Université de Montréal

Insights on interspecies disease tolerance mechanisms through comparative and functional genomics

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Thèse présentée à la Faculté des études supérieures
en vue de l'obtention du grade de PhD.
en Biochimie et Médecine Moléculaire

June 2021

Université de Montréal

Faculté des études supérieures

Cette thèse intitulée :

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Résumé

La sensibilité des primates aux pathogènes et aux maladies inflammatoires chroniques varie considérablement. Par exemple, les singes (tels que les humains et les chimpanzés) sont très sensibles à de très petites doses de lipopolysaccharide (LPS), une molécule mimétique d'agent pathogène, qui cause de graves lésions tissulaires en raison de l'immunopathologie tandis que les singes africains et asiatiques clades sœurs AAM (tels que les macaques et les babouins) sont beaucoup plus tolérants à des doses beaucoup plus élevées de LPS. Cet écart entre l'homme et les autres primates est connu pour être, au moins partiellement, dû à la différence interspécifique de la réponse immunitaire. Dans cette thèse, j'ai effectué une analyse comparative de la réponse immunitaire à travers différentes lignées de primates pour obtenir des informations supplémentaires sur l'évolution de la réponse immunitaire. J'ai trouvé que les singes provoquent une réponse immunitaire beaucoup plus forte aux stimulants (bactériens ou viraux) par rapport aux AMM. Une telle réponse plus élevée s'est également avérée corrélée avec la phylogénie du primate, la plus élevée chez le primate supérieur (humain) et la plus faible chez le primate basal (lémurien). Une réponse aussi élevée peut être bénéfique pour la médiation d'une destruction efficace des agents pathogènes, mais elle est probablement accompagnée de lésions tissulaires plus élevées, ce qui pourrait expliquer pourquoi les humains sont plus sensibles aux maladies immunopathologiques telles que la septicémie. J'ai également caractérisé le paysage réglementaire de la réponse immunitaire chez ces primates. J'ai trouvé que l'activité des éléments régulateurs était significativement différente entre les différentes espèces de primates après une stimulation immunitaire mettant en évidence le rôle de l'épigénétique dans la conduite du changement de la réponse immunitaire chez les primates. De plus, j'ai trouvé une signature d'évolution adaptative sur les régions actives associées aux gènes qui ont la réponse la plus élevée chez l'homme par rapport aux AMM révélant le rôle de la sélection naturelle sur le façonnement de la réponse immunitaire chez les primates.

Mot-clé: lipopolysaccharide - évolution de la réponse immunitaire - éléments régulateurs - évolution des primates

Abstract

Primates vary remarkably in their disease susceptibility to pathogens and chronic inflammatory diseases. For instance, apes (such as humans and chimps) are highly sensitive to very small doses of lipopolysaccharide (LPS), a pathogen mimicry molecule, that causes severe tissue damage due to immunopathology while sister clade African and Asian monkeys AAMs (such as macaque and baboon) are far more tolerant to much higher doses of LPS. This discrepancy between humans and other primates is known to be, at least partially, due to interspecies differences of the immune response. In this dissertation, I performed comparative analyses of immune responses across different primate lineages to gain further insights on the evolution of immune response. I found that apes elicit a much stronger immune response to stimulants (bacterial or viral) relative to AMMs. Such a higher response was also found to be correlated with the primate phylogeny, highest in the higher primate (human) and lowest in the basal primate (lemur). Moreover, this high response may be beneficial in mediating effective pathogen killing but it is likely accompanied by higher tissue damage, which might explain why humans are more susceptible to immunopathological diseases such as sepsis. I also characterized the regulatory landscape of immune response across these primates. I found the regulatory elements activity to be significantly different between different primate species after immune stimulation highlighting the role of epigenetics in driving the immune response change across primates. In addition, I found a signature of adaptive evolution on active regions associated with genes that have the highest response in humans versus AMMs revealing the role of natural selection in shaping the immune response in primates.

Keyword: lipopolysaccharide – immune response evolution – regulatory elements – primate evolution

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List of abbreviations

AAMs	African and Asian monkeys
AIDS	Acquired immunodeficiency syndrome
AMP	Antimicrobial peptides
APC	Antigen presenting cell
AUC	Area under the curve
CCR5	C-C chemokine receptor type 5
c-DRGs	Clade differentially responsive genes
CPM	Count per million
CRISPRs	Clustered regularly interspaced palindromic repeats
DAPs	Differentially accessible peaks
DEGs	Differentially expressed genes
DS	Divergence score
EID	Emerging infectious disease
FACS	Fluorescence-activated cell sorting
FDR	False discovery rate
FBS	Fetal bovine serum
GARD	Gardiquimod
GEM	Gel Bead-in-Emulsion
GSEA	Gene set enrichment analysis
GO	Gene ontology
GTF	Gene transfer format
LPS	Lipopolysaccharide
NC	Negative control
NK	Natural killer
NOD	Nucleotide oligomerization and binding domain
NLRs	NOD-like receptors
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffer saline

PC   Principal component

pDC   Plasmacytoid dendritic cells

PBMCs   Peripheral blood mononuclear cells

PRR   Pattern recognition receptor

RBC   Red blood cells

S/HIV   Simian/human immunodeficiency virus

ATAC-seq   Assay for transposase-accessible chromatin using sequencing

scATAC-seq   Single cell ATAC-seq

RNA-seq   Ribonucleic acid sequencing

scRNA-seq   Single cell RNA-seq

ssRNA   Single-stranded RNA

TLRs   Toll-like receptors

Tregs   Regulatory T cell

UMAP   Uniform Manifold Approximation and Projection for Dimension Reduction

UMI   Unique molecular identifier

Acknowledgment

I would like to thank my supervisor Luis Barreiro for guiding me through my journey of this important stage on my life. Luis is very knowledgeable and helpful with great attitude to his students. Such encouraging and cheerful atmosphere in the laboratory made the research going smooth and easy.

I would like also to thank my colleagues in the laboratory for helping me and having good scientific discussions around various topics which made me aware of other perspectives of different research discipline that deepened my knowledge.

I would like to thank my family and my mother, Omaima El-Zohairy, for always encouraging and pushing me to seek what I always dreamed of, to be a scholar in this field of academia.

1. Introduction

Immune response is central in all living organisms to protect against pathogens, e.g., bacteria and viruses, which are dependent on the host for nutrients and reproduction. Starting with the defense mechanisms in prokaryotes such as clustered regularly interspaced palindromic repeats (CRISPRs) until the highly sophisticated immune response orchestrated by innate and adaptive immune effectors in mammals, immune mechanisms had extensive diversification along evolution course (Kimbrell and Beutler, 2001; Boehm, 2012; Buchmann, 2014). In this chapter, I will first introduce the three major immune strategies the hosts have evolved to fight pathogens: disease tolerance, avoidance and resistance. My major research question is to understand the differences of disease susceptibility across species. Specifically, I aim to explore the role of disease tolerance in driving disease susceptibility across species. I will discuss the major mechanisms of tolerance and resistance, then I will explain the different factors that might lead certain species to evolve a specific strategy to fight the pathogen such as, pathogen virulence or environmental factors. I will also explain tolerance from an evolutionary genetic perspective. Finally, I will conclude by summarizing the importance of interspecies comparative studies in disease tolerance research.

1.1. Immune strategies (resistance and tolerance)

Innate and adaptive immunity has been extensively studied by immunologists for decades. All innate and adaptive immune mediators belong to a specific strategy of immune response known as "resistance". The other immune strategies are "avoidance" and "tolerance" (Medzhitov et al, 2012; Ayres and Schneider, 2012; McCarville and Ayres, 2018). Resistance mechanisms mediate direct killing of the incoming pathogen while tolerance is the strategy by which the host alleviates the harsh impact of the pathogen. Tolerance mechanisms include tissue repair and wound healing mediators (Read et al, 2007; Soares et al, 2017; Martins et al, 2019). Avoidance is an immune strategy that minimizes the chances of the host be in contact with the pathogen through behavioral mechanisms (Medzhitov et al, 2012).

The term "tolerance" was first coined in the late 1800s to describe the ability of the host to reduce the adverse effects of pathogens on plant hosts (Martins et al, 2019). Ecologically, tolerance and

resistance can be investigated as parameters of host health and pathogen load reaction norm (Schneider and Ayres, 2008). Reaction norm is the measurement of the phenotypes for given genotypes under different environmental conditions. Given any reaction norm, resistance is defined as the opposite of pathogen load while tolerance is the slope of the reaction norm (Figure 2) (Simms and Triplett, 1994). The first experimental setup that provided evidence of tolerance as an immune strategy in animals was in 2007 by Raberg et al. In this study, five different strains of mice were infected with three different strains of *Plasmodium chabaudi* (a rodent malaria parasite). Weight loss and anaemia were measured as indicators for health. Reaction norms of mice health and parasite loads identified differences in the slopes of the different mice strains meaning different tolerance potential between different strains. Raberg et al (2007) study unlocks the door for a new branch of immunology that is now fast growing and receiving greater attention. Several mechanisms have been discovered of tolerance in animals which revolutionized our understanding of immune responses in animals (Martins et al, 2019). I will discuss some of the mechanisms below.

I would like to note that tolerance is also referred as "disease tolerance" or "resilience" in literature (Warren et al, 2010). In this thesis, I will use those terms synonymously.
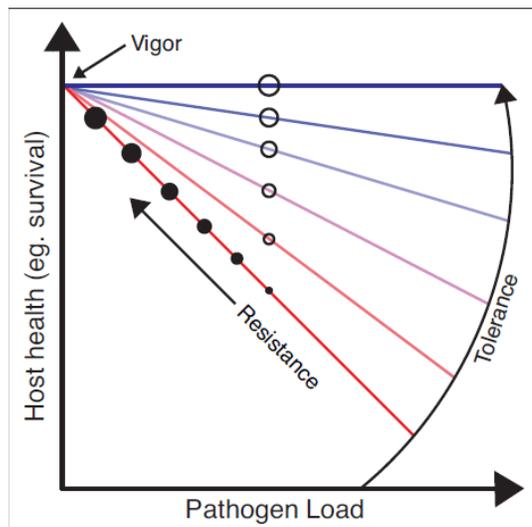
Figure 1. Reaction norm of disease tolerance. A relationship between pathogen load on the x axis and health parameter on the y axis. The slope of the curve measures the tolerance potential, the flatter of the slope, the higher tolerance of the host (denoted by the size of the hollow circles).

Resistance is the opposite of the pathogen load (denoted by the size of solid circles) (from McCarville and Ayres, 2018).

1.2. Mechanisms of resistance

The resistance of the host against pathogens is mediated by innate and adaptive immune pathways. Innate immune mechanisms are the first triggered defense pathways and are relatively less specific such as antimicrobial peptides and complement system (Koenderman et al, 2014). On the other hand, adaptive immune pathways are triggered later after infection and are highly specific to pathogen antigens. Herein, I will give a brief overview on the major mechanisms of the two resistance arms.

1.2.1. Innate immune defense mediators

Innate immune defenses start upon the recognition of the pathogen by specific receptors expressed on host immune cells known as pattern recognition receptor (PRR). PPR recognizes a specific moiety of the pathogen known as pathogen-associated molecular patterns (PAMP). Once interaction between the two molecules occurs, the host elicits immune mediators to kill the pathogen (Janeway and Medzhitov, 2002). An example of PAMP is lipopolysaccharide (LPS), which is a conserved molecule in the outer monolayer of gram-negative bacteria that is recognized by a specific receptor on the host cell, toll-like receptor 4, TLR4 (Figure 1). The recognition process starts with the binding of a complex composed of LBP (LPS binding protein), CD14 (membrane receptor for LPS-LPD), TLR4, and MD-2 (a protein responsible for LPS binding). Then, TLR4 recruits single or multiple adaptors such as MYD88 that trigger different cascades which finally activate transcription of immune effectors e.g., AMPs (Gomez et al, 2015).

Innate immune killing mechanisms are humoral-mediated defenses such as antimicrobial peptides, reactive oxygen species, complement system and cell-mediated defenses such as phagocytosis (Koenderman et al, 2014). Antimicrobial peptides (AMPs) are a set of highly conserved molecules found in many invertebrate and vertebrate hosts (Buchmann et al, 2014; Riera Romo et al, 2016). Several families of AMPs have been characterized in humans such as defensins, histatins and cathelicidins (Wang, 2014). AMPs mechanism of action includes targeting outer pathogen cell

wall, cell membranes and Intracellular molecules such as pathogen DNA (Wang et al, 2014). For instance, AMPs may bind to bacterial cell wall to block the synthesis of the cell wall or may disrupt the cell membrane by forming pores on the membrane leading to bacterial cell lysis (Wang, 2014). Moreover, AMPs may bind to pathogen DNA to stop pathogen molecular biosynthesis processes or to induce apoptosis (Wang, 2014). Beside the innate immune cells that secrets AMPs upon encountering a pathogen, AMPs are also constitutively expressed by cells that are continuously exposed to microbes of the outer environment such as skin and intestinal mucosa (Zhang and Gallo, 2016). Another mechanism of defense involves the generation of reactive oxygen species (ROS). ROS are reactive chemicals derived from molecular oxygen O_2 by produced from certain enzymes such as NADPH or within mitochondria as a byproduct of the metabolic electron transport chain (ETC) (Al-Shehri, 2021). ROS is engaged in direct killing of phagocytosed pathogens by creating oxidative stress, which leads to the destruction of pathogen's cell components (Spooner and Yilmaz, 2011; Shekhova, 2020). In addition to their direct pathogen killing function, AMPs and ROS also exert an immune-modulatory effect on different innate immune cells (Wang, 2014; Spooner and Yilmaz, 2011).

The complement system is another important innate immune system that serves in pathogen clearance, either directly or by priming adaptive immune mechanisms (Dunkelberger and Song, 2009). The complement system is a complex network of serum proteins secreted by the liver and the complement receptors expressed on immune cell surfaces (Merle et al, 2015). There are three major pathways of complement activation namely, classical pathway, lectin pathway and alternative pathway (Merle et al, 2015). These pathways are composed of multilayered regulators that trigger their initiation and activation (reviewed by Merle et al, 2015). All the pathways of activation will primarily lead to three complement effectors 1) proinflammatory molecular and anaphylatoxins, that further activate immune response by attracting and priming immune cells 2) opsonins, which are complement molecules that bind to the pathogen cell surface to facilitate their targeting by phagocytic cells such as macrophage, and 3) membrane attack complexes (MACs), which is a structure assembled on the surface of the pathogen to mediate cellular lysis through pores (Dunkelberger and Song, 2009).

Phagocytosis is a major cell-mediated innate immune process by which host cells such as neutrophils and macrophages uptake and digest the pathogens (Gordon, 2016). The phagocytic

process is tightly regulated by various receptors that mediate recognition of different bacterial or fungal PAMPs followed by cytoskeleton modifications to absorb the pathogen into the phagosome for digestion (Hirayama et al, 2018). Phagocytes can also recognize opsonin-coated pathogens, such as complement molecules and antibodies, that act as adaptors for uptake and digestion (Hirayama et al, 2018).
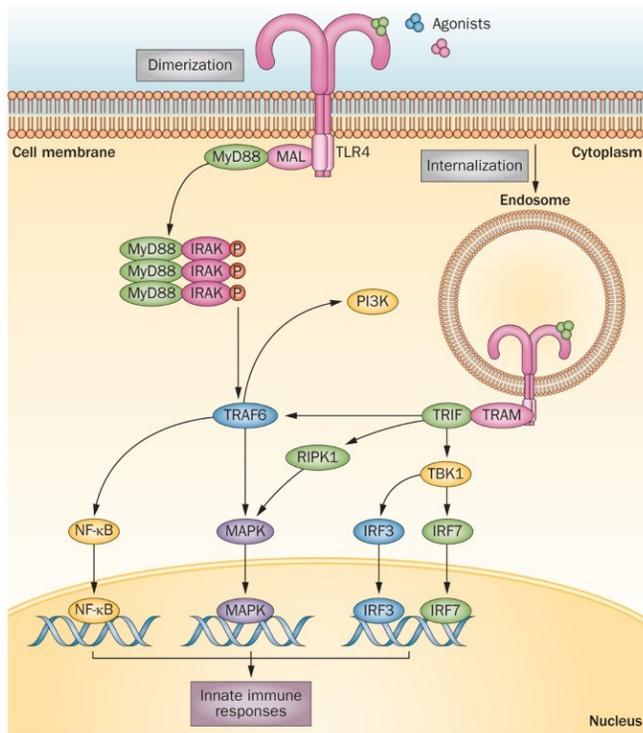
Figure 2. Innate immune response signaling by Toll-like receptor 4 (TLR4). Representation of immune signaling by an innate receptor, TLR4. When the receptor is activated by the ligand, it mediates downstream signaling by adaptors such as MYD88. The receptor can also be internalized and mediate signaling by TRIF adaptor (from Gomez et al, 2015).

1.2.2. Adaptive immune defenses

The adaptive immune system can be broadly grouped into humoral and cellular-mediated defenses, which are regulated by B and T lymphocytes, respectively. Upon activation, mature B cells

differentiate to plasma cells that produce antibodies (immunoglobulin proteins). The signals of activation of B cell come from two immune signals to ensure specificity of response to the pathogen. The first signal is triggered by the binding of the B cell receptor (BCR) to the pathogen. B cell then engulfs the pathogen and digest it to present the antigen by major histocompatibility complex (MHC) on its surface. The second signal originates either from previously activated T cells that recognize specifically the same antigen presented on the B cell (T dependent) or through certain antigens such as LPS (T independent) (Bonilla and Oettgen, 2010; Tsai et al, 2019). Antibody killing mechanisms are either direct by neutralizing the pathogen or indirect by facilitating cell-mediated phagocytosis or cytotoxicity (Forthal, 2014). Direct binding of antibodies to the pathogen paralyzes and reduces the mobility of the pathogen and can also destabilize the pathogen's outer surface blocking the attachment of the pathogen to the host cells (Forthal, 2014). Antibodies can also bind with their receptors on immune cells (known as Fc receptors) which result in cell lysis or apoptosis of pathogens or (cells harboring the pathogen), a process known as Antibody-dependent cellular cytotoxicity (ADCC). Fc receptor interaction may also enhance the phagocytosis process by professional phagocytes (Forthal, 2014).

Cell-mediated adaptive immune mechanisms are orchestrated mainly by T cells. T cells are also activated through interaction with APC, which results in the activation of cytotoxic T cells or helper T cells, also known as CD8 and CD4 T cells respectively, through interaction of T cell receptors (TCR) and MHC molecules expressed on the cell surface of APC (Zajac and Harrington, 2008). CD8 T cells are implicated in direct killing of infected cells whereas CD4 T cells secret cytokines and mediators that enhance the inflammatory response.

1.3.Mechanisms of disease tolerance

Tolerance mechanisms work on alleviating the harsh impact of the pathogen infection. Damage upon infection results from two sources, damage due to the pathogen burden itself (e.g., toxins released by the pathogen) or damage due to the immune response, known as immunopathology (Figure 3). Tolerance mechanisms vary depending on the pathogens (bacteria, viruses or protozoans) and the hosts. Mechanisms of tolerance include neutralization and detoxification,

immunological ignorance, and tissue repair and inflammatory control (Ayres and Schneider, 2012; Soares et al, 2017; Martins et al, 2019).

1.3.1. Neutralization and detoxification

Soon after a pathogen invades the host, several toxic substances are released either by the pathogen such as virulence factors or as a by-product of fighting the pathogen. These toxins reduce host fitness by draining the available resources from other physiological processes of the host. Also, they exacerbate the inflammatory response, potentially leading to immunopathology. For instance, heme is one of the prevalent toxins that are generated from several blood infections (Anzaldi and Skaar, 2010; Sachla et al, 2014; Martins et al, 2019). Blood pathogens (e.g. *Plasmodium* spp.) cause haemolysis which leads to the release of haemoglobin in the blood stream. The free haemoglobin is oxidized to free heme by reactive oxygen species. The free heme is highly toxic to the host either directly by inducing cell death of endothelial cells and destabilizing cytoskeleton or indirectly through activation of polymorphonuclear cells (PMN), which further aggravate tissue damage (Kumar and Bandyopadhyay, 2005; Martins et al, 2019). Heme-oxygenase-1 is an enzyme, encoded by HMOX1 gene, secreted by the host that detoxifies the free heme and prevents the collateral damage of free heme and thus increases tolerance (Ferreira et al, 2010; Soares et al, 2017). Another example is xenobiotics which are chemical foreign molecules that, without detoxification, reach a lethal concentration in the host. Xenobiotics were found to stimulate conserved innate immune pathways that enable detoxification of these molecules (Melo and Ruvkun, 2012; Pukkila-Worley et al, 2014). One of the major receptors of xenobiotics is Aryl hydrocarbon (AhR), which has a major role in neutralizing toxins secreted by bacteria and xenobiotics (Bessede et al, 2014; Moura-Alves et al, 2014). Nrf2 is a core regulator of tolerance which has also a central role in detoxification and neutralizing xenobiotics (Motohashi and Yamamoto, 2004; Martins et al, 2019).

1.3.2. Immunological ignorance

A significant source of the damage during pathogen infection comes from the immune response of the host due to excess inflammation. Hence, a mechanism to avoid this damage is to prevent the response to the pathogen, especially if such a pathogen does not represent a serious threat to the organism. Immunological ignorance is similar to the detoxification mechanism with the difference

that it implies continuous switching off the immune response to the stimulus (Ayres and Schneider, 2012). For instance, the absence of receptors that sense the pathogen or changes of receptor conformation makes the receptor blind to the stimulus. The C-C chemokine receptor type 5 (CCR5) is a coreceptor expressed on immature or memory T cells, macrophages and monocytes that recognizes cytokines such as proinflammatory cytokines CCL3/4/5 (Lopalco, 2010). CCR5 represents a major entry point for simian/human immunodeficiency virus (S/HIV) viruses. At the beginning of infection, S/HIV preferentially infects mucosal CD4 T-cells that express CCR5 resulting in a rapid depletion of CD4 T-cells by the virus (Veazey and Lackner, 2017). Low expression of CCR5 was found to be correlated with low pathology of S/HIV severity (Veazey and Lackner, 2017) since the virus cannot enter the cells and hijack the cellular machinery for its reproduction. Another example is the deformations of the cytoplasmic part of Toll-like receptor 4 (TLR4) required for the downstream signaling. These deformations were suggested to be associated with reduced tissue damage upon excessive immune stimulations by LPS in some mammalian species (Vaure and Liu, 2014). Additional example is the human-specific bacterial infection of *Neisseria gonorrhoeae* that causes gonorrhea, a sexually transmitted disease (Landig et al, 2019). *N. gonorrhoeae* was found to recognize receptors on human immune cells but not on cells from one of our most closely related species, the chimpanzee (Landig et al, 2019). On the other hand, some pathogens were found to rarely be pathogenic in humans while are pathogenic in other animals. For instance, *Bordetella bronchiseptica* infection is pathogenic in cats (Egberink et al, 2009), dogs (Schulz et al, 2014) and rabbits (Deeb et al, 1990) but rarely affects humans (Woolfrey and Moody, 1991). Comparative studies identified major differences of TLR4 receptor that can sense LPS of *B. bronchiseptica* between mice and humans which may result in reduced sensitivity of humans to the pathogen LPS (Melvin et al, 2014).

1.3.3. Immune regulation and tissue repair mechanism

Tight regulation of immune response is required to avoid unnecessary immune response which may result in tissue damage (Goldszmid and Trinchieri, 2012). Several layers of regulations are interconnected to fine-tune the inflammatory immune response and avoid collateral damage including inhibitory proteins and cytokines that negatively regulate inflammatory signaling (Murray and Smale, 2012). Regulatory T cells (Tregs) are specialized T-cells that have a central

role in immune response regulation through a variety of mechanisms such as secretion of immune regulatory cytokines e.g. IL10 and TGF-β (Christoffersson and von Herrath, 2019). Other mechanisms such as inhibiting cytolysis and suppressing metabolic disruption have been also mediated by Tregs (Vignali et al, 2008). All these mechanisms suppressing the intense immune response and increase tolerance.
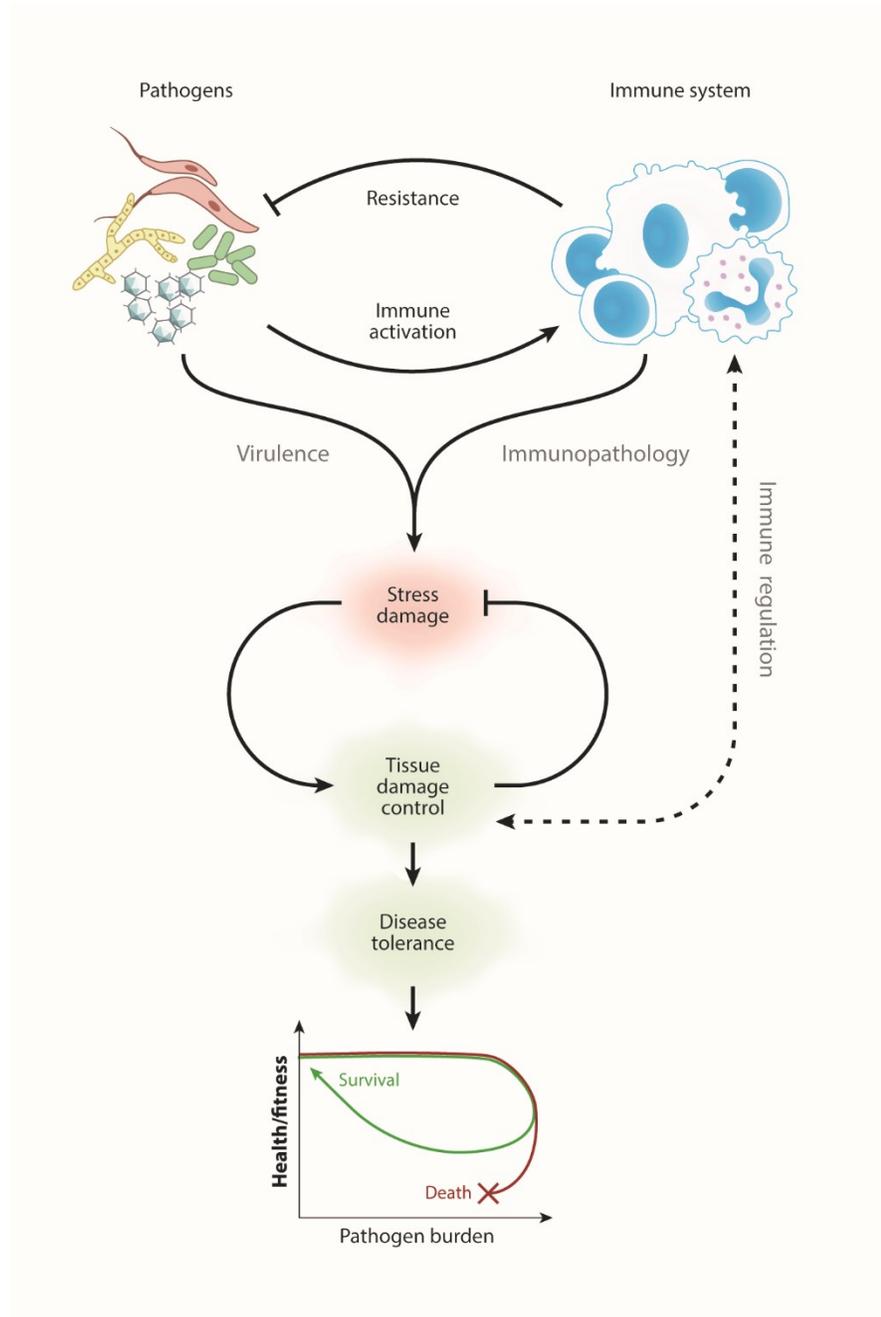
Figure 3. Strategies of immune response against pathogens. Immune cells from the host sense the pathogen and respond by resistance mechanism to restrict it. Disease manifestations will arise due to molecules of pathogen origin such as virulence factors or due to tissue damage by the innate immune mediators (immunopathology). Such stress is alleviated by tolerance mechanisms which increase host survival/fitness (figure from Martins et al, 2019).

1.4. Why different species show different disease susceptibility?

While some species were found to be more affected by specific pathogen infections, other species show fewer pathological manifestations. Humans differ in their susceptibility to different pathogens when compared to other species including birds, bats, rodents and non-human primates (Redl et al, 1993; Warren et al, 2010; Chahroudi et al, 2012; Chan et al, 2013; Mandl et al, 2018). Different mechanisms employed by these species were found to mediate disease tolerance. For instance, natural hosts of SIV, sooty mangabeys and African green monkeys, rarely show pathological symptoms upon infection. These species were found to have low expression of CCR5 in their CD4+ T-cells indicating disease tolerance by immune ignorance (Veazey and Lackner, 2017). Also, distorted receptors due to sequence frameshift and structure-changing variants were found to be associated with reduced response to a stimulant in sooty mangabeys which also mediate immune ignorance (Palesch et al, 2018). Sooty mangabey was found to elicit little activation of the innate immune response upon SIV infection unlike non-natural reservoir hosts e.g., humans. For instance, plasmacytoid dendritic cells (pDC) were found to have little activation and produce low amount of interferon alpha (IFN-α), type I IFN, in sooty mangabeys (Mandl et al, 2008). An example of tolerance through immune regulation was observed in bats where NK cells have an inhibitory role on immune signaling (Pavlovich et al, 2018). NK is an innate immune cell type that is responsible for antiviral response and was found to express several inhibitory receptors in Egyptian rousette which may inhibit intensive immune response and collateral damage.

