MODELING HUMAN GENETIC VARIATION IN NON-CODING DNA IN HUMANIZED MICE

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ABSTRACT

The increasing availability of sequencing data from genome-wide association studies and whole genome sequencing of the human genome has enabled rapid identification of genetic variations—mainly single nucleotide polymorphisms (SNPs)— in non-coding DNA of the human genome. However, it has been difficult to find the biological functions of the numerous SNPs in the genome. This gap in knowledge can be explained in part by our poor understanding of the function of non-coding DNA, and by the challenge of experimentally assigning function to SNPs that map to these non-coding regions. To clearly define the function of non-coding SNPs, we created genetically humanized mice to model human genetic variation in non-coding DNA *in vivo*. To generate the mice, we used a bacterial artificial chromosome (BAC) system harboring two genetically different human *IL10* SNP haplotypes. The *IL10* SNP haplotypes are "ATA" and "GCC," which have been associated with differential IL-10 levels and disease susceptibility in humans. We found a robust allele-specific human IL-10 expression in both macrophages and CD4+ T cells. Specifically, GCC-hIL10BAC encodes for a high human IL-10 level relative to ATA-hIL10BAC in CD4+ T cells both *in vitro* and *in vivo*. The reverse was observed in macrophages. Accordingly, by complementing *Il10* null mice with the GCC-hIL10BAC, namely *Il10-/-*/GCC-hIL10BAC mice, we were able to completely reverse disease outcome. The *Il10-/-*/GCC-hIL10BAC mice were susceptible to persistent leishmania infection as evidenced by a high parasite burden in the liver and spleen. In contrast, like *Il10* null mice, the *Il10-/-*/ATA-hIL10BAC mice were refractory to disease. Therefore, our data demonstrate that human *IL10* promoter SNP haplotypes alone can modulate IL-10 levels and disease risk. In the second part of this dissertation, we examined the regulation of IL-10 and its homolog, IL-24, as a means to indirectly demonstrate that we are not missing important regulatory elements within the

hIL10BAC. We chose IL-24 from the remaining cytokines within the *Il10* gene cluster because the gene encoding for IL-24 is localized at the extreme end of the *Il10* locus in both mouse and man and also human *IL24* gene is not included in the hIL10BAC. Thus, finding co-regulation of IL-10 and IL-24 expression would suggest that the two homologs share common regulatory elements. Interestingly, we found that IL-10 and IL-24 are regulated by distinct cell-type-specific regulatory pathways. Optimal IL-24 expression requires Stat6 and Stat4 in macrophages and NK cells; meanwhile, IL-10 expression is independent of Stat6 and dependent on Stat4 only in IL-12-treated NK cells. We also discovered an unexpected role for Type-I Interferons in mediating differential regulation of IL-10 and IL-24 expression in macrophages and NK cells. Thus, our results suggest that IL-24 and IL-10 are unlikely to share common regulatory elements within the *Il10* locus. Altogether, our results undoubtedly demonstrate that we can model human genetic variation in non-coding DNA *in vivo* using genetically humanized hIL10BAC mice. In the future, the hIL10BAC approach can be extended to other human genes to accelerate rational development of safe and efficient personalized therapies, including vaccines.

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Chapter 1

General Introduction

1. Humanized mice

1.1. Humanized mice: Rational and model systems

Laboratory mice have been instrumental in advancing our basic understanding of mammalian biological systems. Most current paradigms of human biology have been elucidated primarily in mice because inbred mouse strains are more amenable to genetic manipulation for studying the effects of specific genes in the absence of environmental factors. In fact, ~99% of mouse genes have detectable homologues in the human genome. Thus, many orthologous mouse and human genes such as receptors, growth factors, and transcription factors have similar functionalities. Similarly, global gene-expression profiles are conserved between mouse and human cells, particularly in lineage-specific genes(1), strengthening the utility of mice as the model of choice to study human biology and disease.

Despite these similarities, there are many instances where mouse studies do not provide accurate model systems to study human biology, particularly in the field of gene regulation. For example, unlike coding regions which are well conserved between species, regulatory regions in non-coding DNA, which coordinate interspecies differences in gene expression between mammals(2), are highly variable between mouse and human genomes(3). In addition, ~80 million years of evolutionary distance separate mice and humans; thus, a number of species-specific differences between the mouse and human immune systems have been noted(4). These inter-species differences in gene expression are underscored by the observation that numerous pathogens—including HIV, hepatitis viruses, and the malaria pathogen *Plasmodium falciparum*—have a specific tropism for human tissues but not for mice. Thus, new drugs to treat these human-specific pathogens are most often hindered by lack of reliable, cost-effective, and predictive animal models that fully mirror human disease phenotypes. Accordingly, ~90% of new therapeutics that show potency in traditional mouse studies fail in clinical trials(5).

For several years, non-human primates (NHP) such as chimpanzees have been used as an alternative to small animal models in drugs and vaccines development because chimpanzees are the closest living animals to humans. Genetic variability between human and chimpanzee DNA sequence is ~5%[6], suggesting that preclinical studies in chimpanzee are more likely to yield a more reliable surrogate for subsequent human trials. The use of chimpanzees in biomedical research, however, has been forbidden in many countries in Europe due to ethical issues, and in 2011 the U.S. National Institutes of Health (NIH) stopped funding new research on chimpanzees in the United States. Therefore, chimpanzee studies are now limited to a handful of outbred animals which (as in humans) are hampered by inter-individual variability and hence are barely reproducible.

To address the limitations of using small animals as preclinical model systems, researchers have developed novel mouse models—referred to as "humanized mice"—that closely recapitulate human biological systems. A humanized mouse is a mouse in which functional human cells, tissues, or gene cassettes have been implanted. The most common models are mice with a functional human immune system (MHIS). MHIS are made by reconstituting immunodeficient mice with human peripheral blood cells (PBLs), human hematopoïc stem cells (HSCs), or lymphoid tissues that support the generation of a human immune system[7-9]. Examples are immunodeficient mice engrafted with human bone marrow, fetal liver and thymus fragments (BLT mice)[10,11], or mice with a defective IL-2 receptor common gamma chain gene ($Il2r\gamma^{null}$), which is required for T, B and NK cell development, reconstituted with the aforementioned human cells or tissues[12-14].

Although MHIS have been very useful for studying human-specific pathogens and for testing human therapeutics, they have many limitations. The main limitation is that they do not

account for inter-individual variability in gene expression in humans. Current MHIS are generated on inbred mouse strains, which do not account for genetic differences in people. Thus, MHIS can be used for preclinical testing of drugs and vaccines, but cannot be used to investigate how different individuals respond differently to specific compounds and vaccines. This major drawback of MHIS can be overcome by having a mouse system in which one can model human genetic variation in non-coding DNA. To our knowledge that mouse does not exist yet; thus, the main goal of this dissertation is to generate a genetically humanized mouse model to investigate the impact of inter-individual variability on gene expression and disease susceptibility.

Other limitations of MHIS include the finding that species-specific factors such as HLA, cytokines, growth factors, and homing factors for tissues trafficking are structurally different between human and mice. For example, current MHIS cannot support development of human innate immune cells, including NK and myeloid cells(15). Additionally, the effects of mouse innate immunity on engrafted xenogeneic human cells can diminish the efficiency of the engraftment(7,9). The absence of mature lymph nodes, disorganization of lymphoid structure, and poor antibody responses due to impaired affinity maturation and class-switching have also been noted in MHIS(7,9). These limitations can be overcome by injecting exogenous soluble factors(16,17) or by hydrodynamic tail vein injection of DNA plasmid encoding human cytokines(18,19). Additional technologies include transduction of human stem cells (HSCs) with mouse-specific factors such as mouse CD47, which is recognized by SIRPα expressed on phagocytic cells as a "do not eat me signal" to prevent engulfment of human cells by mouse macrophages(20-23). Transgenic expression of human-specific factors(21,24) and genetic alteration of mouse-specific genes to reduce host innate immunity have also been described(7,25).

1.2. Transgenic humanized mice

Transgenic humanized mice have been made to solve some of the limitations of MHIS. Examples of Transgenic humanized mice include mice carrying an exogenous gene cassette such as transgenic expression of cDNA constructs, Knock-in systems, and bacterial artificial chromosome (BAC) technology(9,26). For the first approach, cDNA of a human gene is expressed under the influence of tissue-specific or ubiquitous promoters(27). This is achieved by directly injecting the cDNA construct into fertilized eggs of a female donor. The approach is often used for overexpression of a specific human gene to study function not regulation (because the cDNA construct lacks regulatory regions). The second approach is to replace the entire mouse gene (including exons and introns) with the human counterpart. Therefore, the human gene is expressed under the control of mouse regulatory DNA, which is not ideal for assessing human gene regulation. In contrast to the cDNA approach, Knock-in system relies on the use of embryonic stem cells (ES) as the basis to replace the mouse locus by the corresponding human gene by homologous recombination. Then, modified ES cells are implanted into the blastocysts to generate chimeras. Chimeric mice are later bred with appropriate mouse strains to obtain the desired genotype. Therefore, the knock-in approach is technically challenging and time-consuming (at least 1-2 years)(26). Moreover, ES cells are not available for all mouse strains, which limit the application of this strategy to only a few mouse strains. A third approach is to use large human genomic DNA cloned into a BAC vector. BACs are single-copy, F-plasmid cloning vectors that are often used to generate genomic libraries since they can support faithful segregation of giant inserts (100-300kb) into daughter cells(26,28). Unlike knock-in mice, the expression of the human gene in the BAC is controlled by human regulatory DNA which is more appropriate for studying human gene regulation. Because the BAC insert is large enough, it is

more likely to contain most if not all distal regulatory elements to facilitate gene expression at physiological levels[26]. Additionally, the mouse gene is not replaced; thus, both mouse and human genes are expressed, providing the opportunity to study species-specific differences in gene expression within the same mouse.

There are many benefits in using BACs over Knock-in technology, including speed and ease to make BAC transgenic mice. For example, when the appropriate BAC clone is identified, it takes only 3-6 months to get the founder mouse. There is no need for ES cells because the BAC insert is directly microinjected into fertilized eggs that are later implanted in the oviducts of pseudo-pregnant mothers. Furthermore, because of its size, the BAC transgene is insulated from chromatin interference, hence minimizing the positional effects on transgene expression[29]. Limitations of BAC transgenesis include difficulty assessing structural integrity of the construct once it is inserted into the mouse genome, copy-number effects on transgene expression, and bystander transgene effects due to expression of genes within the BAC other than the gene of interest[26].

In the following sections, I will present current humanized mouse models to study human gene regulation and genetics to set the stage for the work done in this dissertation.

1.3. Humanized mouse models for modeling human gene regulation

Studying human gene regulation can be challenging not only because of the inter-individual variability in gene expression among people but also because of the difficulty of getting tissue samples from healthy subjects. In fact, most human studies are limited to PBMCs that do not necessarily recapitulate tissue microenvironment of a living organism.

The mouse model of Down syndrome (also known as trisomy 21) is one of the first successful humanized mice for studying human gene regulation *in vivo*[30]. Down syndrome is a genetic disorder caused by the presence of an additional copy or part of human chromosome 21. This syndrome can be mimicked in mice by generating trans-species aneuploidy mouse strains harboring a copy of human chromosome 21 in addition to the entire set of mouse chromosomes[31]. Studies using this model have shown that more than 80% of human genes are expressed in mouse tissues[31,32]. The authors also confirmed that hepatocyte-specific gene expression and transcription factors binding patterns in this mouse mimic that of normal murine hepatocytes[30]. Because human chromosomes are studied exclusively in the context of murine transcriptional machinery and tissue microenvironment, the investigators clearly showed that DNA sequence rather than interspecies differences in epigenetics programs is responsible for driving species-specific gene expression[30]. Thus, this mouse was instrumental in demonstrating the utility of humanizing mice for studying not only human gene regulation but also human genetics.

BAC transgenic mice are currently the most popular model for examining human gene regulation *in vivo* because of the aforementioned advantages, but also because efficient homologous recombination technologies are now available to modify BAC construct in *Escherichia coli*[33]. These modifications include fusion of overlapping BACs, truncation of

putative regulatory elements within a BAC insert, and insertion of a reporter gene. An example of human BAC transgenic mice is the *IFNG* BAC mouse developed by Aune and colleagues to determine the role of distal regulatory regions within the human *IFNG* locus[34]. Using these mice, they confirmed that human IFN-γ is appropriately expressed in T lymphocytes and regulated by T-bet and Stat4, as one would expect. When they generated several transgenic strains with specific deletion of the BAC construct, they discovered a conserved non-coding region (CNS) located 30kb upstream of the transcription start site of the *IFNG* gene is necessary for human IFN-γ expression in T cells and NKT cells but not in NK cells. The CNS site was the target of transcription factor Runx3 and it is required for recruitment of RNA polymerase II to the *IFNG* locus. Thus, this study demonstrated the usefulness of BAC mice in studying human gene regulation.

About 10 years ago, our own group generated a human *IL10* BAC transgenic mouse (also referred as hIL10BAC) to study human IL-10 regulation and function *in vivo*[35]. Because human IL-10 is biologically active in mice, we showed that we can faithfully study both regulation and function of the human gene *in vivo*. We demonstrated that regulation of human IL-10 expression is cell-type-specific. Specifically, human IL-10 was appropriately expressed in the myeloid compartment, which was sufficient to protect mice from sepsis[35]. Similarly, IL-10 was also expressed by regulatory T cells in the gut, which confers protection from colitis induced by gut microflora[36]. Human IL-10, however, was weakly produced in splenic Th1 cells compared to mouse IL-10. Low human IL-10 in T_H1 cells protected the mice from persistent leishmaniasis (as IL-10 plays a pathogenic role during leishmaniasis)[35]. Because the hIL10BAC carries a human IL-10 promoter haplotype associated with low IL-10 levels in humans[37-39] and also resistance to cutaneous leishmaniasis, there was a possibility that human IL-10 expression in the hIL-10BAC

mouse is genetically controlled. Yet, at that time, we were not able to address this hypothesis due to the lack of a complementary mouse strain hosting a different human *IL10* haplotype. Accordingly, in the next section, I will discuss the concept of humanizing mice for studying human genetics.

1.4. Humanized mouse models for modeling human genetics

Studies looking at the association between genetic variants in the whole genome and human disease phenotypes are increasing dramatically. However, it is not clear whether these genetic variants—particularly those in non-coding DNA—have a functional/regulatory role. Therefore, there is a need for better tools to validate candidate variants in the context of human regulatory DNA to clearly link genotype to phenotype. Genetically humanized mice can be used for multiple purposes: (1) to model inter-individual variability in gene regulation patterns, (2) to identify subtle changes in transcription factor binding sites, (3) to assess allele-specific gene expression in specific cells or tissues, (4) investigate changes in mRNA splicing and stability, and (5) to test new therapies and vaccines at the preclinical stage.

In the literature, most of the successful genetically humanized mice produced to investigate human genetics were generated to model common monogenic disorders such as cancers, hemoglobinopathies, and Huntington's disease[40]. These mice are generally made by complementing a mouse strain that is null for a specific mouse gene with wild-type or mutant human genes. Because human and mouse genes have similar functionalities and tissue expression patterns, the disease phenotype (most often embryonic lethality) is monitored in mice carrying one of the human alleles. Examples of such mice are the human BRCA1 transgenic

mouse[40-42], the human beta-globin transgenic mouse[43], and the human Huntington transgenic mouse[44].

Emerging technologies that have the potential to advance the field of genetically humanized mice include zinc-finger nuclease (ZFN)[45,46],transcription activator-like effector nuclease (TALEN)[47-49], and Clustered Regularly Interspaced Short Palindromic Repeats CRISPR/Cas9 (CRISPR) technologies[49-51]. Briefly, these nucleases are used to create double-stranded DNA breaks at specific locations of the genome, thus allowing introduction of the desired genetic modifications to target DNA sequence in the presence of a Donor DNA[7]. A key advantage of using these technologies is that DNA modification can be directly performed in fertilized oocytes, thus there is no need for ES cells (which are not available in all mouse backgrounds)[7]. Conversely, major limitations include low efficiency when dealing with large modifications, off-target edition of the genome, and mosaic gene expression due to the remaining nuclease activity[7]. A successful example of genetically modified mice using CRISPR was recently made for human Tyrosenemia Type I (HTI)[52]. HTI is a fatal genetic disease caused by a deficit in Fumarylacetoacetate hydrolase (FAH), an enzyme which catalyzes the last step of the tyrosine degradation pathway in hepatocytes and renal tubal cells. The authors employed an existing mouse model of HTI that carries the same homozygous single base exchange G/A in the last base pair of exon 8 of the human *FAH* gene (the mouse is known as *Fah$^{mut/mut}$*)[53] . This mutation creates a splice variant that gives rise to a truncated and unstable *Fah* protein. The *Fah$^{mut/mut}$* mice died from severe liver damage due to accumulation of toxic metabolites. By injecting a large volume of CRISPR components to correct the defective mutation, they were able to rescue the *Fah$^{mut/mut}$* mice from disease as measured by the loss of body weight[52]. Specifically, in mice in which the mouse *Fah* gene has been repaired, the hepatocytes can

expand and repopulate the liver similar to normal wild-type mice. Thus, this study demonstrated the feasibility of genome correction *in vivo* in adult animals to model a human genetic disorder.

1.5. Utility of genetically humanized mice in post-GWAS era

The introduction of genome-wide association studies (GWAS) and whole genome sequencing has enabled rapid identification of genetic variants, mainly single nucleotide polymorphisms (SNPs) in non-coding DNA, that are enriched in diseased subjects compared to traditional candidate gene analysis[54]. However, it has been challenging to determine the biological function of the myriad of non-coding SNPs in the human genome. This gap in knowledge can be explained in part by the difficulty in defining the function of non-coding DNA, but also controlling for confounding variables in human studies. The confounding variables can be environmental factors, gene-gene interactions, or other SNPs in the human genome that are potentially in linkage disequilibrium with each other over several hundred kilobases across the genome[55]. GWAS variants also tend to have a small effect size, which means that they can explain only a small fraction of known disease heritability[55]. Furthermore, most human studies are limited to surrogate *in vitro* systems such as PBMCs or cell culture. Altogether, these limitations have led to a number of inconsistencies between studies. For this reason, the 2nd chapter of my dissertation will focus on developing a genetically humanized mouse model that would be employed to clearly define effects of non-coding SNPs on gene expression and disease risk.

Since human IL-10 is the target gene in our BAC transgene, the following section will briefly introduce what is known about genetic control of IL-10 expression.

2. Genetic control of human Interleukin-10 expression

2.1. Polymorphisms in the *IL10* gene

IL-10 is a potent immune-regulatory cytokine that plays a key role in controlling excessive inflammation and autoimmune pathologies[56]. IL-10 exerts its anti-inflammatory function mostly through blockade of the production of pro-inflammatory cytokines by innate antigen-presenting cells[57]. IL-10 also inhibits the development of CD4+ Helper T cells, including T_H1, T_H2, and T_H17, which are the cells that mediate host-protective immunity but also immuno-pathology[57-59]. IL-10 has been associated with various human diseases, including infectious and autoimmune diseases. These studies are based either on levels of IL-10 or on genetic polymorphisms in the *IL10* gene. In fact, there is wide inter-individual variability in IL-10 levels, leading to the hypothesis that differential IL-10-producing capacity among people may be genetically determined. Accordingly, heritability in IL-10 production—the proportion of variability in IL-10 levels that can be solely explained by genetics—can be as high as 75%[60].

Several polymorphisms, including microsatellites and single-nucleotide polymorphisms (SNPs) in non-coding DNA, have been discovered in the *IL10* locus[61]. The microsatellites (*IL10*-R and *IL10*-G) are two CA repeats at ~1.2kb and 4kb from the transcription start site[62]. The focus among the SNPs has been on three common polymorphisms at the proximal promoter of *IL10*, including -1082 G>A (rs1800896), -819 C>T (rs1800871), and -592 C>A (rs1800872)[39]. The alleles at position -819 and -592 are in complete linkage disequilibrium with each other, which means that they are inherited together. They make up three common haplotypes in human populations: GCC, ACC, and ATA. Frequencies of the haplotypes in Caucasians are 51% (GCC), 28% (ACC), and 21% (ATA)[39]. However, haplotype frequencies

also vary depending on ethnicity. For example, the GCC haplotype is found in only ~5% in Asian descents while it can be as high as ~33% among African-Americans[63].

So far, 21 SNPs have been identified within the *IL10* locus (spanning ~4kb from the transcription start site to the last exon)[64]. Gibson and colleagues initially described three non-coding SNPs among them as being distal *IL10* haplotypes (65): rs1800890 (-3575 T>A), rs6703630 (-2849 G>A), and rs6693899 (-2763 C>A). Together, they form 8 distal haplotypes out of which 3 are common in Caucasian populations: TGC, AAA, and AGA at the frequency of 51%, 26% and 13%, respectively[65]. Therefore, the 3 proximal SNPs may belong to a larger haplotype that may extend several kilobases away from the transcription start site. A graphical summary of the genetic variation in *IL10* locus discussed in this chapter is presented in Figure 1.1.

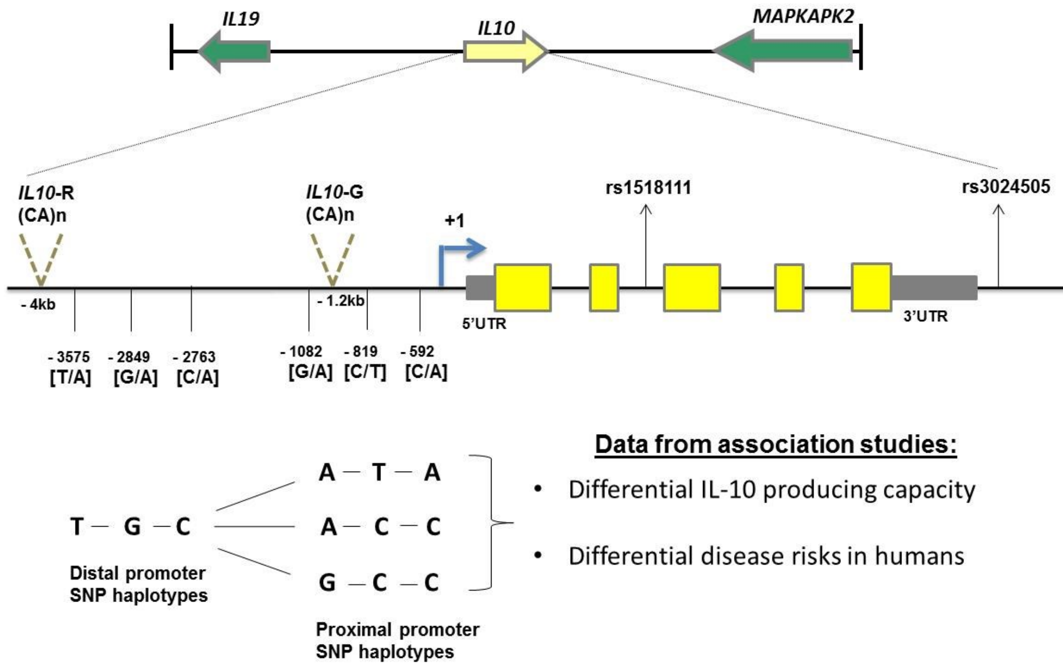


Figure 1.1. A graphical summary of common genetic variations in non-coding DNA of the *IL10* gene discussed in this chapter. The variants are microsatellites (*IL10*-R and *IL10*-G) and single-nucleotide polymorphisms (SNPs) in the promoter (distal and proximal SNPs), in intron 2 (rs1518111) and near the 3'UTR (rs3024505). Yellow boxes are exons 1-5 of *IL10*, gray boxes are UTRs.

2.2. *IL10* promoter SNP haplotypes and IL-10 expression

Crawley et al. were the first to investigate functional consequences of *IL10* promoter haplotypes on IL-10 expression using a luciferase reporter assay[66]. They transfected a monocytic cell line (U937) with a pGL3 vector carrying part of the promoter (from -1137 to +25 upstream of the transcription initiation site) of homozygous individuals for the *IL10* promoter haplotypes GCC, ACC, and ATA. The ATA construct had the weakest IL-10 transcriptional activity, ACC was medium level, and the GCC had the highest expression level. In agreement with this data, when they assayed IL-10 levels in whole blood stimulated with LPS, they found that IL-10 levels were low in individuals homozygous for ATA when compared to GCC carriers.

Another group recently carried out similar studies to understand the molecular basis of human *IL-10* transcription in macrophages[38]. They transfected a murine macrophage-like cell line (RAW264.7) with a luciferase reporter construct containing a region of *IL10* promoter (-1105 and +30). The constructs were engineered to harbor one of the -1082 SNP alleles (A or G, representing ATA and GCC haplotypes, respectively). When they stimulated the cells with LPS or apoptotic cells, -1082G had significantly more transcriptional activities than -1082A. Allele-specific IL-10 expression in macrophages stimulated with apoptotic cells but not LPS was found to be regulated by poly(ADP-ribose) polymerase 1 (PARP-1), a transcriptional repressor that physically interacts with the *IL10* promoter.

Along the same line, Steinke et al. also examined functionality of the -592C>A allele (formerly called -571) in Raji cells (a human B cell line that constitutively expressed IL-10) using similar luciferase reporter construct[67]. The presence of an A allele increases *IL-10* promoter activity by 3.1-fold compared to a construct carrying a C allele at the same position. Remarkably, when they generated reporter constructs with specific deletions of

the *IL10* promoter, the construct that includes -592C was found to have a reduced transcriptional activity (3.3-fold less) than any other constructs, suggesting the presence of a transcriptional repressor that is created by the C allele at this position. They also found that both alleles can bind to Sp1 and Sp3 transcription factors with similar affinity by means of EMSA and Supershift assays. Additionally, reconstitution of Sp1 expression in a Drosophila cell line lacking Sp family of proteins restored *IL10* promoter competency in all constructs but not the one carrying the C allele. Thus, the findings indicate that a C to A mutation at position -592 increases IL-10 production in human B cells, which is not in agreement with studies focused on human monocytes.

Despite these discrepancies between studies, the majority of the literature points to an increased expression of IL-10 in GCC (considered as a high IL-10 producer) over ATA carriers (low IL-10 producer). For instance, when Suárez and colleagues assayed PBMCs from 128 healthy Caucasian donors, they found a higher constitutive mRNA expression in individuals homozygous for GCC compared to ATA carriers[(68)]. Similarly, healthy blood donors from Turkey and the United States were classified as high and low IL-10 producers based on an intronic *IL10* SNP (rs1518111 G>A) known to be in tight linkage disequilibrium with *IL10* promoter haplotypes[(69)]. In the study, the donors carrying one or two G alleles of the intronic SNP expressed a more robust IL-10 level in PBMCs stimulated with LPS but also purified monocytes activated with TLR2 agonists, relative to A allele carriers.

2.3. *IL10* polymorphisms and disease susceptibility

Most of our current understanding regarding the role of IL-10 in diseases has been established in mice. In contrast, the majority of studies investigating the role of IL-10 in human diseases have been done by means of association studies. In fact, dysregulation of IL-10 production has been associated with numerous human diseases, including infectious and autoimmune diseases. These associations are based on either levels of IL-10 or non-coding SNPs in the *IL10* gene. Some examples of these studies will be presented in the following paragraphs.

2.3.1. Infectious diseases

Human *IL10* promoter SNPs have been linked to susceptibility to various chronic viral infections (HIV/AIDS and HBV), parasitic diseases (leishmaniasis and malaria), and bacterial infections (pneumonia and gastritis). In HIV-infected persons, individuals carrying the ATA haplotype (the low IL-10 producer) have an augmented risk of acquiring HIV and, once infected, progress more rapidly to AIDS[(64,70)]. These patients have lower levels of plasma IL-10, high viral load, and low CD4 count[(71,72)]. In contrast, the ATA haplotype is more enriched in asymptomatic HBV carriers compared with subjects experiencing chronic liver disease[(73)]. In leishmaniasis, the presence of a C allele at position -819 is associated with an increased risk of developing cutaneous lesions[(74)]. Conversely, the A allele (-819A) augments the risk of gastritis in humans colonized with *Helicobacter pylori.* In addition, individuals carrying -1082A are less susceptible to develop cerebral malaria[(75)] or septic shock following infection with *Streptococcus pneumoniae*[(76)]. These observations confirm the importance of IL-10 in regulating immune responses to infectious pathogens.

2.3.2. Autoimmune diseases

Polymorphisms in the *IL10* gene have been implicated with many autoimmune diseases. For example, the ATA haplotype has been associated with susceptibility to Type-I diabetes(77), ulcerative colitis(78), Sjogren's disease(79), severe juvenile rheumatoid arthritis(66) and psoriasis(80), suggesting that having the ATA haplotype predisposes to inflammatory diseases. In addition, recent genome-wide studies (GWAS) have demonstrated strong association between polymorphisms in the *IL10* gene with Bechet's disease (BD)(69,81) and systemic lupus erythematosus (SLE)(82). The A allele of rs1518111 of *IL10* has been associated with an impaired mRNA transcription and protein levels in BD patients(69). Similarly, the T allele of rs3024505, a variant found near the 3'-untranslated region of *IL10* (UTR), increased the risk of ulcerative colitis(78), Crohn's disease(83) and SLE(82). Therefore, these studies strongly implicate genetic variants in non-coding DNA that are associated with low levels of IL-10 as risk factors of autoimmune diseases.

2.4. Genomic boundaries of the human *IL10* locus

When the initial hIL10BAC mouse was generated, we found a weak human IL-10 expression in CD4+ T cells. One possibility was that we may be missing regulatory elements required for normal human IL-10 expression in CD4+ T cells in the hIL10BAC. Since the exact genomic boundaries of the IL-10 locus in both mouse and human (Figure 1.2) —which also contains IL-19, IL-20, and IL-24 (all members of the IL-10 family of cytokines)—remains to be defined and also that our hIL10BAC does not contains *IL20* and *IL24*, we took the approach of studying co-expression of IL-10 along with IL-24 which is located at the far end of the locus. The idea being if IL-10 and IL-24 are co-expressed or co-regulated, they may be sharing common regulatory elements within the locus that govern their cell-type-specific co-expression. For this reason, I will give some background information about IL-24 (in the next section) and characterize its co-expression with mouse IL-10 in Chapter 3 of this dissertation.

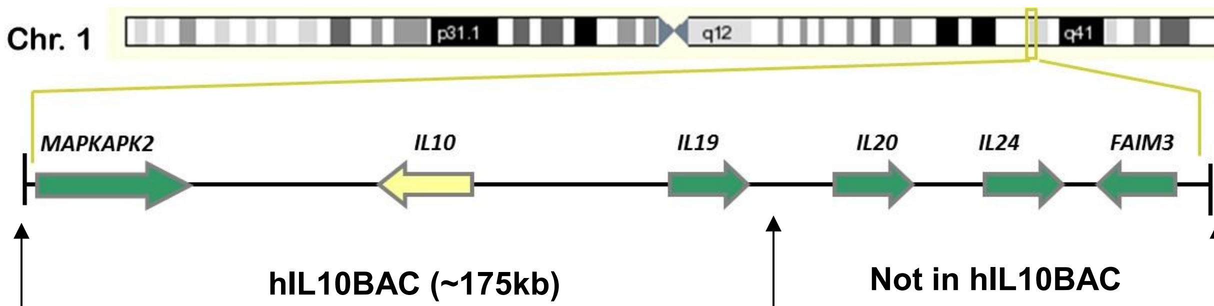


Figure 1.2. The IL-10 locus in mouse and human in chromosome 1 (Chr. 1)

3. Interleukin 24

3.1. Cellular sources

IL-24 was first isolated by subtraction hybridation of cDNA libraries of human melanoma cells and originally named melanoma differentiation-associated gene 7 (MDA-7)[84]. Because of its genomic localization in chromosome 1 and sequence and structural homology with IL-10, it was later renamed as IL-24 and classified among the so-called IL-10 family of cytokines, including IL-10, IL-19 and IL-20[85]. IL-24 is expressed in various immune cells including T lymphocytes, monocytes, and B cells. Among these cells, IL-24 is robustly expressed in T_H2 but not in T_H1 cells after T cell receptor (TCR) activation with anti-CD3 and anti-CD28 or PMA and Ionomycin[86,87]. Thus, IL-24 is a prototypical Th2 cytokine and has been shown to be one of top Stat6 target genes in T_H2 cells[88]. IL-24 expression can be induced in human monocytes stimulated with lipopolysaccharide, concavalin A, or cytokines[89,90]. Meanwhile, B cell-specific IL-24 induction is achieved by cross-linking of the B cell receptor (BCR) with CD40 ligand and anti-IgM[91]. In addition to immune cells, IL-24 protein is secreted by non-hematopoïc cells including keratinocytes, melanocytes, and colonic sub-epithelial myofibroblasts (SEMFs) following stimulation with cytokines such as TNF-α, IFN-β, IL-2, or IL-1β[84,92-95].

3.2. Biological functions and role of IL-24 in disease pathogenesis

IL-24 signals through two heterodimeric receptor complexes, namely IL-20R1/IL-20R2 and IL-20R2/IL-22R, which are mostly present in non-immune cells[96]. The interaction of IL-24 with these receptors activates Stat3 within the target cells[97]. To date, the exact function of IL-24 is not well defined because *Il24* null mice have no unique phenotype[98] ;however, the current literature points to two main functions for IL-24 protein: (1) a classical cytokine and (2) a tumor suppressor gene[99].

As a cytokine, ectopic expression of IL-24 induces secretion of IFN-γ, IL-6, and TNF-α in human PBMCs, suggesting that IL-24 may be a pro-inflammatory cytokine as opposed to IL-10[(100)]. IL-24 has been shown to mediate migration of neutrophils and monocytes *in vitro* as well as the recruitment of CD11b positive myeloid cells *in vivo*[(101)].IL-24 also blocks the differentiation of germinal center B cells to plasma cells through down-regulation of transcription factors such as IRF4, Blimp1, and Bcl6 expression, which are critical for plasma cell differentiation[(102)]. Additionally, transgenic overexpression of IL-24 in the skin results in neonatal lethality, epidermal hyperplasia, and dysregulation of keratinocyte differentiation, suggesting a role for IL-24 in epidermal functionality such as tissue remodeling and wound healing[(103)].

