Transcriptional Analysis of T_H2 Primed Dendritic Cells and T cells

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Abstract

Allergy is a condition affecting between 10 and 30% of the world's population, with incidence rising every year. It is primarily mediated by T_{Helper} (T_H) 0 cells reacting to an ordinarily harmless environmental antigen to induce an adaptive T_H2 response. T_H0 cells are presented the antigen by dendritic cells (DC), the immune systems most proficient antigen presenting cell, which act as the bridge between the innate and adaptive immune system. Dendritic cells specific to this study termed Triple Negative (TN) and CD11b+ are able to prime T cells to become T_H2 cells, but current research has been unable to fully determine the proteins that mediate this T_H2 priming. TN and CD11b+ DC exhibit transcriptional and functional distinction within the T_H2 response, but the individual functions they take on during T_H2 responses have not fully been determined. Some evidence suggests that the cell surface protein OX40L and the secreted protein TSLP are capable of inducing T_H2 priming, but this is not conserved across all T_H2 models. In an effort to determine other specific proteins that induce T_H2 priming, RNA-sequencing has been utilized on TN and CD11b+ dendritic cells in T_H2 inducing conditions. This thesis aims to analyse RNA-sequencing data generated from purified T_H2 antigen positive TN and CD11b+ dendritic cells that have taken up a T_H2-inducing stimulus – fluorescently labelled (AF488) non-viable *Nippostrongylus brasiliensis*. Due to the majority of DC-T_H0 interactions occurring at the cell surface interface, the bioinformatic analysis was focused on genes belonging to the surface and secreted compartments.

Here I show that AF488-*Nippostrongylus brasiliensis* positive TN and CD11b+ DC are transcriptionally distinct from each other. Functional roles of differentially expressed genes (DEG) were also markedly distinct. Superfamily analysis revealed TN genes associated with signal transduction and proteases, whereas CD11b+ DEG were linked to cell adhesion and immune responses. This suggests that the different DC subsets have different roles in an immune response, and potentially different roles in the induction of T_H2 immune responses. Network analysis of DEG from DC subsets and proteins expressed by T_H0 and T_H2 cell surfaces identified over 300 predicted interactions. Notably, 33 identified were known interactions – validating the bioinformatic methods used. Finally, I have been developing a method to assess

novel interactions via flow cytometry methods that allows detection of binding and identification of the cell population that is bound. This has shown promise with the detection of generated proteins bound to TN and CD11b+ DC during T_H2 stimulating conditions, paving the way for future novel interaction analyses.

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Abbreviations

AIM	Absent in melanoma
aLN	Auricular lymph node
APC	Antigen presenting cells
BATF3	Basic leucine zipper transcription factor ATF-like 3
BCL6	B cell lymphoma 6
cAMP	Cyclic adenosine monophosphate
CC	C-C chemokine
CCL	CC ligand
CCR	CC receptor
cDC	Conventional dendritic cells
cDNA	Coding DNA
CDP	Common dendritic cell progenitor
CLR	C-type lectin-like receptor
cSMAC	Central supramolecular activation cluster
CTLA4	Cytotoxic lymphocyte associated protein 4
CXCR	C-X-C chemokine receptor
DAG	Diacylglycerol
DAMPs	Damage associated molecular patterns
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DC	Dendritic cells
FMO	Fluorescence minus one
FOXP3	Forkhead box P3
GATA3	GATA binding protein 3
HDM	House dust mite
HEV	High endothelial venules
ICOS	Interaction of inducible costimulatory
ID2	Inhibitor of DNA binding 2
IDE	Integrated development environment

IFN	Interferon
Ig	Immunoglobulin
I_KB	Inhibitor of $_KB$
IL	Interleukins
IL1rap	IL1 receptor accessory protein
ImmGen	Immunological Genome project
IP3	Inositol triphosphate
IRF	Interferon regulatory factor
IS	Immunological synapse
ITAM	Immunoreceptor tyrosine-based activation motif
iT_{reg}	inducible T regulatory
KLF4	Kruppel-like factor 4
Lck	Leukocyte-specific tyrosine kinase
LTA	Lipotechoic acid
Ltβ	Lymphotoxin beta
LPS	Lipopolysaccharide
MDP	Macrophage and dendritic cell precursor
MHC	Major histocompatibility complex
Ms	*Mycobacterium smegmatis*
Nb	*Nippostrongylus brasiliensis*
NETs	Neutrophil extracellular traps
NFAT	Nuclear factor of activate T cells
NF_KB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NILF3	Nuclear factor interleukin 3 regulated
NOD	Nucleotide-binding and oligomerization domain
OD	Optical density
OX40L	OX40 ligand
PAMPs	Pathogen associated molecular patterns
PBS	Phosphate buffered solution
pDC	Plasmacytoid dendritic cells

PI3K	Phosphatidylinositol-3 kinase
PIP_2	Phosatidylinositol diphosphate
PKCΘ	Protein kinase-C theta
PLCγ	Phosholipase-C gamma
PPIs	Protein-protein interaction networks
pSMAC	Peripheral supramolecular activation cluster
PRR	Pattern recognition receptors
RIG-I	Retinoic acid inducible gene I
RORγT	Retinoic acid receptor related orphan receptor gamma T
SMAD	Mothers against decapentaplegic homolog
STAT	Signal transducer and activation of transcription
Tbet	T box expressed in T cells
T_C	T cytotoxic
TCR	T cell receptor
T_{FH}	T follicular helper
TGF	Transforming growth factor
T_H	T helper
TLR	Toll-like receptor
TN	Triple Negative
TNF	Tumour necrosis factor
TSLP	Thymic stromal lymphopoietin
Zap70	Zeta-activated protein 70 kDa

1. General Introduction

1.1 Dendritic cells

Dendritic cells (DC) are important antigen presenting cells (APCs) that act as the innate immune systems most proficient APC, cells capable of taking up, processing and presenting antigens to activate T cells – immune cells of the adaptive immune system. DC were first described in 1974 by R. M. Steinman as large cells with long pseudopods that are uniform in length. Their lineage was determined some years after their discovery and are one of the youngest members of the hematopoietic cell lineage. Produced in the bone marrow, the common dendritic cell progenitor (CDP) expresses high levels of CX3CR1, CSF-1R, FLT3, and is dependent on interferon regulatory factor (IRF) 8, Ikaros, and PU.1 transcription factor to differentiate from macrophage and dendritic cell precursor (MDP)[63]. CDP differentiate into plasmacytoid DC (pDC) and pre-DC, which are dependent on FLT3L and PU.1 to differentiate. Pre-DC then further differentiate into conventional DC (cDC) 1 and 2. cDC1 express XCR1 and are dependent on IRF8, basic leucine zipper transcription factor ATF-like 3 (BATF3), nuclear factor interleukin 3 regulated (NFIL3), and inhibitor of DNA-binding 2 (ID2)[20-23]. cDC2 express CD11b and SIRPα, and are dependent on IRF2, IRF4, NOTCH2, kruppel-like factor 4 (KLF4), RELB, and lymphotoxin beta (Ltβ)[25,26,63].

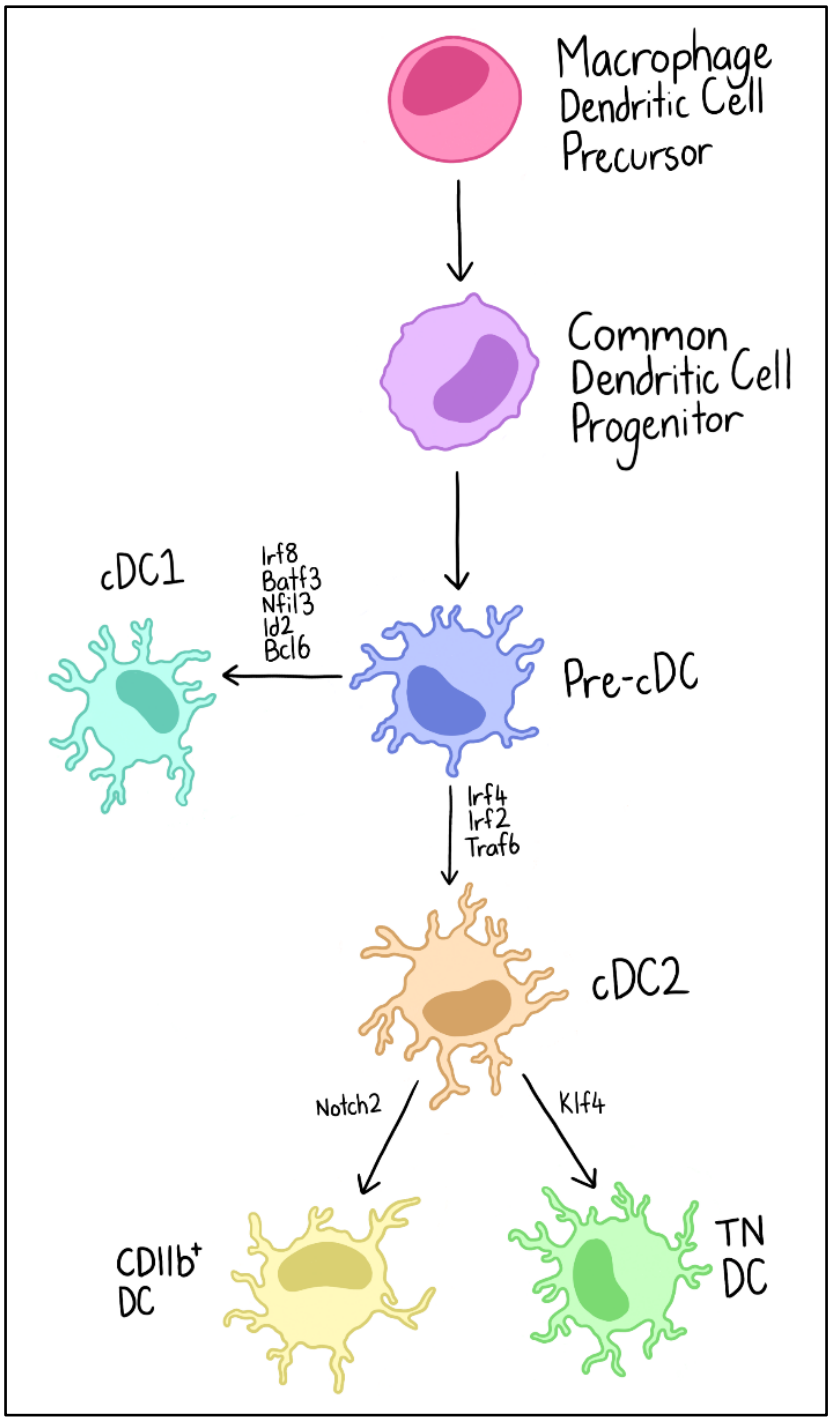


Figure 1.1: Dendritic Cell Lineage Differentiation. Macrophage-Dendritic Cell Precursor cells go through a series of lineage differentiations to produce CD11b^{+} and TN conventional Dendritic Cell 2 cells. Labels by arrows are known transcription factors required for particular cell to cell differentiation[63,162].

DC are a relatively small population of the lymphoid organs, consisting of up to 1.6% of the spleen, 0.5% of the mesenteric lymph nodes, 0.3% of the axillary and cervical lymph nodes, and 0.2% of the peyer's patches. DC were originally only found to express MHC molecules on their surface but have since been found to express many other proteins that pertain to their various functions, such as SIRPα, CD103,

and CD11b. DC functions are primarily to present antigen to other immune cells, typically to cells of the adaptive immune system. It's with these interactions that DC act to shape subsequent immune reactions and tailor them to the specific antigen that DC have presented. Despite the population of DC having a specialised antigen presenting function, they have a large degree of heterogeneity. DC are generally separated into two populations: Migratory DC and Resident DC. Migratory DC are DC found in tissues, typically in barriers of the body such as the skin and the mucosa. They are surveillance cells that are the body's first detection method of microbial invasion. Once migratory DC have taken up pathogens, they rapidly upregulate c-c chemokine (CC) receptor type 7 (CCR7) in order to migrate to the nearest lymph node. CCR7 ligands, CC ligand type 19 (CCL19) and CCL21, are constitutively expressed within the T cell zone of the lymph nodes and enable activated migratory DC to move through the high endothelial venules (HEVs) to the T cell zone[65]. Pathogens can also enter the lymph nodes by disseminating through the lymph vessels. There they can be taken up by resident DC, the second population of DC. Resident DC are stationary within the lymph nodes, and act to survey the circulating lymph fluid. Additionally, resident DC can receive antigen from migratory DC through a poorly understood mechanism[64]. Steady state migratory and resident DC, and those from tissue-localised immunization, differ primarily in their expression of major histocompatibility complex (MHC) II and CD11c. Migratory DC have high expression of MHCII and variable expression of CD11c, with resident DC being the reverse. Migratory and resident DC can be separated into cDC1 and cDC2 which have distinct presentation functions. cDC1 are superior in cross-presenting antigen on MHCI to CD8+ T cells which directly kill virally infected and cancerous cells[139,184], whilst cDC2 are superior in priming on MHCII to CD4+ T_{Helper} (T_H) cells which mediate responses against both intracellular and extracellular pathogens[155].

Every interaction DC have with another immune cell is built around an immunological synapse (IS). ISs are defined as a spatiotemporal, stimulus driven segregation of molecules that participate in immune cell activation. There are multiple forms of IS, including exploratory IS, cytotoxic IS, and perhaps most importantly, activation IS. Activation ISs are formed between DC and T_H cells that are long lived, lasting for hours or days, and highly dynamic. They are divided into two differing portions, the

central supramolecular activation cluster (cSMAC) and the peripheral supramolecular activation cluster (pSMAC). The cSMAC is the section that contains MHC molecules, T cell receptors (TCR), and various costimulatory and coinhibitory molecules[185]. There are various costimulatory molecules, a notable pair of which are CD80 and CD86, partner molecules to CD28 on T cells. The cSMAC is a very stable portion of the IS, as T_H cell activation requires relatively long-lived stimulatory signals. The pSMAC, however, is dynamic, consisting of adhesion molecules such as cadherins and integrins[186]. These molecules act to help to stabilise the IS, but are not exceedingly strong, and are consistently formed and broken over the course of the entire activation IS.

Molecules involved in the activation IS typically cause intracellular signalling cascades, both on the DC side and the T cell side, leading to T cell activation and additional DC activation. The most important signalling molecule on the T cell is the TCR. The TCR colocalises with CD4, found on CD4+ T_H cells, or CD8, found on CD8+ $T_{Cytotoxic}$ (T_C) cells, and CD3, a protein that assists in activation of all T cells[187]. Upon stimulation, this colocalization results in immunoreceptor tyrosine-based activation motif (ITAM) phosphorylation. Several pathways are set into motion after ITAM phosphorylation, but the end result is the activation of the phosphatidylinositol-3 kinase (PI3K) pathway. The PI3K pathway leads to intracellular Ca^{2+} increase and the accumulation of inositol triphosphate (IP_3), which in turn controls the activation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF_KB) and nuclear factor of activated T-cells (NFAT). These nuclear factors are important for the control of T cell activation and differentiation. Research has determined that CD80/CD86 binding to CD28 leads to additional DC activation and cytokine release[188]. However, the effects of other stimulatory molecules on DC are not well understood, leaving a large gap in the effects of T cell priming on DC. As initial DC activation and function is different depending on the pathogen DC detect, this additional activation by costimulatory molecules may be pathogen-class dependant as well, potentially activating DC to prime T cell responses toward that class of pathogen.

1.2 Innate Immune system

DC act as a part of the innate immune system to detect pathogens through the use of germ-line encoded pattern recognition receptors (PRRs), which bind to specific

pathogen-associated molecular patterns (PAMPs). This binding activates the DC, leading to the secretion of various factors including proinflammatory cytokines such as interleukin (IL)-1, IL-6, IL-12, tumour necrosis factor (TNF) alpha, or anti-inflammatory cytokines such as IL-10 and transforming growth factor (TGF) beta. This is essential in the function of the adaptive immune system, as DC secrete these factors and present PAMPs as antigen to activate adaptive immune cells. PRRs are proteins utilized by DC, as well as other innate cells, to recognise PAMPs and DAMPs and are an ancient class of immune recognition, conserved across many distantly related species, including *Drosophila melanogaster* flies and humans. There are five classes of PRRs: Toll-like receptors (TLRs), C-type Lectin-like Receptors (CLRs), Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), and Absent in Melanoma 2 (AIM2)-like receptors (ALRs)[59-62,130]. An important class of PRRs are the TLRs, which were named after the Toll family of proteins, another class of immune proteins, found in *Drosophila melanogaster*. They bind to a wide range of PAMPs and dimerise to activate differing intracellular signalling pathways to cause a variety of immune responses based upon what TLR has bound to its target. A majority of TLRs cause the release of inflammatory molecules through the NF_KB and NFAT pathways and help lead to the induction of all T_H adaptive immunity[129].

PAMPs are molecular structures conserved amongst pathogens as they are essential for the pathogen's survival. There are a wide variety of PAMPs that are recognised, such as components of bacterial and fungal cell walls, single-stranded and double-stranded RNA and DNA that occur outside of the nucleus during viral infections, and bacterial virulence factor components such as flagellin, a protein component of flagella[126,127]. As well as a wide variety of pathogens recognised, the specific type of pathogen can also be distinguished, such as gram-positive vs gram-negative bacteria. Gram-positive bacterial cells walls contain lipoteichoic acid (LTA) which binds to TLR2, whilst gram-negative bacterial cell walls contain lipopolysaccharide (LPS) which binds to TLR4[127]. While downstream intracellular signalling events from both PRRs result in immune activation through Myd88, TLR4 signalling can also activate Myd88-independent pathways to induce production of type-I interferons[127,128]. The second variety of signals typically recognised by DC are damage-associated molecular patterns (DAMPs), which are derived from self-

made molecules. DAMPs are produced by damaged and dying cells, and are typically intracellular molecules that have seeped into the extracellular compartment[58]. They can be detected by all five families of PRRs, and typically cause the induction of inflammatory cytokines to induce localised inflammation. Stressed and dying cells under a state of necrosis have particular morphological changes that allow the change of intracellular molecule locations. This involves the swelling and rupture of organelles, destruction of nucleic acid, and eventually the rupture of the cell itself[133]. This releases proteins, nucleic acids, uric acid salts, and small molecules such as ATP that are taken up by surrounding APCs and activate a wide variety of PRRs such as TLR2, NLRP3, MDA5, CLEC9A, and AIM2[58-62,130-132]. This binding of DAMPs to the PRRs typically induces a NF_KB response, but can also induce a caspase-1 pathway response, both of which induce secretion of IL-1, TNF proteins, and interferons, causing the activation of surrounding immune cells to phagocytose surrounding cell components to prevent continuous inflammation[134,135]. It is the responses caused by PAMPs and DAMPs that enable the innate immune system to shape the actions of the adaptive immune system, led primarily by DC.

1.3 Adaptive immune system

The second arm of the immune system is the adaptive immune system. Adaptive immunity acts after the innate immune system and relies heavily on antigen presentation by DC after the first encounter with the pathogen to act appropriately. A faster adaptive response occurs when the pathogen is encountered again There are two major components of the adaptive immune system, the humoral immune system and the cellular immune system.

1.3.1 T cells

The cellular response is led in large by T_H cells. In the periphery, naïve CD4+ T_H cells are able to interact with DC to recognise the unique antigen that their TCR binds to. CD4 and CD3 colocalise with the TCR, and once the TCR has recognised antigen bound to MHCII, ITAMs become phosphorylated by the protein leukocyte-specific tyrosine kinase (Lck). Phosphorylated ITAMs on CD3 recruit zeta-activated protein 70 kDa (Zap70), which activates phospholipase-C γ (PLCγ). Through PLCγ activation, phosphatidylinositol biphosphate (PIP_2) is cleaved into IP_3 and diacylglycerol (DAG). IP_3 contributes to the release of intracellular Ca^{2+}, leading to

the dephosphorylation and nuclear import of NFAT. DAG leads to the activation of protein kinase-C θ (PKCθ), which subsequently leads to the phosphorylation and degradation of inhibitor of κB (IκB), allowing the transport of NFκB into the nucleus. NFAT and NFκB are the main nuclear factors that are involved in T cell activation[77].