The factors that lead certain species to be resilient to infection and not others are complex and interconnected. In essence, the continuous coevolution between hosts and pathogens leads to different outcomes of host and pathogen interactions. The immune strategy embraced by the host

is determined by several factors (summarized in Figure 4) that depends on the pathogen (virulence, transmission), the host (fecundity, life-span and other life traits) and the surrounding environmental factors such as nutrients availability, symbionts and abiotic factors e.g., temperature. I will elaborate on these factors in detail below.

1.4.1. Pathogen intrinsic factors.

Pathogen intrinsic factors include characteristics of the pathogens such as pathogen size and virulence. Pathogens can be either micro-parasites (viruses, bacteria or protozoans) or macro-parasites (helminths and crustaceans). Dependent on the size of the pathogen, the immune strategy differs accordingly. Due to the macroscopic nature of macro-parasites such as helminths, it is argued that costs associated with complete elimination of the parasites would be too high and hence a major defense strategy against macro-parasites is tolerance (Yap and Gause, 2018). The previous notion is supported by a number of observations such as the relatively low mortality and morbidity due to helminth infections despite their high prevalence. For instance, helminth infections of *Trichuris trichiura* and *Ascaris lumbricoides* are symptomless in many cases except in a very high worm burden (King and Li, 2018).

Pathogen virulence (defined by the harm the pathogen caused upon host infection) is another major factor that directs whether tolerance or resistance is the optimal strategy for immune defense. Several traits define the virulence potential of that pathogen such as pathogen life cycle and mode of infection (Brown et al, 2006; Leggett et al, 2017). Leggett and colleagues (2017) examined the correlation of virulence of six different pathogen traits that have been reported to define pathogen virulence such as route, symptoms of infection and immune subversion. Significant correlations were observed between virulence and specific traits such as growth rate and transmission route. The highly virulent pathogen will lead to selection of the costly immune resistance strategy since tolerance will allow free transmission of the pathogens between hosts and if the host cannot reproduce or recover from the virulent pathogen, tolerance will be an evolutionary dead point to the host (Boots et al, 2009). An example from a natural population that supports this notion was observed in the lethal pathogen *Pseudogymnoascus destructans*, a fungus that infects little brown bats, *Myotis lucifugus*. The pathogen was found to cause a severe decline in bat populations (Langwig et al, 2017). Pathogen density was found to be much lower in bat populations that persisted after pathogen infection than in bat populations that suffered severe declination. Hence,

it is predictable that survived bat populations had to kill the pathogen i.e. used resistance strategy to eliminate the pathogen.

1.4.2. Host-intrinsic factors.

Life history theory assumes resources allocated to vital physiological processes such as reproduction and defense to ensure the best consumption of available resources (Wang et al, 2019). One major determining factor of what defense strategy to be used against pathogens is the energy that host will pay in the absence of selective pressure of the pathogen (Schulenburg et al, 2009). Life-history traits such as reproduction and lifespan obligate a trades-off with the immune response due to resource (re)allocation (Bonneaud et al, 2003; McKean et al, 2008; Ye et al, 2009; Rauw, 2012; Donnelly et al, 2017; Parker et al, 2017). For instance, experimental infection of virulent trematode *Ribeiroia ondatrae* to 13 amphibian species (frogs, toads, and salamanders) that have different lifespans either, short "fast-lived" or long lifespan "slow-lived" showed a trade-off between lifespan and immune response (Johnson et al, 2012). Species with short lifespan were found to be affected by the infection more than species with longer life span. Hence, the study backed the notion that fast-lived species invest less in defense response relative to other traits such as reproduction as they succumbed more to infection (Johnson et al, 2012). Another example of the effect of life-history traits on the immune strategy is the tolerance to pathogens in bats. Bats are unique mammalian species in several aspects including their flight ability and prolonged lifespan given their overall body size (Brook and Dobson, 2015; Mandle et al, 2018). Due to these unique life-trait characteristics, it has been suggested that bats evolved a minimized oxidative stress through mitochondrial modifications to accommodate the metabolic demanding trait such as flight (Mandle et al, 2018). Heavy activities such as flight produce high quantities of reactive oxygen species that result in oxidative stress. Hence, bat mitochondria activate autophagy and apoptosis beside antioxidants enzymes to alleviate oxidative stress and prevent immunopathology (Brook and Dobson, 2015). Another example of tolerance to life-history traits was observed in sheep. Tolerance in natural sheep populations to gastrointestinal nematode infections was found to be associated with higher lifetime breeding success suggesting natural selection on tolerance (Hayward et al, 2014). The higher lifetime breeding success in tolerant individuals may be because tolerance saves more resources than resistance and hence these resources were directed to other life-history traits.

1.4.3. Environmental factors.

Environmental conditions and resources are key factors that determine the host-pathogen outcome such as nutrients, temperature and associated microbiota and symbionts (Ferguson and Read, 2002; Lazzaro et al, 2008; McKean et al, 2008; Maze-Guilmo et al, 2014; Schieber et al, 2015; Kutzer et al, 2018). The classical view on the effect of environmental factors is thought to be mainly mediated through the genotype of the host, hence, the interaction between the genotype-environment (environment X genotype) exerts its effect on the host-pathogen relationship (Lazzaro and Little, 2009). An example for environment X genotype was observed between the rodent malaria parasite, *Plasmodium chabaudi*, and its mosquito vector, *Anopheles stephensi* (Ferguson and Read, 2002). Pathogens had different virulence depending upon the host population, as measured by the mortality rates of the host. Virulence in this study was found to be dependent on the environmental conditions of food (glucose water) availability. Recent reports had also pinpointed a direct role of food composition on disease tolerance using murine model. Iron deficient or enriched dietary was found to be a key factor in defining tolerant or non-tolerant mouse strain (Sanchez et al, 2018). Sanchez and colleagues found that the iron dietary selected attenuated strains of the pathogen while virulent strains died revealing an indirect role of environmental factors in manipulating pathogen virulence and host-pathogen relationship. An interesting recent study examined the role of temperature on the defense response and how a mouse host will allocate resources to thermoregulation and inflammation (Ganeshan et al, 2019). The authors found that animals raised at normal temperature of 33°C (thermoneutral) and relative hypothermic conditions (22°C) differ significantly in their tolerance to bacterial infection when challenged with *Listeria monocytogenes* and *Escherichia coli* bacteria. Hypothermic animals underwent energy-saving program (dormancy) where other maintenance physiological processes such as basic metabolic rate, thermogenesis and locomotion had lower activity (measure by oxygen consumption) by a mechanism that involves Q_{10} (the effect of temperature on biochemical processes). Microbiota has been also found to mediate disease tolerance using murine models (Schieber et al, 2015) and invertebrates as in *C. elegance* (Rangan et al, 2016). Mouse host colonized with a commensal strain of *E. coli* O21:H$^+$ preserved the mouse from fat and muscle wasting induced by bacterial infection (*Salmonella*) or gut trauma (by dextran sulfate sodium) (Schieber et al, 2015). Animals protected from wasting were found to express less Atrogin-1 and Murf-1 which are markers of

muscle atrophy. Analysis of the transcriptome of muscle from protected mice identified notable activation of insulin-like growth factor (IGF-1)/phosphatidylinositol 3-kinase (PI3K)/AKT pathway indicating the role of these pathways in increasing tolerance of infected animals.
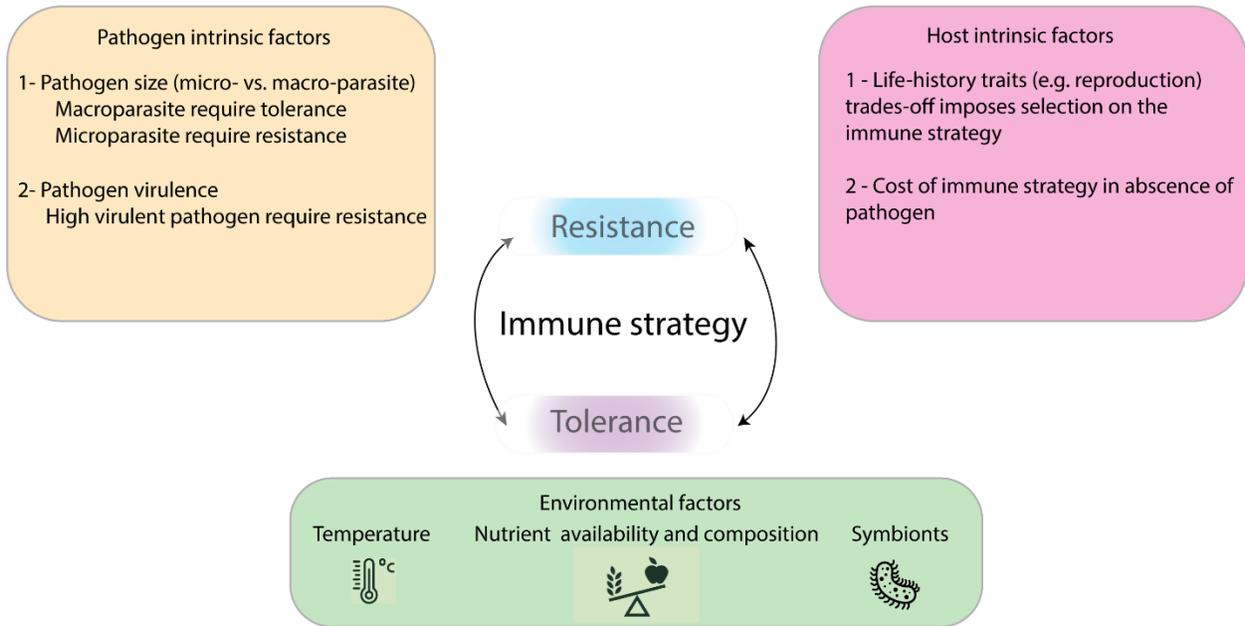
Figure 4. Summary of factors that define immune strategy embraced by a host. The factors can be categorized into three major categories, host-intrinsic factors such as life-history traits trade-off, pathogen-intrinsic factors such as nature of the pathogen or its virulence and environmental factors such as temperature.

1.5. Tolerance from an evolutionary genetic perspective

Host and pathogens evolved through continuous cycles of adaptation and counter-adaptation, an evolutionary arm race known as the "Red queen" dynamic (Terauchi and Yoshida, 2010; Sironi et al, 2015). The term "Red queen" was first coined by Bell (1982) to describe a theory of selection on host genotypes by parasite genotypes (Clay and Kover, 1996). The term was inspired by Lewis Carroll's novel *Through the Looking Glass* when the Red Queen says to Alice "Now, here, you see, it takes all the running you can do, to keep in the same place.". The theory hypothesizes that pathogen genotypes that infect more hosts will be favored and spread. Consequently, on the host

side, rare host genotypes that the pathogen cannot infect will have a selection advantage and start to increase in frequency. In turn, rare pathogen genotypes that infect the new host genotypes will be favored and so on (Figure 5a). An example of the theory on tolerance is given below. For instance, a mutation that causes deformity of recognition receptor to a pathogen may be favored if the cost of immunopathology is high and thus will spread leading to tolerance strategy through ignorance (Sironi et al, 2015) as shown in Figure 5b (Sironi et al, 2015). The virus in turn changes its structure to adapt to the new receptor structure (Figure 5b). Analysis of the DNA from the different species gives insights into the mutations that are favored by natural selection to escape from pathogen infection. For instance, mutations of the TLR4 gene in sooty mangabey made the receptor poorly responsive (Palesch et al, 2018).

Another example of the coevolution between host molecules and pathogens is found in major histocompatibility complex (MHC) proteins. MHC genes are essential genes in vertebrate hosts for triggering host resistance pathways against pathogens as they are implicated in pathogen antigen presentation, inflammatory response, innate and adaptive immune response (Matzaraki et al, 2017). Due to its central role in host pathogen interactions, the genetic polymorphism of these molecules has been extensively studied in humans and other animals (Radwan et al, 2020). A study on natural host pathogen populations, the frog species *Lithobates yavapaiensis* and its obligate fungus parasite *Batrachochytrium dendrobatidis* (*Bd*), showed the effect of MHC polymorphism in driving survival in a natural amphibian population through tolerance to infections (Savage and Zamudio, 2016). The authors found MHC alleles associated with survival (allele Q) through disease tolerance strategy or susceptibility (allele A) to infection. The authors identified directional selection of allele Q in the population with significant survival rate from infection emphasizing the key role played by natural selection in shaping the optimal immune strategy that promote host survival.

A recent study characterized the complete annotation of immunoglobulin heavy chain genomic locus and antibody receptors and complement system proteins repertoire in Egyptian rousette bat (ERB) (Larson et al, 2021). Comparative analysis identified several mutations that render pentraxins unfunctional (Larson et al, 2021). Pentraxins are a set of highly evolutionary conserved molecules that play a key role in pathogen clearance through complement activation and binding to antibody receptors (Du Clos, 2013). The loss of some of these molecules in bats, suggests that

natural selection favored their inactivation as a mechanism to potentially reduce inflammation, and increase tolerance to infection (Larson et al, 2021).

The relationship between tolerance and resistance evolution and the exact tradeoffs between these two strategies remain poorly understood. Several reports found contradicted results when studied resistance and tolerance. For instance, a study on the house finch (*Haemorhous mexicanus*) explored the immune strategy of the host against the emerging pathogen *Mycoplasma gallisepticum* (Bonneaud et al, 2019). The authors found disease tolerance to accompany resistance mechanisms suggesting cooperation between resistance and tolerance strategies in fighting the emerging pathogen. Another study explored the relationship between different immune strategies of Atlantic salmon (*Salmo salar*) to its helminthic parasite *Diplostomum pseudospathaceum* and found an opposite relationship between resistance and tolerance indicating a tradeoff (Klemme et al, 2020). A third study on the *Leuciscus burdigalensis,* a freshwater fish and its crustacean parasite *Tracheliastes polycolpus* found no relationship between tolerance and resistance mechanisms in host populations (Mazé-Guilmo et al, 2014). The discrepancy between studies on the relationship may be due to the different factors that I mentioned previously that dictate the optimal use of defense strategy.
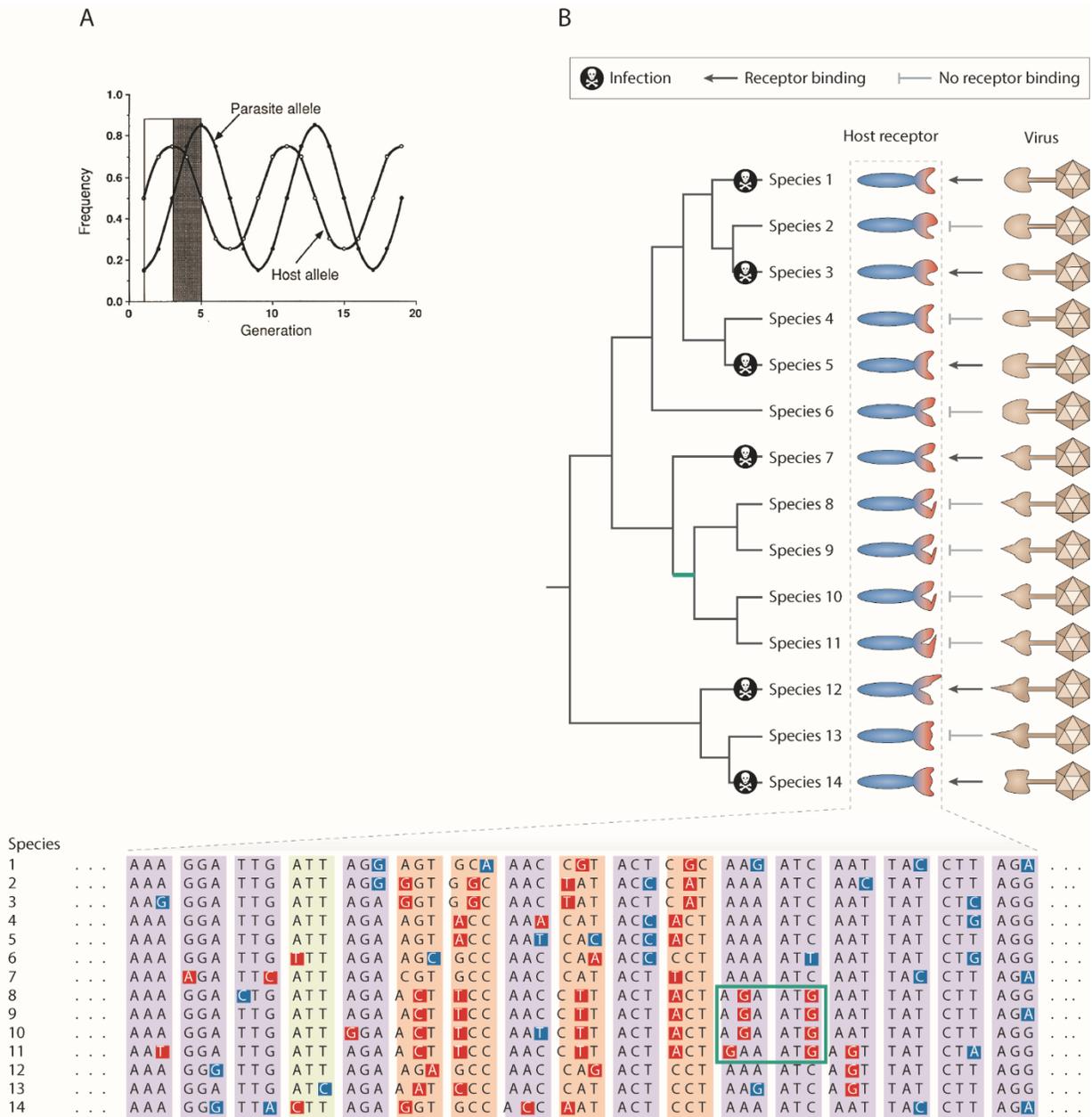
Figure 5. Example of a host pathogen coevolution revealed by interspecies comparative study. **A**. Red queen dynamic relationship between host and parasite genotypes (from Clay and Kover, 1996). **B**. A hypothetical example where a virus uses a receptor on host cells to infect it. To prevent viral binding and infection, selection favors mutations that modify the sequence and structure of the host receptors; on the other side, the virus adapts to such changes by gaining mutations that keep re-establishing host receptor binding (adapted from Sironi et al, 2015).

1.6. Why using an interspecies comparative approach to study disease tolerance?

Disease tolerance is a very important immune strategy that has received increased attention over the last decade. The major focus of current research directions has been to explore potential tolerance mechanisms using mouse models (Martin et al, 2019). Although this strategy has proved to be valuable at discovering several interesting mechanisms of disease tolerance, mouse models only allow discovering tolerance mechanisms that are highly conserved in mammals. Specific tolerance pathways that evolved later in other mammals' clades such as primates are unlikely to be discovered using mouse models. Hence, the interspecies comparative approach is a complementary approach that already provided many valuable insights on disease tolerance mechanisms. I will explain from my point of view why interspecies comparative approaches should be used for disease tolerance research.

Firstly, interspecies comparative studies give a valuable opportunity to uncover potential new mechanisms of tolerance that have been changed during the long course of evolution. For instance, HIV infection and the resilience mechanisms to the virus have been successfully uncovered through comparative analysis of primates (Mandl et al, 2008; Chahroudi et al, 2012; Veazey and Lackner, 2017).

Secondly, a considerable proportion of immune system components is conserved across wide clades of species which facilitates direct comparisons between species. Vertebrates share several components of innate immune effectors such as antimicrobial peptides (AMPs), lytic enzymes (e.g., lysozymes) and complement proteins which are conserved molecules that fight different microbes through various mechanisms (Boehm, 2012; Riera Romo et al, 2016). Cytokines are another central class of effectors that orchestrate vertebrate's immune responses, which responses to immune activation can easily be compared across species. Major constituents of cellular immune components are identified among wide classes of vertebrates including monocytes, basophils and neutrophils; immune cells responsible for phagocytosis and secretion of antimicrobial defensive molecules (Buchmann, 2014; Riera Romo et al, 2016). Immune signaling pathways involved in sensing and responding to pathogens such as toll-like receptors (TLRs) and nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) are highly conserved in animals (Yuan et al, 2014). Indeed, the first member of PPRs was discovered in

Drosophila, TRL4, followed by characterization of different families of PRRs in all vertebrates (Kimbrell and Beutler, 2001; Yuan et al, 2014).

Thirdly, the majority of infectious diseases in humans came originally from animals (Wang and Crameri, 2014; Belay et al, 2017). The significant morbidity and mortality due to pathogen burden stem from emerging infectious disease (EID) such as Ebola virus and severe acute respiratory syndrome 1 and 2 coronaviruses (SARS-CoV 1/2), which are zoonotic diseases caused by pathogens transmitted from animals and, collectively, account for nearly 75% of all EID infections (Belay et al, 2017). Reservoir hosts that harbor these infectious disease agents are in several cases tolerant to these infections. For instance, as pointed previously, HIV has little pathology in the natural reservoir primate hosts albeit the presence of high viremia pinpointing to the tolerance potential of these hosts (Chahroudi et al, 2012). Also, several members of coronaviruses emerged from bats that cause high mortality and morbidity besides severe economic losses (Rasmussen, 2021).

Comparative genomic and functional transcriptomic approaches have been applied to study tolerance in species such as primates and bats highlighting important features in genome evolution that may account for the different disease susceptibility as mentioned previously (Palesch et al, 2018; Pavlovich et al, 2018). Despite the anecdotes of interspecies comparative studies in deciphering mechanisms of pathogens such as HIV in natural and non-natural hosts of human and non-human primates, similar approaches have not been used in a systematic manner. Several other bacterial and viral pathogens cause different pathology between human and other primates. For instance, humans are susceptible to bacterial genital infection by *Neisseria gonorrhoea* bacteria unlike monkeys such as baboons (McGee et al, 1990). Another important example is the interspecies sensitivity to Lipopolysaccharide (LPS) in primates. Humans and chimpanzees are susceptible to immunopathology and septic shock upon administration of a minute amount (5 ng/kg) of LPS while other old world monkey primates, such as macaque and baboon, require much higher amount of LPS to observe similar pathological effect (Redl et al, 1993; Brinkworth et al, 2012; Chen et al, 2019). Little is known about the possible causes for these differences which I will explore through my thesis studies.

1.7. Research goals

The major aim in my thesis is to explore the differences of disease susceptibility in primates by characterizing the immune response and its regulatory elements. Mainly, two studies were conducted to answer this broad question.

Article I. Primate innate immune response to bacterial and viral pathogens reveals an evolutionary trade-off between strength and specificity.

In this article, I characterized the immune response using whole blood stimulation by bacterial and viral stimulants at two time points. I used bulk RNA-seq for functional characterization of the immune response from 4 primates namely, human, chimpanzee, macaque and baboon.

Article II. Adaptive evolution shaped interspecies differences of immune response across primates.

In this article, I characterized the immune response and the regulatory landscape of stimulated and unstimulated cells using single cell RNA-seq (sc-RNA seq) and single cell ATAC-seq (scATAC-seq). I used peripheral blood mononuclear cells (PBMCs) from 4 species namely human, macaque, baboon and lemur.

2. **Article I**

Primate innate immune responses to bacterial and viral pathogens reveals an evolutionary trade-off between strength and specificity.

Mohamed Bayoumi Fahmy Hawash, Joaquin Sanz-Remón , Jean-Christophe Grenier , Jordan Kohn , Vania Yotova, Zach Johnson, Robert E. Lanford, Jessica F. Brinkworth, Luis B. Barreiro

Note: Due to the length of the supplementary tables of this article, they are not included here but they were provided with the supplementary information at the journal website (https://www.pnas.org/content/suppl/2021/03/26/2015855118.DCSupplemental).

Primate innate immune responses to bacterial and viral pathogens reveals an evolutionary trade-off between strength and specificity.

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Abstract

Despite their close genetic relatedness, apes and African and Asian monkeys (AAMs), differ in their susceptibility to severe bacterial and viral infections that are important causes of human disease. Such differences between humans and other primates are thought to be a result, at least in part, of inter-species differences in immune response to infection. However, due to the lack of comparative functional data across species, it remains unclear in what ways the immune systems of humans and other primates differ. Here, we report the whole genome transcriptomic responses of ape species (human, chimpanzee) and AAMs (rhesus macaque and baboon) to bacterial and viral stimulation. We find stark differences in the responsiveness of these groups, with apes mounting a markedly stronger early transcriptional response to both viral and bacterial stimulation, altering the transcription of ~40% more genes than AAMs. Additionally, we find that genes involved in the regulation of inflammatory and interferon responses show the most divergent early transcriptional responses across primates and that this divergence is attenuated over time. Finally, we find that relative to AAMs, apes engage a much less specific immune response to different classes of pathogens during the early hours of infection, upregulating genes typical of anti-viral and anti-bacterial responses regardless of the nature of the stimulus. Overall, these findings suggest apes exhibit increased sensitivity to bacterial and viral immune stimulation, activating a broader array of defense molecules that may be beneficial for early pathogen killing at the potential cost of increased energy expenditure and tissue damage.

Introduction

Despite being close evolutionary relatives, humans, chimpanzees and African and Asian monkeys exhibit inter-species differences in sensitivity to and manifestation of certain bacterial and viral pathogens that are major causes of mortality in humans (e.g. HIV/AIDS, Hepatitis C Virus, broad range of commensal Gram-negative bacteria commonly implicated in sepsis) (Redl et al, 1993; Munford, 2008; Chahroudi et al 2012; Sandmann and Ploss, 2013; Vaure and Liu, 2014). Humans, for example, are highly sensitive to stimulation by the Gram-negative bacterial cell wall component hexa-acylated lipopolysaccharide (LPS), miniscule amounts of which (2-4ng/kg) can provoke inflammation, malaise and fever, and a slightly higher dose, septic shock (15 ug/kg) (Redl et al, 1993, Kumar et al, 2004; Taveira da Silva et al, 1993). In contrast, baboons and macaques require doses nearly 10 fold higher in concentration to trigger similar symptoms (Vaure and Liu, 2014; Haudek et al, 2003; Yin et al, 2005). Pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) play a central role in the mediation of innate immune responses to pathogens (Akira, 2009). The limited number of studies comparing leukocyte function after stimulation with TLR-detected pathogen-associated molecules (PAMPs) suggest that the differences in infectious diseases susceptibility noted between apes and AAMs is, in part, the outcome of lineage-specific evolution of early innate immune system regulation and signaling (Barreiro et al, 2010; Brinkworth et al, 2012; Mandl et al, 2008). Indeed, innate immune components responsible for detecting pathogens, including TLRs that sense Gram-negative bacteria and single-stranded RNA viruses, have been found to be under positive selection in primates (Wlasiuk and Nachman, 2010; van der Lee et al, 2017).

Despite stark differences in the manifestation of severe infections between apes and African and Asian monkeys (AAMs), there are few reports directly comparing the gene expression response across species to bacterial and viral pathogens (Barreiro et al, 2010; Brinkworth et al, 2012). Further, previous studies relied mainly on isolated cell types to characterize immune responses across primates (Barreiro et al, 2010; Danko et al, 2018), which does not faithfully reflect the nature of the innate immune response that is a product of the interaction between several cell populations (Rivera et al, 2016). To better understand the evolution of the primate immune system, this study compares the early responses of apes (humans and common chimpanzees) and AAMs

(rhesus macaques and olive baboons) to bacterial and viral stimulants. Here, we report on the whole genome expression of total blood leukocytes from these four primate species responding to bacterial and viral stimulation during the first 24 hours of challenge. Our results show that apes and AAMs have diverged in sensitivity to specific microbial assaults, such that ape leukocyte responses favor robust antimicrobial power over pathogen specificity at the potential cost of increased energetic expenditure and bystander tissue damage.

Results

Evolutionary relationships explain most of the transcriptional response variation in primates to bacteria or viral stimulation.

To assess differences in innate immune function between higher order primates in as close an approximation to *in vivo* as possible, we challenged whole blood from humans (*Homo sapiens*; N=6), common chimpanzees (*Pan troglodytes*; N=6), rhesus macaques (*Macaca mulattta*; N=6) and olive baboons (*Papio anubis*; N=8) with bacterial or viral *stimuli* via venous draw directly into a media culture tube containing either lipopolysaccharide (LPS) from *Escherichia coli* O111:B4, gardiquimod (GARD), a single-stranded RNA viral mimetic, or endotoxin-free water, as a negative control (Control). Blood was stimulated for 4 and 24 hours before the total leukocytes were isolated, and RNA extracted for RNA-sequencing (**Figure 1A**). We chose these two molecules because in mammals they are broad signals of infection by pathogen types for which there are well established differences in disease manifestation between apes and AAMs (e.g., immunodeficiency viruses, Hepatitis C, common commensal bacteria that cause Gram-negative bacterial sepsis) (Redl et al, 1993; Chahroudi et al 2012; Sandmann and Ploss, 2013). Following quality control filtering, we analyzed 151 high-quality RNA-sequencing profiles across species and treatment combinations (see Methods, **Table S1**). We focus our comparative analyses on the expression levels of 14,140 one-to-one (1:1) orthologous genes, taking into account potential biases in expression estimates due to differences in mappability between species (Methods).