Most of the published literature about IL-24 focuses on its tumor suppressor activities on cancer cells because overexpression of IL-24 induces selective killing of tumor cells but not normal cells[(104)]. The mechanisms involved in IL-24-induced cancer cell death include activation of apoptotic pathways, regulation of the cell cycle, anti-angiogenesis effects, and increased sensitivity of tumor cells to radiation and chemotherapy[(105-107)]. Also, in a phase I clinical trial, Adenovirus delivery of IL-24 (Ad.MDA-7) induced the expansion of T cells, increased the circulating levels of IL-6, IL-10 and TNF-α, thus promoting anti-cancer immune responses[(108)].

IL-24 has been implicated in the pathogenesis of autoimmune diseases such as psoriasis, inflammatory bowel disease, and rheumatoid arthritis. Up-regulation of IL-24 expression in keratinocytes through a TNF-dependent pathway promotes human psoriasis-like skin lesions in mice[(94)]. In agreement with these findings, IL-24 is highly expressed in the epidermis of psoriatic patients[(94,98)], colonic epithelial cells of patients with active ulcerative colitis and Crohn's disease[(109,110)] as well as synovial fluids and plasma of rheumatoid arthritis patients[(111)]. The exact

role played by IL-24 in these autoimmune conditions; however, remains to be elucidated. Recent studies also suggest a protective role for IL-24 during intracellular bacterial infection such as *Mycobacterium tuberculosis*[112,113] and *Salmonella typhimurium* infections[114] through induction of IFN-γ production by CD8+ T cells and neutrophils.

3.3. Regulation in immune cells

Regulation of *Il24* expression in immune cells is not well characterized. Sahoo et al. recently identified the *Il24* proximal promoter responsible for T_H2-specific IL-24 expression[115]. This region (-157 to +95 from the transcription start site) was trans-activated through cooperative binding of Stat6 (which acts downstream of the IL-4 receptor system) and c-Jun (an AP1 family of transcription factors) to turn on *Il24* gene expression. Binding of Stat6 and c-Jun to this site was facilitated by an open chromatin structure defined by an increased binding of active histone marks such as H3AcK9/14 and H3K4Me2[115]. The active chromatin architecture has also been confirmed to be Stat6-dependent because Stat6-deficient mice have a dramatically decreased binding of active histone proteins such as H3K4me3 and H3K36me3[116]. In contrast to Th2 cells, *Il24* promoter was occupied by histone deacetylase 1 (HDAC) in Th1 cells, which could explain why IL-24 is not expressed in these cells[117]. Together, these studies suggest that *Il24* gene is regulated at both transcriptional and epigenetic levels in T_H2 cells; however, further studies are needed to clearly define the molecular mechanisms that govern regulation of IL-24 expression in other immune cell types.

In Chapter 3 of this dissertation, I will investigate the co-expression of mouse IL-24 and IL-10 expression to assess whether they share common regulatory pathways. This work would indirectly provide new insights regarding genomic boundaries of the IL-10 locus. Table 1.1 shows key characteristics of IL-24 and IL-10 for purposes of this dissertation:

Table 1.1: Comparison between IL-24 and IL-10 cellular sources, receptor systems, and biological functions

Characteristics	IL-24	IL-10
Chromosomal location	1q32	1q32
Structure	Monomer	Dimer
Receptors	IL-20R1/IL-20R2 IL-20R2/IL-22R	IL-10R1/IL-10R2
Major cellular sources	T Lymphocytes (T_H2 cells) Keratinocytes Melanocytes	Leukocytes
Cellular targets	Epithelial cells	Leukocytes
Biological functions	Tumor suppression Tissue remodeling Wound healing Anti-bacterial response	Immune suppression

Chapter 2

Elucidating the impact of single nucleotide polymorphisms in non-coding DNA on cell-type-specific human IL-10 expression and disease susceptibility in genetically humanized mice

2.1. ABSTRACT

Genome-wide association studies (GWAS) have revealed that the majority of disease-associated single nucleotides polymorphisms (SNPs) are found in non-coding DNA, yet little is known about the functionality of non-coding SNPs in the context of complex human diseases. To investigate the influence of non-coding SNPs on cell-type-specific gene expression and disease susceptibility, we created genetically humanized mouse strains based on a bacterial artificial chromosome (BAC) transgenic system. Each mouse was designed to harbor one of the two common human *IL10* promoter SNP haplotypes: "ATA-hIL10BAC mouse" and "GCC-hIL10BAC mouse." By reconstituting *Il10*$^{-/-}$ mice with either the ATA-hIL10BAC or the GCC-hIL10BAC construct, we were able to examine the functionality of human *IL10* promoter SNP haplotypes using IL-10-dependent mouse models of human diseases. In response to LPS stimulation, we found a differential human IL-10 production in myeloid compartment both *in vitro* and *in vivo*. The allele-specific human IL-10 expression in myeloid cells, however, was not sufficient to change the outcome of LPS-induced septic shock as evidenced by 100% survival rate for both *Il10*$^{-/-}$/ATA-hIL10BAC and *Il10*$^{-/-}$/GCC-hIL10BAC. Conversely, in response to IL-27*,* an IL-10-promoting cytokine, CD4+ T helper cells from GCC-hIL10BAC mice produce a significantly higher level of human IL-10 compared to ATA-hIL10BAC mice. In the *Leishmania donovani*, a mouse model of persistent infection, *Il10*$^{-/-}$/GCC-hIL10BAC mice (like WT mice) had a high parasite burden in target organs in sharp contrast to *Il10*$^{-/-}$/ATA-hIL10BAC mice that cleared the parasites. Furthermore, IL-10+IFN-γ+ CD4+ T cells—a cellular subset that has been associated with pathogen persistence during leishmaniasis—were significantly enriched in the spleen of *Il10*$^{-/-}$/GCC-hIL10BAC but not in *Il10*$^{-/-}$/ATA-hIL10BAC mice. Thus, we generated for the first time a robust experimental tool to functionally annotate human genetic variation in non-coding DNA *in vivo*. Our results also strongly demonstrate that human *IL10* promoter SNP

haplotypes alone can modulate cell-type-specific human IL-10 expression which subsequently alters susceptibility to persistent infection, but not to LPS-induced septic shock.

2.2. INTRODUCTION

When the first draft of the human DNA sequence was released about a decade ago, the rate of single nucleotide polymorphisms (SNPs) between two haploid human genomes was estimated to be 1 SNP for every ~1250 base pairs[118,119]. Additionally, 99% of sequence variation in the genome was mapped to non-coding regions, which also constitute the majority of the human genome[118]. This project was followed by the International HapMap and 1000 Genomes project consortium, which together provided extensive databases of genetic variation in the human genome[120,121]. Despite these major accomplishments in the field of genomics, the functional significance of the millions of SNPs in the human genome is still not clearly defined. Numerous studies have linked non-coding SNPs to phenotypic variation and disease susceptibility among people by means of genome-wide association studies (GWAS)[122,123]. Similarly, non-coding SNPs have been associated with a change in gene expression in various human cells[124-126]. Nevertheless, it has been a daunting task to move from genetic association studies to causality for complex human diseases and traits. This is due in part to the lack of robust research tools for accomplishing the following: (1) to distinguish functional non-coding SNPs from other genetic variants that are in strong linkage disequilibrium with other large genomics regions across the genome, and (2) to define the function of important non-coding regions of the genome within which one or more important SNPs may be co-localized.

Expression quantitative trait locus (eQTL) mapping is commonly used to identify genomic regions and variants that influence gene expression[127,128]. However, genotyping platforms used for eQTL analysis are designed to detect regions of linkage disequilibrium containing multiple SNPs rather than a single variant. Thus, eQTL mapping is not suitable to discriminate functional SNPs from innocent bystanders. In addition, computational predictions

and conservation-based methods have been employed to identify putative functional regulatory DNA but with limited success [129,130]. For example, computational predictions are available to identify transcription-factor-binding-site motifs near or within GWAS-associated SNPs, yet the presence of a putative motif does not guarantee that a transcription factor will bind *in vivo*[130]. Furthermore, conservation-based approaches are used to define conserved non-coding sequences (CNS), which are highly conserved non-coding DNA that have been preserved between evolutionary distant vertebrate species, thus likely to harbor functional regulatory elements to drive cell-specific gene expression programs[130,131]. Still, one has to experimentally establish that the CNS of interest truly encode for functional cis-regulatory elements. This is usually done by cloning and testing their impact on gene expression using cell-based reporter assays or using a transgenic construct[131]. Additionally, conservation-based methods would likely fail to detect important species-specific regulatory elements [132,133].

To provide a direct experimental approach for assessing the function of non-coding regions of the human genome, the ENCODE project (Encyclopedia of DNA Elements) was initiated to annotate non-coding regions in various cell types[134-136]. The project used whole-genome sequencing approaches to catalog regulatory features across the genome. The features mapped are coding and non-coding transcripts (by RNA-Seq), protein-coding regions (by mass spectrometry), transcription-factor-binding-sites (by ChIP-Seq), chromatin structure (by DNAse-Seq, FAIRE-Seq, histone ChIP-Seq and MNase-Seq), DNA methylation sites (by Reduced Representation Bisulfite Sequencing assay)[136]. This effort has shed light on important findings about the structure and function of the human genome. Experimental data from ENCODE was also integrated to additional resources such as GWAS, eQTL information, computational predictions, and manual annotations to identify functional regulatory regions and sequence

variations[134]. Some major findings related to the topic of this dissertation include: (a) many non-coding SNPs overlap with predicted functional regions, (b) disease-associated SNPs are enriched within ENCODE-annotated functional regions, and (c) disease phenotypes can be linked to a specific cell type or transcription factor[128,129,134,136].

Despite this large advance in our understanding of how the genome is organized into discrete cell-type-specific non-coding regulatory units, the ENCODE project has many limitations: (1) the use of transformed cell lines, (2) ENCODE-annotated regulatory variants are based on correlative studies and were not validated, and (3) information on how ENCODE-annotated regions may be working in an *in vivo* context was not provided. Because the broader goal for both the Human Genome Sequencing and ENCODE projects is to decipher the content of the genome and how it is organized and regulated to better understand the molecular basis for disease susceptibility, new research tools will be needed to advance our ability to link genomic sequences (genotypes) with functional outcomes (phenotypes).

Several years ago, as a proof of principle to provide a clear experimental link between non-coding SNPs, gene expression and disease susceptibility, we took the approach to physically isolate one locus away from other genetic and environmental factors (the human *IL10* locus)[137]. This approach is based on a transgenic system generated by introducing a bacterial artificial chromosome (BAC) into a mouse[137]. The BAC used to generate the mice (hIL10BAC) contains not only *IL10* but also its neighboring genes: *MAPKAPK2* and *IL19*. Additionally, as human IL-10 can signal through the mouse IL-10 receptor[27], we previously validated that human IL-10 is functional and appropriately regulated *in vivo* using well-established IL-10-dependent mouse models of human diseases[137,138]. Specifically, we found that hIL10BAC mice rescue *Il10*$^{-/-}$ mice from LPS-induced septic shock and colitis, which was associated with sufficient *IL-10*

production by macrophages and $CD4^+FoxP3^+$Tregs, respectively[137,138]. However, hIL10BAC failed to promote persistent *Leishmania donovani* infection in *Il10*$^{-/-}$ mice because only a small fraction of splenic Th1 cells co-expressed human IL-10 (which is the pathogenic population in the context of leishmaniasis)[139] induced after infection[137]. Because the hIL10BAC has an *IL10* promoter proximal SNP haplotype (ATA) associated with low IL-10 production in humans[37-39], our data suggested that the impact of SNPs on human IL-10 expression may not be universal (i.e., same effects in all cells) as generally assumed, but rather differential between cell lineages or restricted to only certain cell types.

In this study, we sought to establish a proof-of concept experimental tool as a means to assign causality between genetic variants and human disease phenotypes *in vivo* and to determine the impact of genetic variation on non-coding DNA on cell-type-specific human IL-10 expression and disease outcomes. Our working hypothesis is that non-coding SNPs in the *IL10* locus modulate IL-10 expression patterns in different cell types and thereby influence disease susceptibility. To this end, we developed a new humanized mouse harboring the alternate "GCC" haplotype block, which has been linked to high IL-10 levels in humans [37-39]. We compared human IL-10 levels between the two mice in CD4+T cells and macrophages. Then, we confirmed the impact of cell- and allele-specific human IL-10 expression *in vivo* by employing the sepsis and Leishmania models. In LPS-stimulated macrophages, the GCC-hIL10BAC mice exhibit a lower IL-10 producing capacity when compared to ATA-hIL10BAC mice. However, the lower human IL-10 level in LPS-stimulated macrophages was sufficient to rescue *Il10*$^{-/-}$ mice reconstituted with the GCC-hIL10BAC transgene from LPS-induced septic shock similar to *Il10*$^{-/-}$/ATA-hIL10BAC mice. On the other hand, human IL-10 was robustly produced in *in vitro*-generated CD4+ T helper cells from GCC-hIL10BAC relative to ATA-hIL10BAC mice. Unlike

in macrophages, high IL-10-producing capacity in Th1 cells was sufficient to restore susceptibility of *Il10*$^{-/-}$ mice from persistent leishmaniasis. Therefore, by assessing two divergent immune cell populations—CD4+ T cells and macrophages—we strongly demonstrate that we can model human genetic variation in non-coding DNA *in vivo* in mice. We also found that the effect of *IL10* promoter SNP haplotypes on human IL-10 levels is highly cell-type-specific.

2.3. MATERIALS AND METHODS

2.3.1. Generation of a GCC-hIL10BAC transgenic mouse

2.3.1.1. Selection of BAC clones:

The University of California Santa Cruz (UCSC) Genome Browser (http://genome.ucsc.edu) was used to identify BAC libraries that have similar genomic organization and size as the RP11-262N9 (Existing ATA-hIL10BAC). DH10B *E. coli* strain carrying BAC clones were purchased from Invitrogen (Carlsbad, CA) and grown on LB agar plates under chloramphenicol selection (12.5 μg/ml) (Affymetrix, Santa Clara, CA). Three separate single bacterial colonies were selected from each plate and amplified with primers specific for two of the human *IL10* proximal promoter SNPs: -1082G>A (rs1800896) and -592C>A (rs1800872). PCR products were sub-cloned into a TOPO-TA cloning vector (Invitrogen, Carlsbad, CA) followed by sequencing. Two BAC clones were found to bear a "GCC" Human *IL10* haplotype (CTD-3174K1 and CTD2563L3), but only one was the closest in length (CTD-3174K1) to the RP11-262N9. To ensure that the new BAC would be the same length as the existing BAC, a second BAC clone was used (CTD-2563L3) as a donor BAC to extend the length of the acceptor BAC (CTD-3174K1), which was missing approximately 12,6kb of genomic DNA. A screenshot of this step is shown in Appendix 2.1 and sequences as well as the annealing temperature of the primers are shown in Table 2.1 below.

Table 2.1: List of primers used to genotype *IL10* promoter

Name	Sequence	Annealing Temperature
-1082G>A (rs1800896)	Forward: 5'-GGGAAGGTGAAGGCTCAATC-3' Reverse: 5'-TTGGTTTCCTCACCCTACTG -3'	52°C
-592C>A (rs1800872)	Forward: 5'-TACTCTTACCCACTTCCCCC-3' Reverse: 5'-TGAGAAATAATTGGGTCCCC-3'	58°C

2.3.1.2. Modification of the CTD3174K1 acceptor BAC clone by homologous recombination

We designed a strategy in collaboration with Gene Bridges, the recombineering company (Heidelberg, Germany), to modify the CTD-3174K1 BAC clone by means of homologous recombination. A replacement cassette carrying 50bp homology arms was generated by PCR and cloned into a vector hosting a kanamycin resistance gene (Kan^R). Homologous recombination was induced between the donor BAC and replacement cassette to obtain recombinant clones hosting 31kb of the 3' far end (near *IL19*) of the Donor BAC (CTD-2563L3). Recombinant derivatives (Donor/Kan^R) were screened by PCR and confirmed by sequencing. A 15.6kb containing the "missing 12.6kb portion" and Kan^R gene of Donor/Kan^R was transferred to a shuttle vector (pAmp). Recombinant subclones (truncated Donor/Kan^R) were identified by PCR and confirmed by sequencing. Correct recombinants were subjected to restriction digestion to release the DNA insert from the pAmp backbone. The insert (truncated Donor BAC/Kan^R) was used as a modification cassette to extend the end of the Acceptor BAC (CTD-3174K1) by homologous recombination. The modified BAC clone (i.e., CTD-3174K1 BAC plus 12.6kb) were selected under chloramphenicol and kanamycin (Affymetrix, Santa Clara, CA) and screened by PCR followed by sequencing. The integrity of the construct was verified by PCR, restriction digestion, and sequencing. Glycerol stocks were made and stored at -80ºC for future use. A map of the Modified BAC made by Gene Bridges is shown in Appendix 2.2.

2.3.1.3 Preparation of BAC clones for Microinjection

DH10B *E. coli* hosting the Modified BAC clone were rescued on an LB agar plate and grown overnight in LB liquid media under chloramphenicol (15 µg/ml) and kanamycin (15 µg/ml) selection. The BAC clone was purified by cesium chloride gradient purification and then digested with NotI digestion to obtain an insert of 175kb in length (as the ATA-hIL10BAC insert). The finalized construct was then microinjected into fertilized eggs of C57BL/6 background at the NCI transgenic core facility.

2.3.1.4 PCR Screening of founder mice

The VISTA Genome Browser (http://genome.lbl.gov/vista/index.shtml) was utilized to find regions of low homology between the mouse and human *IL10* loci. PCR primers (5'-CAGGCAAATCTGCATGGGATG-3' and 5'-AGCTGTTGGACAGGCTCTACTG-3') were generated to cover that region, which was an intergenic region between human *IL10* and *IL19*. A tail sample was clipped from each founder mouse. DNA was isolated using a DNeasy Blood and tissue kit (Qiagen, Valencia, CA). Tail DNA specimens from wild-type and ATA-hIL10BAC transgenic mice were used as negative and positive controls, respectively. PCR results were also confirmed by Southern blot analysis.

2.3.1.5 Estimation of transgene copy number

Tail snips of at least 3 mice from 3 separate litters were obtained to assess the transgene copy number. Genomic DNA was isolated from tail tissues as described above and subjected to quantitative real-time PCR (qPCR) by targeting a region within exon 5 of the human *IL19*. qPCR primers were also designed to amplify a single copy mouse gene (coding portion of Jun

oncogene) and four homologous mouse genes of Bcl2a1 family (Bcl2a1a, Bcl2a1b, Bcl2a1c, and Bcl2a1d, which together represent 4 copies endogenous mouse gene). Estimates of the transgene copy numbers (RCN) were generated by normalizing the hIL10BAC to that of the mouse genes using the Delta CT method (2^- [target–standard]). The RCN was then multiplied by 2 for Jun and 4 for Bcl2a1 to infer transgene copy number. Primers and probes sequences are as follows:

BAC-F: GAGTTGGCAATGCTGATTTT

BAC-R: GGACAGGGTGTTCAAGGATCAT

BAC-Probe: CAGGAGCCAAACCCCA

(Jun) 001-Forward: GAGTGCTAGCGGAGTCTTAACC

(Jun) 001-Reverse: CTCCAGACGGCAGTGCTT

(Jun) 001-Probe: GAACTGGGGAGGAGGGCTCAGGGGC

(Bcl2a1) 004-Forward: GTTGCTTTCTCCGTTCAGAAGGA

(Bcl2a1) 004-Reverse: GCCATCTTCAAACTCTTTTTCCATCA

(Bcl2a1) 004-M2: ATTCCACGTGAAAGTC

2.3.2. Mice

The followings mouse strains were used in this study: WT, *Il10*$^{-/-}$, ATA-hIL-10BAC, *Il10*$^{-/-}$/ATA-hIL10BAC, GCC-hIL10BAC, and *Il10*$^{-/-}$/GCC-hIL10BAC. All mice were bred and maintained on C57BL/6J background under specific pathogen-free conditions at the Johns Hopkins University animal facilities. Gender- and age-matched mice were used for all experimental procedures, which were approved by the Johns Hopkins Animal Care and Use Committee.

2.3.3. Antibodies and Cytokines

Purified hamster anti-mouse CD3ε (145-2C11) and purified hamster anti-mouse CD28 (37.51) antibodies were purchased from BD Bioscience (San Diego, CA). Anti-mouse IL-4 monoclonal antibody (11B.11) was obtained from the NCI Preclinical repository and anti-mouse IFN-γ antibody (XMG 1.2) was purchased from eBioscience (San Diego, CA). Recombinant murine IL-2 was obtained from the NCI repository as a donation. Recombinant IL-27 was purchased from R&D Systems (Minneapolis, MN). The following fluorochrome-labeled antibodies for flow cytometry were purchased from BD Bioscience (San Diego, CA): PercP-conjugated anti-CD3, FITC-conjugated anti-CD4, PE-conjugated anti-mouse IL-10, PE-conjugated anti-human IL-10*,* and APC-conjugated anti-IFN-γ.

2.3.4 Preparation of media for primary cell culture

Naïve T cells were cultured in RPMI 1640 medium (Lonza, Walkersville, MD) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS) from Atlanta Biologicals, Inc. (Flowery Branch, GA), 2 mM of L-glutamine (Cellgro®, Mediatech, Inc., Manassas, VA), 1X of non-essential amino acids (Gibco®, Life Technologies, Grand Island, NY), 1mM of sodium pyruvate (Gibco®, Life Technologies, Grand Island, NY), 10mM of 2-mercaptoethanol (Gibco®, Life Technologies, Grand Island, NY), 100 U/ml of penicillin (Cellgro®, Mediatech, Inc., Manassas, VA), and 100 mg/ml of streptomycin (Cellgro^{R}, Mediatech, Inc., Manassas, VA). Bone marrow-derived macrophages (BMM) were cultured in DMEM medium (Lonza, Walkersville, MD), supplemented with 10% of heat-inactivated FBS, 2 mM of L-glutamine, 10 mM of 2-mercaptoethanol, 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 50 µg/ml of gentamycin (Quality Biological, Inc., Gaithersburg, MD).

2.3.5. Isolation and culture of naïve mouse $CD4^+$ T cells

Magnetic beads and antibodies from R&D Systems (Minneapolis, MN) were used to isolate naïve $CD4^+CD62L^+$ T cells from splenocytes by negative selection. All assay procedures were done according to the manufacturer's protocols (R&D Systems, Minneapolis, MN). Three ml of cells at 10^6 cells/ml were seeded in a six-well plate that had been coated overnight with anti-CD3 and ant-CD28 antibodies. To block naïve $CD4^+$T cells polarization toward T_H1 or T_H2, IL-4 and IFN-γ were neutralized with anti-IL-4 and anti-IFN-γ antibodies. Recombinant IL-2 (50 UI/ml) was added as a survival signal. Recombinant mouse IL-27 (50 ng/ml) was added where indicated to induce IL-10 expression (140). On day 3, cells were harvested and spun down to collect supernatants. The cells were then washed, counted, and reseeded at 10^6 cells/ml into a fresh plate with no anti-CD3 or anti-CD28 antibodies but in media supplemented with anti-IL-4, anti-IFN-γ, IL-2, with or without IL-27. On day 4, cells were stimulated with 10 ng/ml of phorbol12-myristate 13-acetate (PMA) (Calbiochem, La Jolla, CA) and 1 µg/ml of Ionomycin (Calbiochem) for 8 hours and 24 hours. Supernatants were collected for human and mouse ELISA and cell lysates for mRNA analyses. Cells with no stimulation were used as negative control.

2.3.6. Isolation and culture of bone marrow-derived macrophages

Bone marrow cells were flushed from femurs and tibias of mice with the BMM culture media. The cells were then passed through a cell strainer, pelleted down and resuspended in the BMM media supplemented with 30% of L929-conditioned media from the American Type Culture collection (ATCC, Manassas, VA). The cells were seeded at $1x10^6$ per ml in 3 ml in a 6-well plate and maintained for 5 days with media change at day 2 and day 4. At day 5, fully differentiated cells were washed three times with 1X PBS then maintained in BMM media

overnight. Fresh media was added at day 6 and the cells were stimulated with LPS (10 ng/ml) (Sigma, St. Louis, MO), IL-4 (10 ng/ml), and IFN-γ (10 ng/ml).

2.3.7 mRNA Analyses

Total RNA was extracted with TRIzol® reagent (Ambion, Life Technologies, Carlsbad, CA). One microgram of RNA was used to make complementary DNA (cDNA) using the SuperScript® First-Strand Synthesis System (Invitrogen™, Life Technologies, Carlsbad, CA). SYBRGreen assay real-time PCR (qPCR) assay from Applied Biosystems (Life Technologies, Grand Island, NY) was employed to amplify the cDNA. Mouse β-2-microglobulin was used as a housekeeping gene, thus all mRNA expression was normalized to that of β-2-microglobulin. The primers used for gene expression analysis were the following: 5'-CAGGCAAATCTGCATGGGATG-3' and 5'-AGCTGTTGGACAGGCTCTACTG-3' for human *IL10,* 5'-TCGGCCAGAGCCACATG-3' and 5'-TTAAGGAGTCGGTTAGCAAGTATGTTG-3' for mouse *Il10*, and 5'-AAATGCTGAAGAACGGGAAAA-3' and 5'-ATAGAAAGACCAGTCCTTGCTGAAG-3' for mouse β-2-microglobulin. Data are shown as fold induction over non-stimulated cells (NS).

2.3.8. Determination of human and mouse IL-10 production *in vitro*

Cell-free supernatants were obtained by centrifugation at 1600 rpm for 5 minutes. Human and mouse IL-10 levels were quantified using the BD OptEIA human or mouse IL-10 ELISA (BD Bioscience, San Diego, CA). All assays were conducted according to the manufacturer's protocols.

2.3.9 *In vivo* LPS challenge

Mice were challenged by intraperitoneal injection (i.p.) with 50 µg of LPS (List Biologicals, Campbell, CA) extracted from *E. coli* 0111:B4 strain. A set of mice (n=3) were sacrificed 2 hours after challenge to collect serum for human IL-10 quantification by ELISA. The remaining mice (n=5) were monitored for at least 2 weeks to determine survival rate after LPS-induced septic shock.

2.3.10 Leishmania infection and parasites

Rag$^{-/-}$ mice (B6.129S7-Rag1^{tm1Mom}/J) were used to maintain *L donovani* amastigotes (LV9) *in vivo,* as previously described (141). Each mouse was infected with 2x10^7 amastigotes by lateral tail vein injection. Parasite burdens in the liver and spleen were determined from methanol-fixed, tissue impression smears stained by the Giemsa method. Data are shown as *Leishmania donovani* Units (LDU).

2.3.11 Assessment of Leishmania-specific CD4$^+$ T cells by flow cytometry

Mice were euthanized at the indicated time points (day 21 and day 28). Single cell suspensions were obtained and stimulated for 2 hours with PMA (20 ng/ml) and Ionomycin (1 µg/ml). Brefeldin A (10 µg/ml) was added for 4 hours. The cells were collected then stained with anti-CD3 (PercP), anti-CD4 (FITC), anti-IFN-γ (APC), anti-mouse IL-10 (PE), and anti-human IL-10 (PE) (all from BD Bioscience). Samples were acquired (350,000 events total) on a BD LSRII (Becton Dickinson) and analyzed with FlowJo software (Tree Star, Ashland, OR).

2.3.12 Statistical analysis

Data were analyzed using GraphPad Prism version 5.0. Software (La Jolla, CA). Unpaired Student's *t*-tests were used for statistical analysis. A two-tailed p-value less than 0.05 was used as a cut-off for statistical significance.

2.4. RESULTS

2.4.1. *Generation and initial characterization of GCC-hIL10BAC mice*

To generate a genetically humanized mouse with a "GCC" *IL10* promoter SNP haplotype, we employed the University of California Santa Cruz (UCSC) genome browser that contains the list of available BAC clones. We identified a BAC clone (CTD-3174K1) in which human *IL10* gene was centrally positioned and flanked by *MAPKAPK2* and *IL19* genes (as in the existing ATA-hIL10BAC). We used PCR analysis followed by sequencing to confirm that the BAC clone harbors the "GCC" *IL10* promoter SNP haplotype. To directly compare the effects of SNPs on gene expression and disease phenotypes, it was important that both transgene constructs have the same length. Thus, we modified the CTD-3174K1 clone by means of homologous recombination with a donor BAC (CTD-2563L3) followed by restriction digestion to obtain a construct of 175kb in size as in RP11-262N9 (Figure 2.1A). Founder mice were screened by Southern blot and PCR analyses (Figure 2.1B and 2.1C). Fourteen founder mice (named A through O, excluding K) were positive for human *IL10* by Southern blot analysis; however, only seven lines were expanded for future characterization. The remaining lines were discontinued for 3 main reasons: (1) Southern blot and PCR data were discordant, (2) no transgene expression *in vivo*, (3) no litters from breeder pairs after several months. All the mice were in good health with no visual signs of diseases or immunological abnormalities for more than a year of follow-up.

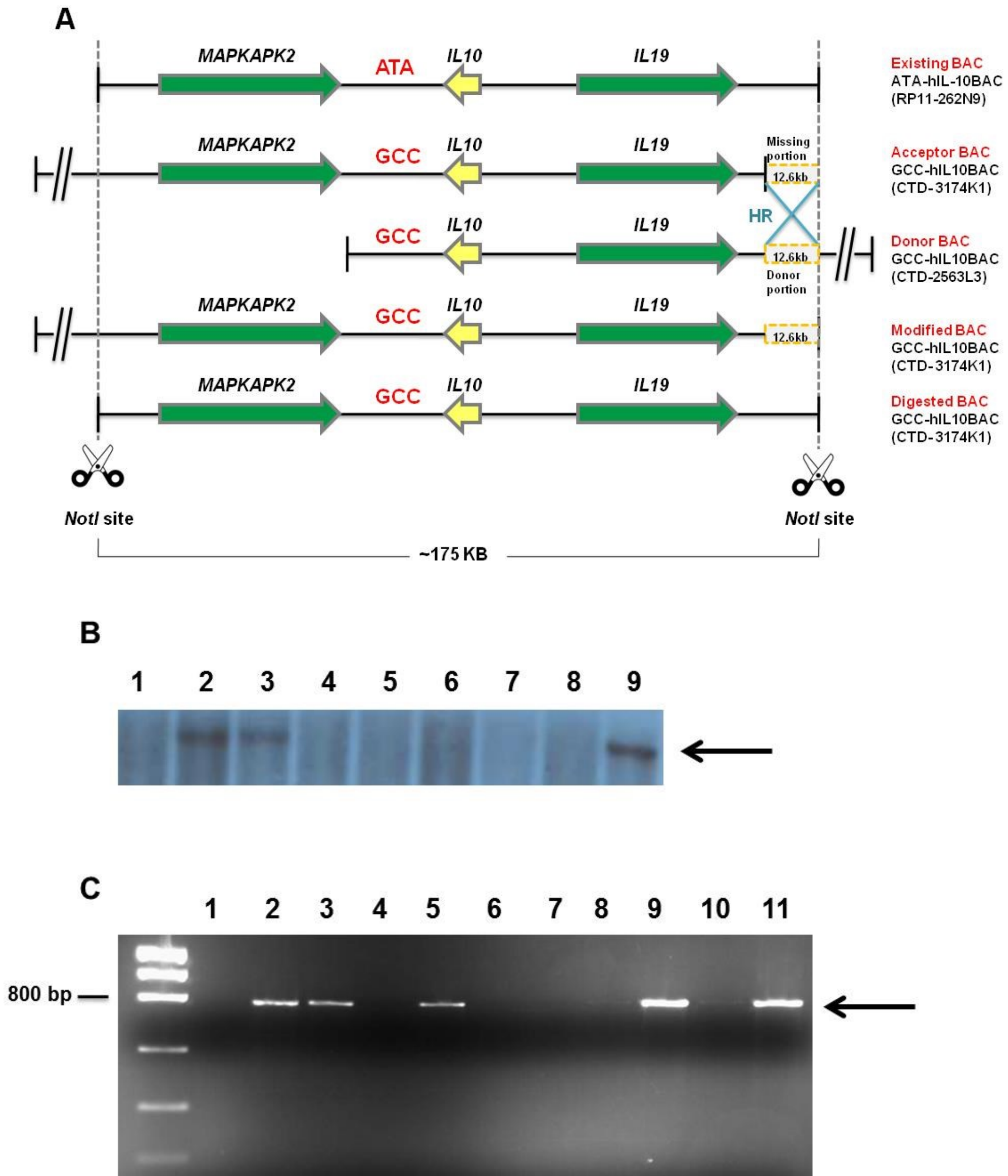


Figure 2.1. Generation of GCC-hIL10BAC mice

(A) Strategy to develop a GCC-hIL10BAC construct by homologous recombination (HR). (B) Southern blot analysis of founder mice. (C) Genotyping of founder mice by polymerase chain reaction (PCR). Arrows indicate an example of hIL10BAC positive mouse for the human *IL10* BAC transgene.

2.4.2. *Human IL-10 expression in the GCC-hIL10BAC mice is copy-number independent*

Previously, we demonstrated that expression of human *IL10* transgene in ATA-hIL10BAC mice is not impacted by the number of transgene copies (i.e., copy number effects)[137]. To verify that human IL-10 is regulated similarly in ATA-hIL10BAC and GCC-hIL10BAC mice, we performed copy number analysis of the GCC-hIL10BAC transgene by real-time PCR (qPCR). As in the ATA-hIL10BAC mice, copy numbers in the seven GCC-hIL10BAC founder lines ranged from 1 to 9 copies (Figure 2.2A). To assess copy number effects on transgenic human IL-10 expression, we challenged GCC-hIL10BAC mice with LPS for 2h to measure human IL-10 production *in vivo*. As expected, human IL-10 was produced at similar levels in all transgenic lines (Figure 2.2B), suggesting that there are no copy number effects on human GCC-hIL10BAC transgene expression *in vivo*. To confirm this finding in a different tissue sample, we stimulated single cell suspension from spleenocytes *in vitro* with LPS for 24 hours. No substantial differences in transgenic human expression or endogenous mouse IL-10 levels among the transgenic lines were observed (Figure 2.2C). Thus, as in the ATA-hIL10BAC mice, human *IL10* is expressed in a copy-number-independent manner in GCC-hIL10BAC mice.

