.594 0.2315 -1.107 -2.253 -	0.131 0.587 0.252 1.104 1.018 0.325 0.223 -0.265 0.076 0.205 0.418 0.184 2.972 -1.32 -0.275	1.4505 Ni 0.2228 3A -0.1122 3A 0.247 ** -0.1075	N		ND ND ND ND	NO NO	1
Dop	DAG1 ACKR1 DBP DCK	0.3155 0.6135 0.874	0.926 0.68 0.463	0.77 0.194 0.703		2	NO NO NO YES	NO	NO NO	4 0 0
Ddk1 Ddit3 Ddx39	DCLK1 DDIT3 DDX39A	0.9735 0.481 0.7405 -0.1205 -0.0705 -0.0695 0.459 0.4372 1.0075	0.8382 1.006 0.116 -0.17 0.019 nan nan 0.7103 0.554 0.469	0.5 0.017 0.298 nan nan 0.766 0.44 0.697	0.32 3A 0.101 3A -0.0374 **		NO NO	NO NO	NO NO	1 1 0
Ddx39 Dk1 Dl4	DDX39A DLC1 DLL4 DOCK2	0.459 0.4372 1.0075 -0.3177 -0.4195 -0.5545 -0.3835 -0.0925 -0.4925 0.312 -0.435 0.8025	-0.4137 -0.504 nan nan -0.444 -0.784 -0.584 -	nan nan 0.658 -0.566 -0.379 0.572 0.215 0.543	0.32 3A 0.101 3A -0.0374 ** 0.0002 1A -0.1215 3A 0.114 1B		NO NO NO	NO NO NO	NO NO NO	1
Dock2 Dok2 Dsn1 Dusp1	DOK2 DSN1 DUSP1	0.647 0.833 1.281 nan nan 0.587 0.056 -0.569 -1.459	0.766 0.728 0.897 0.367 0.16 nan nan	0.702 0.586 0.607 nan nan -0.151 -0.581 -0.437	0.315 1B 0.136 -0.143 3B	4	NO NO NO	ND ND ND	NO NO NO	1
Ear1 Ear2 Ear3	RNASE2; RNASE3 RNASE2; RNASE3	-0.874 -1.172 -2.114 0.153 0.022 0.748 0.499 0.278 1.136	-0.54 -1.296 0.302 - -0.19 -0.823 -0.523 - -0.155 1.21 -0.047 -0.151 1.254 0.346	0.151 -0.581 -0.437 -0.378 -0.212 -0.398 0.445 -0.009 0.149 0.416 0.1 0.456	-2.09 1A;3A -0.05 1A;3A		NO NO NO	ND ND ND	NO NO NO	3 25 0
Ecsit Edem1	ECSIT EDEM1	0.166 0.065 -0.146 0.4515 0.3905 1.0065	-0.018 nan -0.394 - 0.4435 1.891 0.651	-0.102 -0.184 0.3 1.051 0.465 0.759	0.04 1A	N	NO NO	NO NO	NO NO	0
Efnd2 Efnb2 Egf17	EFHD2 EFNB2 EGFL7	0.224 0.2275 0.8095 -0.373 -0.169 -0.9887 -0.3117 -0.2567 -0.561 0.6175 -0.269 0.376 1.2275 1.1075 1.199	0.4035 0.516 0.574 -0.7007 -1.319 -0.763 - -0.4213 -0.728 -0.339 0.4225 1.116 0.257 1.251 1.539 0.852	0.343 0.448 0.348 0.383 -0.505 -0.375 0.416 -0.442 -0.226 0.136 0.783 -0.139 0.954 0.733 0.533	0.3025 3A -0.0418 3A -0.159 3A 0.94 3A 0.5825 1A		NO NO NO	YES NO NO	NO NO	0
Egin3 Egr2 Ehd4	EGLN3 EGR2 EHD4	0.6175 -0.269 0.376 1.2275 1.1075 1.199 -0.17 -0.165 -0.291	0.4225 1.116 0.257 1.251 1.539 0.852 -0.432 -0.562 -1.008 -	0.136 0.783 -0.139 0.954 0.733 0.533 -0.294 -0.318 -0.139	0.94 3A 0.5825 1A -0.006 3A		NO NO NO NO	NO NO NO NO	NO NO NO NO	0
Elfta Elf3 FIK3	EIF1AX ELF3 ELK3 EMILIN1	0.2185 0.26 0.4955 0.437 0.436 0.211 .0.3048 .0.205 .0.183	0.1385 0.214 0.611 0.635 0.284 0.551 - 0.2293 .0.195 .0.321	0.426 0.785 0.224 0.004 0.158 -0.129 0.221 -0.383 -0.056	0.204 ** 0.675 3A		NO NO	NO NO	NO NO	0
Elk3 Emilin1 Emp2	EMILIN1 EMP2 EMP3	-0.3048 -0.2705 -0.183 0.53 0.27 0.4055 -0.2527 -0.2227 -0.5683 0.468 0.203 1.067	-0.2293 -0.195 -0.321 - 0.157 0.237 0.408 -0.5463 -0.999 -0.267 - 0.608 0.474 0.371	0.121 -0.133 -0.056 0.183 0.194 0.119 0.523 -0.384 -0.173 0.682 -0.05 0.353	-0.1658 1A 0.193 3A -0.0553 3A 0.628 3A		NO NO NO	NO NO NO	NO NO NO	0
Emp3 Emr1 Eng	EMR1 ENG ENPP1	0.468 0.203 1.067 0.744 0.492 1.362 -0.2793 -0.2387 -0.6523 0.302 -0.063 0.733			0.698 18 -0.453 3A 0.373 **		NO NO NO	ND ND ND	NO NO NO	1
Eng Enpp1 Entpd1 Epas1 Epb4.1I4b	ENTPD1 EPAS1 EPB41L4B	0.302 -0.063 0.733 0.1557 -0.0333 0.2703 -0.196 -0.196 -0.8025 -0.275 -0.141 -0.885	-0.1723 0.392 0.563 -0.1723 0.392 0.563 -0.9195 -0.663 -0.257 -0.3107 -0.972 -0.492	0.372 -0.474 -0.121 0.55 0.267 0.233 0.428 0.19 0.245 0.925 -0.209 -0.756 0.576 -0.487 -0.299	0.0427 -0.0905 3A -0.086 **	4	NO NO NO	ND ND ND	NO NO NO	0
Ephx1 Ephx2	EPHX1 EPHX2	-0.372 -0.391 -0.693 -0.101 -0.207 -0.49	-0.613 -1.185 -0.493 - 0.137 0.05 -0.235 -	-1.115 -0.638 -0.23 -0.749 -0.079 0.013	0.031 3A		NO NO	NO NO	NO NO	1 5 1
Eps8 Epsti1 Erap1	EPS8 EPSTI1 ERAP1	0.2313 0.151 0.632 0.06 0.383 1.529 0.373 0.0725 0.4075	0.1703 0.366 0.31 1.2763 0.43 0.41 0.459 0.256 nan nan	0.366 0.186 0.285 0.386 0.341 1.071 nan nan	0.3253 3A 0.2145 1A 0.128 3A		NO NO NO	ND ND ND	NO NO	0
Erbb2 Errfi1 Esm1	ERBB2 ERRF11 ESM1	-0.185 -0.057 -0.096 -0.0795 -0.0655 -1.501 -0.815 -0.035 -1.058	-0.007 -0.219 -0.379 - -0.857 -0.99 0.787	0.285 -0.377 0.143 -0.37 -0.736 -0.184 -0.771 -0.331 -0.83	0.149 3A -0.0735 3A -1.211 **		YES NO		YES NO	2
Espl1	ESPL1	0.686 0.492 1.088	1.147 0.73 nan nan	nan nan 0.582 0.272 0.135	0.384	4	NO NO	NO NO NO NO NO NO NO NO	YES NO NO NO NO NO NO NO NO	0
Etv5 Evi2a Evi	ETV5 EV12A EVL	-0.1157 -0.3573 -0.1555 0.25 0.2945 1.6615 0.165 0.1123 0.9827	-0.0643 -0.2925 -1.665 0.8615 1.1295 0.803 0.2687 0.4975 -0.278	-0.171 -1.137 -0.03 0.887 0.413 0.892 0.243 -0.01 0.559	0.4857 3A 0.133 18 -0.0948 18		NO NO	NO NO	NO NO	0
Eya3 F10 F13a1	EYA3 F10 F13A1 F3	0.1655 0.242 0.167 1.1715 0.822 1.4705 0.476 0.253 -0.867 0.781 0.541 -0.144	0.2447 0.1155 -0.023 - 1.4617 2.031 0.99 0.483 -0.116 1.176 -0.443 0.301 0.273	-0.071 -0.094 -0.286 1.412 0.933 1.01 0.237 0.854 0.162 0.384 0.777 0.139	0.0377 1B 0.687 ** 0.472 3A		NO NO NO	ND NO	ND ND ND	1
F10 F13a1 F3 F5 F7 Fabp1	F3 F5 F7 FABP1	0.781 0.541 -0.144 0.8385 0.4335 1.264 0.757 0.672 2.02 -2.017 -1.5325 -1.768	0.483 -0.116 1.176 -0.443 0.301 0.273 -0.1705 0.924 0.667 1.019 1.6 0.356 -1.8455 -0.518 -0.442	1.411 0.854 0.162 0.237 0.854 0.162 0.384 0.777 0.139 0.405 0.199 0.022 0.879 0.134 0.775 -0.85 -0.138 -0.877	0.214 18 0.131 3A 0.823 3A -2.733 3A		NO NO	NO NO	NO NO NO NO	2 1 0
Fabp3 Extent	FABP1 FABP3 FABP4	-0.2145 -0.3495 -1.36	1.019 1.6 0.356 -1.8435 -0.518 -0.442 -0.192 -0.439 0.263 - -0.0155 0.388 -0.431 -	-0.85 -0.138 -0.877 -1.896 -0.245 0.042 -1.178 0.407 -0.131	-2.733 3A -0.0555 3A 0.9815 3A		NO NO	ND ND ND ND ND ND	NO NO	2
Fabp5 Faim3 Fam174b Fam49b Fas	EADINE			0.323 0.648 0.542			NO NO	NO NO	NO NO	0
Fam174b Fam49b Fas	FAIM3 FAM1748 FAM408 FAS	-0.828 -0.294 0.42 -0.118 -0.047 -0.652 0.263 0.232 0.819 0.046 0.116 -0.348	0.851 0.904 nan nan -0.336 -0.732 -0.469 0.4335 0.4455 0.731 -0.355 nan 0.762	-0.735 -0.711 -0.102 0.838 0.619 0.51 0.247 0.529 -0.002	1.264 3A -0.364 1A -0.22 ** -0.0285 ** -0.172 3A 0.954 1B		NO NO YES	NO NO NO NO NO	NO NO NO NO NO	0
	FCER1G FCER2 FCGBP	-0.122 -0.165 1.011 2.059 2.923 2.479	1.626 0.659 1.016 1.1735 1.24 0.569 0.321 3.303 1.363	0.998 0.49 0.49 0.547 0.744 0.527 1.465 0.691 1.420	1.078 1A		YES NO NO	NO NO	NO NO	12 15 0
Foer2a Fogbp Fogr1 Fogr2b	FCGR1A FCGR2B FCGR2B	-0.475 0.222 0.7505 1.641 0.903 1.8803 0.673 0.627 1.783	1.6265 nan 0.626 1.6395 1.4435 2.971 1.232 1.564 1.583	0.623 0.467 0.614 3.405 2.504 2.223 1.372 1.218 1.22	0.088 1B 0.8466 1B 1.07 1B		NO NO	ND ND ND ND ND ND	NO NO NO NO	4
Fogr3 Fen1 Fermt3	FERMT3	0.329 0.449 0.647 0.3817 0.294 1.1763	0.5225 0.715 0.597 0.6983 0.6765 0.563	0.22 0.202 0.72 1.037 0.252 1.317	0.3777 18	4	NO NO	NO NO	NO NO	0
Fetub Fga Fgf1	FETUB FGA FGF1	1119 0.193 0.537 0.775 0.181 nan -0.2853 -0.1497 -0.8617	0.112 0.721 -0.02 - -1.482 nan 0.693 -0.7387 -0.582 -0.474 -	0.342 0.56 0.321 -0.24 0.806 0.114 -0.626 -0.433 -0.111	2.166 3A 0.837 3A -0.4033 3A		115 10 20 20 20 20 20 20 20 20 20 20 20 20 20	NO NO NO NO	NO NO NO NO NO NO	0 1 0
Fga Fgf1 Fgf7 Fgffep1 Fgfr3 Fgfr4 Fgr Fig4 Fmo1 Fmo1 Fmo3	FGF1 FGF7 FGF8P1 FGFR3	-0.2853 -0.1497 -0.8617 -0.145 -0.2755 -0.7105 -0.596 -0.31 -1.334 -0.2245 -0.186 -0.6335	-0.7735 -0.803 -0.879 -	0.626 -0.433 -0.111 -0.02 -0.181 -0.166 0.917 -0.601 -0.426 0.379 -0.533 -0.137 -0.645 -0.618 -0.006	-0.4033 3A -0.234 3A -0.392 3A -0.0525 3A -0.0072 3A		NO NO NO	ND ND ND	NO NO	1 0 0
fgfr4 fgl2 fgr	FGFR4	-0.34 -0.0788 -0.5327					NO NO	NO NO		0 0 2
Fig4 Fmo1 Fmo3	FGR FIG4 FMO1 FMO3	0.291 0.184 0.325 0.4485 0.2875 0.6495 0.491 0.191 0.353 -0.107 -0.034 -1.311 -0.943 -0.64 -1.901	-0.42 -0.732 -0.304 -0.304 -0.325 -0.325 -0.345 -0.	-0.585 -0.085 -0.066 1.209 0.954 0.142 0.574 0.372 0.146 0.241 0.225 0.668 -0.114 -0.355 -0.082 -0.585 -0.764 -0.213	0.324 18 0.535 38 -0.359 3A -1.1 3A		NO NO NO	NO NO NO NO NO	NO NO NO NO	0
	FN1 FOLR2 FOVF2	0.253 0.47 1.1705 0.563 0.253 0.574 0.423 0.104 0.12			0.548 1B		YES NO	ND ND ND	NO	3
Folf2 Foxf2 Foxp1 Fpr2 Frmd6	FOXF2 FOXF1 FPR2 FRMD6	-0.451 -0.104 -0.13 -0.3555 -0.22 0.0216 -0.542 -0.101 -0.646 -0.171 0.083 -0.663	-0.179 -0.316 -0.901 -0.32 -0.164 -0.188 - 1.35 -1.32 1.113 -0.628 -0.586 -0.848 -	0.621 0.103 0.266 0.35 -0.543 -0.306 0.317 -0.383 0.087 0.892 0.669 0.167 0.269 -0.485 0.107	-0.221 ** -0.0794 1A -1.436 1A -0.502 **		NO NO	ND NO NO	NO NO NO NO	1
Frmd6 Fscn1 Fxyd4 Fyb Fzd2	FSCN1 FXYD4	0.2843 0.201 0.863 2.259 1.085 1.436	0.527 0.638 0.775	0.743 0.85 1.241 0.702 0.232 0.933	2.703 **		NO NO	NO NO	NO NO	1
Fytb Fzd2 G0x2	FYB F2D2 6052	-0.125 0.038 1.13 -0.2587 -0.2017 -0.276 -0.318 -0.47 -0.93 -0.065 -0.125 -0.0355 0.917 1.015 0.124	0.785 0.388 1.046 -0.3917 -0.205 -0.6 -0.325 -0.245 -1.27 -0.0275 nan 0.038 -0.036 1.224 2.379	0.67 0.551 0.303 -0.433 -0.618 0.036 -0.379 -0.351 0.128	-0.139 18 -0.1883 3A -0.161 3A		NO NO NO NO NO NO NO NO NO NO	ND ND	NO NO NO NO	1 0 0
G0x2 Gaa Gadd45g Gaint7 Gatm	G052 GAA GADD45G GAUNT7	0.4767 0.35 1.4153	-0.325 -0.245 -1.27 -0.0255 nan 0.058 - 0.036 1.224 2.379 0.6343 0.908 0.175	0.433 -0.551 0.128 0.453 -0.358 0.016 1.425 1.243 0.917 0.324 0.141 0.241 1.768 0.531 1.358	-0.161 3A 0.3625 3C 0.735 3A 0.5473 **		NO NO NO	ND ND ND ND ND	NO NO NO	0 1 0
Gatm Gbp1 Gbp2	GATM GBP3*-GBP1 GBP2	1.478 1.742 3.3 0.084 0.096 nan 0.104 0.221 1.541	1.024 1.68 0.496 1.281 nan 0.708	1.768 0.531 1.358 0.601 1.767 0.616 0.479 1.42 0.552	1.338 -0.116 ** 0.0205 1A	2	NO NO NO	NO NO NO	NO NO NO	0 1 0
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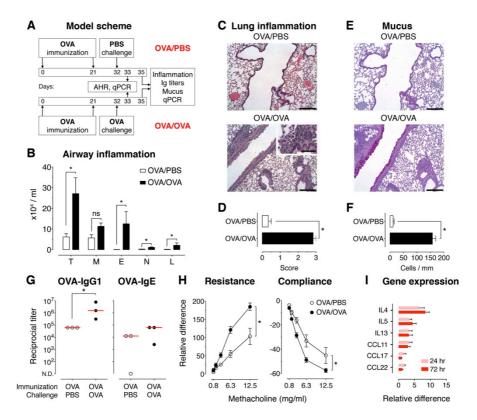
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Gbp3 Gbp8 Gclc	GBP4 GBP6 GCLC	0.104 0.094 1.156 1.598 nan 0.000 0.257 1.07 0.315 -0.403 1A NO -0.34 0.054 1.816 1.14 0.471 0.298 0.258 1.024 1.112 -0.479 JA NO 0.722 0.3735 0.5665 0.134 1.231 0.484 0 0.219 0.445 0.8055 JA NO	NO NO NO NO NO NO
Gda Ghr Glipr1	GDA GHR GUIRT1	0.252 0.2957 0.11 0.4017 0.67 0.549 0.2921 0.621 0.375 0.6001 3.8 NO -0.1853 -0.2867 -0.7063 -0.38 -0.214 -0.388 -0.217 -0.139 1.19 1.6 NO -0.574 -0.424 1.512 0.695 1.197 0.637 0.468 0.338 0.197 0.306 19 NO	NO NO NO NO NO NO
Glipr2 Glirx	GUPR1 GUPR2 GLRX	0.461 0.4915 0.51 0.57 0.317 0.832 0.507 0.789 0.392 0.345 18 NO 0.536 0.2015 0.867 0.5335 0.926 0.918 0.557 0.617 0.561 0.8365 3A NO	NO NO NO NO
Gm13889 Gpm6a Gpnmb	C11orf96 GPM6A GPNMB	0.393 0.43 0.001 0.069 0.333 1.537 0.724 1.067 0.003 0.058 ** NO -0.4295 -0.073 -1.002 -0.6005 -1.088 -0.3 -0.248 -0.208 -0.212 -0.2015 ** NO -0.455 -0.277 2.52 2.6665 1.5065 0.058 0.535 -0.077 0.669 2.093 3A NO	NO NO NO NO NO NO
Gpr125 Gpr3sp1	GPR125 GPRASP1	0.455 0.277 2.92 2.0665 13065 0.058 0.515 0.007 0.069 2.093 3A NO 0.1165 0.184 0.078 0.456 0.542 0.358 0.393 0.654 0.004 0.0065** NO 0.423 0.284 0.933 0.1113 0.506 0.313 0.317 0.213 0.354 0.2055 3A NO 0.2007 0.264 0.3647 0.563 0.685 0.264 0.422 0.621 0.044 0.2055 3B NO	NO NO NO NO NO NO
Gpsm1 Gpx1	GP5M1 GPX1		NO NO NO NO NO NO
Gpx2 Grb14 Gria1	GPX2 GRB14 GRA1		NO NO
Gria1 Gsta2 Gsta3	GRIA1 GSTA5* GSTA3	47775 4.659 4.055 4.061 4.022 4.021 4.121 4.212 M NO 4.002 3.034 -1.240 4.654 4.051 4.212 M NO 4.002 3.034 -1.240 4.654 4.051 -5.024 4.009 4.012 4.018 NO 4.0465 4.2055 -1.636 4.057 -1.64 4.009 4.012 4.018 NO 4.0456 4.2085 -1.636 4.048 -1.22 4.641 4.075 -4.048 4.021 4.018 NO	NO NO NO NO NO NO
Gstm1 Gsto1 Gucv1a3	GSTM1 GSTD1 GUCY1A3	0.999 0.651 0.646 0.4805 0.73 0.541 0.058 0.42 0.094 0.5955 3A NO	NO NO NO NO NO NO
Gucy1a3 Gucy1b3 Gusb	GUCY183 GUS8 HUA-DQA1 HUA-DQ82	0.618 0.291 1.299 0.709 1.058 0.294 0.539 0.034 0.507 0.8545 3A NO	NO NO NO NO NO NO NO NO YES NO
H2-Aa H2-Ab1 H2-DMa	HLA-DMA		NO NO
H2-DMb2 H2-Eb1 H2-M3	HLA-DMB HLA-DRB5	Collist Open Table State Logs Logs Collist Collist <td>NO NO ND NO NO NO NO NO ND NO ND NO ND NO</td>	NO NO ND NO NO NO NO NO ND NO ND NO ND NO
H2-Oa H2-Ob	HIA-G* HIA-DOA HIA-DOB	4070 0.029 6.01 0.50 0.338 0.388 0.347 0.019 0.196 -0.011 h. NO 4.584 -0.55 1.316 0.577 0.651 0.518 0.277 h. NO 4.644 -0.211 0.755 0.413 1.646 and Tam Am 0.073 h. NO 4.649 -0.211 0.755 0.413 1.646 and Tam Am 6.466 0.273 h. NO 4.12 1.257 0.279 for m Tam Am Am<	NO NO NO NO
H2-Q7 H2-Q8 H2afx	HLA-A* #N/A H2AFX	-0.28 0.142 1.247 0.892 1.049 0.75 0.75 0.468 0.017 -0.549 0.4 NO -0.142 0.125 1.179 1.079 nan nan nan nan nan nan nan NiN NO -0.69 0.669 0.041 0.231 0.456 nan nan nan nan nan NiN NO	NO NO
H2afz Hamp	H2AF2 HAMP	0.3125 0.3565 0.9525 0.617 0.553 0.46 0.093 0.503 0.328 0.3763 4 YES -0.1375 -0.747 -0.755 -0.6595 -0.46 1.432 -0.675 0.065 0.087 0.0455 3A NO	NO NO NO NO NO NO