As whole blood contains a variety of leukocyte cell subtypes, we first characterized differences in total blood leukocyte composition between species using fluorescence-activated cell sorting

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(FACS). Leukocyte composition differs between species for all major subtypes measured, with the most notable differences an increase in the proportion of monocytes in humans (CD14$^+$, $P < 0.003$) and helper T cells in chimpanzees (CD3$^+$, CD4$^+$; $P = 0.0006$ to 0.065), relative to other primates (**Figure 1B**, **Table S2**). Using linear models that account for variation in cell composition, we next identified genes that respond to LPS and GARD in each of the species, at each of the time points (see Methods). In all species, both treatments led to the up- or down-regulation of hundreds to thousands of genes (FDR<0.05, **Figure 1C**, **Table S3**). As expected, the transcriptional response to either stimulus was highly concordant across primates (Spearman's r range 0.5 to 0.87 across all pairwise comparisons; **Figure S1**), with stronger correlations between closely related primates than between more distantly-related pairs of species (e.g., at LPS 4 hours Spearman's r□human vs chimpanzee = 0.84, human vs baboon = 0.50). Consistently, the first principal component (PC) of the log2 fold-change responses to both LPS and GARD accounted for ~20% of the total variance in our dataset and separated apes (human and chimpanzee) from AAM (macaque and baboon) (t-test; $P < 1 \times 10^{-10}$ for both 4h and 24h, **Figure 1D**). The second PC captured differences in immune response to bacterial or viral stimulation (t-test; $P < 1 \times 10^{-8}$ for 4 and 24h; **Figure 1D**). We identified a set of 648 and 257 genes that early after stimulation (4 hours) showed a consistently strong response across all species to LPS or GARD, respectively (defined as genes with |log2 FC| > 1 and FDR < 0.05 in all species, **Table S4**). These genes include most of the key transcription factors involved in the regulation of innate immune responses to bacterial (e.g., *NFKB1/2*) and viral pathogens (e.g., *IRF7/9*), as well as several effector molecules involved in the regulation of inflammatory responses to infection (e.g., *IL6*, *TNFα* and *IL1b*).
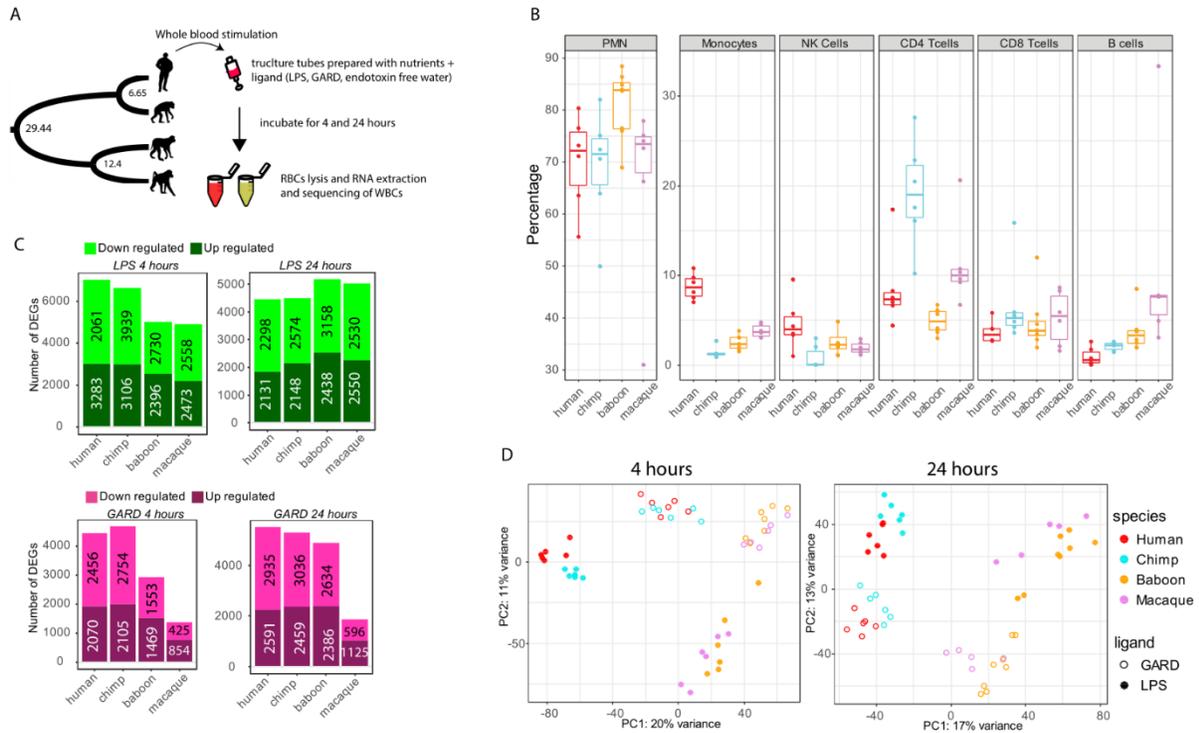
Figure 1. Characterizing innate immune response upon viral and bacterial stimulation of primate's white blood cells. (A) Schematic representation of the study design. Whole blood samples from humans, common chimpanzees, rhesus macaques and olive baboons were stimulated with bacterial or viral stimuli via venous draw directly into a media culture tube containing either lipopolysaccharide (LPS), single stranded RNA viral mimetic gardiquimod (GARD), or endotoxin-free water, as a negative control (Control). At 4- and 24-hours post-stimulation white blood cells were isolated, and RNA extracted for RNA-sequencing. (B) Cell proportion of 6 populations of innate immune cells for all species. Species are indicated on x axis and proportions this population from total leukocytes is on y axis. Abbreviations are PMNs for polymorphonuclear cells and Natural killer for NK cells (C) Number of differentially expressed genes (DEGs; FDR<0.05) in response to LPS (top panels) and GARD (bottom panels) in each of the species at 4- and 24-hours post-stimulation The exact number of up- and down-regulated genes in each condition in each species are indicated on the bar charts. (D) Principal component analyses (PCA) performed on the log2 fold-change responses observed at 4 hours post LPS and GARD stimulation. PC1 primarily separates apes (human and chimpanzee) from AAM (macaque and baboon), and PC2 captures differences in immune response to bacterial or viral stimulation.

Stronger early innate immune response in apes than in AAM

Next, we sought to characterize differences in immune responses across species. To do so, we first looked at overall differences in the magnitude of the transcriptional responses to LPS and GARD across species (see Methods). We found that, at early time points, both ape species (human and chimpanzee) engage a much stronger transcriptional response to both stimuli as compared to rhesus and baboons (in average ~2-fold higher, Wilcoxon test $P < 10^{-10}$, **Figure 2A**). Next, we identified genes for which the magnitude of the transcriptional response to LPS or GARD was significantly different between apes and AAM (FDR<0.10 for *all* pair-wise contrasts between an ape and an AAM species and an average |log2 FC| > 0.5). Hereafter, we refer to these genes as Clade Differentially Responsive Genes, or c-DRGs. We identified a total 831 and 443 c-DRGs, in the early response (4 hours) to LPS and GARD, respectively (**Figure 2B, Table S5**). Among c-DRGs, 83-92% showed a stronger response in apes as compared to AAM, consistent with the genome-wide pattern of an overall more robust transcriptional response to immune stimulation in apes. Importantly, the stronger response observed in apes is not explained by higher baseline expression levels of the receptors involved in the recognition of LPS (*TLR4*, *CD14*, *LY96* and *CASP4*) and GARD (*TLR8*) (**Figure S2**). Next, we focused our analyses on a manually curated list of 1079 genes belonging to different modules of the innate immune system (Deschamps et al, 2016). and that were found to change gene expression in at least one of our experimental conditions, in at least one of the species from our dataset. These genes include sensors (n=188), adaptors (n=36), signal transducers (n=209), transcription (factors) (n=74), effector (molecules) (n=115), accessory molecule (n=54) and secondary receptors (n=50). All modules show similar divergence between clades, with ~15% of the genes within each module classified as c-DRGs with a stronger response in apes, as compared less than 5% showing a significantly stronger response in AAM (**Figure 2C**).

To further characterize functional differences in immune regulation between apes and AAM we devised a new score of transcriptional divergence at the pathway level. We focused on the set of 50 "hallmark pathways", which capture well-defined and curated biological states or processes (Liberzon et al, 2015). Briefly, for each gene in these pathways, a divergence score between apes and AAM was computed by calculating the average difference between the fold-change estimates

between all pairs of species of the two clades, while taking into account variance in transcriptional response within each species. The pathway divergence score reflects the average divergence scores across all genes of a given pathway (see Methods for details). In the early response to LPS, the most divergent pathways between apes and AAM were "Interferon alpha response" and "Interferon gamma response" ($P \leq 0.01$, **Table S6**), indicating that the regulation of interferon responses has significantly diverged since the separation between apes and AAM. In the early response to GARD, pathways directly related to the regulation of inflammatory responses, notably TNF-□ signaling, were the most divergent ($P \leq 0.01$) (**Figure 2D**). These results are consistent with recent finding showing that the transcriptional response of cytokines and chemokines to immune stimulation are amongst the most divergent across mammals (Hagai et al, 2018).
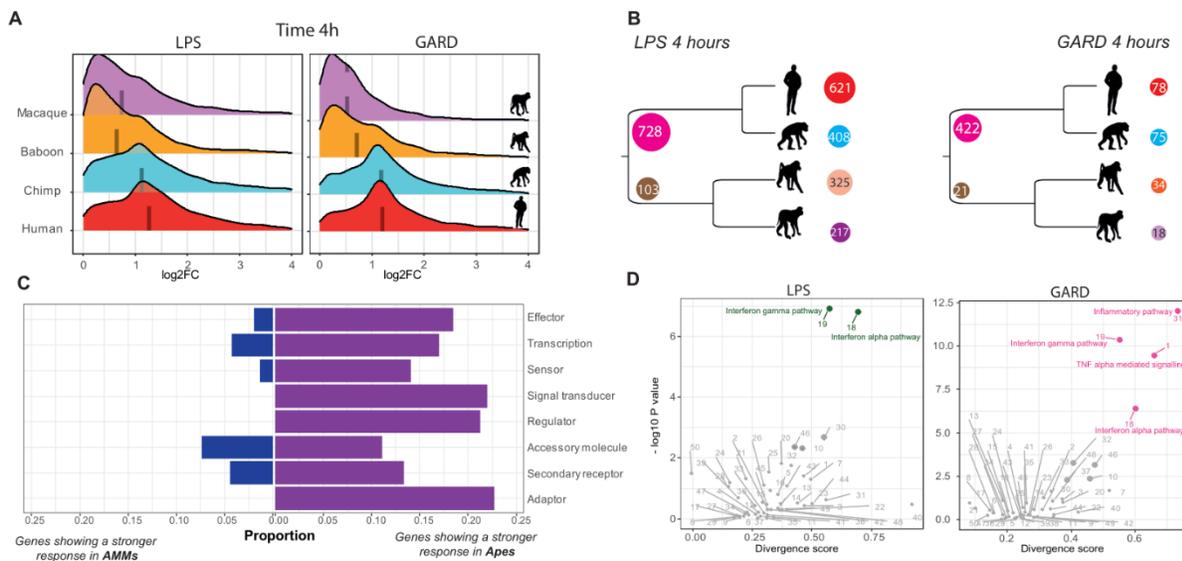
Figure 2. Stronger early innate immune response in apes than monkeys. (A) For each combination of stimulus and time-point we show the distribution of the log2 fold changes (x-axis) among genes that response to that treatment in at least one of the species. The median log2 fold change responses in each species is represented by a dashed line. (B) Number of differentially responsive genes that are clade- or species-specific differently regulated genes at 4 hours post LPS (left) and GARD (right) stimulation. For clade differently regulated genes (c-DRG) we report number of genes that show a stronger response in specific clade at the beginning of ancestral branch of the tree. For example, in response to LPS we identified 831 c-DRGs from which 728 show a stronger response in apes and 103 in AAMs. For species-specific responsive

genes numbers are given in front of each species. The color codes for each species are red for human, cyan for chimp, orange for baboon and violet for macaque. (C) Bar plots represent the proportions of different classes of innate immunity genes that are classified as c-DRGs with a higher response in apes (dark violet) or in AAMs (dark blue). (D) Scatter plot displaying total divergence scores of hallmark pathways for LPS (green) and GARD (pink) at 4h stimulations. For a given pathway, the total divergence is given by divergence score (DS) on the x-axis and -log10 p values for each DS is on the y-axis. The pathways names, DS values, and corresponding p values are shown in **Table S6**. We highlight the pathways showing the most significant divergence scores for both the response to LPS and GARD.

Species-specific immune responses reflect unique immune regulation mechanisms and lineage-specific divergence.

Next, we sought to identify genes that respond to immune stimulation in a species-specific fashion. These were characterized as genes for which the magnitude of the response to LPS or GARD in one species was significantly different to that observed in all other species (see Methods). Across time-points and immune stimulations we identified a total of 980, 726, 425 and 655 species-specific responsive genes in human, chimpanzees, macaques and baboons, respectively (**Table S7**). Among baboon-specific responsive genes the vast majority (69%) showed a weaker magnitude of the response to 4-hours of LPS stimulation in baboons as compared to all other primates (**Table S7**). Gene ontology (GO) enrichment analyses (**Table S8**) revealed that these genes were enriched among defense response genes (FDR= 4.8×10^{-14}) and a variety of other GO-associated immune terms (**Figure 3B**), including several key transcription factors (e.g., *STAT1*, *IRF7/9*), major inflammatory cytokines (e.g., *IL1B* and *CXCL8*), and a number of genes directly involved in LPS sensing and recognition (adaptor molecules *IRAK2*, *3* and *4*, and the primary LPS receptor, *TLR4*) (**Figure 3A; Table S7**). The weaker response observed in baboons appears to be, at least in part, due to a higher baseline expression level of many of these innate immunity genes (**Figure S3**). Baboons have been suggested to bear higher pathogen loads than apes due to their mating promiscuity, and so it is tempting to speculate that increased baseline might represent a mechanism of protection against frequent microbial infections (Nunn et al, 2000; Nunn, 2002). In rhesus macaques, the other AAM species, genes showing a stronger response to LPS at both 4 and 24 hours than that observed in all other species (N=157, **Table S7**) were mostly enriched among

genes involved in the regulation of inflammatory responses (FDR = 0.002, **Table S8**), including *TREM2* a known suppressor of PI3K and NF-kappa-B signaling in response to LPS.

Among chimpanzees-specific genes the most notable GO enrichments were observed among genes showing a weaker response to LPS at 24 hours relative that observed in all other primates. These genes were significantly enriched for GO terms associated with viral defense mechanisms, including "response to virus", or "type I interferon signaling pathway" (FDR<1×10^{-9}, **Figure 3C**). Further inspection of these genes revealed that the vast majority are strongly up-regulated at 4 hours post-LPS stimulation – at similar levels to those observed in other species - but that chimpanzees have a unique ability to shutdown these genes at later time points. For example, the prototypic interferon responsive gene *MX1* is up-regulated by over 5-fold in all primates at 4 hours but by 24 hours *MX1* levels have revert to baseline uniquely in chimpanzees (**Figure 3E**), suggesting that chimpanzees are particularly divergent in the regulatory circuits associated with the control of viral responsive genes.

In contrast to the pattern observed for baboons, human-specific responses were associated with genes showing a stronger response to immune stimulation as compared to that observed in other primates. Gene ontology analyses revealed that these genes are over-represented among terms related to the regulation of cytokine production involved in immune response (FDR = 0.045), and T cell activation involved in immune response (FDR = 0.06) (**Table S8**). Notable examples of human-specific responding genes include the canonical T cell co-stimulatory molecule CD80 (average 5-fold increase in response to both stimuli relative to other species) and *IFN☐*, a cytokine central for protective immunity against a large number of infectious agents and the key determinant of the polarization of T cells towards a pro-inflammatory Th1 phenotype (Bradley et al, 1996). (**Figure 3D**). The higher production of *IFNγ* and *CD80* in humans may mediate more effective killing of viral and bacterial pathogens. Further, as these molecules are important regulators of cytokine production and T cell activation, it also suggests significantly different regulation of T cell responses (Kak et al, 2018; Zha et al, 2017).
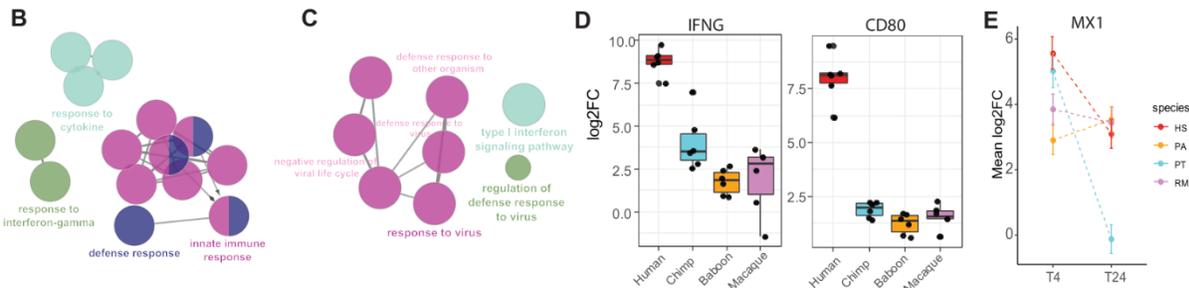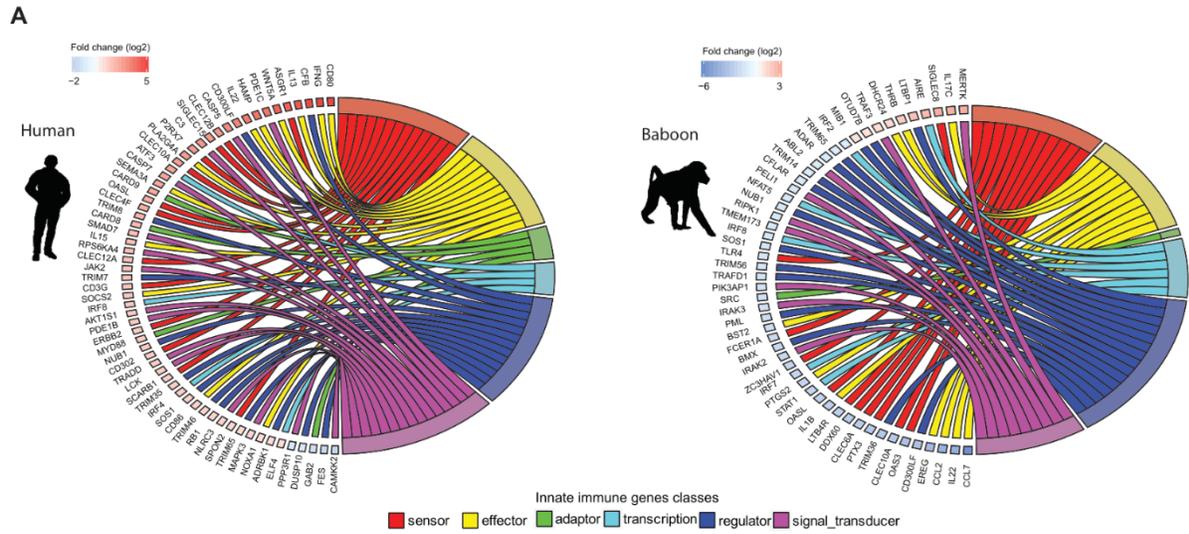
Figure 3. Species specific immune response reflect unique immune regulation mechanisms and lineage specific divergence. (A) Circos plots showing different classes of innate immune genes (clustered using different color codes) classified as species-specific responsive at 4 hours post-LPS stimulation in humans (left) and baboons (right). The log2FC key represent the average difference between species response versus all other species where the positive (red color) and negative (blue) values indicate the magnitude of the stronger and weaker absolute response in this species vs. all others, respectively. (B) Gene ontology (GO) enrichment analysis for genes showing an weaker response to LPS at 4 hours in baboons as compared to all other species. (C) Gene ontology (GO) enrichment analysis for genes showing an weaker response to LPS at 24 hours in chimpanzees as compared to all other species. For B and C only top GO terms are presented. The full list of significant GO terms can be found in Table S8. (D) Boxplot represent the log2FC of IFN☐ and CD80 genes which, at 4 hours post-LPS stimulation, were found to have a significantly stronger response in human than in other primates. (E) Estimates of the mean fold changes response for MX1 (+/- SE) at the two time points across the four primate species studied.

Regulatory divergence decreases as infection proceeds

Next, we compared the transcriptional divergence between early (4 hour) and late (24 hours) immune responses. We observed a marked reduction in divergence scores at 24 hours post-stimulation of most hallmark pathways in the response to both LPS (P=8x10^{-6}) and GARD (P=6x10^{-9}) (**Figure 4A**). In LPS-stimulated cells, the most striking differences were observed for interferon-related pathways, which show a reduction in divergence score of ~6-fold between the two time points. In GARD-simulated cells, the largest reduction in divergence scores was observed among pathways related to the regulation of inflammatory responses (**Figure 4A**). These findings indicate that most transcriptional divergence in immune responses among primates occurs during the initial response to pathogens followed by an overall convergence to similar response levels at later time point, specifically among genes involved in the regulation of inflammation and viral-associated interferon responses (**Figure 4B**). In apes (but not in AAMs), genes involved in the regulation of inflammation are strongly enriched among those for which the response to GARD significantly decreases at the later time point, whereas those decreasing in response to LPS are enriched for viral response genes (**Figure 4C**; **Table S9**).
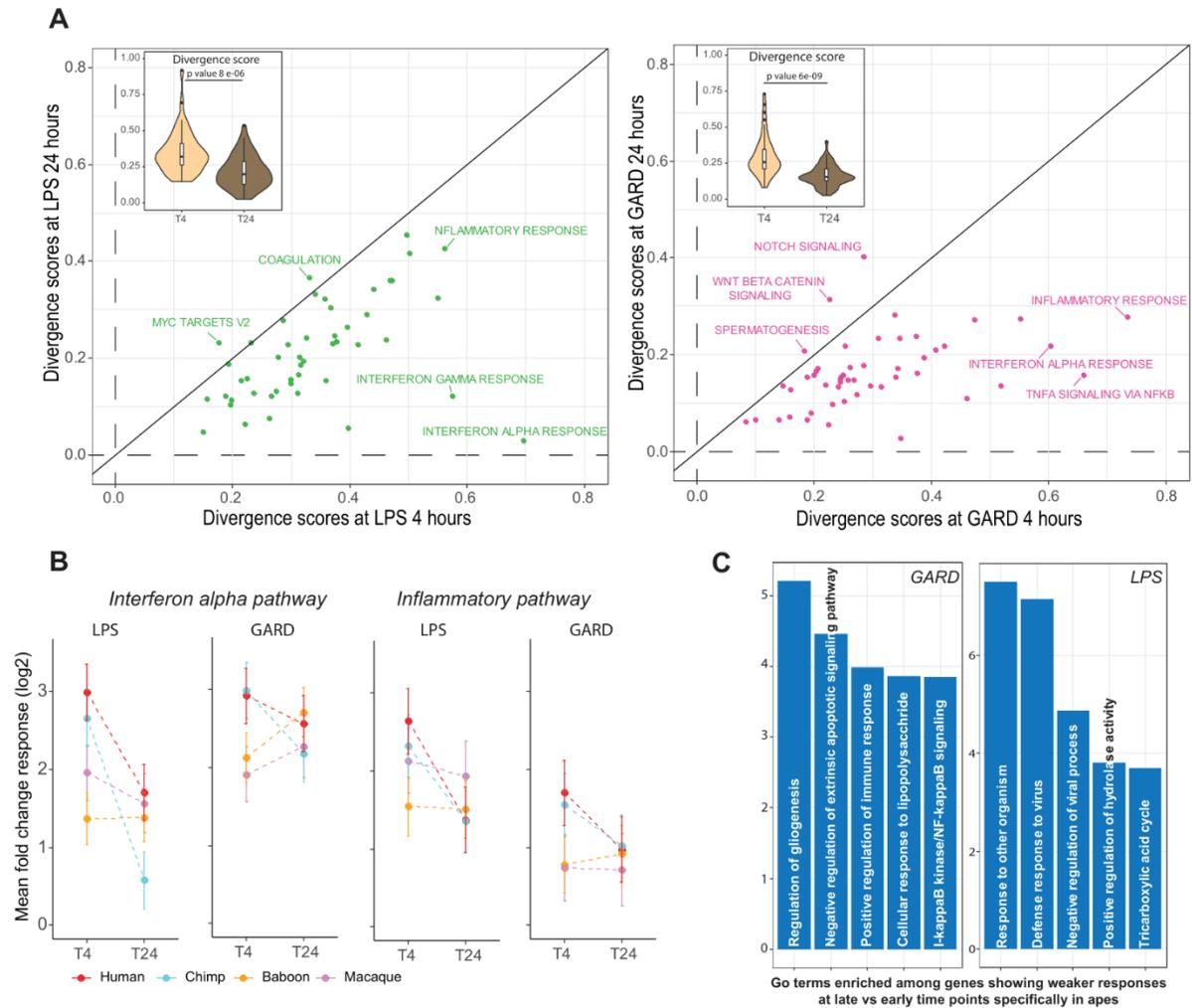
Figure 4. Divergence of immune response is reduced at later time point. (A) Scatter plots of divergence scores of hallmark pathways at early (x-axis) and later time points (y-axis) for LPS (green) and GARD (maroon) stimulations. The inset boxplots contrast the distribution of divergence scores among all pathways between the two time points. P values were obtained using Mann Whitney test. (B) Estimates of the mean response at the two time points for each species (+/- SE) across genes bellowing to the interferon alpha and inflammatory response hallmark pathways. (C) GO enrichment analysis for genes that showed significant decrease in response in apes only (FDR < 0.05 in apes and FDR > 0.05 in monkeys) for LPS and GARD. Top significant GO terms are given as indicated by –log10 p value on the x axis.

Apes engage a less specific innate immune response than AAM

An aspect of innate immunity central to its success during microbial assault is its ability to recognize pathogens and initiate the most appropriate defense against them by type. The specificity of the innate immune response to infection is mediated by pattern recognition receptors that detect the presence of danger signals via conserved molecular patterns associated with subtypes of pathogens and host damage (e.g. penta- and hexa-acylated LPS from Gram-negative bacteria detected by *TLR4-LY96* receptors) (Janeway, 1989; Matzinger. 1994). Signals of viral danger such as GARD are expected to activate a response mainly controlled by transcription factors prominent in antiviral defense such as interferon regulatory factors (IRFs), which limit viral replication and dissemination through the upregulation of interferons and interferon-regulated genes (Brubaker et al, 2015; Fitzgerald and Kagan, 2020). By contrast, recognition of Gram-negative bacteria via cell-wall component LPS stimulates a broader array of cytokine responses that tends towards expression of pro-inflammatory cytokines regulated by transcription factors *NFκB* and *AP1*, but can also include interferon expression regulated by transcription factors such as *IRF3* and JAK-STAT (Brubaker et al, 2015; Kanevskiy et al, 2013; Ghosh et al, 2015; Kagan et al, 2008).

Two major lines of evidence indicate that early transcriptional immune responses are less specific in apes than in AAM. First, we found the transcriptional responses to LPS and GARD were more similar to each other in apes (humans r = 0.87, chimpanzees r=0.83) than they were in baboons (r = 0.44) or macaques (r = 0.65) (**Figure 5A**). Accordingly, we found about three times more genes that respond *uniquely* to either LPS or GARD (i.e., "ligand specific" genes) in AAM as compared to apes (chi^2 test; P=2.2x10^{-16}) (**Figure 5B,** Methods for details on the statistical model used to characterize ligand specific and shared genes). The second piece of evidence comes from the nature of the genes that are differentially activated in response to LPS and GARD. The fact that apes show a higher correlation in GARD and LPS responses compared to AAMs predicts that they will tend to activate both antibacterial and antiviral defenses mechanisms regardless of the nature of the stimuli. Supporting this notion, the genes that exhibited a stronger response in apes than in AAMs after stimulation with the viral mimic GARD for four hours were most significantly enriched genes involved in the regulation of inflammatory responses (P= 7.1x10^{-6}; FDR = 0.008,

Table S8), whereas genes involved in the response to viruses (GO term "response to virus") were enriched upon bacterial LPS stimulation (*P*= 0.0023; FDR = 0.15, **Table S8)**.

To explore these differences in more detail, we focused on genes involved in the interferon alpha pathway (viral-associated response) or inflammatory response (bacterial-associated response). In AAMs, inflammatory response genes tended to be more strongly up-regulated in response to LPS compared to GARD (*P*≤ 0.0027), suggesting that their transcription is particularly sensitive to receipt of a bacterial danger signal compared to a viral one. No significant differences in upregulation of these same genes were noted between LPS and GARD cells in apes () (**Figure 5C**). For example, the canonical pro-inflammatory cytokine *TNF*, which in macaques and baboons is strongly up-regulated only in response to LPS, is potently up-regulated in response to both stimuli in humans and chimpanzees (by over ~4-fold, **Figure 5D**). Other examples of this pattern include the classical pro-inflammatory cytokines *IL1A* and *IL1B* (**Figure S4**). Likewise, interferon-associated genes were more strongly up-regulated in response to GARD compared to LPS in AAM ($P<=7.7 \times 10^{-6}$), while in apes these genes showed more concordant levels of up-regulation between stimuli (**Figure 5C**). Interferon-induced and potent antiviral genes, including *MX1* and *OAS1*, were much more strongly upregulated in response to GARD than to LPS in AAMs compared to apes (**Figure S4**).