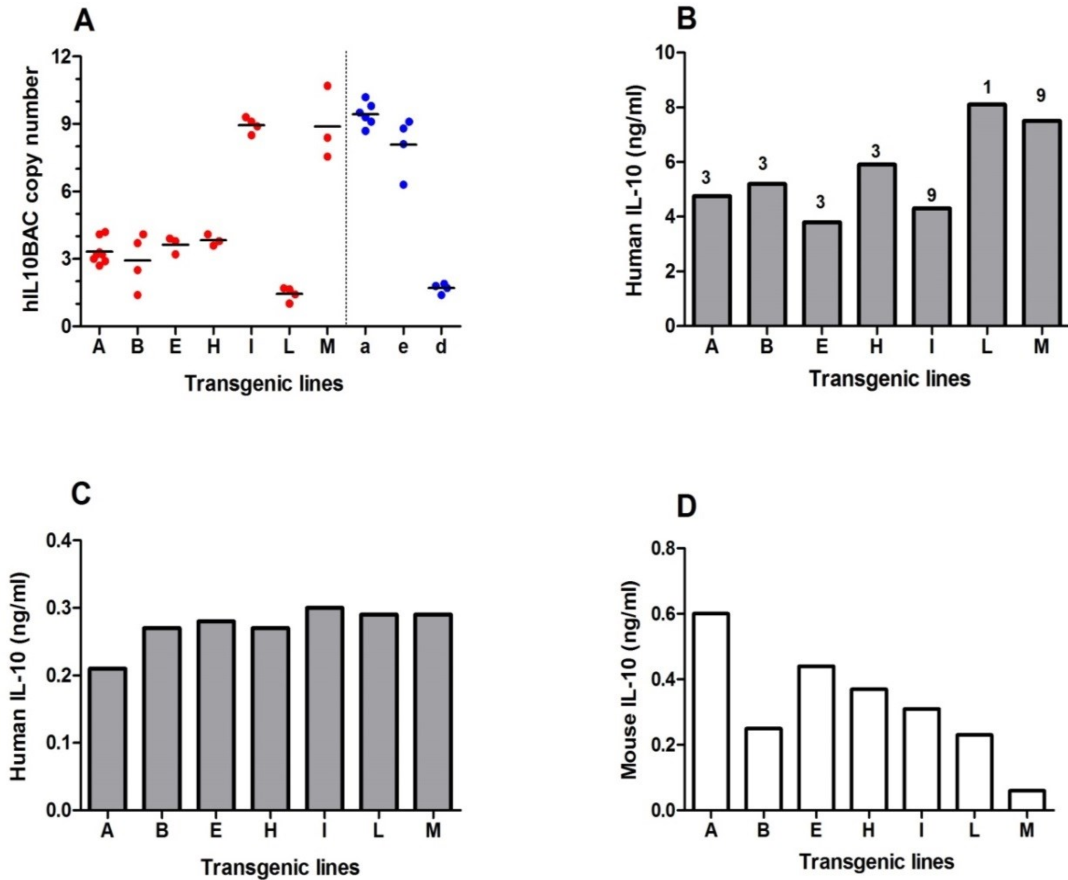


Figure 2. 2. Characterization of the GCC-hIL10BAC mice

(A) Human IL-10 copy number estimation of GCC-hIL10BAC mice (red dots) and existing ATA hIL10BAC mice (blue dots). (B) Human IL-10 production in serum 2 hours after LPS challenge (one of two independent experiments). Number on the top of each bar graph represents copy number of the transgene. (C) and (D) are human and mouse IL-10 levels, respectively in spleenocytes stimulated *in vitro* with LPS for 24 hours (one of two independent experiments).

2.4.3. Tissue-specific human and mouse IL-10 expression patterns in the GCC-hIL10BAC mice

Since we verified that human IL-10 expression is independent of copy number, we concentrated our analysis on one of the founder lines of the GCC-hIL10BAC mice, namely line A which has 3 copies of the human transgene. To determine whether the GCC-hIL10BAC transgene expresses appropriate tissue-specific human IL-10 expression, we assessed basal levels of human and mouse IL-10 mRNAs in tissues known to express human IL-10. The ATA-hIL10BAC mouse (Line a) was used as a comparator. Human IL-10 transcript was constitutively expressed in all tissues assayed, but with varying degrees of expression (Figure 2.3). We observed similar levels of human IL-10 transcripts between ATA-hIL10BAC and GCC-hIL10BAC mice in all tissues assayed, excluding the brain. In contrast, mouse IL-10 transcripts were barely detectable in the tissues tested (Figure 2.3), as previously shown(137). Together these data suggest that, similar to the ATA-hIL10BAC transgene, the GCC-hIL10BAC construct supports faithful tissue-specific human regulation.

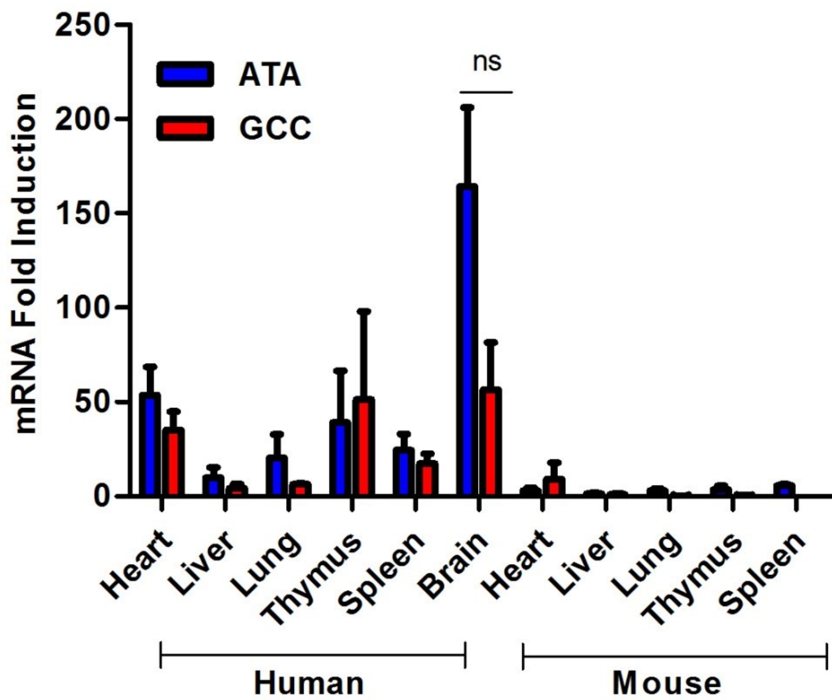


Figure 2. 3. Endogenous IL-10 expression in various tissues

Human and mouse IL-10 mRNA expression was assessed for ATA-hIL10BAC and GCC-hIL10BAC mice. The data was normalized to mouse β-2 microglobulin and expressed as Fold change over mouse IL-10 mRNA level in the brain. ns is non-statistically significant.

2.4.4. Human IL10 promoter SNP haplotypes influence human IL-10 expression in macrophages

We and others have demonstrated that stimulation of bone marrow-derived macrophages (BMM) with TLR4 agonist (such as LPS) induces significant IL-10 production *in vitro*[136,137]. Thus, we used this knowledge to determine whether human *IL10* promoter SNP haplotypes modulate human *IL10* expression in macrophages. We treated BMM isolated from ATA-hIL10BAC and GCC-hIL10BAC mice with LPS alone or LPS in combination with IL-4 and IFN-γ. We used these cytokines because we have previously shown that LPS and IL-4 co-treatment resulted in a synergistic increase of human IL-10 expression, while co-stimulation with IFN-γ inhibited LPS-dependent IL-10 production[137]. We found that human IL-10 levels were significantly higher in LPS treated-BMM of ATA-hIL10BAC mice compared to GCC-hIL10BAC mice at both mRNA and protein levels (Figure 2.4A and 2.4B). Predictably, mouse IL-10 levels in the two transgenic lines were the same (Figure 2.4C and 2.4D). Differential human IL-10-producing capacities were also observed after co-stimulation with LPS and IL-4 or LPS and IFN-γ (Figure 2.4A and 2.4B). These data suggest that the "GCC" promoter SNPs haplotype produces relatively lower levels of human IL-10 in macrophages compared to the "ATA" promoter SNP haplotype.

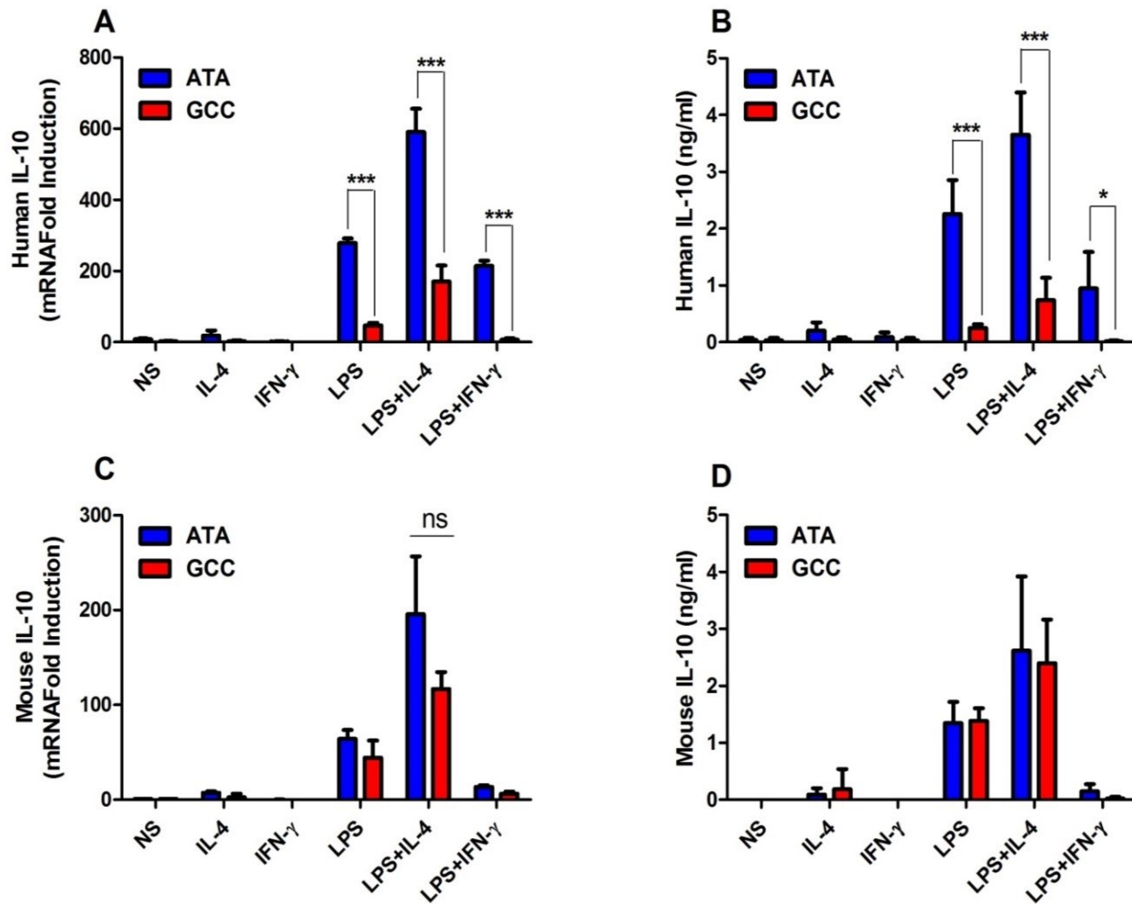


Figure 2. 4. Human and mouse IL-10 expression in macrophages

Human (A) and mouse IL-10 (C) production by bone-marrow derived macrophages (BMM) in ATA-hIL10BAC (blue bar) and GCC-hIL10BAC (red bar) mice 6 hours after stimulation with the indicated stimuli. (B) and (C) show human and mouse IL-10 mRNA expression in BMM 3 hours post -stimulation. *p<0.05 and ***p<0.0001 (student t-test) comparing ATA-hIL10BAC to GCC-hIL10BAC mice. NS is Not stimulated cells (when upper case is used) and non-statistically significant (when lower case is used).

2.4.5. Allele-specific human IL-10 expression in myeloid cells does not alter susceptibility to LPS-induced septic shock

To verify whether the allele-specific human IL-10 expression in macrophages that we observed *in vitro* can be recapitulate *in vivo*, we challenged the two hIL10BAC mice with LPS for 2 hours, as this treatment is known to induce IL-10 production in all myeloid cells, including macrophages. In response to LPS treatment, the ATA-hIL10BAC mice expressed significantly higher amounts of human IL-10 compared to GCC-hIL10BAC mice (Figure 2.5A). Conversely, mouse IL-10 levels were the same in both transgenic strains (Figure 2.5A).

It has been established that myeloid-derived IL-10 controls susceptibility to LPS-induced septic shock, a well-established mouse model of human sepsis[(142)]. In this experimental system, *Il10*$^{-/-}$ animals died due to excessive secretion of pro-inflammatory cytokines[(143)]. To determine whether the allele-specific human IL-10 expression in myeloid compartment influences susceptibility to sepsis, we generated mice which express only the human transgene by reconstituting *Il10*$^{-/-}$ mice with either ATA-hIL10BAC or GCC-hIL10BAC in order to obtain *Il10*$^{-/-}$/ATA-hIL10BAC and *Il10*$^{-/-}$/GCC-hIL10BAC mice. We challenged these mice with low-dose LPS (50 μg). WT and *Il10*$^{-/-}$ mice were used as controls. Then, we monitored survival rate of the mice at 12-hour intervals for 2-week periods. *Il10*$^{-/-}$ mice died by 48 hours post-challenge (Figure 2.5B), while all WT mice were resistant to death (Figure 2.5B). We have previously shown that *Il10*$^{-/-}$/ATA-hIL10BAC mice are resistant to disease (100% survival similar to WT mice). Remarkably, *Il10*$^{-/-}$/GCC-hIL10BAC mice were also refractory to septic shock. Thus, similar to ATA-hIL10BAC and WT mice, the amount of human IL-10 expressed by the GCC-hIL10BAC cassette was sufficient to protect mice from fatal outcome during sepsis. Our results suggest that allele-specific human *IL10* expression in myeloid cells does not influence susceptibility to LPS-induced septic shock.

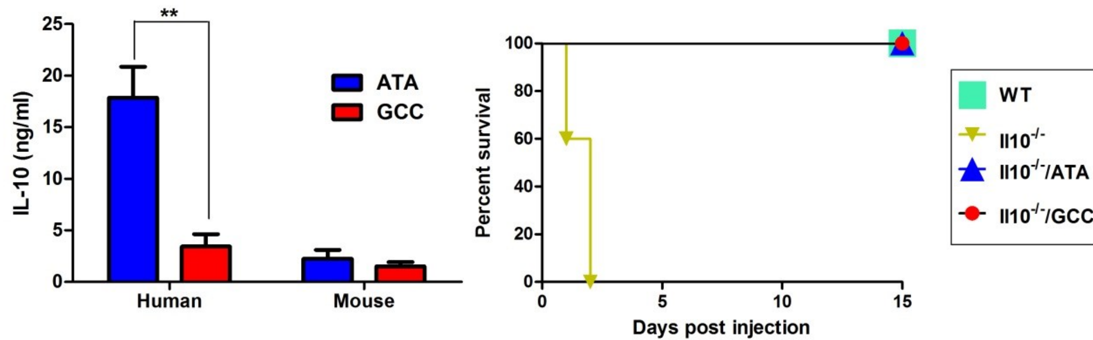


Figure 2.5. Allele-specific human IL-10 expression in myeloid compartment does not influence susceptibility to LPS-induced septic shock

(A) Human and mouse IL-10 production in serum of ATA-hIL10BAC (blue bar) and GCC-hIL10BAC (red bar) transgenic mice challenged with LPS for 2 hours. (B) Survival of WT, *Il10-/-*, ATA-hIL10BAC, and GCC-hIL10BAC mice after i.p. injection with LPS (n=5 for each strain, one of two independent experiments). **p<0.001 (student t-test) comparing ATA-hIL10BAC mice to GCC-hIL10BAC mice.

2.4.6. Human IL10 promoter SNP haplotypes influence human IL-10 expression in CD4⁺ helper T cells

We previously established that human IL-10 expression in $CD4^+$ helper T cells in the ATA-hIL10BAC mouse is weak[137]. To determine whether this low IL-10-producing capacity in $CD4^+$ helper T cells is genetically controlled, we compared human IL-10 levels in splenic $CD4^+$ T cells isolated from ATA-hIL10BAC and GCC-hIL10BAC mice. Specifically, we cultured naïve $CD4^+CD62L^+$ under neutral conditions (Th0) or in the presence of IL-27 (which is known to promote IL-10 production in these cells)[140]. At day 3 of culture, IL-27-dependent human IL-10 was strongly produced by GCC-hIL10BAC mice relative to ATA-hIL10BAC mice (Figure 2.6A). In contrast, there was no difference between mouse IL-10 levels in the two transgenic strains (Figure 2.6B). In addition, when we stimulated the cells at day 4 of culture with PMA and Ionomycin (PI), we observed a robust allele-specific human IL-10 production (high in GCC and low in ATA) between the two transgenic mice at both protein (Figure 2.6C) and mRNA levels (Figure 2.6E). As expected, mouse IL-10 protein and transcript levels were the same between the mice (Figure 2.6D and Figure 2.6F). Thus, our results demonstrate that human *IL10* promoter SNP haplotypes alone can control IL-10 expression in $CD4^+$ T helper cells.

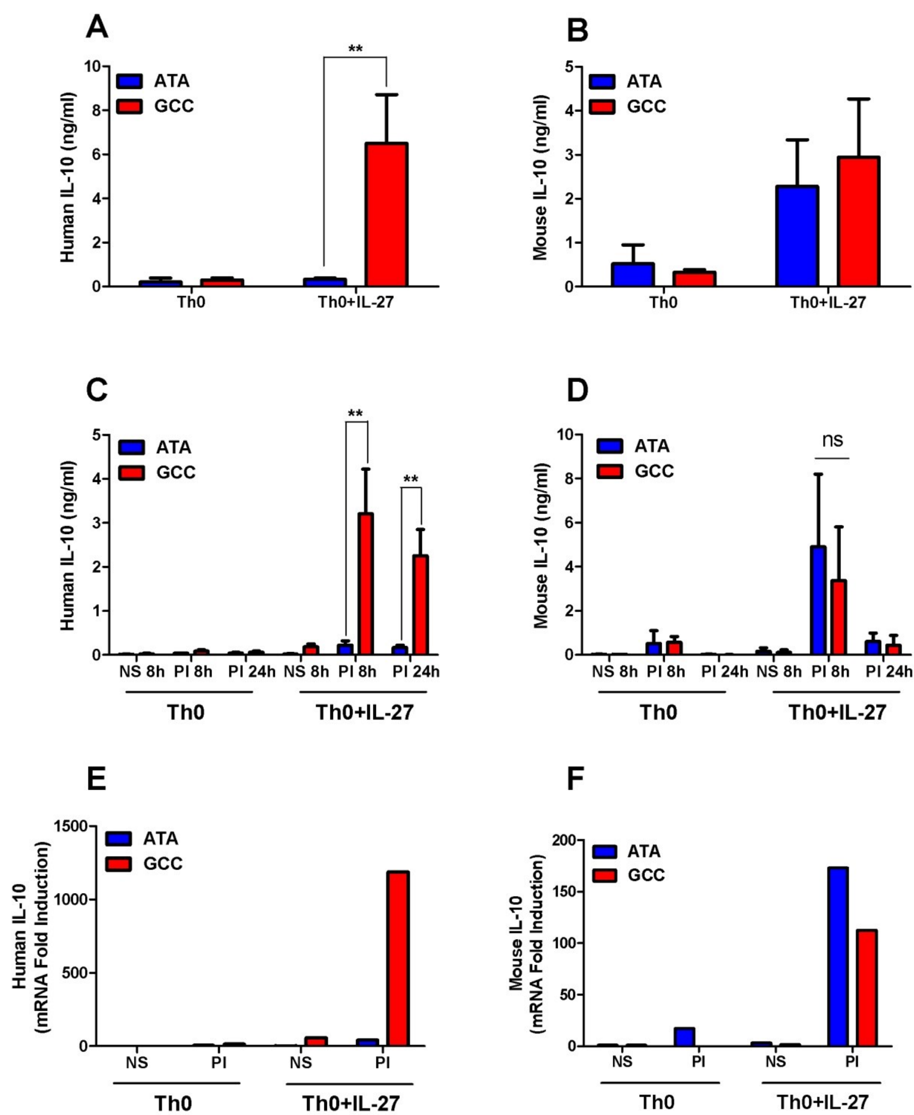


Figure 2.6. Human and mouse IL-10 expression in CD4+ T cells

Human (A) and mouse IL-10 (B) production by naïve $CD4^{+}CD62L^{+}$ T cells cultured in either neutral condition (Th0) or in presence of IL-27 (Th0+IL-27) for 3 days. (C) and (D) represent human and mouse IL-10 production in naive $CD4^{+}$ $CD62L^{+}$ Th0 cells cultured +/- IL-27 for 4 days and stimulated with +/- PI (PMA and Ionomycin) for 8 hours and 24 hours. (E) and (F) show human and mouse IL-10 mRNA induction in naïve $CD4^{+}$ $CD62L^{+}$ Th0 cells cultured and stimulated as mentioned above (one of two representative experiments). **p<0.001 (student t-test) comparing ATA-hIL10BAC to GCC-hIL10BAC mice. NS is Not stimulated cells (when upper case is used) and non-statistically significant (when lower case is used).

2.4.7. Allele-specific human IL-10 expression in $CD4^+$ helper T cells confers susceptibility to leishmaniasis

Infection with *L donovani*, a protozoan parasite responsible of visceral leishmaniasis, results in persistent infection in WT mice while *Il10*$^{-/-}$ mice clear the parasites[139]. In this setting, Th1-derived IL-10 is thought to promote pathogen persistence[139]. Also, our group has shown that *Il10*$^{-/-}$ mice reconstituted with the ATA-hIL10BAC transgene (*Il10*$^{-/-}$/ATA-hIL10BAC mice) are refractory to persistent infection due to low human IL-10 expression by Th1 cells[137]. Because GCC-hIL10BAC mice harbor a transgene which encodes for high human IL-10 levels in $CD4^+$ helper T cells, we questioned whether allele-specific human IL-10 expression in $CD4^+$ helper T cells is sufficient to alter disease risk. To address this question, we infected WT, *Il10*$^{-/-}$, *Il10*$^{-/-}$/ATA-hIL10BAC, and *Il10*$^{-/-}$/GCC-hIL10BAC with *L donovani,* and we followed the course of disease at day-21 and day-28 post-infection. We confirmed that WT mice were susceptible to chronic infection and that *Il10*$^{-/-}$ mice were refractory to disease (Figure 2.7A and 2.7B). In both WT and *Il10*$^{-/-}$/GCC-hIL10BAC mice, parasitemia in the liver reached its maximum at day-21 post-infection and began to drop afterward (Figure 2.7A). Similarly, parasite burden increased steadily in the spleen of these mice during the first month of infection (Figure 2.7B). In contrast, *Il10*$^{-/-}$ and *Il10*$^{-/-}$/ATA-hIL10BAC mice were not susceptible to chronic infection, as predicted. These data clearly demonstrate that the GCC-hIL10BAC and ATA-hIL10BAC mice have a differential susceptibility to *L donovani* infection.

Several groups, including ours, have implicated IL-10^+IFN-γ^+-producing $CD4^+$ T cells for being the pathogenic source of IL-10 that mediates persistent infection during leishmaniasis[137,139]. Accordingly, like *Il10*$^{-/-}$, mice in which *Il10* gene has been altered only in CD4+T cells are also resistant to disease, demonstrating that CD4+ T cells are the major cellular source of pathogenic IL-10 during visceral leishmaniasis[144]. Thus, we performed ICS analysis

by FACS to detect IL-10^{+}IFN-γ^{+}-producing CD4^{+} T cells in the spleens of WT, *Il10*$^{-/-}$, *Il10*$^{-/-}$/ATA-hIL10BAC, and *Il10*$^{-/-}$/GCC-hIL10BAC mice at day-21 and day-28 post-infection. We confirmed the presence of IL-10^{+}IFN-γ^{+}-producing CD4^{+} T cells in WT mice as well as the opposite in *Il10*$^{-/-}$ mice (Figure 2.7C). Consistent with previous studies, this cell population was barely detected in *Il10*$^{-/-}$/ATA-hIL10BAC mice (Figure 2.7C). In contrast, IL-10^{+}IFN-γ^{+}-producing CD4^{+} T were found at significantly higher frequency in *Il10*$^{-/-}$/GCC-hIL10BAC during the course of infection (Figure 2.7C). We also found similar results when we assessed total human IL-10^{+}-producing CD4^{+} T cells in the spleen (Figure 2.7D). Additionally, IFN-γ^{+}-producing CD4^{+} T cells that are involved in host protective immunity were also enhanced in *Il10*$^{-/-}$/ATA-hIL10BAC relative to *Il10*$^{-/-}$/GCC-hIL10BAC, but only at day-21 post-infection (Figure 2.7E). Together, the results demonstrate that allele-specific human IL-10 expression in Th1 cells alone can mediate susceptibility to persistent leishmaniasis.

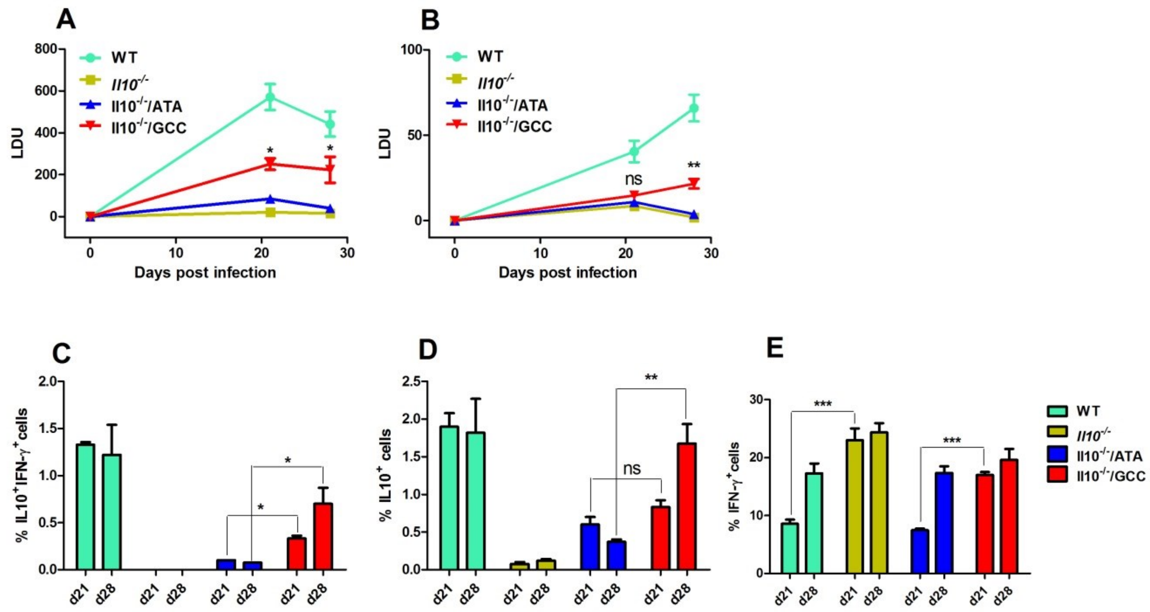


Figure 2.7. Allele-specific human IL-10 expression in CD4+ T cells mediate differential susceptibility to persistent infection

(A) and (B) represent parasite burden in liver and spleen, respectively, of WT, *Il10-/-*, ATA-hIL10BAC, and *Il10-/-*, GCC-hIL10BAC mice (n=5 for each strain, one of two independent experiments). LDU indicates *Leishmania donovani* unit (LDU). (C-E) Intracellular cytokine staining of splenic $CD4^+$ T cells restimulated *in vitro* with PI (PMA and Ionomycin) at day-21 and day-28 post-infection for $IL\text{-}10^+IFN\text{-}\gamma^+$ (C), IL-10 (D) and $IFN\text{-}\gamma^+$ (E). *p<0.05, **p<0.001, and ***p<0.0001 (student t-test) comparing ATA-hIL10BAC mice to GCC-hIL10BAC mice. ns is non-statistically significant.

2.5. DISCUSSION

The increasing numbers of GWAS, which link genetic variation in DNA to specific human diseases, have revealed that the majority of disease-associated SNPs lie in non-coding regions of the genome[122,123]. However, the ability to experimentally distinguish functional non-coding SNPs from other genetic noise has remained a challenge. In this study, we sought to develop a genetically humanized mouse model to establish the biological role of non-coding SNPs on gene expression and disease outcomes. We focused on human *IL10* gene, an essential gene that limits inflammatory responses[145]. We postulated that non-coding SNP haplotypes in the *IL10* locus control cell-type-specific human IL-10 expression and thereby disease susceptibility. We found that human IL-10 was produced in an allele-specific manner in macrophages both *in vitro* and *in vivo* following LPS stimulation. However, *Il10*$^{-/-}$ mice expressing either the ATA-hIL10BAC or GCC-hIL10BAC transgene were resistant to septic shock. Conversely, in the presence of IL-27, CD4+ T cells isolated from GCC-hIL10BAC mice produced significantly higher levels of human IL-10 compared to that of the ATA-hIL10BAC mice. Additionally, *Il10*$^{-/-}$ mice carrying the GCC-hIL10BAC construct was susceptible to persistent leishmaniasis due to heightened human IL-10 levels in Th1 cells co-expressing IL-10 and IFN-γ relative to *Il10*$^{-/-}$ mice reconstituted with the ATA-hIL10BAC.

To our knowledge, we have developed for the first time, an experimental system that can be used to directly interrogate whether genetic variation in non-coding DNA of an endogenous human locus are functional *in vivo*. Our approach is based on a BAC transgenic model that has been proven to be a reliable approach to model tissue-specific gene regulation *in vivo* in many settings[146]. Unlike human studies, our genetically humanized hIL10BAC mice allow us to

control for extraneous genetic and environmental effects. Also, the hIL10BAC allows us to access cells types and tissues relevant to disease pathogenesis.

In line with our approach, Chakravati et al. recently published an essay in which they propose a theoretical set of genetic criteria, based on the Koch's postulates in microbiology, to assign causality between genetic variation in non-coding DNA and complex human diseases[147]. In this essay, he proposed a set of 4 criteria to establish a causal link between non-coding SNPs and disease risk, as shown Box 1.

Box 1. Koch's Postulates for Complex Human Diseases and Traits

(1) Candidate gene variants are enriched in patients.
(2) Disruption of the gene in a model system gives rise to a model phenotype that is accepted as relevant and "equivalent" to the human phenotype.
(3) The model phenotype can be rescued with the wild-type human alleles.
(4) The model phenotype cannot be rescued with the mutant human alleles.

From: A. Chakravarti et al.
Cell. September 26, 2013

Together, the criteria recommended combining data from genetic epidemiology in humans with experimental evidence from model systems such as genetically engineered mice to define the function of genetic variants in the human genome. In this dissertation, we focused on human *IL10* gene as a proof of principle. In agreement with the first criteria, IL-10 is implicated in the pathogenesis of many human diseases, including leishmaniasis[148-152]. Also, disruption of the

Il10 gene in mice recapitulates phenotypes that are relevant in humans, thus fulfilling the second criteria[139,142,143,153,154]. Finally, by generating two genetically different hIL10BACs, we demonstrated that we can faithfully change disease outcome in *Il10* null mice carrying one of the human *IL10* alleles, thus providing a proof for criteria 3 and 4[147,155].

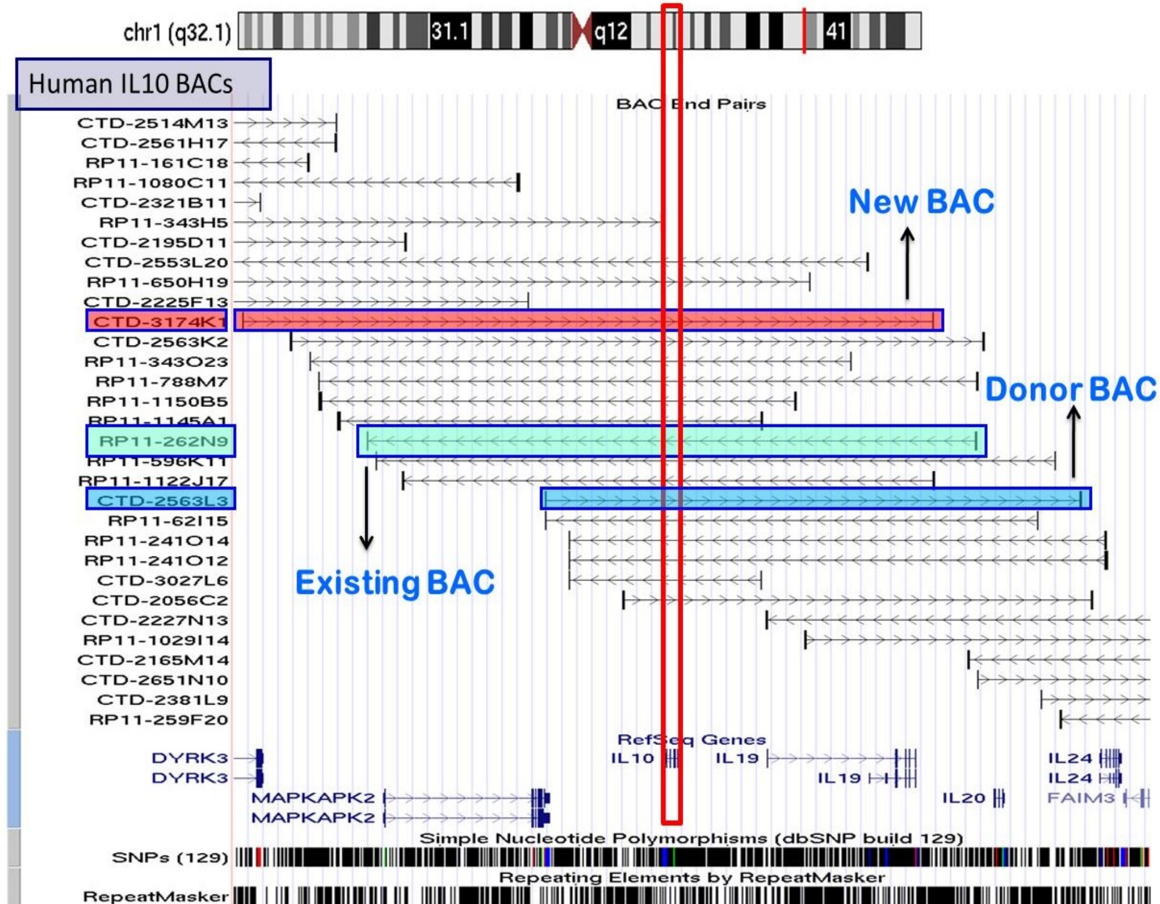
In addition to providing a new approach to functionally annotate non-coding SNPs *in vivo*, we also demonstrated that the impact of *IL10* SNP haplotypes on gene expression is cell-type-specific. Thus, our data validate some of the major findings of the ENCODE data as well as work from independent investigators using human cell-based studies. Specifically, the ENCODE project (like in our study) revealed that non-coding SNPs overlap with putative functional units, sometimes in a cell-type-specific manner, suggesting that these sequence variants may be causally linked to disease phenotypes[128,134-136]. Similarly, when Fairfax and collaborators sampled primary human monocytes and B cells from healthy donors, they discovered that 80% of non-coding SNPs that are linked to change in gene expression are specific to only one of the two cell types[124]. Similarly, in diseased donors, they found that GWAS-associated SNPs for systemic lupus erythematosus (SLE) were predominantly found in genes expressed by B cells, while variants implicated with IBD were enriched in genes expressed by monocytes, suggesting an important role for non-coding SNPs in modulating cell-type-specific gene activity during both health and disease. Furthermore, two concurrent papers recently studied the influence of genetic variation on gene expression in resting and activated immune cells. The results from these studies also suggest that the functional consequence of genetic variation in non-coding DNA may be only detected in a context/cell-type-specific manner[125,126].