Hc Hck Hcls1	C5 HCX HCLS1	0.271 0.28 0.372 -0.089 0.486 0.48 0.435 0.089 0.037 0.473 IA NO 0.129 0.164 0.642 0.909 man 0.335 0.481 0.319 0.217 0.119 IB NO 0.066 0.343 1.207 1.056 1.035 0.956 0.751 0.548 0.594 0.845 IB YES	NO NO NO NO NO NO
Hdac5 Hdc Hexb	HDACS HDC HEXB	-0.082 -0.225 0.043 -0.306 -0.2795 -0.385 -0.584 -0.306 -0.005 -0.212 IA NO	NO NO NO NO NO NO
Hey1 Hey1 Heyl	HEY1 HEYL	0.100 0.099 0.144 0.79 0.731 1.532 0.229 0.41 0.248 0.712 No 0.186 0.279 1.582 0.593 1.387 0.513 0.714 0.248 0.712 No 0.386 0.271 0.484 0.471 0.471 0.248 0.725 No 0.31 0.271 0.484 0.402 0.416 1.197 0.44 0.417 0.277 0.314 No 0.212 0.419 0.402 0.416 1.197 0.44 0.417 0.277 0.314 No 0.212 0.717 0.184 0.020 0.414 0.116 0.247 0.217 0.314 No 0.222 0.217 0.318 0.020 0.444 0.18 0.247 0.314 No	NO NO
Hfe Hist3h2a Hix	HFE HIST3H2A HLX	0.1225 0.177 0.388 -0.052 0.414 0.185 0.246 0.391 0.362 0.069 3A NO -0.211 -0.163 -0.078 -0.3125 -0.199 -1.412 -0.105 -0.691 -0.312 -0.0157 ** NO -0.33 0.002 -0.182 -0.368 nam 0.024 -0.442 0.05 -0.22 0.315 ** NO	NO NO NO NO NO NO
Hmgb2	HMGB2	0.3075 0.297 1.425 0.7137 0.503 0.228 0.393 0.224 0.313 0.181 4 NO	NO NO ND NO ND NO ND NO ND NO
Hingb3 Himox1 Hin1 Hoxb5	HMGB3 HMOX1 HN1 HOX85		NO NO
Hpgd Hpx Hspala	HPGD HPX HSPA1A		NO NO NO NO NO NO
Hspa1b	HSPA1A HSPA1B HSPB1	α 3975 0.0212 α.74 4.5376 1.34 1.480 3.283 4.5335 4.5355 M. NO 0.695 6.2218 1.15 2.225 4.255 3.66 4.5355 M. MO 0.696 6.2218 1.15 2.225 4.255 3.66 4.556 4.575 MA MO 0.7867 0.383 4.785 4.725 0.256 5.466 4.565 0.671 1.556 M MO 0.7867 0.383 4.786 4.725 0.246 4.566 0.571 1.557 M NO 0.2026 5.2055 4.021 4.225 4.226 0.426 0.566 0.681 0.534 M NO	NO NO NO NO NO NO
Hspb1 Htt Hvcn1	HTT HVCN1	0.2413 0.2003 0.1673 0.0115 0.151 nan nan nan nan -0.2015** NO	NO NO NO NO NO NO
icam1 id3 ido1	ICAM1 ID3 ID01	0.101 0.083 0.111 0.045 0.342 0.735 0.023 0.057 0.256 0.413 M Y5 0.201 0.083 0.111 0.045 0.342 0.735 0.023 0.057 0.256 0.413 M Y5 0.266 0.415 0.50 0.55 0.815 0.149 0.252 0.384 0.255 0.143 M N5 0.266 0.055 0.143 M N5	NO NO
Ifi2712a Ifi30	#N/A IFI30	-0.245 0.23 1.54 2.138 -0.133 0.529 0.613 0.14 0.43 0.057 NN NO 0.924 0.595 1.806 0.879 1.243 0.677 1.119 0.116 0.88 0.947 1A NO	NO NO NO NO
ifi47 ifitm1 ifng	#N/A IFITM1;IFITM2;IFITM3 IFNG	-0.113 0.291 1.09 1.393 0.254 1.176 0.45 1.569 0.65 -0.083 NiN NO 0.56 0.36 0.07 0.973 0.060 0.693 0.699 0.458 0.296 0.296 NiN NO	NO NO NO NO NO NO
ifngr2 igf1	IFNG IFNGR2 IGF1	0.349 0.156 0.45 0.4215 0.719 0.641 0.254 0.628 0.287 0.329 IA NO 0.84 0.2897 1.7572 1.1313 1.391 0.74 0.991 0.078 0.336 1.4527 3A YES	NO NO NO NO
igf1r igfbp3 igfbp6	IGFBP3 IGFBP6	-0.313 -0.1375 -0.5393 -0.429 -0.1895 0.238 -0.323 -0.167 -0.344 -0.4298 3A NO	NO NO
lighig lighim	an/A IGHM	-0.408 1.284 2.86 0.204 0.969 0.567 0.934 0.877 0.064 0.475 NiN NO 0.092 -0.2316 2.5359 0.091 2.9055 4.191 5.361 1.666 0.822 0.2462 ** NO	NO NO ND NO ND NO ND NO ND NO
40 lgk lgkv15-103	IGJ #N/A #N/A	0.2e5 - 0.2e05 1.9e4 0.29 2.78 1.677 2.311 1.44 0.94 0.2e55 NiN NO 1.12 - 1.729 2.483 - 0.653 1.577 2.005 1.388 0.933 0.034 1.066 NiN NO	NO NO NO NO
igkv6-15 iglv1 igsf6	#N/A #N/A IGSF6	1.017 0.187 2.541 0.409 2.744 2.507 1.05 0.614 0.1612 NN NO 0.2655 0.181 2.527 2.512 2.512 2.512 2.512 0.816 0.142 NN NO 0.2655 0.181 2.527 2.512 2.512 0.816 0.142 0.78 NN NO 0.2044 0.109 1.111 1.415 0.423 1.229 0.8 0.49 0.548 0.0056 18 NN NO 0.012 0.3 1.407 1.56 0.423 1.219 0.8 0.49 0.348 -0.0056 18 NN NO	NO NO NO NO NO NO NO NO
lgtp	IRGM #N/A	0.010 1.111 1.435 0.425 1.129 0.8 0.09 0.54 -0.096 10 175 0.012 0.3 1.487 1.56 0.287 1.331 0.393 2.051 0.972 0.051 1A NO 0.4085 0.501 2.312 1.834 0.301 2.25 1.161 2.576 1.324 0.0205 NiN NO	NO NO NO NO
ligp1 110 113	IL10 IL13	nan nan nan nan 0.199 1.453 0.4555 0.468 0.212 nan 1A NO	ND NO NO NO NO NO
#16 #18 #18bp #18r1	IL16 IL18 IL188P IL1881	μα μχ μχ <thμχ< th=""> μχ μχ μχ</thμχ<>	NG NG NG NG NO NG NG NG NG NG NG NG NG NG NG NG NG NG
1114	IL18R1 IL1A IL18		NO NO
111b 111f5 111r1	IL36RN IL1R1	0.962 -0.539 -0.224 0.1925 0.147 -0.099 -0.446 0.079 0.009 0.0575** NO	NO YES NO NO NO NO
ilirii Ilirn Ilira	ILIRL1 ILIRN IL2RA	nan nan nan 0.7155 0.185 0.433 0.445 0.38 0.115 0.185 1A NO	NO NO NO NO NO NO NO NO
112rg 1133	11.28G 11.33	0.216 0.1115 0.7255 0.711 0.449 0.535 0.605 0.313 0.514 0.125 IA NO 1.307 0.739 1.348 0.806 1.499 1.707 1.223 1.799 0.82 1.507 IA NO nan nan nan nan 0.463 1.312 0.254 0.37 0.18 0.092 IA NO	NO NO
14 14ea 116	IL4 IL4R IL6	0.0925 0.1085 0.1703 0.1397 nan 1.56 0.879 0.965 0.386 0.2897 1A NO nan nan nan 1.82 0.374 3.516 0.343 0.818 0.006 1.121 1A YES	NO NO NO NO NO YES
II7r Incenp Inmt	IL7R INCENP INMT*	0.2025 0.3615 2.649 1.363 1.173 0.893 0.917 0.288 0.165 0.147 IA NO 0.677 0.813 0.5805 0.4753 0.1975 nan nan nan nan 0.332 4 NO 0.293 0.108 1.128 0.429 0.866 -0.333 0.552 0.376 0.253 0.202 3A NO	NO NO NO NO NO NO
Inpp5d Inpp11 Irf4	INPP5D INPPL1 IRF4	C. 154 C. 155 C. 155 C. 155 C. 156 C. 177 C. 166 18 NO -0.143 -0.255 0.255 0.255 0.451 0.413 NO 0.105 0.107 0.106 18 NO -0.143 -0.256 0.257 -0.261 -0.417 -0.103 0.107 0.108 18 NO -1.143 -0.276 0.276 -0.274 and -0.203 0.107 0.108 18 NO -1.257 1.026 0.798 0.411 A NO 0.411 NO	NO NO NO NO NO NO
irf5 irf7	IRF5 IRF7	0.505 0.285 1.316 0.714 0.577 0.624 0.37 0.411 0.384 0.342 1A NO 0.047 0.353 1.314 3.102 -0.164 0.749 0.825 0.372 0.441 0.142 1A NO	NO NO NO NO
irf8 irg1 iram1	IRF8 IRG1 IRGM*	0.0965 0.0945 1.3275 0.7065 0.604 0.473 0.244 0.487 0.684 0.275 IA NO nan nab 1.396 4.384 0.383 2.274 0.651 1.389 0.022 0.841A NO 0.04 0.263 1.51 1.515 0.374 1.592 0.488 0.6851A NO	NO NO ND NO NO NO NO NO ND NO ND NO
irg1 irgm1 irs1 itga4	IRS1 ITGA4	-0.102 -0.019 -0.76 -0.482 -0.657 -1.41 -0.359 -0.816 -0.158 -0.349 3A NO 0.1423 0.0617 0.5197 0.061 0.2465 nan nan nan nan -0.2868 IA NO	NO NO NO NO NO NO
itga7 itga8 itgai itgam	ITGA7 ITGA8 ITGAL	0.425 0.134 0.72 0.641 min 0.437 0.178 0.573 0.22 0.625 3A NO -0.427 -0.257 -1.08 -0.657 -1.159 -0.25 -0.858 -0.494 -0.24 -0.385 3A NO	NO NO NO NO NO NO
itgam itgax itgb1	ITGAM ITGAX ITGB1	-0.024 -0.025 0.4425 0.4495 0.138 hun man man man man man multi5.34 NO 0.864 0.225 1.442 1.056 0.446 1.122 0.988 0.531 0.263 0.651 NO 1.469 0.682 2.597 1.539 2.245 0.539 0.75 0.121 0.768 1.739 18 NO 0.4085 0.020 0.217 0.1355 0.217 0.102 0.276 0.038 0.279 0.1155 3. YES	NO NO NO NO NO NO NO NO NO NO NO NO YES NO
Iteb1bo2	ITGB1BP2 ITGB2	0.372 -0.42b -0.852 -0.087 man -0.055 -0.083 -0.072 0.038 -0.349 3A NO 0.489 0.15 1.643 1.23 0.957 0.774 0.838 0.242 0.489 1.149 18 YES	ND NO YES NO NO NO NO NO
itgb2 itgb7 itgb1 itln1	ITGB7 ITGBL1 ITLN1	0.033 0.111 0.467 0.454 0.229 nan nan nan nan 0.010 JA NO 0.337 -0.255 -0.552 -0.542 -0.654 40.051 and nan nan 0.664 NO 3.802 0.788 1.124 3.53 3.05 4.604 4.526 4.527 0.394 1.568 JA NO 0.2196 -0.073 -0.424 -0.464 -0.529 nan nan nan nan -0.277 0.394 1.568 JA NO	NO NO ND NO ND NO ND NO ND NO
Itpata Jak2	ITPKB JAK2		NO NO NO NO NO NO
Junb Kank3 Koni15	JUNB KANK3	0.402 0.087 0.363 0.037 0.394 1.676 0.278 0.94 0.255 0.304 4 NO -0.2567 0.2727 0.732 0.689 0.722 -1.489 0.415 0.3669 0.075 0.3017*** NO	NO NO
Konj15 Konj8 Konk2	KCNU15 KCNU8 KCNK2	1.2927 0.2767 0.2007 -0.4687 0.219 -1.119 0.2611 -0.2649 0.2469 -0.2409 NO 4.114 0.015 -0.214 -0.12 -0.007 -0.4687 0.246 -0.217 -0.4687 NO -0.136 0.224 -0.471 -0.468 -0.276 -0.216 -0.217 -0.4687 NO -0.356 0.224 -0.471 -0.468 -0.276 -0.216 -0.218 -0.214 -0.214 NO	NO NO NO NO NO NO
Kdr Kif22 Kitl Kif15	KDR KIF22 KITLG KLF15	-0.344 -0.017 -0.921 -0.382 -0.388 -0.477 -0.58 -0.414 -0.25 -0.418 5A YES 0.881 0.678 1.1987 1.4483 0.311 nan nan nan nan 0.3803 4 NO 0.7016 0.0157 0.6592 1.073 1.016 0.56 0.450 0.450 0.450 0.450 0.000	NO NO NO NO NO NO
KH15 KH2 KH4 Krt19	KLF15 KLF2 KLF4 KRT19	Qarges Qarray Qaray </td <td>NO NO ND NO NO NO NO NO NO NO NO NO NO NO</td>	NO NO ND NO NO NO NO NO NO NO NO NO NO NO
Lag3	LAG3	nan nan 0.364 0.431 0.426 0.124 0.612 -0.013 0.19 0.021 IA NO	NO NO NO NO NO NO
Lair1 Lama3	LAIR1	0.2495 0.26 0.878 1.076 0.315 0.256 0.526 0.355 0.325 0.488 ** NO 0.176 0.174 0.461 0.329 0.826 0.402 0.708 0.376 0.2488 0.0515 3A NO 0.988 0.486 1.156 0.61 0.698 0.413 0.096 0.454 0.992 0.994 3a NO	NO NO ND NO ND NO ND NO ND NO ND NO ND NO
Lap3 Laptm5 Lat	LAP3 LAPTMS LAT	0.246 0.25 0.276 0.255	NO NO NO NO
	LATZ	0.153 0.245 1.527 0.4845 0.4065 nan nan nan nan nan 0.2523 18 NO -0.0135 0.0975 0.396 0.2115 -0.122 0.18 0.143 0.367 0.21 0.4695 1A NO -0.151 0.157 1.010 1.07763 0.0755 0.691 1.1666 0.373 0.872 -0.164 1A YES	NO NO NO NO
Lbp Lck Lcn2 Lcp1	LCN2 LCP1 LCP2	031 0357 108 0.794 0.795 0.897 1.066 0.79 1.072 2.944.9. Y15 1.646 0.497 1.090 1.091 2.049 1.011 1.012 2.019 M 1.646 0.491 1.021 2.049 1.021 1.039 0.039 M M 0.641 0.641 1.021 0.049 1.021 0.049 1.021 0.059 M M M 0.641 <t< td=""><td>NO NO NO NO NO NO</td></t<>	NO NO NO NO NO NO
Lepr Lepr	LEPR LGALS1	-0.108 -0.151 -0.9165 -0.6383 -1.0965 -0.074 -0.096 -0.221 -0.441 -0.12215 IA NO 0.835 -0.4885 -1.056 -0.6875 -1.225 -0.317 -0.698 -0.115 -0.915 -0.803 3A NO	NO NO NO NO NO NO NO NO
Lgals3 Lgals3bp Lgmn	104152	0.734 0.33 1.585 1.082 1.773 0.215 0.366 0.091 0.374 1.312 3A NO 0.275 0.282 1.327 1.608 0.225 0.391 0.526 0.113 0.409 0.783 3A NO	NO NO NO NO
Likb3	LGALS38P LGANN LIG1 LILRB3*		NO NO NO NO NO NO NO NO
Limch1 Lime1 Lims2	UMCH1 UME1 UME2	-0.2713 -0.1965 -0.6873 -0.2658 -0.653 -0.355 -0.254 -0.48 -0.078 -0.4402 ** NO 0.0777 -0.1283 -0.232 -0.129 -0.027 nan nan nan -0.657 18 NO -0.27 -0.207 -0.75 -0.751 -0.876 -0.793 -0.558 -0.669 -0.061 -0.125 ** NO	NO NO NO NO NO NO
Linis2 Lipa Litaf Limib1	LIMS2 LIPA LITAF LIMNB1	d.2713 d.218 d.819 d.819 d.213 d.24 d.01 d.027 MO 0.717 d.219 d.816 d.718 d.836 d.666 d.816 d.716 d.917 d.917 </td <td>NO NO NO NO NO NO</td>	NO NO NO NO NO NO
Lpin2	LMNB1 LPIN2 LPXN	0.2025 0.2535 1.009 0.4425 0.52 1.079 0.021 0.512 0.241 0.5955 4 NO -0.173 -0.012 -0.647 -0.8085 -0.337 0.019 -0.281 -0.279 -0.379 -0.418** NO -1.101 0.887 2.385 1.451 1.74 1.578 1.212 0.881 0.99 1.007.18 NO	NO NO NO NO
Lpan Lrg1 Lrmp Lrrc33 Lst1	18/01	0.775 0.301 1.712 1.230 0.692 0.75 0.997 0.774 0.420 1.069.24 NO	NO NO NO NO
Lrrc33 Lst1 Ltb	LRMP NRROS LST1* LTB	0.249 0.125 1.33 0.593 0.795 0.813 0.755 0.138 0.521 0.179.91 NO 0.207 0.546 0.948 0.838 0.736 0.631 0.779 0.529 0.671 0.35* NO -0.005 0.11 0.358 0.838 0.786 0.648 0.545 0.11 0.199 0.699 NO -0.409 0.099 1.323 0.807 0.628 0.525 0.14 0.199 0.699 NO	NG NG NO NO NO NO NO NO
Ltbp4	LTBP4	-0.429 0.039 1.523 0.872 1.186 0.028 0.752 0.194 0.862 0.172 M NO -0.225 0.159 -1.103 0.98 1.285 0.4453 0.453 0.445 0.411 0.155 3A NO 0.012 0.35 0.743 2.307 0.065 0.667 0.394 0.654 0.943 0.397 JA NO	ND NO NO NO NO NO
Ly86 Lyst Mad211	LY86 LY87 MAD2L1	0.644 0.415 1.69 1.543 0.963 1.141 0.056 1.04 0.604 H NO 0.694 0.437 0.491 1.343 0.963 1.141 0.056 1.04 0.604 H NO 0.095 0.0255 0.247 0.3417 nan 0.522 0.437 0.35 0.28 0.144 NO 0.798 0.6042 1.5 1.072 0.513 0.202 0.433 0.211 0.341 4 YES	NO NO NO NO NO NO
Mafb Malat1 Man1a	MAFB #N/A MAN1A1	0.477 0.349 1.116 0.4575 1.372 2.222 1.25 1.191 0.657 0.6115 18 NO -0.2944 -0.1426 0.0352 -0.5992 -0.3105 0.214 -0.829 0.044 -0.165 -0.547 NiN NO	NO NO NO NO
Manf Map2k3	MANE	0.555 0.305 0.404 0.357 0.070 0.300 0.555 0.405 0.557 0.535** NO	NO NO NO NO NO NO
Map4k1 Mapt Marcks11	MAP2K3 MAP4K1 MAPT* MARCKSL1	0.55 0.0115 0.027 0.1255 0.041 -0.138 -0.165 0.046 0.275 M. NO 0.222 0.331 1.164 0.654 0.71 0.167 0.128 1.04 NO 0.424 0.233 1.164 0.654 0.71 0.037 0.128 1.04 NO 0.448 0.2058 1 0.026 0.265 0.144 0.00 0.288 1.05 0.122 3.04 NO 0.4447 0.061 0.960 1.255 0.226 1.255 0.226 1.052 0.168 0.422 3.4 NO 0.4447 0.061 0.960 1.255 0.226 1.255 0.226 1.055 0.165 0.222 3.4 NO	NO NO NO NO NO NO NO NO
Marco Mcm2 Mcm3	MARCO	0.3265 -0.553 -0.449 1.7605 -0.043 -0.191 0.846 0.538 1.085 1.0995 18 NO	NO NO NO NO NO NO
Mcm4 Mcm5	MCM3 MCM4 MCM5 MCM5	0.5573 0.4662 0.4133 0.6903 0.529 0.155 0.155 0.518 0.527 0.295 4 NO 0.955 0.3735 0.4565 0.4135 0.391 0.391 0.155 0.312 0.203 4 NO 0.955 0.3715 0.456 0.415 0.391 0.467 0.251 0.719 0.312 0.033 4 NO 0.7165 0.217 1.254 1.75 0.45 0.407 0.255 0.155 0.450 0.033 4 NO	
Mcm6 Mcm7 Me1	MCM6 MCM7 ME1	0.7425 0.667 13.465 0.791 0.93 -0.122 0.032 0.051 0.809 0.0365 4 NO 0.3167 0.4975 0.7355 0.5785 0.568 -0.588 -0.134 -0.128 0.64 0.1447 4 NO 0.514 -0.343 0.5665 0.0455 1.086 0.061 -0.066 0.305 0.52 1.248 ** NO	NO NO NO NO NO NO