Figure 5. Apes engage a less specific innate immune response than AAMs. (A) Correlation plots of the magnitude of the fold change responses between LPS (x-axis) and GARD-stimulated cells (y-axis). For each of the species, we only include genes that were differentially expressed (FDR < 0.05) in response to at least one of the stimuli (N= 7862, 7874, 6585 and 5430 genes for human, chimp, baboon and macaque, respectively). High correlation was found in apes (~ 0.85) while modest correlation was found in baboon (0.44) and moderate in macaque (0.65). (B) Proportion of ligand-specific (i.e., genes that respond uniquely to either bacterial or viral stimuli) and shared genes (i.e., genes equally activated by both immune stimuli) across species. (C) Violin plots comparing (scaled) log2 fold-change responses to 4 hours of LPS and GARD stimulation between genes belonging the hallmark pathways "Interferon (IFN) alpha" and "inflammatory response". The p-values shown have been Bonferroni corrected for the number of tests performed. "NS" stands for non-significant (i.e., p value > 0.05) (D) Boxplots of the log2 fold change response (y-axis) of TNF in response to LPS and GARD stimulations across primates.

Discussion

Our study provides a genome-wide functional comparison of variation in innate immune responses between species belonging to two closely related clades of primates. Ape (human and chimpanzee) total blood leukocytes were significantly more responsive to bacterial and viral stimulation compared to total blood leukocytes obtained from AAM (rhesus macaques and baboons) during the early hours of challenge, mounting generally stronger and less specific transcriptional responses. This increased response suggests apes maintain increased sensitivity to particular types of microbial assaults compared to AAM, a phenomenon likely to come with considerable energetic cost (Redl et al, 1993; Vaure and Liu, 2014). From an evolutionary standpoint investment in increased sensitivity to pathogens can limit the negative effects of pathogen exposure on reproductive fitness. Humans and chimpanzees participate in a comparatively slower life history than rhesus macaque and olive baboon monkeys – they live decades longer, take longer to reach sexual maturity, nurture their young longer and maintain a larger body size (Harvey and Clutton-Brock, 1985; Ross, 1992; Wich et al, 2004). A long life at a large size increases risk of pathogen exposure both in terms of number of exposures and absolute load, over the course of a life that will have long periods of time between the birth of offspring. A slow life history strategy can be concomitant with and increase risk in pathogen-mediated limitations in reproductive success, making a more substantial investment in robust early pathogen detection and elimination evolutionarily beneficial, compared to the ordinary metabolic costs of launching those responses (Johnson et al, 2012; Cronin et al, 2010).

However, serious bystander tissue damage is a cost for immune protection during severe infections. Pathogen virulence may play a significant role in the evolution of high energy low specificity early immune responses. The primate genera in this study substantially differ in their evolutionary exposure to particular pathogens (e.g. dengue virus, immunodeficiency viruses, Zika virus) (Buechler et al, 2017; Gao et al, 1999; Hirsch et al, 1989; Vasilakis et al, 2011). Exposure to pathogens of high virulence may lead to a low cost-benefit ratio for primate hosts, since the

reproductive and evolutionary benefit of a transiently demanding immune response outweighs its energetic and tissue costs (Okin and Medzhitov, 2012; Sorci et al, 2013). Under this rubric, a robust but less specific early response to pathogens is effective and beneficial most of the time. Any contribution that response might make to immunopathology in apes through potentially increased risk of sepsis or chronic inflammatory disease is evolutionarily negligible compared to the persistent risk of infection. Interestingly, among the most divergently responding pathways between apes and AAMs, several were associated with the regulation of interferon responses and responses to viruses. These findings are consistent with growing body of literature that pathogens and, specifically, viruses have been important drivers of adaptive evolution in humans and other mammals (van der Lee et al, 2017; Ito et al, 2020; Enard et al, 2016; Harrison et al, 2019).

Regardless of initial strength and divergence of transcriptional response to LPS and GARD, we show that the transcriptional activity of antiviral (interferon) and inflammatory pathways became attenuated over time and more similar between species. While acute-phase and early proinflammatory responses are typically later countered by a later anti-inflammatory response to lessen host damage and maintain homeostasis, the dampening of this initial powerful antimicrobial response over time, is profound (Morris et al, 2014). Remarkably, in apes the pathways that underwent the most pronounced attenuation after 24h tended to be ones not expected to be strongly engaged in the response to the pathogen type in the experiment. For instance, the typically antiviral type I IFN pathway response was found to be markedly reduced in apes after 24 hours of bacterial but not viral stimulation. While the initial response of apes to immune stimulus is very strong, temporal regulation of responding pathways may reduce the energetic costs of such an immune strategy. What gene regulatory and immunological mechanisms are involved in such temporal regulation will require further investigation.

In conclusion, we show initial antibacterial and antiviral responses of apes to be highly correlated, and strongly responsive when compared to close relatives African and Asian monkeys. Apes appear to have adopted an immune strategy that emphasizes sterilization over specificity, strongly transcribing a greater number of genes in response to immune stimulation and releasing very

similar immune transcriptomic "arsenals" regardless of pathogen-type. This powerful response dramatically shifts during the opening hours of infection, to involve significantly fewer genes after 24 hours, which may help limit bystander tissue damage. The energetically costly approach apes initiate in response to immune stimulation may be favored by this primate family's adoption of slower life history with increased risk of pathogen exposure over reproductive life span, or past pathogen exposure. The addition of more primate species, combined with the use of single-cell RNA sequencing methods are important next steps to study the evolution of the immune system and more precisely map the immune cell types that contribute the most to divergence in immune response across primates.

Materials and methods

Sample collection and blood stimulation

We measured innate immune responses on a panel of 6 humans, 6 chimpanzees, 6 rhesus macaques, and 8 olive baboons (three females and 3 males for each species, 4 females, 4 males for baboon). Human samples were obtained via informed consent, with the approval of the Research Ethics Board at the Centre Hospitalier Universitaire Sainte-Justine (Research Ethics Board approved protocol #3557). Non-human primate blood samples were humanely collected in accordance with the animal subject regulatory standards of the Texas Biomedical Research Institute and Emory University Institutional Animal Care and Use Committees. Chimpanzee samples were collected prior to the NIH ban on chimpanzee research.

We drew 1 mL of whole blood from each animal directly into a TruCulture tube (Myriad RBM) that contained: (i) cell culture media only ("control"), (ii) cell culture media plus 1 μg/mL ultra-pure LPS from the *E. coli* 0111:B4 strain ("LPS"),or (iii) cell culture media plus 1 μg/mL of Gardiquimod ("GARD"). Samples were incubated for 4 and 24 hours at 37°C. Following incubation, we separated the plasma and cellular fractions centrifugation, and lysed and discarded the red cells from the remaining cell pellet by applying red blood cell lysis buffer (RBC lysis solution, 5 Prime Inc.) for 10 minutes followed by centrifugation and washing with 1x PBS. The remaining white blood cells were lysed in Qiazol and frozen at -80C until library construction

(Qiagen, San Diego, CA, USA). To control for variation in cellular composition in downstream analyses, we used flow cytometry to quantify the proportions of leukocyte subtypes, accounting for polymorphonuclear (CD14dim/SSC-A>100K/FSC-A>100K/CD66+), classical monocytes (CD14+/CD16-), CD14+ intermediate monocytes (CD14+/CD16+), CD14- non- classical monocytes (CD14-/CD16+), helper T cells (CD3+/CD4+), cytotoxic T cells (CD3+/CD8+), double positive T cells (CD3+/CD4+/CD8+), CD8- B cells (CD3-/CD20+/CD8-), CD8+ B cells (CD3-/CD20+/CD8+), natural killer T lymphocytes (CD3+/CD16+), and natural killer cells (for monkeys: CD3-/CD16+ in the lymphocyte scatter, for apes: CD3-/CD16+/CD56+ in the lymphocyte scatter) Samples for FACS were simultaneously cleared of red blood cells vis lysis and fixed by application of BD FACS-lyse for 2 minutes, prior to washing with 1x PBS, staining with fluorochrome conjugated monoclonal antibodies (**Table S10**), before washing with 1x PBS and suspending in a 1x PBS and paraformadelhyde solution for analysis on the BD LSRFortessa platforms. Proportional analysis was completed in FlowJo X, using BD FACSBeads individually stained with the antibodies to calculate compensation.

RNA-seq data generation

Library construction. Total RNA was isolated from cell lysate by phenol::chloroform extraction and spin-column (miRNAeasy kit, Qiagen, San Diego, CA, USA), quantified by spectrophotometry and assessed for quality using the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alta, CA). Samples with no evidence of RNA degradation (Integrity number >8) were then used for RNA library development. Messenger RNA (mRNA) was isolated by magnetic bead and converted into RNA libraries using the Illumina TruSeq RNA Library preparation kit v2 according to the manufacturer's instructions (Illumina, San Diego, CA, USA). Libraries were sequenced on a HiSeq 2100, producing 151 transcriptomes, at 25-30 million reads per sample

Reads mapping on 1:1 orthologs

Following sequencing, we trimmed Illumina adapter sequence from the ends of the reads and remove bases with quality scores < 20 using Trim Galore (v0.2.7). We used STAR to align the reads to an orthologous reference genome for all four species (Dobin et al, 2013). We developed

this genome using the XSAnno pipeline which combines whole genome alignment, local alignment and multiple filters to remove regions with difference in mappability between species (Zhu et al, 2014). XSAnno pipeline identifies orthologous genes across two species using three major filters namely LiftOver to carry annotation of one species over the other, BLAT aligner to compare orthologous exons identity between the two species and simNGS to identify exons that have different lengths between the species. We used the genome assemblies of hg19 for human, CHIMP2.1.4 for chimpanzee, MMUL 1.0 for macaque and PapAnu2.0 for olive baboon species. We used human annotation as a reference. The pairwise alignment chains between human and each species were obtained from UCSC genome browser (Kent et al, 2002). We used different thresholds to define orthologous regions between the two genomes to carry annotation from one species to another using AnnoConvert program that utilize LiftOver according to simulations using liftOverBlockSim PERL script from XSAnno pipeline (Hinrichs et al, 2006). The values were 0.98, 0.92 and 0.91 for chimp, baboon and macaque respectively that were used to assign -minMatch argument in AnnoConvert. Second step is using reciprocal whole genome alignment using BLAT through BlatFilter software of the pipeline using annotations files generated previously (Kent, 2002). This step will filter exons that are highly divergent between the two species. The last filter is using simNGS to simulate reads for exons assuming they are not differentially expressed. Then, differential expression analysis is performed and if exons were found to be differentially expressed, these will be filtered out as it reflects differential length of the exons between species.

Gene expression estimates were obtained by summing the number of reads that mapped uniquely to each species annotated genome using HTSeq-count (v0.6.1) (Anders et al, 2015). After excluding samples that did not produce sequenceable libraries and post-sequencing quality control, we analyzed read counts for 151 samples (Humans: 12 controls, 12 LPS, 12 GARD; Chimpanzee: 12 controls, 12 LPS, 12 GARD; Rhesus: 11 controls, 12 LPS, 11 GARD; Baboons: 16 controls, 14 LPS, 15 GARD; **Table S1**). We confirmed the identity of all samples based on genotype information derived from SNP calls made from the RNA-seq reads.

Read normalization and filtering lowly expressed genes

Prior to RNA-seq data analysis, we first filtered out genes that were very lowly or not detectably expressed in our samples. Specifically, we only kept genes whose expression was higher or equal to one count per million (CPM) in all the individuals from at least one species, and one of the experimental conditions. This procedure yielded a total of 12,441 genes used for further analysis. Normalization for sequencing depth and library sizes was done using Trimmed Mean of M-values (TMM) method (Robinson and Oshlack, 2010). We normalized the resulting read count matrix using the function *voom* from the R package limma to allow using linear models by limma package (Law et al, 2014). The voom algorithm models mean-variance trend of logCPM for each gene and uses it to compute the variance as a weight of logCPM values. We then modeled the normalized expression values as a function of the different experimental factors in the study design such as species, ligand and time points.

Statistical analysis

All statistical analysis was done on R version 3.6.2. Differential expression analysis was done using limma package v.3.34.9 (Ritchie et al, 2015). We employed linear regression to identify DEGs according to different questions asked by designing different models. We designated a model to test for differences of gene expression across species and treatments, ~ covariates + species + species:Time.point.stimulant. The arm Time.point.stimulant is the samples for each experimental condition i.e. LPS.4h, LPS.24h, GARD.4h and GARD.24h. From this design, one can retrieve ligand responses in each species right away, while responses to ligands at 24h are built from linear combinations, such us (LPS.24h -NC.24h) and (GARD.24h -NC.24h). To take into account the paired structure of the data, with different samples coming from the same individuals, we used the duplicateCorrelation function. The used covariates are the different cell proportions collected by the FACS data. The cell proportions covariates are aimed to correct for the different proportion of white blood cells in different primate species since we conducted the transcriptomic characterization on all immune white blood cells. Genes with different magnitude of response between clades, referred to as clade differentially responsive (c-DR) genes were characterized. We established two filters to characterize significant c-DR genes in each treatment. Firstly, we required the genes not to be differentially responsive to the treatment, even marginally, between within clade species pairs (chimp vs human and baboon vs macaque showing FDR>0.25). Second, we required that any pairwise comparison involving species from different clades to be significant at

FDR<0.1. Third, we also computed the average differences in reponses between apes and AAMs, as follows: the absolute difference between the average response in apes vs AAMs, as follows:

$$\frac{logFC.human + logFC.chimp}{2} - \frac{logFC.macaque + logFC.baboon}{2}$$

And required that contrast to be significant at FDR<0.1, with genes featuring

$$\frac{|logFC.human + logFC.chimp|}{2} - \frac{|logFC.macaque + logFC.baboon|}{2} > 0.5$$

being labeled ape-specific; and AAM-specific for those for which:

$$\frac{|logFC.human + logFC.chimp|}{2} - \frac{|logFC.macaque + logFC.baboon|}{2} < (-0.5)$$

Species-specific differentially responsive (s-DR) genes were identified using pairwise comparisons at FDR < 0.01; consistent direction of expression in all contrasts, and systematic differences corresponding to stronger, or weaker responses in the species of interest with respect to the any of the other three. Finally, we also required genes to show a logFC in response to the stimulus whose absolute differs in more than 1 log2FC with respect to the average of the other three animals. For humans, as an example, this means that:

$$\left| logFC.human - \frac{logFC.macaque + logFC.baboon + logFC.chimp}{3} \right| > 0.5$$

Ligand specific genes in each species are genes that are respond to one ligand (FDR<0.05), but not to the other (FDR>0.25); and whose responses to both ligands are in turn significantly different (FDR<0.05). Shared genes are those whose responses to ligands are both significant (FDR<0.05 in both), and, at the same time, not significantly different between them (FDR>0.25)

Correction of multiple testing was done using false discovery rate, FDR, as described by Benjamini-Hochberg (Benjamini and Hochberg, 1995).

Divergence scores

For each time-point and stimulus, species were compared pairwise to retrieve the absolute differences between species′ responses to the stimulus under analysis. For the pair chimp vs human, for example, we can define:

$$\delta_{human.chimp} = |logFC.human - logFC.chimp|$$

Comparing these differences for pairs of animals within versus across clades, we obtained divergence scores as follows:

$$DS = \frac{\delta_{human.macaque} + \delta_{human.baboon} + \delta_{chimp.macaque} + \delta_{chimp.baboon}}{4}$$
$$- \frac{\delta_{human.chimp} + \delta_{macaque.baboon}}{2}$$

The analysis was conducted for all 50 hallmark pathways. We restrict the analysis in a given pathway to responsive genes (FDR < 0.05 in any species), whose average DS is reported. A p value for each DS of a given pathway was calculated by contrasting the DSs of genes of this specific pathway against the DSs of all responsive genes using Wilcoxon test.

Functional characterization

We conducted the functional characterization using gene ontology (GO) enrichment implemented in CluGO application (2.5.5) of Cytoscape (v.3.7.2) (Bindea et al, 2009). Benjamini-Hochberg

method for multiple correction was used and all orthologous genes, 12441 genes, were used as a background. Default values were used for the rest of the parameters. FDR cut off use was below 0.15.

Data Availability

The RNA-seq data generated in this study have been deposited in Gene Expression Omnibus (accession number GSE155918).
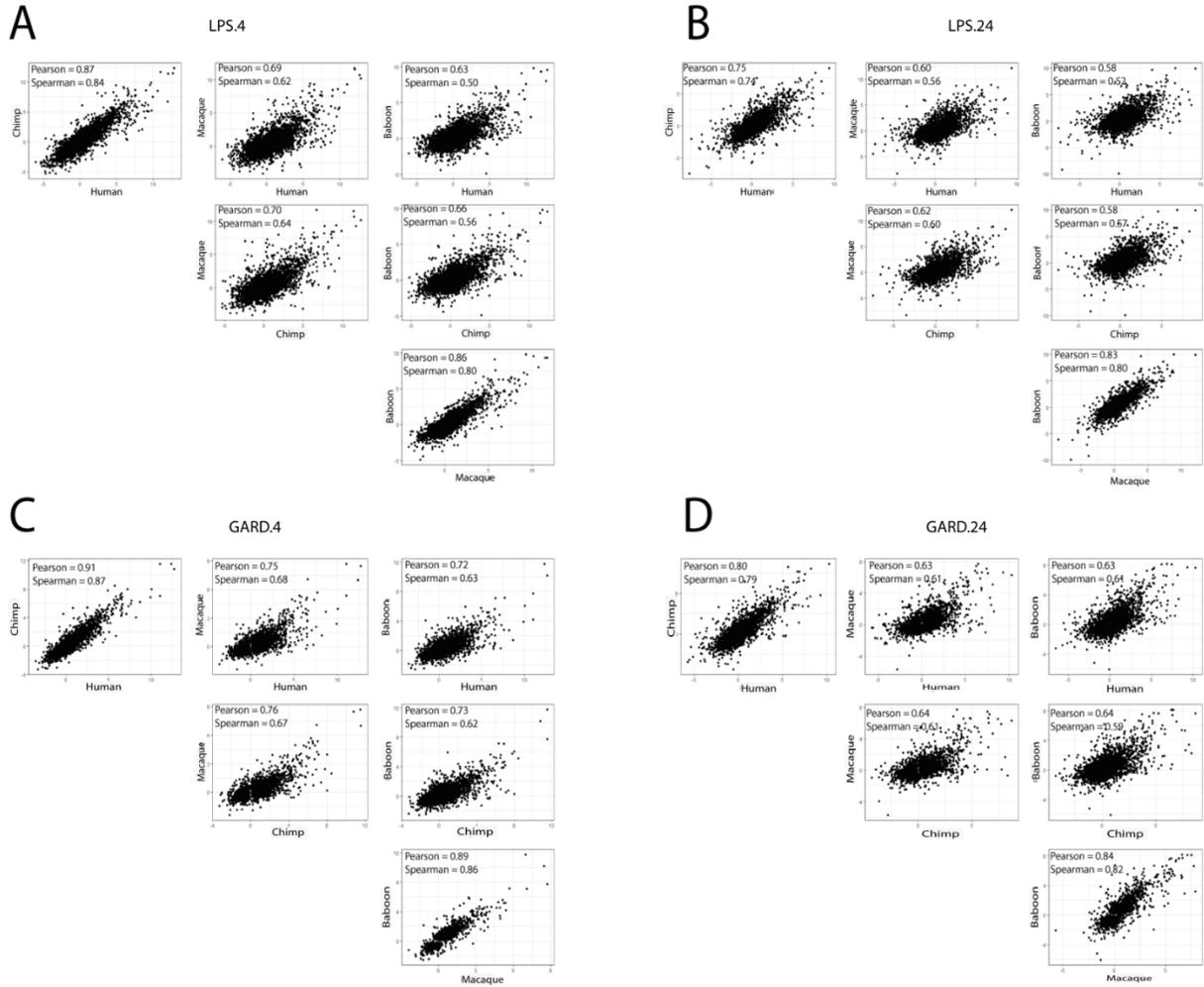
Author Contributions

L.B.B. and J.F.B. designed research, M.B.F.H., J.F.B., J.K., J.S., J.C.G., Y.V., and L.B.B. performed the research, M.B.F.H., J.C.G., J.F.B. and L.B.B. analyzed the data, M.B.F.H., J.F.B. and L.B.B. wrote the paper with contributions from all authors.

Acknowledgements

Supplementary Figures

Supplementary figure S1. Correlation of immune response across species. Correlation across species between fold-change (log2 scale) responses among genes that are either significant in LPS treatment at 4 and 24h (A and B respectively) or GARD treatment at 4 and 24h (C and D respectively) at FDR < 0.05 in at least one of the species studied. Spearman and Pearson correlation coefficients are indicated on each plot.
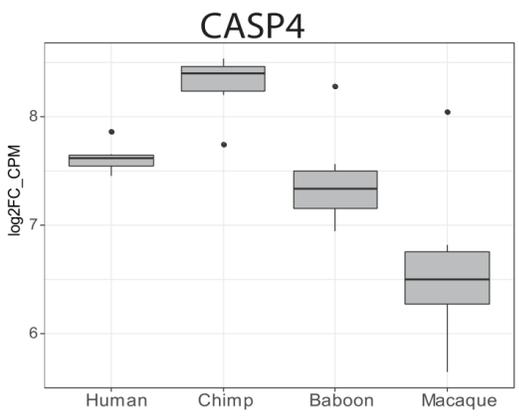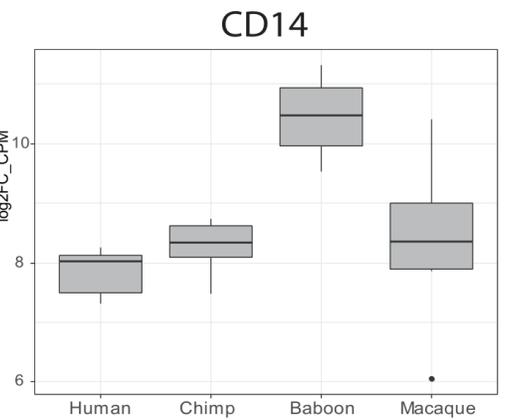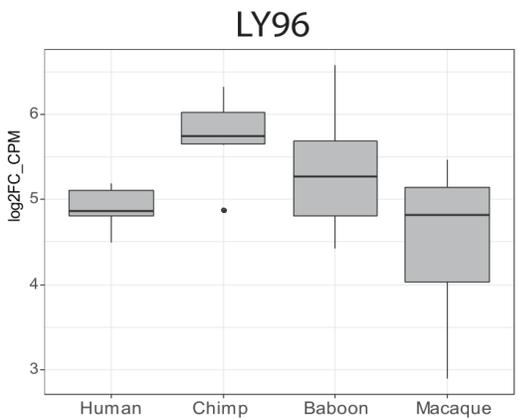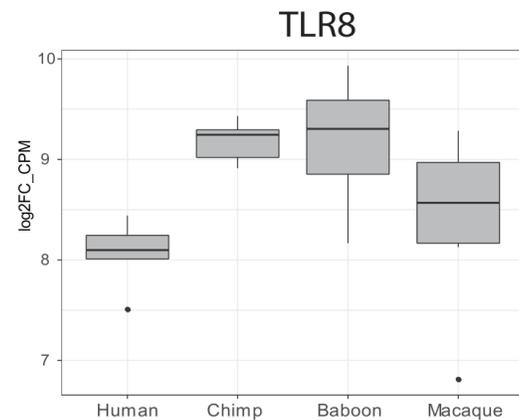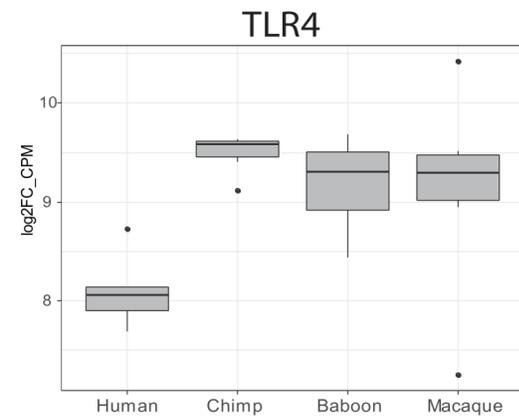
Supplementary figure S2. Expression of key sensors for LPS and GARD in all species.
Boxplots representing expression values across species (log2 count per million; log2CPM) of
key sensors involved in the recognition of the ligands used in this study, LPS and GARD.
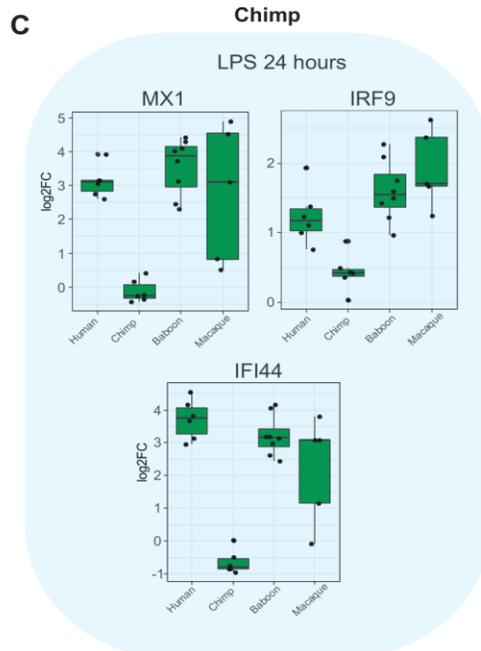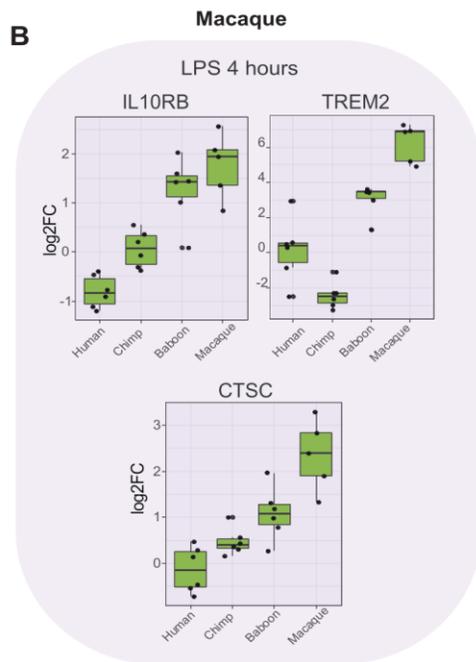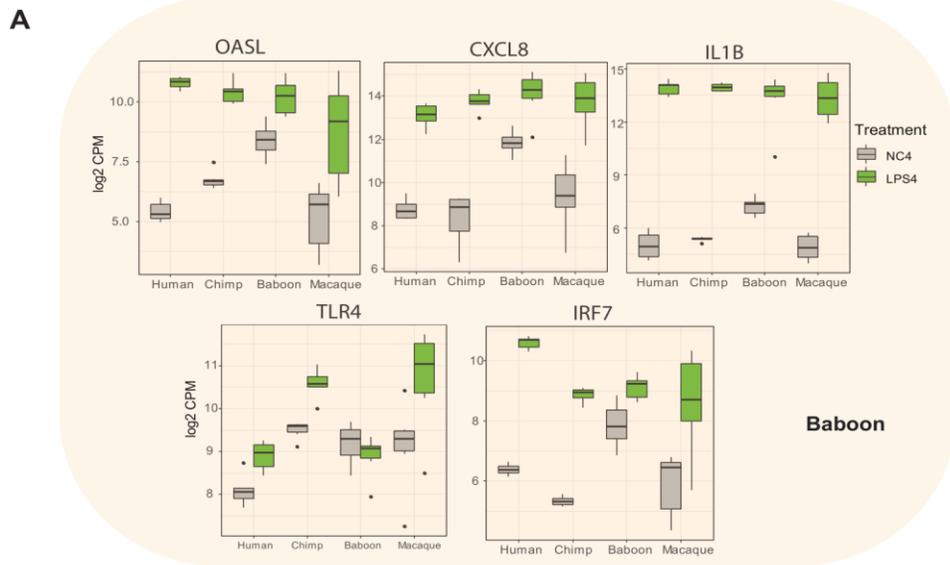
Supplementary figure S3. Examples of species-specific immune response. (A) Example of baboon-specific genes. The boxplots represent the expression values across species in non-stimulated cells (gray) and cells stimulated with LPS for 4 hours (green). In all cases showed herein baboons show a weaker response to LPS compared to all other species, which is primarily due to an increased baseline expression of these genes. **(B)** Example of rhesus-specific genes at 4 hours post LPS stimulation. **(C)** Example of chimpanzee-specific genes at 24 hours post LPS stimulation.
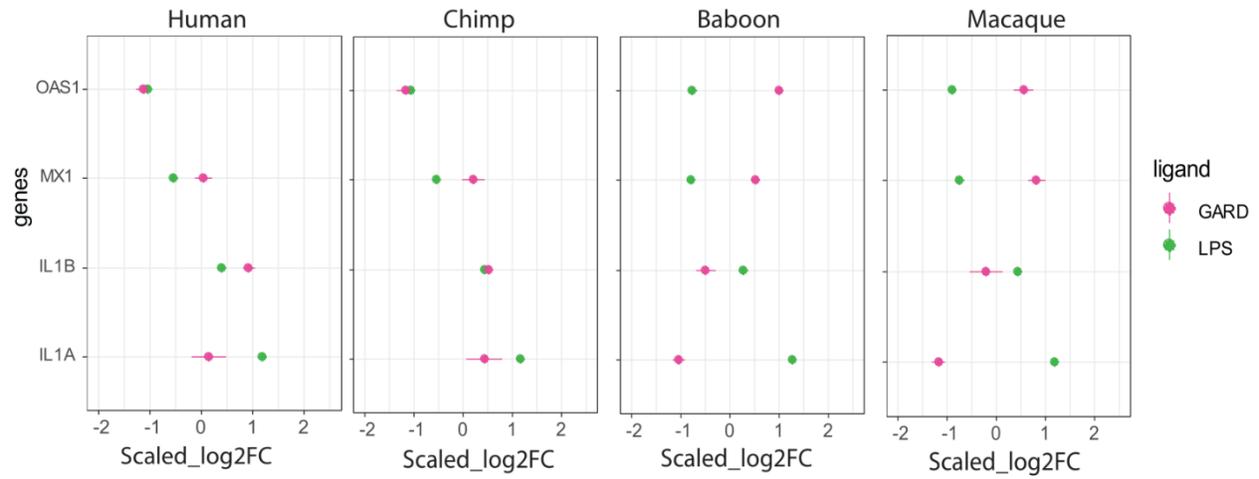
Supplementary figure S4. Scaled log2FC of number of key innate immune genes that showed distinct response to bacterial or viral ligands in monkeys vs apes at 4h time point.