Although our genetically humanized hIL10BAC mice provide a robust experimental tool to investigate human genetic variation in non-coding DNA, some limitations of this study should

be noted. First, we found high human IL-10 levels in macrophages from ATA-hIL10BAC compared to GCC-hIL10BAC mice. These findings are not in agreement with PBMCs-based or cell lines studies in which the "GCC" human *IL10* haplotype was associated with high IL-10-producing capacity[38,39,69]. Thus, more investigations are warrantied to determine whether the allele-specific human IL-10 expression that we observed in macrophages is dependent on the stimuli or receptor systems used to assess macrophage-specific human IL-10 expression *in vitro*. Secondly, we assayed human IL-10 production in CD4+T cells and macrophages only; thus, further studies are needed to assess allele-specific gene expression in other cellular sources of IL-10. Thirdly, in the septic shock model, we used a low-dose LPS that was not lethal to WT mice (our control group). Thus, a sub-lethal dose of LPS could have revealed differences in survival rate between ATA-hIL10BAC and GCC-hIL10BAC mice. Finally, it is possible that the difference in survival and/or immune responses between the two genetically humanized mice is pathogen specific. This could have been addressed by investigating additional disease models such as *Toxoplasma gondii* and Influenza virus infections. Another possibility is the effect of the host (i.e., the genetic background of the mouse which is C57BL/6 in this study) on disease outcomes. We already have these mice backcrossed to BALB/c animals to test that likelihood in the future.

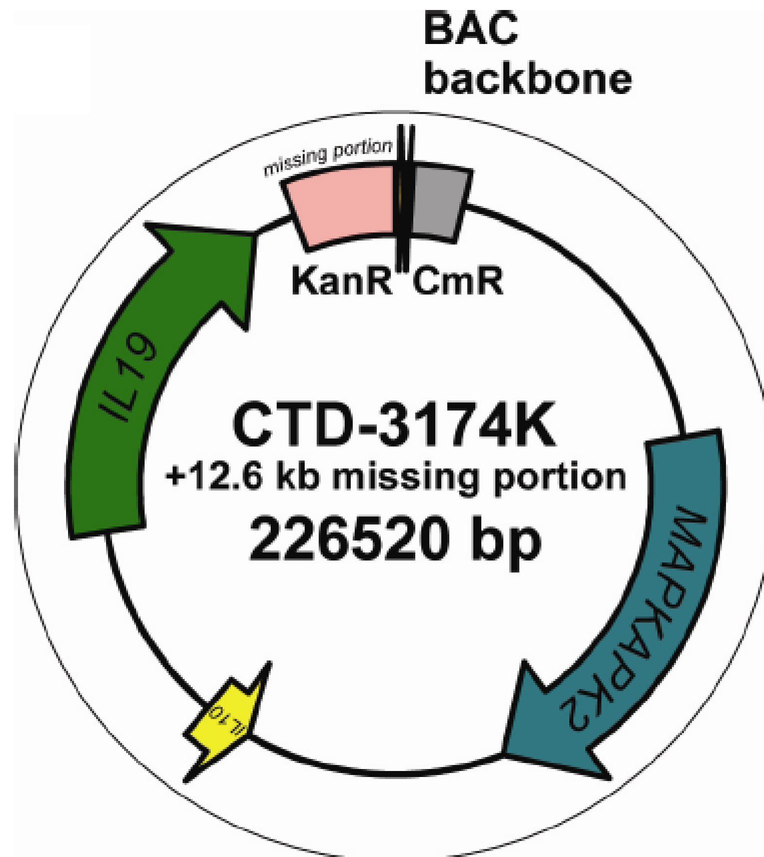
In summary, we successfully developed a genetically humanized mouse model to study the influence of non-coding SNPs on human IL-10 expression and disease susceptibility. These mice would allow us to define in the near future the molecular basis of allele-specific human IL-10 expression such as allele-specific DNA binding and chromatin architecture (in and around the SNPs) that coordinately regulate *IL10* transcriptional activity. By combining immunological and molecular biology strategies to further characterize these mice, we believe we will gain new

insights on how genetics regulates human IL-10 expression. We also believe that our approach can be extended to other human genes to accelerate rational development of personalized therapies and vaccines at both basic and preclinical stages.



Appendix 2.1. Bioinformatics search of the BAC clones used in this study

This screenshot shows the search we performed using the University of California Santa Cruz (UCSC) Genome Browser (http://www.genome.ucsc.edu) to identify BAC clones which not only harbor human *IL10* gene but also have the same size as our Existing BAC (RP11-262N9 colored in light green). Human chromosome 1 is represented at the top of the diagram. Chromosomal location of *IL10* is marked with a red bar. The Left end panel shows the list of human BAC clones that overlap with *IL10*. The BAC clones used in this study are as follows: Acceptor BAC (CTD-3174K1; pink; chromosomal location: chr1:206,816,360-207,021,737; length: 205,378 bp), Existing BAC (RP11-262N9; light green, chromosomal location chr1:206,853,401-207,034,489; length: 181,089 bp), and Donor BAC (CTD-2563L3; light blue, chromosomal location: chr1:206,906,203-207,065,343; length: 159,141 bp).



Appendix 2.2. A circular map of the Modified GCC-hIL10BAC clone

Chapter 3

Cell-type-specific regulatory control of IL-10 and IL-24 expression in murine macrophages and NK cells

3.1. ABSTRACT

The IL-10 locus of cytokines include of *Il10*, *Il19*, *Il20*, and *Il24* genes which together form a cytokine cluster spanning ~145kb on both mouse and human chromosome 1q32. Like the prototypical T_H2 locus, clustering of these four cytokine genes is thought to coordinate their expression through shared genomic elements as well as their biological functions. However, in the *Il10* locus, only IL-10 and IL-24 are co-expressed in T_H2 cells. The regulation of IL-24 in other cellular sources of IL-10 and its role in immune responses are not well defined. To determine whether *Il10* and *Il24* share common regulatory pathways, we examined IL-24 expression in macrophages and NK cells, which are important cellular sources of IL-10. In LPS-stimulated macrophages, optimal IL-24 expression requires IL-4 and Stat6 while IL-10 expression is not mediated by Stat6. Similarly, in IL-2-stimulated NK cells, IL-10 expression is independent of IL-4, but IL-24 is robustly induced by IL-4/Stat6. We found five putative Stat-binding elements, including one at the proximal promoter of the *Il24* gene. Each site interacted with IL-4-induced Stat6 in macrophages and NK cells. Remarkably, Stat4 was also enriched to these sites in IL-12-stimulated NK cells, but not in macrophages. In NK cells, IL-24 induction required type I IFN receptor signaling regardless of the stimuli being added; meanwhile IL-10 expression required type I IFNs only in macrophages. Post-translational histone modifications in the *Il24* gene were mediated by IL4/Stat6 in macrophages but not in NK cells. Thus, we demonstrated that although IL-10 and IL-24 are co-expressed in macrophages and NK cells, they are not co-regulated, suggesting that they might not share common genomic regulatory elements.

3.2. INTRODUCTION

Homologous genes in clusters can be coordinately regulated to synchronize gene expression programs and biological functions. In the eukaryotic genome, there are several important complex loci containing multiple genes such as the β–globin gene cluster and the Th2 locus. Numerous reports have demonstrated that these genes are co-regulated through cooperative actions of distal regulatory elements with promoters and trans-acting factors in a cell-type-specific manner [156-158]. On the other hand, some cytokine gene clusters have distinct gene regulatory programs despite being in relatively close proximity. The human *IFNG* locus which includes *IFNG*, *IL26*, *IL22* genes for instance, have unique gene expression patterns governed by discrete, gene-specific regulatory elements[159]. Therefore, these loci can serve as models to study the regulation of other gene clusters such as the murine *Il10* locus which has not been well studied.

The *Il10* gene is localized in chromosome 1 (1q32) along with other members of the IL-10 family of cytokines: IL-19, IL-20, and IL-24. The latter was the first IL-10 homologous gene identified and historically named melanoma differentiation-associated gene 7 (mda-7)[84]. Although *Il24* and *Il10* genes are at the opposite end of the locus and transcribed in different directions, they are co-expressed in T_H2 cells[86-88,160]. In contrast, *Il19* and *Il20* gene products are mostly produced by myeloid and epithelial cells but not by T lymphocytes[161]. These observations suggest that IL-10 and IL-24 expression may be synchronized in T_H2 cells and other IL-10-producing cell types through shared genomic elements like in the classical T_H2 cytokine locus, consisting of the *Il4*, *Il5* and *Il13* genes[157] and the *IL17A*/*IL17F* locus[162].

Unlike IL-10, of which the expression patterns have been well described in many cell types[160], we are just beginning to characterize the cellular sources of IL-24. In mice, IL-24 is

robustly expressed in T_H2 cells and has been shown to be one of the strongest IL-4-induced Stat6 target genes in these cells[86-88]. In addition to T_H2 cells, TCR stimulation with anti-CD3 alone or in combination with IL-2 promotes IL-24 expression in murine T lymphocytes[90,163]. Similarly, cross-linking of B cell receptor with CD40 ligands and anti-IgM triggers IL-24 expression in B lymphocytes[91]. In human PBMCs, IL-24 is induced in polyclonally activated T lymphocytes and in monocytes/macrophages[89,90]. IL-24 expression has also been noted in non-immune cells such as melanocytes and keratinocytes stimulated with cytokines, including IFN-β, IL-2, IL-1β, and TNF-α[84,93,95].

In this study, we hypothesized that IL-10 and IL-24 are regulated by similar pathways such as shared signal transduction, transcription factors requirements, and epigenetic modifications. Thus, we characterized IL-10 and IL-24 expression pattern in macrophages and NK cells. We chose to study macrophages and NK cells because expression of IL-24 in these cells is not well defined, but also because macrophages and NK cells are classical innate immune cells and important cellular sources of IL-10[160]. In fact, the biological importance of macrophage-specific IL-10 on disease susceptibility has been well established in mice. For example, macrophage-derived IL-10 confers protection from LPS-induced septic shock[164]. In contrast, the role of NK-specific IL-10 is not completely understood. Though NK-derived IL-10 has been implicated in protecting from immunopathology during infection with rapidly disseminating pathogens such as Toxoplasma gondii and Yersinia pestis[165], as well as in chronic mouse cytomegolavirus (MCMV) infection[166].

Because *Il10* and *Il24* genes are localized at the extreme ends of the locus, we thought that studying IL-10 and IL-24 co-expression would also be helpful in defining the genomic boundaries of the *Il10* locus. We found that IL-24 and IL-10 can be co-expressed in NK cells and

macrophages but only in the presence of IL-2 and LPS, respectively. We also found that the effects of IL-2 and LPS on IL-24 expression could be enhanced by addition of IL-12 (in NK cells) or IL-4 (in both NK cells and macrophages) in Stat4 and Stat6-dependent manners. IL-4-induced Stat6 and IL-12-induced Stat4 were recruited to multiple intronic sites of the *Il24* locus. Histone modifications were also dependent on Stat6 and Stat4 in macrophages and NK cells, respectively. Interestingly, type I IFNs regulate IL-24 expression in NK cells but not in macrophages. The opposite was observed for NK- and macrophage-specific IL-10. Most importantly, our results clearly demonstrate that IL-24 and IL-10 are not co-regulated, as opposed to the well-defined T_H2 locus, which suggests that they do not share common regulatory elements in the *Il10* locus.

3.3. MATERIALS AND METHODS

3.3.1. Mice

Wild-type (WT) and Stat6$^{-/-}$ mice on the C57BL/6 background were maintained at the Johns Hopkins University animal facility. WT and Stat4$^{-/-}$ mice on BALB/c background were purchased from the Jackson Laboratory and bred at the same facility. All mice were maintained under specific pathogen-free conditions and were used between 8 and 12 weeks of age. All experimental procedures were approved by the Johns Hopkins University Institutional Animal Care and Use Committee.

3.3.2. Cytokines and antibodies

Recombinant human IL-2 was obtained from the NCI repository as a donation. Recombinant murine IL-4, IL-12, and IL-13 were purchased from PeproTech (Rocky Hill, NJ). Purified Lipopolysaccharide (LPS) in lyophilized powder from Escherichia coli 0111:B4 was purchased from Sigma (St. Louis, MO). Stat6 (Sc-981) and Stat4 (Sc-486) monoclonal antibodies were obtained from Santa-Cruz Biotechnology (Santa Cruz, CA) and Stat5 antibody from R&D Systems (Minneapolis, MN). Acetylated histone H3 (AcH3; 06-599B) and Histone H3 lysine 27 trimethylation (H3K27me3; 07-449) were obtained from Upstate Biotechnology (Millipore, Billerica, MA).

3.3.3. Media for cell culture

NK cell tissue culture media was prepared with RPMI 1640 medium (Lonza, Walkersville, MD) supplemented with the following: 5% or 10% heat-inactivated Fetal Bovine Serum (FBS) from Atlanta Biologicals, Inc. (Flowery Branch, GA), 2 mM of L-glutamine

(Cellgro®, Mediatech, Inc., Manassas, VA), 1X of Non-essential amino acids (Gibco®, Life Technologies, Grand Island, NY), 1mM of Sodium pyruvate (Gibco®, Life Technologies), 10mM of 2-mercaptoethanol (Gibco®, Life Technologies, Grand Island, NY), 100 U/ml of penicillin (Cellgro®, Mediatech, Inc., Manassas, VA), and 100 mg/ml of streptomycin (Cellgro^R, Mediatech, Inc., Manassas, VA). The culture media for the bone marrow-derived macrophages (BMM media) were made as follows: DMEM medium (Lonza, Walkersville, MD), 10% of heat-inactivated FBS, 2 mM of L-glutamine, 10mM of 2-mercaptoethanol, 100 U/ml of penicillin, 100 mg/ml of streptomycin, and 50 µg/ml of gentamycin (Quality Biological, Gaithersburg, MD).

3.3.4. Isolation and culture of murine NK cells

Freshly isolated spleens were mashed in 5% fetal bovine serum (FBS) RPMI 1640 and passed through a cell strainer (BD) to obtain single-cell suspensions as previously described[(167)]. The cell suspensions were pelleted down by centrifugation. The red blood cells (RBC) were lysed from the pellet with ACK lysis buffer (Quality Biological, Gaithersburg, MD). RBC-free cells were washed and resuspended in 5% FBS RPMI 1640. The suspensions were passed through a sterile, pre-wetted nylon wool column and incubated for 50 mn at 37°C. Cells that were not bound to the nylon wool were eluted out with the 5% FBS RPMI 1640, washed, counted and resuspended in 10% FBS RPMI media supplemented with high-dose IL-2 (10,000 U/ml). IL-2-conditionned cells were cultured for 6 days at a density of 1×10^6 per ml to obtain mature NK cells as evidenced by expression of NK1.1 in 70-95% of the cells in culture. At day-6 of culture, adherent cells were harvested following treatment with 1 nM of EDTA treatment (Quality Biological, Gaithersburg, MD), washed two times and starved for 3 hours to remove

any trace of IL-2. The cells were seeded at $3x10^6$ per well in a 6-well plate at a concentration of $1x10^6$ cells per ml and stimulated with the following cytokines: IL-2 (100 UI/µl), IL-12 (10 ng/ml), and IL-4 (10 ng/ml).

3.3.5. Isolation and culture of bone marrow-derived macrophages

Bone marrow cells were flushed from femurs and tibias of mice with the BMM culture media. The cells were then passed through a cell strainer, pelleted down and resuspended in the BMM media supplemented with 30% of L929-conditioned media from the American Type Culture collection (ATCC, Manassas, VA). Three ml of the cells at $1x10^6$ per ml were seeded in a 6-well plate and maintained for 5 days with media change at day-2 and day-4. At day-5, fully differentiated cells were washed three times with 1X PBS then maintained in BMM media overnight. Fresh media was added at day 6 and the cells were stimulated with LPS (100 ng/ml), IL-4 (10 ng/ml), and IL-13 (10 ng/ml).

3.3.6. Messenger RNA isolation and analyses

Total RNA was isolated with TRIzol® reagent (Ambion, Life Technologies, Carlsbad, CA). One microgram of mRNA was used as a template to generate complementary DNA (cDNA) using a SuperScript® First-strand Synthesis System (Invitrogen™, Life Technologies, Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed by SYBRGreen assay (Applied Biosystems, Life Technologies, Grand Island, NY). Murine β-2-microglobulin was used as a housekeeping gene and all mRNA expression was normalized to its levels. The primers used for gene expression analysis were the following: 5'-ACTTCAGCAGGCTGTGGG-3' and 5'-GATGACATCACAAGCATCCG-3' for mouse *Il24*, 5'-TCGGCCAGAGCCACATG-3' and

5’-TTAAGGAGTCGGTTAGCAAGTATGTTG-3’for mouse Il10, and 5’-AAATGCTGAAGAACGGGAAAA-3’ and 5’-ATAGAAAGACCAGTCCTTGCTGAAG-3’ for mouse β-2-microglobulin. Data are shown as fold induction over non-stimulated cells (NS).

3.3.7. Chromatin Immunoprecipitation assay (ChIP)

ChIP assay was conducted using an EZ-Magna ChIP kit from Upstate Biotechnology (Millipore, Billerica, MA). The assay was performed according to the manufacturer’s protocol with minor modifications. NK cells and BMM were stimulated for 2 hours with the appropriate stimuli and fixed with 1% paraformaldehyde (Fisher Scientific, Fair Lawn, NJ) for 10 mn. Nuclear lysates were obtained from the fixed cells and the DNA was sonicated on ice to obtain small fragments. Sonicated DNA was diluted in assay diluent in the presence of Protein A beads and protease inhibitors (all provided in the kit) and immunoprecipitated with specific antibodies for 4 hours. The crosslink between DNA and proteins was reversed by protease K digestion at 62ºC for 2 hours followed by incubation at 95ºC for 10 mn. The DNA was then purified in a spin-column and used as a template for qPCR. The primers used for qPCR are the following:

Site 0: 5’-GGTCATGCTTCCCTGGAGAA-3’ and 5’-ACCCCCCTGTCTAAGAGCAAA-3’ which was initially published by Wei and al. in 2010[88]

Site 1: 5’-CAGTTAACCCTGCTACCTTG-3’ and 5’-CAGGCCAACTTAAGCAG-3

Site 2: 5’-CTGCTTAAGTTGGCCTG-3’ and 5’-CATCAAGAGGTTCTAGACTC-3

Site 3: 5’-CCCCTGTGTGGTGTAGCTTCA-3’ and 5’- AAAGCCCTGCCTCTCATCCT-3

Site 4: 5’-CAGAGGCCATTCCACACA-3’ and 5’-GGGGTCAGGTATGTTAATG-3’

Non-IP control DNA was also treated on the side and used as Input. The results are shown as Percent of the Input (% Input).

3.4. RESULTS

3.4.1. IL-24 and IL-10 have unique expression profile in NK cells and macrophages

Previous studies have identified receptor pathways which regulate IL-10 expression in NK cells and macrophages[167,168]. The control of IL-24 expression in those cells types, however, is not well established. Utilizing IL-2-cultured splenic NK cells and bone marrow-derived macrophages (BMM) as models, we sought to define common pathways leading to IL-24 and IL-10 induction in NK cells and macrophages. We stimulated NK cells and bone marrow-derived macrophages with stimuli that are known to induce IL-10 expression in these cell types. In NK cells, expression of IL-24 mRNA was dependent on IL-2 stimulation (Figure 3.1A). The combination of IL-2 plus IL-12 was highly synergistic in inducing IL-24 and IL-10 mRNAs (Figure 3.1A). Unlike IL-10, IL-24 expression was also enhanced upon stimulation with IL-2 plus IL-4 in NK cells (Figure 3.1A). In macrophages, cytokines alone did not enhance IL-24 and IL-10 expression. Stimulation with LPS alone modestly turned on IL-24 expression, yet was sufficient for optimal IL-10 expression (Figure 3.1B). Conversely, co-stimulation with LPS and IL-4 or LPS plus IL-13 was required for optimal IL-24 expression in macrophages (Figure 3.1B). These results indicate that IL-24 and IL-10 can be co-expressed in NK cells and macrophages, but are regulated independently by different pathways.

We next compared the kinetics of IL-24 and IL-10 expression using stimuli that induce both genes in NK cells (IL-2+IL-12) and BMM (LPS+IL-4). The cells were harvested every hour for up to 6 hours, and at 12 hours and 24 hours post-stimulation. Transcript levels were analyzed for each time point and the mRNA was normalized to non-stimulated controls of the same time points. As shown in Figure 3.1C, IL-24 mRNA expression in NK cells was weakly induced at 3 hours after stimulation but reached its maximal levels at 6 hours post-stimulation. In contrast, IL-

10 mRNA peaked earlier (3h) in NK cells followed by a second burst at 6 hours. In macrophages, IL-24 and IL-10 expression peaked at 3 hours post-stimulation and was sustained at lower levels up to 6 hours, and then were back to baseline level by 12 hours of stimulation (Figure 3.1C). These data demonstrate that IL-24 expression is delayed in NK cells compared not only to IL-10 mRNA but also to macrophage-derived IL-24 and IL-10 mRNAs.

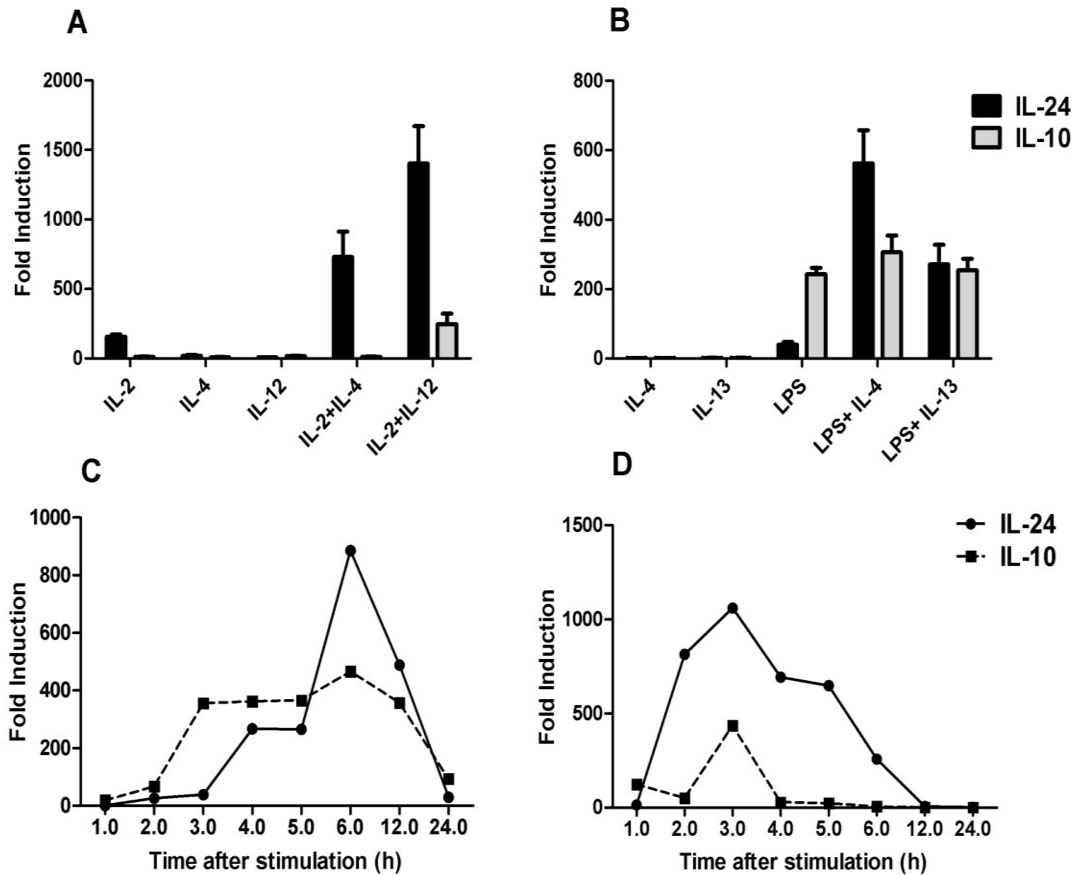


Figure 3.1. IL-24 and IL-10 are co-expressed in NK cells and macrophages

NK cells (A) and bone-marrow derived macrophages (BMM) (B) were stimulated with the indicated stimuli for 6 hours and 3 hours, respectively. IL-24 (black) and IL-10 (gray) mRNA expression was determined by quantitative real time PCR (qPCR) in both cell types. Data are represented as fold increase over non-stimulated cells. Results in (A) and (B) are mean ± sem of three representative experiments. (C) and (D) show one of two independent kinetics studies in which IL-24 and IL-10 mRNA levels were assessed in NK cells stimulated with IL-2 plus IL-12 (C) and BMM treated with LPS plus IL-4 (D) at the indicated time points.

3.4.2. IL-24 and IL-10 mRNA have similar half-life in NK cells but not in macrophages

Having established the synergistic effects of cytokines such as IL-4 on IL-2-dependent and LPS-dependent IL-24 expression in NK cells and macrophages, respectively, we questioned whether this synergy is mediated through mRNA stabilization, which is known to be important in regulating IL-24 expression in keratinocytes[95]. Thus, we determined the stability of IL-24 and IL-10 mRNAs by treating the cells with the transcriptional inhibitor Actinomycin D (ActD), after 3 hours of stimulation. Stimulated cells with no ActD treatment were used as control. Transcripts levels were assessed at 2h and 4h post-ActD treatment and normalized to control cells. IL-24 and IL-10 mRNA half-life were relatively short (less than 2h) in NK cells (Figure 3.2A). Remarkably, IL-24 and IL-10 mRNA levels were reduced by 80% after 2 hours of ActD treatment in NK cells (Figure 3.2A). The addition of IL-4 or IL-12 did not have a stabilizing effect on IL-2-induced IL-24 and IL-10 mRNAs expression. In macrophages however, IL-24 mRNA was highly stable and remained at the same level as non-treated controls for up to 4 hours following ActD treatment independently of IL-4 co-stimulation (Figure 3.2C). In contrast to IL-24, up to 70% of IL-10 transcription was abolished after 2h of transcriptional blockade with ActD relative to control cells (Figure 3.2D). Thus, in NK cells, IL-24 and IL-10 mRNAs have similar mRNA stability, whereas, IL-24 mRNA is more stable than IL-10 in BMM. These data indicate that cytokine-dependent enhancement of IL-24 mRNA expression is not mediated through mRNA stabilization

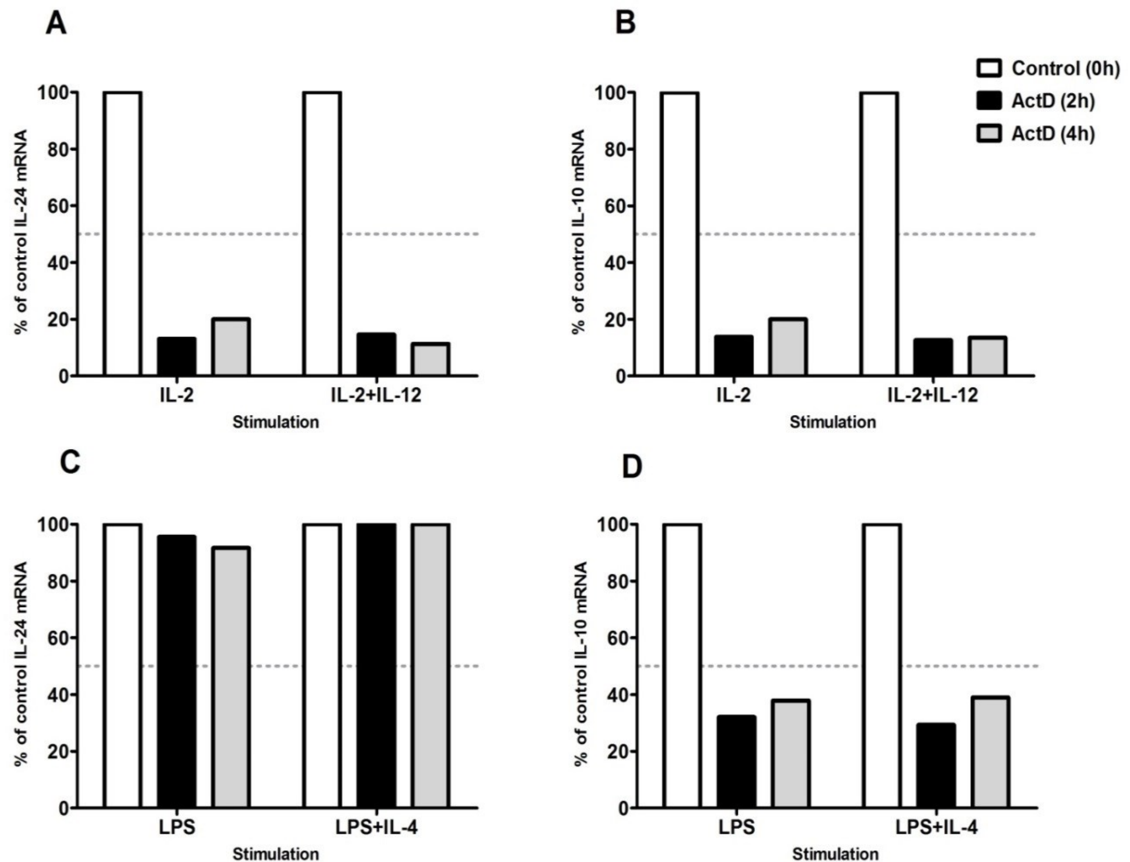


Figure 3.2. IL-24 and IL-10 mRNA stability in NK cells and macrophages
NK cells (A and B) and BMM (C and D) were treated with the indicated stimuli for 3 hours. The cells were then treated with ActD for 2 hours (black) and 4 hours (gray) to block transcription and follow half-life of mRNAs already made. Transcript levels were measured and normalized to non-stimulated cells for each time point. Data are represented as a percent of control mRNA expression which are cells with no ActD treatment (control: 0h; white) that were therefore set at 100% mRNA expression. The data is based on one of two independent experiments. Dashed line shows 50% of control mRNA expression.

3.4.3. Stat6 is required for optimal IL-4-induced IL-24 expression in NK cells and macrophages

Several groups have established that IL-4/Stat6 is a potent regulator of IL-24 and IL-10 expression in T_H2 cells[86,88]. Given the synergistic effects of IL-4 on IL-2-dependent IL-24 in NK cells and LPS-induced IL-24 expression in macrophages, we investigated the molecular basis of IL-4-induced IL-24 expression in NK cells and macrophages. Because IL-4 signals through Stat6 downstream of IL-4R, we examined IL-24 and IL-10 expression in WT and *Stat6*$^{-/-}$ mice. In NK cells, the synergistic effect of IL-2 plus IL-4 co-stimulation on IL-24 induction was greatly reduced in the absence of Stat6 (Figure 3.3A). Interestingly, IL-24 expression is not completely dependent on Stat6 in NK cells as IL-24 induction by IL-2+IL-12 remained intact in *Stat6*$^{-/-}$cells. In macrophages, the effects of LPS plus IL4/IL-13 were also substantially diminished in *Stat6*$^{-/-}$ relative to WT controls (Figure 3.3C). In contrast, Stat6 was largely dispensable for IL-10 expression in both cell types (Figures 3.3B and 3.3D). The data demonstrate that Sta6 plays an important role in regulating IL-24 expression in both NK cells and macrophages. The results also highlight a key difference between IL-24 and IL-10 induction pathways.