Mef2a	MEF2A	-0.1863 -0.1108 -0.304	-0.27 -0.406 0.075	-0.249 -0.307 -0.066	-0.4175 3A	NC	NO	NO	0
Meis1 Metrol Metrol	MEFV MEIS1 METRNL METTL7A CLEC10A	0.22 0.554 0.046 -0.1497 -0.1073 -0.673 0.67 0.4865 1.0015 0.352 0.215	0.708 0.233 0.931 -0.488 -0.5405 -1.88 0.2565 0.605 1.058	0.623 0.643 0.359 -0.293 -0.67 -0.213 0.738 0.464 0.754	0.12 1A -0.615 3A 0.413 **	NC NC NC	NO NO NO	NO NO NO	2 0 0 0
Mgl2 Mif Mki67 Mmp12 Mmp19	MIF MKI67 MMP12 MMP19	1.65 1.695 1.13 0.3 0.089 0.644 1.568 1.557 2.636 2.554 1.99 6.505 1.015 0.14 2.3745	0.301 1.002 0.95 0.54 0.429 -0.066 2.092 1.732 0.812 5.083 4.892 1.575 0.8545 1.116 0.077	0.615 0.619 1.006 -0.122 0.012 0.427 0.591 0.55 0.976 1.051 0.324 0.477 0.876 0.294 0.254	1.255 3A 0.632 1A 0.429 5.549 3A 1.3215 3A	4 NC 4 NC NC	NO NO	NO NO NO NO	1 7 3 11
Mmp8 Mpa2i Mpp6 Mrc1	MMP8 GBP6 MPP6 MRC1	-0.107 0.145 -0.36	1.022 0.118 2.018	0.665 1.128 0.08	0.214 3A	NC	NO NO	NO NO	2 0 0 4
Ms4a4b Ms4a4c Ms4a4d Ms4a6b	#N/A #N/A #N/A MS4A6A:MS4A6E	-0.237 0.199 1.445 0.164 0.5225 0.436 -0.447 -0.299 -0.744	0.345 0.649 0.341 0.136 1.405 0.838 0.788 0.293 1.337 2.416 0.457 0.734 -0.149 -0.553 -0.25 1093 0.32 0.89	0.311 0.527 0.57 1.671 0.9 0.958 0.724 0.679 1.303 0.253 1.042 1.073 -0.635 0.611 1.069	0.416 3B 1.079 1B -0.719 NN -0.241 NN -0.739 NN -0.411 18	NC NC NC NC	NO	NO NO NO NO	1 0 0
Ms4a6c Ms4a6d Ms4a7	MSAAGA,MSAAGE MSAAGA,MSAAGE MSAA7 MSAA8	0.344 0.46 0.352 1.3865 1.71 2.261 0.405 0.613 3.001	1.093 0.32 0.89 1.591 0.662 0.542 2.1415 2.106 2.21 2.225 1.387 0.901 0.417 0.945 0.583 0.074 nan -0.211 1.4215 0.950 0.938	0.876 0.611 1.069 0.999 0.681 0.534 2.366 1.859 1.774 0.74 0.296 0.301 1.043 0.298 0.301 -0.113 -0.078 0.32	-0.401 18 0.228 18 1.7805 18 1.477 ** 1.75 18 -0.028	NC NC NC NC 4 NC	ND ND ND ND	NC NC NC NO NO	0
Ms4a8a Msh2 Msr1 Mt1 Mt2	MSH2 MSR1 MT1A*;MT1E MT3Y*;MT1E1,MT1H*	0.2267 0.2263 1.1087 0.245 0.1575 0.1515 0.722 0.557 0.646	0.4 0.423 1.52	1.012 0.597 0.303 0.813 0.809 0.191 1.548 1.05 0.528	0.541 3A	NC NC	NO NO	NO NO NO	0 1 0
Mt2 Mthfd2 Mtpn Muc4 Muc5ac Muc5b	MTHFD2 MTPN MUC4 MUCSAC	0.8845 0.622 0.995 0.0874 -0.0948 0.2602 0.4845 0.7655 1.2225 2.779 2.772 3.685	1.1695 0.862 -0.664 0.1792 0.011 0.225 1.3335 0.159 nan nan 0.794 1.63 1.631	0.404 0.273 0.442 0.422 0.217 0.606 nan nan 1.247 0.234 0.539	0.665 0.0113 1A 0.8015 3C 1.563 **	4 NC NC NC	NO YES NO NO	NO NO NO	0 0 3 34
Myc Myc1 Myd88	MUCS8* MYC MYCT1 MYD88	1283 1.535 1.96 0.685 0.641 0.351 -0.346 -0.2885 -0.546 0.350 0.332	0.991 1.444 1.298 0.434 nan 1.184 -0.5805 -0.395 -0.901 0.658 nan 1.24	1.24 0.655 0.876 0.338 0.516 0.229 -0.393 -0.28 -0.077 0.45 0.789 0.215	1.287 3C 0.307 3A -0.062 ** 0.422 1A	NC NC NC	NO YES NO	NO NO NO	11 2 0 24
Myh7 My44 Myo1b Myo1f Myo1g	MYH7 MYL4 MYO18 MYO1F	-0.356 -0.198 -0.284 -0.179 -0.303 -0.743 -0.273 -0.1268 -0.6556 0.302 0.247 1.5563 0.45 0.285 1.285	0.562 -0.905 -0.068 -0.021 -0.663 -0.135 -0.6296 -1.1005 -0.451 -0.751 0.8945 nan nan 0.669 0.736 0.433	-0.713 -0.323 -0.076 -1.4 -0.766 0.265 -0.295 -0.289 -0.094 nan nan 0.568 0.188 0.722	-1.002 38 -0.194 38 -0.3466 3A 0.3057 18	NC NC NC	NO NO YES NO	NO NO NO NO	0
Myo1g Myo5a Myocd Myo2 Ncf1	MY01G MY05A MY0CD MY022 NCF1	0.45 0.285 1.285 0.4547 0.39 1.9847 0.412 0.615 0.151 -0.085 -0.938 -1.704 0.6913 0.167 1.515 0.524 0.383 1.315	0.669 0.736 0.433 0.778 0.929 0.66 -0.042 0.812 0.217 -0.239 -0.416 0.039 1.0567 1.107 0.318 0.841 1.141 0.954	0.588 0.388 0.722 0.669 0.263 0.368 0.147 0.099 0.437 -1.065 -0.355 0.376 0.213 0.129 0.359 0.649 0.385 0.536	0.183 18 0.908 ** 0.01 18 -0.629 38 0.446 18 0.653 18	NC NC NC	NO NO	ND ND ND ND ND	0
Ncf2 Ncf4 Nckap11 Ndc80	NCF2 NCF4 NCF4 NCF4P1L NDC80	0.524 0.383 1.315 0.507 0.29 1.194 0.403 0.4445 1.448 nan nan 0.655	0.841 1.141 0.954 1.286 1.282 1.041 0.549 1.158 0.476 0.811 0.346 nan nan	0.649 0.185 0.536 1.24 0.64 0.851 0.78 0.417 0.465 nan nan	0.653 1B 0.401 1B 0.4865 1B 0.352	NC	NO NO	NO NO	2 1 0
Ndrg1 Ndst1 Nest1 Nesk2 Nesk5	NDRG1 NDST1 #N/A	-0.1482 -0.0874 -0.0886 -0.2777 -0.3927 -0.5647 -0.252 -0.057 -0.0890 0.402 0.502 0.9595 0.473 0.405 0.822	0.2358 nan 0.781 -0.5543 -0.414 0.198 -0.467 -0.779 -1.65 1.525 0.461 nan nan	-0.129 0.14 0.169 -1.003 0.328 -0.331 0.073 -0.724 -0.354	0.334 0.0668 3A -0.0105 3A -0.356 NIN 0.2333 0.6537 **	4 NC NC NC 4 NC	ND ND ND ND	NC NC NC NO NO	0 2 0 0
Net1 NRb2 Mbbis	NEK2 NEK6 NET1 NEK82 NEK8A	-0.327 -0.211 -0.416 0.326 0.1545 0.42 0.2026 0.346 0.3204	-0.414 -0.38 -0.467 0.314 0.394 0.655	0.345 0.474 0.121 -0.698 -0.312 -0.281 0.164 0.417 0.376 0.192 0.646 0.302	0.574 3A 0.4455 1A	NC NC	NO NO	NO NO NO	0 0 3 4
Nikbie Nikbiz Niacr1 Nme1	NFKBIZ NFKBIZ HCAR2 NME1	0.7185 0.382 1.4665 -0.1733 -0.0317 -0.0333 0.145 -0.114 0.349 0.3715 0.3295 0.504	0.0236 nan 1.176 1.243 0.9485 0.889 0.445 0.2 2.16 1.634 0.151 1.185 0.364 0.604 0.161	0.549 0.762 0.34 1.023 1.022 0.197 0.651 0.577 0.241 0.463 0.247 0.654	1.025 1A 0.0827 1A 1.08 ** 0.2875	NC NC 4 YES	NO NO NO NO	NO NO NO	0 0 0
Notch1 Npr2 Npr3 Npy Nr1d1	NOTCH1 NPR2 NPR3 NPY	-0.153 0.0885 -0.3675 -0.075 -0.073 -0.47 -0.3 -0.2883 -1.3077 -0.47	-0.505 -0.59 0.27 -0.647 -0.566 -0.309 -1.0633 -1.263 0.168	-0.102 -0.367 -0.337 -0.249 -0.555 -0.239 -1.002 0.02 -0.097	-0.335 3A -0.301 3A -0.4873 3A	YES NO NO	NO NO	NO NO NO	3 0 3
Nr2f1 Nr2f1 Nr2f2	NR1D1 NR1D2 NR2F1 NR2F2	-0.711 -0.421 0.54 -0.005 -0.2585 1.12 -0.073 0.002 -0.574 -0.2984 -0.1686 -0.1177	-0.289 -1.336 -0.532 -0.2007 -1.15 -0.768 -0.154 nan nan nan -0.3308 -0.606 -1.501	-0.357 -0.451 -0.173	0.13 -0.0737 -0.868 ** -0.421	2 NC 2 NC 2 NC 2 NC 2 NC	NO NO NO NO NO	NO NO NO NO YES	0 0 0
Nr3c1 Nrp1 Nrp2 Nt5dc2 Ntrk2 Nuak2	NR3C1 NRP1 NRP2 NTSDC2 NTSDC2	-0.1517 -0.1268 -0.408 -0.1967 0.077 -0.1613 0.0737 -0.0367 0.5415 -0.099 -0.14 -0.409	-0.1942 -0.763 -0.536 -0.3947 -0.47 -0.527 0.2103 0.3145 0.205 -0.453 -0.559 -0.684 0.1882 0.029 -0.006	-0.286 -0.13 -0.187 -0.008 -0.194 -0.172 0.126 0.361 0.129 -0.429 -0.989 0.195 0.022 -0.025 -0.056	-0.1468 1A -0.3315 3A 0.1027 3A 0.335 **	NC	NO NO	NO NO	28 1 0 0
Nut2 Nup37	NTRK2 NUAK2 NUF2 NUP37 NUPR1	-0.099 -0.14 -0.409 -0.199 -0.14 -0.409 -0.1295 -0.241 0.001 -0.43 0.287 0.175 -0.394 1.028 1.522 -0.425 -0.1045 0.0265	0.303 0.352 nan nan 1.257 0.411 nan nan 0.408 0.203 nan nan	nan nan nan nan nan nan	0.335 ** 0.372 3A 0.516 ** 0.572 -0.083	NC NC 4 NC 4 NC	NO NO	NO NO NO NO	2 0 0
Nupr1 Nusap1 Oasl2 Olfm1	NUSAP1 #N/A OLFM1	1252 1.232 1.639 -0.036 0.374 1.11 0.5323 0.4103 0.6117	-0.1505 0.594 -0.395 0.7905 0.922 0.054 2.312 -0.362 0.325 0.248 0.684 0.826 1.251 1.309 -0.249 2.068 nam 0.59	-0.604 0.258 -0.485 0.103 0.022 0.308 0.655 0.316 0.392 0.516 0.784 0.687 0.326 0.303 0.236 1.143 1.084 0.708	0.456 3A 0.395 0.009 NIN 0.2297 ** 1.736 1A 1.223 3A	4 NC 4 NC NC NC NC	ND ND ND	NO NO NO	0
Oir1 Orm1 Osmr Otub1 92rx1	OLR1 ORM1;ORM2 OSMR OTUB1 P2RX1	0.2355 0.3435 0.245 0.094 0.1685 0.1045 0.4285 0.291 0.4995	0.0935 -0.232 1.197 0.0505 0.044 0.008 0.0145 0.504 0.159	0.49 0.957 0.288 -0.976 0.281 -0.072 0.211 0.042 0.28	0.474 1A 0.453 **	NC NC NC	NO	ND ND ND ND ND ND ND	2 0 1 0
P2ry14 P2ry6 Papss2 Pard3	P2RY14 P2RY16 PAPSS2 PARD3	0.485 -0.011 1.248 0.613 0.451 2.057 -0.3257 -0.293 -0.3363 -0.109 -0.1342 -0.4457	0.959 0.953 0.06 0.935 1.47 1.551 -0.6793 -0.434 -0.633 -0.3017 -0.125 0.126	0.515 0.256 0.2 0.929 0.499 1.08 -0.332 -0.063 -0.091 -0.216 -0.321 -0.257	0.494 1A 0.742 1B 0.0133 ** -0.1908 3B	NC NC	NO NO NO	NO NO NO	0
Park7 Pbk Pcna	PARK7 PBK PCNA	-0.0005 0.017 -0.1035 1.307 1.16 1.759 0.249 0.4 0.577	0.0295 -0.139 0.084 2.052 0.829 0.006	-0.332 -0.244 0.322 -0.199 0.288 0.226 0.072 0.084 0.471 -0.96 -0.476 -0.188 0.692 0.707 0.316	-0.045 0.774	4 NC 4 NC	NO NO	NO NO	2 0 25 0
Pcolce2 Pdcd1lg2 Pde4d Pde8b Pdia5 Pdia5	PCOLCE2 PDCD1LG2 PDE4D PDE8B PDIAS	nan nan 2.269 -0.077 -0.216 -0.835 -0.4525 0.027 -0.615 0.393 0.405 -0.094	-0.373 -0.383 nan nan -0.726 -0.808 nan nan -0.103 nan 0.206	nan nan nan nan 0.094 -0.218 0.187	-0.336 3A 0.812 1A -0.264 -0.2425 1A -0.032 **	4 NC 4 NC NC NC NC	ND ND ND ND ND ND	ND ND ND ND ND	10 11 0 0
Pdio4 Per1 Per2 Per3 Pf4	PDK4 PER1 PER2 PER3 PF4	-0.275 -0.231 -1.882 0.347 0.585 -1.172 0.3497 0.1043 -0.5143 -0.0908 -0.1092 0.551 0.194 0.116 0.236	-0.212 -0.434 0.242 -0.658 -0.776 0.101 -0.4667 -0.4775 -0.767 -0.3005 -0.513 -0.949	-0.851 0.192 -0.186 -0.358 -0.907 -0.28 -0.281 -0.783 -0.197 -0.607 -1.017 -0.477	0.23 3A -0.387 3A 0.0667 -0.1238	NC 2 NC 2 NC 2 NC NC	NO NO	NO NO	0 1 2 0
Pglyrp1 Pgrmc1 Pigr	PF4 PGLYRP1 PGRMC1 PIGR PIM1	-0.326 -0.169 -0.747 1.291 1.7965 2.2405	-0.3005 -0.513 -0.940 0.732 nan 0.543 1.522 1.667 1.528 -0.504 -0.673 -0.219 1.52 2.072 2.092 0.79 0.933 1.705	-0.607 -1.017 -0.477 1.133 0.099 0.341 0.865 1.109 1.41 -0.72 -0.185 -0.245 1.769 1.622 0.818	-0.1238 0.395 1A 1.479 1A -0.276 3A 3.4065 1A 0.3325 **	NC	NO NO	NO NO NO NO	2 2 0 1
Pim1 Pkd2 Pla2g7 Plaa Plac8	PIM1 PHD2 PLA2G7 PLAA PLAC8	0.574 0.3665 0.4825 -0.187 -0.1215 -0.568 0.416 0.078 2.81 0.0585 0.0825 0.356 0.263 0.253 0.502 -0.2635 -0.186 -0.528	-0.449 -0.698 -0.593 1.314 2.011 0.967 0.1785 nan -0.018	0.808 1.404 0.738 -0.337 -0.329 -0.277 1.19 0.68 0.172 0.225 0.193 0.188 1.242 0.943 0.769 -0.854 -0.527 -0.565	-0.383 3A 1.158 1B -0.258 3A -0.006 ** -0.269 **	NC NC NC	NO NO	NO NO NO	1 0 5 0
Plag1 Plat Plbd1 Plce1	PLOBLI PLAT PLBD1 PLCE1	-0.037 0.488 -0.354 0.493 0.439 1.285 0.324 0.355 1.020	-0.1705 0.087 0.832 0.319 0.499 0.924 0.617 0.835 0.205	-0.854 -0.527 -0.565 0.503 0.199 -0.069 -0.086 0.454 0.603 -0.576 -0.269 -0.016	-0.167 3A 0.26 1B	NC NC NC NC	NO NO	ND ND ND ND ND ND	0
Picg2 Piek Pin Pixdc2	PLCG2 PLEK PLN PLXDC2	0.158 0.163 0.77 0.4297 0.073 0.9447 -0.0723 -0.5193 -0.956 -0.258 -0.3093 -0.627	-0.625 0.568 nan nan 1.3743 0.791 1.844 0.3337 -0.208 0.21 -0.5733 -0.6285 0.071	nan nan 1.178 1.451 0.415 -0.684 -0.554 0.129 -0.727 -0.264 0.03	0.183 1B 0.8637 1B -0.4973 3B -0.3443 1B	NC YES NC	NO NO NO	NO NO NO	0 0 1 0
Pnp Polr3k Pon1 Pou2af1	PNP POLR3K PON1 POU2AF1	0.246 0.223 0.242 0.1835 0.1853 0.2632 -0.859 -0.645 -2.771 0.022 0.252 1.803	0.519 0.209 0.332 0.208 nan nan nan	0.60 0.443 0.240	0.29 1A 0.209	4 NC 4 NC NC YES	NO	ND ND ND ND	0 1 4 0
Pou2af1 Ppara Ppp1r14a Prickle1 Prickb Prickb	POUZAF1 PPARA PPPIRIAA PRICKE1 PRICE PRICE	-0.022 0.152 1.803 -0.2527 -0.0837 -0.1485 -0.177 -0.016 -0.772 -0.291 -0.0745 -0.347 -0.054 0.103 1.2855 -0.351 0.3885 0.926	-0.75 -1.26 -0.95 0.656 2.215 2.196 -0.17 -0.127 0.065 -0.568 -0.818 -0.62 -0.3635 -0.739 -1.713 0.6185 0.5885 0.838 0.336	2.237 1.602 1.386 -0.666 -0.216 0.167 -0.434 -0.987 0.339 -0.297 -0.425 0.244 0.56 0.324 0.743 0.377 0.252 0.169	-0.416 1A -0.04 3A -0.371 1B -0.1245 1B -0.1297 1B 0.2465 3A	NC NC YES	YES	NO NO NO NO YES	3 4 0 1
Prikci Prikg2 Procr	PRKCI PRKG2 PROCR PRO2 PRS58	-0.1265 -0.262 -0.5455 -0.4037 -0.213 -1.083 -0.411 -0.128 1.772	-0.468 -0.786 -0.512 -0.6607 -0.9335 -0.882 1.821 -0.139 0.389	-0.433 -0.423 -0.143 0.049 -0.578 -0.18 0.528 -0.204 0.344 0.049 0.576 -0.029 -0.596 -0.436 -0.09	-0.235 38 -0.271 0.473 3A 1.409 3A 0.123 3A	4 NC 100 NC	NO NO	NO NO NO NO	1 0 0
Proz Prss8 Psap Psmb8 Psmb9 Psmb9	PSAP PSMB8 PSMB9	-0.372 -0.116 0.157 0.2115 0.081 1.2165 0.021 0.263 1.371 -0.024 0.12 1.274 0.117 0.143 0.625	0.282 1.326 0.388 0.911 0.4275 0.193 1.03 0.589 -0.007	0.31 -0.197 0.357 0.558 0.318 0.901 0.204 0.382 0.633	1.3275 18 0.275 1A 0.242 1A	NC NC NC NC	NO NO	NO NO NO NO	0 1 2 0
Psme2 Ptch1 Ptger4	PSME1 PSME2 PTCH1 PTGER4	0.023 0.207 0.636 -0.0903 -0.0883 -0.389 1.101 0.824 1.468	0.738 nan 0.134 -0.252 -0.305 -1.588 0.681 1.041 1.33	0.245 0.377 0.547 -0.429 -0.721 0.163 0.931 0.697 0.802	0.583 1A 0.282 1A -0.1777 3A 0.167 1A		80	NO	0 0 2 7
Ptges Ptgs1 Ptgs1 Ptgn2 Ptgn2 Ptgn6 Ptgnc Ptgr Ptgr	PTGES PTGS1 PTPN1 PTPN2	0.495 0.2107 0.34 0.3725 0.1945 0.8985 0.1375 0.1995 0.392 0.3505 0.281 0.55	0.3385 0.329 1.202	0.47 0.775 0.234 0.61 0.322 0.64 0.46 0.392 0.609 0.582 1.176 0.33	0.5683 3A 0.6515 3A 0.2545 3A 0.2012 1A	NC YES NC	NO NO NO	NO NO NO	2 7 3 2
Ptpn5 Ptpn6 Ptprc Ptrf Ptre1	PTPN2 PTPN22 PTPN6 PTPRC PTRF PTTG1	0.1375 0.1395 0.390 0.3505 0.281 0.55 0.149 0.26 1.613 0.054 0.095 0.640 0.123 0.274 0.7075 0.1797 0.2903 0.236 0.2387 -0.0327 0.329	0.676 0.884 1.533 0.9665 0.635 0.153 0.572 0.6025 0.163 -0.5027 -0.736 0.063 1.1163 0.384 nan nan	0.582 1.176 0.33 0.673 0.217 0.394 0.509 0.197 0.624 1.122 0.738 0.684 -1.333 0.334 -0.369 nan nan	-0.05 1A -0.0325 1A -0.586 1A 0.1023 3A 0.0277	NC YES NC 4 YES	NO NO NO	NO NO NO	9 68 0
Pttg1 Pvrl3 Pycard Pybin1 Qiox1 Rab3il1 Rac2	PVRL3	-0.194 -0.111 -0.793		-0.247 -0.052 -0.148	-0.4038 38	NC NC	NO NO NO	NO NO NO	0 3 1 0
Rad51	PIGNU PIGNU RABILI RAC2 RAD51 RAM92	1.161 1.187 1.473	0.592 0.896 -0.015	1.017 0.14 0.49 0.096 0.141 0.379 0.544 0.299 0.204 0.016 0.678 0.587 0.016 0.085 0.587 0.181 -0.6 -0.251	-0.511 1A 0.743 ** 0.402 ** 0.45 1B 0.537 0.128 1A	NC YES 4 YES NC	NO NO NO	NO NO NO	0 1 0
Rapgel3 Rasal2 Rbp4 Rbpj Rbpms	RAPGEF3 RASAL2 RBP4 RBPJ RBPMS	-0.1298 0.0198 -0.5378 -0.1553 -0.061 -0.8087 0.376 0.142 -0.031 0.071 0.129 0.4307 -0.0738 -0.0516 -0.4896	-0.5664 -0.3075 -0.423 -0.7277 -0.5305 -0.495 -0.198 1.675 -0.61 0.5637 0.3295 0.366 -0.4474 -0.4075 -0.457	-0.123 -0.287 -0.091 -0.497 -0.527 0.057 -0.686 -0.164 0.16 0.417 0.343 0.324 -0.449 -0.435 0.038	-0.655 18 -0.3547 ** 1.879 3A 0.0427 3A 0.0168 **	NC NC NC	NO NO NO	NO NO NO	1 1 2 4
Reck	RBPMS RCAN2 RECK REG3G RELA	-0.0738 -0.0516 -0.4896 -0.14 -0.213 -0.8005 -0.235 -0.196 -1.087 1.095 1.556 1.827 -0.046 0.034 -0.036	-0.4474 -0.4075 -0.457 -0.595 -0.669 -0.222 -0.701 -0.911 -0.638 2.149 2.386 1.222 -0.109 -0.259 0.507	-0.449 -0.435 0.038 -0.581 0.068 -0.044 -0.602 -0.573 -0.332 0.73 1.747 1.219 -0.376 0.014 -0.057	0.0168 ** -0.132 3A -0.652 3A 1.495 1A 0.102 1A	NC NC NC		ND ND ND ND	0
Reg3g Refa Refb Retnla Rgs1 Rgs4 Rhoh	RELB RETNLB RGS1 RGS4	0.455 0.31 0.539 3.254 2.747 4.073 0.936 0.557 2.556	0.728 0.426 0.934 2.878 2.517 2.264 1.846 0.535 1.686	0.392 0.589 0.283 1.716 1.746 1.789 0.651 0.459 -0.006	0.545 1A 3.044 1A 1.364 1A	VES NC	ND ND ND ND ND ND ND	YES NO NO	4 2 7 0
Rhoh Rhou Ripk2 8xf128	RHOH RHOU RHOU	-0.216 0.14 0.67 0.4895 0.192 0.117 0.1565 0.092 0.021		0.434 0.53 0.183 0.456 0.274 0.335 0.22 0.589 0.083 0.178 0.335 -0.086 0.381 0.557 0.036	0.7833 1A 0.218 1B 0.5595 1B 0.234 1A 1 723 **	NC NC NC	NO NO NO	NO NO NO	0 0 2
Rhou Ripk2 Rnf128 Rnf186 Rnf19b Rora Rrfp1	RNF128 RNF126 RNF196 ROFA ROFA RRBP1	-0.1785 -0.1455 -0.4191 0.4793 0.394 0.5595	0.3095 0.021 0.034 0.3095 0.177 1.934 0.038 nan 0.44 1.4685 1.402 0.444 0.027 0.491 1.077 0.6805 0.949 2.207 -0.3525 0.4 -0.524 0.3622 0.276 0.239	0.178 0.333 -0.086 0.381 0.535 -0.086 -0.464 -0.844 -0.198 0.774 1.552 0.533 -0.255 -0.283 -0.164 0.487 0.23 0.227	0.228 18 0.5595 18 0.234 1A 1.723 ** 0.154 ** 0.709 ** -0.1946 0.1382 3A	NC 2 NC 2 NC	NO NO NO	NO NO NO	0 6 0
8rm2 Rsad2 Rtin2 S100a4 S100a8 S100a9	RRM2 RSAD2 RTKN2 S100A4 S100A8 S100A9	1.5387 1.3127 2.2067 -0.3927 0.194 -0.06 -0.426 -0.115 -0.913 1.174 0.892 1.565 -0.856 -0.142 -0.652 -0.878 -0.146 -0.462	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	0.457 0.109 0.874 0.315 -0.078 0.334 -0.39 0.745 -0.162 1.052 0.685 1.228 2.892 1.681 0.535 2.747 1.786 0.405	0.765 0.297 1A 0.188 ** 1.093 3A -0.205 1B 0.305 1B	4 YES NC NC	NG N	NO NO NO	0
Saa1	SAA1 SAA1	nàn nàn nàn nàn nàn nàn	1.27 nan 0.882	1.477 0.526 0.681 0.616 0.409 -0.105 ni 2.072 3.367 3.34	0.734 3A in 3A	NC NC	NO NO NO	ND NO NO NO	4 3 0
Saa3 Samhd1 Samsn1 Sash3 Scara5	SAMHD1 SAMSN1 SASH3 SCARAS	-0.0032 0.1384 0.4904 0.4435 0.1315 0.259 0.129 0.174 1.18 -0.672 -0.603 -0.574	0.2056 0.2315 0.366 0.337 0.4485 1.765 0.733 0.546 0.139 0.095 -0.659 -0.292	0.226 0.369 0.416 0.974 0.846 0.754 0.645 0.344 0.423 -0.842 -0.087 -0.872	3.805 NN -0.1342 1A 0.495 1B 0.286 1B -0.055 ** -0.775 **	<pre></pre>	NO NO NO NO NO NO NO NO NO	10 10 10 10 10 10 10 10 10 10 10 10 10 1	- 0 0
Scel Scnn1b Scube2 Sdc4	SCEL SCNN1B SCUBE2 SDC4	-0.512 -0.121 -0.983 -0.386 -0.235 -0.823 -0.569 -0.188 -1.297 -0.2365 -0.071 -0.0605	-0.818 -0.681 -0.69 -0.537 -1.247 -0.441 -0.849 -1.1 -0.495 -0.094 0.377 1.416	-0.668 -0.532 -0.49 -0.511 -0.545 -0.188 -0.97 -0.353 -0.274 -0.012 -1.195 -0.365	-0.775 ** 0.034 1B 0.05 ** 0.45 3A	NC NC	ND ND ND	NO NO NO	0 1 0 1