Once activated, naïve CD4+ T_H cells can differentiate into at least 5 main effector lineages depending on the antigen and the microenvironment produced by DCs. These lineages are termed T_H1, T_H2, T_H17, T follicular helper ($_{FH}$), and inducible T regulatory (iT_{reg}) cells depending on the phenotype they exhibit. The T_H1 lineage is dependent on the presence of interferon (IFN) γ and IL-12 and are controlled by the nuclear factor T-box expressed in T cells (Tbet) via signal transducer and activation of transcription (STAT) 1 and STAT4. Th1 cells differentiate in response to intracellular pathogens and induce the activation of macrophages and cause the production of opsonising antibodies via the secretion of IFN-γ and TNFβ[54,78,82]. This enables the efficient coating, engulfment, and destruction of the pathogen by macrophages before it can invade cells. T_H17 lineage differentiation requires IL-6 and TGFβ and is under the control of retinoic acid receptor related orphan receptor gamma t (RORγt) via STAT3[79]. T_H17 cell differentiation is induced by the presence of extracellular pathogens such as fungi, and secrete IL-17, IL-21, and IL-22 to promote neutrophil differentiation and chemotaxis[80,81]. Neutrophils are able to control and destroy extracellular pathogens through the expulsion of neutrophil extracellular traps (NETs) that contain anti-microbials, phagocytosis of the pathogen, and degranulation of numerous granules containing a plethora of toxic proteins[83]. T_{FH} cells develop in the lymph nodes T cell zone and act to help B cells undergo affinity maturation. T_{FH} development begins with the interaction of inducible costimulator (ICOS) with ICOS ligand. This interaction is crucial for the early induction of B cell lymphoma 6 (Bcl6) to upregulate C-X-C chemokine receptor type 5 (CXCR5)[84]. CXCR5 allows T_{FH} to migrate into the lymph node follicles to interact with B cells presenting antigen, and secrete IL-4 and IL-21 to promote affinity maturation[84,85]. iT_{reg} cells differentiate when naïve T_H cells are stimulated via antigen presentation in the presence of TGF-β and IL-2. This activates the gene regulator forkhead box P3 (FOXP3) via STAT5 and mothers against decapentaplegic homolog (SMAD) 2 and SMAD3[86]. The antigen presented is generally self-antigen or antigen that is constitutively present in the body such as commensal bacteria. The

role of iT_{reg} is to dampen immune responses through methods such as secretion of TGF-β and preventing DC maturation via cytotoxic lymphocyte associated protein 4 (CTLA4)[87].

1.3.2 Th2 responses

T_H2 responses are another T cell lineage that can develop, primarily in response to helminth infection, and focus on the destruction and expulsion of such parasites. T_H2 cells are known to require IL-4, but other cytokines have been associated with T_H2 differentiation, such as thymic stromal lymphopoietin (TSLP) in allergen-induced T_H2 responses, and type I interferons[5,17,88]. T_H2 responses are unusual compared to other T_H responses, given the lack of a well-defined initiation condition. The source of which the required IL-4 is produced from is unclear, and whilst TSLP can induce T_H2 differentiation in response to allergens, some helminthic infections can bypass the need for TSLP[46]. The major intracellular pathways of IL-4 signalling have been identified, with IL-4 receptor stimulation leading to the increase of STAT6 within the cell nucleus. This ultimately leads to the induction of the transcription factor GATA binding protein 3 (GATA3)[89]. GATA3 induction leads to the production of the main cytokines of T_H2 responses: IL-4, IL-5, and IL-13. IL-4, despite acting as a major initiation cytokine, also acts downstream of T_H2 cell initiation to induce B cell affinity maturation. This affinity maturation causes B cells to produce IgE and IgG_1, which are the major immunoglobulins (Igs) involved in extracellular parasite destruction. IgE binds to its high affinity receptor $Fc_\varepsilon RI$ expressed by basophils and mast cells[152]. When antigen binds to IgE on $Fc_\varepsilon RI$, the receptors become cross-linked and stimulate the degranulation of these cells, releasing numerous toxic compounds such as histamine. IgG_1 has been shown to bind to $Fc_\gamma RII$ expressed by eosinophils and enhances eosinophil survival and activation[90]. IL-5 is a cytokine that is primarily involved in eosinophil maturation and migration from the bone marrow. Eosinophils themselves are a highly important cell in T_H2 responses. They selectively migrate to infected tissue and become stimulated to release toxic compounds from granules such as major basic protein and hydrogen peroxide[90]. IL-13, the last major cytokine of T_H2 responses, acts on smooth muscle and goblet cells[91]. By signalling through IL-13 receptor on smooth muscles, IL-13 modifies the contractility of such muscles to contract at higher frequencies. This contributes to intestinal helminth expulsion, but also is attributed to airway hyperreactivity in asthma[92]. IL-13 also acts on goblet

cells, which are the cells that produce mucus in the body. Acting through the IL-13 receptor and subsequently STAT6, IL-13 induces goblet cell metaplasia, causing the overproduction of mucus[93,94]. This mucus production is proposed to help with the entrapment and expulsion of helminths.

1.3.3 Dendritic cell subtypes in T_H2 responses

After their discovery, DC were viewed as a cell type capable of universal antigen presentation and had been shown to present antigen to CD4+ T cells to induce their activation[153,154]. However, as subpopulations of DC were discovered using a growing range of antibodies, it was revealed that these subpopulations were specialised towards presentation of specific antigen[155]. Indeed, there are currently a multitude of DC subpopulations described with particular antigen presentation niches[3,13]. cDC2 are a subpopulation of DC that are highly heterogenous, with markers such as CD11b, CD301b, CD206 and PDL2 having varying expression across the population[16,25,45,156,157]. As research teased apart the various adjuvant that induced DC subpopulations to preferentially present innocuous antigen, it was revealed that CD301b$^+$ cDC2 were important in the induction of T_H2 responses[16]. Additionally, genetic ablation of IRF4 from CD11b$^+$ DC revealed these IRF4$^+$CD11b$^+$ DC to be critical for T_H2 responses.[25] There are two subpopulations of cDC2 that are particularly important in T_H2 responses[17,18]. These DC are found as part of the skin immune population and are termed CD11b$^+$ DC and triple negative (TN) DC. CD11b$^+$ DC are defined as CD301b$^+$ PDL2$^+$ CD326$^-$ CD103$^-$ CD11b^{high}, TN DC are CD301b$^-$ PDL2^{var} CD326$^-$ CD103$^-$ CD11b^{low}. Both of these cell types can be activated by *Nippostrongylus brasiliensis* (Nb), a potent T_H2 inducing helminth, and DBP-FITC, which induces contact hypersensitivity. It is known that they are required for T_H2 responses as removing TN via a CD11c-Flox KLF4-Cre system results in reduced, but not ablated, T_H2 responses[26]. These cells upregulate a type I interferon signature after Nb, which was important in the response as anti-IFNAR antibody prevented particular activation molecule upregulation such as Ly6A/E and PDL1[17]. Additionally, treatment of anti-IFNAR antibody reduced the subsequent T_H2 response after Nb immunization, showing a role for type I interferons in T_H2 responses[17], which matched trends in previous research as type I interferons had been implied in the induction of T_H2 responses, demonstrated using *Ifnar1*$^{-/-}$ mice[88].

The full extent of CD11b^{+} and TN DC in T_H2 responses is far from fully understood, but they are required for fully functional T_H2 responses alongside type I interferons.

1.4 Helminth vs. Allergen

Even with all the mechanisms the immune system has to prevent infection of the body, it can be inappropriately activated in the absence of an infection. Allergy is a common example of this as it is a reaction of the immune system toward innocuous proteins in the environment and there is a rising prevalence of allergy in western civilization[95], with little evidence to definitively show the cause. In areas with high helminthic burden, individuals show a reduction in the prevalence of allergy, despite having high T_H2 cytokine markers, showing an inverse correlation between helminth infection and allergy[96]. Regardless of this inverse correlation, both helminth and allergen induce the same type of immune response, being a T_H2 response.

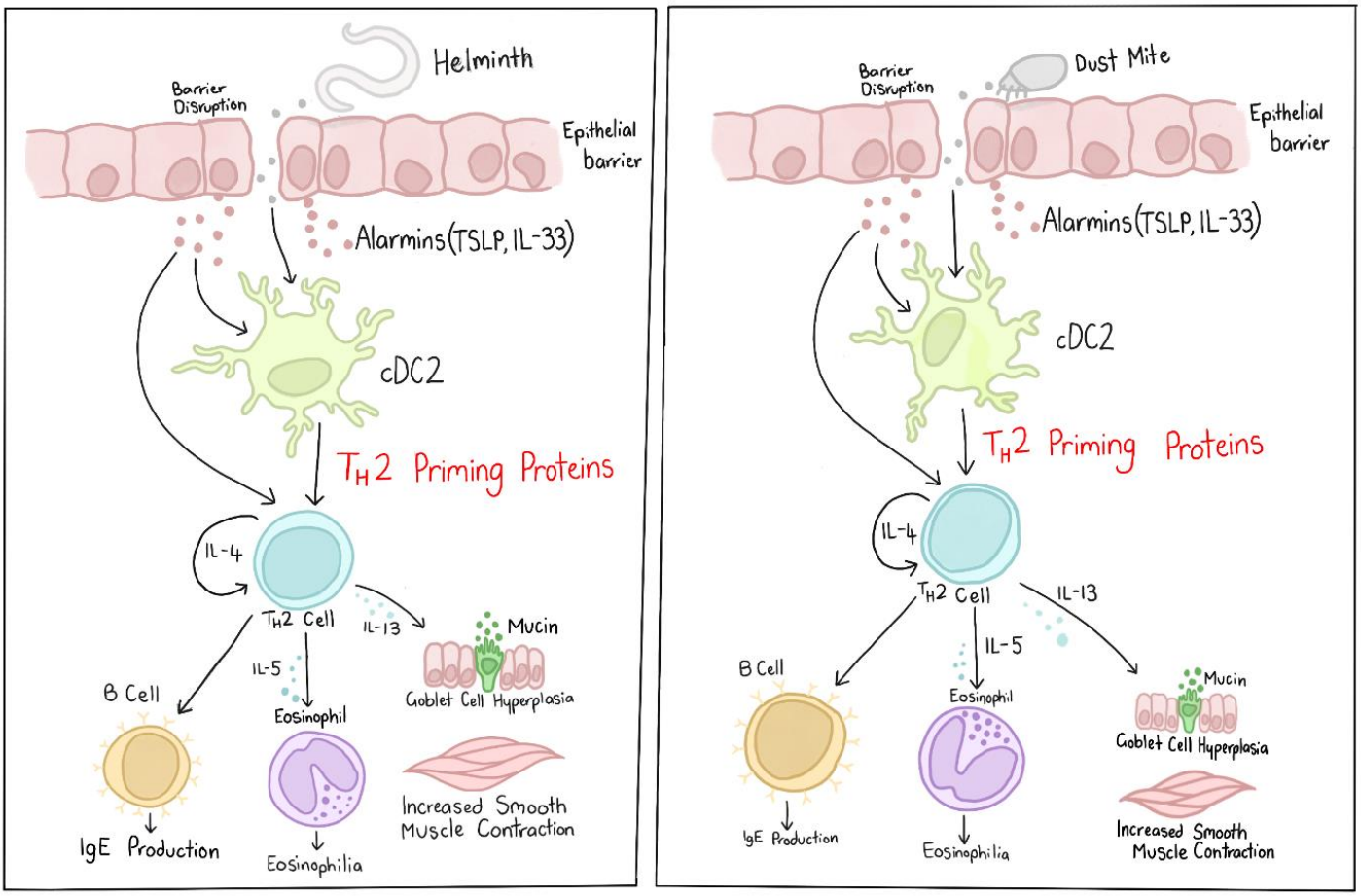


Figure 1.2: Helminths and allergens are thought to cause similar immune responses via DC mediated T_H2 priming. As the allergen or helminth breaks the epithelial barrier, alarmins are released which act on cDC2 and T cells. cDC2 also take up the antigen and present it to T cells to induce T_H2 differentiation. T_H2 cells then release soluble factors such as IL-4, IL-5, and IL-13 to cause downstream effects.

Allergens appear to mimic various helminth proteins in their structure and chemical properties, with many being proteases that induce local barrier damage. This damage causes the release of various cytokines called alarmins, which can induce T_H2 responses in some instances[47,48,113]. The key distinction between allergen and helminth is the longevity in the body. Helminths are often parasites that are relatively long-lived within the body and require the host for their lifecycle. In order to complete this long lifecycle, helminths often modulate the host immune system through a poorly understood mechanism, so that a chronic infection dampens the immune reaction towards the helminth. Some chronic parasitic infections have been shown to rely on the induction of T_{reg} cells, also causing suppression of allergen-induced effector cells[99,100]. Without this immune dampening chronic infection that is becoming exceedingly rare, it is a possibility that the immune system becomes overreactive to substances resembling helminth proteins such as allergens, which are short lived in the body and are unlikely to induce immune tolerance. However, the similarity between helminth and allergen in the response they cause is what enables helminths to be used as a tool in research to investigate the cause of allergy. Helminths such as Nb are potent inducers of T_H2 response, and rarely drive T_H1 or T_h17 responses, as opposed to allergens, which can induce a mixed response. This is an ideal model to illuminate the mechanisms involved in T_H2 immune induction, as it lacks confounding influence of other types of immune responses.

1.5 Dendritic Cell Dependent and Independent Pathways in Allergy

In allergic diseases, there are two general pathways that intercellular signalling can go through to prime naïve cells to become T_H2 cells. The allergens, which frequently have protease activity, act directly on cells to release a host of structurally unrelated cytokines called alarmins. These alarmins can either act in concert with DC to prime T_H2 cells, or can act directly on T cells, eosinophils, basophils, and/or mast cells to induce T_H2 immunity. There are many kinds of alarmins, such as α- and β-defensins that are stored in neutrophils and epithelial cells, respectively[101]. The function of alarmins is to act rapidly in the event of tissue damage and pathogen invasion to activate the immune system[101,102]. There are two alarmins that appear to be specifically involved in the activation of T_H2 responses: TSLP and IL-33.

TSLP is an IL-7 like protein that functions through the high affinity TSLP receptor, a heterodimeric protein consisting of the TSLP receptor, similar to the common cytokine receptor γ chain, and the IL-7 receptor α chain[103,104]. The TSLP receptor is expressed throughout the body, but TSLP itself is released from keratinocytes and other epithelial cells in response to barrier damage[47]. By acting directly on T_H cells in the presence of TCR stimulation, TSLP has been shown to activate CD4+ T cells[107] and specifically induce the upregulation of IL-4, a critical T_H2 cytokine[105,106]. TSLP can also act on differentiated T_H2 cells and drive the increased production of T_H2 molecules[108,109]. IL-33 is an IL-1 like protein that is found throughout the body in the nucleus of endothelial and epithelial cells. It functions through the ST2 receptor, consisting of ST2 and IL-1 receptor accessory protein (IL-1RAP), which is expressed on eosinophils, mast cells, basophils, and T_H2 cells[110,111,112]. By acting through these cells, IL-33 can induce the activation of T cells to differentiate into T_H2 cells through the release of inflammatory mediators and T_H2 cytokines, and amplify an already existing T_H2 response[110,113-115]. However, the specific role for IL-33 in allergy remains unclear as mice deficient in ST2 are still capable of mounting an allergic T_H2 response, presumably due to the presence of TSLP[118].

Despite alarmins being capable of initiating T_H2 responses through non-APCs, they likely act in concert with DC mediated antigen presentation to T_H cells to induce allergic disease. TSLP receptor is expressed by DC and upon binding induces the maturation of DC, seemingly towards a T_H2 priming phenotype[116,117], and although TSLP binds to T_H cells, it requires TCR stimulation to activate them[107], suggesting a role for DC to either initiate the T_H2 response and/or compliment the alarmin-induced activation. Alarmins undeniably have an essential role in allergen-induced T_H2 responses, but their role in parasite driven responses is not as clear. Certain helminth infections can bypass TSLP to directly act on DC to induce T_H2 cells, whereas others cause TSLP signalling to occur[46]. There is evidence to show that IL-33 has a positive effect on helminth elimination and expulsion, but the extent of this effect varies between helminth infections[115,119,120]. DC are clearly required for appropriate T cell responses to occur in the presence of a T_H2 stimulus[14,15,17,18], however there is little understood about the priming event that occurs between the DC and T cell. Studies point to OX40 ligand (OX40L) being a protein with the

capacity to prime DC to induce T_H2 responses[48], but these results are limited to the DC being activated by TSLP. Given that helminth infections do not necessarily require TSLP to induce T_H2 responses, this leaves a gap in the T_H2 priming mechanism that need to be filled in order to further understand how T_H2 responses are induced.

1.6 Data Development

For decades, cell populations have been defined by the proteins that they express during steady state or inflamed conditions. These proteins allow cells to interact with one another to create particular responses, with thousands of proteins being present in the body. Despite DNA being shown to be the genetic material in cells in 1944, the advent of technology that allows the examination of changes in gene expression was only developed in the mid-2000's[121,122]. These genetic changes underlie the various proteins expressed by cells during steady state and different responses, mirroring their phenotype and providing input to their molecular functions.

As the complexity of cells has become further appreciated with the discovery of many sub populations within existing populations, genomic studies are becoming more popular alongside to functional studies to uncover the functions of differing cells. RNA sequencing is one such technique, taking the mRNA present in cells, reverse transcribing it to coding DNA (cDNA), then sequencing cDNA to determine the genes present and the amount of each gene transcript. This provides potential target proteins based upon their transcript amount for further examinations without the need for considerably more difficult surface or intracellular proteomic screens by mass spectrometry. With the advent of RNAseq, the need for databases to store all this new information grew. This led to the development of large, open-access, online databases such as the Database for Annotation, Visualisation and Integrated Discovery (DAVID) in 2003 and the Immunological Genome Project (ImmGen) in 2008[123-125]. This allowed streamlined identification of genes found in particular cell types, as well as gene functional compartments and pathways, opening up many new targets for the biological functional analysis of cells. This method of examining immunological populations was in large led by Miller et al (2012)[15]. Using RNA microarray analysis, the pipeline developed by the ImmGen database, and flow cytometry, this paper clearly and convincingly shows the transcriptional lineage of DC, which had not been shown in full before. This work was a large breakthrough in

showing clearly the different populations of DC, one of which is explored in this thesis (CD11b+ DC) and paved the way for future functional analyses to be performed.

1.6.1 Th2:DC transcriptome

In spite of their ability to intake and present any pathogen, the transcriptomic changes that occur in DC after pathogen recognition and uptake were not extensively described and published until the early 21st century[158-161]. The various transcriptomic libraries developed for DC after pathogen uptake led to the next decade of research into new proteins involved in the development of the various T cell effector states seen under differing pathogen invasion. Even with this range of new targets to explore, T_H2 responses remained unsolved in their priming kinetics. With investigations into various DC present in the skin, a particular subset of DC showed bias toward priming T_H2 responses, and with transcriptomic profiling, led to a more focused T_H2:DC transcriptome library developed[44]. However, T_H2 priming details remained obscure after this discovery, evident from proteins implicated in the induction of T_H2 responses such as TSLP being unable to be applied to all T_H2 inducing scenarios[46]. The dermal T_H2 skewing DC population was separated into CD11b$^+$ and TN DC, with TN DC showing importance in the induction of functional T_H2 responses[18]. Transcriptomic profiling of these DC subpopulations T_H2 inducing stimuli showed that these cells were highly divergent from each other in their transcriptomic profile under the same stimulus[17]. This provided additional T_H2:DC transcriptomic libraries, and showed the divergence of DC involved in the T_H2 response.

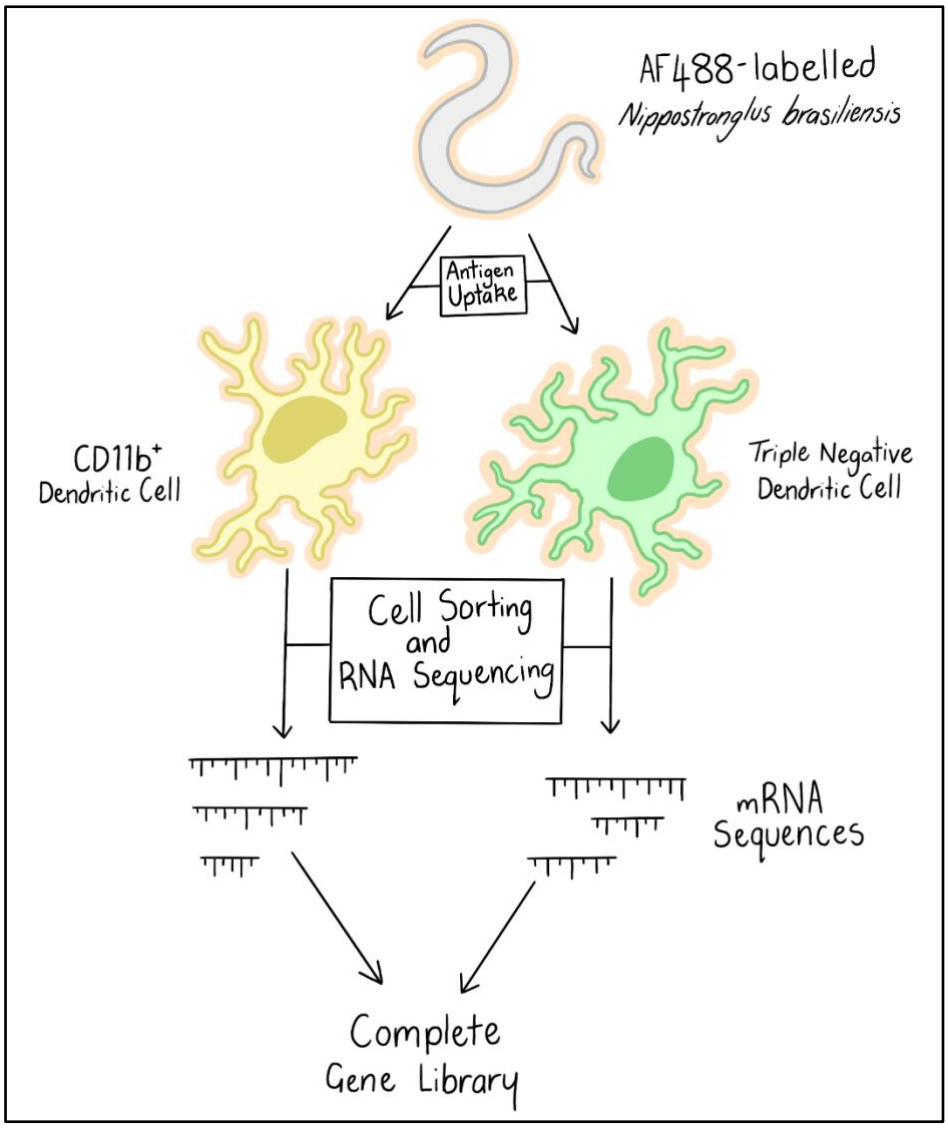


Figure 1.3: Generation of RNA sequencing library from antigen positive DC. AF488-labelled (depicted by an orange halo) *Nippostrogylus brasiliensis* is taken up by $CD11b^+$ and TN DC. $AF488^+$ DC are then sorted and sequenced to gather the mRNA sequences used to build the complete gene library.