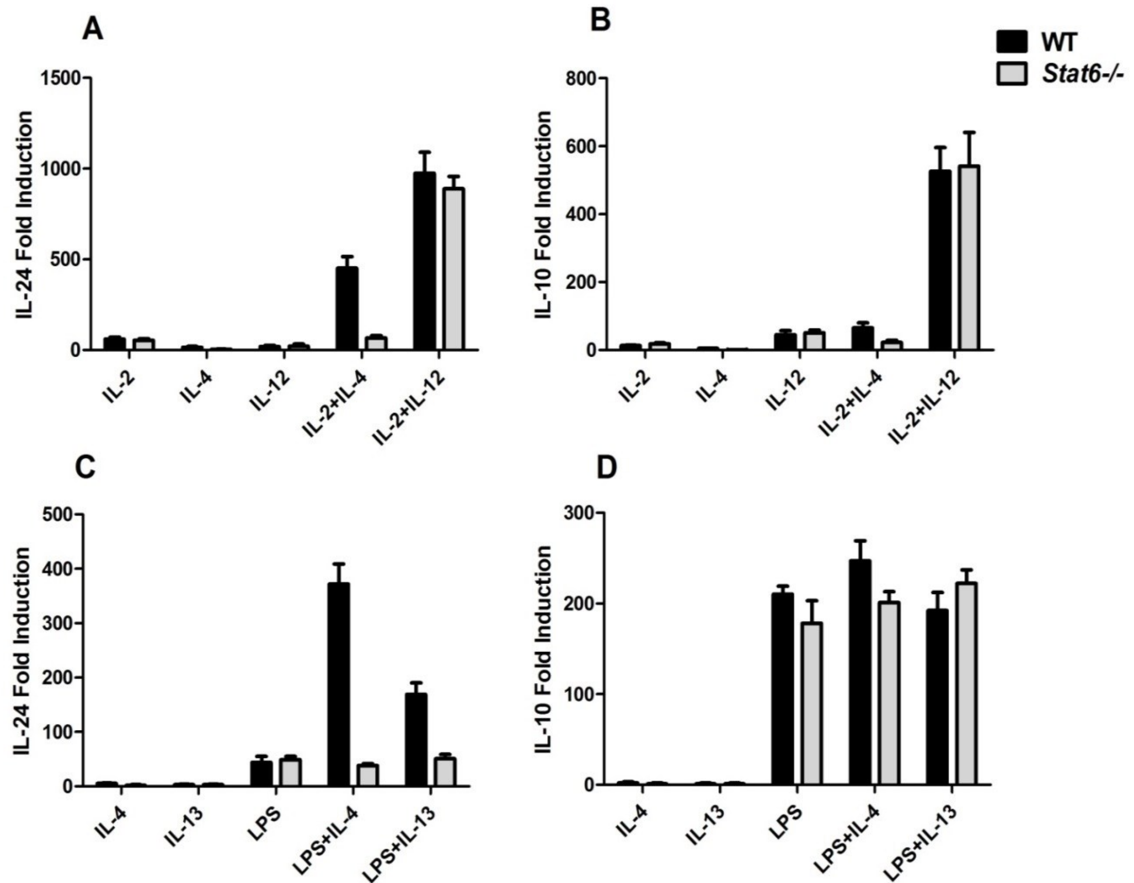


Figure 3.3. IL-4-induced Stat6 mediates IL-24 expression in NK cells and macrophages
Cultured NK cells (A and B) and BMM (C and D) were generated from WT (black) and Stat6 deficient cells (gray). Cells were stimulated for 6h for NK cells and 3h for macrophages as previously described. IL-24 (A and C) and IL-10 (B and D) mRNA levels were measured by qPCR in each cell type and normalized to non-stimulated cells. Results show mean ± sem of tree representative experiments. The data are represented as fold induction over non-stimulated cells.

3.4.4. Stat4 is required for maximal IL-24 and IL-10 co-expression in NK cells

We have previously demonstrated that IL-12-induced Stat4 is required for optimal induction of IL-10 expression in NK cells[167]. Because IL-2 and IL-12 co-stimulation strongly induced IL-24 and IL-10 expression in NK cells, we hypothesized that Stat4 may be involved in NK-specific IL-24 expression. We measured IL-24 and IL-10 expression in $Stat4^{-/-}$ and WT mice. As with IL-10, IL-24 expression was substantially reduced in Stat4-deficient NK cells (Figure 3.4A). Unexpectedly, in sharp contrast to IL-10, IL-24 mRNA expression was also diminished even in the absence of IL-12 stimulation (Figure 3.4A and 3.4B). The effect of Stat4-deficiency on IL-10 expression was only evident under IL-12 stimulation conditions (Figure 3.4B). In *Stat4*$^{-/-}$ macrophages, there was a slight reduction of IL-24 mRNA expression in cells treated with LPS and IL-4, meanwhile IL-10 expression was unaffected by Stat4-deficient in macrophages (Figure 3.4C and 3.4D). These data suggest that Stat4 may be necessary for NK-specific IL-24 induction, independent of IL-12.

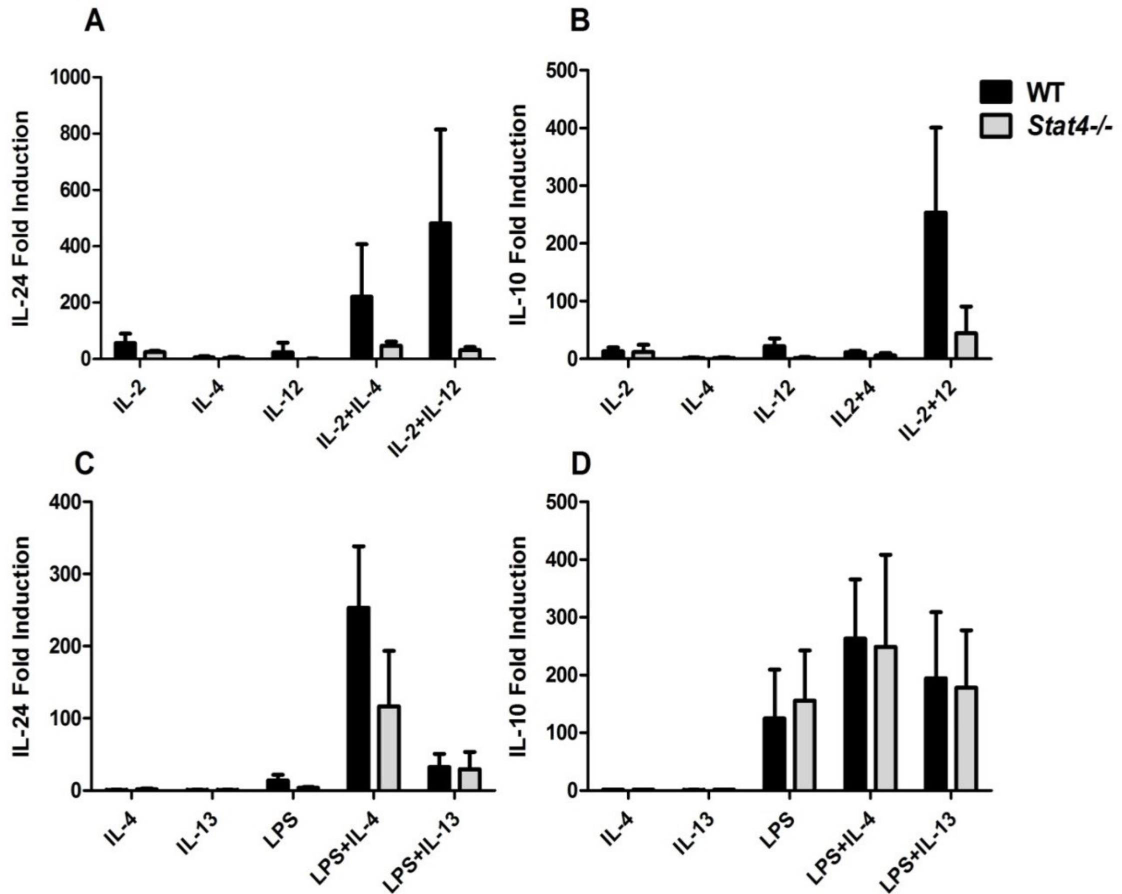


Figure 3.4. Stat4 is essential for optimal induction of IL-24 and IL-10 in NK cells
WT (black) and Stat4 deficient mice (gray) were used to generate and NK cells (A and B) and BMM (C and D). The cells were stimulated as previously described. IL-24 (A and C) and IL-10 (B and D) mRNA levels were assayed by qPCR and normalized to cells with no stimulation. Results are mean ± sem of three representative experiments. The data are represented as fold increase over non-stimulated cells

3.4.5. IL-24 and IL-10 expression is differentially regulated by Type-I Interferons

To identify stimuli or receptor systems involved in Stat4-dependent, IL-12-independent IL-24 expression, we turned to type I interferon (IFN) signaling because IFN are known to activate multiple pathways, including Stat4[(169)]. Additionally, recombinant INF-β has been shown to induce IL-24 expression in melanoma cells[(84)] and IL-10 in macrophages[(168)]. We hypothesized that NK cells and macrophages express IFN in response to cytokine and LPS stimulation, which acts in an autocrine loop to enhance IL-24 expression in a Stat4-dependent manner. Therefore, we examined mRNA levels in cells lacking IFN-receptor-α (*Ifnra*$^{-/-}$). WT cells were used as controls. In *Ifnra*$^{-/-}$ NK cells, IL-24 expression was drastically reduced under all stimulation conditions in contrast to IL-10 which was not affected by the loss of IFN signaling (Figure 3.5A and 3.5B). We also examined the capacity of IFNs in directly inducing IL-24 and IL-10 mRNAs and found that IFN-β alone did not induce IL-24 expression in NK cells but synergize with IL-2 (Appendix 3.1A). In *Ifnra*$^{-/-}$ macrophages, IL-24 expression was not affected, whereas IL-10 mRNA was reduced by half (Figure 3.5C and 3.5D). When we added recombinant IFN-β to WT macrophages, IL-10 mRNA was induced in contrast to IL-24 (Appendix 3.1B). The data point to a differential regulation of IL-24 and IL-10 expression between NK cells and macrophages with Type-I IFN playing a central role in this process.

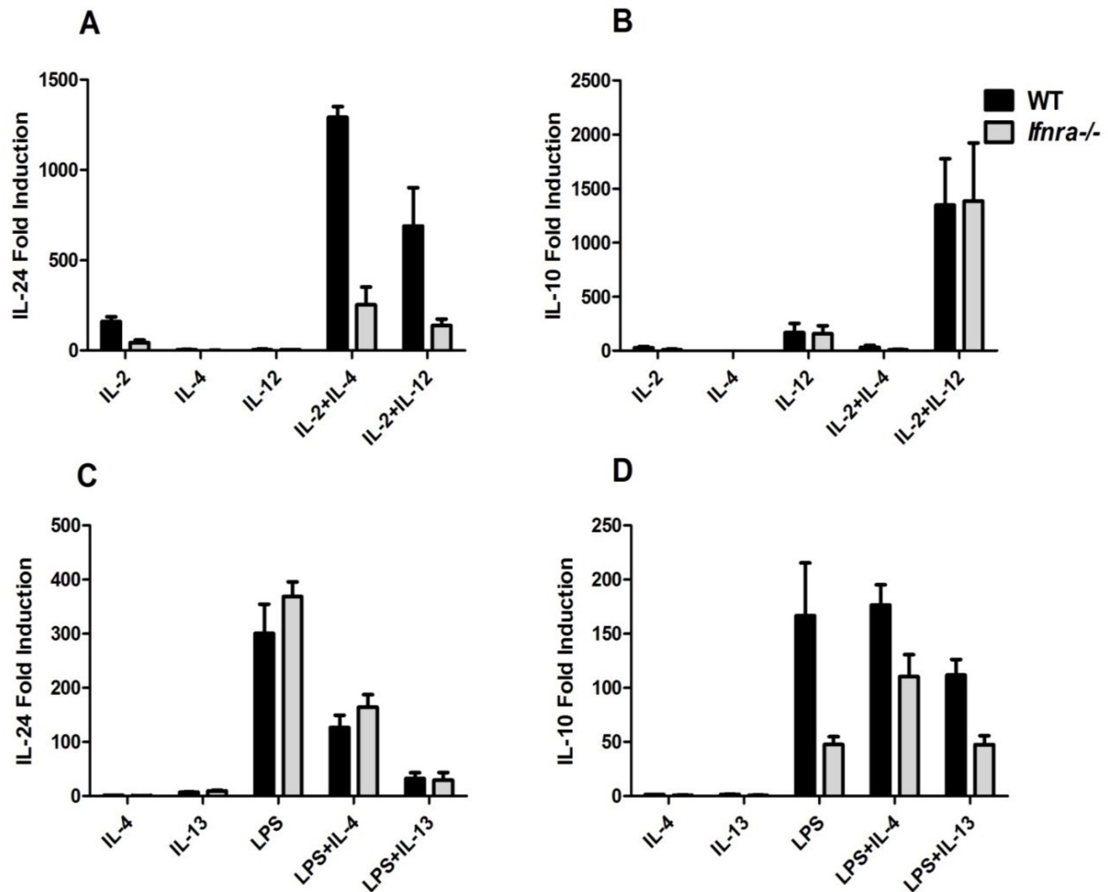


Figure 3.5. Cell-type-specific expression of IL-24 and IL-10 is mediated by Type-I IFNs
Transcripts of IL-24 (A and C) and IL-10 (B and D) were determined in WT (black) and *Ifnra-/-* (gray) in NK cells (A and B) and macrophages (C and D). Results indicate mean ± sem of three representative experiments. The data are represented as fold increase over non-stimulated cells.

3.4.6. Cytokine-induced Stat4 and Stat6 are recruited to the Il24 locus in NK cells and macrophages

We have previously shown that IL-12-induced Stat4 binds to an intronic site of *Il10* in NK cells[167] . We postulated that Stat4 and Stat6 may interact with the endogenous *Il24* gene in NK cells and macrophages in a signal-specific manner. We first performed a bioinformatics search by scanning through the *Il24* gene body looking for Stat-binding elements. Our search string identified five putative sites, including the previously identified Stat6 element at the proximal promoter of the locus[86,88] . Genomic localization of the putative Stat-binding elements is shown in Figure 3.6. The four putative sites are located in intron 1, 2 and 5. The two sites that are next to each other in intron 1 are 23bp apart. We employed Chromatin Immunoprecipitation (ChIP) assay to examine Stat6 and Stat4 recruitment in NK cells and macrophages. In NK cells, IL-4-induced Stat6 and IL-12-induced Stat4 were enriched at the promoter and at the four intronic sites (Figures 3.7A and 3.7B), however, higher levels of Stat4 binding were found in comparison to Stat6 in NK cells (Figure 3.7B and 3.7A). Conversely, Stat6 and Stat4 binding was barely detectable in the absence of IL-4 or IL-12 stimulation. In LPS-stimulated macrophages, Stat6 but not Stat4 was recruited to the Stat-binding elements in response to IL-4 stimulation. As expected, Stat6 binding was not observed in the absence of IL-4. The results demonstrate that Stat6 and Stat4 can physically interact with the endogenous *Il24* gene in macrophages and NK cells, respectively in a signal-specific manner.

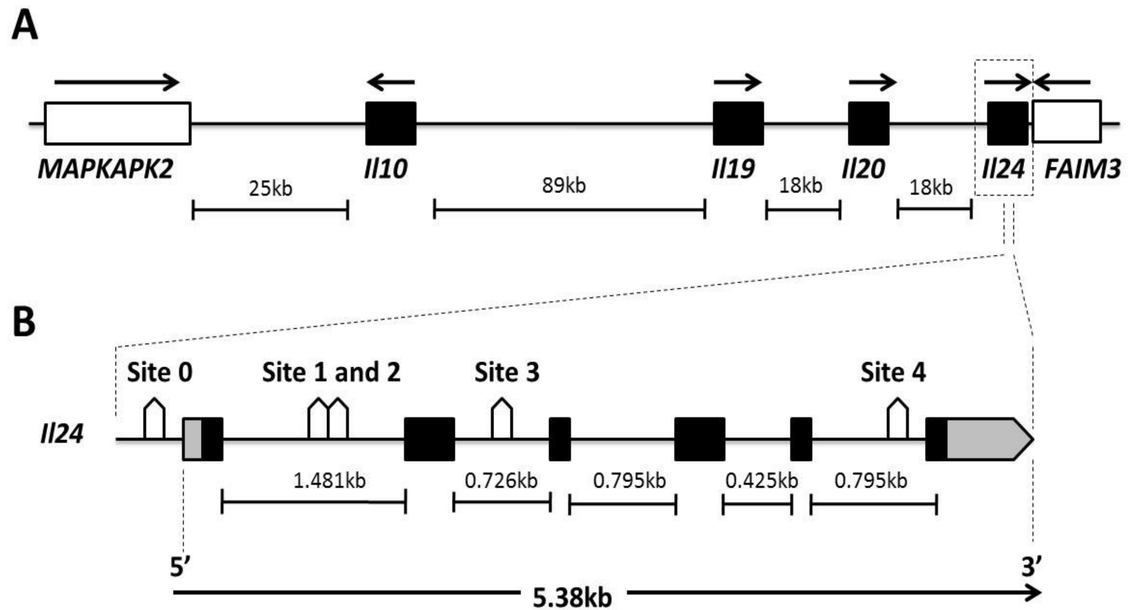


Figure 3.6. Map of the mouse *Il10* locus displaying putative Stat-binding sites within *Il24*
Panel A shows *Il10* and its neighboring cytokines flanked by MAPKAP2 (3’ of *IL10*) and FAIM3 (5’ of *IL10*). Panel B displays genomic organization of the *Il24* gene including the 6 exons (blue boxes) and the 5’ and 3’ UTRs (gray boxes). Putative stat-binding elements are represented in pentagon (red).

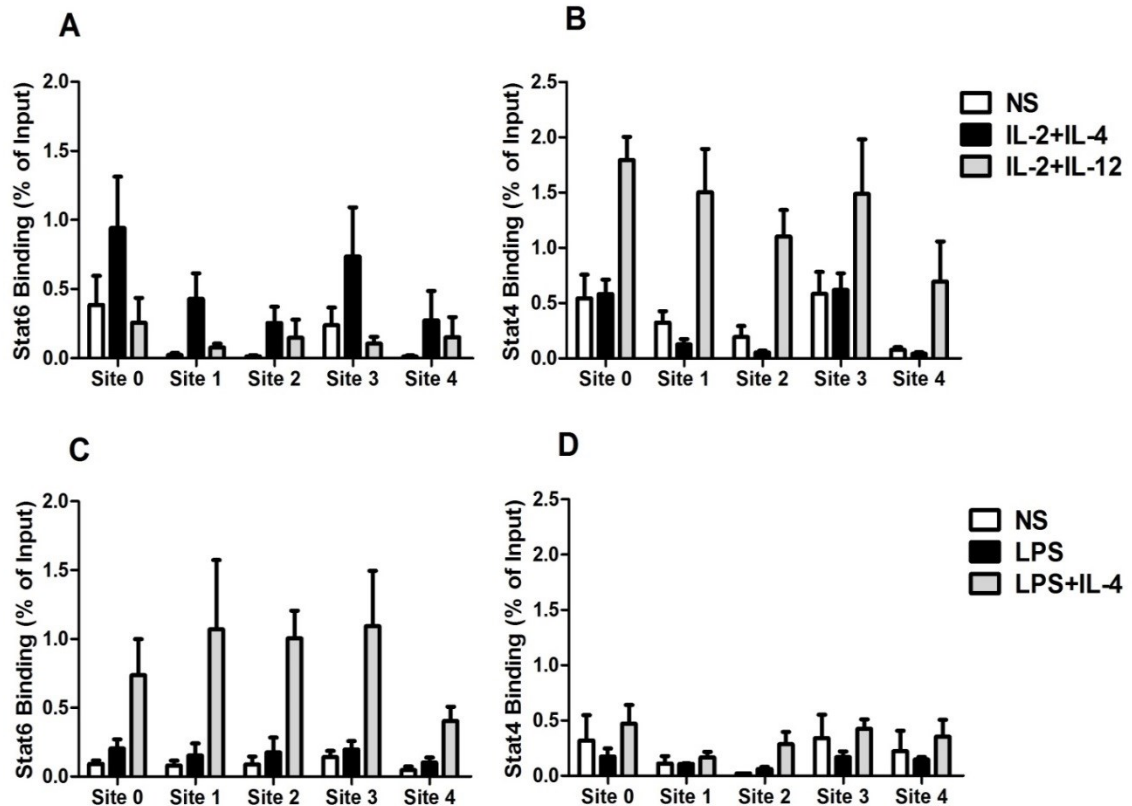


Figure 3.7. Stat6 and Stat4 are recruited to *Il24 gene* in NK cells and macrophages
NK cells (A and B) and BMM (C and D) were stimulated for 2h with the indicated stimuli. Nuclear lysates were obtained to immunoprecipitate the DNA with antibodies specific for Stat6 (A and C) and Stat4 (B and D). The DNA was then purified and amplified using primers targeting Stat-binding elements within *Il24* gene. Data are presented as percent of Input DNA (mean ± sem) of three to four representative experiments.

3.4.7. Stat6 and Stat4 are involved in cytokine-induced histone modifications of Il24

Previous reports found a link between Stat6 recruitment at the promoter and epigenetic changes such as histone modifications of IL-24 and IL-10 in T_H2 cells[86,88]. We questioned whether the accessibility of the chromatin at the *Il24* locus would change in the absence of Stat6 and Stat4. We performed ChIP assays to look for enrichment of AcH3 (an active histone mark) and H3K27me3 (a silent histone mark) in WT, *Stat6*$^{-/-}$, and *Stat4*$^{-/-}$ cells. In NK cells, the chromatin was poised in the absence of either Stat6 or Stat4, which was evidenced by enrichment of both AcH3 and H3K27me3 in Stat6- and Stat4-deficient cells (Figure 3.8A and 3.8B and Figure 3.9A and 3.9B). In macrophages, we observed lower levels of the active histone mark (AcH3) at the promoter and at the first intron in *Stat6*$^{-/-}$ relative to WT macrophages (Figure 3.10A). Conversely, higher levels of H3K27me3 were detected at the same site in *Stat6*$^{-/-}$ compared to WT macrophages (Figure 3.10B). Unlike Stat6, Stat4 deficiency had no impact on the state of the chromatin in macrophages (Appendix 3,2). Thus, the effect of Stat6 on histone marks is mostly observed in macrophages, while Stat4 acts primarily in NK cells. The results suggest that cytokine-induced Stat proteins are involved in epigenetic tuning of the *Il24* gene in NK cells and macrophages.

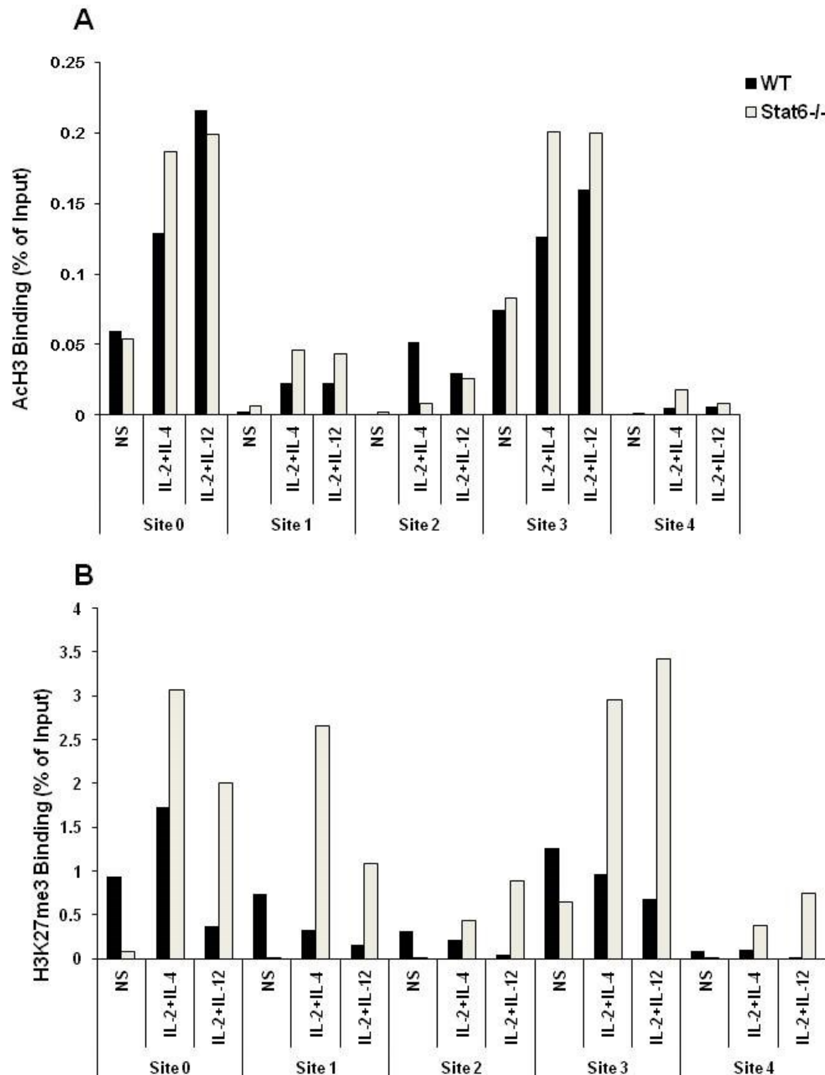


Figure 3.8: Stat6-dependent histone modifications in NK cells
AcH3 (A) and H3K27me3 (B) binding was measured by ChIP assay in WT and $Stat6^{-/-}$ NK cells. Results are presented as percent of Input DNA of one of the two independent experiments.

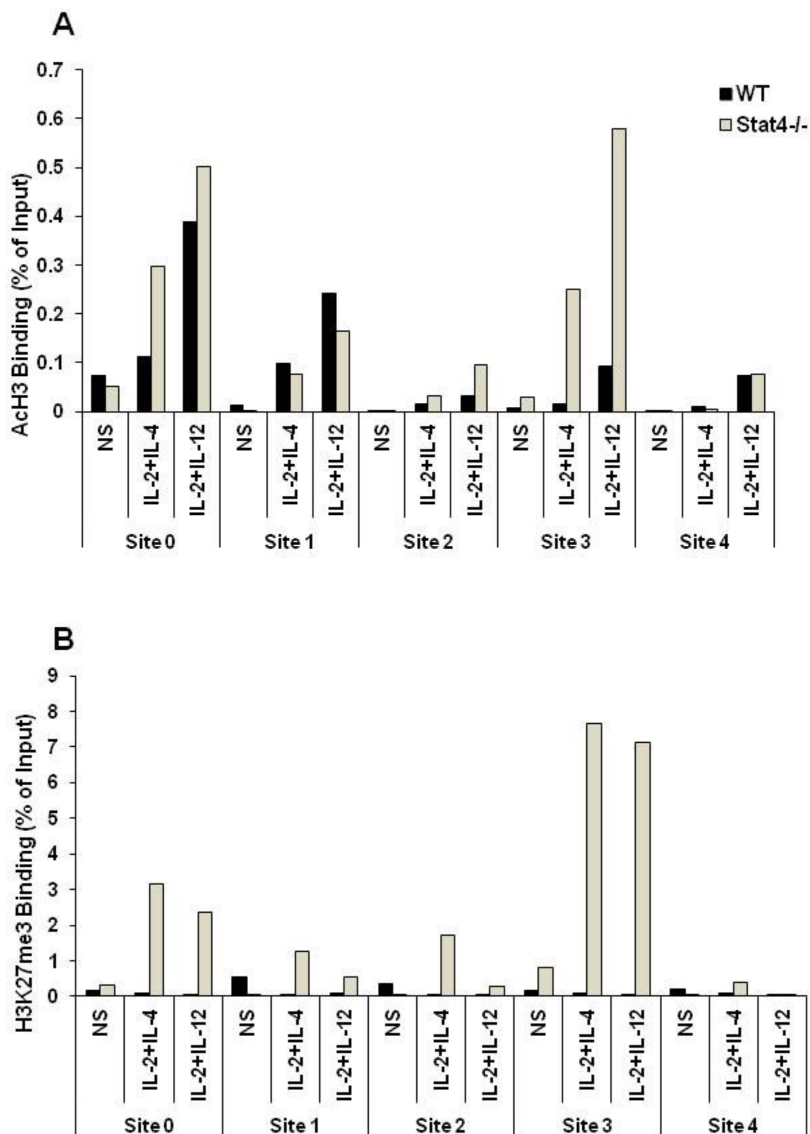


Figure 3.9: Stat4-dependent histone modifications in NK cells

NK cells were derived from WT and Stat4$^{-/-}$ mice. AcH3 (A) and H3K27me3 (B) antibodies were used for ChIP assay. The DNA was then purified and amplified using primers targeting Stat-binding elements within *Il24* gene. Data are presented as percent of Input DNA of one of the two independent experiments.

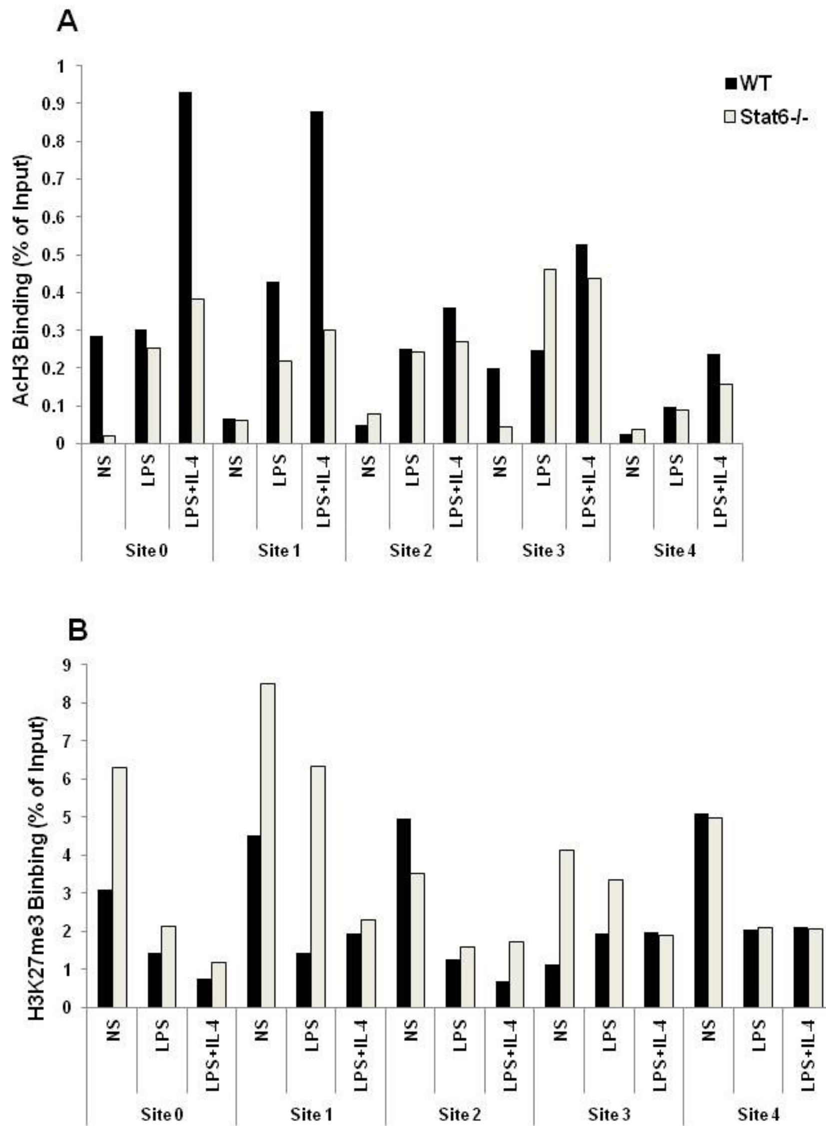


Figure 3.10: Stat6-dependent histone modifications in macrophages
BMM were generated from WT and Stat6-/- mice. ChIP assay was performed using antibody specific for AcH3 (A) and H3K27me3 (B). The DNA was then purified and amplified using primers targeting Stat-binding elements within *Il24* gene. Data are presented as percent of Input DNA of one of the two independent experiments.

3.5. DISCUSSION

In this study, we considered the hypothesis that IL-10 and its homolog IL-24, share common regulatory features such as similar stimuli and transcription factor requirements that mediate their cell-type-specific expression. This hypothesis was based on the findings that IL-10 and IL-24—but not IL-19 and IL-20 (also members of the *Il10* gene cluster)—are co-expressed in murine T_H2 cells[161]. Focusing on NK cells and macrophages, which are important innate cells, we demonstrated that IL-10 and IL-24 are co-expressed in a cell-type and stimuli-specific manner, yet not co-regulated. Although we found some similarities in IL-10 and IL-24 expression patterns, such as their Stat4-dependency in NK cells and their co-expression in LPS-treated macrophages, we predominantly uncovered more disparities outweighing their parallels. For example, we demonstrated that Stat6 mediates optimal expression of IL-24 in NK cells and macrophages, but is dispensable for IL-10 expression. Additionally, Stat6 binds to *Il24* in a cell-type and signal-specific fashion. We also found that IL-10 and IL-24 co-expression are under different regulatory constraints that are largely governed by Type-I IFNs. Together, our data suggest that—unlike the classical T_H2 locus—IL-24 and IL-10 are regulated by distinct cell-type-specific regulatory pathways, thus unlikely to share common regulatory elements.

To determine which transcription factors are involved in cytokine-induced IL-24 and IL-10 expression, we examined IL-24 and IL-10 mRNA induction in cells lacking Stat6 or Stat4, which are activated downstream of IL-4 and IL-12, respectively. We found that IL-4-induced Stat6 enhanced IL-24 but not IL-10 expression in IL-2-stimulated NK cells or in LPS-activated macrophages. Conversely, Stat4 regulates IL-24 expression in NK cells in the presence or absence of IL-12 stimulation, whereas IL-10 induction in NK cells is mainly controlled by IL-12-induced Stat4. Thus, the effect of Stat6 is restricted to IL-24 in both NK cells and

macrophages, while Stat4 mediates NK-specific IL-10 and IL-24 co-expression. These findings are in contrast to the regulation of IL-10 and IL-24 expression in T_H2 cells in which IL-24 and IL-10 co-expression is mediated by IL-4-induced Stat6 and TCR stimulation[(88)]. Conversely, IL-10 is produced by Th1 cells under Stat4 and ERK pathways (but not IL-24)[(170)] as opposed to Stat4-dependent IL-24 and IL-10 co-expression in NK cells. Hence, to our knowledge, our findings provide for the first time, molecular mechanisms, governed by cytokines and their cognate transcription factors (Stat6 and Stat4), in regulating IL-24 and IL-10 co-expression in innate cells.