5df211	SDF2L1	1 0.616 1.136	0.55 1.523 0.205	0.058 0.333 0.35	1.088 **	NO	NO	NO	0
Sdpr Seclic Selenbpl Selp Selplg	SDPR SEC11C SELENBP1	-0.2687 -0.1087 -0.8277 0.1755 0.2095 0.94	0.050 1.523 0.000 -0.5757 -1.1375 0.2888 0.2495 0.787 0.673 0.496 1.435 -1.054 0.4065 0.663 1.554 0.711 0.69 0.724	0.086 0.33 0.35 0.494 0.78 0.286 0 0.599 0.534 0.568 0 0.747 0.593 0.044 0.747 0.059 0.438 0.899 0.222 0.501	1.028 ** 0.3443 18 0.0505 ** -0.216 2 0.201 1A 0.496 1A				0
Selenbp1 Selp	SELENBP1 SELP	-0.351 -0.182 -0.792 1.174 1.074 0.303 0.354 0.206 1.095	-0.496 -1.435 -1.054 0.4005 0.603 1.514	-0.207 -0.593 -0.044 0.744 1.005 0.438	-0.216 2 0.201 1A	NO NO NO NO	NO I	NO NO NO NO	0
	SELPLG SELMAJA					NO NO NO	NO I NO I	NO NO NO	3
Sema3c Sema4a Sema4d	SEMA3C SEMA4A	-0.0535 -0.2535 -0.602 0.3545 0.2395 0.7815 -0.041 0.242 1.0995	-0.485 -0.866 -0.091 0.592 0.331 0.673 1.1825 0.676 1.081	0.121 -0.259 -0.129 0.36 0.623 0.391 0.756 0.494 0.674	-0.031 3A 0.3395 1A	ND ND ND	NO I NO I	NO NO NO	2
Sema4d Serpina3c	SEMA4D SERPINA3	-0.041 0.242 1.0995 1.087 0.37 -0.246	1.1825 0.676 1.081 0.279 0.463 1.584 1.624 2.756 2.887	0.756 0.494 0.674 1.245 1.164 0.655	0.3325 3A 0.193 3A	NO NO	NO I	NO NO	3
Serpina3c Serpina3g Serpina3m Serpina3n Serpind1	SERVIAD SERVINAS SERVINAS SERVINAS SERVINAS SERVINDS	-0.041 0.242 1.0955 1.087 0.37 -0.266 2.341 2.302 3.238 0.496 0.913 0.245 1.499 1.184 1.402 0.134 0.202 nan	1.1825 0.076 1.081 0.279 0.463 1.584 1.624 2.756 2.387 0.127 na 0.445 0.345 0.99 1.624 0.225 -0.135 nan nan nan nan	0.36 0.523 0.391 0.756 0.494 0.674 1.245 1.164 0.655 3.411 2.846 2.621 1.819 0.891 0.492 1.779 1.037 1.063	0.193 3A 0.471 3A 0.643 3A 0.914 3A 1.097 3A	NO NO NO NO	NO 1 NO 1 NO 1 NO 1	40 10 10 10	3
Serpind1	SERPIND1	0.134 0.202 nan 0.369 0.375 1.103	-0.135 nan nan nan I 0.9 0.908 0.584		0.5974 3A 1.097 3A 0.687 1B	NO NO	NO I	NO	0
Sfpi1 Sfrp1 Seol1	SPI1 SFRP1 SGOL1	0.2376 0.0896 0.2202				ND ND	NO I NO I	NO NO NO	1
Sgol1 Sgol2 Sh2b2	500L2 5H282	0.32 0.406 0.791 0.707 0.392 1.079 0.135 0.0315 0.018	0.9565 0.209 nan nan 1.464 0.465 nan nan 0.29 0.42 0.772	nan nan 0.057 0.636 0.263	0.0005.00	ND ND ND	NO I NO I	NO NO NO	0
Sh3bgrl3 She Siglec5	SH3BQRL3 SHE SIGLECE	0.135 0.0315 0.018 0.478 0.067 1.140 -0.135 -0.228 -0.744 0.065 -0.552 0.647 0.6147 0.512 0.7692 0.299 0.192 1.299	0.0568 0.009 and nan nan 0.1464 0.465 nan nan nan 0.29 0.617 0.605 0.42 0.772 0.617 0.605 0.42 0.715 0.156 0.455 0.035 0.42 0.156 0.455 0.035 0.42 0.156 0.455 0.038 1.081 1.788 0.787 0.566 1.795	0.037 0.335 0.253 0.235 -0.045 0.454 -0.332 -0.578 -0.073 -0.113 0.004 -0.193 0.908 0.467 0.446 0.521 0.352 0.233	0.836 ** -0.218 ** -1.466 18 0.1738 3A 0.478 18	NO NO NO NO	NO 1 NO 1 YES 1 NO 1	NO NO NO NO	0
Siglec5 Sia Sic11a1	SIGLEC8 SLA SLC11A1	0.065 -0.562 0.647 0.6347 0.512 0.7692 0.299 0.192 1.298	0.156 0.145 0.08	-0.113 0.004 -0.193 0.908 0.467 0.446	-1.466 18 0.1738 3A	NO NO	YES I	NO NO	7
Sic15a3 Sic15a1	SLC15A3 SLC16A1	0.428 0.541 1.573 0.303 0.054 0.508	1 1.357 1.286 1.795 1 0.553 0.3605 0.281	0.704 0.992 0.552 -0.002 0.619 0.637	0.752 1B 0.71 **	YES NO	NO I NO I	40 10	0
SIc26a4	SLC2644	4.402 3.908 4.628	1.754 4.285 2.811	3.494 2.449 3.153 0.265 0.932 0.432	5.509 3C	NO NO	YES I	NO NO	9
51c2a1 51c2a3 51c37a2 51c38a4 51c39a14 51c39a14 51c41a2	SLC2A3 SLC2A3 SLC23A2 SLC38A4 SLC39A14 SLC39A14 SLC39A14	0.365 0.136 0.327 0.244 0.1685 0.3985 0.504 0.267 0.913 -0.077 -0.184 -1.3475 0.566 0.374 0.215 0.217 0.506 0.799	0.0263 1.184 1.499 0.855 0.622 0.184 0.1021 0.633 0.112 0.393 0.144 1.921 0.264 0.45 0.732	$\begin{array}{cccccccccccccccccccccccccccccccccccc$	0.329 3A 0.7617 18 0.355 ** -1.106 18 0.1398 3A 0.646 **	NO NO	NO I	NO NO	0
Slc38a4 Slc39a14	SLC38A4 SLC39A14	-0.077 -0.184 -1.3475 0.566 0.374 0.2115	i -1.021 -0.633 0.112 i 0.393 0.144 1.921 0 0.264 0.45 0.732	-0.262 0.039 -0.153 1 1.127 0.397	-1.105 18 0.1398 3A	NO NO NO NO	NO 1 NO 1 NO 1 NO 1	40 10 10 10	0
Sic5a1 Sic5a20a	SLC5A1 SLC5A1 SLC6A20	0.217 0.506 0.799 2.2165 1.174 1.3365 0.612 1.008 2.447		0.478 0.634 0.281 1.272 1.099 1.022 0.916 1.097 0.781	0.646 ** 3.0035 3A 4.789 1B	NO NO NO	NO I NO I	NO NO	0
Sic7a10 Sic7a2	SLCBA20 SLC7A10 SLC7A2	-0.378 -0.144 -1.591 0.9923 0.726 1.168 -0.382 0.544 0.379	- 0.636 -1.203 0.312 1.1073 1.257 0.917 1.598 0.203 2.529	-0.355 -0.376 -0.208 0.743 0.757 0.057 1.103 1.123 0.594	-0.29 ** 1.4535 ** 0.153 **	NO NO NO	NO I NO I NO I	NO NO NO	0
	CLEMIN 2. CLEMIN 21	-0.382 0.544 0.379 0.319 0.542 1.05	1.598 0.203 2.529 5 1.326 0.518 1.386	0.335 0.376 0.208 0.743 0.757 0.057 1.103 1.123 0.594 0.592 0.747 0.319 1.955 1.115 0.304	0.153 **	NO NO	NO I	NO NO	0
Sifn2 Sifn4 Siit2	SLFN12/SLFN12L SLFN12L SLIT2 SLP1	0.319 0.542 1.06 -0.326 0.31 0.375 -0.3537 -0.1853 -0.128 0.79 -0.818 1.036	5 1.326 0.518 1.386 5 2.559 -0.04 2.454 1 0.1367 -0.068 0.014 5 0.637 1.778 0.99	0.592 0.747 0.319 1.955 1.115 0.304 -0.239 -0.376 -0.039 - 0.643 0.714 0.17	0.324 ** -0.042 ** 0.3433 18 1.215 3A	NO NO NO NO	NO NO NO	ND ND ND	0
Sipi Smad6		-0.202 -0.151 -0.661	-0.719 -1.391 -2.058	-0.239 -0.376 -0.039 - 0.643 0.714 0.17 -0.471 -0.787 -0.191	1.215 3A -0.306 4 -0.124 4	NO NO	NO I	NO NO	2
Smc2 Smc4 Snrk	SMC2 SMC4 SNRK	0.5445 0.6605 1.366 0.173 0.116 0.7563	0.5462 0.2415 -0.482 0.437 0.127 0.008	-0.471 -0.787 -0.191 0.54 0.115 0.431 0.284 -0.278 0.101 -	0.3063 4	NO NO	NO I NO I	NO NO	0
Sec.10	610710	-0.133 0.0745 -0.802 0.112 0.1445 0.6065	-0.6215 -0.806 -0.737 0.5945 0.466 0.909	-0.424 -0.4 -0.179 - 0.282 0.78 0.309 -	0.4215 3A 0.2015 **	NO NO	NO I	10 10	0
Sm5 Socs1 Socs2	50CS1 50CS2	0.5785 0.39 0.383 0.2022 0.214 -0.344	0.6005 0.3745 1.174	0.373 1.463 0.275 0.515 0.638 0.19	0.266 1A	NO NO	NO I	vo vo	6
Socs3 Sod2	5005 50051 50052 50052 50053 5002	0.112 0.1445 0.0006 0.0196 0.132 0.0906 0.0196 0.5785 0.39 0.383 0.2022 0.214 -0.344 0.9437 0.5557 0.381 0.249 0.1127 0.328	0.03945 0.465 0.350 0.4226 1 0.28 0.6005 0.3745 1.174 0.6005 0.3745 1.174 0.6377 0.174 2.566 0.4625 0.155 0.881	-0.424 -0.4 -0.179 - 0.282 0.78 0.309 - 0.877 0.709 0.625 - 0.373 1.463 0.275 - 0.515 0.638 0.19 - 1.228 1.517 0.637 - 0.298 1.052 0.594 -	0.2055 3A 0.266 1A 0.266 1A 0.528 1A 0.487 3A	NO NO	NO 1 NO 1 NO 1 NO 1	40 10 10 10	7 5
Sord Sox17	SORD SOR17	-0.049 -0.3465 -0.174	-0.0315 -0.321 -0.469	-0.628 -0.352 -0.141 -	0.0845 4	NO NO	NO I	vo vo	0
Sox18 Sox2 Sox7	SCIX18 SCIX2 SCIX7	-0.407 -0.273 -0.095 0.338 0.482 -0.03 -0.148 -0.214 -0.493	0.347 -0.698 -2.217 0.495 -0.222 -0.092 -0.372 -1.111 -0.484	-0.429 -0.235 -0.049 -0.371 0.635 0.31 -0.532 -0.31 -0.242 -	-0.052 3A 0.538 3A 0.1865 **	NO NO	NO I NO I	NO NO NO	2
Sox9 Spc25 Sphk1	SCIK9 SCIK9	-0.146 -0.214 -0.493 nan nan 0.252 1.103 0.943 1.741	0.054 0.431 nan nan 0.921 0.646 nan nan	nan nan	0.774 3A	NO NO	NO I	NO NO	2
Sphk1 Son	SCIX9 SPC25 SPHK1 SPN	-0.187 -0.0495 -0.085 -0.0335 0.1745 0.8395	0.054 0.481 nan nan 0.921 0.646 nan nan -0.0315 -0.568 2.104 0.4415 0.302 nan nan	0.227 0.543 -0.172	0.0485 3A 0.0765 1A	NO NO NO	NO NO NO	ND ND ND	4
Spon2 Spp1	SPON2 SPP1	-0.354 -0.196 -0.319 -0.06 -0.339 1.117	-0.02 nan 0.114 1.435 2.347 0.445	0.359 -0.195 0.129 0.579 0.265 -0.159	0.05 18 2.598 **	ND ND ND	YES I NO I NO I	NO NO NO	1 12
Spry1 Sqstm1	SPRY1 SQSTM1	-0.278 -0.202 -0.783 -0.161 -0.203 -0.253 0.185 0.033 0.528	-0.623 -0.741 -0.474 -0.19 -0.236 -0.448 -0.176 0.838 1.482	-0.476 -0.61 -0.246 -0.425 -0.175 -0.182 1.091 0.983 0.449	-0.294 3A 0.426 3B 0.248 1B	ND YES ND	NO I NO I	NO NO NO	0 3
Septema Sragai4 Stabal Stab1 Stat1 Stat5a	SRGN ST3GAL4	-0.276 -0.202 -0.783 -0.161 -0.203 -0.253 0.185 0.033 0.525 0.464 0.454 0.511 -0.2125 0.0095 -0.2195 0.66 0.108 0.049 -0.079 0.2235 1.0668 0.3065 0.1315 0.402 0.2135 0.403	0.176 0.838 1.482 0.125 0.788 1.31 0.264 -0.498 -0.215	-0.425 -0.175 -0.182 1.091 0.983 0.449 0.245 0.824 0.187 -0.54 -0.63 -0.624 0.269 -0.385 0.073 0.364 0.688 0.777 - 0.14 0.75 0.202	0.248 18 1.122 3C	NO NO	NO I	NO NO	1
Stab1	SRON ST3GAL4 ST8SIA2 STAB1 STAT3 STAT3A STAT5A	0.185 0.033 0.522 0.4 0.454 0.511 -0.2125 0.0095 -0.2375 0.06 0.108 0.049 -0.079 0.2235 1.0668 0.3665 0.1315 0.402	0.176 0.338 1.442 0.125 0.788 1.31 0.0254 -0.498 -0.215 0.0258 nim 0.022 1.2257 0.0875 -0.031 0.0251 nim 1.157	0.245 0.824 0.187 -0.54 -0.63 -0.624 0.269 -0.385 -0.073 0.364 0.75 0.202	0.248 18 1.122 3C 0.0535 1A -0.177 18 0.1545 1A 0.472 1A	NO NO YES NO	NO 1 NO 1 NO 1 NO 1	ND ND ND ND ND	0
Stat5a Stat5b	STATSA STATSB	0.3065 0.1315 0.402 0.0215 -0.1025 -0.128	0.261 nan 1.157 0.261 nan 0.128	0.304 0.568 0.777 - 0.34 0.75 0.202 -0.331 -0.014 -0.193	0.472 1A 0.098 1A	NO NO	NO I	NO NO	13 8 4
	FAM47E-STBD1 STEAP2	0.061 0.124 -0.354	-0.144 -0.422 -0.948	-0.462 -0.637 -0.06	0.038 1A 0.057 ** 0.6014 **	NO NO NO	NO I NO I NO I	0 00 00	0
Steap4 Swap70	STEAP4 SWAP70	0.4085 0.321 1.1325 -0.2605 -0.1085 -0.2395	1.266 0.72 2.528 -0.2005 -0.1305 -0.126	1.645 1.897 0.79 -0.206 -0.204 0.146	1.333 ** -0.238 1A 1A	NO NO	NO I	NO NO	0
Steap2 Steap4 Swap70 Sykb Syme2	SWAP70 SYK SYNE2	0.5305 0.3267 0.4065 0.321 1.325 0.2605 -0.1085 -0.2395 0.36 -0.6655 0.7583 -0.192 -0.115 -0.4945 0.3675 0.3395 0.777 -0.16 -0.198 -0.378	L260 U.72 2.528 -0.2005 -0.1305 -0.126 0.4613 nan nan nan 1.05387 -0.5387 -0.463 0.521 0.47 0.465 -0.78 -0.465 -0.78 -0.507		0.6082 4	NO YES NO NO	NO 1 NO 1 NO 1 NO 1	40 10 10 10	13
Syngr2 Tacc2 Tap2	SYNGR2 TACC2 TAP2	-0.16 -0.198 -0.378 -0.217 0.033 0.507	0.356 0.521 0.47 0.463 -0.78 -0.507 0.549 0.035 0.166	-0.616 -0.714 0.086 - 0.176 0.319 0.456 - -0.63 -0.581 -0.013 0.169 0.406 0.419	0.5985 4	NO NO	NO I	V0 V0	0
Tapbp Tbx2	TAPEP TBX2		0.549 0.035 0.166 0.574 0.156 0.362 -0.551 -0.809 -1.44	0.169 0.406 0.419 -0.335 0.157 0.246 -0.472 -0.593 -0.049	0.248 ** 0.369 ** -0.245 3A	NO NO	NO I		1
Tbx3 Tbx2s1	TBX3 TBXAS1	0.1865 0.08/5 0.314 -0.255 -0.11 -0.577 -0.4877 -0.027 -0.4600 0.651 0.354 1.164 0.909 0.683 1.472 0.0063 -0.1867 -0.0945 -0.413 -0.086 -0.594 -0.154 -0.081 -0.682 1.7 0.018 -0.068	-0.551 -0.809 -1.44 -0.1513 -0.9165 -1.132 0.671 0.679 0.082 1 0.922 min min min -0.543 -0.564 -1.23 -0.613 -1.055 -0.519 -0.718 -0.564 -0.564 -0.118 man 0.55	-0.472 -0.593 -0.049 -0.447 -0.115 -0.012 - 0.579 -0.079 0.382	0.0757 3A 1.166 1B	NO NO NO	NO I NO I	NO NO NO	0
Tcfec Tef	TFEC TEF	0.051 0.354 1.104 0.909 0.683 1.472 0.0063 -0.1367 -0.0945 -0.413 -0.1367 -0.0945 -0.354 -0.081 -0.682 1.7 0.018 -0.046	0.922 nan nan nan i -0.6147 -0.564 -1.23		.0.097 2	NO NO NO NO	NO 1 NO 1 NO 1 NO 1	40 10 10 10	0
Tek Tenc1 Tff1	TEK TENC1 TFF1	-0.413 -0.186 -0.984 -0.354 -0.081 -0.682	0.633 -1.055 -0.519 -0.738 -1.233 -0.169 0.138 nan 0.55	nan nan nan nan -0.542 -0.628 -0.185 -0.501 -0.557 -0.165 -0.742 -0.309 -0.227 -0.622 0.082 0.071	-0.433 3A 0 3A 0.566 3A	ND NO	NO I	V0 V0	2
TH2 That2	TFF2 TFP12	3.204 2.295 1.704 2.185 1.279 1.395		1.382 1.171 1.97 1.532 1.152 0.759	0.928 3C -0.007 3A	NO NO	YES I	NO NO	9
Tgfb2 Tgfbi Thbd Thbs1 Thrsp	TGFB2	-0.1708 -0.157 -0.174	-0.3315 -0.2695 0.354 0.8973 0.895 0.654	-0.635 0.109 -0.466	0.3285 3A	NO YES	NO I	NO NO	6
Thbd Thbs1	THED THESI THISP	-0.072 -0.103 -0.693 1.4293 0.612 -0.1663	0.0877 -0.652 -0.706 -0.1207 1.1 2.267 0.08 -0.09 -0.595 -0.491 -0.353 -0.695	-0.338 -0.274 -0.049 1.092 0.779 0.765 -2.606 0.211 1.203 -0.193 -0.407 -0.063	-0.073 3A 0.365 3A	NO NO	NO I	NO NO	1
Thisd1 Thy1	THISP THSD1 THY1	0.2278 0.3222 1.2253 -0.072 -0.103 -0.603 1.4293 0.612 -0.1663 -0.1663 -0.8137 -0.7513 -0.212 -0.1195 -0.49 0.261 0.309 1.384	0.988 1.459 2.189 -0.3135 0.2895 0.354 i 0.8973 0.895 0.554 i 0.597 -0.652 -0.706 i 0.2095 0.154 -0.267 i 0.2097 1.1 2.267 i 0.068 -0.079 -0.595 i 0.086 0.037 man nan i 0.896 0.937 man 1.602	-2.606 0.211 1.205 - -0.193 -0.407 -0.063 -	0.1663 3A -0.073 3A 0.165 3A 0.1973 3A 0.4735 ** 0.339 1A	NO YES NO NO NO NO	NO 1 NO 1 NO 1 NO 1	40 10 10 10	0
Tifa Timo1	TIFA TIMP1	0.143 0.076 1.145	2,412 1,658 1,661	2.867 1.555 1.599	1.376 3A	NO YES	NO I	vo vo	0
Timp3 Tirap Tk1	TIMP3 TIBAP	-0.129 -0.2828 -0.7462 -0.0385 -0.045 0.0595 1.083 1.165 1.304	0.7285 -0.673 -0.202 -0.0585 0.008 0.46 1.565 0.993 -0.112	0.226 -0.26 -0.562 0.051 0.329 -0.081 0.048 -0.055 0.545	0.0715 3A 0.0905 1A	NO NO	NO NO	NO NO NO	1
Tk1 Tir1 Tir2	TK1 TLR1	1.083 1.165 1.304 0.193 0.235 1.674	1.565 0.993 -0.112 1.275 0.926 0.519	-0.216 -0.26 -0.562 0.051 0.329 -0.031 0.048 -0.055 0.545 1.019 0.176 -0.026 0.335 0.593 0.325 0.652 0.242 0.308 0.36 0.313 0.097		NO NO YES YES YES NO	NO I	NO NO	6
Tir2 Tir4 Tir6	TLR1 TLR2 TLR4 TLR6	0.193 0.235 1.674 0.596 -0.071 0.471 0.358 0.366 0.2505 -0.021 0.303 nam	1.505 0.993 -0.112 1.275 0.926 0.519 1.527 -0.282 1.38 0.2197 0.037 0.119 0.31 0.125 0.461	0.019 0.176 -0.026 0.335 0.693 0.325 0.652 0.242 0.308 0.36 0.313 0.097	-0.299 1A 0.346 1A 0.2497 1A 0.136 1A	YES	NO NO NO	ND ND ND	50 81
Tir7 Tmcc2	TLR7 TMCC2	0.059 0.065 0.846	0.645 0.112 0.42	0.208 0.042 0.117		NO NO NO	NO I NO I NO I	NO NO NO	18
Tmcc3 Tmem100 Tmem105a	TMCC3 TMEM100	-0.0245 0.1255 -0.8285 -0.3814 -0.2236 -0.8866 -0.331 -0.1345 -0.827 0.449 0.4555 1.1415	i -0.6725 -0.895 -1.23 i -0.5494 -0.8565 -0.239 i -0.7195 -1.132 -1.231 i 0.8305 0.624 0.219	-0.249 -0.033 -0.198 - -0.64 -0.478 -0.079 - -0.488 -0.858 -0.423 0.55 0.27 0.386	0.2275 3A 0.0674 ** -0.468 ** 0.6805 **	ND NO NO	NO I NO I	40 40 40	0
Tmem105a Tmem173 Tmem51	TMEM106A TMEM173 TMEM51	0.449 0.4555 1.1415 0.9145 0.832 1.4805	0.8305 0.624 0.219 1.159 1.025 0.816	0.55 0.27 0.386 0.613 0.831 0.114	0.6805 ** 0.6755 3A	NO NO	ND I YES I	NO NO	0
Tmem51 Tnc	TMEM51 TNC TNFAIP2	0.449 0.455 1.4405 0.9145 0.832 1.4405 0.169 0.214 0.716 1.069 0.468 1.222 0.447 0.0705 0.4105 0.439 0.203 1.188	0.0345 0.024 0.219 1.159 1.025 0.316 0.475 0.319 0.688 0.9325 0.773 0.392 0.6883 0.236 1.971 0.742 0.6565 0.788	-0.488 -0.858 -0.423 0.55 0.27 0.386 0.613 0.831 0.114 0.216 0.614 0.486 0.661 -0.068 0.482 0.661 1.196 0.485 0.637 0.57 0.496	0.6755 3A 0.275 ** -0.329 3B 0.2087 1A 0.525 3A	NO NO NO NO	YES 1 NO 1 NO 1 NO 1	40 10 10 10	6
Tnc Tnfaip2 Tnfaip8 Tnfrsf13b	TNFAP8 TNFRSF13B	0.439 0.203 1.185 -0.045 0.124 1.125	0.742 0.6565 0.788 0.591 1.113 nan nan	0.637 0.57 0.496	0.525 3A 0.135 1A	NO NO	NO I	vo vo	0
Tnfrsf13c Tnfrsf19	TNFRSF13C TNFRSF19	0.073 0.206 0.813	0.797 0.552 nan nan 0.2723 -0.853 -0.937	nan nan	.0.413.14	NO NO	NO I NO I	NO NO	0
Tnfrsf1b Tnfrsf9	TNFRSF1B TNFRSF9	-0.2863 -0.164 -0.5473 0.208 0.0825 0.5185 1.209 0.466 1.0345	0.797 0.552 nan nan 0.0272 -0.853 -0.937 0.0981 0.512 1.11 1.4155 0.781 1.878 0.0043 -0.128 -0.585 0.0515 0.6235 0.503 0.0515 0.6235 0.503 0.529 0.445 1.056 1.529 0.945 0.951	-0.28 -0.907 -0.021 - 0.586 0.571 0.195 1.03 2.129 0.797	0.1957 3A 0.146 1A 1.804 1A	ND ND	NO I NO I	NO NO NO	9
Tnfsf10 Tnfsf13b Tnfsf9	TNFSF10 TNFSF13B	-0.241 -0.2703 -0.035 0.9 0.415 0.435 0.23 0.341 0.65 0.186 0.146 0.612	1.4155 0.761 1.876 i -0.0043 -0.128 -0.585 i 0.0315 0.6235 0.503 i 0.547 0.042 0.674 i 0.529 0.145 1.056	-0.274 0.066 -0.129 - 1.102 0.341 0.638 - 0.285 0.24 0.064 - 0.189 0.814 0.225 -	0.1527 1A 0.3905 1A 0.233 1A 0.3965 1A	NO NO NO	NO NO NO	ND ND ND	3
Tnip1 Tnab	TNFSF9 TNIP1 TNXB	0.186 0.146 0.612 -0.846 -0.217 -0.762	0.529 0.145 1.056 -1.03 -0.966 -0.31	0.189 0.814 0.225 -0.848 -0.581 -0.252	0.335 1A 0.396 1A -0.63 3A	NO NO	NO I	NO NO	1
Top2a Tppp3	TOP2A TPPP3	1.27 1.0465 1.7085 -0.102 -0.11 -0.967	1.3605 0.921 0.2 -0.392 -1.171 -0.419	0.143 0.257 0.951 -0.77 -0.421 -0.046	0.1953 4	ND ND NO	NO I NO I	NO NO NO	1
Trafti Trf	TRAF6 TF	-0.007 -0.012 -0.0095	-0.049 nan 0.724	0.077 0.146 -0.035	0.0858 1A	NO YES	NO 1	rES NO	3 1
Trill Trim30a	TRIL TRIMS TP63 TSC2	-0.3505 -0.38 -0.829 -0.0565 0.396 0.834 0.273 0.009 nan 0.004 -0.033 -0.325	0.100 0.100 0.110 0.0683 0.787 -1.242 1.4055 0.18 0.64 0.3227 nan nan nan 0.3227 nan nan nan	-0.626 -0.733 -0.231 - 0.579 0.393 0.469	0.5775 ** -0.318 3B	ND ND	NO I NO I NO I	90 90 90	0
Trp63 Tsc2 Tsc22d3	T5C2 T5C22D3	0.273 0.009 nim 0.004 -0.033 -0.325 0.2477 0.16 -0.9967	0.3227 nan nan nan -0.313 -0.419 -0.22 -0.481 -1.133 -0.858	-0.399 -0.486 0.054 0.003 -0.714 -0.167	0.3407 3A 0.188 3A 0.2227 1A	NO NO	NO I	V0 V0	1
Tspan7 Ttc39c	TSPAN7 TTC39C	-0.38 -0.178 -1.2125 0.527 0.299 0.65	-0.719 -1.048 0.029	-0.318 -0.344 -0.322	0.14 3A 0.2987 **		YES I	NO NO	0
Ttil1	TTLL1 TUBB6	-0.004 -0.115 0.019	-0.0023 -0.331 -0.988 0.2895 0.568 1	-0.591 -0.834 -0.114 - 0.835 0.344 0.709	0.0043 **	NO NO	NO I	NO NO	0
Twig1 Tyrobp	TWSG1 TYROBP	-0.0757 -0.317 -0.611 0.367 0.186 1.145 1.305 1.306 1.853	-0.3397 -0.665 -0.27 0.781 1.108 1.101 2.234 1.431 -0.07	-0.297 -0.123 -0.205 0.688 0.396 0.511	0.093 3A 0.785 1B	NO YES	NO I	NO NO	0
Twig1 Tyrobp Ube2c Uchl1 Uhrf1	TWSG1 TWSG1 TWSGP UBE2C UCHL1 UHM/F1*	1.305 1.306 1.853 nan nan nan 1.2885 0.9795 1.1685		0.419 0.217 0.284 0.591 0.584 0.114 0. 0.835 0.344 0.709 0.297 0.223 0.205 0.688 0.396 0.511 0.651 0.062 0.122 0.022 0.0465 0.223 0.040 0.525 0.237 0.04 0.186	0.219 *** 0.093 3A 0.785 1B 1.015 4 0.156 1A 0.5865 4	NO NO	NO I	NO NO	2
Unc93b1	LING	0.002 -0.013 0.621		0.237 0.04 0.185	0.325 1A 0.348 3A	NO NO	NO I	NO NO	0
Unc93b1 Ung Upk3b Usp18 Utm Vav1 Vcam1 Vcam1 Vcan Vdr Vcga Vdr Vegla Vldir	UPK3B USP18 UTRN			-0.827 -0.224 -0.281	0.0105 ** 0.106 1A -0.504 3A	NO NO	NO I	NO NO	0
Utm Vav1	UTRN VAV1	-0.478 -0.332 -1.3483 0.106 0.353 0.766 -0.2167 -0.1753 -0.7155 0.122 0.462 0.633 0.537 0.571 0.1643 1.1045 0.607 -0.0477 0.288 0.094 0.437 -0.278 -0.164 -0.7285 0.441 0.156 0.7285	-0.7813 -0.864 -0.012 0.933 0.255 nan nan	-0.316 -0.415 -0.153 nan nan	-0.504 3A 0.382 1A	NO NO	NO I	NO NO	1
Voam1 Voan	VAV1 VCAM1 VCAN VCR VEGFA	-0.2167 -0.1753 -0.225 0.122 0.462 0.633 0.537 0.571 0.1643 1.1045 0.607 -0.0477 0.288 0.094 0.437 -0.278 -0.164 -0.7285	0.07813 -0.0804 -0.012 0.913 0.255 nan nan 0.03063 0.177 0.776 0.03863 0.183 0.736 0.0445 0.409 0.89 0.65845 -0.914 -0.229	nan nan 1.056 1.096 0.657 0.723 0.514 0.28 0.254 0.332 -0.009 -0.674 -0.187 -0.35	-0.304 3A 0.382 1A 0.0607 1A 0.0438 1A 0.2715 1A -0.29 3A	NO NO	ND I	90 90	16 1 18 45 2 0
Vegfa Vidir	VEGFA VEDER	0.285 0.074 0.437 -0.278 -0.164 -0.7285 -0.404 -0.3164 -0.7865	- 0.6345 -0.914 -0.229 - 0.456 -0.914 -0.229	-0.674 -0.187 -0.35 -0.797 -0.684 -0.009	-0.29 3A 0.7045 3A	YES	NO I	NO NO	45 2
	VLDLR VMP1 VNN1	0.2405 0.277 0.362		-0.351 0.766 -0.17	0.7045 3A 0.3835 ** 1.08 3A	ND ND	NO I	NO NO	0
Vrk1 Vtn	VNN1 VRK1 VTN*	-0.3795 -0.3515 1.4605 0.322 0.3945 0.320 -0.053 -0.2155 -0.8155 0.292 0.056 0.011 0.35 0.0607 0.3027	0.16 0.2135 0.46 -0.638 -0.57 -0.237	0.351 0.766 0.17 0.542 0.366 0.62 -0.206 0.166 0.52 0.485 0.223 0.552 0.282 -0.118 0.168	1.08 3A 0.037 4 -0.229 3A	NO NO	NO I	NO NO	0
Vmp1 Vm1 Vm1 Vm Vm Wm5a Xcr1 Xpc	VWF WNTSA	0.322 0.5945 0.329 -0.053 -0.2155 -0.3155 0.292 0.056 0.011 0.35 0.0607 0.3027 nan nan 0.507 -0.441 -0.296 -0.443	0.2475 0.542 0.471 1.7035 0.266 0.567 0.16 0.2135 0.46 1.0357 0.217 0.217 1.0251 0.516 0.125 0.251 0.416 -0.052 0.17 0.371 0.054 0.377 -0.574 -0.58	-0.206 -0.166 -0.33 0.485 -0.223 0.552 0.282 -0.118 0.168 nan nan	-0.312 3A 0.115 3A	YES NO	ND I	NO NO	1
Xpc Zap70	VRCL VTN* VWF WNTSA XCRL XPC ZAP70 ZBP1 T0015	-0.441 -0.296 -0.443 0.0577 0.1087 0.2887	1 0.172 0.09 nan nan 1 -0.377 -0.574 -0.58 1 0.4077 0.303 0.609	nan nan -0.468 -0.39 -0.187 0.496 0.224 0.283	0.241 1A -0.397 4 0.089 1A	NO NO	NO I	NO NO	1 0 1
Zap70 Zbp1 Zfp91 Zfywi21	28P1 2/P91 2PYVE21	0.3415 0.499 1.324 0.017 0.052 0.0945	2.123 0.373 0.534 -0.0568 0.38 0.436	0.496 0.224 0.283 0.576 0.397 0.516 0.249 0.07 0.293 - -0.39 -1.169 0.401	0.007 4 -0.220 3A 0.115 3A 0.241 1A 0.241 1A 0.099 1A 0.665 4 0.1783 ** 0.055 3A	NG NG NG NG NG NG NG NG NG NG NG NG NG N	115 10 20 20 20 20 20 20 20 20 20 20 20 20 20		1 0 0
29yw21	2010121	-0.2045 -0.028 -0.383	-0.5465 -0.899 -1.642	-0.39 -1.169 0.401	A Cours	ND	NU I	NU	u