1.7 Research Proposal

There are multiple DC lineages with specific roles in immunity, but subpopulations of cDC2 are essential in the initiation of T_H2 responses[14,16-18,25,26]. These T_H2 responses involve the differentiation of T_H2 cells from naïve T cells and develop in response to extracellular parasites and allergens[18,28]. T_H2 responses require IL-4 to develop, and initiate the release of IL-4, IL-5, and IL-13 into the periphery that act on other cells to cause eosinophilia, increased mucus production, and increased smooth muscle contraction[90-93,106]. Despite T_H2 responses are essential in the induction of allergic responses, the exact mechanisms of priming T_H2 cells by dendritic cells is not well understood.

The aim of my thesis is to investigate the transcriptional differences between DC involved in T_H2 responses and identify potential protein interactions between DC and T cells in the context of T_H2 responses. These DC subtypes, termed TN and CD11b+ DC, are known to have distinct transcriptional profiles[17], and we hypothesise that this transcriptional profile extends to the surface and secreted protein compartments, which are essential in signalling between TN/CD11b+ DC and T_H cells to induce T_H2 cell differentiation. Additionally, TN and CD11b+ DC have differing levels of fold change for various genes even when conditioned to the same antigen and being involved in the same response[17]. I hypothesise that TN and CD11b+ DC have different functions in the same response that distinguish them from each other in antigen positive populations. Although many surface and secreted proteins are known for DC and T cells, there are hundreds of genes encoding surface and secreted proteins that are differentially expressed in DC and T cells in T_H2 responses. Many of these proteins have not been examined in the context of T_H2 immunity, so we hypothesise that there are novel protein partners between DC and T cells that could be important in T_H2 cell priming in response to T_H2 inducing antigen.

1.7.1 Hypothesis

In summary, I hypothesise that that TN and CD11b+ DC have distinct functional compartments based upon the differences seen in their transcriptional profiles and fold change of genes. This would cause these DC to exhibit different functions while under the same stimulus context and provide more specific targets for T_H2 priming. The large number of proteins differentially expressed by DC and T cells in T_H2 responses is much larger than what is currently understood. Therefore, I hypothesise

that there are novel protein partners that could be important or essential in T_H2 priming.

1.8 Aims

My thesis aims to characterise the transcriptional difference between TN and CD11b DC, provide a list of potential protein partners between DC and T cells under naïve and T_H2 contexts, and determine the binding profile of selected proteins via flow cytometry.

1. Determine the transcriptional differences between TN and CD11b DC in their surface and secreted protein compartments
2. Determine surface proteins expressed by naïve T cells and T_H2 cells, as well as characterising intracellular pathways associated with those proteins
3. Provide potential protein partners between DC and T cells based upon their distinct protein expression and characterise the binding profile of select proteins.

2. Methods and Materials

2.1 Materials

2.1.1 Laboratory Equipment

Table 2.1 Laboratory Equipment

Product	Catalogue Number	Company
0.1-10µL pipette tips	1036-260	Interlab
0.35mL Insulin needles	326103	Becton-Dickonson
100-1000µL pipette tips	1049-260	Interlab
10-200µL pipette tips	1030-260	Interlab
10mL serological pipette	KJ9102	Interlab
12 well plate	BDAA353043	Bio Strategy
1mL syringe	302100	Becton-Dickonson
25-gauge needles	301805	Becton-Dickonson
25mL serological pipette	KJ9252	Interlab
96 well plate	KJ511-3U10	Interlab
Cell Strainer	144781	Bio Strategy
Custom Plasmids		GeneUniversal
Eppendorf Tube	AXYGMCT-175-C	Bio Strategy
Falcon Tube 15mL	546021	Interlab
Falcon Tube 50mL	KJ326	Interlab
Medium Nitrile gloves	100-252	Interlab
Microcentrifuge tube	500000-N	Interlab

Monarch Plasmid Miniprep kit	T1010L	New England Biolabs

2.1.2 Laboratory Machines

Table 2.2 Laboratory Machines

Supplier/Manufacturer	**Machine**
Benton-Dickinson, San Jose, CA, USA	BD LSRII SORP
Thermo Scientific, New Zealand	ND-1000 Spectrophotometer
Thermo Scientific, New Zealand	Hereaeus Multifuge X3R Centrifuge
Thermo Scientific, New Zealand	Midi 40 CO_2 Incubator
Olympus, Wellington, New Zealand	Olympus CX41 Compound Microscope
Grant Instruments, Cambridge, UK	SUB Aqua 18Plus water bath
Labconco, Missouri, USA	Purifier Biological Safety Cabinet
Marshall Scientific, New Hampshire USA	Sanyo CO_2 MCO-20AIC Incubator

2.1.3 Reagents and Buffers

2.1.3.1 Buffers and Buffer Components

Ampicillin Powder

Irradiated ampicillin powder was purchased from Thermo Fisher Scientific and stored at 4°C (Catalogue Number 11593027). Reconstituted ampicillin was made up with one bottle of ampicillin powder in 20 mL of ddH_2O. This was separated into 1 mL aliquots and stored at -20°C.

BactoAgar

Purchased from Becton-Dickinson (Catalogue Number 214010) and stored at room temperature.

BactoTryptone

Purchased from Becton-Dickinson (Catalogue Number 211705) and stored at room temperature.

Fetal Calf Serum

Manufactured by Invitrogen (New Zealand) and stored at -20°C in 50 mL aliquots.

Glycerol

Purchased from GE Healthcare (Catalogue Number Z191459007) and stored at room temperature.

IMDM

Purchased from Gibco by Life Technologies (Catalogue Number 31980097) in 500 mL bottles and stored at 4°C.

MOPS

Purchased from Sigma Aldrich (Catalogue Number M1254-250G) and stored at room temperature.

Sodium Chloride

Purchased from Sigma Life Science (Catalogue Number 70M027330V) and stored at room temperature.

Potassium Acetate

Purchased from Sigma Life Science (Catalogue Number P1190-500G) and stored at room temperature.

Sodium Azide

Purchased from Sigma Aldrich (Catalogue Number S2002-100G) and stored at room temperature out of light in a secondary container.

Yeast Extract

Purchased from Becton-Dickinson (Catalogue Number 21250) and stored at room temperature.

Ethanoic Acid

Purchased from Sigma Aldrich (Catalogue Number A9967500G) and stored at room temperature in a corrosive's cabinet.

DNase I (Grade II)

Purchased from Sigma Aldrich as a lyophilised powder (Catalogue Number 10104159001). This was made into 100 µL and 200 µL aliquots at 10 mg/mL in IMDM and stored at -20°C.

Dulbecco's Phosphate Buffered Saline

Purchased from Gibco by Life Technologies (Catalogue Number 14190-250) and stored at 4°C.

Ethylenediaminetetraacetic Acid (EDTA)

Purchased from Sigma Aldrich (Catalogue Number EDS-500G) and stored at room temperature. Stock solution was made to 0.5M in 1 L milliQ H_2O and adjusted to pH 8 using sodium hydroxide.

Calcium Chloride

Liberase TL (Research Grade)

Purchased from Sigma Aldrich as lyophilised powder (Catalogue Number 5401020001) and stored at -20°C. This was made into 1 mL aliquots at 1g/mL in IMDM and stored at -20°C.

Dulbecco's Phosphate Buffered Saline Powder

Purchased from Sigma Aldrich in powder form (Catalogue Number D5652-10X1L) and stored at 4°C. 1 packet of DPBS powder in 1 L dH_2O was used to make 1 L bottles of PBS and was adjusted to pH 7.4. DPBS stock was filter-sterilised or autoclaved and stored at 4°C.

Rubidium Chloride

Tetrahydrate Manganese Chloride

Tris

Purchased from Sigma Life Science (Catalogue Number RDD008-2.5KG) and stored at room temperature.

Glycine

Purchased from Sigma Aldrich (Catalogue Number G8898-1KG) and stored at room temperature.

SDS

Purchased from Bio-Rad (Catalogue Number 161-0302) and stored at room temperature.

Methanol

Purchased from Sigma Aldrich (Catalogue Number 322415-2L) and stored in a flammables cabinet.

β-mercaptoethanol

Purchased from Sigma Aldrich (Catalogue Number M6250-100ML) and stored at room temperature in the dark.

Bromophenol Blue

Purchased from Sigma Aldrich (Catalogue Number B5525-5G) and stored at room temperature.

4-20% Mini-PROTEAN TGX Gels

Purchased from Bio-Rad (Catalogue Number 4561096) and stored at 4°C in the dark.

TfbI Buffer

0.588g of potassium acetate, 2.42g of rubidium chloride, 0.294g of dihydrate calcium chloride, 2g of tetrahydrate manganese chloride, and 30 mL of glycerol was added to 200 mL of milliQ H_2O, mixed well, and adjusted to pH 5.8 with dilute acetic acid. This buffer was filtered through a 0.22 µm filter and stored at 4°C.

TfbII Buffer

0.21g of MOPS, 0.121g of rubidium chloride, 1.1g of dihydrate calcium chloride, and 15 mL of glycerol were added to 100 mL of milliQ H_2O, mixed well, and adjusted to pH 6.5 with dilute sodium hydroxide. This buffer was filtered through a 0.22 µm filter and stored at 4°C.

2.1.3.2 Flow Cytometry Buffers and Reagents

Trypan Blue (0.4%)

Purchased from Gibco by Life Technologies (Catalogue Number 15250-061) and stored at room temperature.

UltraComp eBeads™ Compensation Beads

Purchased from Thermo Fisher Scientific (Catalogue Number 01-2222-42) and stored at 4°C.

FACS Buffer

20 mL FBS (2%), 2 mL 5% sodium azide and 4 mL 0.5M EDTA was added to 1 L of PBS. The buffer was mixed well and stored at 4°C.

Rat anti-mouse CD16 (Fc_εRIII)/CD32 (Fc_εRIII) (Clone 2.4G2) (Fc Block)

Fc block was affinity purified at the Malaghan Institute of Medical Research from hybridoma culture supernatants using HI Trap protein G Sepharose Columns and stored at 4°C.

Digestion Buffer

The digestion buffer was used for aLN tissue. It was made by adding 0.1 mg/mL liberase TL and 0.1 mg/mL DNase I to IMDM.

Formalin Solution 10% neutral buffered (contains 4% formaldehyde)

Purchased from Sigma Aldrich (Catalogue Number HT5012-60ml) and stored at room temperature.

Table 2.3 Flow Cytometry Viability Dyes

Specificity	Dilution	Supplier	Catalogue Number
Zombie aqua live dead	1:1000	Mediray	423102

Table 2.4 Flow Cytometry Antibodies

Specificity	Fluorophore	Dilution	Supplier	Clone	Catalogue Number
CD11c	BV786	1:200	BD Biosciences	HL3	563735
CD11b	BUV737	1:200	BD Biosciences	M1/70	564443
I-A/I-E (MHCII)	FITC	1:200	BD Biosciences	K2G9	553623
CD64	PE Cy7	1:500	BioLegend	X54-5/7.1	139314
B220	BV605	1:200	BD Biosciences	RA3-6B2	563708
TCRβ	BV605	1:400	BioLegend	H57-597	109241
Ly6C	AF700	1:200	BioLegend	HK1.4	128024
Sirpα	PE CF594	1:200	BioLegend	P84	144016
CD326	BV711	1:1000	BioLegend	G8.8	118233
CD86	BUV395	1:200	BD Biosciences	GL-1	564199
DYKDDDDK (Flag) Tag	AF647	1:200	BioLegend	L5	637316

2.1.3.5 Mouse Treatment Regime Reagents

DPBS

Purchased from Gibco by Life Technologies (Catalogue Number 14190-144) in 500mL bottles and stored at 4°C.

Anaesthetic

10x stock solution of ketamine/xylazine was provided at 86 mg/mL ketamine and 2.6 mg/mL xylazine and was stored at 4°C covered with tinfoil. Stock solution was diluted with sterile PBS at a 1:10 dilution to a 1x working concentration, also stored at 4°C.

2.1.3.6 Immune System Stimuli

Killed Larval Stage 3 Nb larvae

Nb eggs are grown into larval stage 3 Nb at the Malaghan Institute of Medical Research, washed three times with sterile PBS and three times with an antibiotic buffer, and inactivated by three freeze-thaw cycles.

Killed *Mycobacterium smegmatis* (Ms)

Ms was grown at the Malaghan Institute of Medical Research in LB broth under agitation at 37°C overnight. Bacteria were washed with sterile PBS containing 0.05% Tween, heat-killed for one hour at 75°C, and stored at -70°C.

2.1.4 Mice

Animal Care and Ethics

Mice used in the experiments were housed in the mouse facility in Victoria University of Wellington. Mice were fed autoclaved meat-free rat and mouse food (Specialty Feeds, Western Australia) and acidified autoclaved water in a specific pathogen-free environment with a controlled 12-hour light and 12-hour dark cycle. Food and water were available at all times. The Animal Ethics Committee at Victoria University of Wellington approved all manipulations performed, which followed the Code for Ethical Conduct for the Manipulation of Animals. Mice were sex-matched, and age matched at 6-12 weeks from the start of experiments. Treatment groups were split across cages where possible to try to prevent cage related effects. Experiments fell under the 25790 protocol: Impact of microenvironment on dendritic cell function (approved 14/03/18).

Mouse Strains

C57BL/6J (C57BL/6)

Original breeding pairs were obtained from the Jackson Laboratory (Bar Harbour, ME, USA) and bred in the mouse facility at Victoria University of Wellington.

2.2 Methods

2.2.1 Mouse Manipulations

Mouse manipulations included handling and intraperitoneal injections of anaesthetic. After anaesthetic administration, mice were injected intradermally with 30µL volumes

of dead L3 Nb, inactivated Ms, or phosphate buffered saline (PBS). In some cases, mice were also ear tagged.

Handling

Handling of mice was required to perform intraperitoneal injections of anaesthetic. All injections of anaesthetic were performed by Kaitlin Buick, a research officer within the same lab group as I. Once anaesthetised, mice were laid on their flanks in order to roll the ears over and expose the pinnae for intradermal injection. After injection, mice were returned to their cage, placed side by side to keep warm, and monitored to ensure full recovery from anaesthetic.

Intraperitoneal Injections

Intraperitoneal injections had the needle inserted in the lower left quadrant of the abdomen of the mice. The dosage of anaesthetic was dependant on the weight of the mice in question (100 mg/kg of Ketamine and 3 mg/kg of Xylazine), but generally totalled 200-300 µL per mouse.

Intradermal Injections

After anaesthetic administration, mice were placed on their flank to expose the ear. The ear was rolled back gently on an insulin needle cap to expose the pinnae and stretch the skin. 30 µL volume was carefully injected at a low angle between the layers of skin on both ears. Mice were monitored during and after injection to ensure recovery.

2.2.2 End Point

Euthanasia

Euthanasia was performed by CO_2 asphyxiation and mice were checked to have no reflexes before any further procedures were performed.

2.2.3 Flow Cytometry

Auricular Lymph Node (aLN) Extraction and Processing

After euthanasia, the aLN were extracted from the sides of the neck using the local vasculature as a guide. The aLN were stored on ice in 1 mL of IMDM in a 1.7 mL microcentrifuge tube. To obtain a single cell suspension in experiments examining DC, the LN capsule was broken using two needles in a digestion buffer in a 24-well

plate. This was left for a 25-minute incubation at 37°C. Digestion was stopped with 10 µL of EDTA and left for 5 minutes at 37°C. The aLN were broken further with several resuspensions using a Gilson® pipette. The suspended LN were filtered through a 70 µm cell filter, and the well was washed with 0.5 mL IMDM, resuspended ten times, then filtered. The filter was then washed with 10 mL of IMDM. Samples were centrifuged (250 x g at 4°C for 10 minutes), the supernatant was discarded, and the cells were resuspended in 200 µL of IMDM, ready to transfer into flow assisted cell sorting (FACS) tubes.

Cell Counting

Cell counting was used to calculate the number of cells per aLN. Counting was performed by diluting 10 µL of sample in 90 µL of Trypan Blue stain in 0.7 µL micro centrifuge tubes. After mixing, 10 µL of stained cells was loaded into a haemocytometer. Live cells were counted, with cells touching the left and bottom lines of squares being excluded. If there were too many cells to feasibly count, five squares were counted, and the number was used to average the number of cells across all 25 squares. The concentration of cells was calculated using the equation: concentration (cells/mL) = average number of cells*dilution factor(10)*10^4.

Live Dead Staining and Fc Block

After aLN single cell suspensions were transferred into FACS tubes, the tubes were centrifuged (250 x g at 4°C for 2 minutes) and the supernatant removed for viability staining. Zombie Aqua™ Fixable Viability dye was used at a 1:1000 dilution. Dilution was performed with PBS and 100 µL was added to each sample by resuspending the pellet. After a 30-minute incubation at 4°C, samples were washed with 100 µL of PBS, centrifuged (250 x g at 4°C for 2 minutes), and the supernatant was removed. 100 µL of Fc block (diluted at a 1:300 concentration with FACS buffer) was used to resuspend the cell pellets and left to incubate for 10 minutes at 4°C. After incubation, another wash and centrifuged was performed as described previously.

Fusion Protein Staining

Cell supernatant containing fusion protein is harvested. 300 µL of fusion protein is added to samples and left to incubate at 37°C for 30 minutes. Samples were washed

with 100 µL of FACS buffer, then centrifuged (250 x g at 4°C for 2 minutes) and the supernatant removed. Samples were then washed twice more.

Cell Surface Staining

All antibodies were centrifuged at 17000 RPM at 4°C for one minute before use. The cell surface antibody and fluorescence minus one (FMO) cocktails were made up in FACS buffer according to the dilutions shown in Table 2.4. 50 µL of corresponding cocktail was added to each sample. Samples were then incubated at 4°C for 15 minutes, after which they were washed three times with FACS buffer. Samples were incubated for ten minutes on ice, then washed three times as described previously.

Fixing

In all experiments, cells were fixed then run on the LSRII the following day. Cells were fixed using formalin solution after cell surface staining. 100 µL of formalin solution was added to each sample, then incubated for 20 minutes at 4°C. Samples were then centrifuged (250 x g at 4°C for five minutes), supernatant was removed, then pellets were resuspended in 250 µL of FACS buffer. Samples were covered in parafilm and stored at 4°C overnight.

Compensation Controls

Single stain compensation controls were required to set up the flow cytometers. All markers except for MHCII and live/dead were made using Ultracomp eBeads™. Each stain had half a drop of Ultracomp eBeads™ in 100 µL of FACS buffer and 1 µL of the corresponding antibody was added. Controls were then incubated for ten minutes at room temperature in the dark. Following incubation, beads were washed with 1 mL of FACS buffer, then centrifuged (250 x g at 4°C for five minutes). Supernatant was removed, beads were resuspended in 250 mL of FACS buffer, and then stored at 4°C. Cells were used for MHCII and live/dead compensation controls.

Flow Cytometry

Before samples were run on the flow cytometers, a CST was done by the Hugh Green Cytometry Centre staff at the Malaghan Institute of Medical Research to check cytometer performance and to calculate optimal voltages and laser delays for the day.