3. Article II

Adaptive evolution shaped interspecies differences in immune response across primates.

Hawash MBF, Dumaine A, Lanford RE, Kohn J, Barreiro L

Manuscript in preparation

Adaptive evolution shaped interspecies differences of immune response across primates.

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Note: Due to the length of the supplementary tables associated with this study, they are provided separately in the following link
(https://www.dropbox.com/home/Article%20II%20supp%20tables)

Abstract

Humans are more prone to inflammatory and infectious diseases than other primate species such as macaques and baboons. These differences, at least partially, are expected to result from interspecies differences of immune response. How the innate immune response from these species is functioning and diverges across different immune cell populations remains poorly understood. Moreover, the regulatory elements that dictate the response across these species have not yet been characterized. Herein, we characterized immune response and its regulatory landscape from human, rhesus macaque, olive baboon and one basal primate, ring-tailed lemur upon stimulation with lipopolysaccharide (LPS) at single cell resolution. We found that immune response strength is correlated with the primate phylogeny, the highest in human and the lowest in lemur with monocytes being the most responsive immune cells across species. Also, the regulatory landscape was found to change significantly only in simian primates after stimulation suggesting a major role of epigenetics in directing the immune response across primates. Finally, we found of signature of adaptive evolution on active enhancers associated with differentially responsive genes between humans and monkeys, highlighting the role of natural selection on directing the immune response divergence in primates.

Introduction

Primates vary in their disease susceptibility to several pathogens and inflammatory disease manifestations (Mandl et al, 2008; Chahroudi et al, 2012; Chen et al, 2019). For instance, humans are known to be prone to immunopathology caused by very low concentrations (2-4 μg/kg) of lipopolysaccharide (LPS), a pathogen mimicry molecule for gram-negative bacteria unlike African and Asian monkeys (AAMs) such as macaque and baboon that do not develop pathological manifestation except on 10-fold higher doses (Redl et al, 1993; Warren et al, 2010; Vaure and Liu, 2014). The high sensitivity of human to stimulation and the superfluous immune response causes severe mortality and morbidity in human as evidenced by the fatality rate due to septic shock (Delano and Ward, 2016).

The advent of single cell technology has revolutionized many fields of biology including evolutionary medicine (Shafer, 2019; Liu et al, 2021). Exploring unique traits and their underlying biological mechanisms at a high-resolution cellular level has become feasible for non-model species using single cell sequencing (Hilton et al, 2019; Ren et al, 2020). Moreover, disentangling the heterogeneity of complex tissue and characterizing rare cellular populations involved in core physiological processes in model and non-model species had also been studied using single cell technologies (Crinier et al, 2018; Tosches et al, 2018). Peripheral blood mononuclear cells (PBMCs) are key immune cells in fighting pathogens and are a complex system that involves several specialized cell types (Sen et al, 2017). Whether PBMCs cellular heterogeneity across primates that show different disease susceptibility is playing role in innate immune response has not been investigated. Also, the regulatory landscape that controls the immune response and the evolutionary forces that shaped the differences in disease susceptibility across primates is unknown. Herein, we characterized the transcriptomic and epigenetic landscape of immune response of PBMCs at single cell level from primates that show different LPS sensitivity namely human, macaque, baboon and lemur. We found immune response strength to be correlated with the primate phylogeny (highest in human, lowest in lemur) and monocytes to be the major responsive cells in all primates. We found a signature of adaptive evolution that is associated with the strong immune response in human suggesting a role of natural selection in directing the immune response divergence in primates.

Results

PBMC heterogeneity across primates identifies novel subtypes of immune cells in primates.

We characterized scRNA-seq using 10x Genomics platform from unstimulated (RNAase-free water treated) and stimulated (LPS-treated) PBMC from four primate species namely, human (*Homo sapiens*), rhesus macaque (*Macaca mulatta*), olive baboon (*Papio anubis*) and ring-tailed lemur (*Lemur catta*). PBMC were included from 6 individuals from each species (Figure 1A). Total of 17393, 16364, 15960 and 14328 cells from human, macaque, baboon and lemur were included respectively after filtering out low quality cells and potential doublets (see methods). *Lemur catta* annotated genome is not available in any database. We used the genome of mouse lemur in Ensembl as a proxy after comparison with the other genome of lemurs, the greater bamboo lemur (see Supplementary note 1).

We first investigated the natural heterogeneity of PBMC (prior to stimulation) across the primates. We integrated unstimulated cells (n= 9110, 8440, 8391 and 6982 from human, macaque, baboon and lemur respectively) using Seurat (Butler et al, 2018) followed by UMAP clustering. UMAP clustering of the integrated object distinguished the major clusters of the PBMC which were found to be generally conserved in all primates (Figure 1B). For instance, canonical markers for monocytes, B cells, cytotoxic and T cells such as CD14, MS4A1, NKG7 and CD3E markers respectively identified the different clusters of PBMC which were found in all species (Figure 1C; Supplementary figure S1). However, the clustering identified a potential subcluster in B cells which may represent a subtype of B cells. We calculated the relative proportion of the number of cells in each cluster relative to the total number of PBMCs per individual in each species (Figure 1D). We found that this subtype of B cells of significantly higher proportion in monkeys than others (p < 0.0035, Wilcoxon test) suggesting this B cell subtype to be specific to these species (henceforth, will be referred to as Bcell_II). We identified the genes that show higher expression in Bcell_II compared to all other PBMC cells, i.e., the expression module of this cluster, using the function "FindMarkers" in Seurat package (257 genes) (Supplementary table 1). We found a marker, T Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), to be highly expressed in this cluster specifically in macaque and baboon cells (Supplementary figure 1). TIGIT is a marker of T cells which act as an inhibitory coreceptor (Manieri et al, 2017) indicating Bcell_II to be a

unique type of B cells. CD63 was also found in Bcell_II gene expression module which is a member of tetraspanins family and is known to be highly expressed in endosomes and lysosomes to mediate endocytosis (Pols and Klumperman, 2009). CD63 is also highly expressed in antigen presenting cells (APC) such as monocytes and dendritic cells to mediate phagocytosis for foreign cells. Expression of CD63 across species PBMC identified CD63 to have the highest expression in monocytes, dendritic cells than in Bcell_II especially in monkey cells (Supplementary fig 1). To find the closest cluster to Bcell_II, we did enrichment analysis using Bcell_II gene expression module against all PBMC using VISION (DeTomaso et al, 2019). We found monocytes and dendritic cells to be the closest clusters to Bcell_II followed by Bcell_I as indicated by the area under the curve (AUC) value (Supplementary figure S2; Table S1) suggesting Bcell_II to have similarity to professional APC in monkeys. Consistently, functional characterization of Bcell_II expression module resulted in multiple immune related GO terms, in particular among the top enriched GO terms is "antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent" (Figure 1E; Table S1), which further emphasize on its capacity as APC. Similar analysis of gene expression module of Bcell_I did lead to similar results, showing that APC potential is specific to Bcell_II (Supplementary figure S2; Table S1).

Next, we investigated the most variable genes across all species (within each cluster) to shed light on the internal heterogeneity and highly variable genes across species. For instance, we found S100A8 to be the most variable in monocytes (Supplementary figure S3). Expression of S100A8 was found to be the highest in human than macaque and nearly no expression in baboon and lemur (Figure 1F). S100A8 is a cytoplasmic protein constitutively expressed in monocytes and neutrophils and released during inflammation. S100A8 further enhances the inflammatory response by recruiting leukocytes and enhancing cytokine secretion. Given its key role in potentiating the inflammation, S100A8 has been intensively studied as a therapeutic target for inflammatory disease (Wang et al, 2018). Thus, the high expression of S100A8 in human is in line with the current notion that humans are highly susceptible to inflammatory disease. Among the genes that were highly variable as well are cytotoxic genes such as NKG7, PRF1 and granzymes in the cytotoxic cells, CD8 T cells and NK (Supplementary figure S3). Cytotoxic cells are aimed mainly to kill intracellular pathogens or infected cells through effectors that are stored in cytolytic granules inside these cells. These granules contain perforin-1 (PRF1), Fas-ligand

(FASLG), granzymes such as GZMA/B and NKG7 which all function in the lysis of targeted cells upon activation (Halle et al, 2017; Golstein and Griffiths, 2018; Ng et al, 2020). Expression of NKG7 gene was found to have gradual higher expression in NK cells from lemur (lowest) to human (highest). However, we found that humans showed the lowest expression of all cytotoxic genes in CD8 T cell (Figure 1F; Supplementary figure S3). To better explore the heterogeneity in these clusters, we extracted cells from Cytotoxic CD8 T cell cluster and performed UMAP clustering. We found a subcluster of CD8 T cell in human that did not express any cytotoxic genes, naïve CD8 T cell, unlike other primate cells suggesting naïve CD8 T cell to be circulating with PBMC in human while all CD8 T cells from other primates are functional cytotoxic cells (Figure 1G).

Taken together, we found a heterogeneity of PBMC across primates as evidenced by the distinct PBMC clusters peculiar to specific species which may be involved in species specific defense mechanisms.
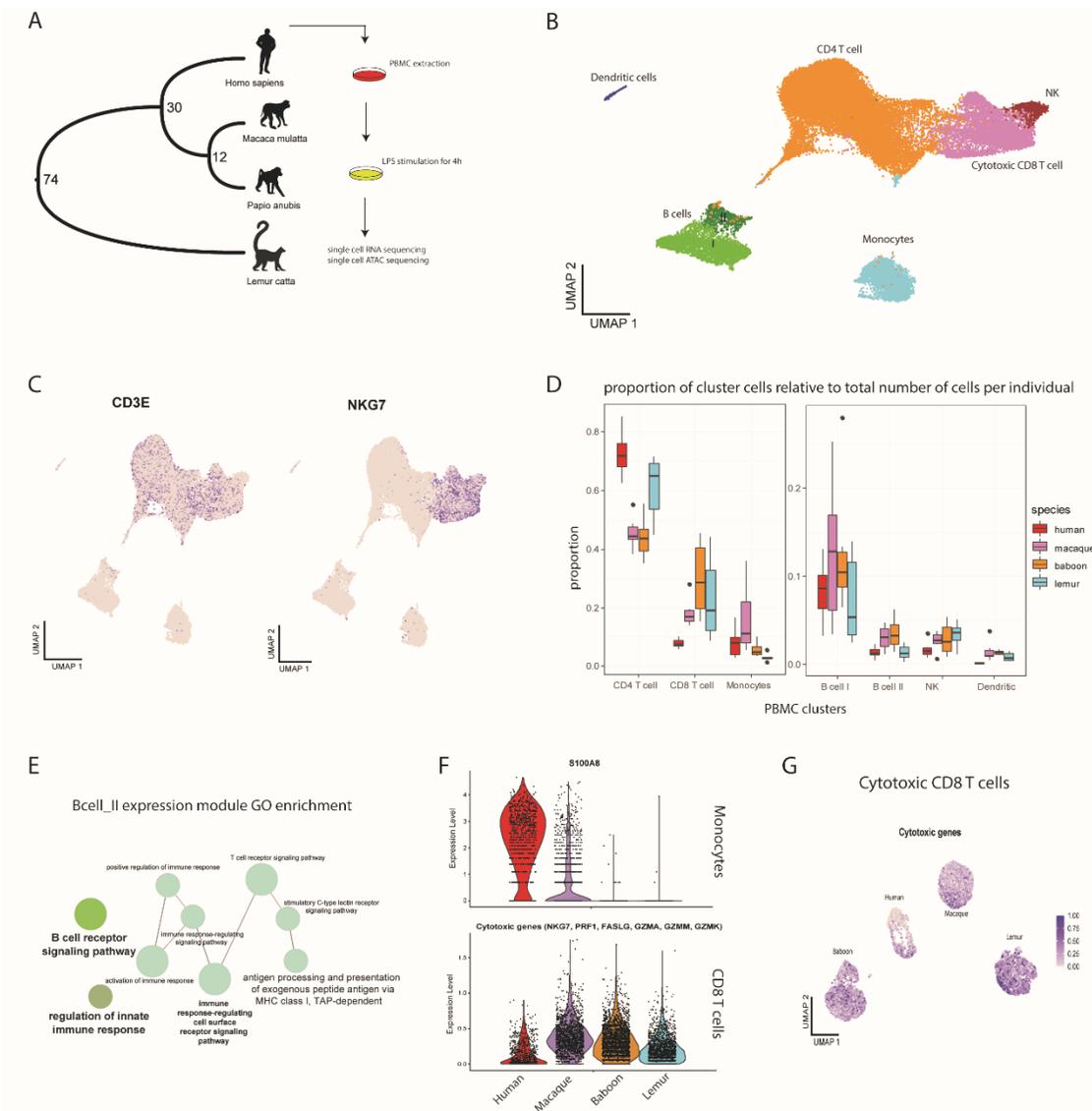
Figure 1: Primate's PBMC heterogeneity identifies novel subtypes of immune cells in primates. A. Study design with phylogenetic tree of species. Time to the most recent common ancestor is indicated on tree nodes. PBMCs from primate species is extracted and stimulated with lipopolysaccharide (LPS) for hours followed by single cell RNA and ATAC sequencing of stimulated and unstimulated cells. **B**. UMAP clustering of all PBMCs from all species after integration of unstimulated condition (NC). B cells were found to be clustered into two main clusters referred as Bcell_I and Bcell_II **C**. Examples of markers that was used to annotate all the PBMC clusters from primates and more examples are shown in supplementary figure S1. **D.** Boxplots represents the proportion of cells in each PBMC cluster relative to total number of cells per individual. Monkeys were found to have higher proportion of Bcell_II than others. **E.** Significant GO terms of Bcell_II cluster (dark green colored cluster in panel B) expression module (FDR < 0.15). **F**. Examples of genes with high variability across species in specific

clusters (shown in Supplementary figure S3), S100A8 in Monocytes and cytotoxic genes (NKG7, PRF1, FASLG and grandzymes) expression in cytotoxic T cells. **G**. UMAP clustering of cells from cluster Cytotoxic CD8 T cell (colored purple in panel B) without integration.

Immune response strength correlates with primate phylogeny.

Next, we studied the immune response upon LPS stimulation in all four primate species. We first did integration across treatments (NC, LPS) per species to annotate stimulated cells using the annotation of unstimulated cells in the previous step (Supplementary figure S4). Integration of the two groups, stimulated and unstimulated samples, will cluster all cells of the same PBMC population regardless of the treatment. Cell counts before and after stimulation did not significantly change when checked. We then used linear models to identify responding genes in each species in PBMC clusters after pseudobulking of major PBMC clusters, i.e., transforming gene-cell count matrix to gene-individual count matrix (see methods). We did pseudobulking for all 5 major PBMC clusters for each treatment namely, monocytes, CD4 T cell, cytotoxic cells (cytotoxic T cells and NK), B cell and dendritic cells, in addition to one matrix that has all PBMCs. We first implemented a simple model which is individual + treatment to identify differentially expressed genes (DEGs) in each cluster at FDR < 0.05. Monocytes followed by CD4 T cells have the highest number of DEGs in all species except for lemur which is monocytes then cytotoxic cells (Figure 2A; Supplementary table S2).

Then, we estimated the total strength of immune response across species (total log2FC values of all DEGs in any species) in each cluster and in all PBMCs. We found that the strength of immune response in total PBMCs correlated with primate phylogeny, the strongest immune response was observed in the higher primate (human) and the weakest response in the basal primate species (lemur). Monkey species (macaque and baboon) showed an intermediate response (Figure 2B). This pattern was also observed for specific cells within PBMC including the cell types most responsive to LPS, monocytes and CD4 T cells (Supplementary figure S5). Several traits are also correlated with primate evolution such as body mass and life span (Kamilar and Cooper, 2013; Vining and Nunn, 2016), which may suggest an association of other traits with immune response evolution.

Next, we aimed to explore functionally the immune response across species and clusters. All PBMC clusters activate an immune response. We first identified shared or specific DEGs among different clusters in each species (Supplementary figure S6). As expected, monocytes and CD4 T cells have the highest number of specific genes in all species. Only a few genes were shared among all clusters in all species (36, 48, 32, 1 in human, macaque, baboon, lemur respectively). GO enrichment of these genes resulted in a very strong enrichment of viral GO terms in all species except lemur (Supplementary Table S3). We also estimated specificity of response across clusters in each species using a more detailed quantitative approach by "specificity index". We used a modified formula of specificity index from what was used previously on gene expression (Tosches et al, 2018). Briefly, specificity index measures the overall distinction of response across clusters by values ranging from 0 to 1 where values closer to 0 mean lower specificity (genes responding in the majority of clusters) and greater values mean higher specificity (see methods). We measured the specificity index of response for each species followed by gene set enrichment analysis (GSEA) using hallmark pathways (Liberzon et al, 2015). We found Type I interferon pathway to be a conserved response in all species from all PBMCs, except lemurs (Figure 2C). Other pathways were found to have high specificity index (FDR < 0.15) such as TNFA signaling in human and IL6 JAK-STAT signaling in macaque (Supplementary Tables S4) where the signal mainly come from monocytes. Lemurs showed a lower total number of shared genes responding in all clusters suggesting evolution of shared response across PBMC clusters occurs later in primate evolution. For instance, ISG20, a type I IFN gene, was activated in all PBMC clusters in simian primates (human, macaque and baboon) while it was only activated in dendritic and specific T cell cluster in lemurs (Figure 2D).

Next, we explored the immune response differences across species. Since monocytes and CD4 T cells are the most responsive, we focused in determining the differentially responsive genes (DRSGs) between species clades in these specific clusters since they are likely the major contributors to immune response divergence. We estimated the overall divergence of response across species in each cluster for all DEGs in any species (see methods). Monocytes and CD4 T cells are the major contributors to immune response divergence not only because of the number of DEGs they activate but also the magnitude of the response observed for these genes. We calculated the average response divergence (per gene) across primates in each cluster and found monocytes

and CD4 T cells to be the most divergent after dendritic cells (Supplementary figure S7). A nested model was designed to identify DRSG between human vs AAM and between simian primates (human, macaque and baboon) vs basal primate (see methods). Considering human vs AAM comparison, a total of 1133 and 99 DRSGs were significant for monocytes and CD4 T cell respectively while 233 and 30 when simian primates vs lemur was compared for the same clusters (Supplementary table S5). Remarkably, GO enrichment analysis for DRSG of monocytes in the two clade comparisons identified "response to bacteria", "response to lipopolysaccharide" and "inflammatory response" was among the top significant GO terms (Figure 2E; Supplementary table S6) indicating the evolution of stronger immune response to fight the invading pathogen along primate evolution.

Taken together, we found immune response strength to correlate with the known phylogenetic status of species and monocytes is the most responsive cells across all species.
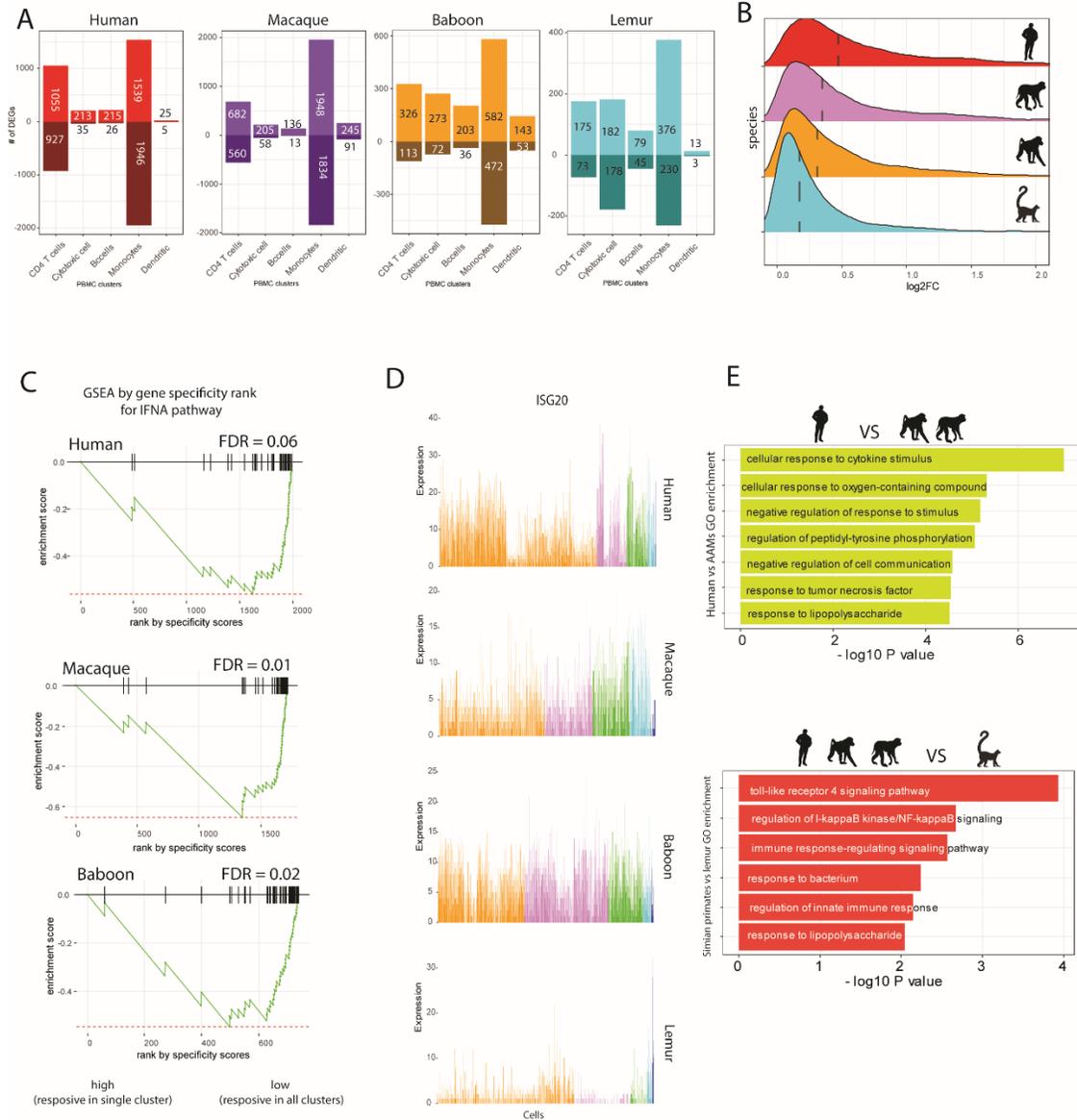
Figure 2. Immune response strength correlates with primate phylogeny. A. Bar plots represent the number of differentially expressed genes (DEGs) in each species for each PBMC cluster (x axis). DEGs are genes significantly changed expression after LPS at FDR < 0.05. The time of stimulation for all species is 4h. Cells from NC or stimulated were kept in the same conditions. **B**. Distribution of log2FC of genes with significant response from all PBMCs (FDR < 0.05) in each species (x-axis). Included genes in these distributions are DEGs in any species (n=4752). Similar plots for each PBMC cluster are provided in supplementary figure S5. **C.** Enrichment plots for gene set enrichment analysis for all species of type I interferon pathway. Genes from each species were ranked based on specificity scores (the response of genes across clusters), genes on top of the list are specific genes (responding in specific cluster) while genes

on the bottom are not specific (respond in most of clusters). Type I IFN in not specific in all simian primates as indicated by FDR. **D.** Normalized UMI counts (y-axis) per cell (x axis) for gene ISG20 in each species. Cells were colored by cluster (orange for CD4 T cell, purple for cytotoxic CD8 T cell, green for B cells, cyan for Monocytes and blue for dendritic). **E.** Bar plots represents the top significant GO terms (-log p value on the x axis) of differentially responsive genes (DRSGs) between human vs. macaque and baboon (top) and between simian primates (human, macaque and baboon) vs basal primate (lemur) (bottom).

Adaptive evolution shaped the interspecies immune response divergence.

A fundamental question for understanding the change of immune response across species is how the regulatory elements have evolved to control the trait? To answer this, we characterized the regulatory elements of stimulated and unstimulated PBMCs from the 4 primates using single cell ATAC-seq (scATAC-seq). For each condition, NC and LPS stimulated for 4 hours, pooled PBMCs from two individual (one male and one female) for each species was used. The total number of cells after QC (see methods) was for NC is 4963, 7136, 6257 and 3829; for LPS is 4949, 6551, 6276 and 3608 for human, macaque, baboon and lemur respectively. Following UMAP clustering, we annotated clusters using canonical markers and by label transfer from RNA expression data implemented in Signac (Stuart et al, 2020) (Figure 1A-C, Supplementary figure S8). There was generally a high concordance between the two methods. Next, we used MACS2 (Zhang et al, 2008) for peak calling. The total number of peaks that were found in either treatment is 133963, 151861, 134127 and 93409 for human, macaque, baboon and lemur respectively. In all species, we found that peaks to be highly cluster specific (~ 40-50% of peaks) with a relatively smaller proportion of peaks shared among all clusters ~ 10-15% (Supplementary figure S9). Then we identified 1:1 conserved peaks across the 4 species using human as reference (see methods). We found a clear trend between the number of conserved peaks and the specificity of peaks to clusters i.e., the more the peak is shared among clusters, the more likely not to be conserved (Supplementary figure S10). Annotation of peaks (based on proximity to genomic features) identified peaks at exon-intron boundaries to be mostly shared among clusters relative to other categories (Supplementary figure S10) suggesting the activity of splice sites to be highly variable among primates. Indeed, the proportion of peaks at intron-exon boundaries have the lowest peak conservation (~ 0.2) compared to other categories (~0.3 in promoters for instance) (Supplementary figure S10)

Next, we did single cell integration across treatment in each species to perform differential accessibility in active regions. Differential accessibility analysis (using "FindMarkers" function, Wilcoxon test) identified monocytes to be the most active cluster in human and macaque and second active cluster in baboon (after Bcell_II) in terms of the number of differentially accessible peaks (DAPs) (FDR < 0.05) (Figure 3D). We found no significant DAPs in lemurs, which corroborates with the weaker transcriptional response to LPS observed in lemurs as compared to other primate species. For instance, IL1B gene region was found to have high activity after stimulation in all primate's monocytes except lemur (Figure 2E). The clustering of unintegrated cells also provided insights on the epigenetically active clusters in each species. In simian primates, monocytes showed a distinction between two conditions compared to other clusters while in human, clusters of T-cells also showed a clear difference between NC and LPS treatment (Supplementary figure S11). Monocytes and CD4 T cells are, transcriptionally, the most responsive cells in all species indicating that epigenetics plays a major role in driving the immune response divergence across primates. Motif enrichment analysis identified hundreds of motifs such as REL and JUND that are significant (FDR < 0.05) in simian primates (Supplementary table S7).