Supplementary Figures

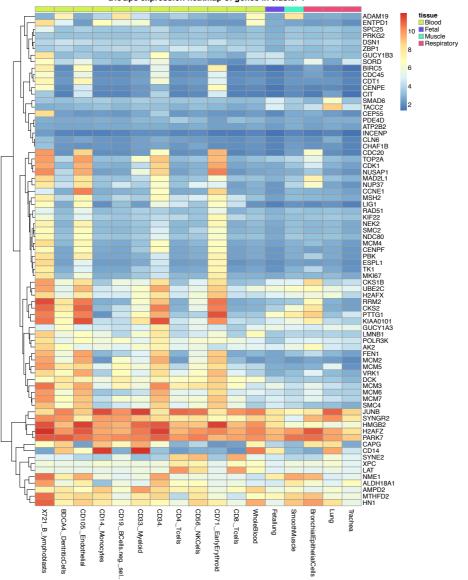


Supplementary Figure E1. Ovalbumin (OVA) immunized and challenged mice have increased airway and lung inflammation, mucus hypersecretion, serum allergen-specific lgG1 and lgE titers, airway hyperresponsiveness, and type-2 related cytokine and chemokine gene expression in the lungs compared to control animals. (A) Schematic representation of acute allergic asthma model. (B) Total and differential cell counts in bronchoalveolar lavage fluid. T - total cells, M - macrophages, E - eosinophils, N - neutrophils, L lymphocytes. (C) Representative photomicrographs of H&E stained lung sections. Inset shows eosinophils in inflammatory infiltrates in asthmatic mice. Scale bars: 200 µm (inset 50 µm), original objective: 10x (inset 40x). (D) Grades for lung inflammation; for description please refer to material and methods. (E) Representative photomicrographs of Periodic-acid Schiff (PAS) stained lung sections showing fuchsia-stained mucus in epithelial goblet cells of asthmatic animals. Scale bar: 200 µm; original objective: 10x. (F) Number of mucus-positive cells per millimeter of basement membrane. (G) Serum titers of OVA-specific IgG1 and IgE. (H) Airway resistance and dynamic compliance in response to methacholine presented as relative difference to base-

line values measured after PBS nebulization. (I) Increased mRNA expression of selected type-2 immune response cytokines and chemokines in whole lungs at 24 and 72h after OVA challenge. Data are expressed as relative difference to corresponding PBS challenged mice. Data in A-G and I are shown as mean \pm SEM and are representative from at least 2 independent experiments (*n* = 3). Data in H show summary for at least 5 mice per group and are shown as mean \pm SEM. (B-H) * *p* < 0.05 unpaired Student's *t*-test.

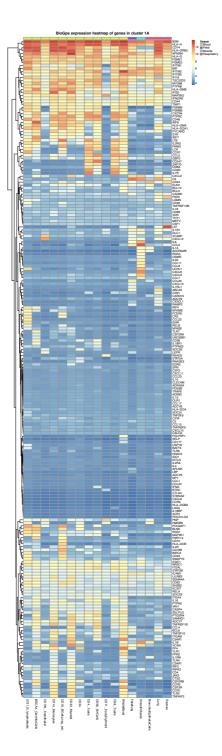
	Gene	Asthma-related	Hub (H) or	qP	CR			м	icroa	rray c	ompa	arisor	IS		
Cluster	symbol	(AR) or -ignorome (AI)	Peripheral gene (P)	at 24h	at 72h	Α	в	С	D	Е	F	G	н	ì.	J
	CARD10	AR													
	CCL11	AR	~			·									
	CCL17	AR	-												
	CCL22	AR	-		1.00										
	CXCL9	AR	н												
	CXCL10	AR	н						1						
1A	IFNG	AR	н												
	IL1B	AR	н										<u> </u>		
	LCK	AR	н												_
	PIGR	AR									÷		k		
	PSME1	AI													
	STAT1	AR	н												
	VAV1	AR													_
	C1QB	AI													
	CLEC5A	AI	Р							_					
	CTSS	AR	н												
	EMR1	AR													
	FCGR2B	AR	-		4							1.1.1.1			
1B	HCLS1	AI	н												
	IGSF6	AI	н		-										
	MAFB	AI													
	PLEK	AI	н								<u>U</u> _,				
	SIGLEC5	AR	Р												
	TYROBP	AR	н												
	ALAS1	AI	н									_			
	NR1D1	AI	-									9 - L.			
2	PER2	AR	-												
	PER3	AI													
	RORA	AR													
	ALPL	AR	н												
	BAMBI	AI	Р												
	EPHX1	AR	-												
	FZD2	AI	-												
3A	HFE	AI				_									
	KLF4	AR	н												
	PPARA	AR	н											_	
	TGFBI	AI	н												
	TIMP1	AR	н									2			-
	TMEM173	AI	P												
	PARD3	AR	н												
3B	PRKCI	AR	н												
	SQSTM1	AR	-				_	_		_					-
	MUC5AC	AR													
3C	MUC5B	AR	-												-
	TFF2	AR	-												
	CDC20	AI	-												
	CDK1	AR	-												
	CDT1	AI	-												
	DCK	AI	н												
	FEN1	AI	~												
4	HN1	AI													
~	MAD2L1	AI	н												
	MCM3	AI													
	MCM7	AI													
	NME1	AI	н												
	RRM2	AI	н												
	TK1	AI	н												

Supplementary Figure E2. Expression profiles of 59 selected genes from 7 clusters were confirmed with quantitative PCR. Data are presented as mean log_2 fold changes of gene expression by quantitative real-time PCR and microarray relative to control mice. Quantitative real-time PCR data were measured in whole lung extracts and are representative from 2 independent experiments (*n*=3), while microarray data are from 6 publicly available datasets broken down into 10 direct comparisons of asthmatic and control mice (please refer to Table1 and Figure 1 for details).

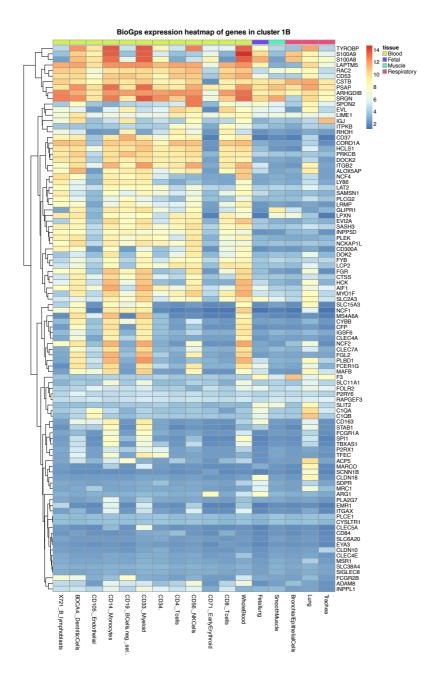


BioGps expression heatmap of genes in cluster 4

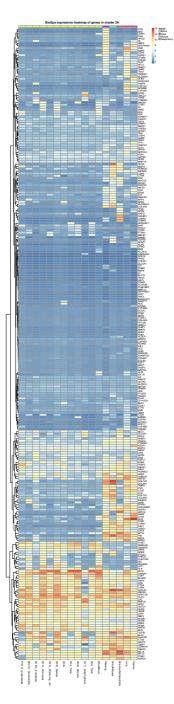
Supplementary Figure E3. BioGPS expression heatmap of cluster 4 genes indicates enrichment for genes highly overexpressed in B lymphoblasts, CD105⁺ Endothelial cells, CD34⁺ cells and CD71⁺ early erythroid cells. Data are shown as expression levels normalized in log scale within selected set of 17 different tissues (12 leukocytes and 5 present in whole lungs). Euclidean distance was used for clustering genes.



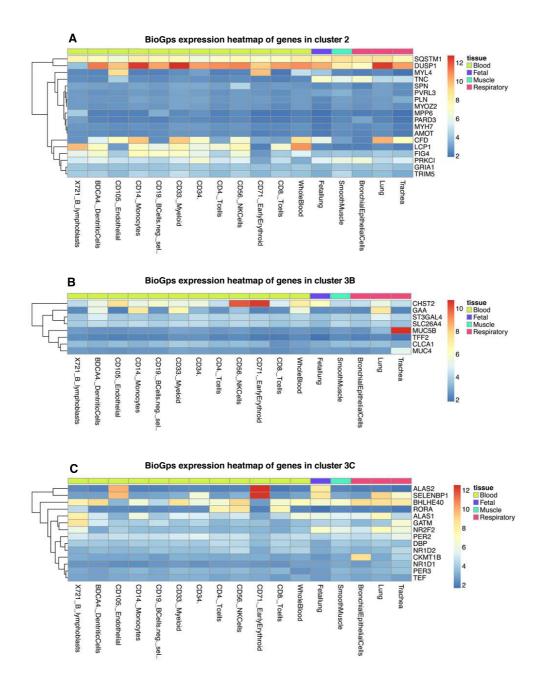
Supplementary Figure E4. BioGPS expression heatmap of cluster 1A genes indicates enrichment for genes overexpressed in leukocytes compared to the lung tissues, indicating that genes in cluster 1A are mainly involved in inflammation. Data are shown as expression levels normalized in log scale within selected set of 17 different tissues (12 leukocytes and 5 present in whole lungs). Euclidean distance was used for clustering genes.



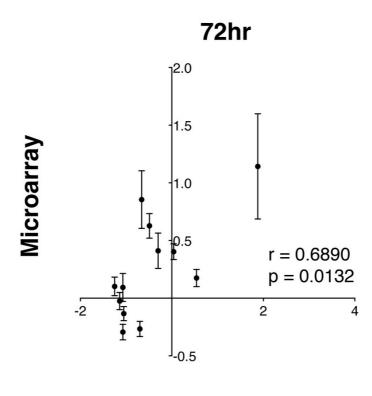
Supplementary Figure E5. BioGPS expression heatmap of cluster 1B genes indicates enrichment for genes overexpressed in the lung tissues compared to leukocytes, suggesting that those genes are involved in lung tissue response to allergic inflammation. Data are shown as expression levels normalized in log scale within selected set of 17 different tissues (12 leukocytes and 5 present in whole lungs). Euclidean distance was used for clustering genes.



Supplementary Figure E6. BioGPS expression heatmap of cluster 3A genes indicates enrichment for genes overexpressed in in leukocytes compared to the lung tissues, indicating that genes in cluster 3A have mainly role in inflammation. Data are shown as expression levels normalized in log scale within selected set of 17 different tissues (12 leukocytes and 5 present in whole lungs). Euclidean distance was used for clustering genes.

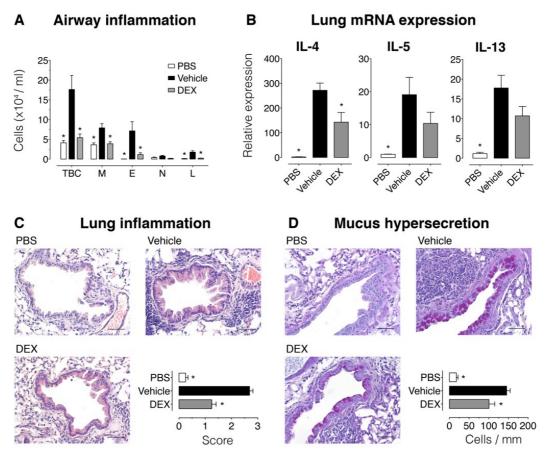


Supplementary Figure E7. BioGPS expression heatmap of (A) cluster 2, (B) cluster 3B and (C) cluster 3C genes. No specific tissue was found enriched in these clusters, probably due to low number of genes in each cluster. Data are shown as expression levels normalized in log scale within selected set of 17 different tissues (12 leukocytes and 5 present in whole lungs). Euclidean distance was used for clustering genes.



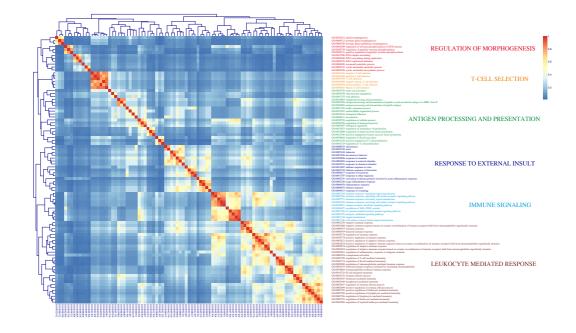
qPCR

Supplementary Figure E8. Microarray data for 12 super-connector genes from 10 comparisons of control and asthmatic mice correlate to quantitative PCR data from our independent mouse asthma model. For qPCR, OVA-sensitized BALB/c mice received PBS (controls) or OVA challenge 72h before extraction of total lung RNA. Pearson r coefficients and *p* values for each correlation are indicated.



Supplementary Figure E9. Dexamethasone significantly inhibits airway and lung inflammation, mucus hypersecretion and lung cytokine mRNA expression. (A) Total and differential cell counts in bronchoalveolar lavage fluid of control (PBS challenged), vehicle and dexamethasone (DEX) treated mice 72h after the last aerosol challenge. T – total cells, M – macrophages, E – eosinophils, N – neutrophils, L – lymphocytes. (B) Dexamethasone treatment suppressed IL-4, IL-5, and IL-13 mRNA expression in whole lungs at 72h after OVA challenge. Data are expressed as relative difference to corresponding PBS challenged mice. (C) Representative photomicrographs of H&E stained lung sections (scale bars: 50 µm, original objective: 40x). Control animals (PBS) have normal lung physiology, while dense eosinophilic inflammatory infiltrates in vehicle-treated mice and are reduced in dexamethasone (DEX) treated group. Grades for lung inflammation were done according to the description in material and methods. (D) Representative photomicrographs of Periodic-acid Schiff (PAS) stained lung sections with fuchsia-stained mucus in epithelial goblet cells (scale bars: 50 µm, original objective: 40x) and numbers of mucus-positive cells per millimeter of basement membrane. Data are

shown as mean \pm SEM and are pooled from 2 independent experiments (*n* = 6). * *p* < 0.05 *vs.* vehicle treated group, one-way ANOVA followed by Tukey's multiple comparison test.



Supplementary Figure E10. Pathway-driven approach: 76 enriched Gene Ontology Biological Processes (GO.BP) terms in 10 microarray comparisons of asthmatic mice *vs.* controls. GO.BP terms are clustered for semantic similarity: red-yellow zones refer to related biological processes.

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3.2. Publication 1

Dendritic polyglycerolsulfate near infrared fluorescent (NIRF) dye conjugate for non-invasively monitoring of inflammation in an allergic asthma mouse model

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Dendritic Polyglycerolsulfate Near Infrared Fluorescent (NIRF) Dye Conjugate for Non-Invasively Monitoring of Inflammation in an Allergic Asthma Mouse Model

Stefania Biffi^{1,2,*}⁹, Simeone Dal Monego³⁹, Christian Dullin⁴, Chiara Garrovo^{1,2}, Berislav Bosnjak⁵, Kai Licha⁶, Pia Welker⁶, Michelle M. Epstein⁵, Frauke Alves⁷

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Abstract

Background: Non-invasive *in vivo* imaging strategies are of high demand for longitudinal monitoring of inflammation during disease progression. In this study we present an imaging approach using near infrared fluorescence (NIRF) imaging in combination with a polyanionic macromolecular conjugate as a dedicated probe, known to target L- and P-selectin and C3/C5 complement factors.