Before a sample was run through the cytometer, it was filtered through a 70 µm filter and vortexed. As the samples were running, the electronic abort rate was monitored to ensure less than 5% of the sample was lost, and flow rate was changed when needed. Samples were run until the entire sample was collected.

LSRII – BD LSRII SORP (Becton Dickinson, San Jose, CA)

The unstained sample was run and voltages were set so that peak fluorescence was at 10^2. A fully stained sample was then used to ensure that positive peaks were on scale. Single stained controls were then recorded, and positive and negative peaks were determined. Fully stained samples and FMOs were then recorded.

2.2.4 Gene Selection

A list of genes that were upregulated in TN and $CD11b^+$ DC when exposed to Nb antigen was acquired from RNA sequencing. These genes were organised from highest differential expression to lowest, with the cut off being a 2-log fold increase or decrease in expression. These were then copied into the online resource DAVID, which grouped the genes based on their function. The genes encoding for surface and secreted proteins were taken, and genes were then examined for their amino acid sequence. Surface genes required a singular extracellular domain labelled, as this would be the point of contact between cells. Secreted genes required the signal peptide to be labelled, as this is not in the protein in the extracellular compartment and may disrupt protein folding if left in. Surface genes without a singular extracellular domain labelled and secreted genes without the signal peptide labelled were separated out, as they would not be able to be used in plasmids. The same process was done for a list of genes collected from naïve T cells and T cells during a T_H2 response, except only surface genes were selected.

2.2.5 Bacteria and Plasmids

Bacterial Transformation

1 µL of plasmid containing the gene of interest and an ampicillin resistance gene was added to 100 µL of transformation competent DH5-α *E. coli*. The bacteria were left on ice for five minutes, then heat shocked at 42°C for 40 seconds. 1 mL of LB broth was added, and bacteria were then placed on a heated rocker for one hour at 37°C. 0.1 µL of bacteria were plated on LB agar with ampicillin (0.1 mg/mL) plates and left in a Gallenkamp incubator at 37°C overnight. Colonies that had grown the next day

contained the plasmid. One colony was collected and placed in 5 mL of LB broth/ampicillin (0.1 mg/mL) for 16 hours at 37°C.

Competent Bacteria Generation

DH5α *E. coli* were streaked onto a LB agar plate that contained no ampicillin and left to grow overnight at 37°C. A lone colony that had grown was collected and placed in LB broth without ampicillin overnight at 37°C. 1 mL of overnight culture was into 100 mL LB broth with no ampicillin and incubated at 37°C. The optical density (OD) was checked using a spectrophotometer until OD 0.9 was reached. The culture was centrifuged (2380 x g at 4°C for 20 minutes) and supernatant was removed. The pellet was resuspended in 40 mL of ice cold TfbI buffer and incubated on ice for one hour. Bacteria were centrifuged (2380 x g at 4°C for 20 minutes) and supernatant was removed. The pellet was resuspended in 3 mL of ice cold TfbII. This was separated into 100 µL aliquots and stored at -80°C.

Miniprep Plasmid Harvest

All minipreps were performed using the New England Biolabs® Inc Monarch® Plasmid Miniprep Kit. Bacteria that had grown for 16 hours were centrifuged (3500 x g at 4°C for ten minutes), and the supernatant was tipped off. 200 µL of Resuspension Buffer B1 was added and used to resuspend the pellet, and the suspension was transferred to a microcentrifuge tube. 200 µL of Cell Lysis Buffer B2 was added, and the tube gently inverted six times. 400 µL of RNAse Buffer B3 was added, and the tube gently inverted until the suspension was fully neutralised (when suspension was a clear yellow colour with white precipitate). The suspension was centrifuged at 13,300g for ten minutes, and the supernatant was transferred to a microcentrifuge DNA column. The supernatant was centrifuged at 13,300g for one minute, and the liquid pulled through was discarded. The column was washed by adding 200 µL of Plasmid Wash Buffer 1 and centrifuging at 13,300g for one minute. The column was washed by adding Plasmid Wash Buffer 2 and centrifuging for 13,300g. The plasmid was eluted by adding 50 µL of DNA Elution Buffer and incubating at room temperature for one minute, replacing the tube below the column with a microcentrifuge tube, and centrifuging at 13,300g for one minute. Plasmid concentration was then determined using a NanoDrop™ 2000.

2.2.6 Transfection and Transient Cell Generation

Expifectamine Transfection

8 µL Expifectamine was diluted in 150 µL of DMEM. This was added together with 4 µg of custom design plasmids at the bottom of wells in a 12 well plate. 2 mL of HEK EXPI cell media containing 1 million cells/mL was added onto the Expifectamine/plasmid mixture and left on a rotating plate at 125 rpm at 37°C 5% CO_2 for four days to generate fusion proteins. The cell supernatant containing fusion protein was collected, spun down and transferred to a new container to remove cells from suspension. Cells were then disposed in accordance to GMO waste disposal.

2.2.7 Protein Validation

SDS Page, Western blot

Protein integrity and molecular weight was confirmed by SDS Page. 10 µL of supernatant was collected and heated to 100°C for 10 minutes to denature proteins. Samples were then loaded into a 4-20% Acrylamide Gel and run for 25 minutes at 200V. Product was transferred to membrane using 100V for 45 minutes and probed with 2µL of goat anti-human-Fc antibody conjugated to horse radish peroxidase (HRP). Amersham Imager 600 machine was used to visualise bands. MW was determined by calculating the weight of the extracellular region using ExPASy and adding the weight of the Fc portion (25 kDaltons).

pNPP Substrate

All fusion proteins used in laboratory experiments in this thesis included a human aminophosphatase region, which allowed detection of protein involving the addition of 20 µL of pNPP substrate to 20 µL of cell media from transfection. This was left at room temperature for at least one hour. If AP-protein was present in the cell media, the solution turned a clear yellow colour.

2.2.8 Code Used for Bioinformatic Analysis

Volcano Plot Generation

Volcano plots were generated by arranging data into columns of Cell Type, Gene Name, p-value, and Log_2 Fold Change, then run through the following code written by myself in Python™ version 3.8:

```
import pandas as pd
from bioinfokit import visuz
df = pd.read_csv("CD11b diff ex. secreted genes vs PBS.csv")
df.head()
visuz.gene_exp.volcano(d=df, lfc= "L2FC", pv="p-value", color=('#E10600FF','#00239CFF'), gstyle=2
                       ,  sign_line=True, xlm=(-6,6,1), ylm=(0,120,20), figtype='svg')
```

Heatmap Generation

Heatmaps were generated by arranging data into columns of Cell Type, Gene Name or VSTPk, and Log_2 Fold Change, then run through the following code written by myself in Python™ version 3.8:

```
import pandas as pd
import seaborn as sns
import matplotlib.pyplot as plt
import numpy as np
import matplotlib

gapminder = pd.read_csv("file_name.csv") #insert file name with file in csv format
print(gapminder.head(3))
df2 = gapminder[['CellType', 'geneName', 'L2FC']] #L2FC is replaced with vstpK for VSTPk values
heatmap1_data_work = pd.pivot_table(df2, values='L2FC', index='geneName', columns='CellType')
matplotlib.pyplot.figure(figsize=(50, 92))
sns.set(font_scale=2)

sns.clustermap(heatmap1_data_work, vmin=('0'), vmax=('20'), col_cluster=False, cmap='YlGnBu', cbar_kws=None, figsize=(15, 30))

plt.yticks(rotation=0.5, fontsize=10)
plt.xticks(fontsize=15)

ylabel=("Gene Name")
xlabel=("Cell Type")
plt.ylabel(ylabel, fontsize=15)
plt.xlabel(xlabel, fontsize=15)
# label plot title and file name with whatever cell type and function is related
plt.title(label='plot_title', fontsize=20)

plt.savefig(fname='plot_title.svg', format='svg')
plt.savefig('plot_title.svg')

plt.show()
plt.close()
```

PCA Plot Generation

Genes in the raw count data were separated in accordance to the cell type, treatment type, and batch of RNA sequencing. These genes were then assigned a sample name in accordance to these conditions. A metadata file was generated that

described the condition and batch number each sample name was under. The data and metadata files were then run through the following code written by myself with help from Sam Old of the Malaghan Institute in R version 4.0.3:

```
library(biomaRt)
library(DESeq2)
library(magrittr)
library(tidyverse)
library(ggfortify)

VSTpk <- read.csv("/Users/cynth/OneDrive/Documents/rnaseq1.csv",
                        row.names = 1,
                        header = TRUE,
                        )

metadata <- read.csv("/Users/cynth/OneDrive/Documents/metadata.csv",
                       row.names = 1,
                       header = TRUE)
dds <- DESeqDataSetFromMatrix(countData = VSTpk,
                              colData = metadata,
                              design = ~ Sample) %>%
  DESeq()

dds <- vst(dds)
rld <- rlog(dds)

gene.exclude <- as.numeric(which(apply(t(VSTpk), 2, var)==0))

pca_res <- prcomp(t(VSTpk)[,-gene.exclude], scale. = TRUE)
autoplot(pca_res)

ggplot(dat = pca_res$x, aes(x = PC1, y = PC2)) +
  geom_point(aes(colour = colouring), size = 3)+
  theme_bw(
  )+xlab(paste("PC1:", round(pca_res$sdev[1]^2/sum(pca_res$sdev^2)*100, 3), "%")
         ) +ylab(paste("PC2:", round(pca_res$sdev[2]^2/sum(pca_res$sdev^2)*100,
                                     3), "%")
         )
colouring <- c(rep("CD11b_pos", 3), rep("CD11b_neg", 3),
               rep("TN_pos", 3), rep("TN_neg", 3))
```

All code can be found in a repository at https://github.com/morgancy711/Codes-for-thesis

2.2.9 Data Analysis

Flow Cytometry Data

FlowJo software (version 10, Treestar Inc, CA, USA) was used to analyse the flow cytometry data. Gates were drawn based upon FMOs and unstained samples. The flow cytometry plots shown were also generated using FlowJo.

Graphical Data

All graphical data was made using the Graph Pad Prism software (version 8, Graph Pad software, CS, USA). All plots made using R or Python was generated using the matplotlib package in Python and the ggplot package in R, and edited using Adobe

Illustrator (version 25.0.1, Adobe Inc, San Jose, CA). A p-value less than 0.05, a $\log_2$ fold change less than -1 or greater than 1, and a VSTPk value greater than 2 were considered significant.

3. Transcriptional analysis of *Nippostrongylus brasiliensis* positive Triple Negative and CD11b+ Dendritic Cells

3.1 Introduction

DC are a heterogeneous population comprising of multiple subsets that can drive pathogen specific T cell responses through cell surface interactions and secreted proteins[1,3]. It is these interactions that determine what lineage naïve T cells will become, all dependant on the type of pathogen DC present[3-6]. Among DC subsets are cDC2 cells, which are the most heterogenous compared to other subsets, but are known to be dependent on IRF4[25]. cDC2 are divided into various subpopulations, two of which are CD11b+ and TN DC. TN DC are dependent on KLF4[26], and it is these two cell subtypes that are particularly important for priming Th2 responses[17,18], which are involved in immunity to helminths and allergic disease.

Specific lineages of DC are shown to control T_H2 responses, such as IRF4 dependant DC and CD301b$^+$ DC[16,25]. In a mouse model of IRF4-Cre CD11c-Flox mice, which are deficient in DC2 subsets (TN and CD11b+ DC), these mice are shown to have an impaired T_H2 immune response to T_H2 conditioning stimuli. Mechanisms involved in DC2 mediated T_H2 priming have been shown to partially require various proteins such as ICOSL, OX40L, IL-33, ST2, TSLP, and CCL17[42,45,48,113,163-165]. These proteins are all at the surface interface between DC2 and T cells, the most likely place for proteins important in T_H2 priming to be located.

Past research has generated libraries of genes involved in T_H2 responses from sequencing DC2 primed with T_H2 inducing stimuli[17,88,161]. This research has uncovered the requirement of type I interferon pathways in priming immune responses in a T_H2 setting and show a distinct difference seen in the transcriptomics of TN and CD11b DC. However, these studies are limited by the use of total TN and CD11b+ DC in the analysis[17], and have yet to discover the key drivers of T_H2 differentiation. Focusing on antigen positive TN and CD11b+ DC will provide a more focused library of DEG in T_H2 stimulating conditions with little background from DC that have not taken up antigen which could blunt the overall observed response. As surface and secreted proteins from DC also contact T cells to provide stimulation and other various effects, this provides another level of focus in order to identify potential proteins that T_H2 priming depend on.

3.2 Aims

I hypothesize that antigen positive TN and CD11b DC will have distinct transcriptomic differences which will showcase their different roles within T_H2 responses under the same stimulus. By focusing on the surface and secreted proteins of Ag+ TN and CD11b, I hypothesize that I will be able to narrow down on specific genes that have potential to be important in T_H2 priming by using a combination of clustermaps, volcano plots, and structural and functional analysis.

Specific Aims:

- To determine the functional differences between Nb+ TN and CD11b+ DC.
- To find genes that could be important to the T_H2 priming event from amongst the surface and secreted genes of Nb+ TN and CD11b+ DC.

3.3 Results

3.3.1 Nb+ TN and CD11b+ DC have highly distinct transcriptional profiles

TN and CD11b+ DC have been shown to have distinct differentially expressed genes (DEG) following Nb treatment, but these studies examined the total DC subset, which included both DC that directly interact with antigen and those that were not involved in the response[17]. Due to this, the transcriptomic signature from non-specific DC with the sample pool could have masked or blunted important gene signatures associated with T_H2 immune induction. It was therefore important to explore the transcriptomes of enriched population of DC that have taken up antigen and likely received signals from the T_H2 stimulus. Transcriptional profiles of TN and CD11b+ DC2 that were positive for AF488 fluorescently labelled Nb antigen were generated previously (performed by my supervisor L. Connor in the laboratory of Prof. Ronchese, Malaghan Institute of Medical Research). Briefly, DC populations were isolated from the skin draining lymph nodes (dLN) of mice 48 hours after Nb-AF488 or PBS intradermal injection, which corresponded with the time when DC were interacting with T cells in the dLN. Antigen positive and negative TN and CD11b+ subsets were purified by flow assisted cell sorting, and RNA was prepared and sequenced using the Illumina RNA sequencing platform. To explore the difference in transcriptional profiles between antigen positive TN and CD11b+ DC, I performed a differential expression gene analysis. DESeq2 had previously been employed on Nb+ and PBS RNAseq data from TN and CD11bhi DC to identify genes that were differentially expressed between samples from Nb and PBS treatments (Log2FC>1, p-value<0.05). Consistent with whole population analysis[17], I found that TN and CD11b+ DC subsets exhibit distinct transcriptional profiles in response to Nb stimulation (**Figure 3.1b**). The number of DEG within the TN population was significantly greater than the number of DEG within the CD11b+ population (TN DEG 1751 vs. CD11b+ DEG 889). Interestingly, while the fraction of shared genes between the two different populations (695 DEG in total) represented less than 40% of the total TN DEG, the majority (78.2%) of DEGs regulated in CD11b+ DC by Nb were shared with TN, suggesting that TN may have a dominant and more specialised role in T_H2 immunity. Lastly, the proportion of genes that were up- and down-regulated was relatively equivalent in both DC populations (TN: 850 up, 898 down; CD11b+: 481 up, 405 down).

To explore the differences in transcriptional profiles of antigen-positive TN and CD11b+ DC driven by Nb treatment, I used the VSTpk values of genes to generate a principal component analysis (PCA) plot (**Figure 3.1 b**) which separates samples based on their variation along PC axes. I found that each individual population clustered closely together, while different treatments and DC subsets could be separated into distinct areas. PC1 axis showed separation for treatment type and accounted for 36.599% of the variation between DEG, whereas the PC2 axis showed separation for cell type and accounted for 25.637% of the variation between DEG.

To determine the extent and pattern of differential expression, I generated volcano plots from genes with differential expression > 1 Log2FC (**Figure 3.1c**) and clustermaps using Log2FC DEG and VSTpk values of all genes expressed by TN and CD11b+ DC (**Figure 3.1d**). I found that TN transcriptional changes in response to Nb represented a much larger range of differential expression than Nb positive CD11b+ DC (Log2FC between 6 and -5 and Log2FC between 4 to -3.5 respectively). Antigen positive TN DC also have more DEG that were biologically significant. Overall, the transcriptional signature of antigen-positive TN and CD11b+ DC responding to Nb treatment is significantly different, suggesting that these populations may play unique and distinct roles in the induction of T_H2 immune responses.

Nb-treated DC subsets express gene signatures associated with activation and T_H2 immunity

My investigation of the transcriptome of Nb-treated DC subsets suggests that a distinct genetic program was occurring in each population. Before conducting additional analyses on the functional role of DEGs, I first wanted to confirm that each DC population was activated and expressed genes commonly associated with T_H2-primed DCs.

To investigate general activation and T_H2 priming signature, I created libraries of known activation- and T_H2-associated proteins and genes to generate clustermaps. I used both log2FC and VSTpk values of antigen-positive TN and CD11b+ DC treated with Nb or antigen-negative DC subsets from PBS treated samples to determine gene profiles (**Figure 3.2a and b**). Antigen-positive TN and CD11b+ DC subsets

showed higher log2FC and VSTpk values of general activation genes (i.e. *Cd40, Cd80, Cd86, Tnfsf4, Dll4, Pd1l1*)[17,178,179,218-222] and T_H2 associated genes (i.e. *Tnfrsf11b, Ccl17, Ccl22*) compared to PBS treatment. I also created gene sets that associate with non-T_H2 immune responses, including T_H1 and T_H17 immunity (**Figure 3.2c**). Interestingly, antigen-positive TN and CD11b+ DC expressed significantly higher levels of *Il6* (TN, 3 log2FC; CD11b+, 2 log2FC) and *Il1rn* (TN and CD11b+ 2 log2FC) than PBS, both of which are genes associated with $T_H1/17$ responses[52]. Conversely, T_H1-associated *Il12b* and T_H17-associated *Tgfb3* expression levels were downregulated in TN DC treated with Nb.

Generation and analysis of a new data set of surface and secreted genes for functional analysis

In order to gain insight from the transcriptome to determine how T_H2-primed DC can drive the differentiation of T_H2 cells, I decided to restrict gene functional analysis to genes expressed on the cell surface or secreted, as they encode proteins more likely to interact with naïve T cells. I created a new sample set of cell surface and secreted genes, based upon the defined region of a cell that gene products are known to inhabit, for Nb-treated TN and CD11b+ DC populations using the online tool, Database for Annotation, Visualization, and Integrated Discovery (DAVID)[123,124]. DEG log2FC > 2 for each DC subsets were uploaded and the Functional Annotation table was used to identify localisation of the gene product. Localisation of DEGs were confirmed using a second database, UniProt.

Genes encoding cell surface and secreted proteins constitute 38.34% and 44.32% of all DEGs from TN and CD11b+ DC respectively (**Figure 3.3a**). Cell surface DEGs were more abundant in TN DC than secreted genes, and a greater proportion were upregulated (**Figure 3.3b**). However, secreted genes in CD11b+ DC exhibited greater changes in expression by Nb treatment and were mostly upregulated. In summary the transcriptional analysis of cell surface and secreted genes suggest that antigen-positive TN exhibit a greater cell surface change than secreted, and have more intracellular DEGs than CD11b+. Conversely, antigen-positive CD11b+ DC show a greater secreted change than their surface change, suggesting a larger secretory than surface interaction role in T_H2 responses.