Next, we investigated the role of DAPs in the activation of the immune response. Proximity to enhancers is a key factor that defines their effect on target genes (Rodelsperger et al, 2011; Quintero-Cadena and Sternberg, 2016). We hypothesized that since DAPs are likely the regions most directly involved in the regulation of the immune response, the closer the gene to DAPs, the more likely this gene to be differentially responsive between species. We focused our analysis here on monocytes since they are the most responsive and the key determinant of the immune response divergence of PBMCs. From the previous analysis, we detected 399 genes that have higher upregulation in human vs AAMs (log2FC > 0.5 and FDR < 0.05). We found 149 DAPs (~ 17% from total DAPs) that were found proximal to the DRSGs, within 50k distance. We found this proportion to be significantly higher than the average proportion of randomly sampled genes (~ 66 DAPs) as detected by permutation test (p = 0) i.e., the more a gene is closer to a DAP, the more likely it is a DRSG. Next, we asked if there is a signature of positive selection on human DAPs that might explain their role in boosting the response in human vs other species. We used INSIGHT algorithm to infer selection on DAPs that are associated with DRSG (hereafter referred as DAPs-

DRSGs) (Arbiza et al, 2013; Gronau et al, 2013). INSIGHT tests for sites that underwent adaptive evolution specifically in human compared to ancestral alleles of 3 species, chimpanzee, orangutan and macaque. To do so, it calculates adaptive evolution parameter $E[D_p]$/kbp, beside other parameters that infer weak and strong purifying selection (inferred from within population polymorphism). We found a strong signal of adaptive evolution as estimates by $E[D_p]$/kbp parameter, =1.05 on DAPs-DRSGs indicating a signature on adaptive evolution compared to $E[D_p]$/kbp on the rest of DAPs (0.00019) (Figure 3F). An example for adaptive substitutions in a DAP that is associated with a DRSG (TMEM132A) (supplementary table S5) is provided (Figure 3G). These results suggest that natural selection in regulatory regions contributed, at least in part, to the observed differences in immune response observed today between human and our closest relatives.

Taken together, we found chromatin remodeling to vary across primates and to play a role in immune response regulation in simian primates. Also, we found adaptive substitution of regulatory elements to be associated with the stronger response in primates.
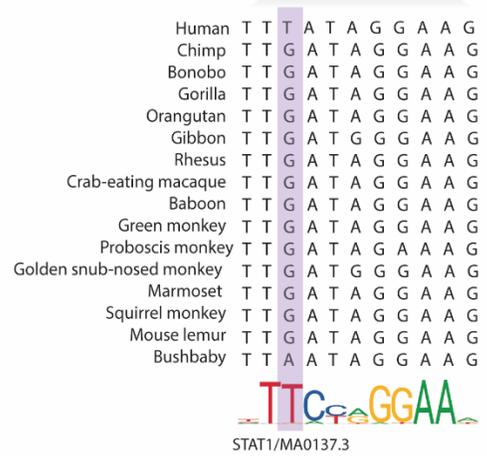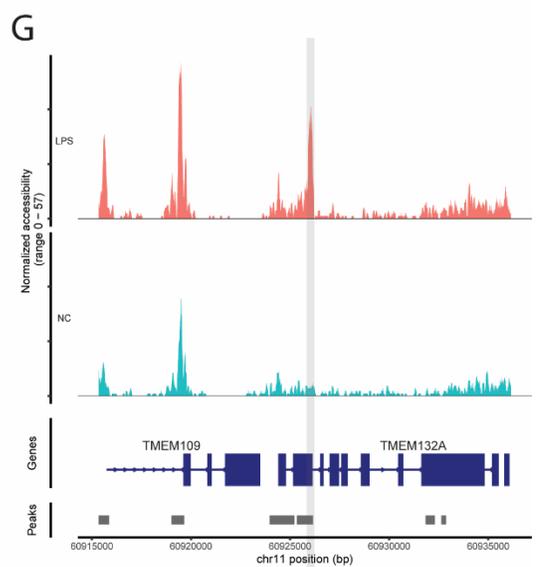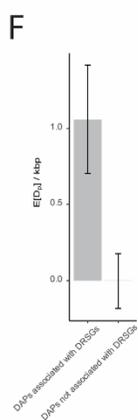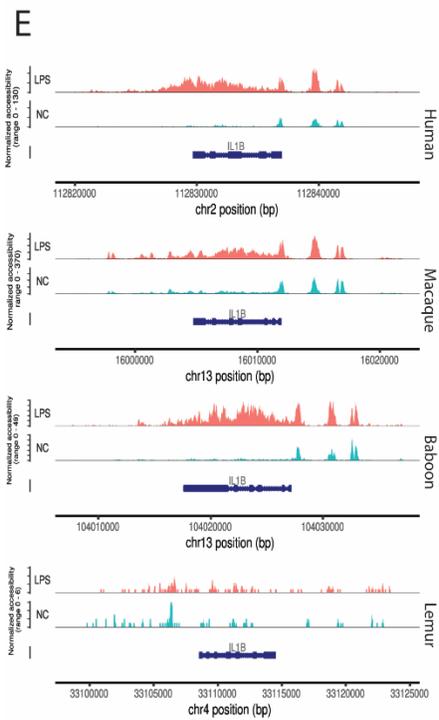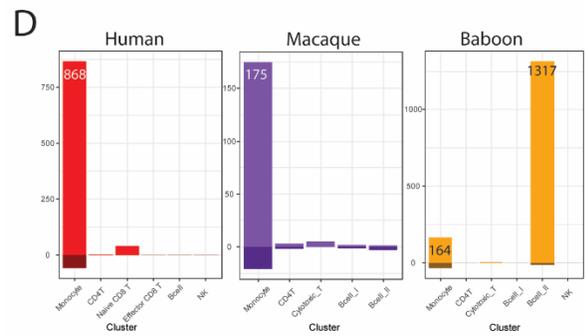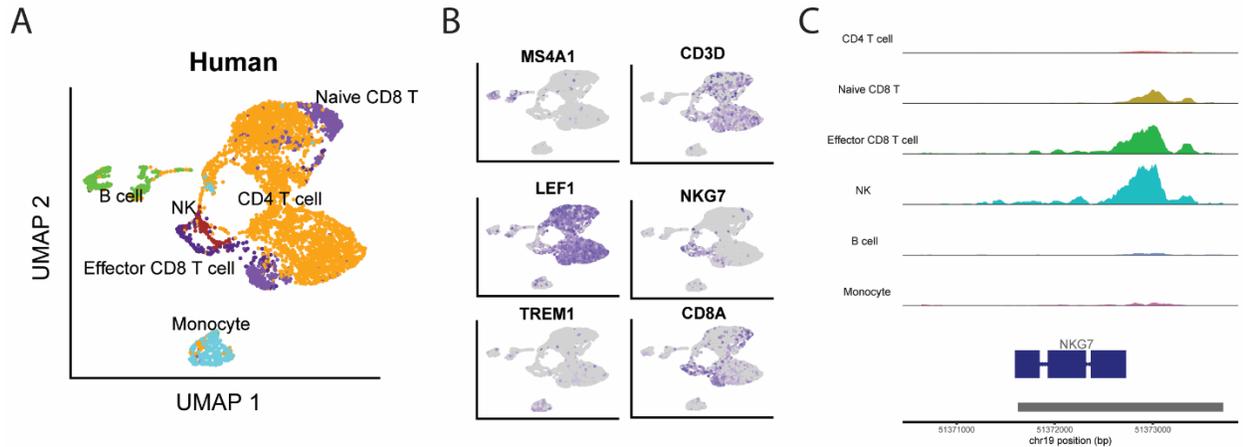
Figure 3. Adaptive evolution shaped the interspecies immune response divergence revealed by single cell ATAC seq. A. UMAP of cells from single cell ATAC seq (scATACseq) from human. **B**. Markers used to annotate the different clusters of scATAC seq assay. UMAP from other species and the markers used for annotation different species is provided in supplementary figure S8. **C**. Coverage plot of one marker, NKG7, where is the peak of the gene promoter is active on only NK and effector CD8 T cells and modest on naïve CD8 T cells while absent in rest of clusters. **D.** Bar plots represent the number of differentially accessible peaks (DAPs) after LPS stimulation (FDR < 0.05 Wilcoxon test) for human, macaque and baboon (no significant peaks were found in lemur) for each cluster (x axis). **E**. Example of conserved DAP in IL1B gene across species except for lemur. **F.** Bar plots summarize the parameters from INSIGHT algorithm for DAPs that are associated with genes had higher upregulation in human vs macaque and baboo (DRSGs) (top) and rest of DAPs (bottom). Scale on the right side represent the values of positive selection ($E[D_p]$/kbp) and purifying selection $E[P_w]$/kbp and on the left side represent the value of total selection (rho). **G.** Example of adaptive substitution in STAT1 transcription factor binding site specific to human. The mutation is in peak region that is closest to TMEM132A, which found to have significant higher upregulation in human vs macaque and baboon (Supplementary Table S5) highlighting role of adaptive mutation in immune response divergence in primates.

Discussion

In this study, we compared immune responses across primate species that show different susceptibility to disease namely, human, macaque and baboon beside lemur as outgroup. We found the immune response strength correlate with the primate phylogeny being the highest in human, the lowest in the basal primate, lemur. Remarkably, we found two major mechanisms that correlated with this increase of immune response namely, epigenetic activity and selection.

The robust innate immune response is widely accepted to be a major cause for inflammatory disease and immunopathology such as sepsis (Delano and Ward, 2017). We observed here that immune response is correlated with primate phylogeny suggesting that immune response trait to be evolved under the influence or connection with other life history traits that shaped the primate evolution. Life history traits often covary and affect each other (Jones, 2011; Zimmermann and Radespiel, 2013). For instance, for the species tested in this study, human is the "slowest-living", i.e., has low reproductive rate with low offspring number and longer life span, lemur is relatively "fast-living" with high reproductive rate and offspring number and short life span while macaque

and baboon are in between the two. Indeed, a study of several life history traits in primates showed a significant phylogenetic signal of the majority of traits studied (Kamilar and Cooper, 2013). Life span was found to be an important trait that affects the immune response and has been experimentally observed (McDade, 2003; Johnson et al, 2012). For instance, it has been shown that species with shorter life span will invest more in reproduction and less in defensive mechanisms as opposite to long-life span species. Hence, the pattern of immune response herein is consistent with these observations. Although, the investment of high response could be beneficial in protecting the organism in its extended lifespan, but it may be associated with the cost of being more prone to tissue damage accompanied by the strong defense response.

The nature of the immune response elicited by primate PBMCs is also remarkable. We found by far monocytes to be the most responsive cells in all species which makes sense since monocytes are key innate immune cells of the PBMCs (Kantari et al, 2008). Also, we found that the increase of immune response across primates in monocytes to be functionally relevant to respond and fight the bacterial pathogen. However, we also found type I IFN pathway to be remarkably conserved in all PBMCs from all simian primates. Type I IFN is known to be mainly antiviral defense pathway (McNab et al, 2015) so why it is a default response from simian primate's PBMCs when responding to a bacterial stimulant? Type I IFN is known to have diverse roles in many types of infections. For instance, low levels of type I IFN initiate a cell-mediated immune response against bacteria (Bogdan et al, 2004; McNab et al, 2015). Also, type I IFNs was found to directly limit bacterial replication and virulence (Boxx and Cheng, 2016).

Several mechanisms have been shown to mediate the change of the immune response across species such as transposable elements, promoter architecture by elements such as TATA boxes and selection (Chuong et al, 2016; Danko et al, 2018; Hagai et al, 2018). We found positive selection to be a major factor that explains the immune response change. We tested TE proportion and promoters TATA boxes and found no significant results (data not shown). We also observed regulatory landscape remodeling to be likely a factor that may explain the pattern of immune response strength across species. In lemur, with the lowest immune response, no difference between stimulated and unstimulated cells while in human majority of PBMCs showed a clear difference. The role of epigenetics has been proposed to play an important role in interspecies difference of other phenotypes such as gene expression across primates (Zhou et al, 2014).

In conclusion, we found immune response to be correlated with primate phylogeny and likely to be interconnected with other life history traits in primates. Adaptive evolution was found to be a major evolutionary mechanism that mediates the divergence of the immune response across primates. Functional editing studies on specific regulatory elements detected here are warranted to illuminate the molecular evolution of the regulatory elements network in primates.

Methods

Peripheral blood mononuclear cells (PBMCs) collection and stimulation.

We obtained PBMCs from 6 individuals from human, macaque, baboon and extracted PBMC from heparinized blood (3 males and 3 females for each species). Human samples were obtained from BioIVT ® (NY, USA). Signed written consent was obtained from each participant and the study was approved by the ethics committee at CHU Sainte Justine. Macaque and baboon blood samples were obtained from Texas Biomedical Research Institute following implemented animal-subject regulatory standards. Samples from lemurs were collected from Duke Lemur Center (Durham, NC, USA). Non-human primate individuals have no history of chronic infection as regularly inspected by the veterinary staff and appeared healthy at the day of sampling.

Cells from all species were either stimulated by lipopolysaccharide (LPS, Sigma) or cell culture media (control). We stimulated 120K cells per treatment for all species. A working concentration of 500 ng/ml LPS was used for stimulation and the incubation time is 4 hours post stimulation.

We first thawed the cryopreserved cells (one vial per species ~ 2 million cells / 2ml). PBMCs were unfrozen nearly 20 hours pre-treatment and cells rested overnight in RPMI 1640 mixed with 10% fetal bovine serum (FBS), 2 mM L-glutamine, and 10 ug/ml gentamycin at 37°C in 5% CO_2 and 20% O_2 incubator. The stimulation experiment was done over 6 days (one individual per day) so that stimulation was done on all species samples simultaneously to avoid confounders that may hinder interspecies comparative analysis. For each day of the experiment, cells were first inspected under light microscope to be viable (bright and intact) then distributed on concentration 120K cells/well prior to stimulation. After stimulation and incubation period, cells were harvested, washed, and prepared for single cell RNA sequencing using 10X Genomics platform. Cells were

pooled from all species into two 10X captures, one for each treatment, in a single day yielding total 12 captures for all individuals. We targeted 2,000 cells per species per treatment resulting in total 8,000 cells per capture that were used for generation Gel Bead-in-Emulsion (GEM) using Chromium Single Cell Reagent (v3) kit (10X Genomics). After GEM formation, reverse transcription (RT) was performed in thermo cycler (53°C for 45 min, 85°C for 5 min) and the resulted cDNA products were stored at -20°C until library preparation and sequencing.

Library preparation and single cell RNA sequencing

Library preparation for single cell RNAseq was conducted according to manufacturer protocol of Chromium Single Cell 3' Reagent Kits v3 user guide (10X Genomics). Briefly, step 2 of the user guide, post GEM-RT Cleanup and cDNA Amplification, started by cDNA cleaning by DynaBeads MyOne SILANE beads (ThermoFisher) followed by PCR amplification according to the program specified in the user guide. cDNA cleanup after amplification was conducted by SPRIselect reagent kit before cDNA quality checking and quantification performed are performed by n Agilent Bioanalyzer High Sensitivity chip. Last step, 3' Gene Expression Library Construction, was performed by following sequential steps namely, fragmentation, end-repair, A-tailing, post fragmentation, end repair & A-tailing double sided size selection – SPRIselect, adaptor ligation and post ligation cleanup – SPRIselect as detailed in the user guide. Libraries were sequenced using Illumina - 10X Chromium single cell – NovaSeq by 100bp cassette at CHU Sainte Justine sequencing core facility.

Reads mapping and demultiplexing.

Cellranger v3 (10X Genomics) pipeline was used for generation of FASTQ files and aligning reads to species genomes using cellranger mkfastq and cellranger count software respectively. For human, we used the available prebuild reference genome GRCh38 on 10X Genomics repository (downloaded January 2020). For the rest of the species genomes which are not available on 10X website, we generated their reference genomes using cellranger mkref pipeline. For each species we used annotation and genome files from Ensembl database. The genome versions used for each species are Mmul_10 for macaque, Panu_3.0 for baboon and Mmur_3.0 for mouse lemur and Prosim_1.0 for greater bamboo lemur. Customized GTF annotation files were generated using

cellranger mkgtf program for each species original GTF files to include only features that are polyadenylated to avoid multi-mapped reads as recommended by algorithm developers.

For demultiplexing cells from different species, we used mitochondrial gene expression as makers to disentangle cells from each species. Mitochondrial DNA is known to be highly divergent across primates (Pozzi et al, 2015) and thus is expected to be a good specific marker. Prior to alignment step, we added mitochondrial genomes FASTA and GTF files from all species that were obtained from NCBI (Accession numbers MG787545 for *Papio anubis*, NC_005943 for M*acaca mulatta* and NC_004025 for *Lemur catta*) (Arnason et al, 2002; Gokey et al, 2004; Roos et al, 2018) which were annexed to genome files of all species.

QC and single cell data integration

We used Seurat v4.0 (Hao et al, 2020) for major processing and single cell data analysis. Firstly, we performed major data processing and clustering steps namely, normalization, PCA analysis using highly variable genes and UMAP clustering to check cells on the dimensional space. Cells were clustered mostly according to species as identified by mtDNA gene expression (Supplementary figure S12). We characterized cells from each species, defined by cell whose mtDNA gene expression > 1% of total counts and < 0.1% of mtDNA from each other species. Nearly quarter of total cells pertains to single species compared to total number cells as expected, 23.7%, 22%, 22% and 20% for human, macaque, baboon and lemur respectively. The total number of cells after filtering is less since some cells contain doublets (cells from two species inside single droplet) (Supplementary figure S12). QC was done on cells from each species to eliminate low quality cells, defined by two parameters mtDNA and number of Features, cells with mtDNA expression < 15-20% and number of Features > 200 and < 3000-4000. We changed values for each parameter according to their distributions in each species since for instance number of features likely to be different across species since it reflects different annotation qualities between species (Supplementary figure S13).

We retrieved 1:1 orthologous genes using human as a reference from Ensembl v.103 (n=10517) and restricted the rest of downstream analysis on them only. Integration of cells across species (on NC condition) was done using Seurat algorithm by first normalizing cells from all species using SCTransform function regressing out individual and mtDNA gene expression variables followed

by identifying "anchors" for integrating cells across species using SelectIntegrationFeatures, PrepSCTIntegration, and FindIntegrationAnchors functions and finally IntegrateData function.

Modelling and linear regression

We first generated individual x genes matrices of PBMC clusters by pseudobulking the original cell x genes matrices. Pseudobulking was performed by summing all UMI counts of cells from a specific cluster per individual in each species. For instance, for a specific PBMC population, we sum counts in cells pertain to one treatment to one individual. At the end, instead of cells x genes matrix, we had individual x genes matrix. Then, linear models were used for comparing immune response across species using limma package (Ritchie et al, 2015). We first filtered out lowly expressed genes as we included only genes which have more than 1 count per million (CPM) in 6 individuals in at least one treatment (NC or LPS). The resulted matrices had 7465, 6557, 6648, 6634 and 5434 in CD4 T cell, cytotoxic cells, B cells, monocytes and dendritic cells respectively. Normalization of library size difference across samples was performed using trimmed mean of M-values method (Robinson and Oshlack, 2010) implemented in calcNormFactors function. The second normalization was done using voom algorithm (Law et al, 2014) which models mean-variance trend of logCPM. The resulted mean-variance trend should stabilize variance of genes with high count showing trend decrease as genes of count increases (Law et al, 2014) except if there is high variation between samples for which algorithm fails to stabilize the variance. We checked these trends before formally using the models. We found this trend is as expected except in one analysis, characterizing differentially expressed genes in human dendritic cells, was showing high variance at high counts in one analysis. We found one individual (HMN306871 at NC condition) has few counts and when removing this individual, the trend showed the expected pattern.

We designed two models to characterize response within species and to compare response between species. A simple model of ~ individual + treatment was used to identify differentially expressed genes (DEGs) in each species. In the interspecies model, we added log2 of the cell counts to regress out the difference of cell counts across species so that the model is ~ log2 cell counts + species + species:treatment. To consider paired nature of the experiment, we used duplicateCorrelation function and blocked individual variable. Differentially responsive genes across species, henceforth referred to as DRSGs, were characterized using pairwise comparisons using the

interspecies model. Two major comparisons between primate clades were considered, one between human (ape) vs. macaque and baboon (AAM) and the other comparison is simian primates (human, macaque and baboon) vs. basal primate (lemur). For instance, a gene is considered DRSG between human and monkeys if it is significantly different between human vs. macaque and between human vs. baboon (FDR < 0.1) and has a consistent direction of expression (up- or down-regulated in both comparisons). Correction of multiple testing was done using FDR, as described by Benjamini-Hochberg (1995).

Specificity index and divergence scores

Specificity index formula which gives a quantitative measure of uniqueness of response across PBMC clusters within each species were adapted from Tosches et al (2018), $S_g = \frac{max_{i \in C}(r_i)}{\sum_{i=1}^{c} r_i}$ where S_g is the specificity index of gene g, r_i is the log2FC of normalized response of gene g in cluster i, C is the set of all PBMC clusters and c is the length of C. We restricted the analysis to genes that are upregulated. Genes showing opposite direction of response across clusters were omitted (~ 10% of total responsive genes). $S_g \in [0,1]$ where higher the value indicates response comes mostly from a specific cluster and the opposite is true. Divergence scores were calculated by summing the differences of the two successive elements of a vector that has log2FC values of all species for a specific gene in a specific cluster i.e., $D_{g,c} = \sum_{1}^{(l-1)} |(L)_{l+1} - (L)_l|$ where $D_{g,c}$ is the divergence score of gene g in cluster c, L is a vector of log2FC of all species of gene g in cluster c and l is the length of vector L

Gene set enrichment analysis and functional annotation

Gene set enrichment analysis was done using "fgsea" package v1.14.0 and using fgsea function where number of permutations, nperm, = 10000, minimum size of gene set to test, minsize, = 15 and maximum, maxsize, = 500. Functional annotation was conducted using gene ontology (GO) enrichment in CluGO app (2.5.5) of Cytoscape (version 3.7.2). The Benjamini-Hochberg method for multiple correction was used for hypergeometric test, the background lists of genes were adjusted according to the cluster used in the analysis. Default values were used for the rest of the parameters.

scATAC seq library preparation and sequencing

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scATAC-seq libraries were prepared according to the Chromium Single Cell ATAC User Guide (v1). The first step it to prepare nuclei suspension by extracting nuclei for specie cells. NP-40 lysis buffer (0.025%) was used for nuclei suspension for 3 minutes. Nuclei were checked to be intact and round to avoid too much lysis. Then the 4 steps were performed sequentially namely, transposition, GEM generation and barcoding, post GEM incubation cleanup and library construction according to manufacturer protocol. Libraries sequencing was performed at sequencing core at Chicago University.

scATAC seq data processing and QC

Same genome versions used for scRNA seq were used for mapping of scATAC seq. Cellranger-atac pipeline was used for data processing using mkfastq and count software of the pipeline. Pre-build reference of the human GRCh38 genome was used from 10X Genomics website (downloaded December 2020). Customized references genomes were configured for the rest of the species using mkref software (10X Genomics). Signac v.1.1.1 (Stuart et al, 2020) was used for data further downstream analysis and processing. QC was performed using peak region fragments to be > 2000-3000 and < 15-30 000 depending on the species, percentage of reads in peaks > 15, nucleosome signal < 5 and transcription starting site (TSS) enrichment score > 2. These values were adjusted from the distribution of each parameter in each species. Normalization of the data was conducted using term frequency inverse document frequency (TF-IDF) implemented in "RunTFIDF" function using default values. Then, finding top variable features (peaks) was identified to be used for dimension reduction using singular value decomposition (SVD) method through functions "FindTopFeatures" and "RunSVD" respectively assigning default parameters. Clustering was done by UMAP using the same function as in scRNA seq.

Cluster annotation and Integration of scATAC.

Integration with RNAseq was performed by first assigning "gene activity" for each scATAC chromatic object by "GeneActivity" function. The function will sum all fragment counts within a gene and up to 2k bp and store it as a proxy of the gene activity. This matrix is then log normalized and used to integrate with scRNA seq object. Firstly, anchors between the two datasets were identified using "FindTransferAnchors" then label transfer was conducted by "TransferData" functions.

For integration between the two treatments within each species, we first created a unified set of ranges using the two treatment peak ranges using "reduce" function of GenomicRanges package v 1.40.0. (Lawrence et al, 2013). A new matrix was produced for the new genomic ranges using "FeatureMatrix" and stored as chromatic assay using "CreateChromatinAssay". Finally, integration was conducted using "FindIntegrationAnchors" and "IntegrateData" functions followed by normalization steps as mentioned above. Annotation of chromatin objects was obtained from AnnotationHub package v 2.20.2 (Morgan and Shepherd, 2020) by extracting Ensembl annotation (release 99) using "ensDbFromAH" and "EnsDb" functions then create annotations using "GetGRangesFromEnsDb".

Conserved peaks across species

Peaks from any species were identified to be orthologous to human peaks by using UCSC chains through "import.chain" and "liftOver" from rtracklayer v1.48.0 (Lawrence et al, 2009). Peaks considered orthologs if it is mapped to only one region in the species genome and this region is overlapped with a peak by length > 1 using "findOverlaps".

Motif enrichment and TF footprinting

Motif enrichment was conducted by first build matrices of motif position frequency for human using JASPAR2020 database (Oriol et al, 2020) which was added as an assay to the object using "AddMotifs". Background peaks from the same cluster where peaks of interest are tested (analysis was performed only for monocytes) were selected using functions AccessiblePeaks to obtain all peaks in the particular cluster and MatchRegionStats to obtain peaks of similar GC content and enrichment was conducted using "FindMotifs".