To understand the mechanisms underlying Stat4-dependent, IL-12-independent IL-24 expression in NK cells, we considered other receptor systems that are known to induce Stat4 phosphorylation. We decided to focus on type I IFN receptor signaling for three main reasons. In resting NK cells, there is a high level of phosphorylated Stat4 (p-Stat4) compared to other lymphocyte subsets, and such high basal p-Stat4 has been shown to be induced by IFNs[(169,171)]. In addition, it has been established that following LPS stimulation, macrophages produce type I IFNs, which then act via an autocrine loop to promote IL-10 transcription in macrophages[(168,172)], hence, similar mechanisms could be in play for NK-derived IL-24 expression. Furthermore, IL-24 cDNA was initially isolated by subtraction hybridation of human melanoma cells with recombinant IFN-β in the presence of mezerein[(84)]. Indeed, we found that while IFN-β did not directly induce IL-24 expression in NK cells, it did synergize with IL-2. Remarkably, IL-24 induction in macrophages was completely independent of IFN signaling. To confirm these findings, we determined the expression of IL-24 and IL-10 in Ifnra-deficient cells and WT controls. In Ifnra$^{-/-}$ mice, IL-24 transcript was substantially diminished in NK cells but not in macrophages. In contrast, IL-10 expression was intact in NK cells lacking IFN receptor

signaling, but was reduced by half in *Ifnra*$^{-/-}$ macrophages. Therefore, our results suggest that IL-24 and IL-10 expression are governed by an interferon-dependent gene-specific and cell-type-specific regulatory pathways.

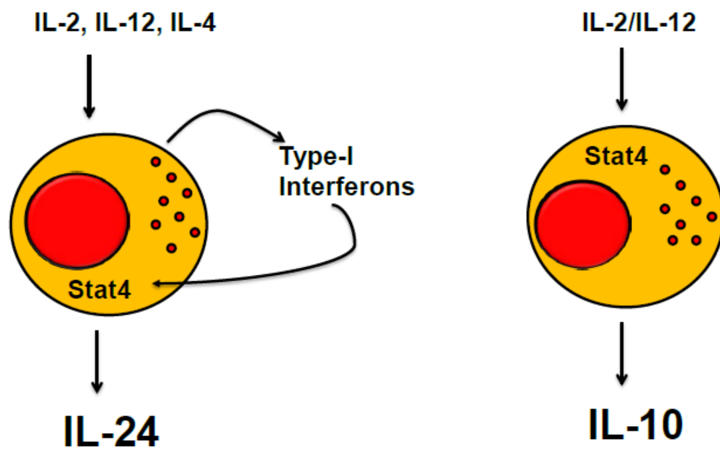
We and others have shown that IL-12-induced Stat4 and IL-4-induced Stat6 bind to the *Il10* gene in NK cells and Th2 cells, respectively[86,88,167]. To determine whether these Stats can directly interact with *Il24* gene in NK cells and macrophages, we performed ChIP assays to assess Stat6 and Stat4 binding across the locus. We found that both Stats can bind to multiple sites across the *Il24* locus, but in a signal- and cell-type-specific manner. Because Stat6 binding has been linked not only to acute transcription but also to epigenetic modifications of *Il24* in T_H2 cells[86-88], we questioned whether binding of Stat6 as well as Stat4 mediates post-translational modifications of the histones in the cell types assayed. As in Th2 cells, Stat6 was associated with recruitment of permissive histone marks (AcH3) at the *Il24* locus in macrophages but not in NK cells. The opposite was observed when we assayed binding of H3K27me3 in Stat6-deficient macrophages. In contrast to Stat6, the lack of Stat4 did not influence the accessible chromatin structure in macrophages (as expected). In NK cells, the chromatin was poised (i.e., enriched for both active and silent marks) in the absence of Stat6 or Stat4. These data suggest that the effects of Stats on the epigenetic landscape of *Il24* in NK cells are may be indirect through induction of other pathways. In addition to work done in T_H2 cells and our own study, epigenetic studies on *Il24* expression are scarce. Nevertheless, in Th1 cells where *Il24* is barely expressed, the *Il24* promoter is occupied by histone deacetylase (HDAC) which has been associated with gene silencing [86]. Similarly, in human melanoma cells, treatment with HDAC4 reduced binding of Sp1 and AcH3 levels at the *Il24* promoter[173].

Although we identified signaling pathways and epigenetic events that govern IL-24 expression in NK cells and macrophages, there are limitations to our study. We focused on changes in gene expression by assessing mRNA levels. We did not assay IL-24 protein because of the lack of commercial ELISA reagents. Thus, further studies are needed to confirm that protein and mRNA data match. In addition, Stat5-dependent *Il24* expression was not investigated in NK cells because Stat5$^{-/-}$ mice are not viable[174] and also our in vitro culture system for NK cells is driven by IL-2/Stat5 signaling. We did investigate Stat5 binding in NK cells, but we did not observe any evidence for Stat5 enrichment to the Stat-binding sites assayed (Appendix 3.3). Nevertheless, *Il24* expression in IL-2 stimulated T lymphocytes was reduced in mice genetically engineered to be deficient of Stat5 tetramers [163]. In the same study, the authors revealed that Stat5 tetramers bind to a region within intron 1 of *Il24* in T cells of WT mice by ChIP-seq. Also, another group recently identified a Stat5-binding element in a distal region upstream of *Il24* in T_H2 cells[175] that was not investigated in this study. Because we found a role for type I IFNs in regulating IL-24 expression in NK cells and no Stat5 binding across the locus in NK cells, we now propose that IL-2 induces IL-24 expression in NK cells through production of IFNs, which in turn feedback to activate Stat4. Therefore, our findings point to cell-type-specific regulatory mechanisms that distinguish IL-2-dependent IL-24 expression in T cells from that of NK cells.

In summary, this study provides insights into the molecular mechanisms that govern IL-24 and IL-10 co-expression in NK cells and macrophages. We established that IL-10 and IL-24 expression are regulated by different cell-type-specific pathways. So far, our working model of IL-24 and IL-10 regulation in NK cells and macrophages is as follows: In NK cells, IL-2 alone or in combination with cytokines induce IFNs production. The IFNs act in an autocrine-fashion to activate Stat4, which is required for potent IL-24 expression in NK cells. Meanwhile, IL-12-

induced Stat4 is sufficient for optimal IL-10 expression in NK cells. In LPS-activated macrophages, IL-4 activates Stat6 downstream of the IL4R. Stat6 binds to the promoter and other sites across the locus which facilitates opening of the chromatin and thereby enhancing expression of IL-24 transcription. In contrast to the regulation of IL-24, IFNs but not IL-4/Stat6 is required for optimal macrophage-derived IL-10 expression. A diagram of this model is presented in Figure 3. 11.

A. NK cells



B. Macrophages

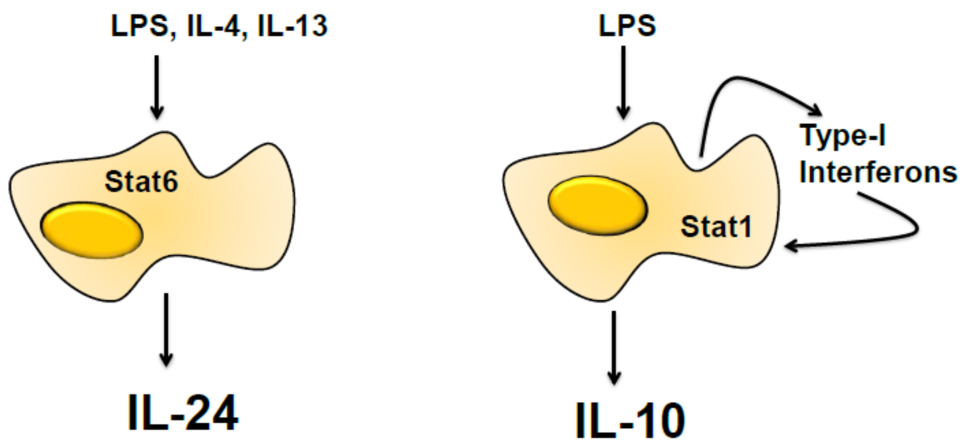
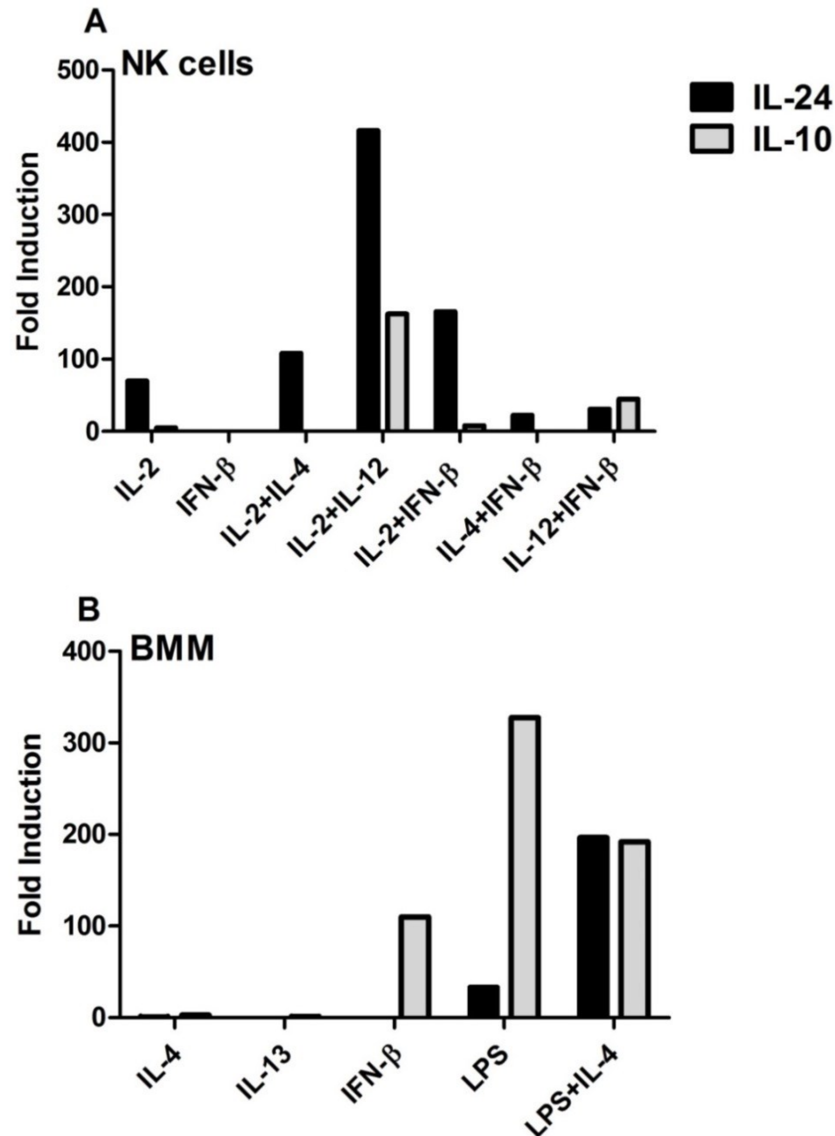


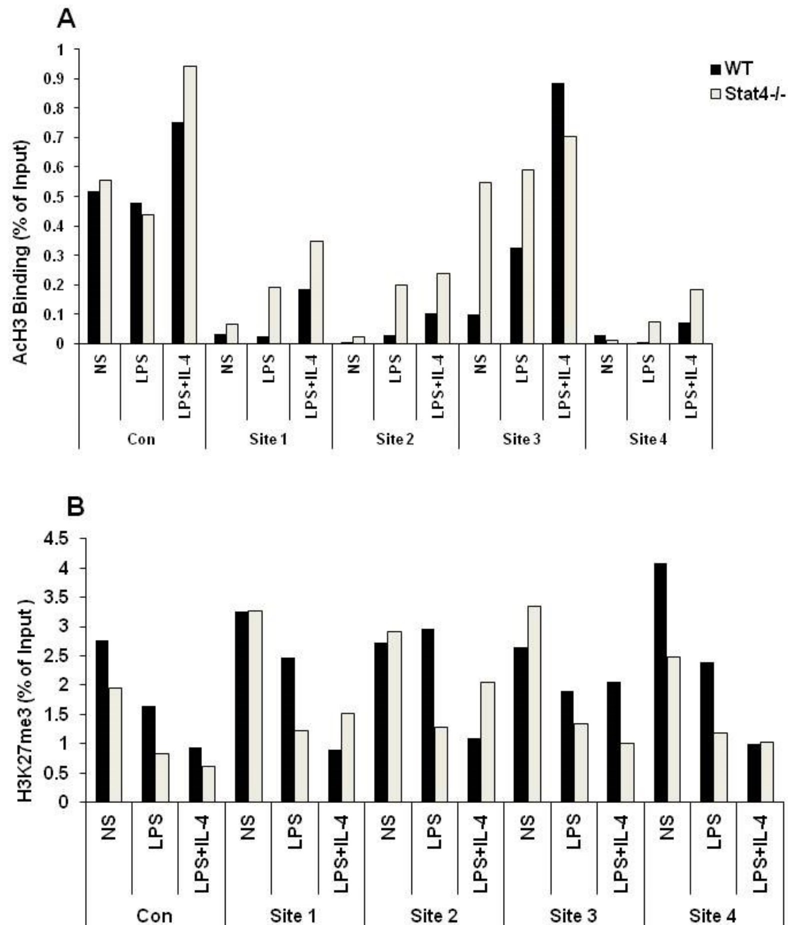
Figure 3.11: A model of IL-24 and IL-10 co-expression in NK cells and macrophages

3.6. Appendix

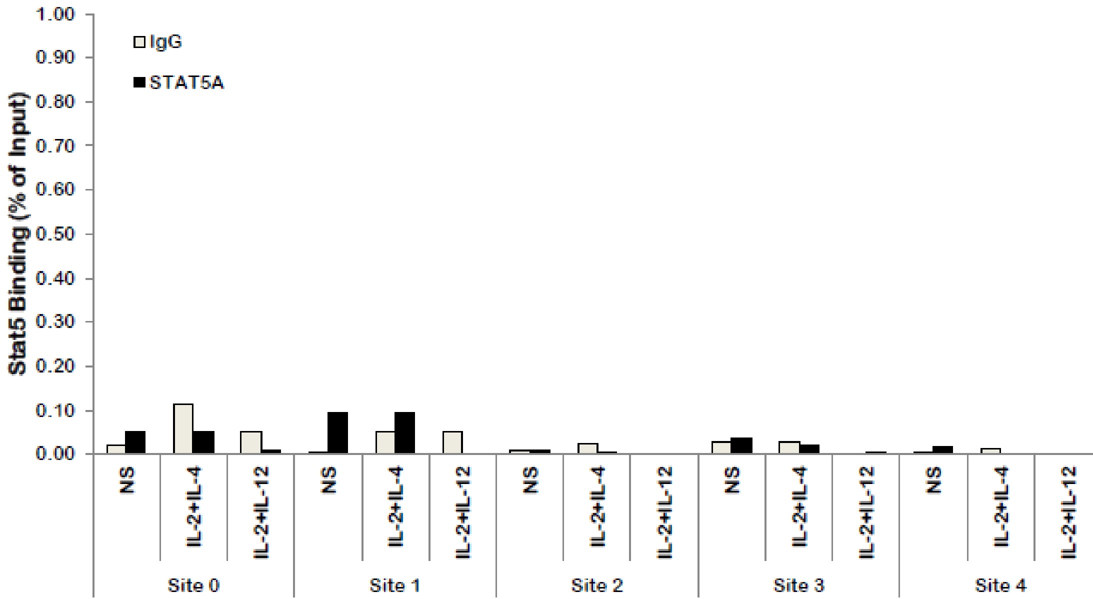


Appendix 3.1: Type-I IFN-dependent expression of IL-10 and IL-24 in NK cells and macrophages

NK cells (A) and macrophages (B) were stimulated with cytokines and/or LPS. IL-24 (black bar) and IL-10 (gray bar) mRNAs were assessed by qPCR and normalized to non-stimulated cells. Data are representative of two independent experiments.



Appendix 3.2: Stat4-dependent histone modifications in macrophages
ChIP assay was used to assess enrichment of AcH3 (A) and H3K27me3 (B) in WT and $Stat4^{-/-}$ macrophages. Data are presented as percent of Input DNA of one of the two independent experiments.



Appendix 3.3: Stat5 binding across the *Il24* gene

ChIP assay was conducted in NK cells to detect Stat5a binding to the putative stat-binding sites. IgG antibody was used as control. Data are representative of two independent experiments.

Chapter 4

General Discussion and Conclusions

GENERAL DISCUSSION AND CONCLUSIONS

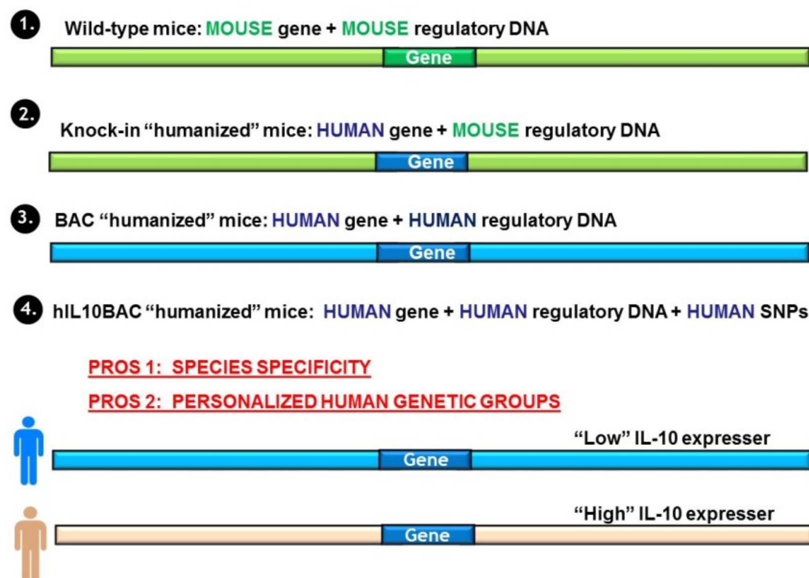
Mouse models have been traditionally used to elucidate the underlying mechanisms of human diseases, as well as for testing candidate therapeutics, including vaccines at the preclinical stage. However, it has been difficult to translate knowledge gained from mouse studies in humans due in part to a number of differences between mice and human immune responses[4]. Thus, there is growing interest in developing faithful mouse models that fully mirror human biology, particularly inter-individual variability in gene expression and disease risk.

One promising approach is the use of humanized mice containing human cells, tissues, organs, or genes. There are a number of successful humanized mice with varying utilities such as humanized mice with functional immune systems (MHIS), humanized mice for studying human gene regulation, and humanized mice for studying human genetics. In Chapter 1, we presented examples of these mice as well as their advantages and limitations. We also argued that existing humanized mice are not ideal for capturing the genetic diversity among people, especially sequence variation in non-coding DNA which constitutes most of the variability in the human genome[118]. Thus, in Chapter 2 we designed and generated for the first time a genetically humanized mouse to study the biological function of non-coding SNPs in the human *IL10* locus.

The hIL10BAC mice were made by introducing a bacterial artificial chromosome (BAC) carrying one of the two different human *IL10* promoter SNP haplotypes into C57BL/6 mice: ATA-hIL10BAC and GCC-hIL10BAC mice. We choose human IL-10, a very important immuno-regulatory cytokine, as our target gene for several reasons: (1) human IL-10 is known to cross-react with the mouse IL-10 receptor, enabling us to study both gene regulation and function *in vivo*; (2) there is a strong genetic component to human IL-10 expression (reviewed in Chapter 1); (3) non-coding SNP haplotypes in *IL10* promoter have been associated with

differential IL-10 levels and disease risk (Reviewed in Chapter 1); (4) Targeted deletion of IL-10 in specific cells in mice demonstrated that cellular sources of IL-10 determine disease outcomes[139,142,144,176]. Thus, by testing these mice using well-established IL-10-dependent mouse models of human diseases (i.e., sepsis and leishmaniasis), we firmly established that *IL10* promoter SNP haplotypes control cell-type-specific human IL-10 expression and disease susceptibility.

Together, the work presented in Chapter 2 contributes significantly to the body of knowledge regarding the utility of hIL10BAC humanized mice for modeling both human genetics and gene regulation *in vivo.* Key differences between the hIL10BAC humanized mice over existing humanized mice for investigating gene regulation is shown below.



To date, the hIL10BAC humanized mouse can be used to elucidate the molecular basis of allele-specific human IL-10 expression. This could be achieved by assessing changes in transcription factor binding patterns in varying cell types in response to different stimuli. In fact, we and others have found allele-specific protein binding at the promoter and intronic *IL10*

SNPs[38,177,178]. Because those studies were conducted using bulk PBMCs or cell lines—which do not necessarily reproduce the epigenetic microenvironment of primary cells—it would be interesting to repeat these experiments in freshly isolated cells/tissues from ATA-hIL10BAC and GCC-hIL10BAC mice. Alternatively, one could perform a ChIP-Seq assay on CD4+ T cells +/- IL-27 to assess allele-specific Stat1 and Stat3 binding on hIL10BAC locus since IL-27-induced IL-10 production in CD4+ T cells has been shown to be dependent on both Stat1 and Stat3[140]. This could also reveal species-specific Stat1/Stat3 binding differences between human and mouse IL-10 genes in CD4+ T cells.

In addition, a comparative sequence analysis between human and mouse IL-10 by our group has found 13 conserved non-coding sequences (CNS) within the hIL10BAC[138]. Some of these CNS sites appear to be important for tissue-specific IL-10 expression[138]. Because *in vivo* functional analysis of CNS has been done with success for certain genes such as *IFNG*[179] and *MYC* (an oncogene involved in many human cancers)[180], it would be interesting to use similar approach in order to determine which CNS or CNS+SNPs are responsible for changes in gene expression and susceptibility to disease. To do this, one could start by identifying SNPs that fall within these sites to refine the list of putative functional SNPs in hIL10BAC and then generate new mice with specific deletion of the CNS sites (ΔCNS#hIL10BAC mice). Data obtained from ΔCNS#hIL10BAC mice would be compared to existing ATA-hIL10BAC or GCC-hIL10BAC transgenes to define the function of the CNS and or CNS+SNPs removed from the hIL10BAC.

Further studies could also investigate the impact of the allele-specific chromatin structure such as post-translational modifications of histone tails (i.e. acetylation and methylation) on gene expression. Finally, because existing hIL10BAC mice allow us to study the global effects of "GCC" and "ATA" SNP haplotypes but not individual SNPs, it would be interesting to generate

a complementary mouse in which the G-C-C promoter SNPs are mutated back to A-T-A by site-directed mutagenesis. This new mouse (ATA+GCC-hIL10BAC) would be critical to determine whether our findings (gene expression and disease phenotype) are caused by the promoter *IL10* SNPs only or by the cumulative effects of all the non-coding SNPs in the hIL10BAC.

In the near term, the ultimate goal is to use the hIL10BAC humanized mice to test the activity of pharmacological compounds that have been designed to induce or inhibit IL-10 production. In addition, our mice can be used to study the efficacy of recombinant human IL-10 in treating various inflammatory diseases associated with dysregulation of IL-10 production. Furthermore, we can also test the effects of allele-specific IL-10 expression on the immune responses that are induced following immunization to vaccine candidates. By deciphering the molecular basis of allele-specific IL-10 expression, we might also be able to categorize people based on their IL-10 genotype to obtain a cohort of subjects ready to be enrolled in clinical trials. Finally, the hIL10BAC approach can be extended to other human genes for which a bacterial artificial chromosome and mouse-null allele are available.

In Chapter 2, we addressed an important question regarding the regulation of cytokines in the mouse *Il10* locus. Because genomic boundaries of the IL-10 gene cluster is not well defined in both humans and mice, we cannot exclude the possibility that we are missing distal regulatory elements within the hIL10BAC. To investigate this possibility, we took the approach of studying the co-expression of the mouse IL-10 and IL-24 mRNAs. We considered the hypothesis that IL-10 and IL-24 share common regulatory elements that govern their cell-type-specific co-expression. Our results clearly demonstrate that IL-10 and IL-24 are co-expressed, but are regulated by distinct cell-type-specific pathways. The major findings of this work are summarized in Table 4.1. We also provided molecular mechanisms for macrophage- and NK-

specific IL-24 expression, which could be useful to pharmacologically modulate IL-24 expression independently of IL-10.

Table 4.1: Cell-type-specific regulatory control of IL-24 and IL-10 expression in innate cells

Signaling pathways	Macrophages		NK cells	
	IL-24	**IL-10**	**IL-24**	**IL-10**
Stat6	Yes	No	Yes	No
Stat4	No	No	Yes	Yes
Type-I IFNs	No	Yes	Yes	No

In the short term, the results from this study can be further expanded to include data from a Chromosome Conformation Capture assay (3C). This assay is often used to detect the frequency at which genomic loci interact with each other in a given cell at a natural state[(157)]. 3C assay has been used with success to investigate chromosomal looping, bringing distal regulatory elements as well as associated transcription factors into close proximity to the gene of interest. This assay has enabled scientists to study many gene clusters, including the globin locus, T_H2 locus, T_H17 locus, and the human *IFNG*[(11)]. Thus, the absence of long-range chromosomal interactions between proximal promoters of *Il10* and *Il24* in macrophages and NK cells would definitely prove that they are not co-regulated in the cell types assayed.

Altogether, in this dissertation, we designed and generated for the first time a genetically humanized mouse to model human genetic variation in non-coding DNA. This mouse enables assessment of inter-individual variability in gene expression and its effect on disease susceptibility, which has been difficult to examine until now. Additionally, the hIL10BAC

mouse would be useful for testing safety and efficacy of novel drugs targeting IL-10-dependent pathways. We also defined the molecular basis governing cell-type-specific expression of cytokines, including *Il10* and *Il24* in the mouse IL-10 locus, which can be useful in defining genomic requirements for faithful IL-10 expression for future studies.

REFERENCES

1. Shay, T., V. Jojic, O. Zuk, K. Rothamel, D. Puyraimond-Zemmour, T. Feng, E. Wakamatsu, C. Benoist, D. Koller, and A. Regev. 2013. Conservation and divergence in the transcriptional programs of the human and mouse immune systems. *Proc. Natl. Acad. Sci. U. S. A* 110: 2946-2951.

2. McLean, C. Y., P. L. Reno, A. A. Pollen, A. I. Bassan, T. D. Capellini, C. Guenther, V. B. Indjeian, X. Lim, D. B. Menke, B. T. Schaar, A. M. Wenger, G. Bejerano, and D. M. Kingsley. 2011. Human-specific loss of regulatory DNA and the evolution of human-specific traits. *Nature* 471: 216-219.

3. Waterston, R. H., K. Lindblad-Toh, E. Birney, J. Rogers, J. F. Abril, P. Agarwal, R. Agarwala, R. Ainscough, M. Alexandersson, P. An, S. E. Antonarakis, J. Attwood, R. Baertsch, J. Bailey, K. Barlow, S. Beck, E. Berry, B. Birren, T. Bloom, P. Bork, M. Botcherby, N. Bray, M. R. Brent, D. G. Brown, S. D. Brown, C. Bult, J. Burton, J. Butler, R. D. Campbell, P. Carninci, S. Cawley, F. Chiaromonte, A. T. Chinwalla, D. M. Church, M. Clamp, C. Clee, F. S. Collins, L. L. Cook, R. R. Copley, A. Coulson, O. Couronne, J. Cuff, V. Curwen, T. Cutts, M. Daly, R. David, J. Davies, K. D. Delehaunty, J. Deri, E. T. Dermitzakis, C. Dewey, N. J. Dickens, M. Diekhans, S. Dodge, I. Dubchak, D. M. Dunn, S. R. Eddy, L. Elnitski, R. D. Emes, P. Eswara, E. Eyras, A. Felsenfeld, G. A. Fewell, P. Flicek, K. Foley, W. N. Frankel, L. A. Fulton, R. S. Fulton, T. S. Furey, D. Gage, R. A. Gibbs, G. Glusman, S. Gnerre, N. Goldman, L. Goodstadt, D. Grafham, T. A. Graves, E. D. Green, S. Gregory, R. Guigo, M. Guyer, R. C. Hardison, D. Haussler, Y. Hayashizaki, L. W. Hillier, A. Hinrichs, W. Hlavina, T. Holzer, F. Hsu, A. Hua, T. Hubbard, A. Hunt, I. Jackson, D. B. Jaffe, L. S. Johnson, M. Jones, T. A. Jones, A. Joy, M. Kamal, E. K. Karlsson, D. Karolchik, A. Kasprzyk, J. Kawai, E. Keibler, C. Kells, W. J. Kent, A. Kirby, D. L. Kolbe, I. Korf, R. S. Kucherlapati, E. J. Kulbokas, D. Kulp, T. Landers, J. P. Leger, S. Leonard, I. Letunic, R. Levine, J. Li, M. Li, C. Lloyd, S. Lucas, B. Ma, D. R. Maglott, E. R. Mardis, L. Matthews, E. Mauceli, J. H. Mayer, M. McCarthy, W. R. McCombie, S. McLaren, K. McLay, J. D. McPherson, J. Meldrim, B. Meredith, J. P. Mesirov, W. Miller, T. L. Miner, E. Mongin, K. T. Montgomery, M. Morgan, R. Mott, J. C. Mullikin, D. M. Muzny, W. E. Nash, J. O. Nelson, M. N. Nhan, R. Nicol, Z. Ning, C. Nusbaum, M. J. O'Connor, Y. Okazaki, K. Oliver, E. Overton-Larty, L. Pachter, G. Parra, K. H. Pepin, J. Peterson, P. Pevzner, R. Plumb, C. S. Pohl, A. Poliakov, T. C. Ponce, C. P. Ponting, S. Potter, M. Quail, A. Reymond, B. A. Roe, K. M. Roskin, E. M. Rubin, A. G. Rust, R. Santos, V. Sapojnikov, B. Schultz, J. Schultz, M. S. Schwartz, S. Schwartz, C. Scott, S. Seaman, S. Searle, T. Sharpe, A. Sheridan, R. Shownkeen, S. Sims, J. B. Singer, G. Slater, A. Smit, D. R. Smith, B. Spencer, A. Stabenau, N. Stange-Thomann, C. Sugnet, M. Suyama, G. Tesler, J. Thompson, D. Torrents, E. Trevaskis, J. Tromp, C. Ucla, A. Ureta-Vidal, J. P. Vinson, A. C. Von Niederhausern, C. M. Wade, M. Wall, R. J. Weber, R. B. Weiss, M. C. Wendl, A. P. West, K. Wetterstrand, R. Wheeler, S. Whelan, J. Wierzbowski, D. Willey, S. Williams, R. K. Wilson, E. Winter, K. C. Worley, D. Wyman, S. Yang, S. P. Yang, E. M. Zdobnov, M. C. Zody, and E. S. Lander. 2002. Initial sequencing and comparative analysis of the mouse genome. *Nature* 420: 520-562.

4. Mestas, J. 2004. Of mice and not men: differences between mouse and human immunology.

5. van der Worp, H. B., D. W. Howells, E. S. Sena, M. J. Porritt, S. Rewell, V. O'Collins, and M. R. Macleod. 2010. Can animal models of disease reliably inform human studies? *PLoS. Med.* 7: e1000245.

6. Britten, R. J. 2002. Divergence between samples of chimpanzee and human DNA sequences is 5%, counting indels. *Proc. Natl. Acad. Sci. U. S. A* 99: 13633-13635.

7. Brehm, M. A. 2014. Generation of improved humanized mouse models for human infectious diseases.

8. Legrand, N. 2009. Humanized mice for modeling human infectious disease: challenges, progress, and outlook.

9. Shultz, L. D. 2012. Humanized mice for immune system investigation: progress, promise and challenges.

10. Lan, P., N. Tonomura, A. Shimizu, S. Wang, and Y. G. Yang. 2006. Reconstitution of a functional human immune system in immunodeficient mice through combined human fetal thymus/liver and CD34+ cell transplantation. *Blood* 108: 487-492.

11. Melkus, M. W., J. D. Estes, A. Padgett-Thomas, J. Gatlin, P. W. Denton, F. A. Othieno, A. K. Wege, A. T. Haase, and J. V. Garcia. 2006. Humanized mice mount specific adaptive and innate immune responses to EBV and TSST-1. *Nat. Med.* 12: 1316-1322.

12. Ito, M., H. Hiramatsu, K. Kobayashi, K. Suzue, M. Kawahata, K. Hioki, Y. Ueyama, Y. Koyanagi, K. Sugamura, K. Tsuji, T. Heike, and T. Nakahata. 2002. NOD/SCID/gamma(c)(null) mouse: an excellent recipient mouse model for engraftment of human cells. *Blood* 100: 3175-3182.

13. Shultz, L. D., B. L. Lyons, L. M. Burzenski, B. Gott, X. Chen, S. Chaleff, M. Kotb, S. D. Gillies, M. King, J. Mangada, D. L. Greiner, and R. Handgretinger. 2005. Human lymphoid and myeloid cell development in NOD/LtSz-scid IL2R gamma null mice engrafted with mobilized human hemopoietic stem cells. *J. Immunol.* 174: 6477-6489.

14. Traggiai, E., L. Chicha, L. Mazzucchelli, L. Bronz, J. C. Piffaretti, A. Lanzavecchia, and M. G. Manz. 2004. Development of a human adaptive immune system in cord blood cell-transplanted mice. *Science* 304: 104-107.

15. Rongvaux, A., H. Takizawa, T. Strowig, T. Willinger, E. E. Eynon, R. A. Flavell, and M. G. Manz. 2013. Human hemato-lymphoid system mice: current use and future potential for medicine. *Annu. Rev. Immunol.* 31: 635-674.

16. Drake, A. C., Q. Chen, and J. Chen. 2012. Engineering humanized mice for improved hematopoietic reconstitution. *Cell Mol. Immunol.* 9: 215-224.

17. Lapidot, T., F. Pflumio, M. Doedens, B. Murdoch, D. E. Williams, and J. E. Dick. 1992. Cytokine stimulation of multilineage hematopoiesis from immature human cells engrafted in SCID mice. *Science* 255: 1137-1141.