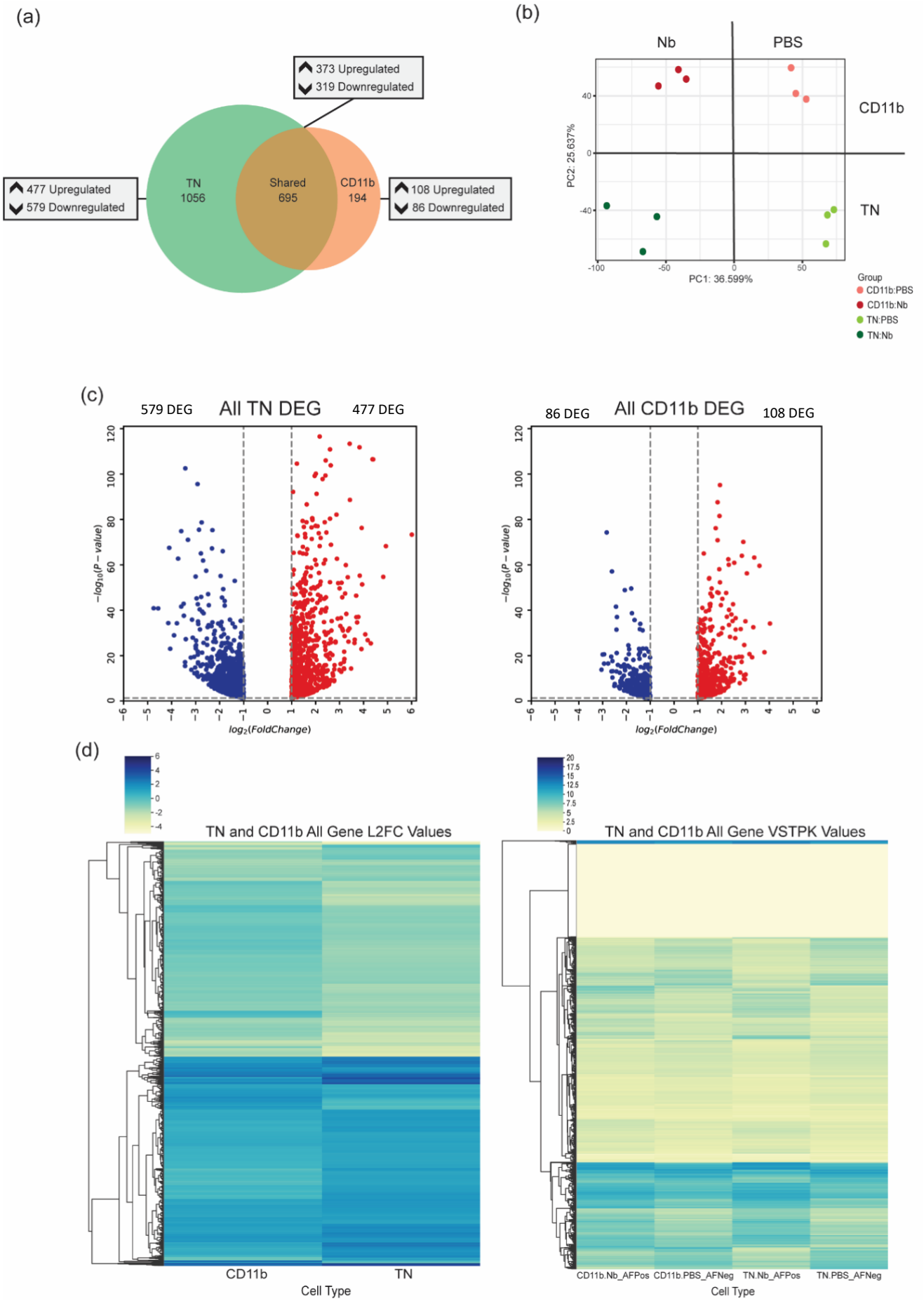

(a)
373 Upregulated
319 Downregulated
477 Upregulated
579 Downregulated
TN
1056
Shared
695
CD11b
194
108 Upregulated
86 Downregulated
(b)
Nb
PBS
CD11b
TN
PC2: 25.637%
PC1: 36.599%
Group
CD11b:PBS
CD11b:Nb
TN:PBS
TN:Nb
(c)
579 DEG
All TN DEG
477 DEG
86 DEG
All CD11b DEG
108 DEG
−log10(P − value)
log2(FoldChange)
(d)
TN and CD11b All Gene L2FC Values
TN and CD11b All Gene VSTPK Values
CD11b
TN
Cell Type
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type

Figure 3.1: Primary Investigation of RNA Sequencing Data from Triple Negative and CD11b^{+} Dendritic Cells. RNA sequencing data and VSTPk values were used to determine transcriptomic differences, patterning of DEG, and DEG breadth within TN and CD11b DC **(a)** RNA sequencing data was separated in accordance to the cells that the genes were associated with. These genes were split again into upregulation and downregulation. **(b)** Raw reads of RNA sequencing were used in a code to generate a PCA plot in R showing the placement the samples of differing treatment groups and cell types in reference to PC1 and PC2. PC1 is in relation to treatment group, and PC2 is in relation to cell type. **(c)** DEG (Nb+ vs. PBS) for each cell type were taken with their Log2 Fold Change and p-values and used to generate volcano plots in Python showing gene distributions for each cell type. **(d)** DEG (Nb+ vs. PBS) with their Log2 Fold Change were used to generate heat maps in Python for both cell types. Genes were clustered based upon Euclidean mathematics in order for genes with similar distances to be clustered together, allowing patterns to be seen within the gene distribution for each cell type. VSTPk values are in short a normalization method of gene counts in which gene counts are normalized with their transcript length taken into account. All genes had a p-value of <0.5.

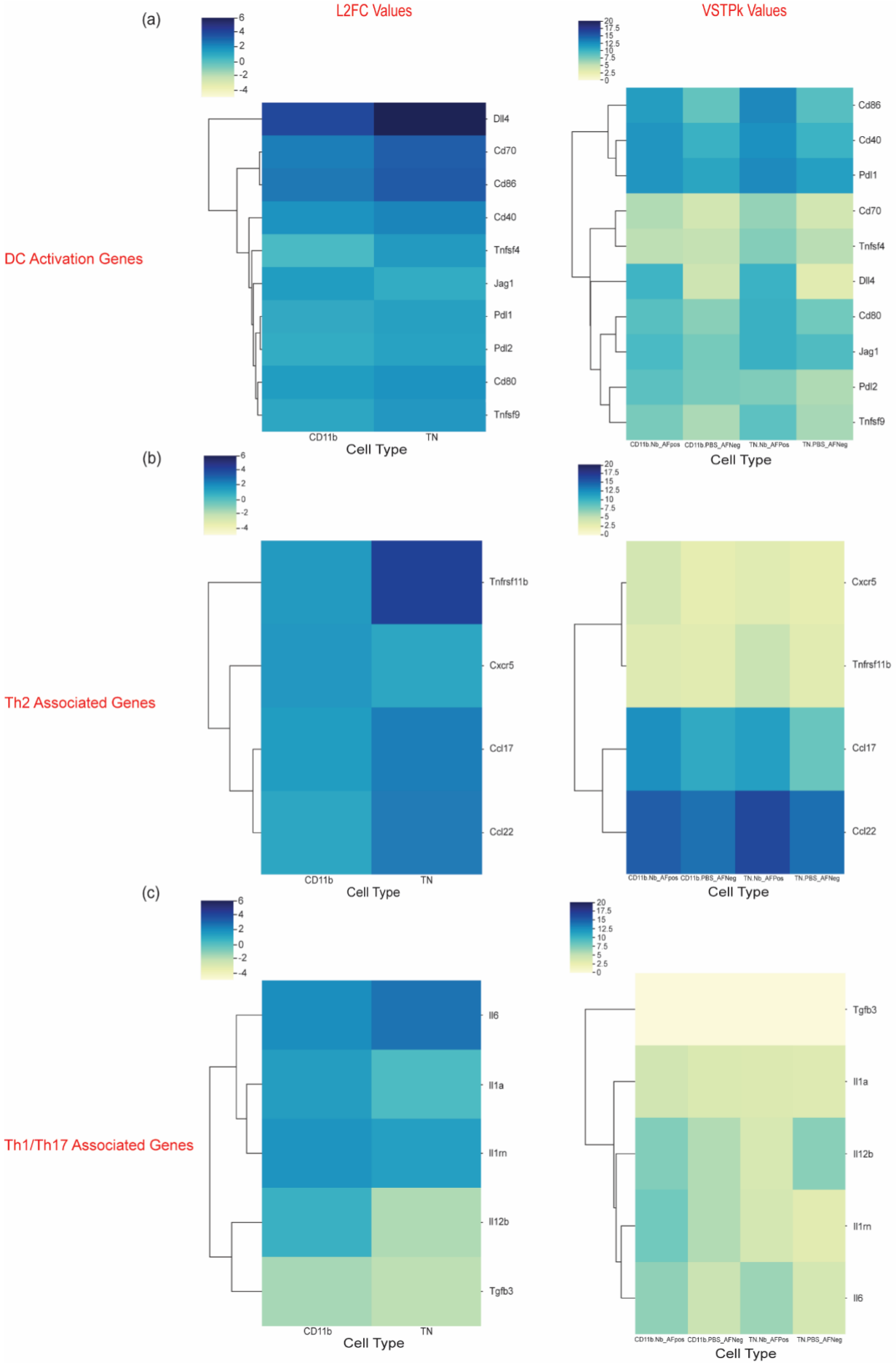

(a)
L2FC Values
VSTPk Values
DC Activation Genes
Dll4
Cd70
Cd86
Cd40
Tnfsf4
Jag1
Pdl1
Pdl2
Cd80
Tnfsf9
Cd86
Cd40
Pdl1
Cd70
Tnfsf4
Dll4
Cd80
Jag1
Pdl2
Tnfsf9
CD11b
TN
CD11b.Nb_AFpos
CD11b.PBS_AFNeg
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type
(b)
Th2 Associated Genes
Tnfrsf11b
Cxcr5
Ccl17
Ccl22
(c)
Th1/Th17 Associated Genes
Il6
Il1a
Il1rn
Il12b
Tgfb3

Figure 3.2: Confirmation of DC activation marker upregulation, Th2 marker upregulation, and Th1/Th17 marker downregulation to determine contextual DC activation. Using markers associated with DC activation and causing various CD4+ T cell responses, TN and CD11b DC were determined to be activated and upregulating Th2 associated proteins, at the expense of Th1 and Th17 proteins. **(a)** Known DC activation markers were extracted from total RNAseq data and used to generate heatmaps in Python. This determines that these DC are activated based on positive Fold Change and mRNA levels with treatment. **(b)** Genes encoding proteins potentially important in Th2 activation were found in literature and used to generate heatmaps in Python. With positive Fold Change and increased mRNA levels, these DC are very likely to be causing a Th2 response. **(c)** Genes encoding proteins known to cause preferential Th1 or Th17 activation were used to generate heatmaps in Python. This showed clear downregulation of these genes in TN DC, and mixed downregulation in CD11b DC. All genes had a p-value of <0.5.

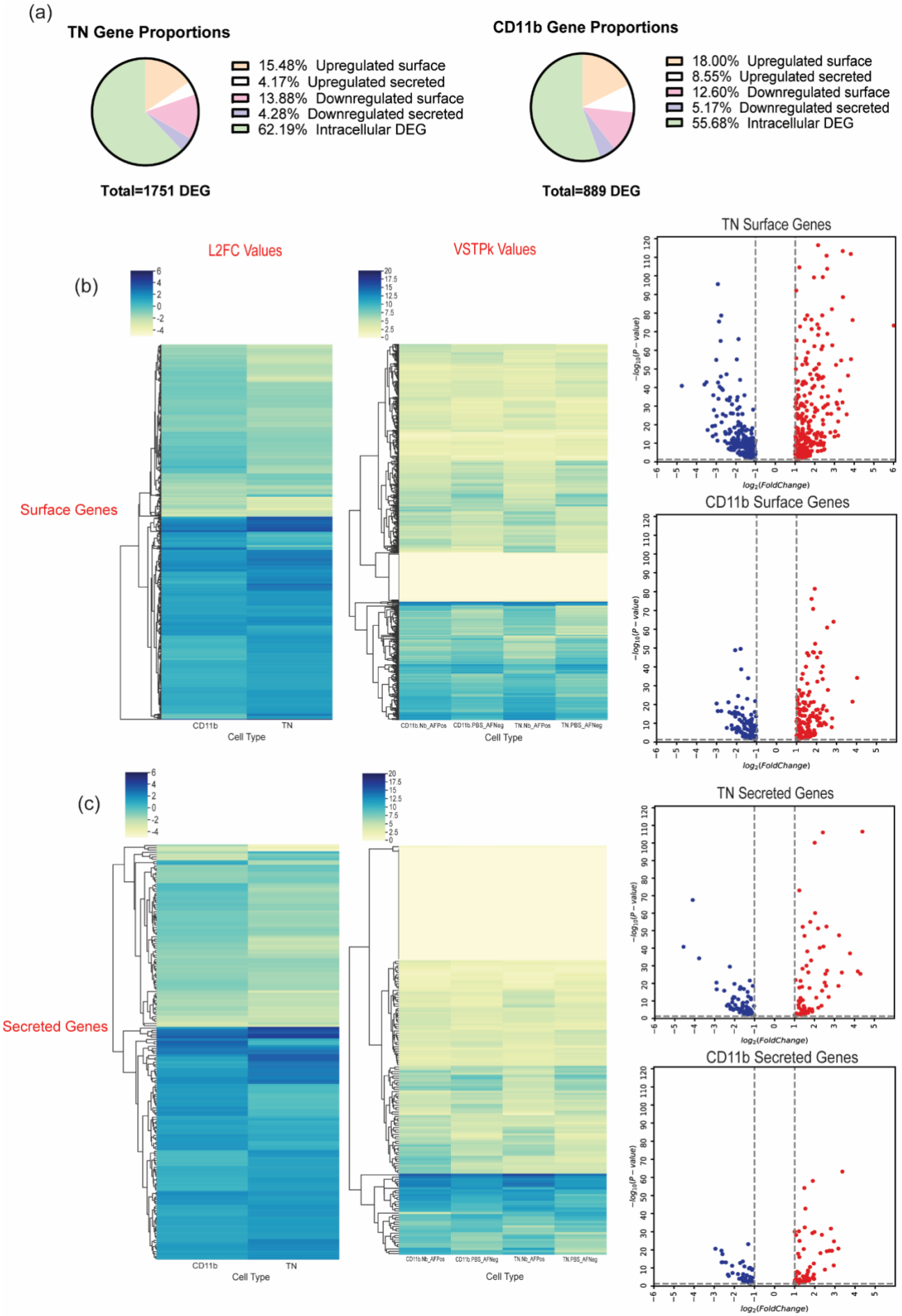

(a)
TN Gene Proportions
15.48% Upregulated surface
4.17% Upregulated secreted
13.88% Downregulated surface
4.28% Downregulated secreted
62.19% Intracellular DEG
Total=1751 DEG
CD11b Gene Proportions
18.00% Upregulated surface
8.55% Upregulated secreted
12.60% Downregulated surface
5.17% Downregulated secreted
55.68% Intracellular DEG
Total=889 DEG
(b)
L2FC Values
VSTPk Values
Surface Genes
CD11b
TN
Cell Type
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
TN.Nb_AFPos
TN.PBS_AFNeg
TN Surface Genes
CD11b Surface Genes
(c)
Secreted Genes
TN Secreted Genes
CD11b Secreted Genes
log2(FoldChange)
−log10(P − value)

Figure 3.3: Proportions, Patterning, and Breadth of Surface and Secreted Differentially Expressed Genes to Highlight Distinction Between Cell Types. Genes were separated into surface and secreted genes, both up and downregulated, with the use of DAVID, an online database, and used to generate pie charts, heatmaps, and volcano plots. **(a)** Upregulated and downregulated surface and secreted gene numbers were used to generate a pie chart of proportions in relation to total gene number using Prism. **(b)** Log2 fold change values, VSTPk values, and p-values of surface DEG (Nb+ vs. PBS) of each cell type were used to generate heatmaps and volcano plots in Python to show distinct patterning of genes between cell types. **(c)** Log2 fold change values, VSTPk values, and p-values of secreted DEG (Nb+ vs. PBS) of each cell type were used to generate heatmaps and volcano plots in Python to show distinct pattering of genes between cell types.

3.3.2 Nb+ TN and CD11b+ DC have distinct superfamily and specific functional compartments

By examining the superfamily and general functions of Nb+ TN and CD11b+ DC, I could gain insight to TN and CD11b+ DCs functional distinction. Superfamilies are a classification used for proteins based on a conserved structure, which grants the protein a general function. I used the online database SupFam to find the superfamily and function of each surface and secreted gene product. The number of each superfamily and function in antigen-positive DC subtypes was then used to generate donut plots, showing the relative proportion of each superfamily from the total amount of superfamilies.

Antigen-positive TN DC had much higher numbers of upregulated surface superfamilies than secreted (114 and 29 respectively) and display a larger amount of downregulated secreted superfamilies over upregulated (50 and 29 respectively) seen in Figure 3.4. Antigen-positive CD11b+ DC show similar amounts of upregulated and downregulated surface and secreted superfamilies (Surface: 28 upregulated and 22 downregulated, secreted: 22 upregulated and 17 downregulated). Additionally, antigen-positive TN DC had a large proportion of upregulated surface gene superfamilies having a signal transduction (21.67%) and cell adhesion (8.37%) function, whilst the upregulated secreted gene superfamilies showed a largely protease function (32.26%). Interestingly, TN also largely downregulated gene superfamilies associated with the same functions as the upregulated superfamilies, signal transduction (20.30%) and cell adhesion (14.85%). CD11b+ DC show largely cell adhesion (38.46%) in the upregulated surface gene compartment, and largely immune response (44.44%) in the upregulated secreted gene compartment. This evidence suggests that antigen-positive TN DC specialise in signal transduction from surface interactions and the release of proteases. Antigen-positive CD11b+ DC show that they are able to adhere to other cells, but secrete a large amount of immune response proteins, suggesting that they take on a more secretory than surface interaction role during T_H2 responses.

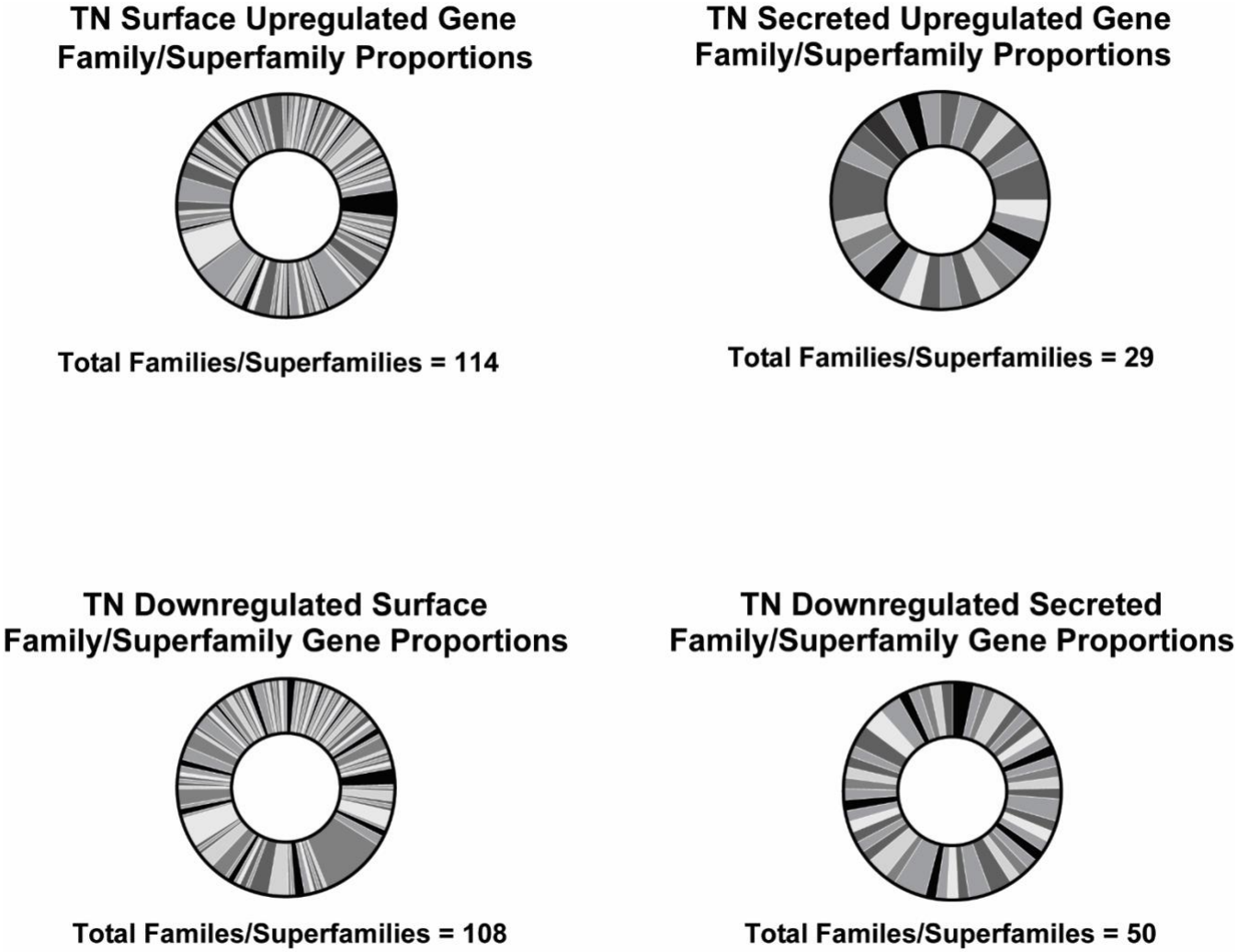


Figure 3.4: Example of Super Family Proportions from Triple Negative Dendritic Cell Upregulated and Downregulated Surface and Secreted Genes. Genes were separated into superfamilies with the use of SupFam, an online database. These were then used to generate donut plots of TN upregulated and downregulated surface and secreted superfamilies.