Selection by INSIGHT pipeline

INSIGHT was conducted on web portal (http://compgen.cshl.edu/INSIGHT/) using default values but peaks were lifted to hg19 genome version first before doing the analysis.
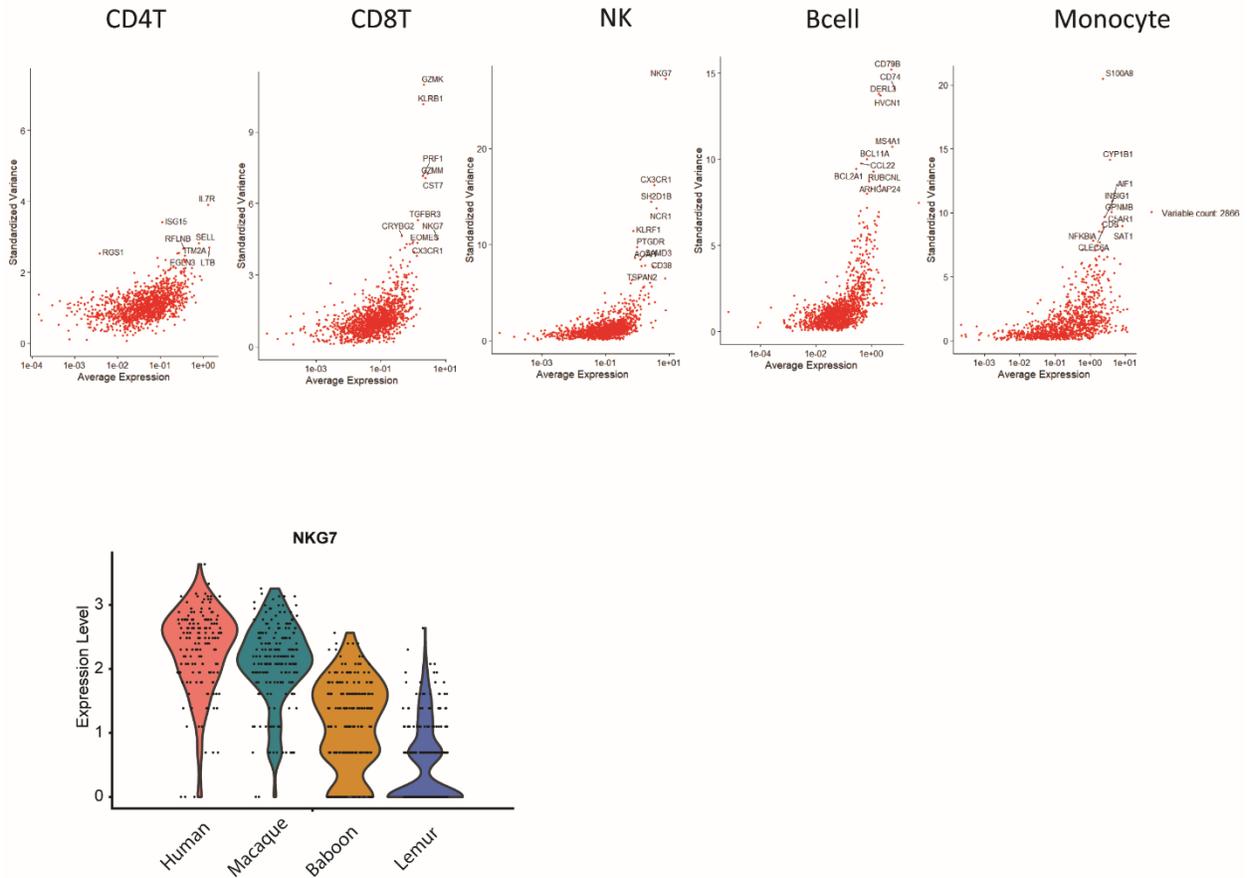
Supplementary Figures
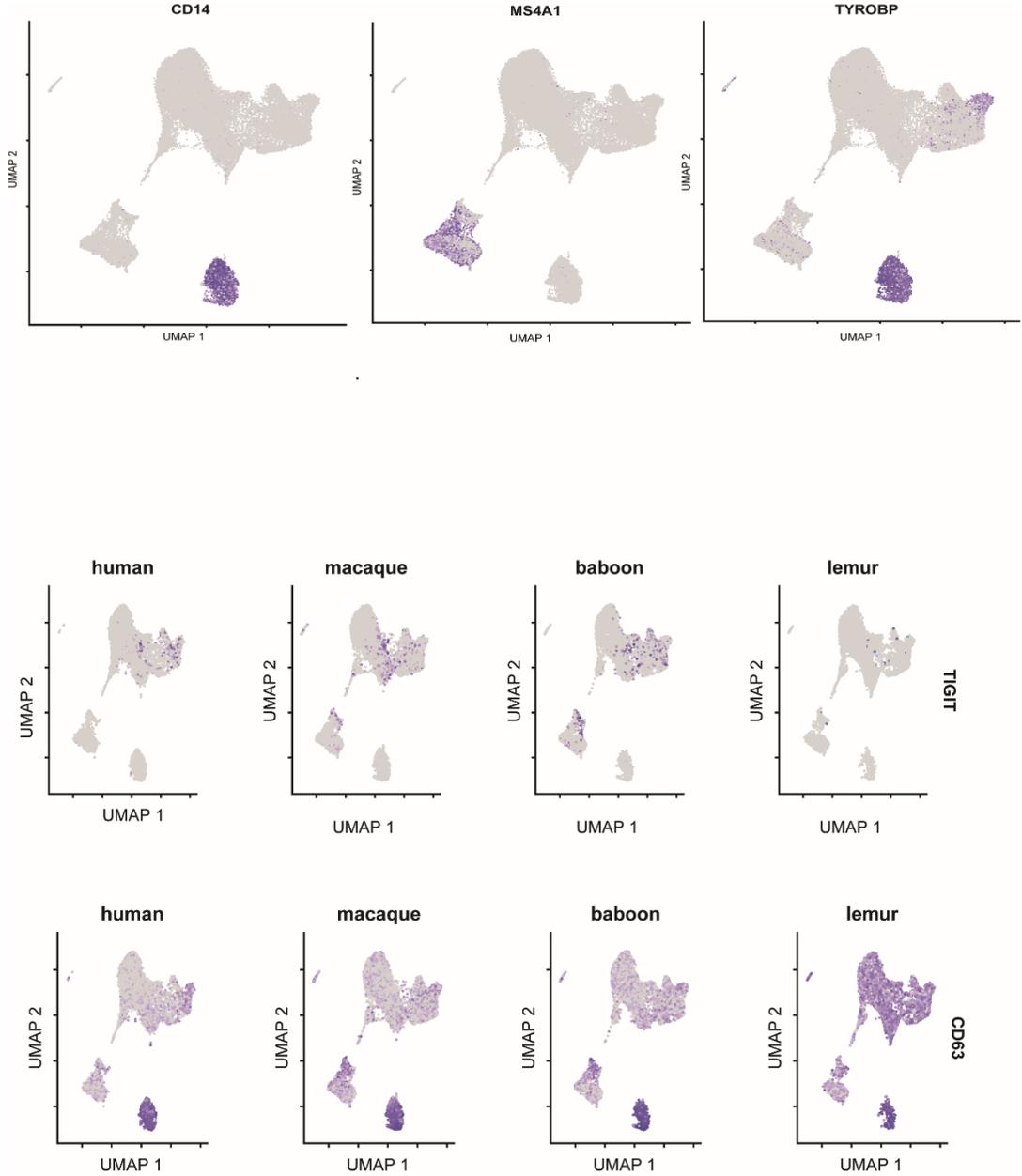
Supplementary figure S1. Markers that were used to annotate the PBMC clusters from primates PBMCs.
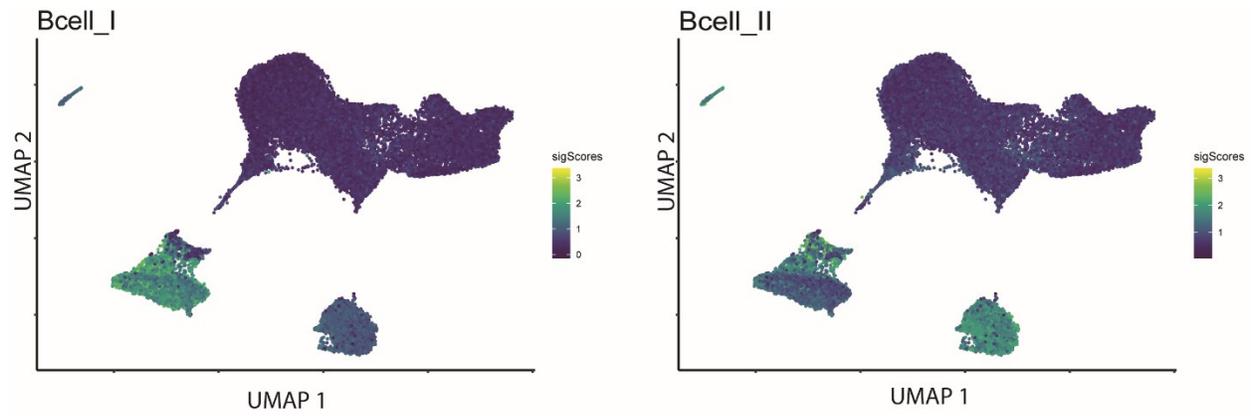
Supplementary figure S2. Enrichment using expression module of cluster Bcell_I and Bcell_II. UMAP clusters of primates PBMC highlighting the enrichment scores per cell using expression module of cluster Bcell_I and Bcell_II

Supplementary figure S3. Gene variability across species in PBMC clusters. Scatter plots for genes that has high variability as indicated by variance (y axis) and the expression on x axis for all PBMC major clusters. Below is an example of genes with high expression variability, NKG7.
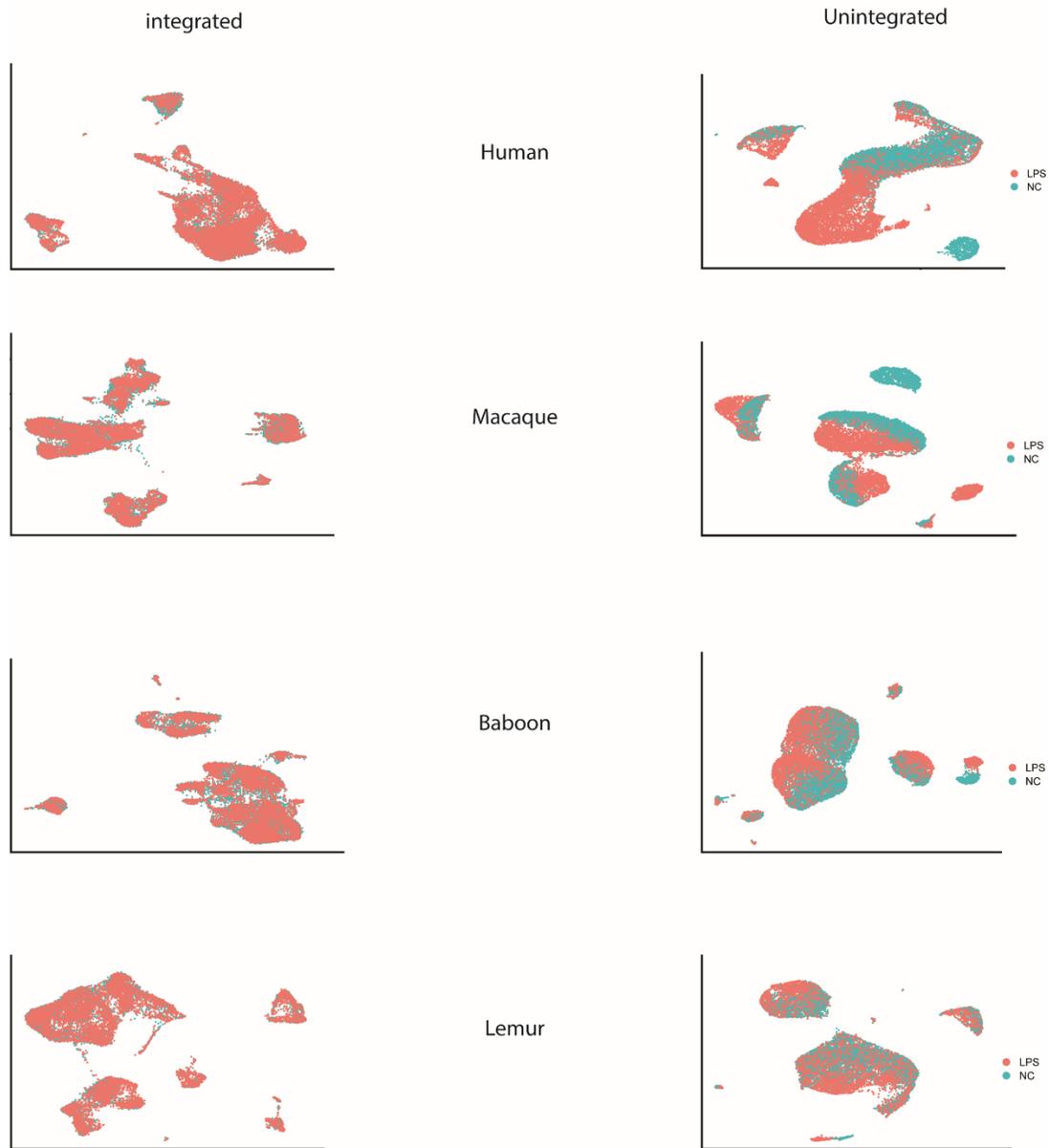
Supplementary figure S4. Cells from all species with and without integration. PBMCs from all species with and without integration highlighting successful integration of PBMC across conditions (LPS and NC for all species).
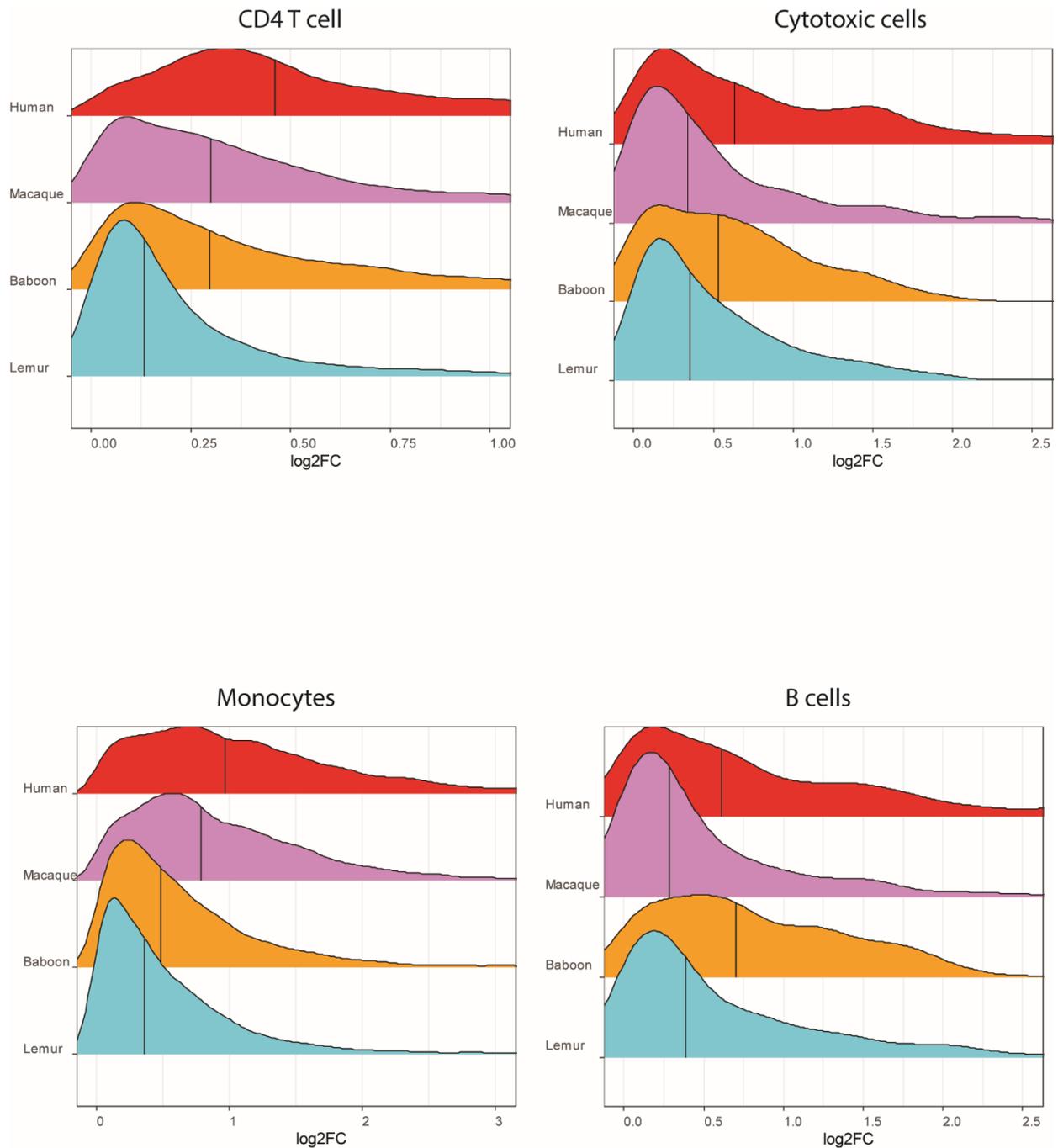
Supplementary figure S5. Distribution of log2FC of genes with significant response.
Distribution of log2FC of genes with significant response from specific cluster (FDR < 0.05) in
each species (y-axis). Included genes in these distributions are DEGs in any species (n=2733 for
CD4 T cell; 512 for Cytotoxic cells; 5467 for monocytes; 403 for Bcells).
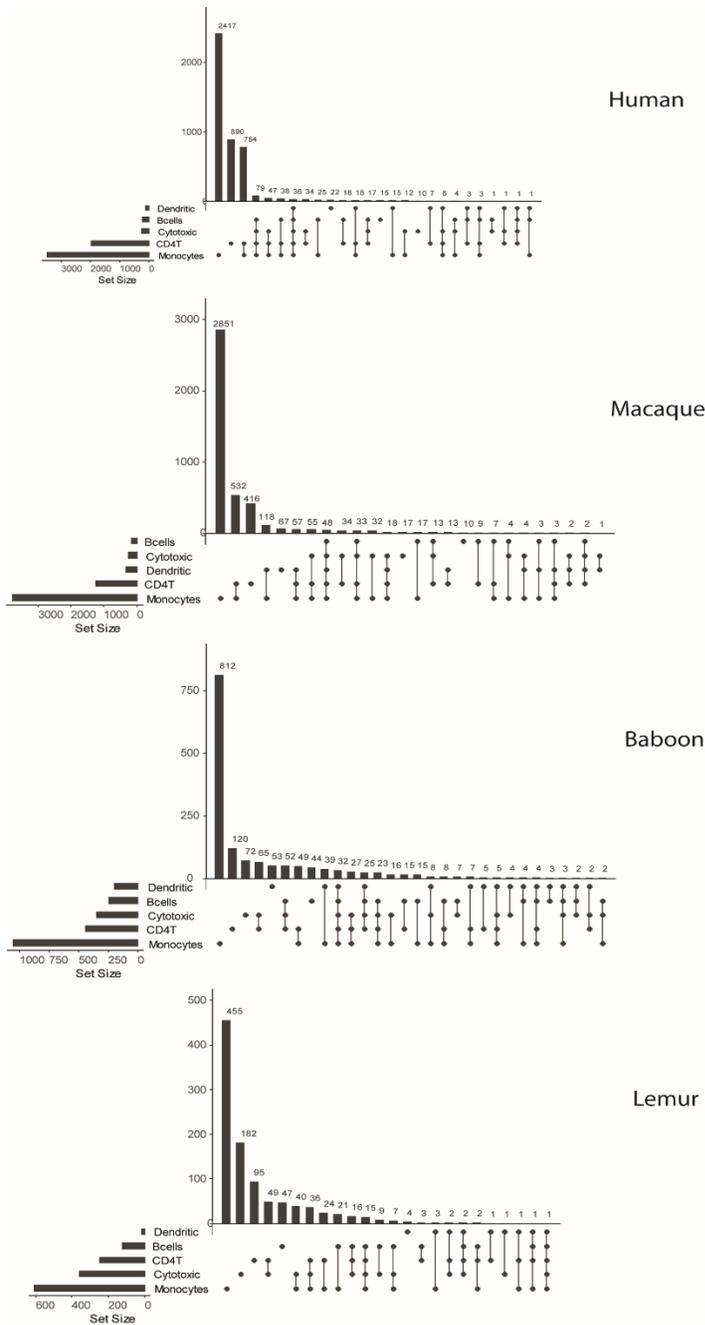
Supplementary figure S6. Specific and shared genes across clusters in each species. Upset plots summarizing the pattern of specific and shared genes across clusters in each species. Monocytes then CD4 T cells have the most specific genes in all species except lemur it is monocytes then CD8 cytotoxic cells.
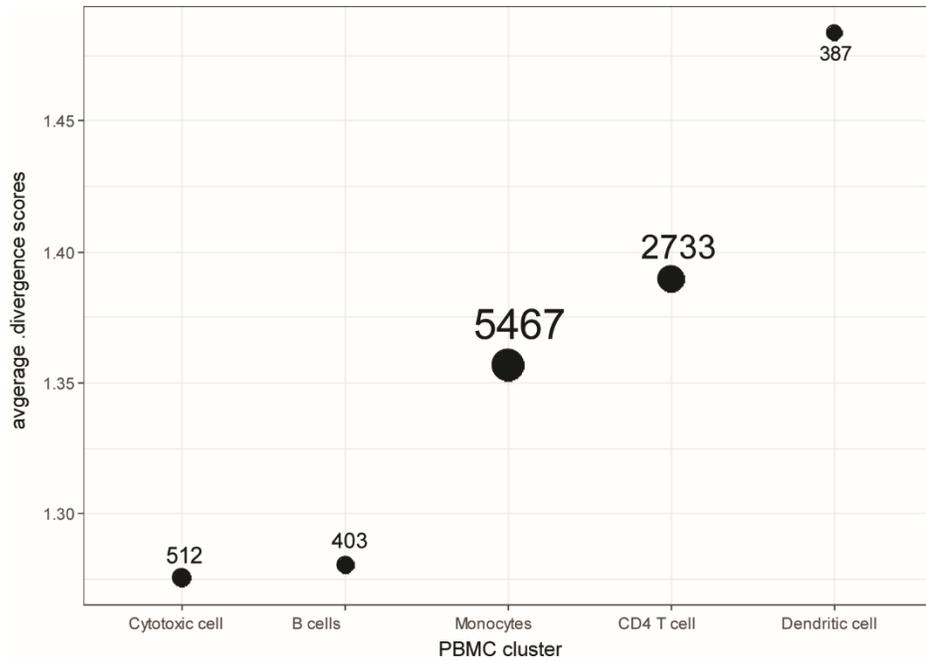
Supplementary figure S7. Average divergence scores for each cluster. Average divergence scores for each cluster (y axis). Divergence scores was calculated for all significant genes in any species (FDR < 0.05) for each cluster (adjacent to the circle) the divided by the total number of these genes (the mean). Circle size is proportional to the number of total significant genes in this cluster.
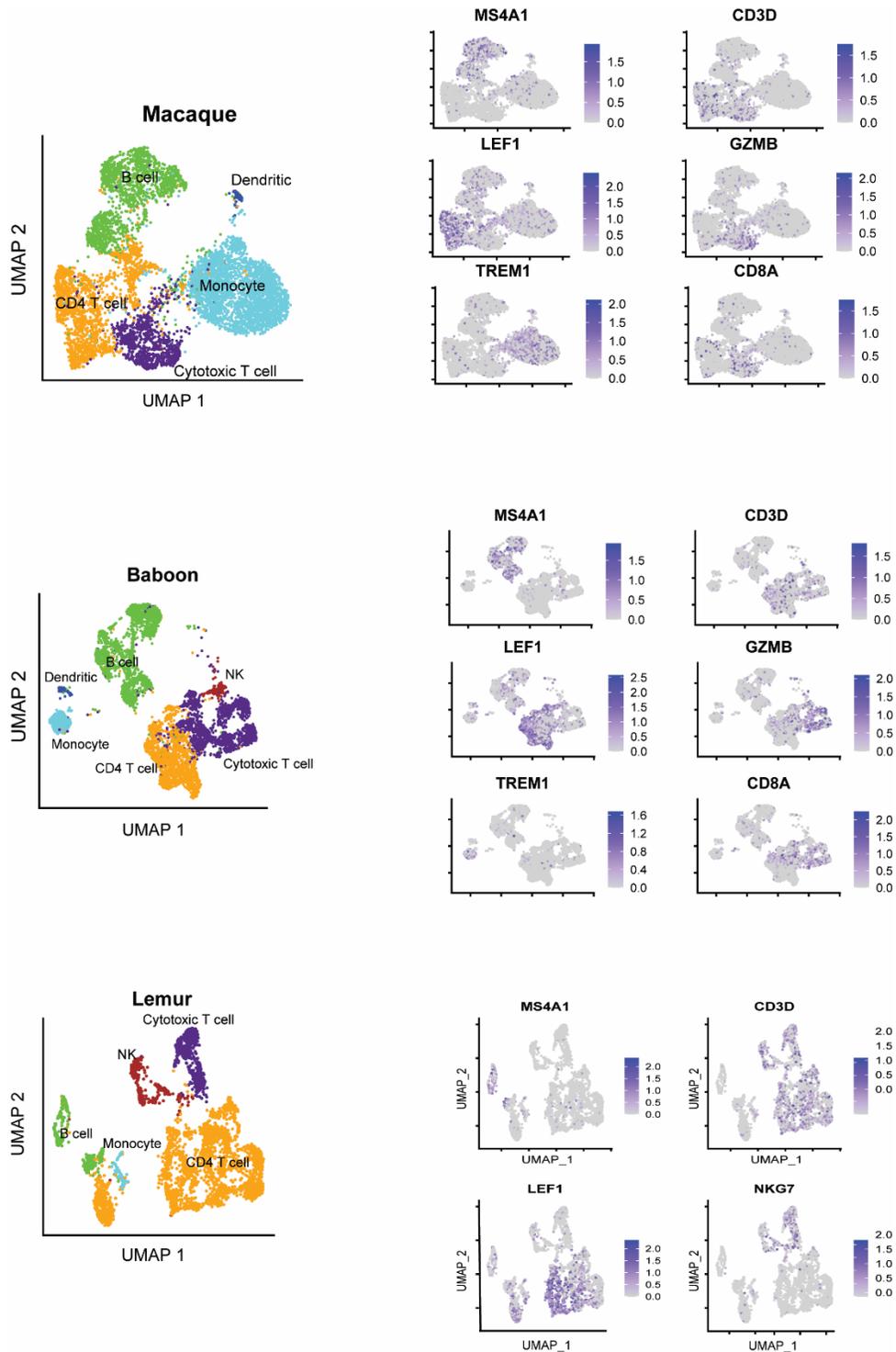
Supplementary figure S8. UMAP cluster of cells from scATAC seq assay. UMAP cluster of cells from scATAC seq assay for macaque, baboon and lemurs with markers used to annotate the PBMCs indicated on the right.
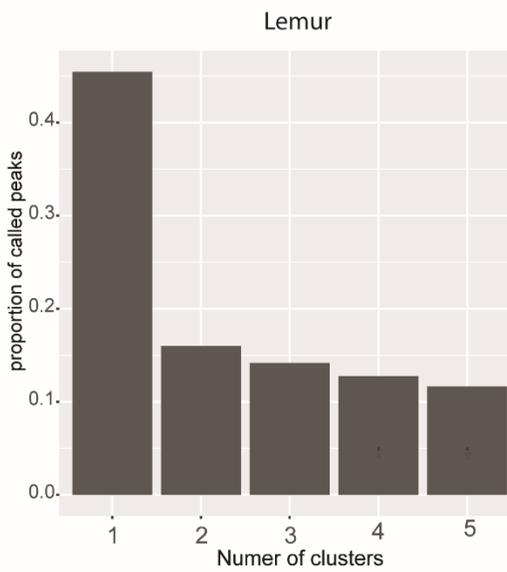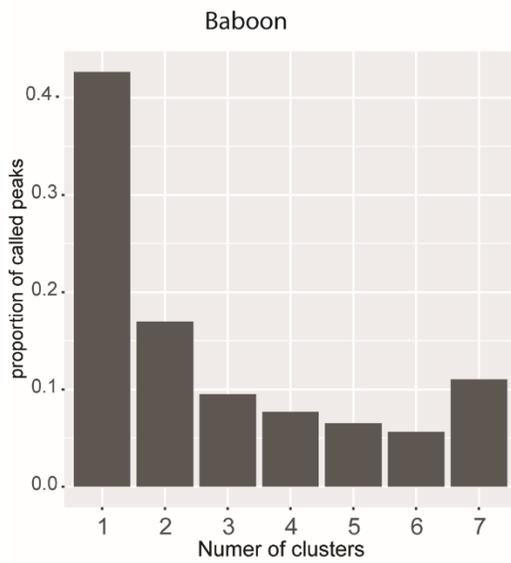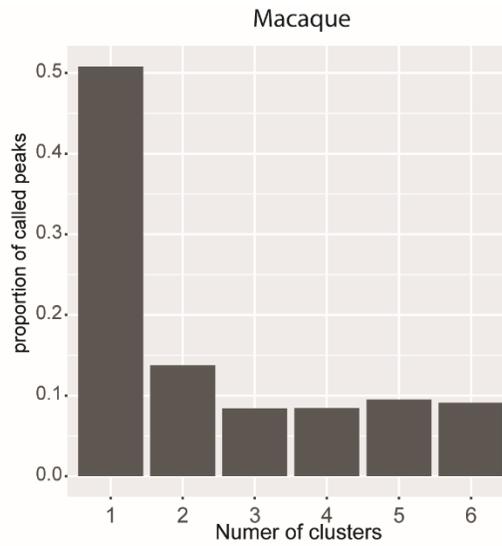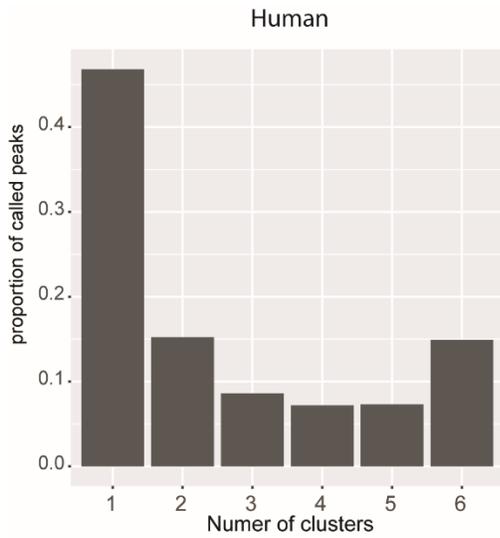
Supplementary figure S9. Relationship between the proportion of called peaks in clusters.
Bar plots represents the relationship between the proportion of peaks that are found in how many
clusters in each species. Majority of peaks are cluster specific (~ 40%) in all species.
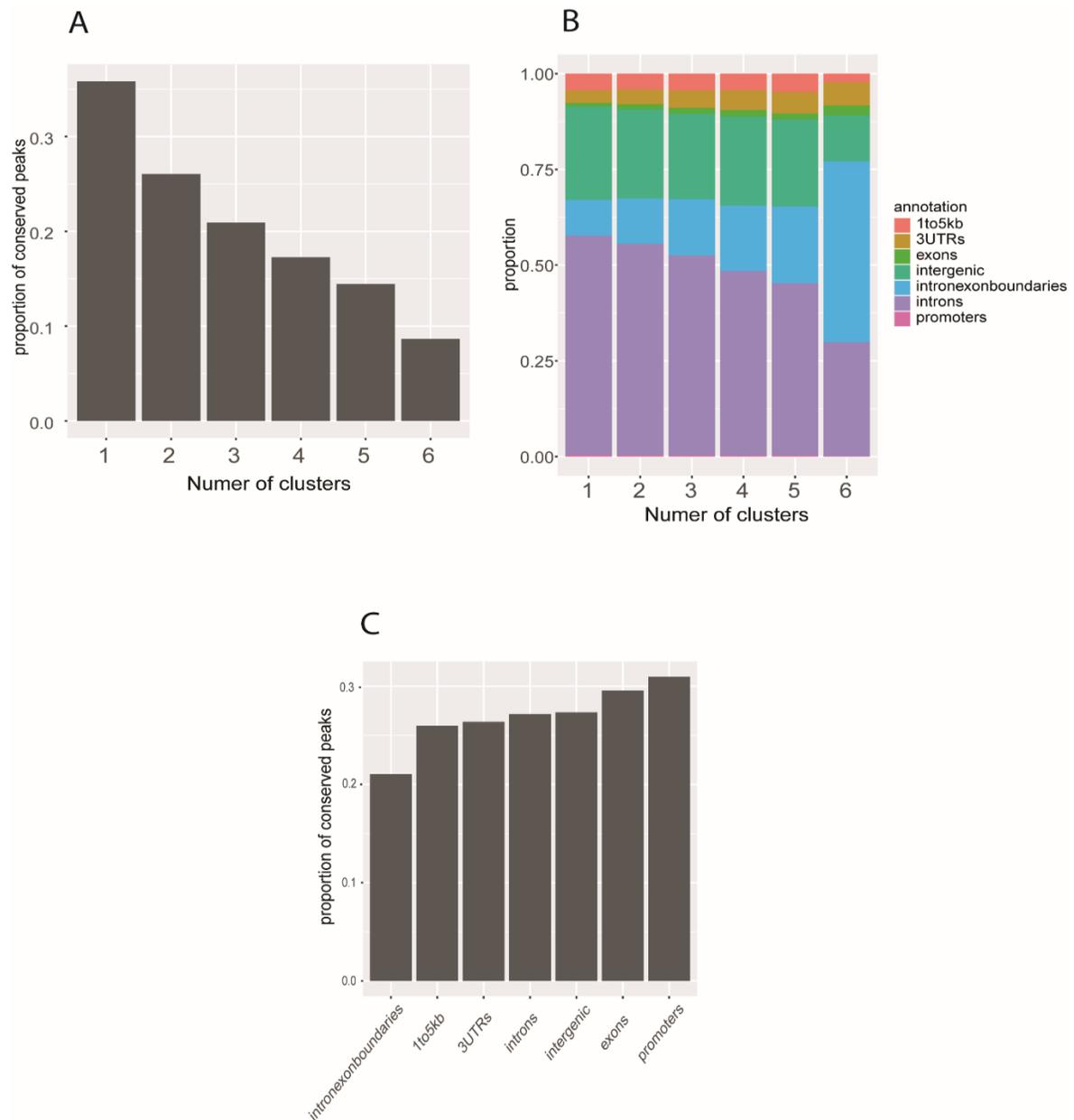
Supplementary figure S10. Relationship between proportion of conserved peaks and number of clusters they called. A. Bar plots represent the relationship between proportion of conserved peaks (y axis) and the number of clusters these peaks were called in (x axis). B. Bar plots summarize the relationship between the number of clusters peaks were called in and their annotation based on genomic features. Peaks that were called in all clusters has higher proportion on exon-intron boundaries than peaks called in lower clusters. C. Bar plots represent the proportion of conserved peaks (y axis) and their annotation based on Genomic features (x axis).
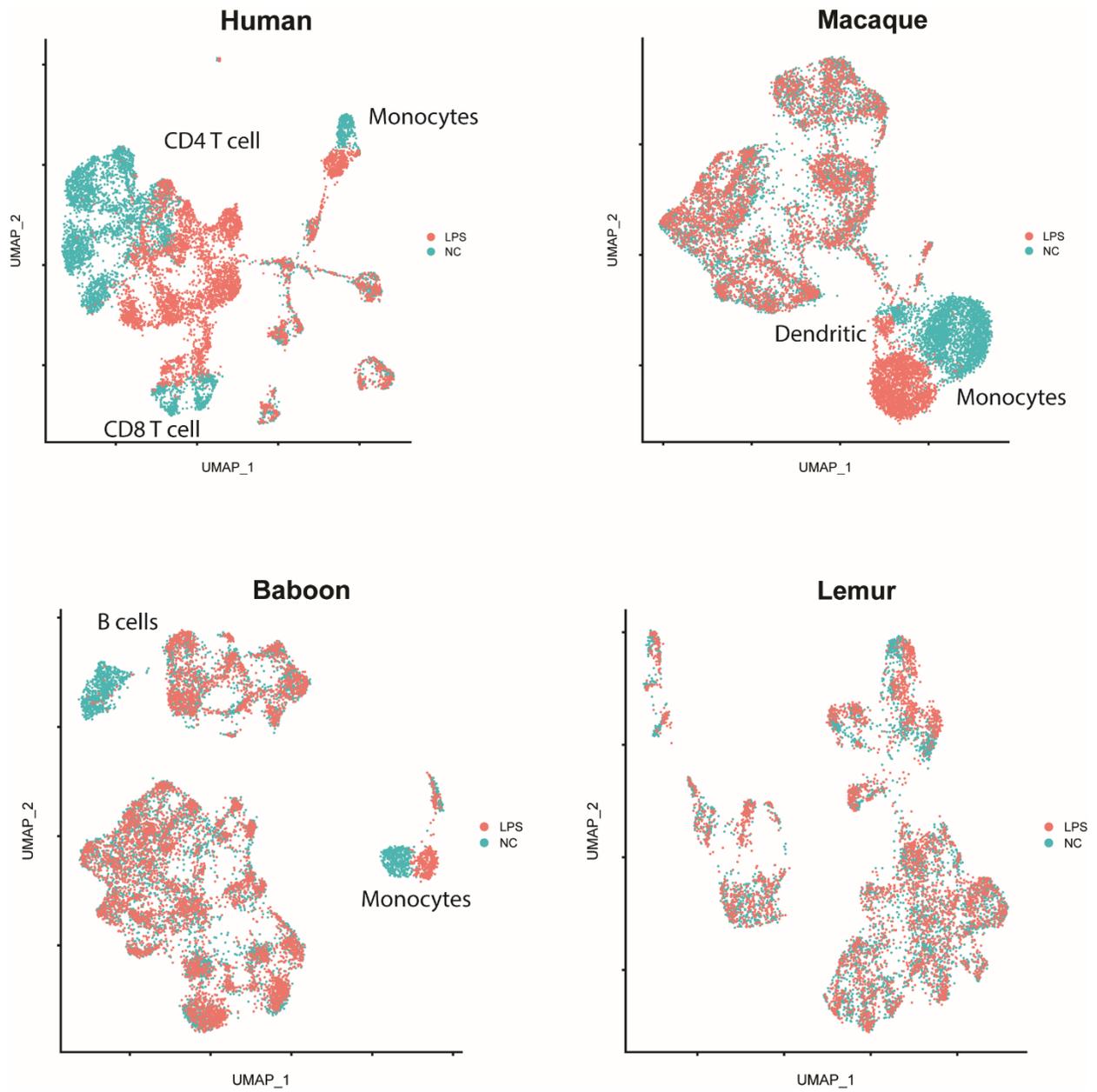
Supplementary figure S11. UMAP of cells from scATAC seq form all species without integration highlighting the difference between the two conditions (NC and LPS).
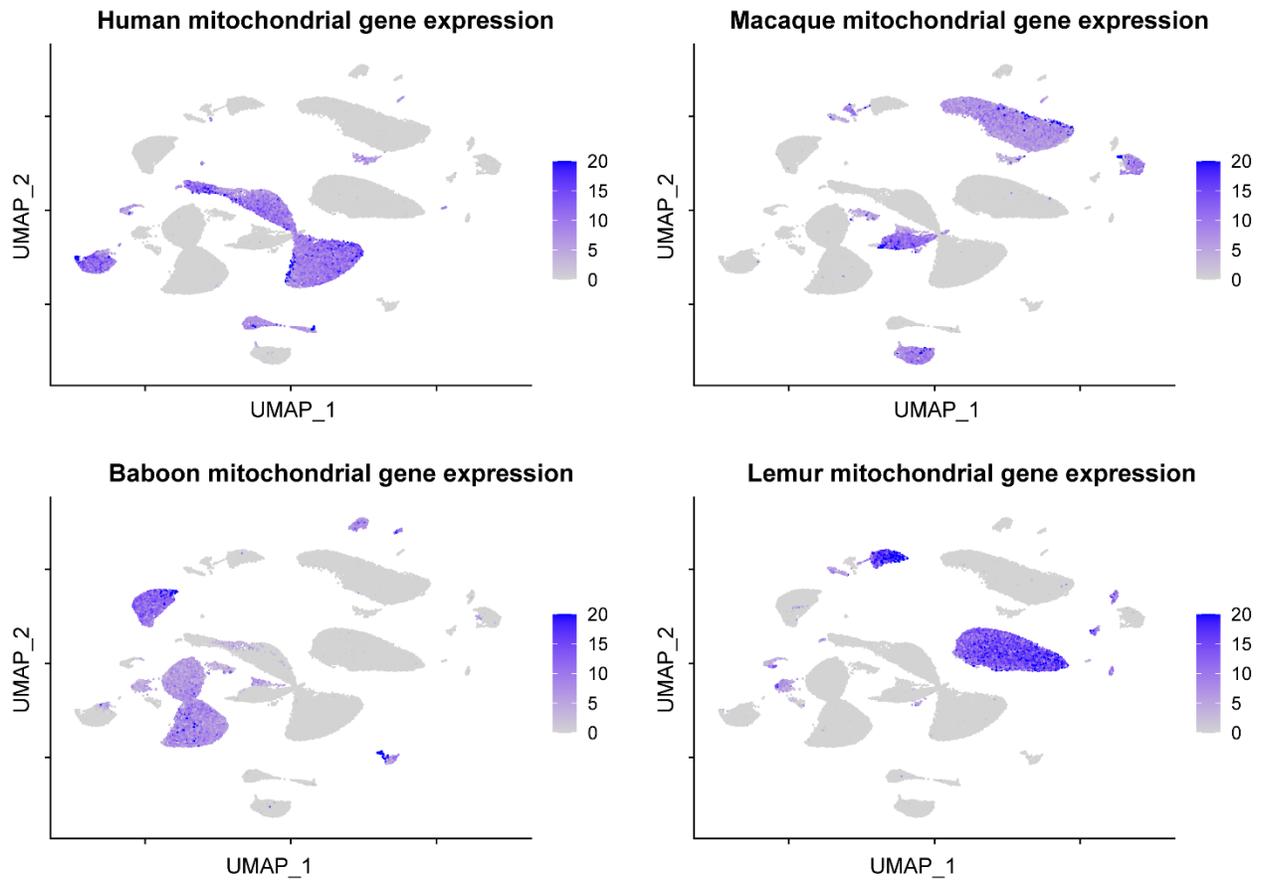
Supplementary figure S12. UMAP of PBMC cell from all species highlighting the uniqueness of mitochondrial gene expression of primate cells.
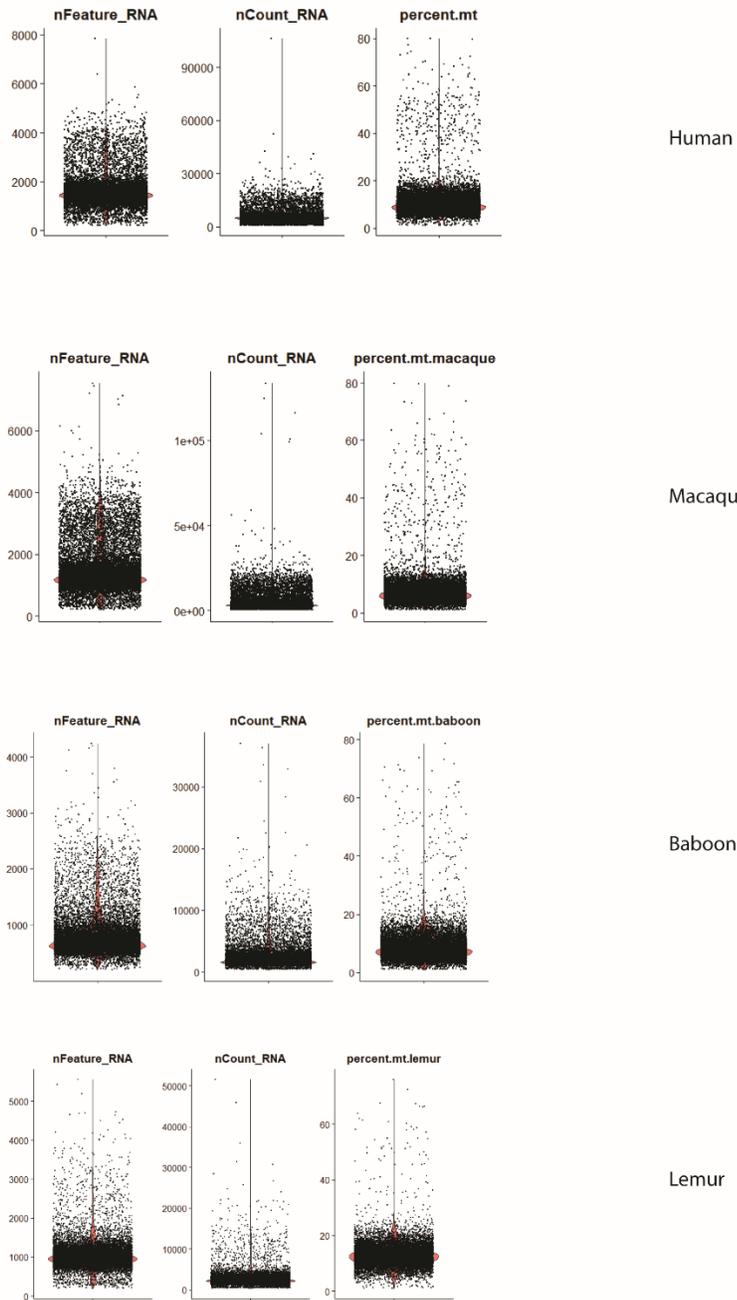
Supplementary figure S13. Distribution of QC parameters for cells from all species.
Distribution of cells from all species using 3 parameters used for quality control of cells namely
number of features (nFeature_RNA), total number of RNA molecules (nCount_RNA) and
percent of mitochondrial gene expression.