Methodology/Principal Findings: We investigated the suitability of dendritic polyglycerol sulfates (dPGS), conjugated with a hydrophilic version of the indocyanine green label with 6 sulfonate groups (6S-ICG) to monitor sites of inflammation using an experimental mouse model of allergic asthma. Accumulation of the NIRF-conjugated dPGS (dPGS-NIRF) in the inflamed lungs was analyzed *in* and *ex vivo* in comparison with the free NIRF dye using optical imaging. Commercially available smart probes activated by matrix metalloproteinase's (MMP) and cathepsins were used as a comparative control. The fluorescence intensity ratio between lung areas of asthmatic and healthy mice was four times higher for the dPGS in comparison to the free dye *in vivo* at four hrs post intravenous administration. No significant difference in fluorescence intensity between healthy and asthmatic mice was observed 24 hrs post injection for dPGS-NIRF. At this time point *ex-vivo* scans of asthmatic most exore used at 4 hrs post injection.

Conclusions/Significance: Compared with smart-probes resulting in a high fluorescence level at 24 hrs post injection optical imaging with dPGS-NIRF conjugates is characterized by fast uptake of the probe at inflammatory sites and represents a novel approach to monitor lung inflammation as demonstrated in mice with allergic asthma.

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Competing Interests: Stefania Biffi, Simeone dal Monego and Chiara Garrovo are employees of Cluster in Biomedicine (CBM Scrl). CBM offers services in different research areas, spanning from genomics and bioinformatics, to optical imaging and advanced microscopy. CBM is also actively involved in research collaborations within the framework of european and national projects, as well as project management and grant writing support. Kai Licha and Pia Welker are employees of Mivenion GmbH. Mivenion is a life-science company focusing on personalized medicine for patients with inflammatory and autoimmune diseases. A network of academia and industry partners is supporting Mivenion. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials. The authors from Academia (Goettingen and Vienna) have declared that no competing interests exist.

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Introduction

Currently, NIRF imaging is a common technology in preclinical studies that obtains functional information *in vivo* over time for assessment of antibody binding, protein expression, enzyme activities, cell tracking etc. [1–3]. Optical imaging provides relatively inexpensive and non-harmful methods and is preferred over other imaging methods used in preclinical research and drug development, such as PET and SPECT that are more complex to perform. However, the penetration depth of typically up to 4 cm

in the near infrared range (NIR) limits its clinical application to endoscopic techniques and structures beneath the skin or fluorescence guided surgery [4]. Crucial for the success of *in vivo* NIRF imaging will be the development of dedicated NIRF probes for distinct targets of molecular events characterizing different diseases. So far, these probes, based on their mechanisms of targetdetection can be divided into four groups: passive probes to image areas with increased blood supply [5], target-specific fluorescent probes which are directed against molecular and/or diseasespecific markers [6], fluorescent labels to track injected fluores-

cence stained cells [7], and application of smart probes activated by enzymes for the detection of molecular events [8].

NIRF imaging in lung disease models has remained challenging due to the high scattering nature of the lung and its comparable deep location. Recent application of novel non-invasive imaging technologies in mouse models of asthma has enabled functional and longitudinal *in vivo* monitoring of disease, validation of novel biomarkers, and direct tracking of immune cells within tissues. Novel methods for *in vivo* monitoring of lung inflammation in mice include the utilization of smart-probes activated by MMPs or cathepsin, enzymes known to be involved in lung inflammation [9–11].

Airway inflammation is a central component of asthma that consists of edema, cellular infiltration, particularly of eosinophils, neutrophils, activated T lymphocytes and mast cells, increased airway secretions, and deposition of excess collagen. Therefore mouse models of asthma present attractive tools for evaluating probes suitable for *in vivo* molecular imaging of lung inflammation [12,13].

Using a model of allergen-induced lung inflammation, we applied fluorescence imaging in combination with near-infrared (NIR) fluorescently-labeled dendritic polyglycerol sulfates (dPGS), a class of compounds that selectively bind to mediators of inflammatory processes such as L- and P-selectin and C3/C5 complement factors [14,15]. The role of selectin-ligand interactions in allergic asthma is well established, making them an attractive target for visualization of inflammation [16-19]. For example, reduced airway hyperresponsiveness in asthma in L-Selectin-deficient mice has been reported [19]. Furthermore, studies show that dPGS is transported into inflammatory cells e.g. in activated mononuclear cells [20,21]. Generally, dPGS consists of a highly branched (dendritic) polyglycerol core, which due to the large amount of hydroxyl end groups enables high functionalization. In our case, sulfate groups were generated from the hydroxyl groups, thereby creating the highly charged, polyanionic dPGS compound (Figure 1). dPGS acts via a multivalent binding mechanism mimicking naturally occurring selectin ligands [20], with a clearly demonstrated dependence of the binding affinity from molecular weight and degree of sulfation [15,21]. Sulfation of the hydroxyl groups in the polymer established a multivalent polyanionic entity with high affinity for L- and P-selectin [22]. Anti-inflammatory property of dPGS in much higher concentrations has been reported to occur as a result of a multivalent interaction enabled by the multitude of sulfate groups. For instance, binding of dPGS to L-selectin on leukocytes and Pselectin on inflamed vascular endothelium reduces leukocyte extravasation by shielding the adhesion molecule [22]. Additionally, inhibition of C5a generation inhibits leukocyte chemotaxis [14,22].

The compound used herein has a core molecular weight of about 6000 Da, imparting high binding affinity of the respective polysulfate [21] and at the same time having a reasonable molecular weight range for sufficient distribution and excretion [20], as well as chemical derivatization in order to conjugate NIRF dyes to the polymeric entity. The aim of the present study was to assess the capacity of dPGS conjugated with a near infrared fluorescent (NIRF) dye related to indocyanine green (dPGS-NIRF) to detect inflammatory sites in lungs by NIRF optical imaging analysis in a mouse model of asthma and to compare dPGS-NIRF to the commercially available smart-probes MMPSense and ProSense.

Results

OVA-immunization and challenge-induced allergic inflammation and extensive mucus hypersecretion in the lungs, and elevated serum OVA-specific IgG1

Allergic asthma inflammation and mucus hypersecretion in mice was induced by two intraperitoneal injections and subsequent intranasal challenges with OVA. Figure 2 illustrates lung histology from H&E and PAS-stained lung sections of asthmatic and healthy control mice. H&E staining revealed that no inflammatory infiltrates were present in lungs from healthy mice (Figure 2A). In contrast, immunized mice had dense inflammatory infiltrates containing predominantly eosinophils, as well as macrophages and lymphocytes surrounding blood vessels, and large and small airways (Figure 2B). The extent of allergic inflammation was evaluated by assessing the total surface area and location of leukocyte infiltration in lung sections (Figure 2C). Mice with allergic inflammation have histological scores of 5.2 ± 0.4 (dPGS-NIRF group) and 4.4 ± 0.3 (dye group) compared to healthy controls with 0.5±0.3 (dPGS-NIRF group) to 0.8±0.3 (dye group), demonstrating that diseased mice have lung inflammation affecting more than two thirds of the examined lung sections with infiltrates present in the hilum extending to the lung periphery.

To assess mucus hypersecretion, adjacent lungs sections were stained with PAS. As expected, only rare mucus producing cells were detected in the central airways of healthy control mice (Figure 2D), whereas numerous mucus producing cells were observed in asthmatic mice (Figure 2E). Histological evaluation revealed that asthmatic mice have histological scores for mucus overproduction of 2.8 ± 0.4 (dPGS-NIRF group) and 2.5 ± 0.5 (dye group) compared to healthy controls with 0.3 ± 0.3 (dPGS-NIRF group) to 0.2 ± 0.3 (dye group) (Figure 2F), indicating that mucus hypersecretion extended to the periphery of the diseased lungs.

We also tested serum OVA-specific Th2-isotype antibody titres. While no OVA-specific antibodies in sera were detected before immunization with OVA, high titres (\geq 1:7812500) of OVA-specific IgG1 were detected in all OVA-sensitizated and challenged mice (results not shown), further supporting presence of allergic immune responses in both investigated groups.

Allergic asthma can be successfully visualized by combination of dPGS-NIRF probe and *in vivo* optical imaging

To visualize allergic inflammation *in vivo*, we injected dPGS-NIRF and the control dye i.v. into the tail vein at 72 hrs after last OVA challenge, when we expected that allergic inflammation in the lung is at its peak. Asthmatic and healthy mice were imaged at 4 and 24 hrs post dPGS-NIRF or unconjugated NIRF dye injection as control.

Figures 3 and 4 illustrate the distribution of the control dye and dPGS-NIRF, respectively, after 4 hrs in the thoracic area of asthmatic in comparison to healthy mice. A slight increase of fluorescent signal was recorded after injection of control dye in asthmatic mice in comparison to healthy mice (Figure 3A). In order to localize the dPGS-NIRF probe within inflamed lung region we applied fluorescence microscopy in combination with immunofluorescence staining of macrophages by the use of an antibody against F4/80, a 160 kDa cell surface glycoprotein that is widely expressed on mature tissue macrophages. As shown in Figure 3B a higher amount of macrophages was clearly detectable in lungs of asthmatic mice in comparison to healthy controls. The Control dye was not detected in lung sections of asthmatic mice using fluorescence microscopy (Figure 3B). In contrast, higher

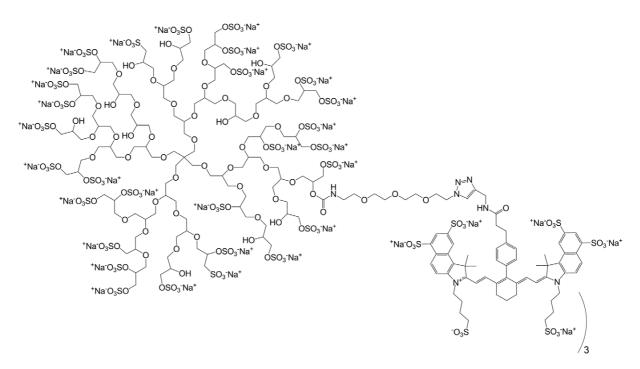


Figure 1. Chemical structure of dPGS-NIRF. The chemical structure indicates the linker structure and connection to the dye (approx. 3 dyes per polymer). Please note that the polymer is not depicted in original molecular weight, but is shown only as principle sketch. doi:10.1371/journal.pone.0057150.g001

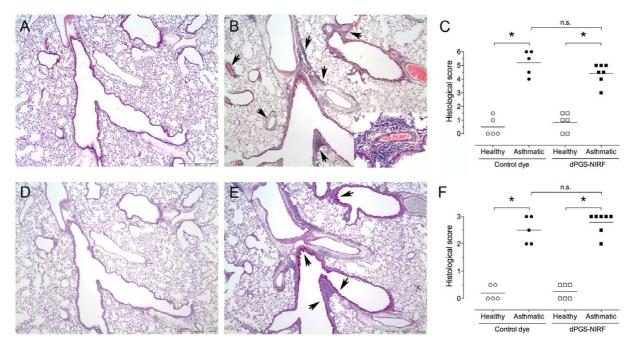


Figure 2. Allergic inflammation and mucus hypersecretion in the lungs of asthmatic, but not control mice. Lungs were harvested 76 hrs after the final ovalbumin (OVA) challenge meaning 4 hrs post i.v. probe injection. Representative H&E (A and B) and PAS (D and E) stained photomicrographs of lungs from healthy (A and D) or asthmatic mice (B and E) are shown (magnification $40 \times$, inset $400 \times$). (B) Arrows indicate inflammation, and in (E) arrows indicate mucus hypersecretion. Allergic inflammation (C) and mucus hypersecretion (F) scores in H&E and PAS stained lung sections, respectively, of healthy (open symbols) or asthmatic mice (filled symbols). Each symbol represents individual mice (n = 5–6 for healthy groups and n = 5–7 for asthmatic groups), and line represents group mean. One-way ANOVA followed by Tukey's multiple comparison test (*P<0.05) was used to compare differences between groups. doi:10.1371/journal.pone.0057150.g002

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fluorescence intensity was detected in the thoracic region of asthmatic mice 4 hrs post dPGS-NIRF probe injection (Figure 4A). Moreover, fluorescence microscopy of lung sections of asthmatic mice confirmed dPGS-NIRF probe localization in areas where F4/80 stained macrophages could be detected, which demonstrated that dPGS-NIRF accumulates especially in the inflamed region of lungs of the pathological model (Figure 4B).

Fluorescence signals obtained with in vivo imaging were quantified and intensity ratios were calculated as described in the Material and Methods. As depicted in Figure 5A, at 4 hrs post injection of control dye, we observed a slight increase in fluorescence signal in asthmatic mice when compared to healthy mice (increase in average $RI_{Dve}(4h) \sim 11\%$, p-value = 0.047), most probably due to an increase in the vascular flow in the inflamed lungs. In contrast, dPGS-NIRF increased the fluorescence signal in the thorax of asthmatic mice dramatically, as seen by an average $RI_{dPGS}(4h) \sim 44\%$ with p-value = 0.004. Moreover, a direct comparison of the contrast (RI) between dPGS-NIRF and free dye in the asthmatic mice revealed a 30% higher $RI_{dPGS}(4h)$ than $RI_{Dye}(4h)$ (p-value = 0.005) at this time point. At 24 hrs post dPGS-NIRF injection, fluorescence signals over the lung areas of healthy and asthmatic mice were not longer distinguishable (average $RI_{dPGS}(24h)$ difference ~8%, p-value = 0.162) (Figure 5B). In vitro analysis of serum binding of ICG as well as of 6S-ICG demonstrate that ICG completely binds to serum proteins (23), whereas less than 40% of 6S-ICG was bound to serum proteins (data not shown).

Ex vivo optical imaging confirmed the in vivo results

To confirm the *in vivo* imaging findings immediately after the last imaging, we imaged the lungs ex-vivo using an Optix MX2 system. Ex-vivo imaging avoids autofluorescence of other organs and absorption and scattering within the body and fur. This increases both specificity and sensitivity of probe detection. In accordance to the *in vivo* results, we found a significant difference between the fluorescence intensity within the lungs of asthmatic and healthy mice 4 hrs post injection of the dPGS-NIRF conjugate (difference of $RI_{dTGS}^{ex-vivo}(4h) \sim 65\%$, p-value = 0.009), but not control dye (difference of $RI_{Dye}^{ex-vivo}(4h) \sim 18\%$, p-value = 0.127) (Figure 6A and 6B). At 24 hrs post administration of dPGS-NIRF, the observed fluorescence intensity over the lungs was reduced to about 30% of the intensity measured 4 hrs post injection. Moreover, the difference in fluorescence intensity between healthy and asthmatic mice dropped down to $\sim 10\%$ and was not significant (difference of $RI_{dPGS}^{ex-vivo}(24h) \sim 10\%$, p-value = 0.323) (Figure 6B).

Comparison of dPGS-NIRF with commercially available smart-probes

Commercially available smart-probes ProSense and MMPSense, activated by cathepsins and MMPs, respectively, were used for imaging lung inflammation [9]. Both smart-probes were injected at 72 hrs after the last OVA challenge and imaged after 24 hrs, according to probe manufacturer's recommendations. The intensity difference from the thoracic region between healthy and asthmatic mice was $\sim 27\%$ (p-value = 0.013) after administration of ProSense and $\sim 83\%$ after injection of MMPSense but with no statistical significance (p-value = 0.093) (Table 1).

Discussion

In this study, we present a novel approach for functional *in-vivo* imaging utilizing a dendritic polyglycerolsulfate conjugated to a NIRF dye related to ICG (dPGS-NIRF) in combination with optical imaging to monitor sites of inflammation in the lung by applying an experimental model of allergic asthma [12].

We successfully demonstrated that the applied dPGS-NIRF probe accumulates to inflammatory sites within the lung already 4 hrs after probe administration. The results show a significant four times stronger contrast of the fluorescence intensity of the dPGS-NIRF probe compared to the free dye in lungs of asthmatic in comparison to healthy mice. At this time point fluorescence

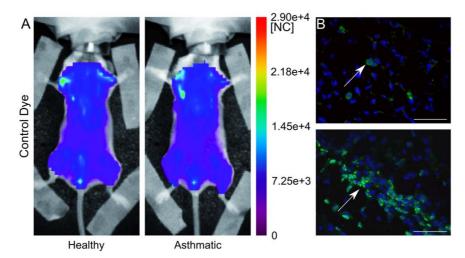


Figure 3. In vivo distribution of free dye (indocyanine green) 4 hours post probe injection and 76 hours post last OVA challenge. Panel A: whole body fluorescence intensity distribution of a representative healthy and asthmatic mouse displayed in normalized counts [NC]. Panel B: Fluorescence microscopy images of F4/80 stained macrophages and DAPI stained cell nuclei of lungs isolated from asthmatic and healthy mice injected with the NIRF labeled control dye and sacrificed 4 hrs post injection demonstrate no fluorescent control dye. F4/80 expression on macrophages are depicted in green, cell nucleus in blue, control dye was not detected (bar = 50 µm). In the healthy model few macrophages have been detected with respect to the asthmatic mouse, where cluster of cells are visible (see white arrows indicating macrophages). In both samples no unconjugated NIRF dye 65-ICG has been visualized. doi:10.1371/journal.pone.0057150.q003

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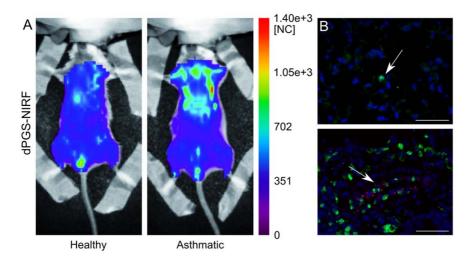


Figure 4. In vivo distribution of dPGS-NIRF 4 hours post probe injection and 76 post last OVA challenge. Panel A: whole body fluorescence intensity distribution in a representative healthy and asthmatic mouse displayed in normalized counts [NC]. Stronger fluorescence intensity over the lung area of the asthmatic mouse can be seen. Panel B Fluorescence microscopy images of F4/80 stained macrophages and DAPI stained cell nuclei of lungs isolated from asthmatic and healthy mice injected with dPGS-NIRF and sacrificed 4 hrs post injection. F4/80 expression on macrophages are depicted in green, cell nucleus in blue, dPGS-NIRF displayed in red (bar = 50 μ m). In the healthy model, few macrophages and no probe localization have been detected. In the asthmatic mouse, cluster of macrophages are detectable (see white arrows) and the dPGS-NIRF probe was visualized in the same region of macrophages. doi:10.1371/journal.pone.0057150.g004

microscopy confirmed the localization of the dPGS-NIRF probe within the inflamed lungs in areas where F4/80 stained macrophages could be detected and histology demonstrated the presence of inflammatory infiltrates in more than two thirds of the examined lung sections. Therefore, dPGS-NIRF is suitable to monitor inflamed lungs by NIRF imaging.

Furthermore, at 4 hrs post injection, the calculated $RI_{ex}(4h)$ was significantly lower in asthmatic lungs after administration of free dye. These results indicate that a specific target in the model appears to be involved. dPGS-NIRF exerts high-affinity binding to

positively charged protein motifs e.g. P- and L-selectin as well as to C3/C5 complement factors [22]. The selectivity is demonstrated by the very low affinity for E-selectin compared to P and L-selectin, which dPGS bind to with nanomolar affinity *in vitro* [20,22]. Furthermore, dPGS accumulates in inflamed tissue by a not yet understood cellular uptake mechanism into macrophages and endothelial cells, but not into lymphocytes. This was shown for example by fluorescence microscopy of liver tissue specimens after dPGS-NIRF application that depicted accumulation in rat liver macrophages (Kupffer cells) and of A549 tumor cells as well

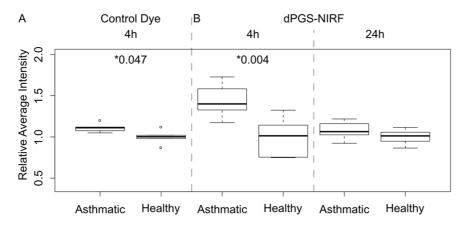


Figure 5. Quantification of in vivo imaging results of dPGS-NIRF and pure dye. Box plots of ratios of average fluorescence intensity over the lung area compared with the mean value of each control group respectively are reported for asthmatic and healthy mice. Mice treated with free dye 4 hrs post injection showed a slight increase in fluorescence signal in asthmatic mice (n = 5) when compared to healthy mice (n = 5; increase in average ~11%, p-value = 0.047, panel A). Mice treated with dPGS-NIRF probe 4 hrs post injection (healthy n = 6, asthmatic n = 6) showed an increased fluorescence signal in the thorax in asthmatic mice (increase in average ~44% with p-value = 0.004, panel B left side). At 24 hrs post injection fluorescence signals over the lung areas of healthy (n = 5) and asthmatic mice (n = 10) shown no difference (difference ~8%, p-value = 0.162, panel B right side). Both control dye and dPGS-NIRF probe were injected 72 hrs after last aerosol challenge. Note, intensity ratios were used to compare probes with different brightness, therefore the box plots are depicted in the same scale.

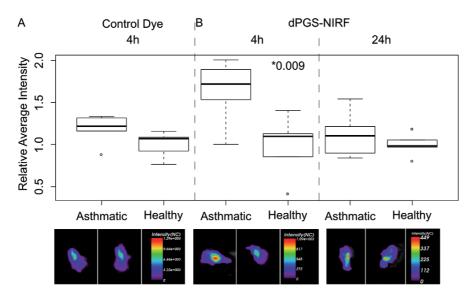


Figure 6. Ex vivo imaging results of dPGS-NIRF and pure dye. Box plots of ratios of average fluorescence intensity over the explanted lungs compared with the mean value of each control group respectively are reported for asthmatic and healthy mice treated with free dye 4 hrs post injection (panel A), and treated with dPGS-NIRF probe 4 hrs (panel B left side) and 24 hrs (panel B right side) post injection. The corresponding fluorescence intensity images of representative lungs are given at the bottom of each box plot. A significant difference between the fluorescence intensity within the lungs of asthmatic (n = 5) and healthy mice (n = 5) was observed 4 hrs post injection of the dPGS-NIRF conjugate (difference of ~65%, p-value = 0.009), but not of the control dye (healthy n = 5, asthmatic n = 5; difference of ~18%, p-value = 0.127). Both control dye and dPGS-NIRF probe were injected 72 hrs after last aerosol challenge. Note, intensity ratios were used to compare probes with different brightness, therefore the box plots are depicted in the same scale. doi:10.1371/journal.pone.0057150.g006

as of activated, LPS-stimulated mononuclear cells, both demonstrating accumulation of dPGS [22].

The underlying chemical structure of the polymer in published studies [22] is based on a polyglycerol core of 6000 Dal, whereas different dyes were attached to the polymer, such as a visible cyanine dye or a NIRF dye in a ratio of approx. 1 dye per polymer, yielding identical selectin-binding properties. The conjugate used herein employs the same polymer, but a more hydrophilic indocyanine dye with 6 sulfonate groups (6S-ICG) added to the fluorophore structure. Coupling to the dPGS could be achieved at a dye-to-polymer ratio of 3 without signs of aggregation known to be induced by more lipophilic indocyanine dyes in bioconjugates, as described in [20].

Previously, studies show also inflammation-specific imaging with dPGS-NIRF in an animal model of collagen induced rheumatoid arthritis using the preceding conjugate with a lipophilic indocyanine green label. Comparable to our study, the authors demonstrated a fast and selective uptake of the probe with a 3.5 fold higher fluorescence difference between healthy and diseased joints and a signal peak at 1 hr after probe administration. Together with a rough estimation of a blood half-life of shorter than 1 hr by employing the eye fluorescence as a provisional solution to monitor blood kinetics, they postulate targeting mechanisms not yet fully understood [20] whereby dPGS-NIRF binds to mediators of inflammation.

Interestingly, the high contrast between the fluorescence intensity of dPGS-NIRF in the asthmatic and healthy groups was not observed after 24 hrs. This might be explained in part by shedding of P- and L-selectins from the cell surface after binding of dPGS-NIRF [23]. Bound dPGS-NIRF probes will be removed from the cells resulting in the reduction of fluorescence intensity to background after 24 hrs.

The MMPSense and ProSense probes, which are activated in the presence of inflammation-associated enzymes such as cathepsin and MMP's that are present in the lungs during allergen challenge are successfully used by others, for example to detect lung inflammation and rapidly screen for new drug effects [9–11] as well as to visualize colon adenomas [8]. Similar to our study,

Table 1. Calculated average fluorescence intensity ratios $RI_{ex}(t)$ between healthy and asthmatic mice after injection of control dye, dPGS-NIRF, or two commercially available probes: ProSense and MMPSense.