(a)

Proportions of TN Upregulated Surface Functions of Families/Superfamilies

Total = 148 genes

- 21.67% Signal Transduction
- 8.87% Diverse Function
- 8.37% Cell adhesion
- 7.88% Ion metabolism and transport
- 6.40% Kinases/phosphatases
- 6.40% Protein interaction
- 5.91% Small molecule binding
- 5.42% Unknown function
- 3.45% DNA Binding
- 2.96% Immune response
- 2.96% Post-translational modification
- 1.48% Lipid metabolism and transport
- 1.48% Phospholipid metabolism and transport
- 1.48% Proteases
- 1.48% Transferase
- 1.48% Transport
- 1.48% Amino acid metabolism and transport
- 1.48% Cell motility
- 0.99% DNA replication/repair
- 0.99% Receptor activity
- 0.99% Redox
- 0.99% Cellular Activation
- 0.99% Lipid Binding
- 0.49% Structural protein
- 0.49% Vesicle Fusion
- 0.49% Cellular anabolism
- 0.49% Carbohydrate metabolism and transport
- 0.49% Coenzyme metabolism/transport
- 0.49% Cell cycle, Apoptosis
- 0.49% Secondary metabolism
- 0.49% Protein Trafficking
- 0.49% Hydrolase

(b)

Proportions of TN Downregulated Surface Functions of Families/Superfamilies

Total = 202 genes

- 20.30% Signal Transduction
- 14.85% Cell adhesion
- 9.41% Ion metabolism and transport
- 7.43% Immune response
- 4.95% Diverse Function
- 4.95% Small molecule binding
- 4.46% Unknown function
- 4.46% Protein interaction
- 3.96% Kinases/phosphatases
- 2.48% Lipid Binding
- 2.48% Cell cycle, Apoptosis
- 2.48% DNA-binding
- 2.48% Transport
- 1.49% Amino acid metabolism and transport
- 1.49% Tranferase
- 1.49% Lipid metabolism and transport
- 1.49% Receptor activity
- 1.49% Proteases
- 0.99% Post-translational modification
- 0.99% Secondary metabolism
- 0.99% DNA replication/repair
- 0.99% Translation
- 0.50% Ion binding
- 0.50% Coenzyme metabolism and transport
- 0.50% Cell motility
- 0.50% Energy
- 0.50% Nucleoside metabolism and transport
- 0.50% Other enzymes
- 0.50% Other regulatory function
- 0.50% Phospholipid metabolism and transport

Proportions of TN Upregulated Secreted Functions of Families/Superfamilies

Total = 25 genes

- 32.26% Proteases
- 6.45% Receptor activity
- 6.45% Secondary metabolism
- 6.45% Small molecule binding
- 6.45% Cell adhesion
- 6.45% Cell cycle, Apoptosis
- 6.45% Immune response
- 6.45% Nucleotide metabolism and transport
- 3.23% RNA binding, metabolism, and transport
- 3.23% Lipid metabolism and transport
- 3.23% Other enzymes
- 3.23% Phospholipid metabolism and transport
- 3.23% Unknown function
- 3.23% Blood clotting
- 3.23% Carbohydrate metabolism and transport

Proportions of TN Downregulated Secreted Functions of Families/Superfamilies

Total = 61 genes

- 16.39% Proteases
- 13.11% Cell adhesion
- 8.20% Redox
- 8.20% Receptor activity
- 6.56% Carbohydrate metabolism and transport
- 4.92% Small molecule binding
- 4.92% Other enzymes
- 3.28% Secondary metabolism
- 3.28% Signal transduction
- 3.28% Electron transfer
- 3.28% Protein interaction
- 3.28% Diverse function
- 3.28% Immune Response
- 1.64% Cell growth
- 1.64% DNA binding
- 1.64% DNA replication/repair
- 1.64% Ion metabolism and transfer
- 1.64% Kinases/phosphatases
- 1.64% Lipid metabolism and transport
- 1.64% Phospholipid metabolism and transport
- 1.64% Post-translational modification
- 1.64% Protein modification
- 1.64% Trafficking/secretion
- 1.64% Unknown function

Figure 3.4: Example of Proportions of General Functions from Upregulated and Downregulated Surface and Secreted Superfamilies Unique to Triple Negative Dendritic Cells. Superfamilies were sorted into their general functions with the use of SupFam, an online database. These were then used to generate donut plots and relative percentages of functions of **(a)** TN upregulated surface and secreted general functions and **(b)** TN downregulated surface and secreted general functions.

To determine specific functions of genes within the superfamily groups, I performed pathway analysis on the genes from the general function groups representing the highest proportions from each DC subset using the online database DAVID.

Both antigen-positive TN and CD11b+ DC expressed genes that did not cluster within a group and these genes showed little similarity in specific function to each other. Among the genes from TN DC that did cluster together, a high proportion of clusters belonged to the signal transduction superfamily. These clusters associated with pathways including: T-cell receptor signalling, VEGF signalling, and GPCR signalling. TN DC had only one cluster in surface cell adhesion which involved upregulated genes involved in the plexin/semaphorin pathway. Clusters involving proteases and protease inhibitors were largely associated with the Serpin protein family. Within the CD11b+ DCs, genes that were upregulated and encode for secreted products exhibited the highest clusters within the immune response functional group. These functions included complement cascade activation, the NOD-like signalling pathway, the chemokine mediated signalling pathway, and lymphocyte chemotaxis. The general functions of genes that TN and CD11b+ share involve more clustering in downregulated genes. This includes clusters involved with metallopeptidase activity and EGF signalling. The shared genes do show clustering in the upregulated immune response compartment similar to what is seen for CD11b+ DC. In summary, the gene cluster and functional analysis revealed highly diverse and markedly different functions between the different DC subsets, which suggests that TN and CD11b+ DCs play unique, or distinct roles in this model of T_H2 immunity.

Individual genes from the most highly represented clusters (signal transduction, cell adhesion, proteases) were incorporated into clustermaps using gene VSTpk values, which provides an indication of the level of gene expression within the cell population.

Almost every clustermap contained two clusters, a high and a low VSTpk cluster. The high VSTpk clusters included genes known to be involved in immune responses, such as receptors involved in general activation, including: signal-regulatory protein alpha (Sirpα) which prevents phagocytosis of healthy cells and antigen-presenting molecule, Cd1d1, both belonging to the TN surface cell adhesion genes (**Figure**

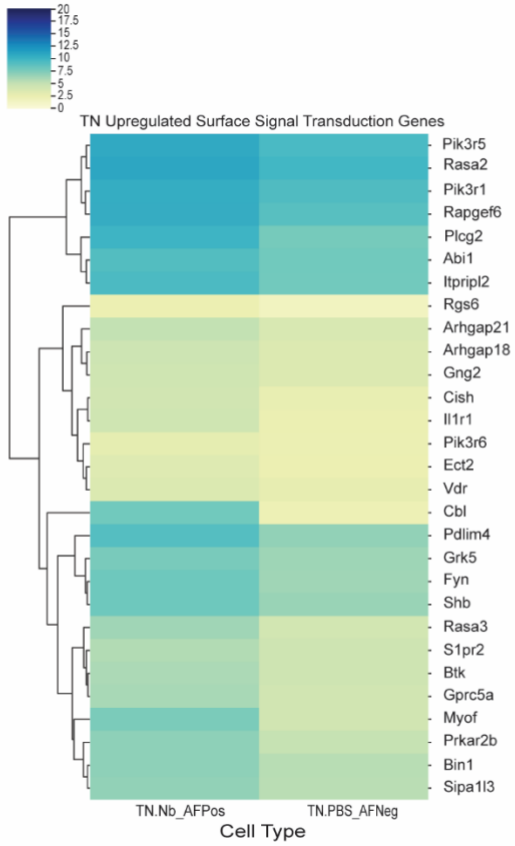
3.5)[180-183]. Cell adhesion genes encoding proteins known in DC maturation and stimulation of T cells were also present, and as predicted were expressed in both TN and CD11b+ DC (Cd274, Cd80, Cd86, and Cd200[199-205]). Of interest, a number of genes present within the clustermaps have indeed been associated with T cell differentiation, and in particular T_H2 differentiation. Among upregulated genes from CD11b+ DC, Jag1 (signal transduction[194-196]), and Il27 in CD11b (immune response[197,198]) were present. Jag1 is a member of the Notch signalling pathway, which plays a major role in T cell differentiation[refs]. IL27 forms a heterodimer with Epstein Barr virus-induced protein 3 (EBI3) when combined binds to IL27 receptor on T cells to promote T cell differentiation[refs]. A second member of the notch signalling pathway, Dll4, is expressed in both TN and CD11b+ DC within the signal transduction superfamily cluster[178,179]. Finally, the T_H2 associated chemokines Ccl17 and Ccl22[169] were also present in both TN and CD11b+ within the immune response cluster (all found in **Supplementary Figure 2**).

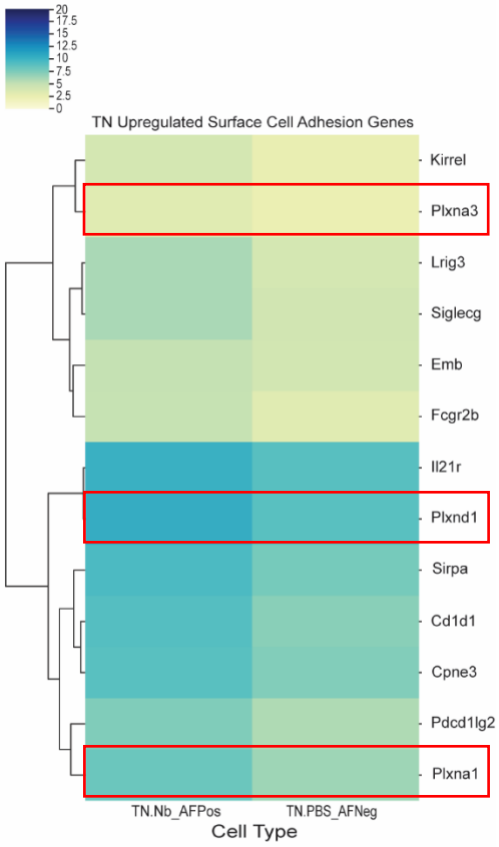
Alongside known immune-involved genes, there were a number of genes with roles not commonly associated with T cell differentiation or DC maturation. *Rasa2*, *Rasa3*, and *Prkar2b* are associated with negative regulation of cyclic adenosine monophosphate (cAMP) concentration, and *Cbl*, *Plcg2*, and *Bin1* are associated with positive regulation of cytosolic Ca^{2+} concentration (**Figure 3.5**). Both are known secondary messenger molecules, however there is evidence to suggest that low cAMP concentration and high Ca^{2+} concentration in DC skews T cell responses to favour T_H2 responses[260-262]. *Plxnd1*, *Plxna1*, *Plxna3* (**Figure 3.5**), and *Sema7a* (**Supplementary Figure 2c**) all encode proteins involved within the semaphorin/plexin system that was clustered during functional analysis (**Table 3.1**). This protein family was first known to be involved during neurogenesis and is required for proper neurological development. However, semaphorins recently have been shown to be involved in certain immune responses, including T_H2 responses[55-57,147,148]. It is particularly interesting that some of these genes also have amongst the highest VSTpk values within their respective clustermaps, as this implicates that they are more likely to be biologically significant, making them good targets for further exploration into their role in T_H2 responses.

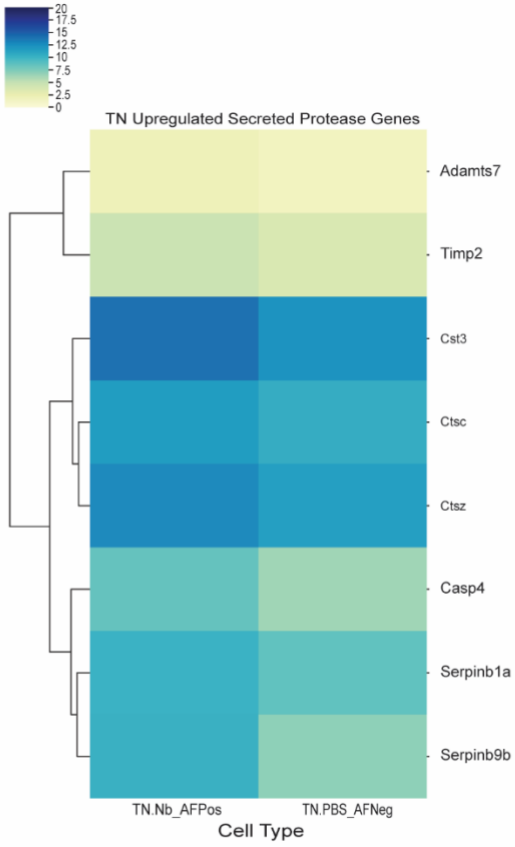
Many genes in the low VSTpk value clusters were not known in their involvement in immune responses, such as *Ect2*, *Vdr*, and *Lrig3*, involved in innate immune

sensing, such as *Fcgr2b*, *Il1r1*, and *Siglecg*, or encoded general adhesion proteins, such as *Kirrel*. Overall, my gene analysis suggests that genes with high VSTpk values are important for supporting direct T cell activation and low VSTpk value genes might be more involved in adhesion or irrelevant immune pathways.

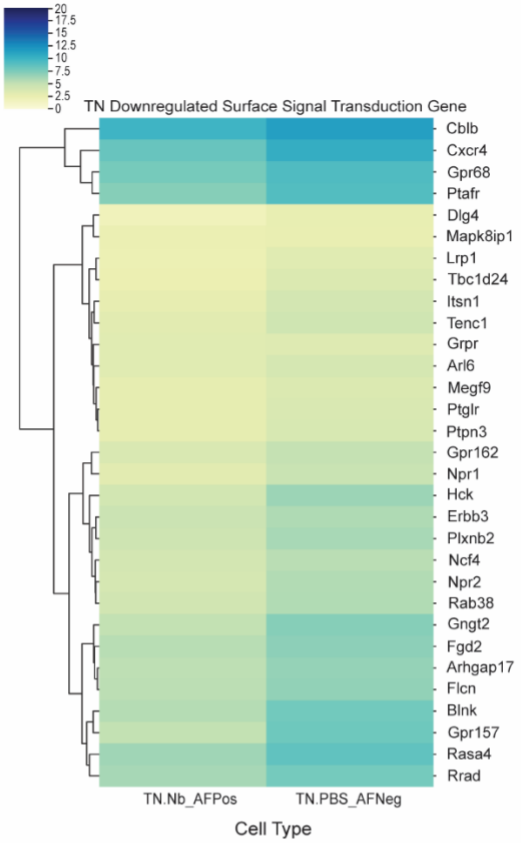
(a)

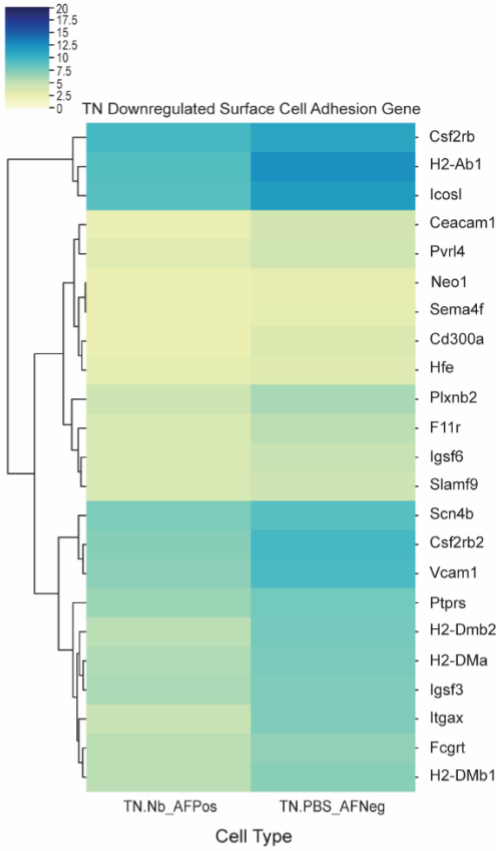

20
17.5
15
12.5
10
7.5
5
2.5
0
TN Upregulated Surface Signal Transduction Genes
Pik3r5
Rasa2
Pik3r1
Rapgef6
Plcg2
Abi1
Itpripl2
Rgs6
Arhgap21
Arhgap18
Gng2
Cish
Il1r1
Pik3r6
Ect2
Vdr
Cbl
Pdlim4
Grk5
Fyn
Shb
Rasa3
S1pr2
Btk
Gprc5a
Myof
Prkar2b
Bin1
Sipa1l3
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type


20
17.5
15
12.5
10
7.5
5
2.5
0
TN Upregulated Surface Cell Adhesion Genes
Kirrel
Plxna3
Lrig3
Siglecg
Emb
Fcgr2b
Il21r
Plxnd1
Sirpa
Cd1d1
Cpne3
Pdcd1lg2
Plxna1
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type


20
17.5
15
12.5
10
7.5
5
2.5
0
TN Upregulated Secreted Protease Genes
Adamts7
Timp2
Cst3
Ctsc
Ctsz
Casp4
Serpinb1a
Serpinb9b
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type

(b)


20
17.5
15
12.5
10
7.5
5
2.5
0
TN Downregulated Surface Signal Transduction Gene
Cblb
Cxcr4
Gpr68
Ptafr
Dlg4
Mapk8ip1
Lrp1
Tbc1d24
Itsn1
Tenc1
Grpr
Arl6
Megf9
Ptglr
Ptpn3
Gpr162
Npr1
Hck
Erbb3
Plxnb2
Ncf4
Npr2
Rab38
Gngt2
Fgd2
Arhgap17
Flcn
Blnk
Gpr157
Rasa4
Rrad
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type


20
17.5
15
12.5
10
7.5
5
2.5
0
TN Downregulated Surface Cell Adhesion Gene
Csf2rb
H2-Ab1
Icosl
Ceacam1
Pvrl4
Neo1
Sema4f
Cd300a
Hfe
Plxnb2
F11r
Igsf6
Slamf9
Scn4b
Csf2rb2
Vcam1
Ptprs
H2-Dmb2
H2-DMa
Igsf3
Itgax
Fcgrt
H2-DMb1
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type

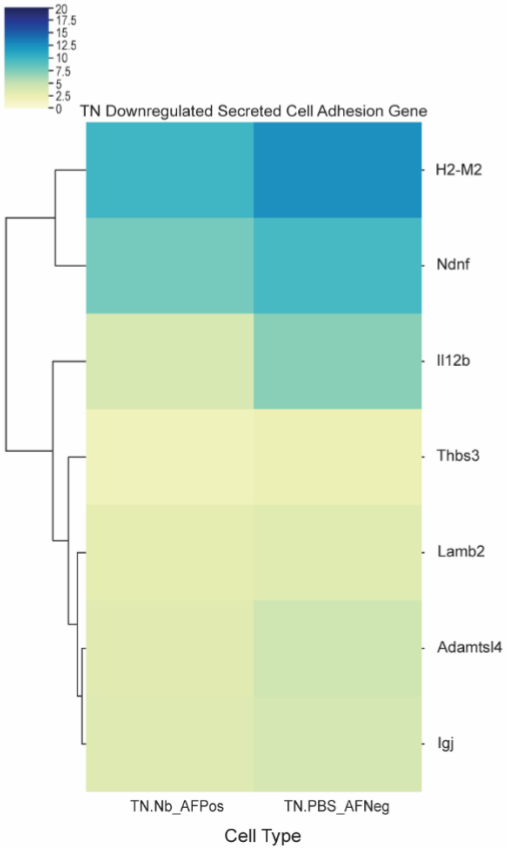

20
17.5
15
12.5
10
7.5
5
2.5
0
TN Downregulated Secreted Cell Adhesion Gene
H2-M2
Ndnf
Il12b
Thbs3
Lamb2
Adamtsl4
Igj
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type

Figure 3.5: VSTPk Heatmaps of Genes from Selected General Functions of Upregulated and Downregulated Surface and Secreted Genes of Triple Negative Dendritic Cells. Proportionally large general functions were selected and used to generate lists of genes whose VSTPk values were used to generate heatmaps using Python. Outlined in red are Plexin protein family genes **(a)** VSTPk heatmaps of TN upregulated surface signal transduction, surface cell adhesion, and secreted protease genes. **(b)** VSTPk heatmaps of TN downregulated surface signal transduction, surface cell adhesion, and secreted cell adhesion genes. Outlined in red are genes of the Plexin protein family, which are receptors for Semaphorin proteins.