4. Discussion and perspectives

Mammals are the most diverse group of vertebrates. From species that fly in the air to ones that live in water, they are considered the most successful and dominant clade of vertebrates. Unique features of mammals such as extended longevity (bats) to species that do not develop cancers (elephants) have puzzled researchers for decades and motivated interspecies comparative studies (Ferris et al, 2018). Disease tolerance is one of those traits that show remarkable differences across mammals, especially primates. For instance, apes (such as humans and chimpanzees) were observed to be highly sensitive to minute amounts of lipopolysaccharide (LPS), a pathogen mimicry molecule, while specie of sister clade of African and Asian monkeys (AAMs), such as macaque and baboon, are far more resilient to high doses of LPS (Redl et al, 1993; Chen et al, 2019). The high sensitivity of humans to pathogens is a cause for several inflammatory diseases such as sepsis which is a leading cause for significant mortalities in humans (Delano and Ward, 2016). Why other primates do not have similar levels of susceptibility to chronic inflammatory disease remains poorly understood. In my PhD studies, I aimed to explore this question using approaches of functional genomics and single cell omics.

Cross activation of immune signaling is a novel cause for interspecies differences of inflammatory disease susceptibility.

I found apes (humans) to elicit the highest immune response to bacterial or viral stimulation. The high immune response is consistent with the observation of increased susceptibility of humans to chronic inflammatory disease and immunopathology relative to AAMs. I also found that apes engage an immune response to pathogens that is less specific than that observed among AAMs. Apes were found to trigger both antibacterial and antiviral response to bacterial or viral pathogens whereas AAMs showed higher specificity in their response, suggesting a tradeoff between strength and specificity (Figure 1). Such cross activation of immune response represents a novel cause for the difference of interspecies susceptibility to pathological manifestations across species. I proposed two possible reasons for this trend. Firstly, it could be a mechanism by host immune response to overcome immune evasion strategies by pathogens. Immune evasion is a key trait shared by many pathogens that involves manipulation of host defense mechanisms to establish

infection in the host body (Schmid-Hempel, 2007). For instance, one mechanism of immune evasion is interfering by secreting molecules that block the defense response signaling by the host (Schmid-Hempel, 2007). The efficiency of the immune evasion with more than one killing mechanism will be less efficient since if one pathway was blocked, the other pathway will mediate pathogen killing. Hence, pathogen targeting by more than one defense pathway results in higher restriction of the pathogen. This notion is supported by the observation that defense pathways can usually target both bacteria and viruses. For instance, interferon pathways, which are known to be mainly antiviral, were found to also target specific bacteria (McNab et al, 2015). However, the role of interferon pathways in bacterial killing remains controversial and limited to certain bacterial strains (Boxx and Cheng, 2016). This discrepancy of interferon pathway efficiency in bacterial targeting may make sense since if the pathway is mainly evolved as antiviral (McNab et al, 2015), bacterial pathogens may evolve counter adaptation for the pathway (Boxx and Cheng, 2016). Another possibility for the higher immune response in apes is that it is an adaptation to their longer lifespan (Zimmermann and Radespiel, 2013). In amphibian species, for example, lifespan has been shown to correlate with the immune strength, with species of a longer lifespan tending to elicit a stronger immune response in amphibian species (Johnson et al, 2012). Apes live longer than AMMs ~ 50 years while AAMs ~ 25-30 years which is consistent with what had been shown in amphibian species (Johnson et al, 2012). This explanation is supported when another primate species included, lemurs, which have a lifespan of ~ 15 years (Zimmermann and Radespiel, 2013), they have even less immune response strength than human and AAMs. Either of these explanations does not rule out the other. Despite the negative impact of the strong immune response as it is considered a major cause for immunopathology in humans, but it is also protective against lethal infections from highly virulent pathogens. One interesting experiment was performed that highlighted the difference between tolerance and resistance in mice. When TLR4-mutant mice were injected with LPS molecules from *Salmonella*, they lived longer than the wild type since the LPS molecule itself is harmless but when challenged with a virulent strain of *Salmonella*, they died faster than the wild type because the cost of virulence is higher than tissue damage by the immune response (O'Brien et al 1980; Ayres and Schneider, 2012). An extrapolation of this experiment reveals the importance of the strong immune response and that the associated tissue damage is a cheaper cost in case of highly virulent pathogens.

The causes for response integration observed here may be due to autocrine/paracrine activation of distinct pathways by cytokines secretion or crosstalk of intracellular pathways (Lee and Kim, 2007; Junger, 2011; Thaiss et al, 2016). For instance, the highly divergent pathway "TNFA signaling through NFKB" is detected in LPS stimulation where TNF is exclusively activated in apes (Caldwell et al, 2014). Further investigations are warranted to explore the molecular mechanism of the response integration.
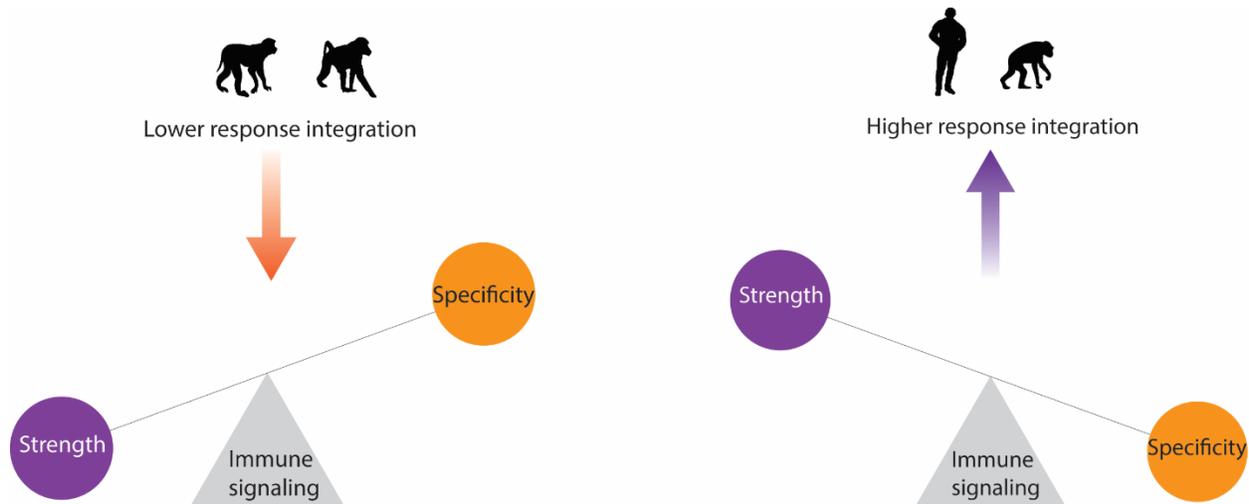
Figure 1. Diagrammatic illustration of a tradeoff relationship between strength and specificity of immune response in primates.

Species specific mechanisms of disease tolerance in primates.

I also found other potential mechanisms of disease tolerance in specific species namely, baboons. I found the major receptor for LPS in baboon, TLR4, not differentially expressed after stimulation unlike what is observed in all other tested primates (human, chimpanzee and macaque). Moreover, adaptors of TLR4, such as IRAK4, were found to be downregulated only in baboons. Baboons specifically were found to have the lowest immune response to LPS among apes and AAMs. Hence, the inhibition of TLR4 signaling may be another factor that contributes to the dampened immune response that baboons engage in response to immune stimulation. Reduction of the receptor expression is a mechanism by which toll-like receptors (TLRs) regulate their activation (Liew et al, 2005). The inhibition of recognizing receptors has been reported in primates such as CCR5 for HIV (Veazey and Lackner, 2017). Another interesting observation in baboons is the

higher base expression of specific innate immune genes. The high base expression of innate immune genes has been observed in certain species of bats that are known to have high disease tolerance (Zhou et al, 2016; Shaw et al, 2017). The high base expression of specific immune effectors in baboons may be a mechanism to partially compensate for the overall reduced response. For instance, while the majority of immune mediators will have low expression after stimulation due to the low response (low logFC) in baboons, the ones with high base expression will have high expression after stimulation since their base expression was already high.

Adaptive evolution drives the interspecies immune response divergence.

One key finding in my thesis is that adaptive substitutions were found to be significantly associated with the divergence of immune response observed across primates. The search for the signature of natural selection has historically been focused on the coding region of the genome (Rogers and Gibbs, 2014). However, more recently, selection on the regulatory region has been shown to drive several phenotypes in human (Gittelman et al, 2015; Dong et al, 2016). The majority of accelerated regulatory regions in human were found to be involved in neuronal functions and development (Gittelman et al, 2015; Dong et al, 2016) while accelerated regions on coding regions were enriched in immune related functions (Dong et al, 2016; Daub et al, 2017; van der Lee et al, 2017). In my study, I found a signal of positive selection on the regulatory regions of the immune response. Several mechanisms have been suggested to affect the immune response regulation across species such as transposable elements, promoter architecture and natural selection (Chuong et al, 2016; Danko et al, 2018; Hagai et al, 2018). Among those, I found adaptive substitutions in regulatory elements to be the factor that explains the immune response divergence which emphasizes on the role of selection in shaping the immune response across primates.

This thesis provides considerable insights on whole genome immune response in primates. This is, however, a first step in the long journey of understanding the molecular mechanisms associated with the observed differences in immune responses between humans and our closest relatives. Towards a more in-depth characterization of the mechanisms associated with the differences in transcriptional response observed additional experiments should follow. For example, one hypothesis to be tested is that the more overt response to immune stimulation observed in apes – humans, notably – as compared to other primates results from paracrine signals induced by specific cytokines (Trinchieri and Sher, 2007). Supporting that view, I found several inflammatory

cytokines that have higher response in apes. Among those, I found tumor necrosis factor alpha (TNF) to have higher response in apes vs. monkeys, especially upon viral stimulation. TNF is a well-known pro-inflammatory cytokine that has been tested for therapeutic treatment of inflammatory diseases including sepsis (Lv et al, 2014). TNF is a member of TNF protein superfamily that triggers TNF-mediated inflammatory pathways (Aggarwal, 2007). Interestingly, I found that TNF mediated signaling was the most divergent pathway between apes and monkeys upon viral stimulation. Hence, it is plausible that TNF plays a major role in driving the integrated response by activating the antibacterial defenses, which would otherwise be turned off during a viral infection.

To test this hypothesis, it would be interesting to conduct whole blood challenge with viral stimulant with and without TNF antagonists (e.g., blocking antibodies (Wong et al, 2008)). If TNF-dependent paracrine signaling is causal for the integrated response, I expect to find lower immune response in apes and more correlated response to monkeys in the absence of TNF signaling. Along the same line, interferons are important cytokines that mediate antiviral defenses and were found to be the most divergent pathways in bacterial stimulation. Analogous to the previous experiment, it would be interesting to evaluate how the immune responses of apes and monkeys would differ when using blocking antibodies against IFN-alpha (Duguet et al, 2021). These experiments should provide insights on the mechanism of integration and the role of released cytokines in such responses. One possibility is that there could be natural inhibitors in the serum of the tolerant species that reduce the inflammatory signal transduction across cell populations. Indeed, an interesting study found that proteins in the serum to mediate tolerance in LPS tolerant species (Warren et al, 2010).

In my studies, I identified several potential regulators linked to pathological conditions. Among the genes that had a higher response in monkeys after LPS treatment at 24h treatment is acyloxyacyl hydrolase, AOAH. AOAH is a well-known lipase enzyme that modulates the response to LPS by disrupting the fatty acid chains of the inner lipid A moiety, a component of LPS molecule hence detoxifies the molecule (Hall and Munford, 1983; Munford and Erwin, 1992). Other potential master regulator to the observed differences in the magnitude of the response engaged upon immune stimulation is TNFAIP3, which also showed higher response in monkeys

as compared to apes. TNFAIP3 is a gene that encodes for a protein that inhibits NFkB signaling and is a major regulator of immunopathology and inflammatory disease (Vereecke et al, 2009). Receptors are also key determinants of disease susceptibility as they are at the origin of pathogen-inducing immune signaling. TREM2, which is a receptor for lipid molecules involved in inhibiting TLR response, protecting therefore against excessive inflammatory responses (Hamerman et al, 2006; Turnbull et al, 2006), was found to be specifically upregulated in monkeys in LPS in both time points. These are just a few of the genes that could contribute to the observed differences in immune response between apes and moneys. To formally test their role to the observed differences, it would be interesting to perform knockout and/or overexpression experiments on these genes and ask the question if in their absence the responses between apes and monkeys would look more alike, which would support their causal role to immune divergence.

In my studies, I have also identified several regulatory elements that may govern the immune response in primates. Importantly, I found several putatively regulatory regions that showed higher epigenetic activity nearby genes that have a higher response in human vs other primates. Interestingly, these regulatory elements were enriched for signatures of adaptive evolution in humans. Divergence of regulatory elements is widely accepted to drive phenotypic differences between species. Point mutations, insertions or deletions are common mechanisms that drive regulatory elements divergence (Wittkopp and Kalay, 2011). It would be fascinating to use genomic editing tools to dissect the role of these regions in driving the stronger immune response in humans. One genomic editing methodology CRISPRpath that target regulatory elements of genes controlling the same pathway was recently developed (Ren et al, 2021). Future efforts should be made to use CRISPRpath to target regulatory elements associated with genes belonging to "response to bacterium" pathway, which were amongst the most significantly enriched in my GO analysis. This pathway was found to be consistently enriched when comparing simian primate vs lemur or human vs all species. Hence, I expect regulatory elements that control this pathway to play a profound role in driving the strong immune response in primates. The genome editing experiments followed by LPS stimulations will provide insights on the relative effect of different epigenetically active regions in driving the higher immune response. Targeting specific regulatory elements have been suggested to be used for medical therapeutics (Antoniani et al, 2017),

therefore, understanding the evolution of regulatory elements of innate immune response may have a direct benefit in translational medicine.

In conclusion, the studies in this thesis provide insights on the disease tolerance mechanisms and evolution across primates. I pointed to cross activity of immune response as a potential novel cause for the interspecies disease tolerance in primates which is, to my best knowledge, was not highlighted before. Also, I provided the most comprehensive transcriptomic and regulatory landscape of immune response of primates at the resolution of single cell in the complex immune cells of PBMC in this thesis.

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6. List of publications

- **Hawash, M.B.F**, Joaquin Sanz-Remón, Jean-Christophe Grenier, Jordan Kohn, Vania Yotova, Zach Johnson, Robert E. Lanford, Jessica F. Brinkworth, Luis B. Barreiro. Primate innate immune responses to bacterial and viral pathogens reveals an evolutionary trade-off between strength and specificity (Proceedings of the National Academy of Sciences Mar 2021, 118 (13) e2015855118; DOI: 10.1073/pnas.2015855118).

- **Hawash, M.B.F**, Azmi Al-Jubury, Mita Eva Sengupta, Tina Vicky Alstrup Hansen, Stig Milan Thamsborg, Peter Nejsum, Evidence for mitochondrial pseudogenes (numts) as a source of contamination in the phylogeny of human whipworms. Infection, Genetics and Evolution, 2020, https://doi.org/10.1016/j.meegid.2020.104627.

- Valerie Abadie, Sangman Kim , Thomas Lejeune , Brad Palanski , Jordan Ernest , Olivier Tastet ,Jordan Voisine , Valentina Discepolo , Eric Marietta , **Hawash, M.B.F**, Cezary Ciszewski ,Romain Bouziat , Kaushik Panigrahi , Irina Horwath , Matthew Zurenski , Ian Lawrence , AnneDumaine , Vania Yotova , Jean-Christophe Grenier , Joseph Murray , Chaitan Khosla , Luis Barreiro,Bana Jabri, 2020. IL-15, gluten and HLA-DQ8 drive tissue destruction in coeliac disease. Nature

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- **Hawash, M.B.F**, Betson. M, Al-Jaburi, A., Hansen, T. V., Xie, L., Ketzis, J., Willingham A.L., Bertelsen, M. F.,Cooper P. J., Littlewood, D. T. J., Zhu, XQ, Nejsum, P. Whipworms in humans and pigs: origins and demography. Parasites & vectors. 2016;9(1):37. doi: 10.1186/s13071-016-1325-8. PubMed PMID: 26800683

- **Hawash M.B.F**, Andersen LO, Gasser RB, Stensvold CR, Nejsum P. Mitochondrial Genome Analyses Suggest Multiple *Trichuris* Species in Humans, Baboons, and Pigs from Different Geographical Regions. PLoS Negl Trop Dis. 2015 9(9): e0004059. doi:10.1371/journal.pntd.0004059

- Meekums, H**., Hawash M.B.F**., Sparks, A., Oviedo Y., Sandoval C., Chico, M. E., Stothard, J. R., Cooper, P. J., Nejsum, P., Betson, M. "A genetic analysis of *Trichuris trichiura* and *Trichuris suis* from Ecuador" Parasites and Vectors 2015; 8: 168. doi: 10.1186/s13071-015-0782-9