	Control Dye	dPGS-NIRF		ProSense	MMPSense
measurement time (hours)	4	4	24	24	24
in vivo	1.11±0.06 (0.047)	1.45±0.20 (0.004)	1.08±0.10 (0.162)	1.27±0.02 (0.013)	1.83±0.50 (0.093)
ex vivo	1.18±0.18 (0.127)	1.65±0.35 (0.009)	1.10±0.22 (0.323)	n.d.	n.d.

Results are shown as mean calculated average fluorescence intensity ratios \pm standard deviation, while statistical significance between each pair of control and asthmatic mice is given by p-value for the Welch-T-Test in brackets. Legend: n.d. – not done. doi:10.1371/journal.pone.0057150.t001

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Cortez-Retamozo et al. demonstrated fluorescence differences between asthmatic lungs and healthy controls by applying the same amount of MMPSense or ProSense however by using fiberoptic bronchoscopy and fluorescence molecular tomography (FMT) [9]. Others also reported that the *in vivo* profile of cysteine protease activation was depicted by FMT in a mouse model of acute airway inflammation by LPS-induction [10,11].

These smart probes exhibit slower kinetics due to their activation mechanism, demonstrating maximal fluorescence intensity within the lungs about 24 hrs after probe injection [9]. The application of these enzymatically activated probes is often hampered by the fact that despite a dramatic increase of their fluorescence intensity over inflammatory areas, the activated probes do not remain at the site of interest for very long and instead produce a strong liver signal due to their excretion pathway.

In conclusion, we present a novel *in vivo* NIRF imaging probe for detection of inflammatory reactions within the lungs of mice, as demonstrated in mice with allergic inflammation, by utilizing a dendritic polyglycerolsulfate NIRF dye conjugate known to bind to selectins and complement factors. The greater fluorescence intensity of dPGS-NIRF in inflammation of mice with allergic asthma in combination with rapid kinetics makes dPGS-NIRF a powerful probe candidate to monitor inflammation processes and responses to therapy in experimental mouse models of lung disease.

Materials and Methods

Mice

Female BALB/c mice (4- to 6-weeks old) were purchased from Charles River and maintained with ad libitum food and water. All the experimental procedures were performed in compliance with the guidelines of European (86/609/EEC) and Italian (D.L.116/ 92) as well as German laws and were approved by the Italian Ministry of University and Research and the Administration of the University Animal Facility, Trieste, as well as by the administration of Lower Saxony, Germany.

Synthesis of a dendritic polyglycerol sulfates NIR dye conjugate probe (dPGS-NIRF)

dPGS was synthesized by anionic polymerization of glycidol and subsequent sulfation using SO3/pyridinum complex according to Türk and colleagues [14]. Conjugation of dPGS to an NIRF dye (based on indocyanine green chromophore; derivative with reactive group for conjugation) are described [20] elsewhere. Briefly, the polyglycerol intermediate was reacted with an aliphatic linker chain followed by the sulfation reaction. To this linker, a novel NIRF dye (6S-ICG propargyl; mivenion GmbH) was conjugated followed by high-performance liquid chromatography (HPLC) purification yielding dPGS-NIRF with a mean dye-topolymer ratio of 3 and an average molecular weight of 19000 Da. The degree of sulfonation was 85% (elementary analysis) and the polydispersity index (PDI) within 1.6-1.8 (measured for the polyglycerol intermediate using GPC). The dye used herein is a hydrophilic version of the previously described indocyanine green label, with 4 additional sulfonate groups in the molecule resulting in a 6-fold sulfonated entity of maximal hydrophilicity for this type of NIR fluorophore. The chemical structure is depicted in Figure 1. Absorption maxima in PBS were 710 and 795 nm, fluorescence emission maximum 810 nm. Unconjugated NIRF dye (6S-ICG molecular weight ~1700 g/mol, free carboxylic acid instead of linkage to polymer) served as control probe in the in vivo experiments.

Mouse Model of Acute Allergic Asthma

Mice were sensitized intraperitoneally (i.p.) at day 0 and day 21 with 10 μ g ovalbumin (OVA) dissolved in 200 μ l PBS. At day 28 and day 29 mice were treated intranasal (i.n.) with a solution of 100 μ g OVA/50 μ l PBS/mouse. Healthy age and gender matched BALB/c mice served as controls. Histology of H&E stained lung sections was performed at 76 hrs post last challenge

Optical Imaging Scan

48 mice were examined by optical imaging (Table 2). Mice were shaved over the lung area prior to the scanning procedure in order to reduce scattering of the signal from fur. Throughout all imaging sessions, mice were anesthetized with vaporized isoflurane at 1.8–2 volume % as described [2]. The anesthetized mice were placed inside an Optix MX2 acquisition system (Advanced Research Technologies, Montreal, Canada) and gently fixed on a heated block (37°C) for the entire duration of data acquisition.

All *in vivo* analyses were preceded by native scans of the mice prior to NIRF probe injection to provide a base line for later analysis. At 72 hrs after the last OVA challenge, mice were injected intravenously (i.v.) via the tail with 100 μ l of one of the following: dPGS-NIRF (2.6 nmol, polymer/dye = 1/3), free NIRF dye (3.6 nmol), 100 μ l (5 nmol) of either MMPSense (MMPSense[®], Perkin Elmer) or ProSense (ProSense[®], Perkin Elmer), all dissolved in 0.9% NaCl. The amount of injected dPGS-NIRF and NIRF solutions was calculated based on the weaker fluorescence signal of dye in the conjugate than in the unconjugated control dye. Exact numbers of animals in each group are shown in Table 2.

In vivo and ex vivo Optical Imaging

Animals with acute asthma and wild type controls were scanned at 4 and 24 hrs post i.v. dPGS-NIRF or NIRF dye administration. For the MMPSense and ProSense, scans were performed 24 hrs after probe administration. According to the supplier (PerkinElmer), this time point constitutes the peak activation of these probes [9]. All in vivo data was acquired by using the small-animal timedomain Optix MX2 preclinical NIRF-imager (Advanced Research Technologies, Montreal, CA), equipped with four pulsed laser diodes and a time correlated single photon counting detector [24]. This system works in reflection mode applying a raster acquisition scheme, measuring and analyzing fluorescence response to pulsed excitation for each excitation spot by creating fluorescence photon time of flight histograms. In all imaging experiments applying the dPGS-NIRF and control dye, a 785 nm pulsed laser diode with a repetition frequency of 80 MHz was used whereas for the MMPSense and ProSense studies a 670 nm pulsed laser diode with a repetition frequency of 80 MHz was applied. Fluorescence emission was accordingly collected with an 800 nm long pass filter for dPGS-NIRF and control dye and a 700 nm long pass filter for both MMPSense and ProSense to block the excitation light. Two-dimensional regions of interest (ROIs) were selected, and laser power, integration time (repetition time of the excitation per raster point), and scan step size were optimized according to the emitted signal. Prior to probe application, mice were scanned to obtain background images. These background signal intensities recorded with the baseline image for each animal before the injection of the probe was subtracted from each post injection image. At the end of the last imaging session, 4 and 24 hrs after dPGS-NIRF/NIRF dye i.v. injection, animals were sacrificed and ex vivo optical imaging of the explanted lungs was performed. To calculate the total lung fluorescence intensity (Ilung) in each scan, fluorescence intensities were normalized with the laser power used for excitation and summed up in ROI's

	Control Dye	dPGS-NIRF		ProSense	MMPSense
Measurement time					
(hours)	4	4	24	24	24
· · · ·					
Healthy mice (number)	5	6	5	2	2

doi:10.1371/journal.pone.0057150.t002

encompassing the whole organ. Lungs were then preserved in formalin for histological analysis.

Image processing

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Image analysis was done using OptiView (2.02.00), the proprietary software developed for the Optix device. All data sets of mice receiving dPGS-NIRF, free NIRF dye control as well MMPSense and ProSense were normalized for different excitation laser power and variations of the used integration time and therefore expressed in normalized counts [NC], an arbitrary unit. Average fluorescence intensity was calculated within a region of interest covering the whole lung for every sample (x) and time point (t) as $\bar{I}(x,t)$ and subtracted by the base line intensity within the same region $\bar{I}_{bg}(x)$. To remove the influence of different brightness of all applied probes, ratios $RI_{ex}(x,t)$ between the average intensity of the sample (x) and the mean average intensity of the control group for each experiment (ex) and time point (t) were calculated and denominated as $RI_{ex}(x,t)$.

$$RI_{ex}(x,t) = \frac{\bar{I}(x,t) - \bar{I}_{bg}(x)}{N_{cnt}^{-1} \sum \bar{I}(x,t)}$$
(1)

 $RI_{ex}(x,t)$ can be interpreted as contrast or probability to distinguish asthma mice from controls at certain time points and was therefore used for comparison of the different studies and statistical calculations.

Histological analysis of lung inflammation

Following ex vivo image analysis performed 76 hrs after the last ovalbumin challenge, tissue samples were fixed in 10% buffered formalin and embedded in paraffin. To evaluate allergic lung inflammation, 3 µm thick lung sections containing main stem bronchi were stained with hematoxylin and eosin (H&E). A blinded grading of the slides was done to evaluate the intensity and extent of inflammation according to our semi-quantitative scoring system. For intensity of inflammation: 0 - no inflammatory infiltrates; 1 - inflammatory infiltrates in central airways; 2 inflammatory infiltrates extending to middle third of lung parenchyma; and 3 - inflammatory infiltrates extending to periphery of the lungs. For extent of inflammation: 0 - no inflammatory infiltrates; 1 - inflammatory infiltrates present in one third of lung surface; 2 - inflammatory infiltrates spreading up to two thirds of lung surface; 3 - inflammatory infiltrates present in more than two thirds of lung surface. Data are presented as histological score calculated as the sum of intensity and extent of inflammation for each sample. For detection of mucus-containing cells in lung tissue, adjacent 3 µm sections containing main stem bronchi from each lung specimen were stained with periodic acid-Schiff (PAS) and counter stained with hematoxylin. Slides were examined blinded for the treatment and mucus overproduction

was scored as: Grade 0 – no mucus producing cells in airways; Grade 1 – few mucus producing cells in central airways; Grade 2 – mucus producing cells detected in middle airways; and Grade 3 – mucus producing cells extending to respiratory bronchioles. In borderline cases, an intermediate grade was used (0.5; 1.5 or 2.5), extending the scoring to a total of seven grades.

Serum OVA-specific immunoglobulin

For the measurement of OVA-specific immunoglobulin (Ig) G1, ELISA plates were coated with OVA at 10 μ g/ml overnight at 4°C. The plates were washed and blocked with 2% bovine serum albumin in PBS with 0.05% Tween 20 for 2 hrs at RT. Then sera were titrated onto the plates and incubated for 24 hrs at 4°C before washing. Plates were incubated for an additional 2 hrs at 4°C with biotinylated anti-IgG1 (Southern biotechnology associates Inc., Birmingham, AL, USA) detection mAb, followed by incubation with streptavidin horseradish peroxidase (Southern biotechnology) for 1 h at RT. Plates were washed and incubated with TMB substrate solution (100 µl/well, BD OptEIATM, Becton Dickinson Biosciences) for 10 min at RT. The reaction was stopped with 100 µl of 0.18 M H2SO4 and the plates were measured at 450 nm.

In vitro analysis of serum binding

The serum binding of 6S-ICG was determined in vitro by incubation with pooled human serum (PAA) with dye concentration of 5 μ g/ml [25]. The sample was placed in a Centriprep micropartition unit NWML 30 kDa (Milipore, Billerica, USA), and centrifuged at 5000 g for 20 min. The protein-bound 6S-ICG and the free dye in the ultrafiltrate was quantified spectrophotometrically (Beckman Coulter, USA).

Fluorescence microscopy

Detection of injected dPGS-NIRF probe or unconjugated NIRF dye 6S-ICG in lungs of control and asthmatic mice was carried out by fluorescence microscopy. In order to correlate NIRF fluorescence signals from the probes to inflammatory sites, lungs were counterstained with at anti-mouse F4/80 antibody. detecting macrophages Two-micrometer-thick sections were cut from paraffin blocks, the slides were first processed for avidin/biotin and protein blocking steps using xylol and decreasing alcohol concentration for deparaffination and rehydration and later incubated with the primary antibody rat anti-mouse F4/80 (AbD Serotec, Oxford, UK), dilution factor 1:100 at 4°C overnight. After the incubation with the primary antibody, the samples were incubated with secondary biotinylated antibody goat anti-rat (BioLegend, San Diego, USA), dilution factor 1:200 at RT for 1 hour, and then with streptavidin- Alexa 555 (Molecular Probes, Life Technologies Corporation, USA) dilution factor 1:400 at RT for 1 hour. DAPI was diluted in the mounting media and used as nuclear counterstaining. Fluorescence was analyzed with a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss,

Germany) equipped with a xenon lamp and a high sensitivity ORCA-AG digital camera (Hamamatsu, Japan). Data were acquired with AxioVs40 software (Carl Zeiss). Filter settings were as followed: DAPI: Ex: BP 365/25 (+/-12.5); FT 395; Em: BP 445/50 (+/-25); Cy7: BP 708/75 (+/-37.5); FT 757; BP 809/81 (+/-40.5); Alexa555: BP 546/12 (±6); FT 580 and LP 590 filter. Subsequent analyses were performed using the java-based image processing program ImageJ.

Statistical Analysis

Statistical verification of the differences of $RI_{ex}(x,t)$ between asthmatic and control mice for each experiment and time point was done using an unpaired Welch Two Sample t-test implemented in the PAST statistic software [26]. A p-value of less than 0.05 was considered significant.

Histological scores between groups were compared using Oneway ANOVA followed by Tukey's multiple comparison test.

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GraphPad Prism (v.5.00, GraphPad Software, San Diego, CA) was used for data analysis.

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Author Contributions

Conceived and designed the experiments: FA SB SDM MME BB. Performed the experiments: SB SDM CD CG BB. Analyzed the data: SB SDM CD MME BB. Contributed reagents/materials/analysis tools: KL PW. Wrote the paper: FA MME SB CD SDM.

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3.3. Publication 2

Tiotropium bromide inhibits relapsing allergic asthma in BALB/c mice

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Tiotropium bromide inhibits relapsing allergic asthma in BALB/c mice \ddagger





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ABSTRACT

Recurrent relapses of allergic lung inflammation in asthmatics may lead to airway remodeling and lung damage. We tested the efficacy of tiotropium bromide, a selective long-acting, muscarinic receptor antagonist as an adjunct therapy in relapses of allergic asthma in mice. We compared the effectiveness of local intranasal administration of tiotropium and dexamethasone in acute and relapsing allergic asthma in BALB/c mice. Although tiotropium at low doses is a potent bronchodilator, we tested higher doses to determine effectiveness on inflammation and mucus hypersecretion. A 5-day course of twice daily intranasal tiotropium or dexamethasone (1 mg/kg (b.w.)) suppressed airway eosinophils by over 87% during disease initiation and 88% at relapse compared to vehicle alone. Both drugs were comparable in their capacity to suppress airway and parenchymal inflammation and mucus hypersecretion, though tiotropium was better than dexamethasone at reducing mucus secretion during disease relapse. Despite treatment with either drug, serum antigen-specific IgE or IgG1 antibody tirres remained unchanged. Our study indicates that tiotropium at higher doses than required for bronchodilation, effectively suppresses inflammation and mucus hypersecretion in the lungs and airways of mice during the initiation and relapse of asthma. Tiotropium is currently not approved for use in asthma. Clinical studies have to demonstrate the efficacy of tiotropium in this respiratory disease.

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1. Introduction

The importance of eosinophilic inflammation in the lungs of individuals with allergic asthma is underscored by their essential role in allergen-induced airway hyperresponsiveness (AHR) and ultimately airway remodeling in mouse models [12,20]. T helper (Th) 2 cytokines IL-4, IL-5, and IL-13 produced by T lymphocytes are important for eosinophil accumulation and activation [3,6,32]. Once present in the lung, eosinophils produce factors such as cytokines that lead to mucus hypersecretion and airway

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remodeling [7,28]. Although it is important to address the longterm adverse effects of chronic eosinophilic lung inflammation in asthmatics, we argue that it is also crucial to recognize the role of eosinophils in recurrent allergen-induced relapses common in individuals with seasonal allergic asthma. Asymptomatic patients between relapses of allergic asthma have lung pathology [25,30,33]. Additionally, mice recovered from one episode of acute allergic asthma have chronic lung inflammation containing allergen-specific Th2 cells without eosinophils and respond vigorously to allergen rechallenge which induces disease relapses [24]. Chronic inflammation, even without eosinophils during asymptomatic remissions and undoubtedly recurrent relapses may lead to airway remodeling.

Anticholinergic drugs, such as tiotropium bromide are important for the treatment of chronic obstructive pulmonary disease, but are generally not used to treat asthma. However, there is clinical evidence revealing that tiotropium, a selective long-acting, muscarinic receptor antagonist treatment reduces relapses in severe asthmatics [27]. The effects of tiotropium on bronchoconstriction are well known [10]. However, its properties

Abbreviations: AHR, airway hyperresponsiveness; AUC, area under curve; BAL, bronchoalveolar lavage fluid; OVA, ovalbumin; PBS, phosphate buffered saline; Th, T helper.

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on inflammation and immunity have not been extensively studied [15,26]. Acetylcholine appears to play a role in the regulation of immune responses [8,11] and the release of pro-inflammatory mediators from airway smooth muscle and epithelial cells [14,18,19]. Moreover, lymphocytes, Th2 cells, eosinophils, alveolar macrophages and mast cells all synthesize and contain acetyl-choline (reviewed in Ref. [34]). Furthermore, muscarinic receptors are expressed on T cells, neutrophils, macrophages and mast cells [11] and the muscarinic receptor antagonist, atropine inhibits antibody production, T cell proliferation and leukocyte migration [31]. Additional evidence comes from animal models showing that tiotropium effectively suppresses inflammation in chronic asthma in mice and guinea pig [16,21,22].

In this study, we sought to further analyze the antiinflammatory properties of tiotropium in a mouse model of relapsing allergic asthma that mimics seasonal allergic disease. Our data suggest that higher doses of tiotropium suppress inflammation and have clinical implications for the prevention of allergic relapses.

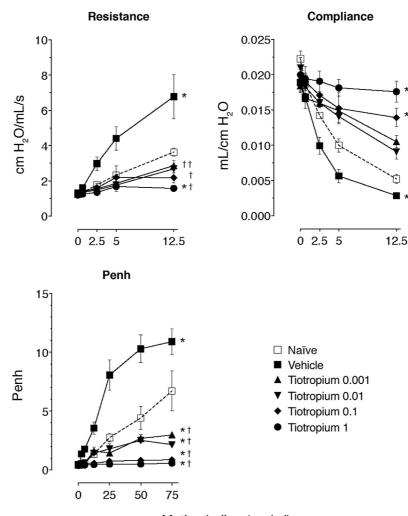
2. Materials and methods

2.1. Mice

Eight week old female BALB/c mice (Charles River, Germany) provided ovalbumin (OVA)-free food (SSNIFF, Soest, Germany) and water *ad libitum* were used in all experiments. All experimental protocols complied with the requirements of the Animal Care Committee of the Austrian Ministry of Science and the ethics committee at Boehringer-Ingelheim, Biberach.

2.2. Induction of allergic asthma

Immunization and challenge of mice in models of acute allergic asthma and disease relapse were done as previously described [24]. Briefly, naïve BALB/c mice were compared with mice immunized with 10 μ g of OVA (Sigma Chemical Co., St. Louis, MO, USA) intraperitoneally (i.p.) on days 0 and 21 and then challenged 1 week later (or as indicated) with nebulized 1% OVA in phosphate buffered saline



Methacholine (mg/ml)

Fig. 1. Effect of tiotropium on methacholine-induced airway hyperresponsiveness. Titrated doses of aerosolized methacholine-induced resistance and compliance measured with resistance and compliance system, as well as enhanced pause measured with whole-body plethysmography. Naïve mice, vehicle-treated, and mice treated with titrated doses of tiotropium from 0.001 to 1 mg/kg were compared 72 h after aerosol challenge. Data are expressed as mean \pm SEM from two independent experiments (n = 6-8). One-way ANOVA followed by Dunnett's multiple comparison test of mean AUC; *p < 0.05 is significant for all groups compared to the naïve group and $\dagger p < 0.05$ is significant when compared to the vehicle-treated group.

(PBS) in a Plexiglas chamber by an ultrasonic nebulizer (Aerodyne, Kendall, Neustadt, Germany) for 60 min twice daily on 2 consecutive days (days 28, 29 unless indicated) to induce acute onset disease (acute). For disease relapse, mice with acute disease were rested for at least 90 days and then rechallenged with a 1% OVA nebulization for 60 min twice daily on 2 consecutive days as indicated.

2.3. Treatment protocols

For the treatment of AHR, we administered dexamethasone (Sigma Chemical Company, St. Louis, USA) at the dose of 1 mg/kg or tiotropium (Boehringer-Ingelheim Pharma, Biberach, Germany) at the doses of 1, 0.1, 0.01 or 0.001 mg/kg for 5 days, starting 3 days before nebulized OVA challenges in the acute model. For the treatment of other disease parameters before the onset of acute disease and before inducing disease relapse, mice received twice daily intranasal instillations of dexamethasone or tiotropium at the dose of 1 mg/kg for 5 days, starting 3 days before nebulized OVA challenges. For all treatments, we administered the drugs 30 min before aerosol challenges. For each application of 1 mg/kg dose, dexamethasone and tiotropium were dissolved in PBS for a final concentration of 0.5 mg/ml. For 0.1, 0.01 and 0.001 mg/kg doses, tiotropium was dissolved in PBS for a final concentration of 50, 5 and 0.5 μ g/ml, respectively. All solutions were administered to the mice in 50 μ l.

2.4. Airway hyperresponsiveness

We measured airway hyperresponsiveness (AHR) as a change in airway function at 72 h after the last challenge with aerosolized methacholine (Sigma) using two different techniques. In conscious, unrestrained mice, we measured AHR by whole-body plethysmography (Buxco Electronics Ltd., NY, USA). PBS, followed by increasing concentrations of methacholine, was nebulized for 3 min and lung function was recorded and calculated as enhanced pause (Penh), which is a dimensionless unit that correlates with pulmonary resistance. In separate experiments, airway resistance and compliance were measured in anesthetized and ventilated animals by Resistance and Compliance System (Buxco Electronics Ltd.). As for whole-body plethysmography, PBS followed by increasing concentrations of methacholine was nebulized for 3 min and lung function was recorded and calculated using FinePoint software (Buxco Electronics Ltd.).

2.5. Airway inflammation

Seventy-two hours after last challenge, mice were terminally anesthetized, tracheostomy was performed and a plastic catheter was clamped into the trachea. The lungs were washed 3 times with PBS in a total volume of 1 ml (0.4, 0.3 and 0.3 ml) to collect bronchoalveolar lavage fluid (BAL). The total number of cells in BAL was enumerated in a Neubauer hemocytometer. The percentage of inflammatory cells was determined by morphological examination of at least 300 cells in cytocentrifuged preparations (Cytospin-4, Shandon Instruments, UK), stained with the Kwik-Diff (Thermo Fisher Scientific Inc., Pittsburgh, PA, USA).

2.6. Lung inflammation and mucus hypersecretion

After bronchoalveolar lavage, lungs were dissected and fixed by immersion in 4% paraformaldehyde. Paraplast-embedded lung sections of 3 μ m were stained with hematoxylin and eosin (H&E) for morphological evaluation, with Luna stain for eosinophil enumeration [23] and with periodic acid-Schiff stain (PAS) for mucopolysaccharide staining intensity of lung. For scoring of inflammatory cell infiltration, sections containing main stem bronchi from each lung specimen stained with H&E were used. An expert respiratory

pathologist blinded to the treatment groups graded the extent of inflammation in the lungs according to a semi-quantitative scoring system: Grade 0 - no inflammatory infiltrates; Grade 1 - inflammatory infiltrates in central airways; Grade 2 - inflammatory infiltrates extending to middle third of lung parenchyma; and Grade 3 inflammatory infiltrates extending to the periphery of the lung [24]. For quantification of eosinophil infiltration, eosinophils were counted on 5 random fields in Luna stained sections (400 \times magnification) containing major airways and vessels (which were selected from low power magnification) and averaged for each lung. For detection of mucus-containing cells in formalin-fixed lung tissue, 4 µm sections containing main stem bronchi from each lung specimen were stained with PAS and counter stained with hematoxylin. The number of mucus-containing cells per millimeter of basement membrane was determined with Cell'B software (Olympus Europa Holding GmbH, Hamburg, Germany).