Superfamily function group	Location: surface or secreted	Up- or downregulated in Th2 response	TN gene functions	CD11b gene function	Shared genes
Signal transduction	Surface	up	Large diversity of signal transduction pathways, including T cell receptor signalling, VEGF signalling, and GPCR signalling	Positive regulation of cytosolic Ca^{2+} concentration	-
		down	-	-	-
Cell Adhesion	Surface	up	Specific clustering of Plexin/ Semaphorin pathway	Cadherin cluster	Positive regulation of cell migration
		down	Cell adhesion pathways for pathogens unrelated in Th2 reactions	-	-
Protease	Secreted	up	-Shows clusters of proteases and protease inhibitors -Specific clustering of Serpin family	Clusters for blood coagulation and complement activation	Serine-type peptidase activity

		down	-	-	Metallopeptidase activity
Immune response	Secreted	up	-	Clusters show: -Complement cascade activation -Positive regulation of ERK1/ERK2 pathway -NOD-like signalling pathway -Chemokine mediated signalling pathway -Cellular response to TNF, IFNγ, and IL-1 -Lymphocyte chemotaxis	Clusters show: -Positive regulation of ERK1/ERK2 pathway -Chemokine mediated signalling pathway -Cellular response to TNF, IFNγ, and IL-1 -Lymphocyte chemotaxis
		down	-	-	Clusters show EGF signalling
Cell Adhesion	Secreted	up	-	-	-
		down	-	-	-EGF cluster -Metallopeptidase cluster

Table 3.1: Summary of functions and pathways of secreted and surface proteins from T_H2 DC subsets. Functional Annotation Clustering was performed on each gene list of selected general functions using the online database DAVID. Unique functions and pathways that appeared in clustering were noted down and compiled together.

3.4 Discussion

CD11b+ and TN DC are cDC2 subsets known to be important for the initiation of Th2 responses[17,18,42-45]. TN DC were first described as CD103^{lo} CD326^{lo} CD11b^{lo} by Ochiai et al (2014)[45], while CD11b DC were first described by M. Kitajima and S.S. Zeigler (2013)[42]. Both cell types were shown to be responsive to TSLP to induce T_H-2 responses[42,45], however current research has not shown how these cells are distinct from each other within the T_H2 response. Connor et al (2017), showed recently that Nb primed TN and CD11b+ DC are highly transcriptionally different[17], yet the proteins TN and CD11b+ DC use to prime Th2 responses are not well known. There are candidate proteins capable of inducing Th2 priming such as TSLP, an epithelial cell protein produced in the response of damage[47,48], and OX40L, a protein upregulated at the surface of DC2 under T_H2 stimulating conditions[48]. OX40L-mediated T_H2 priming used TSLP to activate DC and was performed in vitro[48]. However, there are Th2 responses, such as certain helminth infections[46], that bypass TSLP and directly act on DCs. This limits the applicability of this data to in vivo T_H2 responses, and suggests that additional factors are capable of priming T_H2 responses. In this chapter I showed that Nb positive TN and CD11b+ DC are transcriptionally distinct from each other and propose their specific functional difference using pathway analysis which follows data trends seen by Connor et al (2017). Additionally, I show surface and secreted genes that are potentially important in Nb-mediated T cell activation based on protein superfamily structure and function analysis.

TN and CD11b+ DC from mice primed with Nb are transcriptionally distinct, with 656 DEG in TN DC and 435 DEG in CD11b+ DC[17]. Examining RNA sequencing data specifically of TN and CD11b positive for Nb, I have shown an even larger transcriptional difference, with TN having close to twice the number of DEG than CD11b. I have shown that the extent of the differential expression is much larger in TN than CD11b, which does not match the extent of differential expression of TN and CD11b+ shown by Connor et al (2017), as they show TN and CD11b+ DC having similar extents of differential expression in response to Nb. This suggests that TN DC have a much stronger transcriptional change than CD11b+ DC when Nb positive, but TN and CD11b+ DC that have not taken up Nb respond to a similar degree. There are clear differences in the extent of differential expression and

mRNA levels between individual genes in my data, matching data trends by Connor et al (2017). Additionally, individual gene differential expression between my data and data from Connor et al (2017) show some similarity in type I interferon response genes, a pathway shown to be crucial in the induction of T_H2 responses (Connor et al (2017), Webb et al (2017), Trottein et al (2004)).

DC upregulate multiple markers when they become activated, including CD40, CD80, CD86, and PDL1[164,168]. In addition to these general activation markers, there are proteins associated with helping to induce particular T_H responses, such as CXCR5 and CCL22 for T_H2 responses[49,169], IL12b for T_H1 responses[54], and IL6 and TGFβ for T_H17 responses[51,52]. By examining the differential expression and VSTpk values of such markers, I have shown that TN and CD11b+ DC that are Nb positive are upregulating activation and T_h2 priming associated genes, whilst downregulating a majority of genes associated with T_H1 and T_H17 responses. TN DC also appear to be more activated and have greater expression of T_H2 associated genes when compared to CD11b+ DC, suggesting that TN DC are more capable of inducing T_H2 responses than CD11b+ DC.

Connor et al (2017), showed that TN and CD11b+ DC were transcriptionally distinct after Nb priming. However, the size of the transcriptional profiles of both cell subtypes denied the possibility of examining them thoroughly for genes potentially important in T_H2 priming. This necessitated separating total DEG into surface and secreted DEG, as surface and secreted proteins are important in T cell activation. TN had many more DEG total than CD11b, but their surface and secreted gene proportions remained similar aside from upregulated secreted genes. CD11b+ DC upregulated over double the proportion of secreted genes than TN DC, 8.55% and 4.17% respectively, suggesting that CD11b+ DC perform a more secretory role than TN DC in T_H2 responses. There are patterns of differential expression in surface and secreted DEG similar to, but more distinct than, the total DEG. There are specific clusters of DEG that are different between TN and CD11b, showing that surface and secreted DEG are different on the individual gene level. Additionally, the extent of differential expression between TN and CD11b is different between surface and secreted compartments. Both cell types are similar in the secreted compartment, but TN DC have a larger extent of differential expression of surface DEG than CD11b. This suggests that TN DC have a greater cell-to-cell contact role in T_H2 responses

than CD11b+ DC. Given the involvement of both TN and CD11b+ DC in T_H2 immune responses, the inability of CD11b+ DC to induce optimal T_H2 responses alone, the importance of surface interactions in T cell activation, and the higher proportion of secreted protein associated DEG seen in Nb+ CD11b+ DC, this could indicate that CD11b+ DC take on a mostly secretory role in T_H2 responses and act to support TN DC, who could have more surface cell-to-cell communication with T cells.

Protein superfamilies are a classification tool based upon the structural motif of proteins, which has a particular function. Examining the surface and secreted DEG superfamilies of Nb positive TN and CD11b+ DC showed that TN DC upregulate almost 4 times the number of surface associated superfamilies than secreted associated superfamilies and downregulate almost double the number of secreted superfamilies than the secreted superfamilies they upregulate. CD11b+ DC upregulate slightly more surface superfamilies than secreted superfamilies and conversely downregulate more surface superfamilies than secreted superfamilies. The differences in numbers of surface and secreted superfamily regulation further suggests that TN DC perform a more surface focussed role and CD11b DC perform a more secretory role in T_H2 responses. Nb positive TN DC upregulate a relatively large proportion of surface genes associated with the signal transduction function and secreted genes associated with the protease function. This could indicate that Nb positive TN are more inclined toward specific intracellular modulation from the T_H2 stimulus and explain why Nb primed TN DC are able to prime naïve T_H cells to become T_H2 cells in the absence of additional antigen[18]. Nb positive CD11b+ DC upregulate a relatively large proportion of surface cell adhesion genes and secreted immune response genes. This indicates that CD11b+ DC could be adhering to T cells and providing secretory signals to support the surface signalling that TN DC are likely providing to T cells by the large amount of surface DEG.

The plexin/semaphorin pathway and the serpin protein family were specifically clustered out during functional annotation clustering of Nb positive TN DC. Plexins are receptors for Semaphorin proteins which have been shown to be important in neurological development, and more recently in some immune responses[55-57]. There is limited research into the function of semaphorins in T_H2 responses[171-173], but specific genes such as *Plxnd1* on TN DC and *Nrp1* on both TN and CD11b+ DC are predicted to interact with *Sema4a* by String analysis. TN and CD11b+ DC also

upregulate *Sema7a*, which is predicted to interact with *Itga1* and *Itgb1*, both of which are upregulated by T cells. *Sema7a* has been implicated in the induction of airway hyper-reactivity and contact hypersensitivity[241,242], assumedly through *Itga1* and *Itgb1*, but its role has not been examined in helminth T_H2 models. It would be interesting to determine if this interaction between *Sema7a* and its integrin receptors remains important in helminth induced T_H2 responses. Serpins are inhibitors of proteases which control a large array of biological processes including blood clotting and inflammation[170]. Serpins have been implicated in the protection of T cells from their own toxic proteins[243], but the role of specific serpins in T_H2 priming is not known. As serpins were clustered out during functional analysis, it is likely they have a role in T_H2 priming. It would be interesting to explore the effect various serpins have on T_H2 priming, and if they are required for normal T_H2 responses to develop.

3.5 Conclusions

In this chapter, I have demonstrated that Nb positive TN and CD11b+ DC are transcriptionally distinct from each other. These differences lie primarily in the functional compartments that each cell population possesses. Nb positive TN DC upregulate signal transduction genes for a wide variety of pathways, cell adhesion molecules focusing on the Semaphorin/Plexin pathway, and proteases, including protease inhibitors in the Serpin family. CD11b DC upregulate a larger proportion of cell adhesion molecules and have a large variety of secreted immune response proteins with a multitude of functions. The genes that both cell groups share show a function that is intermediate of the genes unique to the cell populations.

4. Predicted Protein Interaction Analysis of T Cells and Dendritic Cells from T_H2 Stimulating Conditions

4.1 Introduction

T_H cells are an important class of cells that are involved in adaptive immunity. Once activated, T_H cells have a multitude of functions, depending on the subclass of T_H cell, and are highly variable in their surface and secreted protein profile. T_H0 cells are naïve T_H cells that are yet to encounter antigen. Upon activation, T_H0 cells differentiate into one of various different lineages, including T_H2 cells. T_H2 cells are mediators of the highly important T_H2 cell response that is responsible for immune responses towards helminths and inducing allergy. T_H0 differentiation and fate decision is thought to mediated by DC interacting with T cells through the various ways that cells can communicate with each other, the major pathways being cell to cell contact and paracrine signalling[140]. The role of DC in T_H2 differentiation has been shown to involve TSLP/TSLPR, OX40/OX40L, CCL22/CCR4, and IL-33/ST2, although these interactions are model specific.

DC:T cell contact signalling is driven by surface molecules interacting with each other between cells. These interactions often lead to intracellular signal transduction which enables T_H0 cells to become activated and differentiate into T_H2 cells. Proteins that dendritic cells express are dependent on the pathogen that dendritic cells encounter and act to reinforce T_H0 differentiation into T_H effector cells. The surface and secreted signalling pathways dendritic cells use are important in T_H2 cell differentiation and activation[54,89,79]. These include the strength of TCR signalling[248,251], the presence of IL-4 and IL-2[249-250], the absence of IL-12 and IFN-γ[249-250], and the stimulation received by costimulatory pairs such as CD80/86 – CD28 and OX40 – OX40L[252-254]. These pathways lead to an increase of GATA3 through the activation of STAT5, ending in the activation of the NF_KB and NFAT pathways. Examining these intracellular pathways used by T_H0 and T_H2 cell surface genes would help identify potentially important T_H2 pathways and genes.

Protein-protein interaction networks (PPIs) are used to generate predicted interactions of proteins based upon the confidence level assigned to the interaction, which relies on large databases of protein co-occurrence, experimental evidence, and data mining[122,140]. By identifying predicted interacting surface and secreted pairs between TN/CD11b+ DC and T_H0/T_H2 cells, specific pairs can be identified and explored in protein binding analyses, such as flow cytometry, and functional analyses.

4.2 Aims

I hypothesise that by analysing the pathways used by T_H0 and T_H2 surface associated genes in the context of the DC:T cell interactome, I will be able to identify genes that are likely to be important in a T_H2 priming event. In doing so, I will be able to generate predicted interactions between TN/CD11b+ DC surface and secreted genes and T_H0/T_H2 surface genes. Additionally, I hypothesise that I would be able to rank predicted interactions by biological significance, which I will then explore the involvement of in T_H2 responses using flow cytometry.

Specific Aims:

- To select genes from T_H0 and T_H2 surface genes to investigate from pathway analysis.
- To determine predicted interactions between TN/CD11b+ DC and T_H0/T_H2 cells.
- To rank interactions by biological significance and use this to select specific interactions for analysis.

4.3 Results

4.3.1 T_H0 and T_H2 cells have many predicted interactions with Nb positive TN and CD11b+ DC

T_H0 and T_H2 cells use intracellular signalling pathways in response to surface receptor interactions that cause change in their function, particularly T_H0 cells as they become activated. Therefore, examining the signalling profile of T_H0 and T_H2 surfaceome data would be useful in identifying genes with potential importance in T_H2 priming. The T_H0 surfaceome data is derived from a cell surface proteomic screen conducted by Damaris Bausch-Fluck et al (2015)[244]. In short, cell surface proteins were captured and tagged using an oxidative and coupling reaction at the proteins' glycosylated sites using biotin. This allows protein fragments to be detected via mass spectrometry and subsequently quantified to determine the relative abundance of proteins. T_H2 surfaceome data was generated by J. Chandler in the laboratory of Prof. Le Gros, Malaghan Institute of Medical Research. T_H2 cells were extracted from 4C13R mice 7 days after Nb or PBS intradermal immunization, which corresponds to the peak time of T cell responses. 4C13R mice cells express AmCyan when expressing IL-4, and DsRed when expressing IL-13. AmCyan-IL4+ T_H2 cells were purified using flow assisted cell sorting and RNA was prepared and sequenced using the Illumina RNA sequencing platform.

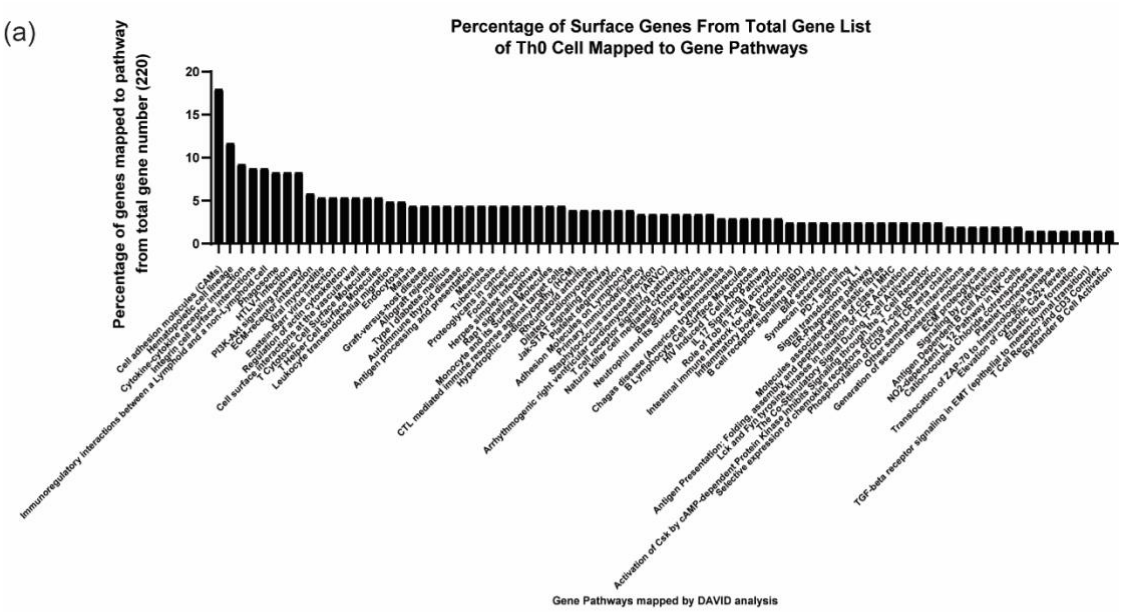
To investigate the intracellular pathways used by T_H0 surface proteins and T_H2 surface associated DEG (Log2FC > 1 P < 0.05), I used the pathway analysis tool from the online database DAVID, which groups genes into intracellular pathways they have previously been associated with. Gene sets from both T_H0 and T_H2 samples were analysed. T_H0 proteins are involved with almost double the number of pathways as T_H2 genes (T_H0 78 pathways vs. T_H2 45 pathways) (**Figure 4.1a** and **b**). Interestingly, 35 pathways are shared between T_H0 and T_H2 surface genes. There are shared pathways that T_H2 cells use at a greater proportion than T_H0 cells, including cytokine-cytokine receptor interaction, the JAK-Stat signalling pathway, PD-1 signalling, and signalling by interleukins (**Figure 4.1c)**. There was a small number of pathways that T_H0 surface genes used at a greater proportion than T_H2 surface genes, including cell adhesion molecules and immunoregulatory interactions between a lymphoid and non-lymphoid cell. Many pathways were irrelevant to the T_H2 response, such as Epstein-Barr virus infection, measles, and proteoglycans in

cancer. I selected pathways from T_H0 and T_H2 analysis to determine the individual genes involved. Shared pathways were: cell adhesion molecules[208-210], cytokine-cytokine receptor interactions[245], ECM-receptor interactions[211-213], JAK-Stat signalling[206,207], PI3K-Akt signalling[214,215], immunoregulatory interactions between a lymphoid and non-lymphoid cell[246,247], integrin cell surface interactions[216,217], and other semaphorin interactions[55-57]. From pathways only T_H0 cells used I chose: the co-stimulatory signal during T-cell activation[223-226], Lck and Fyn tyrosine kinases in initiation of TCR activation[227,228], generation of second messenger molecules[229-231], elevation of cytosolic Ca^{2+} levels[232-234], cation coupled chloride co-transporters[235-237], and Rap1 signalling pathway[238-240]. From pathways only T_H2 cells used I chose: TNFs bind to their physiological receptor[255,256], axon guidance[257], and interleukin-2 family signalling[258,259].

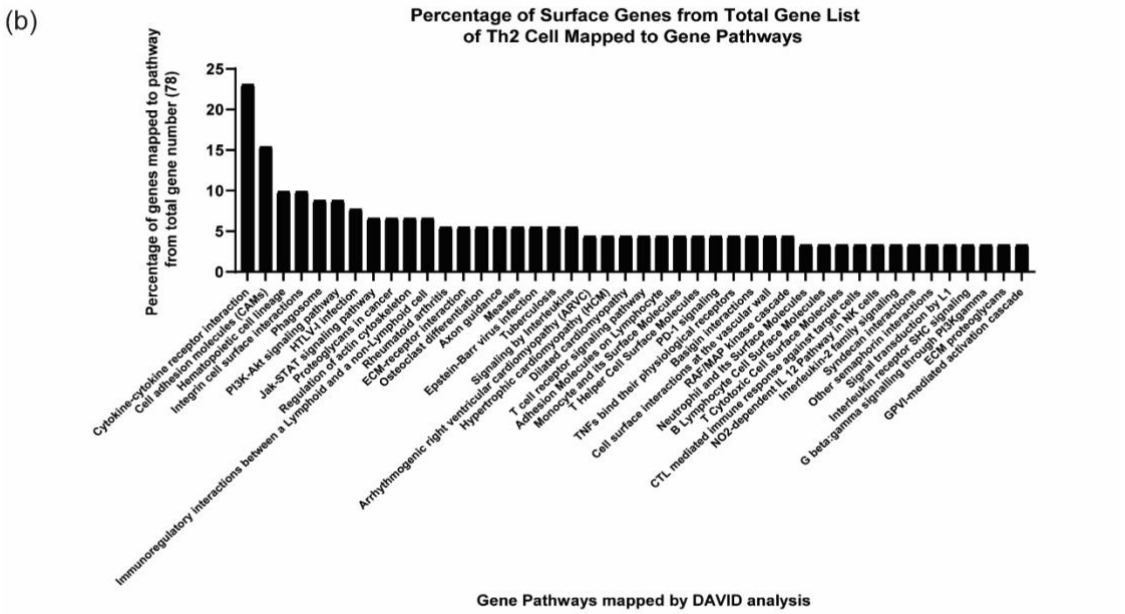
To explore the predicted interactions between surface genes upregulated by T_H0/T_H2 cells and surface/secreted genes upregulated by Nb positive TN and CD11b+ DC, I used the String database through the Cytoscape integrated development environment (IDE). I found that at a high confidence of 0.9 there was 336 total interactions predicted. Some of these predicted interactions have been observed as receptor-ligand pairs such as Pdcd1-Pdcd1lg2 and Sirpα-CD47. However, 91% predicted interactions shown in **Figure 4.2** and **Supplementary Figure 4** are unknown in their role during a T cell immune response if the interaction occurs.

To rank interactions, I calculated the expression product of each predicted interaction (**Figure 4.3** and **Supplementary Figure 4**). The expression product is a simple way to assign biological importance based on the product of Log2FC or VSTpk values of each interaction. The higher the expression product, the higher the importance of the interaction. The surface T_H0 genes were found from proteomic data, which only had values of log2 of protein expression. These were multiplied by the VSTpk values of their predicted interactors. I found that many interactions with high expression products are interactions known to be involved in immune reactions, such as CD80/CD28, Alcam/CD6, and CD200/CD200r1 (**Supplementary Figure 6**). This indicates that interactions with high expression products are more likely to be involved or important in immune reactions, and so represent a more focused list of predicted interactions to begin preliminary analysis on.