18. Chen, Q., M. Khoury, and J. Chen. 2009. Expression of human cytokines dramatically improves reconstitution of specific human-blood lineage cells in humanized mice. *Proc. Natl. Acad. Sci. U. S. A* 106: 21783-21788.

19. Li, Y., Q. Chen, D. Zheng, L. Yin, Y. H. Chionh, L. H. Wong, S. Q. Tan, T. C. Tan, J. K. Chan, S. Alonso, P. C. Dedon, B. Lim, and J. Chen. 2013. Induction of functional human macrophages from bone marrow promonocytes by M-CSF in humanized mice. *J. Immunol.* 191: 3192-3199.

20. Jaiswal, S., C. H. Jamieson, W. W. Pang, C. Y. Park, M. P. Chao, R. Majeti, D. Traver, R. N. van, and I. L. Weissman. 2009. CD47 is upregulated on circulating hematopoietic stem cells and leukemia cells to avoid phagocytosis. *Cell* 138: 271-285.

21. Strowig, T., A. Rongvaux, C. Rathinam, H. Takizawa, C. Borsotti, W. Philbrick, E. E. Eynon, M. G. Manz, and R. A. Flavell. 2011. Transgenic expression of human signal regulatory protein alpha in Rag2-/-gamma(c)-/- mice improves engraftment of human hematopoietic cells in humanized mice. *Proc. Natl. Acad. Sci. U. S. A* 108: 13218-13223.

22. Takenaka, K., T. K. Prasolava, J. C. Wang, S. M. Mortin-Toth, S. Khalouei, O. I. Gan, J. E. Dick, and J. S. Danska. 2007. Polymorphism in Sirpa modulates engraftment of human hematopoietic stem cells. *Nat. Immunol.* 8: 1313-1323.

23. Yamauchi, T., K. Takenaka, S. Urata, T. Shima, Y. Kikushige, T. Tokuyama, C. Iwamoto, M. Nishihara, H. Iwasaki, T. Miyamoto, N. Honma, M. Nakao, T. Matozaki, and K. Akashi. 2013. Polymorphic Sirpa is the genetic determinant for NOD-based mouse lines to achieve efficient human cell engraftment. *Blood* 121: 1316-1325.

24. Brehm, M. A., W. J. Racki, J. Leif, L. Burzenski, V. Hosur, A. Wetmore, B. Gott, M. Herlihy, R. Ignotz, R. Dunn, L. D. Shultz, and D. L. Greiner. 2012. Engraftment of human HSCs in nonirradiated newborn NOD-scid IL2rgamma null mice is enhanced by transgenic expression of membrane-bound human SCF. *Blood* 119: 2778-2788.

25. Thayer, T. C., M. Delano, C. Liu, J. Chen, L. E. Padgett, H. M. Tse, M. Annamali, J. D. Piganelli, L. L. Moldawer, and C. E. Mathews. 2011. Superoxide production by macrophages and T cells is critical for the induction of autoreactivity and type 1 diabetes. *Diabetes* 60: 2144-2151.

26. Sparwasser, T., and G. Eberl. 2007. BAC to immunology--bacterial artificial chromosome-mediated transgenesis for targeting of immune cells. *Immunology* 121: 308-313.

27. Groux, H., F. Cottrez, M. Rouleau, S. Mauze, S. Antonenko, S. Hurst, T. McNeil, M. Bigler, M. G. Roncarolo, and R. L. Coffman. 1999. A transgenic model to analyze the

immunoregulatory role of IL-10 secreted by antigen-presenting cells. *J. Immunol.* 162: 1723-1729.

28. Heintz, N. 2001. BAC to the future: the use of bac transgenic mice for neuroscience research. *Nat. Rev. Neurosci.* 2: 861-870.

29. Giraldo, P., and L. Montoliu. 2001. Size matters: use of YACs, BACs and PACs in transgenic animals. *Transgenic Res.* 10: 83-103.

30. Wilson, M. D., N. L. Barbosa-Morais, D. Schmidt, C. M. Conboy, L. Vanes, V. L. Tybulewicz, E. M. Fisher, S. Tavare, and D. T. Odom. 2008. Species-specific transcription in mice carrying human chromosome 21. *Science* 322: 434-438.

31. O'Doherty, A., S. Ruf, C. Mulligan, V. Hildreth, M. L. Errington, S. Cooke, A. Sesay, S. Modino, L. Vanes, D. Hernandez, J. M. Linehan, P. T. Sharpe, S. Brandner, T. V. Bliss, D. J. Henderson, D. Nizetic, V. L. Tybulewicz, and E. M. Fisher. 2005. An aneuploid mouse strain carrying human chromosome 21 with Down syndrome phenotypes. *Science* 309: 2033-2037.

32. Reynolds, L. E., A. R. Watson, M. Baker, T. A. Jones, G. D'Amico, S. D. Robinson, C. Joffre, S. Garrido-Urbani, J. C. Rodriguez-Manzaneque, E. Martino-Echarri, M. Aurrand-Lions, D. Sheer, F. Dagna-Bricarelli, D. Nizetic, C. J. McCabe, A. S. Turnell, S. Kermorgant, B. A. Imhof, R. Adams, E. M. Fisher, V. L. Tybulewicz, I. R. Hart, and K. M. Hodivala-Dilke. 2010. Tumour angiogenesis is reduced in the Tc1 mouse model of Down's syndrome. *Nature* 465: 813-817.

33. Copeland, N. G., N. A. Jenkins, and Court DL. 2001. Recombineering: a powerful new tool for mouse functional genomics. *Nat. Rev. Genet.* 2: 769-779.

34. Collins, P. L., S. Chang, M. Henderson, M. Soutto, G. M. Davis, A. G. McLoed, M. J. Townsend, L. H. Glimcher, D. P. Mortlock, and T. M. Aune. 2010. Distal regions of the human IFNG locus direct cell type-specific expression. *J. Immunol.* 185: 1492-1501.

35. Ranatunga, D., C. M. Hedrich, F. Wang, D. W. McVicar, N. Nowak, T. Joshi, L. Feigenbaum, L. R. Grant, S. Stager, and J. H. Bream. 2009. A human IL10 BAC transgene reveals tissue-specific control of IL-10 expression and alters disease outcome. *Proc. Natl. Acad. Sci. U. S. A* 106: 17123-17128.

36. Ranatunga, D. C., A. Ramakrishnan, P. Uprety, F. Wang, H. Zhang, J. B. Margolick, C. Brayton, and J. H. Bream. 2012. A protective role for human IL-10-expressing CD4+ T cells in colitis. *J. Immunol.* 189: 1243-1252.

37. Crawley, E., R. Kay, J. Sillibourne, P. Patel, I. Hutchinson, and P. Woo. 1999. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum.* 42: 1101-1108.

38. Kang, X., H. J. Kim, M. Ramirez, S. Salameh, and X. Ma. 2010. The septic shock-associated IL-10 -1082 A > G polymorphism mediates allele-specific transcription via poly(ADP-Ribose) polymerase 1 in macrophages engulfing apoptotic cells. *J. Immunol.* 184: 3718-3724.

39. Turner, D. M., D. M. Williams, D. Sankaran, M. Lazarus, P. J. Sinnott, and I. V. Hutchinson. 1997. An investigation of polymorphism in the interleukin-10 gene promoter. *Eur. J. Immunogenet.* 24: 1-8.

40. Schmouth, J. F., R. J. Bonaguro, X. Corso-Diaz, and E. M. Simpson. 2012. Modelling human regulatory variation in mouse: finding the function in genome-wide association studies and whole-genome sequencing. *PLoS. Genet.* 8: e1002544.

41. Chandler, J., P. Hohenstein, D. A. Swing, L. Tessarollo, and S. K. Sharan. 2001. Human BRCA1 gene rescues the embryonic lethality of Brca1 mutant mice. *Genesis.* 29: 72-77.

42. Lane, T. F., C. Lin, M. A. Brown, E. Solomon, and P. Leder. 2000. Gene replacement with the human BRCA1 locus: tissue specific expression and rescue of embryonic lethality in mice. *Oncogene* 19: 4085-4090.

43. Vadolas, J., H. Wardan, M. Bosmans, F. Zaibak, D. Jamsai, L. Voullaire, R. Williamson, and P. A. Ioannou. 2005. Transgene copy number-dependent rescue of murine beta-globin knockout mice carrying a 183 kb human beta-globin BAC genomic fragment. *Biochim. Biophys. Acta* 1728: 150-162.

44. Hodgson, J. G., D. J. Smith, K. McCutcheon, H. B. Koide, K. Nishiyama, M. B. Dinulos, M. E. Stevens, N. Bissada, J. Nasir, I. Kanazawa, C. M. Disteche, E. M. Rubin, and M. R. Hayden. 1996. Human huntingtin derived from YAC transgenes compensates for loss of murine huntingtin by rescue of the embryonic lethal phenotype. *Hum. Mol. Genet.* 5: 1875-1885.

45. Carbery, I. D., D. Ji, A. Harrington, V. Brown, E. J. Weinstein, L. Liaw, and X. Cui. 2010. Targeted genome modification in mice using zinc-finger nucleases. *Genetics* 186: 451-459.

46. Cui, X., D. Ji, D. A. Fisher, Y. Wu, D. M. Briner, and E. J. Weinstein. 2011. Targeted integration in rat and mouse embryos with zinc-finger nucleases. *Nat. Biotechnol.* 29: 64-67.

47. Boch, J. 2011. TALEs of genome targeting. *Nat. Biotechnol.* 29: 135-136.

48. Hockemeyer, D., H. Wang, S. Kiani, C. S. Lai, Q. Gao, J. P. Cassady, G. J. Cost, L. Zhang, Y. Santiago, J. C. Miller, B. Zeitler, J. M. Cherone, X. Meng, S. J. Hinkley, E. J. Rebar, P. D. Gregory, F. D. Urnov, and R. Jaenisch. 2011. Genetic engineering of human pluripotent cells using TALE nucleases. *Nat. Biotechnol.* 29: 731-734.

49. Moscou, M. J., and A. J. Bogdanove. 2009. A simple cipher governs DNA recognition by TAL effectors. *Science* 326: 1501.

50. Jinek, M., F. Jiang, D. W. Taylor, S. H. Sternberg, E. Kaya, E. Ma, C. Anders, M. Hauer, K. Zhou, S. Lin, M. Kaplan, A. T. Iavarone, E. Charpentier, E. Nogales, and J. A. Doudna. 2014. Structures of Cas9 endonucleases reveal RNA-mediated conformational activation. *Science* 343: 1247997.

51. Yang, H., H. Wang, C. S. Shivalila, A. W. Cheng, L. Shi, and R. Jaenisch. 2013. One-step generation of mice carrying reporter and conditional alleles by CRISPR/Cas-mediated genome engineering. *Cell* 154: 1370-1379.

52. Yin, H., W. Xue, S. Chen, R. L. Bogorad, E. Benedetti, M. Grompe, V. Koteliansky, P. A. Sharp, T. Jacks, and D. G. Anderson. 2014. Genome editing with Cas9 in adult mice corrects a disease mutation and phenotype. *Nat. Biotechnol.* 32: 551-553.

53. Aponte, J. L., G. A. Sega, L. J. Hauser, M. S. Dhar, C. M. Withrow, D. A. Carpenter, E. M. Rinchik, C. T. Culiat, and D. K. Johnson. 2001. Point mutations in the murine fumarylacetoacetate hydrolase gene: Animal models for the human genetic disorder hereditary tyrosinemia type 1. *Proc. Natl. Acad. Sci. U. S. A* 98: 641-645.

54. Hindorff, L. A., P. Sethupathy, H. A. Junkins, E. M. Ramos, J. P. Mehta, F. S. Collins, and T. A. Manolio. 2009. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc. Natl. Acad. Sci. U. S. A* 106: 9362-9367.

55. Manolio, T. A., F. S. Collins, N. J. Cox, D. B. Goldstein, L. A. Hindorff, D. J. Hunter, M. I. McCarthy, E. M. Ramos, L. R. Cardon, A. Chakravarti, J. H. Cho, A. E. Guttmacher, A. Kong, L. Kruglyak, E. Mardis, C. N. Rotimi, M. Slatkin, D. Valle, A. S. Whittemore, M. Boehnke, A. G. Clark, E. E. Eichler, G. Gibson, J. L. Haines, T. F. Mackay, S. A. McCarroll, and P. M. Visscher. 2009. Finding the missing heritability of complex diseases. *Nature* 461: 747-753.

56. Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10: 170-181.

57. Moore, K. W., M. R. de Waal, R. L. Coffman, and A. O'Garra. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu. Rev. Immunol.* 19: 683-765.

58. Hawrylowicz, C. M., and A. O'Garra. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat. Rev. Immunol.* 5: 271-283.

59. Huber, S., N. Gagliani, E. Esplugues, W. O'Connor, Jr., F. J. Huber, A. Chaudhry, M. Kamanaka, Y. Kobayashi, C. J. Booth, A. Y. Rudensky, M. G. Roncarolo, M. Battaglia, and R. A. Flavell. 2011. Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner. *Immunity.* 34: 554-565.

60. Reuss, E., R. Fimmers, A. Kruger, C. Becker, C. Rittner, and T. Hohler. 2002. Differential regulation of interleukin-10 production by genetic and environmental factors--a twin study. *Genes Immun.* 3: 407-413.

61. Smith, A. J., and S. E. Humphries. 2009. Cytokine and cytokine receptor gene polymorphisms and their functionality. *Cytokine Growth Factor Rev.* 20: 43-59.

62. Eskdale, J., G. Gallagher, C. L. Verweij, V. Keijsers, R. G. Westendorp, and T. W. Huizinga. 1998. Interleukin 10 secretion in relation to human IL-10 locus haplotypes. *Proc. Natl. Acad. Sci. U. S. A* 95: 9465-9470.

63. Hoffmann, S. C., E. M. Stanley, E. D. Cox, B. S. DiMercurio, D. E. Koziol, D. M. Harlan, A. D. Kirk, and P. J. Blair. 2002. Ethnicity greatly influences cytokine gene polymorphism distribution. *Am. J. Transplant.* 2: 560-567.

64. Oleksyk, T. K., S. Shrestha, A. L. Truelove, J. J. Goedert, S. M. Donfield, J. Phair, S. Mehta, S. J. O'Brien, and M. W. Smith. 2009. Extended IL10 haplotypes and their association with HIV progression to AIDS. *Genes Immun.* 10: 309-322.

65. Gibson, A. W., J. C. Edberg, J. Wu, R. G. Westendorp, T. W. Huizinga, and R. P. Kimberly. 2001. Novel single nucleotide polymorphisms in the distal IL-10 promoter affect IL-10 production and enhance the risk of systemic lupus erythematosus. *J. Immunol.* 166: 3915-3922.

66. Crawley, E., R. Kay, J. Sillibourne, P. Patel, I. Hutchinson, and P. Woo. 1999. Polymorphic haplotypes of the interleukin-10 5' flanking region determine variable interleukin-10 transcription and are associated with particular phenotypes of juvenile rheumatoid arthritis. *Arthritis Rheum.* 42: 1101-1108.

67. Steinke, J. W., E. Barekzi, J. Hagman, and L. Borish. 2004. Functional analysis of -571 IL-10 promoter polymorphism reveals a repressor element controlled by sp1. *J. Immunol.* 173: 3215-3222.

68. Suarez, A., P. Castro, R. Alonso, L. Mozo, and C. Gutierrez. 2003. Interindividual variations in constitutive interleukin-10 messenger RNA and protein levels and their association with genetic polymorphisms. *Transplantation* 75: 711-717.

69. Remmers, E. F., F. Cosan, Y. Kirino, M. J. Ombrello, N. Abaci, C. Satorius, J. M. Le, B. Yang, B. D. Korman, A. Cakiris, O. Aglar, Z. Emrence, H. Azakli, D. Ustek, I. Tugal-Tutkun, G. Akman-Demir, W. Chen, C. I. Amos, M. B. Dizon, A. A. Kose, G. Azizlerli, B. Erer, O. J. Brand, V. G. Kaklamani, P. Kaklamanis, E. Ben-Chetrit, M. Stanford, F. Fortune, M. Ghabra, W. E. Ollier, Y. H. Cho, D. Bang, J. O'Shea, G. R. Wallace, M. Gadina, D. L. Kastner, and A. Gul. 2010. Genome-wide association study identifies variants in the MHC class I, IL10, and IL23R-IL12RB2 regions associated with Behcet's disease. *Nat. Genet.* 42: 698-702.

70. Shin, H. D., C. Winkler, J. C. Stephens, J. Bream, H. Young, J. J. Goedert, T. R. O'Brien, D. Vlahov, S. Buchbinder, J. Giorgi, C. Rinaldo, S. Donfield, A. Willoughby, S. J. O'Brien, and M. W. Smith. 2000. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc. Natl. Acad. Sci. U. S. A* 97: 14467-14472.

71. Naicker, D. D., L. Werner, E. Kormuth, J. A. Passmore, K. Mlisana, S. A. Karim, and T. Ndung'u. 2009. Interleukin-10 promoter polymorphisms influence HIV-1 susceptibility and primary HIV-1 pathogenesis. *J. Infect. Dis.* 200: 448-452.

72. Naicker, D. D., B. Wang, E. Losina, J. Zupkosky, S. Bryan, S. Reddy, M. Jaggernath, M. Mokgoro, P. J. Goulder, D. E. Kaufmann, and T. Ndung'u. 2012. Association of IL-10-promoter genetic variants with the rate of CD4 T-cell loss, IL-10 plasma levels, and breadth of cytotoxic T-cell lymphocyte response during chronic HIV-1 infection. *Clin. Infect. Dis.* 54: 294-302.

73. Miyazoe, S., K. Hamasaki, K. Nakata, Y. Kajiya, K. Kitajima, K. Nakao, M. Daikoku, H. Yatsuhashi, M. Koga, M. Yano, and K. Eguchi. 2002. Influence of interleukin-10 gene promoter polymorphisms on disease progression in patients chronically infected with hepatitis B virus. *Am. J. Gastroenterol.* 97: 2086-2092.

74. Salhi, A., V. Rodrigues, Jr., F. Santoro, H. Dessein, A. Romano, L. R. Castellano, M. Sertorio, S. Rafati, C. Chevillard, A. Prata, A. Alcais, L. Argiro, and A. Dessein. 2008. Immunological and genetic evidence for a crucial role of IL-10 in cutaneous lesions in humans infected with Leishmania braziliensis. *J. Immunol.* 180: 6139-6148.

75. Apinjoh, T. O., J. K. Anchang-Kimbi, C. Njua-Yafi, A. N. Ngwai, R. N. Mugri, T. G. Clark, K. A. Rockett, D. P. Kwiatkowski, and E. A. Achidi. 2014. Association of candidate gene polymorphisms and TGF-beta/IL-10 levels with malaria in three regions of Cameroon: a case-control study. *Malar. J.* 13: 236.

76. Schaaf, B. M., F. Boehmke, H. Esnaashari, U. Seitzer, H. Kothe, M. Maass, P. Zabel, and K. Dalhoff. 2003. Pneumococcal septic shock is associated with the interleukin-10-1082 gene promoter polymorphism. *Am. J. Respir. Crit Care Med.* 168: 476-480.

77. Barrett, J. C., D. G. Clayton, P. Concannon, B. Akolkar, J. D. Cooper, H. A. Erlich, C. Julier, G. Morahan, J. Nerup, C. Nierras, V. Plagnol, F. Pociot, H. Schuilenburg, D. J. Smyth, H. Stevens, J. A. Todd, N. M. Walker, and S. S. Rich. 2009. Genome-wide association study and meta-analysis find that over 40 loci affect risk of type 1 diabetes. *Nat. Genet.* 41: 703-707.

78. Franke, A., T. Balschun, T. H. Karlsen, J. Sventoraityte, S. Nikolaus, G. Mayr, F. S. Domingues, M. Albrecht, M. Nothnagel, D. Ellinghaus, C. Sina, C. M. Onnie, R. K. Weersma, P. C. Stokkers, C. Wijmenga, M. Gazouli, D. Strachan, W. L. McArdle, S. Vermeire, P. Rutgeerts, P. Rosenstiel, M. Krawczak, M. H. Vatn, C. G. Mathew, and S. Schreiber. 2008. Sequence variants in IL10, ARPC2 and multiple other loci contribute to ulcerative colitis susceptibility. *Nat. Genet.* 40: 1319-1323.

79. Hulkkonen, J., M. Pertovaara, J. Antonen, N. Lahdenpohja, A. Pasternack, and M. Hurme. 2001. Genetic association between interleukin-10 promoter region polymorphisms and primary Sjogren's syndrome. *Arthritis Rheum.* 44: 176-179.

80. Kingo, K., S. Koks, H. Silm, and E. Vasar. 2003. IL-10 promoter polymorphisms influence disease severity and course in psoriasis. *Genes Immun.* 4: 455-457.

81. Mizuki, N., A. Meguro, M. Ota, S. Ohno, T. Shiota, T. Kawagoe, N. Ito, J. Kera, E. Okada, K. Yatsu, Y. W. Song, E. B. Lee, N. Kitaichi, K. Namba, Y. Horie, M. Takeno, S. Sugita, M. Mochizuki, S. Bahram, Y. Ishigatsubo, and H. Inoko. 2010. Genome-wide association studies identify IL23R-IL12RB2 and IL10 as Behcet's disease susceptibility loci. *Nat. Genet.* 42: 703-706.

82. Gateva, V., J. K. Sandling, G. Hom, K. E. Taylor, S. A. Chung, X. Sun, W. Ortmann, R. Kosoy, R. C. Ferreira, G. Nordmark, I. Gunnarsson, E. Svenungsson, L. Padyukov, G. Sturfelt, A. Jonsen, A. A. Bengtsson, S. Rantapaa-Dahlqvist, E. C. Baechler, E. E. Brown, G. S. Alarcon, J. C. Edberg, R. Ramsey-Goldman, G. McGwin, Jr., J. D. Reveille, L. M. Vila, R. P. Kimberly, S. Manzi, M. A. Petri, A. Lee, P. K. Gregersen, M. F. Seldin, L. Ronnblom, L. A. Criswell, A. C. Syvanen, T. W. Behrens, and R. R. Graham. 2009. A large-scale replication study identifies TNIP1, PRDM1, JAZF1, UHRF1BP1 and IL10 as risk loci for systemic lupus erythematosus. *Nat. Genet.* 41: 1228-1233.

83. Franke, A., D. P. McGovern, J. C. Barrett, K. Wang, G. L. Radford-Smith, T. Ahmad, C. W. Lees, T. Balschun, J. Lee, R. Roberts, C. A. Anderson, J. C. Bis, S. Bumpstead, D. Ellinghaus, E. M. Festen, M. Georges, T. Green, T. Haritunians, L. Jostins, A. Latiano, C. G. Mathew, G. W. Montgomery, N. J. Prescott, S. Raychaudhuri, J. I. Rotter, P. Schumm, Y. Sharma, L. A. Simms, K. D. Taylor, D. Whiteman, C. Wijmenga, R. N. Baldassano, M. Barclay, T. M. Bayless, S. Brand, C. Buning, A. Cohen, J. F. Colombel, M. Cottone, L. Stronati, T. Denson, V. M. De, R. D'Inca, M. Dubinsky, C. Edwards, T. Florin, D. Franchimont, R. Gearry, J. Glas, G. A. Van, S. L. Guthery, J. Halfvarson, H. W. Verspaget, J. P. Hugot, A. Karban, D. Laukens, I. Lawrance, M. Lemann, A. Levine, C. Libioulle, E. Louis, C. Mowat, W. Newman, J. Panes, A. Phillips, D. D. Proctor, M. Regueiro, R. Russell, P. Rutgeerts, J. Sanderson, M. Sans, F. Seibold, A. H. Steinhart, P. C. Stokkers, L. Torkvist, G. Kullak-Ublick, D. Wilson, T. Walters, S. R. Targan, S. R. Brant, J. D. Rioux, M. D'Amato, R. K. Weersma, S. Kugathasan, A. M. Griffiths, J. C. Mansfield, S. Vermeire, R. H. Duerr, M. S. Silverberg, J. Satsangi, S. Schreiber, J. H. Cho, V. Annese, H. Hakonarson, M. J. Daly, and M. Parkes. 2010. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat. Genet.* 42: 1118-1125.

84. Jiang, H., J. J. Lin, Z. Z. Su, N. I. Goldstein, and P. B. Fisher. 1995. Subtraction hybridization identifies a novel melanoma differentiation associated gene, mda-7, modulated during human melanoma differentiation, growth and progression. *Oncogene* 11: 2477-2486.

85. Huang, E. Y., M. T. Madireddi, R. V. Gopalkrishnan, M. Leszczyniecka, Z. Su, I. V. Lebedeva, D. Kang, H. Jiang, J. J. Lin, D. Alexandre, Y. Chen, N. Vozhilla, M. X. Mei, K. A. Christiansen, F. Sivo, N. I. Goldstein, A. B. Mhashilkar, S. Chada, E. Huberman, S. Pestka, and P. B. Fisher. 2001. Genomic structure, chromosomal localization and expression profile of a novel melanoma differentiation associated (mda-7) gene with cancer specific growth suppressing and apoptosis inducing properties. *Oncogene* 20: 7051-7063.

86. Sahoo, A., C. G. Lee, A. Jash, J. S. Son, G. Kim, H. K. Kwon, J. S. So, and S. H. Im. 2011. Stat6 and c-Jun mediate Th2 cell-specific IL-24 gene expression. *J. Immunol.* 186: 4098-4109.

87. Schaefer, G., C. Venkataraman, and U. Schindler. 2001. Cutting edge: FISP (IL-4-induced secreted protein), a novel cytokine-like molecule secreted by Th2 cells. *J. Immunol.* 166: 5859-5863.

88. Wei, L., G. Vahedi, H. W. Sun, W. T. Watford, H. Takatori, H. L. Ramos, H. Takahashi, J. Liang, G. Gutierrez-Cruz, C. Zang, W. Peng, J. J. O'Shea, and Y. Kanno. 2010. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity* 32: 840-851.

89. Poindexter, N. J., E. T. Walch, S. Chada, and E. A. Grimm. 2005. Cytokine induction of interleukin-24 in human peripheral blood mononuclear cells. *J. Leukoc. Biol.* 78: 745-752.

90. Wolk, K., S. Kunz, K. Asadullah, and R. Sabat. 2002. Cutting edge: immune cells as sources and targets of the IL-10 family members? *J. Immunol.* 168: 5397-5402.

91. Maarof, G., L. Bouchet-Delbos, H. Gary-Gouy, I. Durand-Gasselin, R. Krzysiek, and A. Dalloul. 2010. Interleukin-24 inhibits the plasma cell differentiation program in human germinal center B cells. *Blood* 115: 1718-1726.

92. Andoh, A., M. Shioya, A. Nishida, S. Bamba, T. Tsujikawa, S. Kim-Mitsuyama, and Y. Fujiyama. 2009. Expression of IL-24, an activator of the JAK1/STAT3/SOCS3 cascade, is enhanced in inflammatory bowel disease. *J. Immunol.* 183: 687-695.

93. Jen, E. Y., N. J. Poindexter, E. S. Farnsworth, and E. A. Grimm. 2012. IL-2 regulates the expression of the tumor suppressor IL-24 in melanoma cells. *Melanoma Res.* 22: 19-29.

94. Kumari, S., M. C. Bonnet, M. H. Ulvmar, K. Wolk, N. Karagianni, E. Witte, C. Uthoff-Hachenberg, J. C. Renauld, G. Kollias, R. Toftgard, R. Sabat, M. Pasparakis, and I. Haase. 2013. Tumor necrosis factor receptor signaling in keratinocytes triggers interleukin-24-dependent psoriasis-like skin inflammation in mice. *Immunity* 39: 899-911.

95. Otkjaer, K., H. Holtmann, T. W. Kragstrup, S. R. Paludan, C. Johansen, M. Gaestel, K. Kragballe, and L. Iversen. 2010. The p38 MAPK regulates IL-24 expression by stabilization of the 3' UTR of IL-24 mRNA. *PLoS One* 5: e8671.

96. Wang, M., Z. Tan, R. Zhang, S. V. Kotenko, and P. Liang. 2002. Interleukin 24 (MDA-7/MOB-5) signals through two heterodimeric receptors, IL-22R1/IL-20R2 and IL-20R1/IL-20R2. *J. Biol. Chem.* 277: 7341-7347.

97. Dumoutier, L., C. Leemans, D. Lejeune, S. V. Kotenko, and J. C. Renauld. 2001. Cutting edge: STAT activation by IL-19, IL-20 and mda-7 through IL-20 receptor complexes of two types. *J. Immunol.* 167: 3545-3549.

98. Chan, J. R., W. Blumenschein, E. Murphy, C. Diveu, M. Wiekowski, S. Abbondanzo, L. Lucian, R. Geissler, S. Brodie, A. B. Kimball, D. M. Gorman, K. Smith, M. R. de Waal, R. A. Kastelein, T. K. McClanahan, and E. P. Bowman. 2006. IL-23 stimulates epidermal hyperplasia via TNF and IL-20R2-dependent mechanisms with implications for psoriasis pathogenesis. *J. Exp. Med.* 203: 2577-2587.

99. Hofmann, S. R., A. Rosen-Wolff, G. C. Tsokos, and C. M. Hedrich. 2012. Biological properties and regulation of IL-10 related cytokines and their contribution to autoimmune disease and tissue injury. *Clin. Immunol.* 143: 116-127.

100. Caudell, E. G., J. B. Mumm, N. Poindexter, S. Ekmekcioglu, A. M. Mhashilkar, X. H. Yang, M. W. Retter, P. Hill, S. Chada, and E. A. Grimm. 2002. The protein product of the tumor suppressor gene, melanoma differentiation-associated gene 7, exhibits immunostimulatory activity and is designated IL-24. *J. Immunol.* 168: 6041-6046.

101. Buzas, K., J. J. Oppenheim, and O. M. Zack Howard. 2011. Myeloid cells migrate in response to IL-24. *Cytokine* 55: 429-434.

102. Maarof, G., L. Bouchet-Delbos, H. Gary-Gouy, I. Durand-Gasselin, R. Krzysiek, and A. Dalloul. 2010. Interleukin-24 inhibits the plasma cell differentiation program in human germinal center B cells. *Blood* 115: 1718-1726.

103. He, M., and P. Liang. 2010. IL-24 transgenic mice: in vivo evidence of overlapping functions for IL-20, IL-22, and IL-24 in the epidermis. *J. Immunol.* 184: 1793-1798.

104. Jiang, H., Z. Z. Su, J. J. Lin, N. I. Goldstein, C. S. Young, and P. B. Fisher. 1996. The melanoma differentiation associated gene mda-7 suppresses cancer cell growth. *Proc. Natl. Acad. Sci. U. S. A* 93: 9160-9165.

105. Kreis, S., D. Philippidou, C. Margue, and I. Behrmann. 2008. IL-24: a classic cytokine and/or a potential cure for cancer? *J. Cell Mol. Med.* 12: 2505-2510.

106. Lebedeva, I. V., L. Emdad, Z. Z. Su, P. Gupta, M. Sauane, D. Sarkar, M. R. Staudt, S. J. Liu, M. M. Taher, R. Xiao, P. Barral, S. G. Lee, D. Wang, N. Vozhilla, E. S. Park, L. Chatman, H. Boukerche, R. Ramesh, S. Inoue, S. Chada, R. Li, A. L. De Pass, P. J. Mahasreshti, I. P. Dmitriev, D. T. Curiel, A. Yacoub, S. Grant, P. Dent, N. Senzer, J. J. Nemunaitis, and P. B. Fisher. 2007. mda-7/IL-24, novel anticancer cytokine: focus on bystander antitumor, radiosensitization and antiangiogenic properties and overview of the phase I clinical experience (Review). *Int. J. Oncol.* 31: 985-1007.

107. Menezes, M. E., S. Bhatia, P. Bhoopathi, S. K. Das, L. Emdad, S. Dasgupta, P. Dent, X. Y. Wang, D. Sarkar, and P. B. Fisher. 2014. MDA-7/IL-24: Multifunctional Cancer Killing Cytokine. *Adv. Exp. Med. Biol.* 818: 127-153.

108. Cunningham, C. C., S. Chada, J. A. Merritt, A. Tong, N. Senzer, Y. Zhang, A. Mhashilkar, K. Parker, S. Vukelja, D. Richards, J. Hood, K. Coffee, and J. Nemunaitis. 2005. Clinical and local biological effects of an intratumoral injection of mda-7 (IL24; INGN 241) in patients with advanced carcinoma: a phase I study. *Mol. Ther.* 11: 149-159.

109. Andoh, A., M. Shioya, A. Nishida, S. Bamba, T. Tsujikawa, S. Kim-Mitsuyama, and Y. Fujiyama. 2009. Expression of IL-24, an activator of the JAK1/STAT3/SOCS3 cascade, is enhanced in inflammatory bowel disease. *J. Immunol.* 183: 687-695.

110. Fonseca-Camarillo, G., J. Furuzawa-Carballeda, J. Granados, and J. K. Yamamoto-Furusho. 2014. Expression of interleukin (IL)-19 and IL-24 in inflammatory bowel disease patients: a cross-sectional study. *Clin. Exp. Immunol.* 177: 64-75.

111. Kragstrup, T. W., K. Otkjaer, C. Holm, A. Jorgensen, M. Hokland, L. Iversen, and B. Deleuran. 2008. The expression of IL-20 and IL-24 and their shared receptors are increased in rheumatoid arthritis and spondyloarthropathy. *Cytokine* 41: 16-23.

112. Ma, Y., H. D. Chen, Y. Wang, Q. Wang, Y. Li, Y. Zhao, and X. L. Zhang. 2011. Interleukin 24 as a novel potential cytokine immunotherapy for the treatment of Mycobacterium tuberculosis infection. *Microbes. Infect.* 13: 1099-1110.

113. Wu, B., C. Huang, M. Kato-Maeda, P. C. Hopewell, C. L. Daley, A. M. Krensky, and C. Clayberger. 2008. IL-24 modulates IFN-gamma expression in patients with tuberculosis. *Immunol. Lett.* 117: 57-62.