2.7. Serum OVA-specific immunoglobulin

For the measurement of OVA-specific IgG1 and IgE, ELISA plates were coated with OVA at 10 μ g/ml overnight at 4 °C. The plates were washed and blocked with 2% bovine serum albumin in PBS with 0.05% Tween 20 for 2 h at room temperature. Then sera were titrated onto the plates and incubated for 24 h at 4°C before washing. Plates were incubated for an additional 2 h at 4°C before washing. Plates were incubated for an additional 2 h at 4°C with biotinylated anti-IgG1 (Southern Biotechnology Associates Inc., Birmingham, AL, USA) or anti-IgE (Becton Dickinson Biosciences, Franklin Lakes, NJ, USA) detection antibodies, followed by incubation with streptavidin horseradish peroxidase (Southern Biotechnology) for 1 h at room temperature. Plates were washed and incubated with TMB substrate solution (100 μ l/well, BD OptEIATM,

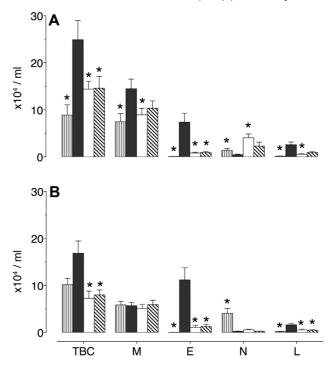


Fig. 2. Effect of tiotropium on airway inflammation. Total inflammatory cell count and differential counts in BAL of naïve, vehicle, dexamethasone and tiotropium treated mice 72 h after the last aerosol challenge at (A) disease initiation and at (B) disease relapse. Results are expressed as mean cell counts \pm SEM from two independent experiments (n = 6-10). One-way ANOVA followed by Dunnett's multiple comparison test; *p < 0.05 is significant for all groups compared to the naïve group and †p < 0.05 is significant when compared to the vehicle-treated group. TBC: Total BAL cell count, M: Macrophages, E: Eosinophils, N: Neutrophils, L: Lymphocytes.

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3. Results

asthma

Becton Dickinson Biosciences) for 10 min at room temperature. The reaction was stopped with 100 μ l of 0.18 M H₂SO₄ and the plates were measured at 450 nm.

ANOVA followed by Dunnett's multiple comparison test. Histology scores for inflammation were analyzed with Chi-squared test for trend. All analyses were done using GraphPad Instat v.5.0 (GraphPad Software Inc.) and p values were considered significant at <0.05.

2.8. Statistical analysis

To compare AHR data, area under curve (AUC) was calculated for each experimental animal on the graph of resistance, compliance or Penh (*y* axis) vs. the methacholine concentration (mg/ml; *x* axis). Calculated AUC values from AHR data, airway inflammation data, as well as mucus-positive cell and eosinophil counts in the lungs were log-transformed to equalize variances and analyzed with one-way

3.1. Tiotropium inhibits airway hyperresponsiveness in allergic

Tiotropium as a muscarinic receptor antagonist in chronic obstructive pulmonary disease inhibits bronchoconstriction. To test

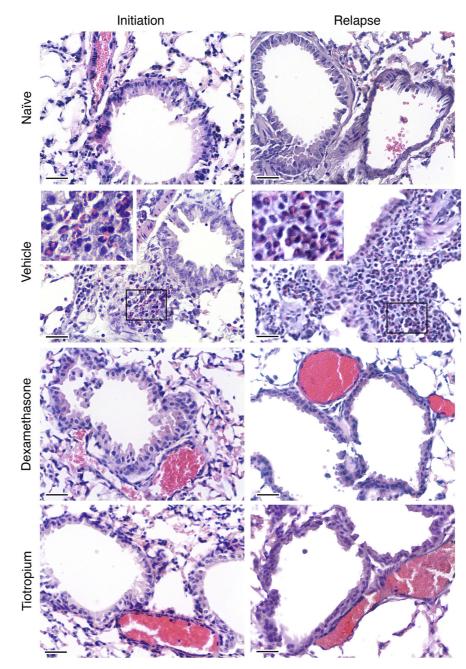


Fig. 3. Effect of tiotropium on lung inflammation. Representative photomicrographs of lung tissue on H&E stained sections (bar 100 μ m) from naïve, vehicle, dexamethasone and tiotropium treated mice 72 h after the last aerosol challenge (n = 6-10). Naïve mice have normal tissue without evidence of inflammation. At initiation and relapse, inflammatory infiltrates containing eosinophils (insets) are observed, but are reduced in the lungs of dexamethasone and tiotropium treated mice.

bronchodilator efficacy in acute allergic asthma, we instilled mice with the drugs intranasally for 5 days starting 3 days before the first challenge and then measured methacholine-induced AHR by unrestrained plethysmography. Tiotropium significantly reduced AHR at doses starting at 0.001 mg/kg (Fig. 1). Tiotropium treatment continued to inhibit AHR for 72 h after treatment at all doses, showing a long-acting effect on AHR even at low doses. However, at higher doses of 1 mg/kg, AHR was inhibited for over 1 week and airway eosinophils were suppressed (data not shown). We also measured airway resistance and compliance in anesthetized and ventilated allergen-treated animals and found that tiotropium at the same doses effectively and dose-dependently inhibited airway resistance and compliance (Fig. 1) an effect that was also seen when the mice were treated with dexamethasone (data not shown). Furthermore, in naïve mice tiotropium also reduced methacholineinduced AHR, but not as strongly as seen in the allergen treated mice (data not shown).

3.2. Tiotropium has anti-inflammatory effects in allergic asthma

Dexamethasone is one of the most effective anti-inflammatory drugs used in allergic asthma. To address whether tiotropium exhibited similar anti-inflammatory properties, we compared the effect of dexamethasone and tiotropium on inflammatory cell infiltrates in the airways and lungs of mice. At the initiation of allergic asthma, tiotropium and dexamethasone significantly inhibited overall airway inflammation by 41.1% and 42.1%, respectively compared to vehicle controls (Fig. 2). Both treatment regimens significantly inhibited airway eosinophilia from 7.4 \pm 1.9 \times 10⁴ cells/ ml in the vehicle, to $0.84 \pm 0.17 \times 10^4$ and $0.9 \pm 0.18 \times 10^4$ cells/ml in dexamethasone and tiotropium treated mice, respectively. Both drugs also significantly reduced the number of lymphocytes. An additional effect of dexamethasone treatment was the reduction of macrophages and increase in neutrophils, a feature not observed with tiotropium treatment. To evaluate the drug efficacy during a relapse of allergic asthma, mice were treated with either tiotropium or dexamethasone just before and at the time of OVA-aerosol rechallenges (Fig. 2). We found that, similar to the effect on acute initiation of disease, tiotropium and dexamethasone inhibited the extent of inflammation in the airways by 52.6% and 56.8%, respectively, and specifically reduced the number of infiltrating eosinophils by 88.4% and 90.2% and lymphocytes by 69.0% and 66.0%, respectively.

Further evaluation of inflammation was achieved by examining H&E stained lung sections (Fig. 3). These lung sections were graded for the severity of inflammation and eosinophils were enumerated in the tissues (Fig. 4). At disease initiation, drug treatment suppressed overall inflammation in the parenchyma by up to 43.2% and eosinophils were reduced by tiotropium by 82.0% and dexamethasone by 85.2% compared to vehicle alone treatment. Tiotropium and dexamethasone also prevented eosinophilic lung inflammation upon OVA-induced disease relapse (Fig. 4). Taken together, these data demonstrate that tiotropium at higher doses than required to inhibit AHR, has anti-inflammatory properties comparable to dexamethasone.

3.3. Tiotropium effectively prevents mucus hypersecretion in acute and relapsing allergic asthma

To determine whether tiotropium inhibits mucus production in allergic disease, we evaluated the extent of mucus secretion of goblet cells in bronchial epithelium (Fig. 5). We compared the efficacy of tiotropium with dexamethasone and vehicle treatments and observed that both drugs markedly suppressed mucus overproduction at the initiation of allergic asthma compared to vehicle-treated control mice (Fig. 6). Naïve mice have the occasional mucus producing goblet cell whereas vehicle-treated mice have their airway epithelia filled with mucus producing cells. In mice with acute allergic asthma, tiotropium reduced the number of mucuspositive cells by 50.3% compared to 56.7% by dexamethasone, indicating that tiotropium is as good as dexamethasone at suppressing mucus production. In experiments in which mice were treated before disease relapse, tiotropium treatment decreased the number of mucus-positive cells by 33.7%, which was greater than dexamethasone which suppressed mucus-positive cell number by 16.6%. These data demonstrate that tiotropium inhibits mucus hypersecretion as well as and even better than dexamethasone in acute and relapsing allergic asthma.

3.4. Tiotropium effect on OVA-specific IgE and IgG1

To determine whether B cell immune responses were influenced by treatment, we tested serum OVA-specific antibody titers 5 days after the last aerosol challenge (Fig. 7). We did not detect OVAspecific antibodies in naïve mice, however, vehicle-treated animals had high titres of OVA-specific IgE and IgG1 antibodies. Neither treatment with dexamethasone nor tiotropium reduced antibody titres.

4. Discussion

Here, we compared the potential of tiotropium in the prevention of the onset of acute asthma and a subsequent disease

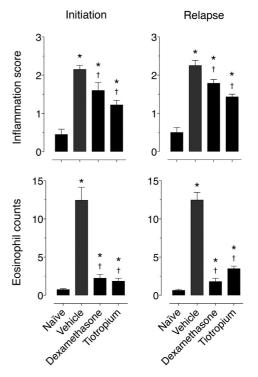
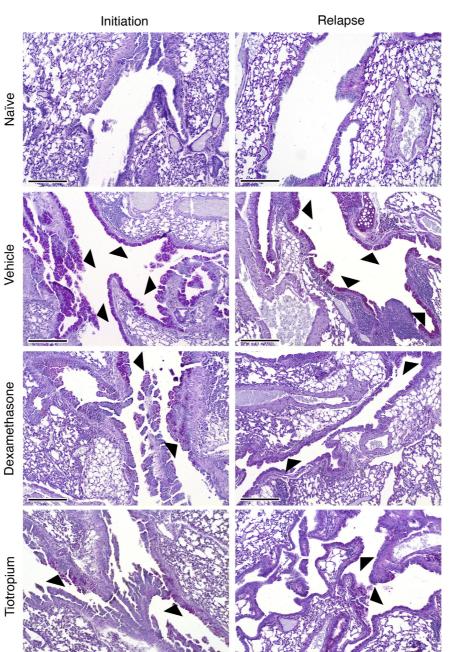


Fig. 4. Effect of tiotropium on inflammation and eosinophila in lung parenchyma. Inflammation scores and eosinophil counts in lungs of naïve control mice, vehicle, dexamethasone and tiotropium treated mice before initiation and relapse of asthma. Luna stained lung sections (per 400×) sampled at 72 h after final challenge. Results are expressed as mean inflammation score and eosinophil count per high power field ± SEM from two independent experiments (n = 6-10). One-way ANOVA followed by Dunnett's multiple comparison test for eosinophil counts and a Chi-square test for trend for inflammation scores. *p < 0.05 compared with naïve group; †p < 0.05 compared with vehicle-treated group.



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Fig. 5. Effect of tiotropium on mucus hypersecretion. Representative photomicrographs of lung tissue on PAS stained sections (bar 200 μ m) from naïve, vehicle, dexamethasone and tiotropium treated mice 72 h after the last aerosol challenge (n = 6-10). Naïve mice have normal tissue with very rare mucus-positive cells. At initiation of disease and relapse in the vehicle-treated sections, almost all cells in the epithelium are positive for mucus. At initiation and relapse, mucus-positive cells are observed, but are reduced in the lungs of dexamethasone and tiotropium treated mice. Arrowheads point to the areas of the epithelium with mucus-positive cells.

relapse. We observed a powerful effect on AHR at low doses and at higher doses tiotropium was as effective as dexamethasone at suppressing mucus hypersecretion and eosinophilic lung inflammation. Tiotropium was as effective at preventing the onset of acute disease as it was at preventing disease relapse, suggesting the potential use of tiotropium in patients with seasonal allergic asthma. Our findings support the use of tiotropium as a bronchodilator, mucolytic and anti-inflammatory in the treatment of relapses of allergic asthma. Tiotropium successfully reduced AHR at very low doses by either decreasing the reactivity of airway smooth muscle or blocking the binding of methacholine to its receptor. There is evidence that non-bronchodilating mechanisms of tiotropium prevent airway hyperreactivity in a guinea pig model of allergic asthma [4]. Antigen-induced hyperreactivity was completely blocked by tiotropium treatment and partially blocked with atropine treatment and that tiotropium blocked bronchoconstriction induced by intravenous acetylcholine but did not inhibit vagally-induced

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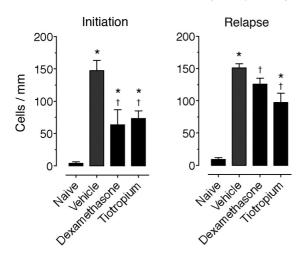


Fig. 6. Effect of tiotropium on the extent of mucus hypersecretion. Number of mucuspositive cells per millimeter of basement membrane in naïve control mice, vehicle, dexamethasone and tiotropium treated mice before initiation and relapse. Results are expressed as mean \pm SEM from two independent experiments (n = 6-10). *p < 0.05compared with naïve group; †p < 0.05 compared with vehicle-treated group (Chisquare test for trend).

bronchoconstriction in sensitized controls. These data suggest that tiotropium does not block hyperreactivity by blocking receptors for vagally-released acetylcholine and suggests that tiotropium may work via anti-inflammatory mechanisms because it also inhibited eosinophilia in the lungs and around nerves.

Our findings illustrate that at high doses, tiotropium is as effective an anti-inflammatory as dexamethasone in the initiation and relapse of allergic asthma. Our data support previous reports showing that 4 daily doses of 50 μ l of 0.1 mM tiotropium given by aerosol over 3 min had anti-inflammatory effects in the airways of mice with acute onset allergic asthma [26] and a study in guinea pigs revealing that tiotropium (1 μ g/kg) suppresses lung eosinophilia in acute allergic asthma [4]. In contrast, low doses of 50 μ l of 0.1 nM tiotropium (corresponding to a dose of ~0.1 μ g/kg) given intranasally for 5 days (on the same days that the animals were challenged with allergen) actually increased airway inflammation with an increase in macrophages and eosinophils [15]. Not only do our results

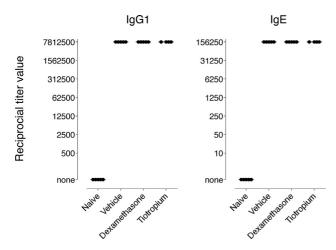


Fig. 7. Tiotropium effect on serum OVA-specific antibody at disease initiation. Analysis of serum OVA-specific IgG1 and IgE antibody titres from naïve, vehicle, dexamethasone and tiotropium treated mice 5 days after the last aerosol challenge for the initiation of allergic asthma. Results are shown as reciprocal titres for individual mice (dots) and group mean (line) from two independent experiments (n = 4-5). *p < 0.05 compared with naïve group by one-way ANOVA.

with high doses illustrate that acute initiation of eosinophilic airway inflammation is suppressed by tiotropium, but that parenchymal lung inflammation is also significantly suppressed.

Tiotropium and dexamethasone almost completely prevent eosinophil infiltration of the airways at disease relapse. Previous mouse studies reveal that mice recovered from allergic asthma have long-lived memory Th2 cells, macrophages, B cells and dendritic cells residing in chronic infiltrates, but lack eosinophils and neutrophils [24]. These memory cells respond rapidly to allergen rechallenge with recruitment of eosinophils that treatment with both tiotropium and dexamethasone prevent. Although, acute onset eosinophilic airway inflammation and eosinophilia in the parenchyma were prevented, we observed that inflammatory infiltrates in the lungs remain, i.e. the lungs are not like those from naïve mice. Unfortunately, these infiltrates are difficult to eliminate and if maintained, the cells within the infiltrates will continue to respond to allergen rechallenges leading to relapses (M. Epstein, unpublished data). Generally, parenchymal infiltration and airway inflammation correlate. However, we have encountered other situations in which they do not [13]. In this study, parenchymal infiltrates were eliminated while airway inflammation persisted after treatment with dexamethasone. In contrast, tiotropium appears to reduce airway inflammation more rapidly than parenchymal inflammation and seems to target eosinophils earlier than macrophages and lymphoctyes, which are the predominant cells within the infiltrates. It is possible that for complete elimination of lung infiltrates, chronic treatment is necessary, even in asymptomatic individuals with infrequent relapses. This is especially relevant in lieu of the studies showing that a longer duration of treatment with low dose tiotropium was effective at reducing inflammation and reduced airway remodeling in chronic models in mice [15,26] and in guinea pigs [2,9].

Clinical evidence of tiotropium treatment reducing the number of relapses in severe asthmatics [27] suggests that tiotropium is beneficial in allergic asthma, yet the mechanisms underlying a potential anti-inflammatory effect remain unclear. There is evidence for a role of muscarinic receptors on immune and inflammatory cells [8,11,31], however, our studies suggest that it is necessary to increase the dose of tiotropium to increase efficacy, at least in mice. Having determined that inflammation is suppressed by treatment with tiotropium, that Th2 cytokines are concomitantly reduced [15,26], and being aware that B cells express muscarinic receptors [34], we anticipated that high doses of tiotropium would inhibit B cell responses resulting in reduced allergen-specific antibody titres. However, we observed no effect of either tiotropium or dexamethasone treatment on antibody production. It is not clear why titres remained unaffected but a possible explanation is that the duration of treatment was too short to observe a change in the titres. In a previous study, no changes in allergen-specific antibody titres were observed in a chronic model with chronic tiotropium treatment [26].

The observed anti-inflammatory mechanism may be related to the bronchodilatory properties of tiotropium. However, preclinical evidence suggests anti-inflammatory properties of tiotropium beyond bronchodilation. Cholinergic stimulation was shown to trigger the release of chemotactic factors in bronchial epithelial cells *in vitro* [5,19]. Supernatant taken from epithelial cells stimulated with acetylcholine induced chemotaxis in primary human eosinophils [19] or neutrophils [5]. This migratory response of neutrophils was absent in experiments where the epithelial cells were pretreated with tiotropium [5]. Neutrophil migration was inhibited directly by an LTB4 antagonist suggesting that this mediator may play a role in cholinergic inflammation. Taken together, these data suggest that tiotropium may also have direct anti-inflammatory effects in our mouse model of allergic B. Bosnjak et al. / Pulmonary Pharmacology & Therapeutics 27 (2014) 44-51

inflammation. Further research is needed to elucidate if and by which mechanism tiotropium causes direct anti-inflammatory effects.

Tiotropium and dexamethasone treatment suppressed mucus secretion by over 50% at the initiation of disease, but they were less effective during a relapse and dexamethasone reduced mucus production half as well as tiotropium. Our results demonstrate that both treatments do not abolish mucus production and this may relate to duration of therapy, however, long-term experiments in mice with chronic treatment reduced mucus from approximately 60 to 40%, but this might be because mucus metaplasia is more severe in chronic disease and therefore, more difficult to treat [26].

Our study indicates that tiotropium at higher doses than required for bronchodilation effectively suppresses inflammation and mucus hypersecretion in the lungs and airways of mice during the initiation and relapse of asthma. Recent studies show that tiotropium is effective as an adjunct in uncontrolled asthma at doses of $5-50 \mu$ g daily [1,17,29]. The doses used in the studies are not as high as the doses used in mice. It is likely that in this dose level, the effect is on airway smooth muscle. It is not yet known whether higher doses would further increase effectiveness by reducing inflammation. These studies have not been done. Taken together, our studies in addition to others indicate that tiotropium has anti-inflammatory activity at higher doses and that tiotropium is effective in patients. More studies are needed to understand the mechanism underlying the tiotropium effect in asthma.

Authorship contributions

Participated in research design: Bosnjak, Erb, Pieper, Epstein. Contributed new reagents or analytic tools: none.

Conducted experiments: Bosnjak, Tilp, Tomsic, Dekan.

Performed data analysis: Bosnjak, Erb, Tilp, Tomsic, Dekan, Epstein.

Wrote or contributed to the writing of the manuscript: Bosnjak, Dekan, Pieper, Erb, Epstein.

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4. CONCLUDING DISCUSSION

This thesis presented research that (i) led to detection of novel genomic biomarkers involved in disease pathogenesis, (ii) contributed to development of novel imaging approach to monitor lung inflammation and (iii) successfully suppressed all aspects of asthma relapse in mice using novel treatment, tiotropium bromide.

Detailed understanding of gene expression is one of keys for understanding complex biological processes such as asthma (113,114). Moreover, detailed transcriptional profiling of diseases facilitates biomarker and drug discovery enabling target identification and validation, side-effect profiling, pharmacogenomics, biomarker development, clinical trial evaluation and toxicology (68,79,115). In the first manuscript presented in this thesis, we used a powerful bioinformatic analyses to prioritize asthma-related and -ignorome genes within the clusters and biological domains and provide important insights into interactions between lung, immune and inflammatory response genes and networks. These data provide important insights into asthma pathogenesis and provides new opportunities for better classification of the disease and development of novel biomarkers and treatment modalities.

Longitudinal monitoring of pathological processes *in vivo* revolutionized the ability to develop new treatments (116). Non-invasive monitoring of cellular and molecular processes not only allows detailed visualization of pathological processes, but also can serve as a biomarker for early indications of efficacy of the treatment response (117). In pulmonary research, improvements of imaging techniques such as PET, SPECT and MRI allowed anatomical, molecular and functional visualization of the lungs in preclinical models (95,118). In publication 1, we developed an imaging approach that combines near infrared fluorescence dye (allowing visualization of mice lungs due to penetration depth of 4 cm) with specificity of probe (dendritic polyglycerol sulfates that bind L- and P-selectins and C3 and C5 complement factors) to non-invasively monitor allergic asthma in mice. The combination of relatively inexpensive and

non-harmful imaging method with specific probe makes this novel imaging approach suitable for monitoring inflammation processes and responses to therapy in experimental mouse models of lung disease.

Selection of optimal experimental *in vivo* model is crucial for preclinical drug discovery (119). In asthma, many different models are in use, each mimicking different aspects of the disease (39,53,89,90). Our laboratory developed model of asthma relapse to mimic intermittent reversible course of the disease and to investigate importance of allergen-specific Th2 memory cells for its maintenance (48,120,121). In publication 2, we successfully used the asthma relapse model to evaluate efficacy of novel treatment modality, tiotropium bromide, in asthma.

A prerequisite for successful development of new anti-asthmatic therapies is detailed understanding of asthma phenotypes and endotypes. Achievement of this goal is, however, impossible without discovery of biomarkers from body fluids and/or affected tissues (16,68). This thesis, therefore, provided a stepwise approach towards detection of novel biomarkers and anti-asthmatic medications: (i) our detailed gene expression profile of an acute allergic asthma provides numerous novel biomarker and treatment targets; (ii) our novel imaging technology enables elegant way for visualization of allergic lung inflammation *in vivo*; while (iii) our data do not only provide supportive evidence for the use of anticholinergic drug tiotopium bromide in asthma, but also describe an elegant experimental setting for testing novel drugs or validation of novel biomarkers.

5. DECLARATION OF CONTRIBUTION

Revealing the acute asthma ignorome: characterization and validation of uninvestigated gene networks

Berislav Bosnjak participated in selection of studies for this microarray metaanalysis, contributed to its data interpretation, as well as designed validation experiments using quantitative real-time PCR on total lung RNA samples from mice in allergic asthma model. He participated in manuscript preparation and approved its final version.

Dendritic Polyglycerolsulfate Near Infrared Fluorescent (NIRF) Dye Conjugate for Non-Invasively Monitoring of Inflammation in an Allergic Asthma Mouse Model

Berislav Bosnjak contributed to the study with the analysis of lung inflammation on H&E-stained lung sections and mucus hypersecretion on PAS-stained lung sections, as well as determining of serum OVA-specific IgG1 titers. He also read and approved the final version of the manuscript.

Tiotropium bromide inhibits relapsing allergic asthma in BALB/c mice

Berislav Bosnjak was involved in the design of experiments included in this study, he performed immunization, treatment and aerosol challenges of animals, collected BAL, lung and sera and analyzed airway inflammation data, eosinophil counts in lungs, as well as allergen-specific IgG1 and IgE titers in sera. He also contributed to manuscript preparation and approved its final version.

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