114. Ma, Y., H. Chen, Q. Wang, F. Luo, J. Yan, and X. L. Zhang. 2009. IL-24 protects against Salmonella typhimurium infection by stimulating early neutrophil Th1 cytokine production, which in turn activates CD8+ T cells. *Eur. J. Immunol.* 39: 3357-3368.

115. Sahoo, A., C. G. Lee, A. Jash, J. S. Son, G. Kim, H. K. Kwon, J. S. So, and S. H. Im. 2011. Stat6 and c-Jun mediate Th2 cell-specific IL-24 gene expression. *J. Immunol.* 186: 4098-4109.

116. Wei, L., G. Vahedi, H. W. Sun, W. T. Watford, H. Takatori, H. L. Ramos, H. Takahashi, J. Liang, G. Gutierrez-Cruz, C. Zang, W. Peng, J. J. O'Shea, and Y. Kanno. 2010. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity.* 32: 840-851.

117. Sahoo, A., C. G. Lee, A. Jash, J. S. Son, G. Kim, H. K. Kwon, J. S. So, and S. H. Im. 2011. Stat6 and c-Jun mediate Th2 cell-specific IL-24 gene expression. *J. Immunol.* 186: 4098-4109.

118. Venter, J. C., M. D. Adams, E. W. Myers, P. W. Li, R. J. Mural, G. G. Sutton, H. O. Smith, M. Yandell, C. A. Evans, R. A. Holt, J. D. Gocayne, P. Amanatides, R. M. Ballew, D. H. Huson, J. R. Wortman, Q. Zhang, C. D. Kodira, X. H. Zheng, L. Chen, M. Skupski, G. Subramanian, P. D. Thomas, J. Zhang, G. L. Gabor Miklos, C. Nelson, S.

Broder, A. G. Clark, J. Nadeau, V. A. McKusick, N. Zinder, A. J. Levine, R. J. Roberts, M. Simon, C. Slayman, M. Hunkapiller, R. Bolanos, A. Delcher, I. Dew, D. Fasulo, M. Flanigan, L. Florea, A. Halpern, S. Hannenhalli, S. Kravitz, S. Levy, C. Mobarry, K. Reinert, K. Remington, J. Abu-Threideh, E. Beasley, K. Biddick, V. Bonazzi, R. Brandon, M. Cargill, I. Chandramouliswaran, R. Charlab, K. Chaturvedi, Z. Deng, F. Di, V, P. Dunn, K. Eilbeck, C. Evangelista, A. E. Gabrielian, W. Gan, W. Ge, F. Gong, Z. Gu, P. Guan, T. J. Heiman, M. E. Higgins, R. R. Ji, Z. Ke, K. A. Ketchum, Z. Lai, Y. Lei, Z. Li, J. Li, Y. Liang, X. Lin, F. Lu, G. V. Merkulov, N. Milshina, H. M. Moore, A. K. Naik, V. A. Narayan, B. Neelam, D. Nusskern, D. B. Rusch, S. Salzberg, W. Shao, B. Shue, J. Sun, Z. Wang, A. Wang, X. Wang, J. Wang, M. Wei, R. Wides, C. Xiao, C. Yan, A. Yao, J. Ye, M. Zhan, W. Zhang, H. Zhang, Q. Zhao, L. Zheng, F. Zhong, W. Zhong, S. Zhu, S. Zhao, D. Gilbert, S. Baumhueter, G. Spier, C. Carter, A. Cravchik, T. Woodage, F. Ali, H. An, A. Awe, D. Baldwin, H. Baden, M. Barnstead, I. Barrow, K. Beeson, D. Busam, A. Carver, A. Center, M. L. Cheng, L. Curry, S. Danaher, L. Davenport, R. Desilets, S. Dietz, K. Dodson, L. Doup, S. Ferriera, N. Garg, A. Gluecksmann, B. Hart, J. Haynes, C. Haynes, C. Heiner, S. Hladun, D. Hostin, J. Houck, T. Howland, C. Ibegwam, J. Johnson, F. Kalush, L. Kline, S. Koduru, A. Love, F. Mann, D. May, S. McCawley, T. McIntosh, I. McMullen, M. Moy, L. Moy, B. Murphy, K. Nelson, C. Pfannkoch, E. Pratts, V. Puri, H. Qureshi, M. Reardon, R. Rodriguez, Y. H. Rogers, D. Romblad, B. Ruhfel, R. Scott, C. Sitter, M. Smallwood, E. Stewart, R. Strong, E. Suh, R. Thomas, N. N. Tint, S. Tse, C. Vech, G. Wang, J. Wetter, S. Williams, M. Williams, S. Windsor, E. Winn-Deen, K. Wolfe, J. Zaveri, K. Zaveri, J. F. Abril, R. Guigo, M. J. Campbell, K. V. Sjolander, B. Karlak, A. Kejariwal, H. Mi, B. Lazareva, T. Hatton, A. Narechania, K. Diemer, A. Muruganujan, N. Guo, S. Sato, V. Bafna, S. Istrail, R. Lippert, R. Schwartz, B. Walenz, S. Yooseph, D. Allen, A. Basu, J. Baxendale, L. Blick, M. Caminha, J. Carnes-Stine, P. Caulk, Y. H. Chiang, M. Coyne, C. Dahlke, A. Mays, M. Dombroski, M. Donnelly, D. Ely, S. Esparham, C. Fosler, H. Gire, S. Glanowski, K. Glasser, A. Glodek, M. Gorokhov, K. Graham, B. Gropman, M. Harris, J. Heil, S. Henderson, J. Hoover, D. Jennings, C. Jordan, J. Jordan, J. Kasha, L. Kagan, C. Kraft, A. Levitsky, M. Lewis, X. Liu, J. Lopez, D. Ma, W. Majoros, J. McDaniel, S. Murphy, M. Newman, T. Nguyen, N. Nguyen, and M. Nodell. 2001. The sequence of the human genome. *Science* 291: 1304-1351.

119. Lander, E. S., L. M. Linton, B. Birren, C. Nusbaum, M. C. Zody, J. Baldwin, K. Devon, K. Dewar, M. Doyle, W. FitzHugh, R. Funke, D. Gage, K. Harris, A. Heaford, J. Howland, L. Kann, J. Lehoczky, R. Levine, P. McEwan, K. McKernan, J. Meldrim, J. P. Mesirov, C. Miranda, W. Morris, J. Naylor, C. Raymond, M. Rosetti, R. Santos, A. Sheridan, C. Sougnez, N. Stange-Thomann, N. Stojanovic, A. Subramanian, D. Wyman, J. Rogers, J. Sulston, R. Ainscough, S. Beck, D. Bentley, J. Burton, C. Clee, N. Carter, A. Coulson, R. Deadman, P. Deloukas, A. Dunham, I. Dunham, R. Durbin, L. French, D. Grafham, S. Gregory, T. Hubbard, S. Humphray, A. Hunt, M. Jones, C. Lloyd, A. McMurray, L. Matthews, S. Mercer, S. Milne, J. C. Mullikin, A. Mungall, R. Plumb, M. Ross, R. Shownkeen, S. Sims, R. H. Waterston, R. K. Wilson, L. W. Hillier, J. D. McPherson, M. A. Marra, E. R. Mardis, L. A. Fulton, A. T. Chinwalla, K. H. Pepin, W. R. Gish, S. L. Chissoe, M. C. Wendl, K. D. Delehaunty, T. L. Miner, A. Delehaunty, J. B. Kramer, L. L. Cook, R. S. Fulton, D. L. Johnson, P. J. Minx, S. W. Clifton, T. Hawkins, E. Branscomb, P. Predki, P. Richardson, S. Wenning, T. Slezak, N. Doggett, J. F. Cheng,

A. Olsen, S. Lucas, C. Elkin, E. Uberbacher, M. Frazier, R. A. Gibbs, D. M. Muzny, S. E. Scherer, J. B. Bouck, E. J. Sodergren, K. C. Worley, C. M. Rives, J. H. Gorrell, M. L. Metzker, S. L. Naylor, R. S. Kucherlapati, D. L. Nelson, G. M. Weinstock, Y. Sakaki, A. Fujiyama, M. Hattori, T. Yada, A. Toyoda, T. Itoh, C. Kawagoe, H. Watanabe, Y. Totoki, T. Taylor, J. Weissenbach, R. Heilig, W. Saurin, F. Artiguenave, P. Brottier, T. Bruls, E. Pelletier, C. Robert, P. Wincker, D. R. Smith, L. Doucette-Stamm, M. Rubenfield, K. Weinstock, H. M. Lee, J. Dubois, A. Rosenthal, M. Platzer, G. Nyakatura, S. Taudien, A. Rump, H. Yang, J. Yu, J. Wang, G. Huang, J. Gu, L. Hood, L. Rowen, A. Madan, S. Qin, R. W. Davis, N. A. Federspiel, A. P. Abola, M. J. Proctor, R. M. Myers, J. Schmutz, M. Dickson, J. Grimwood, D. R. Cox, M. V. Olson, R. Kaul, C. Raymond, N. Shimizu, K. Kawasaki, S. Minoshima, G. A. Evans, M. Athanasiou, R. Schultz, B. A. Roe, F. Chen, H. Pan, J. Ramser, H. Lehrach, R. Reinhardt, W. R. McCombie, M. de la Bastide, N. Dedhia, H. Blocker, K. Hornischer, G. Nordsiek, R. Agarwala, L. Aravind, J. A. Bailey, A. Bateman, S. Batzoglou, E. Birney, P. Bork, D. G. Brown, C. B. Burge, L. Cerutti, H. C. Chen, D. Church, M. Clamp, R. R. Copley, T. Doerks, S. R. Eddy, E. E. Eichler, T. S. Furey, J. Galagan, J. G. Gilbert, C. Harmon, Y. Hayashizaki, D. Haussler, H. Hermjakob, K. Hokamp, W. Jang, L. S. Johnson, T. A. Jones, S. Kasif, A. Kaspryzk, S. Kennedy, W. J. Kent, P. Kitts, E. V. Koonin, I. Korf, D. Kulp, D. Lancet, T. M. Lowe, A. McLysaght, T. Mikkelsen, J. V. Moran, N. Mulder, V. J. Pollara, C. P. Ponting, G. Schuler, J. Schultz, G. Slater, A. F. Smit, E. Stupka, J. Szustakowski, D. Thierry-Mieg, J. Thierry-Mieg, L. Wagner, J. Wallis, R. Wheeler, A. Williams, Y. I. Wolf, K. H. Wolfe, S. P. Yang, R. F. Yeh, F. Collins, M. S. Guyer, J. Peterson, A. Felsenfeld, K. A. Wetterstrand, A. Patrinos, M. J. Morgan, J. P. de, J. J. Catanese, K. Osoegawa, H. Shizuya, S. Choi, and Y. J. Chen. 2001. Initial sequencing and analysis of the human genome. *Nature* 409: 860-921.

120. Abecasis, G. R. 2012. An integrated map of genetic variation from 1,092 human genomes.

121. Altshuler, D. M. 2010. Integrating common and rare genetic variation in diverse human populations.

122. Hindorff, L. A. 2009. Potential etiologic and functional implications of genome-wide association loci for human diseases and traits.

123. Manolio, T. A. 2009. Finding the missing heritability of complex diseases.

124. Fairfax, B. P. 2012. Genetics of gene expression in primary immune cells identifies cell type-specific master regulators and roles of HLA alleles.

125. Fairfax, B. P. 2014. Innate immune activity conditions the effect of regulatory variants upon monocyte gene expression.

126. Lee, M. N. 2014. Common genetic variants modulate pathogen-sensing responses in human dendritic cells.

127. Stranger, B. E., A. C. Nica, M. S. Forrest, A. Dimas, C. P. Bird, C. Beazley, C. E. Ingle, M. Dunning, P. Flicek, D. Koller, S. Montgomery, S. Tavare, P. Deloukas, and E. T.

Dermitzakis. 2007. Population genomics of human gene expression. *Nat. Genet.* 39: 1217-1224.

128. Schaub, M. A. 2012. Linking disease associations with regulatory information in the human genome.

129. Vernot, B., A. B. Stergachis, M. T. Maurano, J. Vierstra, S. Neph, R. E. Thurman, J. A. Stamatoyannopoulos, and J. M. Akey. 2012. Personal and population genomics of human regulatory variation. *Genome Res.* 22: 1689-1697.

130. Vavouri, T., and G. Elgar. 2005. Prediction of cis-regulatory elements using binding site matrices--the successes, the failures and the reasons for both. *Curr. Opin. Genet. Dev.* 15: 395-402.

131. Nardone, J., D. U. Lee, K. M. Ansel, and A. Rao. 2004. Bioinformatics for the 'bench biologist': how to find regulatory regions in genomic DNA. *Nat. Immunol.* 5: 768-774.

132. Dermitzakis, E. T., and A. G. Clark. 2002. Evolution of transcription factor binding sites in Mammalian gene regulatory regions: conservation and turnover. *Mol. Biol. Evol.* 19: 1114-1121.

133. Schmidt, D., M. D. Wilson, B. Ballester, P. C. Schwalie, G. D. Brown, A. Marshall, C. Kutter, S. Watt, C. P. Martinez-Jimenez, S. Mackay, I. Talianidis, P. Flicek, and D. T. Odom. 2010. Five-vertebrate ChIP-seq reveals the evolutionary dynamics of transcription factor binding. *Science* 328: 1036-1040.

134. Boyle, A. P. 2012. Annotation of functional variation in personal genomes using RegulomeDB.

135. Ecker, J. R. 2012. Genomics: ENCODE explained.

136. Bernstein, B. E. 2012. An integrated encyclopedia of DNA elements in the human genome.

137. Ranatunga, D. 2009. A human IL10 BAC transgene reveals tissue-specific control of IL-10 expression and alters disease outcome.

138. Ranatunga, D. C., A. Ramakrishnan, P. Uprety, F. Wang, H. Zhang, J. B. Margolick, C. Brayton, and J. H. Bream. 2012. A protective role for human IL-10-expressing CD4+ T cells in colitis. *J. Immunol.* 189: 1243-1252.

139. Anderson, C. F., M. Oukka, V. J. Kuchroo, and D. Sacks. 2007. CD4(+)CD25(-)Foxp3(-) Th1 cells are the source of IL-10-mediated immune suppression in chronic cutaneous leishmaniasis. *J. Exp. Med.* 204: 285-297.

140. Stumhofer, J. S., J. S. Silver, A. Laurence, P. M. Porrett, T. H. Harris, L. A. Turka, M. Ernst, C. J. Saris, J. J. O'Shea, and C. A. Hunter. 2007. Interleukins 27 and 6 induce STAT3-mediated T cell production of interleukin 10. *Nat. Immunol.* 8: 1363-1371.

141. Stager, S. 2006. Distinct roles for IL-6 and IL-12p40 in mediating protection against Leishmania donovani and the expansion of IL-10+ CD4+ T cells.

142. Siewe, L. 2006. Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA.

143. Berg, D. J. 1995. Interleukin-10 is a central regulator of the response to LPS in murine models of endotoxic shock and the Shwartzman reaction but not endotoxin tolerance.

144. Schwarz, T. 2013. T cell-derived IL-10 determines leishmaniasis disease outcome and is suppressed by a dendritic cell based vaccine.

145. Jankovic, D. 2010. IL-10 production by CD4+ effector T cells: a mechanism for self-regulation.

146. Sparwasser, T. 2007. BAC to immunology--bacterial artificial chromosome-mediated transgenesis for targeting of immune cells.

147. Chakravarti, A. 2013. Distilling pathophysiology from complex disease genetics.

148. Engelhardt, K. R. 2014. IL-10 in Humans: Lessons from the Gut, IL-10/IL-10 Receptor Deficiencies, and IL-10 Polymorphisms.

149. Gautam, S. 2011. IL-10 neutralization promotes parasite clearance in splenic aspirate cells from patients with visceral leishmaniasis.

150. Naicker, D. D. 2009. Interleukin-10 promoter polymorphisms influence HIV-1 susceptibility and primary HIV-1 pathogenesis.

151. Portugal, S. 2014. Exposure-dependent control of malaria-induced inflammation in children.

152. Wilson, E. B. 2011. The role of IL-10 in regulating immunity to persistent viral infections.

153. Jankovic, D. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection.

154. Kuhn, R. 1993. Interleukin-10-deficient mice develop chronic enterocolitis.

155. MacArthur, D. G. 2014. Guidelines for investigating causality of sequence variants in human disease.

156. Palstra, R. J., L. W. de, and F. Grosveld. 2008. Beta-globin regulation and long-range interactions. *Adv. Genet.* 61: 107-142.

157. Spilianakis, C. G., and R. A. Flavell. 2004. Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. *Nat. Immunol.* 5: 1017-1027.

158. Falvo, J. V., L. D. Jasenosky, L. Kruidenier, and A. E. Goldfeld. 2013. Epigenetic control of cytokine gene expression: regulation of the TNF/LT locus and T helper cell differentiation. *Adv. Immunol.* 118: 37-128.

159. Collins, P. L., M. A. Henderson, and T. M. Aune. 2012. Lineage-specific adjacent IFNG and IL26 genes share a common distal enhancer element. *Genes Immun.* 13: 481-488.

160. Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10: 170-181.

161. Ouyang, W., S. Rutz, N. K. Crellin, P. A. Valdez, and S. G. Hymowitz. 2011. Regulation and functions of the IL-10 family of cytokines in inflammation and disease. *Annu. Rev. Immunol.* 29: 71-109.

162. Wang, X., Y. Zhang, X. O. Yang, R. I. Nurieva, S. H. Chang, S. S. Ojeda, H. S. Kang, K. S. Schluns, J. Gui, A. M. Jetten, and C. Dong. 2012. Transcription of Il17 and Il17f is controlled by conserved noncoding sequence 2. *Immunity* 36: 23-31.

163. Lin, J. X., P. Li, D. Liu, H. T. Jin, J. He, R. M. Ata Ur, Y. Rochman, L. Wang, K. Cui, C. Liu, B. L. Kelsall, R. Ahmed, and W. J. Leonard. 2012. Critical Role of STAT5 transcription factor tetramerization for cytokine responses and normal immune function. *Immunity* 36: 586-599.

164. Siewe, L., M. Bollati-Fogolin, C. Wickenhauser, T. Krieg, W. Muller, and A. Roers. 2006. Interleukin-10 derived from macrophages and/or neutrophils regulates the inflammatory response to LPS but not the response to CpG DNA. *Eur. J. Immunol.* 36: 3248-3255.

165. Maroof, A., L. Beattie, S. Zubairi, M. Svensson, S. Stager, and P. M. Kaye. 2008. Posttranscriptional regulation of II10 gene expression allows natural killer cells to express immunoregulatory function. *Immunity* 29: 295-305.

166. Lee, S. H., K. S. Kim, N. Fodil-Cornu, S. M. Vidal, and C. A. Biron. 2009. Activating receptors promote NK cell expansion for maintenance, IL-10 production, and CD8 T cell regulation during viral infection. *J. Exp. Med.* 206: 2235-2251.

167. Grant, L. R., Z. J. Yao, C. M. Hedrich, F. Wang, A. Moorthy, K. Wilson, D. Ranatunga, and J. H. Bream. 2008. Stat4-dependent, T-bet-independent regulation of IL-10 in NK cells. *Genes Immun.* 9: 316-327.

168. Iyer, S. S., A. A. Ghaffari, and G. Cheng. 2010. Lipopolysaccharide-mediated IL-10 transcriptional regulation requires sequential induction of type I IFNs and IL-27 in macrophages. *J. Immunol.* 185: 6599-6607.

169. Miyagi, T., M. P. Gil, X. Wang, J. Louten, W. M. Chu, and C. A. Biron. 2007. High basal STAT4 balanced by STAT1 induction to control type 1 interferon effects in natural killer cells. *J. Exp. Med.* 204: 2383-2396.

170. Saraiva, M., J. R. Christensen, M. Veldhoen, T. L. Murphy, K. M. Murphy, and A. O'Garra. 2009. Interleukin-10 production by Th1 cells requires interleukin-12-induced STAT4 transcription factor and ERK MAP kinase activation by high antigen dose. *Immunity.* 31: 209-219.

171. Mack, E. A., L. E. Kallal, D. A. Demers, and C. A. Biron. 2011. Type 1 interferon induction of natural killer cell gamma interferon production for defense during lymphocytic choriomeningitis virus infection. *MBio.* 2.

172. Chang, E. Y., B. Guo, S. E. Doyle, and G. Cheng. 2007. Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production. *J. Immunol.* 178: 6705-6709.

173. Pan, L., H. Pan, H. Jiang, J. Du, X. Wang, B. Huang, and J. Lu. 2010. HDAC4 inhibits the transcriptional activation of mda-7/IL-24 induced by Sp1. *Cell Mol. Immunol.* 7: 221-226.

174. Yao, Z., Y. Cui, W. T. Watford, J. H. Bream, K. Yamaoka, B. D. Hissong, D. Li, S. K. Durum, Q. Jiang, A. Bhandoola, L. Hennighausen, and J. J. O'Shea. 2006. Stat5a/b are essential for normal lymphoid development and differentiation. *Proc. Natl. Acad. Sci. U. S. A* 103: 1000-1005.

175. Wurster, A. L., P. Precht, K. G. Becker, W. H. Wood, III, Y. Zhang, Z. Wang, and M. J. Pazin. 2012. IL-10 transcription is negatively regulated by BAF180, a component of the SWI/SNF chromatin remodeling enzyme. *BMC. Immunol.* 13: 9.

176. Jankovic, D., M. C. Kullberg, C. G. Feng, R. S. Goldszmid, C. M. Collazo, M. Wilson, T. A. Wynn, M. Kamanaka, R. A. Flavell, and A. Sher. 2007. Conventional T-bet(+)Foxp3(-) Th1 cells are the major source of host-protective regulatory IL-10 during intracellular protozoan infection. *J. Exp. Med.* 204: 273-283.

177. Sakurai, D., J. Zhao, Y. Deng, J. A. Kelly, E. E. Brown, J. B. Harley, S. C. Bae, M. E. Alarcomicronn-Riquelme, J. C. Edberg, R. P. Kimberly, R. Ramsey-Goldman, M. A. Petri, J. D. Reveille, L. M. Vila, G. S. Alarcon, K. M. Kaufman, T. J. Vyse, C. O. Jacob, P. M. Gaffney, K. M. Sivils, J. A. James, D. L. Kamen, G. S. Gilkeson, T. B. Niewold, J. T. Merrill, R. H. Scofield, L. A. Criswell, A. M. Stevens, S. A. Boackle, J. H. Kim, J. Choi, B. A. Pons-Estel, B. I. Freedman, J. M. Anaya, J. Martin, C. Y. Yu, D. M. Chang, Y. W. Song, C. D. Langefeld, W. Chen, J. M. Grossman, R. M. Cantor, B. H. Hahn, and B. P. Tsao. 2013. Preferential binding to Elk-1 by SLE-associated IL10 risk allele upregulates IL10 expression. *PLoS. Genet.* 9: e1003870.

178. Shin, H. D., C. Winkler, J. C. Stephens, J. Bream, H. Young, J. J. Goedert, T. R. O'Brien, D. Vlahov, S. Buchbinder, J. Giorgi, C. Rinaldo, S. Donfield, A. Willoughby, S. J. O'Brien, and M. W. Smith. 2000. Genetic restriction of HIV-1 pathogenesis to AIDS by promoter alleles of IL10. *Proc. Natl. Acad. Sci. U. S. A* 97: 14467-14472.

179. Collins, P. L., M. A. Henderson, and T. M. Aune. 2012. Diverse functions of distal regulatory elements at the IFNG locus. *J. Immunol.* 188: 1726-1733.

180. Sur, I. K. 2012. Mice lacking a Myc enhancer that includes human SNP rs6983267 are resistant to intestinal tumors.

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PERSONAL DATA

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EDUCATION AND TRAINING

2009-present Ph.D. Candidate (Expected graduation date February 2015), Department of Molecular Microbiology and Immunology Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

2007-2009 Master of Science, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

2007-2009 Certificate in Vaccine Science and Policy, Department of International Health, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

1997-2004 Doctorate in Pharmacy (Pharm.D), Department of Pharmaceutical sciences School of Medicine and Pharmacy, University of Bamako, Mali

PROFESSIONAL EXPERIENCE

Graduate research assistant (PhD): August 2009-present, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health

Responsibilities: Conducted independent graduate level research, trained junior graduate students and technicians (6 PhD students, 2 master students, 3 technicians), maintained inventory of lab supplies and reagents for ordering, and managed a large colony of transgenic and knock-out mice.

Primary research projects

- **Impact of genetic variation on human *IL10* gene expression and disease susceptibility**
  Designed and generated a humanized transgenic mouse model to determine if non-coding SNPs in the *IL10* locus affect IL-10 expression and subsequently disease outcomes. Results indicate that non-coding SNPs in the *IL10* locus control human IL-10 expression in $CD4^+$ T cells. Specifically, allele-specific human IL-10 producing capacity in T_H1 cells was sufficient to reverse susceptibility to persistent infection.

- **Cell-type-specific regulatory control of IL-10 and IL-24 expression in innate immune cells**
  Characterized IL-10 and IL-24 expression patterns in murine NK cells and macrophages to determine if the two cytokines share regulatory features (such as common signal transduction pathways and transcription factors requirements). Results demonstrated that IL-10 and IL-24 are co-expressed but regulated by distinct pathways in NK cells and macrophages.

Graduate research assistant (Master): August 2007-May 2009, Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health

Primary research projects

- **Evaluation of high-sensitivity multiplex cytokine platform performance characteristics and potential impact on human immune monitoring studies**
  - Developed and validated a novel multiplex assay to simultaneously measure cytokines in human biological fluids in collaboration with Meso Scale Discovery (MSD), Gaithersburg, MD.
  - Compared performance parameters of the MSD cytokine multiplex assay with commercially available Cytometric Bead Array in term of sensitivity, recovery and precision in healthy and diseased donors.
- **Association of circulating inflammatory biomarkers with disease progression**
  - Profiled serum cytokines as biomarkers of disease progression in infectious diseases such as HIV/AIDS.

Associate Scientist: (October 2003 – August 2007); HIV/TB Research and Training Center, the SEREFO project, International Center for Excellence in Research (ICER/NIAID/NIH), University of Bamako, Mali

Responsibilities: Involved in research projects focused on studying the pathogenesis of Tuberculosis in HIV positive and negative patients from Mali

Primary research projects

- **"CD4+ T Cell Immune Responses to Mycobacterium Tuberculosis"**
- **"Correlation of the Precursor Frequency of CD4+ Effector Memory T cells with Induration measured in the Tuberculin Skin Test (TST)"**
- **"Establishment of Normal Parameters for Blood and Sputum with Samples Obtained From Volunteers in Bamako"**

In all three projects, I conducted the following duties:

- Performed laboratory assays such as routine immunophenotyping, intracellular cytokine staining, T cell proliferation assay, Isolation and storage of human PBMCs, HIV serological testing and viral load testing
- Analyzed data generated from immunological and virological tests
- Trained juniors scientists and technicians
- Coordinated weekly journal club and research seminars
- Edited manuscripts submitted for publications

Research Assistant: (June 2002 – July 2003); Applied Molecular Biology Laboratory, School of Science and Technologies, University of Bamako, Mali

Responsibilities: Conducted doctoral level research in the field of HIV vaccinology, supervised assays performed in the immunology section of the laboratory, and trained Pharm.D candidate, Bachelor students and technicians to run assays and analyze data.

Primary research project:

- **Evaluation of the immunogenicity of HIV-1 epitopes for the development of a "universal HIV vaccine"**
  Employed ELISPOT assay to test the secretion of IFN-γ by CD4+ and CD8+ T cells from PBMCs stimulated with HIV epitopes. Results indicate that epitopes derived from core proteins (i.e. Env and Gag) are immunogenic and could be used to design a therapeutic vaccine to control HIV disease progression.

HONORS AND AWARDS

- American Association of Immunologists (AAI) Trainee Abstract Award (2014)
- Student Conference Fund Recipient, Johns Hopkins Bloomberg School of Public Health (2013)
- Eleanor Bliss Honorary Fellowship, Johns Hopkins Bloomberg School of Public Health (2010)
- Master Tuition Scholarship, Johns Hopkins Bloomberg School of Public Health (2008)
- NIH/NIAID Intramural Scholarship for Pre-doctoral Studies (2007-2015)

TEACHING EXPERIENCE

Biological Basis of Vaccine Development: (2009 – 2013) – Teaching assistant, course ID: 223.689.01, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD, USA

Responsibilities: tutored students, proctored and graded exams, managed the course web site, and provided administrative assistance to principal instructors

Public Health Perspectives on Research: (2010 – 2013) – Teaching assistant, course ID: 550.865.81, Johns Hopkins Bloomberg School of Public Health,
Baltimore, MD, USA

Responsibilities: graded exams, managed the course web site, organized group discussions, and provided administrative assistance to principal instructors

PUBLICATIONS

Bhavsar NA, Bream JH, Meeker AK, Drake CG, Peskoe SB, **Dabitao D**, De Marzo AM, Isaac WB, Platz EA. 2014. A Peripheral TH1 profile is inversely associated with Prostate Cancer risk in CLUE II . Cancer Epidemiol Biomarkers Prev. PMID: 25150281

Dabitao D, Margolick JB, Lopez J, and Bream JH. 2011**.** Multiplex measurement of proinflammatory cytokines in human serum: comparison of the Meso Scale Discovery electrochemiluminescence assay and the Cytometric Bead array. Journal of Immunological Methods. PMID: 21781970

Hedrich CM, Ramakrishnan A, **Dabitao D**, Wang F, Ranatunga D, Bream JH. 2010. Dynamic DNA methylation patterns across the mouse and human IL-10 genes during CD4 (+) T cell activation; influence of IL-27. Mol Immunol. PMID: 20952070

Redd AD, **Dabitao D**, Bream JH, et al. 2009. Microbial Translocation, the Innate Cytokine Response, and HIV-1 Disease Progression in Africa. Proc Natl Acad Sci USA. PMID: 19357303

Imamichi H, Koita OA, **Dabitao D**, et al. 2009. Identification and characterization of CRF02_AG, CRF06_cpx and CRF09_cpx recombinant subtypes in Mali, West Africa. AIDS and Human Retrovirus Journal. PMID:19182920

Diallo S, Toloba Y, Coulibaly SA, **Dabitao D**, Diop S, et al. 2008. Male Circumcision and HIV in the Malian Military. Mali Med. PMID:19437815

Koita OA, **Dabitao D**, Mahamadou I, Tall M, et al. 2006. Confirmation of immunogenic consensus sequence HIV-1 T-cell epitopes in Bamako, Mali and Providence, Rhode Island. Human Vaccine. PMID: 17012903

Manuscript in preparation

Dabitao-Keita D, Hedrich CM, Huska J, Wang F, Anderson SK, Bream JH. Cell-type-specific regulatory control of IL-24 and IL-10 expression in innate immune cells. (In Preparation)

Dabitao-Keita D, Feigenbaum L, Stäger S, Bream JH. A personalized genomics approach to study the impact of allele-specific human IL-10 expression on disease susceptibility. (In Preparation)

Dabitao-Keita D, Margolick JB, Bream JH. High-sensitivity multiplex cytokine analysis as a tool to monitor human immune responses. Invited Review. (In Preparation)

PRESENTATIONS

Oral presentation

1. IL-10 and IL-24 expression is regulated by distinct pathways in macrophages and NK cells,
   Immunology 2014, Annual meeting of the American Association of Immunologists (AAI), Pittsburgh, PA, 2014

2. A personalized genomics approach to study the impact of allele-specific human IL-10 expression on disease susceptibility, HIV/TB Research and Training Center, the SEREFO project, International Center for Excellence in Research (ICER/NIAID/NIH), University of Bamako, Bamako, Mali, 2012

3. Repopulation of lymph node-homing CD4 T cell subsets after suppression of HIV-1 Viremia, 6th Scientific Annual Research Meeting, Accra, Ghana, 2006.

4. Immunogenic T cell epitopes for a global HIV-1 Vaccine: 6th International Congress of the Federation of African Immunological Societies (FAIS), Dakar, Senegal, 2006

Poster Presentation

1. IL-10 and IL-24 expression is regulated by distinct pathways in macrophages and NK cells, Immunology 2014, Annual meeting of the American Association of Immunologists (AAI), Pittsburgh, PA, 2014

2. Performance evaluation of two multiplex technologies for the measurement of serum cytokines in HIV-infected individuals, 96th AAI Annual Immunology Meeting, Baltimore, MD, 2010.

3. Reconstitution of immune responses occurs very rapidly after initiation of therapy for tuberculosis, 94th AAI Annual Immunology Meeting, Miami, FL, 2007.

Conference Participation

1. 7th Annual Vaccine Renaissance Conference, Providence, RI 02903, 2013
2. High Throughput Biology (HiT) Center Symposium on Human System biology, Baltimore, MD 21205, 2012

PROFESSIONAL DEVELOPMENT

Laboratory Skills

- ELISA
- Various multiplex cytokine assay systems
- ELISPOT
- Flow cytometry
- Chromatin Immunoprecipitation (ChIP)
- Molecular cloning
- Plasmid and bacterial artificial chromosome (BAC) isolation/manipulation
- PCR and RT-qPCR
- DNA and RNA extraction
- Microscopy
- Cell transfection
- Tissue culture (including isolation and differentiation of primary mouse and human cells)
- Bacterial culture
- Viral load quantification
- *In vivo* animal models (including transgenic systems)

- Mouse colony management (including mouse husbandry, breeding, backcrossing, and genotyping)

Software Proficiency: FlowJo, Clone Manager, Primer Express, VISTA Genome Browser, Integrative Genomics Viewer (IGV), STATA and GraphPad Prism

Languages: French, English, and Bambara **(fluent in all)**

MEMBERSHIP IN PROFESSIONAL SOCIETIES

- American Association for the Advancement of Science (2012 – present)
- American Society for Microbiology (2008 – present)
- American Association of Immunologists (2007 – present)

RESEARCH INTERESTS

- Vaccines and therapeutics development and implementation in developing countries
- Immunology of infectious diseases
- Biomarkers of diseases
- Impact of allelic variation on disease susceptibility and response to therapies
- Development and evaluation of immunological methods

REFERENCES

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