(a)


Percentage of Surface Genes From Total Gene List of Th0 Cell Mapped to Gene Pathways
Percentage of genes mapped to pathway from total gene number (220)
Gene Pathways mapped by DAVID analysis

(b)


Percentage of Surface Genes from Total Gene List of Th2 Cell Mapped to Gene Pathways
Percentage of genes mapped to pathway from total gene number (78)
Gene Pathways mapped by DAVID analysis

(c)

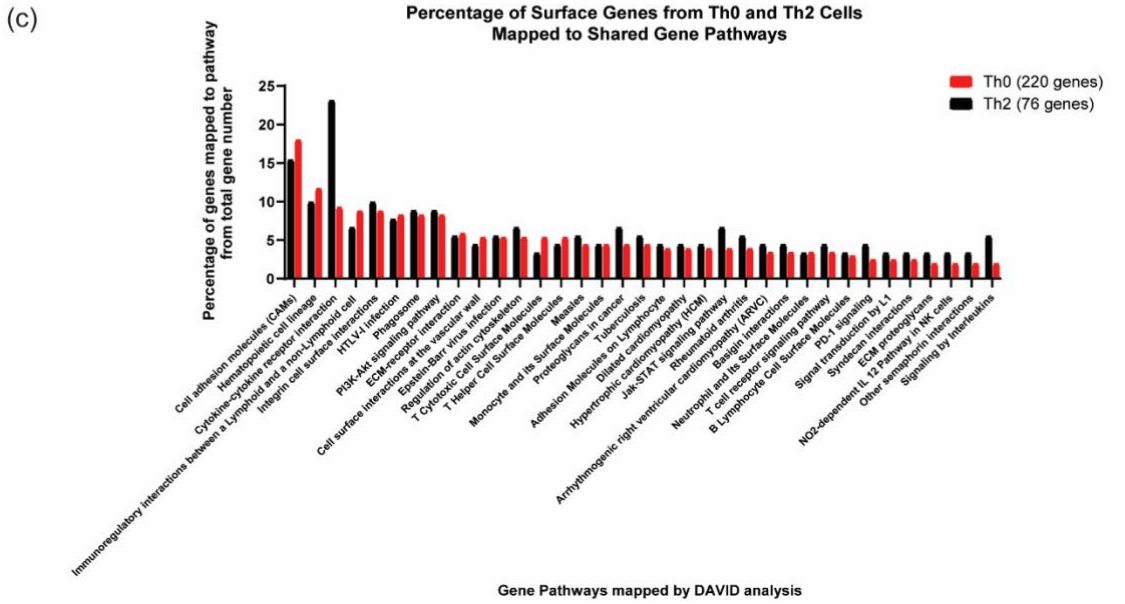

Percentage of Surface Genes from Th0 and Th2 Cells Mapped to Shared Gene Pathways
Th0 (220 genes)
Th2 (76 genes)
Percentage of genes mapped to pathway from total gene number
Gene Pathways mapped by DAVID analysis

Figure 4.1: Pathway Analysis of T_H0 proteins and T_H2 Genes using the DAVID Database. Genes were uploaded to DAVID and used to perform pathway analysis using the KEGG, Reactome, and Biocarta databases. (a) T_H0 and (b) T_H2 pathway proportions were plotted and used to generate (c) the proportions of shared pathways between T_H0 and T_H2 cells.

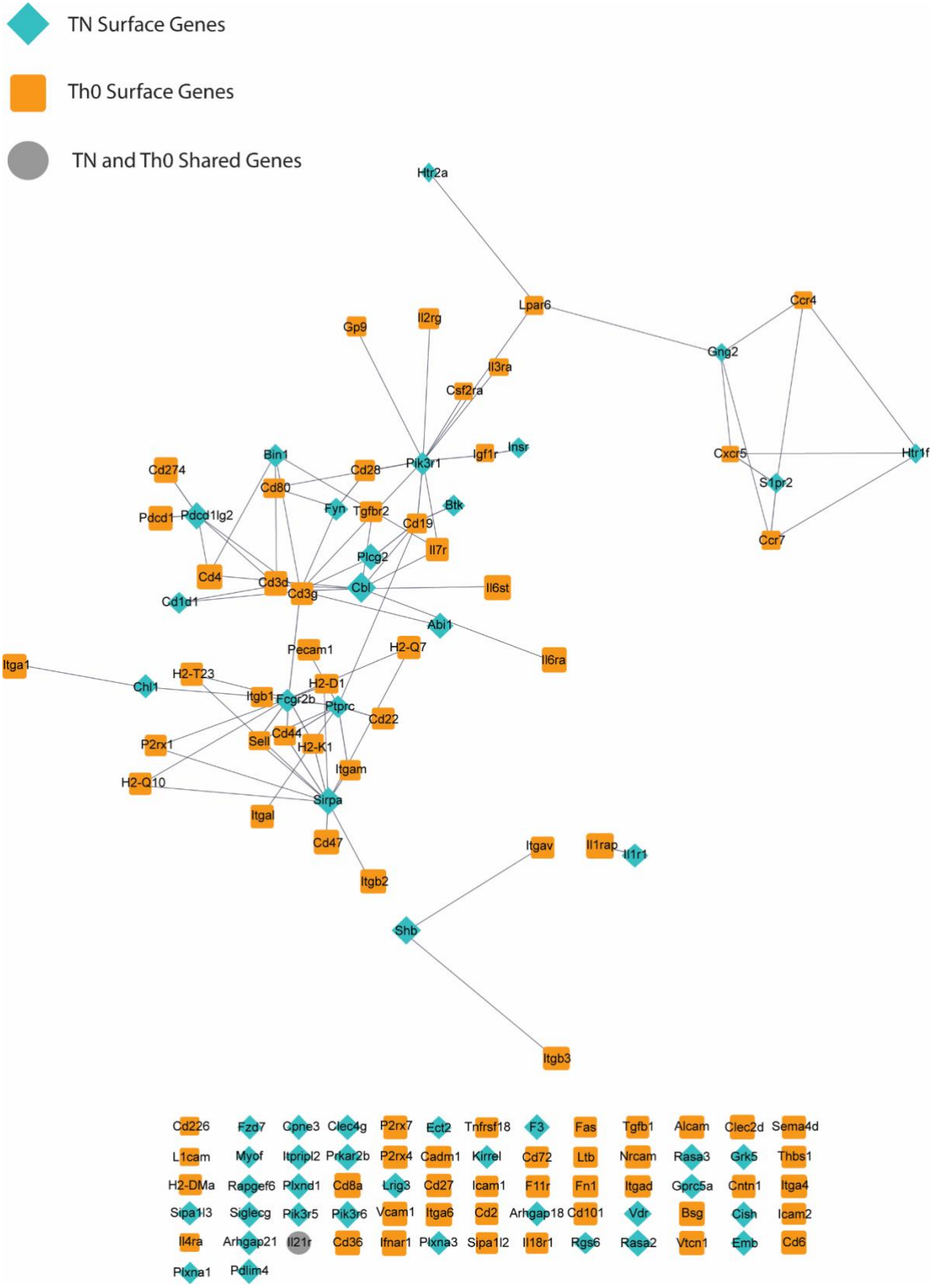

TN Surface Genes
Th0 Surface Genes
TN and Th0 Shared Genes
Htr2a
Lpar6
Ccr4
Il2rg
Gp9
Gng2
Il3ra
Csf2ra
Bin1
Igf1r
Insr
Pik3r1
Cxcr5
Htr1f
Cd274
Cd28
S1pr2
Cd80
Pdcd1
Pdcd1lg2
Fyn
Tgfbr2
Btk
Cd19
Ccr7
Il7r
Plcg2
Cd4
Cd3d
Cbl
Il6st
Cd3g
Cd1d1
Abi1
Pecam1
H2-Q7
Itga1
Il6ra
H2-T23
Chl1
H2-D1
Itgb1
Fcgr2b
Ptprc
Cd22
Cd44
Sell
P2rx1
H2-K1
Itgam
H2-Q10
Sirpa
Itgal
Cd47
Itgav
Il1rap
Il1r1
Itgb2
Shb
Itgb3
Cd226
Fzd7
Cpne3
Clec4g
P2rx7
Ect2
Tnfrsf18
F3
Fas
Tgfb1
Alcam
Clec2d
Sema4d
L1cam
Myof
Itpripl2
Prkar2b
P2rx4
Cadm1
Kirrel
Cd72
Ltb
Nrcam
Rasa3
Grk5
Thbs1
H2-DMa
Rapgef6
Plxnd1
Cd8a
Lrig3
Cd27
Icam1
F11r
Fn1
Itgad
Gprc5a
Cntn1
Itga4
Sipa1l3
Siglecg
Pik3r5
Pik3r6
Vcam1
Itga6
Cd2
Arhgap18
Cd101
Vdr
Bsg
Cish
Icam2
Il4ra
Arhgap21
Il21r
Cd36
Ifnar1
Plxna3
Sipa1l2
Il18r1
Rgs6
Rasa2
Vtcn1
Emb
Cd6
Plxna1
Pdlim4

Figure 4.2: Triple Negative and T_H0 surface PPI as an example PPI. TN surface genes and T_H0 surface genes were loaded into the String database using Cytoscape as an IDE. All interactions internal to each list were removed and using a confidence of 0.9, all interactions between gene lists were mapped to generate the PPI.

TN Gene Name	TN VSTPk	Th0 Gene Name	Th0 L2Ex	Expression Product
Pik3r1	10.51333333	Cd3g	23.737527	249.5605303
Plcg2	10.11333333	Cd3g	23.737527	240.0655196
Sirpa	9.596666667	H2-K1	23.426957	224.8206942
Abi1	9.273333333	Cd3g	23.737527	220.1259973
Cd1d1	9.19	Cd3g	23.737527	218.1478701
Cd1d1	9.19	Cd3d	21.854907	200.8465923
Pik3r1	10.51333333	Cd28	18.863337	198.3165461
Pik3r1	10.51333333	Gp9	18.831909	197.9861387
Sirpa	9.596666667	H2-Q10	20.491523	196.6503189
Fyn	8.143333333	Cd3g	23.737527	193.3025921
Cbl	8.11	Cd3g	23.737527	192.5113413
Pik3r1	10.51333333	Igf1r	17.97635	188.9913596
Pik3r1	10.51333333	Il3ra	17.47775	183.7494116
Ptprc	10.1	Pecam1	18.021217	182.0142883
Sirpa	9.596666667	Cd47	18.931083	181.6752964
Sirpa	9.596666667	H2-T23	18.76508	180.0822177
Ptprc	10.1	Sell	17.79839	179.763739
Pik3r1	10.51333333	Il7r	16.904243	177.7199449
Cbl	8.11	Cd3d	21.854907	177.2432931
Pdcd1lg2	7.433333333	Cd3g	23.737527	176.4489482
Ptprc	10.1	Itgal	17.40488	175.789288
Sirpa	9.596666667	Sell	17.79839	170.805216
Sirpa	9.596666667	Itgb2	17.290583	165.9319647
Bin1	6.986666667	Cd3g	23.737527	165.8461863
Sirpa	9.596666667	H2-Q7	17.19818	165.0452007
Pdcd1lg2	7.433333333	Cd3d	21.854907	162.4548062
Insr	8.96	Igf1r	17.97635	161.068096
Pik3r1	10.51333333	Il2rg	15.31709	161.0336728
Pik3r1	10.51333333	Cd19	15.21213	159.9301933
Pik3r1	10.51333333	Csf2ra	14.86541	156.2850104
Pik3r1	10.51333333	Lpar6	14.84406	156.0605508
Plcg2	10.11333333	Cd19	15.21213	153.8453413
Ptprc	10.1	Cd19	15.21213	153.642513
Fyn	8.143333333	Cd28	18.863337	153.6104382
Bin1	6.986666667	Cd3d	21.854907	152.6929479
Cbl	8.11	Cd4	18.286483	148.3033798
Ptprc	10.1	Itgam	14.606347	147.5241013
Sirpa	9.596666667	H2-D1	15.12411	145.1410423
Ptprc	10.1	Cd22	14.148143	142.8962477
Sirpa	9.596666667	Itgam	14.606347	140.1722402
Sirpa	9.596666667	P2rx1	14.44438	138.6179001
Cbl	8.11	Il6st	17.079175	138.5121093
Ptprc	10.1	Itgb1	13.584917	137.2076583
Cbl	8.11	Il7r	16.904243	137.0934134
Pdcd1lg2	7.433333333	Cd4	18.286483	135.9295261
Pdcd1lg2	7.433333333	Pdcd1	17.76249	132.034509
Shb	8.133333333	Itgav	16.14171	131.285908
Pdcd1lg2	7.433333333	Cd274	17.522837	130.2530859
Cbl	8.11	Il6ra	16.0313	130.013843
Pik3r1	10.51333333	Cd80	12.223	128.5044733
Bin1	6.986666667	Cd4	18.286483	127.7615636
Ptprc	10.1	Cd44	12.48885	126.137385
Cbl	8.11	Tgfbr2	15.32177	124.2595547
Cbl	8.11	Cd19	15.21213	123.3703743
Fcgr2b	5.056666667	Cd3g	23.737527	120.0327599
Sirpa	9.596666667	Cd44	12.48885	119.8513305
Fcgr2b	5.056666667	H2-K1	23.426957	118.4623109
Bin1	6.986666667	Il7r	16.904243	118.1043134
Shb	8.133333333	Itgb3	13.43568	109.276864
S1pr2	5.74	Ccr4	18.08422	103.8034228
Fcgr2b	5.056666667	H2-Q10	20.491523	103.618803
S1pr2	5.74	Cxcr5	17.42894	100.0421156
Fyn	8.143333333	Cd80	12.223	99.53596333
Fcgr2b	5.056666667	H2-T23	18.76508	94.88875454
S1pr2	5.74	Ccr7	15.999023	91.83439393
Btk	5.96	Cd19	15.21213	90.6642948
Fcgr2b	5.056666667	Sell	17.79839	90.00052544
Fcgr2b	5.056666667	H2-Q7	17.19818	86.96546354
Gng2	4.413333333	Ccr4	18.08422	79.81169093
Gng2	4.413333333	Cxcr5	17.42894	76.91972186
Fcgr2b	5.056666667	H2-D1	15.12411	76.47758291
Fcgr2b	5.056666667	P2rx1	14.44438	73.04041487
Gng2	4.413333333	Ccr7	15.999023	70.60902297
Gng2	4.413333333	Lpar6	14.84406	65.5117848
Fcgr2b	5.056666667	Cd44	12.48885	63.1519515
Il1r1	4.423333333	Il1rap	13.12965	58.0768185
Htr2a	0	Lpar6	14.84406	0
Htr1f	0	Ccr4	18.08422	0
Htr1f	0	Ccr7	15.999023	0
Htr1f	0	Cxcr5	17.42894	0
Chl1	0	Itga1	15.17914	0
Chl1	0	Itgb1	13.584917	0

Figure 4.3 Example of Expression Products from TN DC Surface Gene and T_H0 Surface Gene Predicted Interaction Expression Products. All TN DC surface gene VSTpk values were multiplied by the log2 of expression of T_H0 surface genes within each predicted interaction. The expression products of all interactions were arranged from highest to lowest, with documented receptor ligand pairs highlighted in yellow.

4.3.2 Flow cytometry analysis of Nrp1-AP fusion protein binding

Nrp1 is a gene upregulated by T_H2 cells that is predicted to bind to Plxna1 and Plxna3 on Nb positive TN DC. Plxna1 and Plxna3 are proteins of the Plexin family which are known as receptors for the Semaphorin family of proteins, certain family members of which have been implicated as being involved during certain T_H responses[55-57,147,171,172]. The semaphorin/plexin family was identified as a family of interest during cluster analysis of Nb positive TN DC signal transduction genes (**Figure 3.6**). As Nrp1 appears to be involved in this system, I wanted to determine whether it was expressed and enriched in a T_H2 immune response. Our laboratory has recently established a technique to generate fusion proteins of cell surface receptors, which can be used to assess expression and binding patterns of specific receptors during different immune responses. If Nrp1-AP showed preferential binding to migratory cDC2 cells, in particular TN DC, under T_H2 stimulating conditions, it would be a good candidate for further functional analysis to determine its role in T_H2 priming.

4.3.2.1 Production of Recombinant NRP1 and CTLA4 fusion proteins

I generated Nrp1-AP and CTLA4-AP fusion proteins. CTLA4-AP was used as a positive control of protein staining, as it had showed significant staining in previous experiments and CTLA-4 is a well-documented molecule known to be important in DC-T cell interactions[263]. Generation of fusion proteins was validated previously using CTLA4-Fc fusion protein via SDS-Page western blot (**Figure 4.4**), with the approximate molecular weight shown by western blot confirmed to be correct using the ExPasy database. NRP1-AP and CTLA4-AP fusion proteins were confirmed to have been generated via cell transfection by adding pNPP- substrate to cell supernatant. The AP portion of the fusion proteins breaks down pNPP to produce para-nitrophenol, creating a a clear yellow solution (**Figure 4.5**). Proteins were assumed to have been generated at the correct molecular weight due to protein generation and validation shown in **Figure 4.4**.

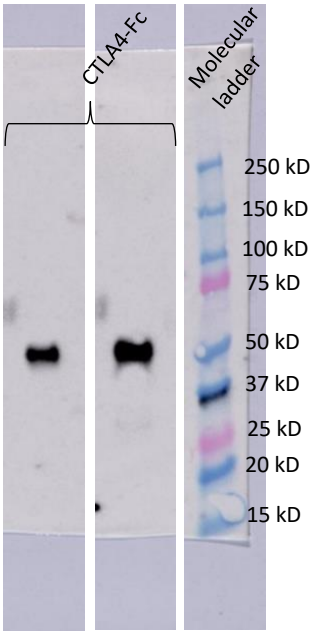


Figure 4.4 Western blot validation of CTLA4 fusion protein. CTLA4 fusion proteins were generated from custom plasmids and validated via SDS-Page Western blot. The approximate molecular weight of CTLA4 fusion protein was validated using the ExPasy database.

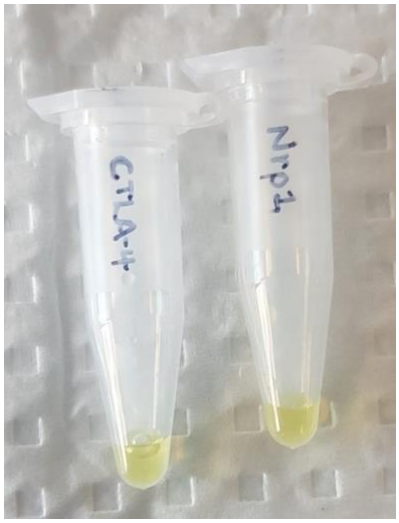


Figure 4.5 NRP1-AP and CTLA4-AP fusion protein validation. pNPP substrate was added to cell supernatant and left at room temperature for at least one hour. A clear yellow solution indicates the presence of fusion protein.

4.3.2.2 NRP1 fusion protein binding to DCs follow distinct types of immune responses

To determine whether Nrp1-AP showed preferential binding to DC that had been exposed to a T_H2 stimulus, I used fusion proteins generated to label DC isolated from skin draining lymph nodes of C57BL6 mice that were either treated with PBS, 300 non-viable L3 larvae (Nb, T_H2 stimulus) or heat-inactivated *Mycobacterium smegmatis* (Ms, T_H1 stimulus) by intradermal injection. On day 2 (peak of DC accumulation in LN), draining LN were isolated and processed for flow cytometry analysis. To compare AP-protein binding across cell types, each cell type analysed was gated out after gating on singlets, live cells, and cells of interest (**Figure 4.6a**) and were subsequently gated on to identify CD86/BST2 positive and AP-protein positive cells. Monocytes were determined as $Ly6C^+CD64^+$, resident DC were determined as $Ly6C^-$ $CD64^-$ $MHCII^{int}$ $CD11c^{hi}$, cDC1 were determined as $Ly6C^-$ $CD64^-$ $MHCII^{hi}$ $CD11c^{int}$ $Sirp\alpha^-$ $CD326^-$, CD11b+ DC were determined as $Ly6C^-$ $CD64^-$ $MHCII^{hi}$ $CD11c^{int}$ $Sirp\alpha^+$ $CD326^-$ $CD11b^+$, and TN DC were determined as $Ly6C^-$ $CD64^-$ $MHCII^{hi}$ $CD11c^{int}$ $Sirp\alpha^+$ $CD326^-$ $CD11b^{lo}$. The final CD86+ CTLA4-AP+, BST2+ CTLA4-AP+, CD86+ Nrp1-AP+, and BST2+ Nrp1-AP+ gates were determined by CD86, BST2, and AP-protein FMOs.

Ms immunization caused significantly higher cell counts of migratory DC, resident DC, and monocytes as compared to Nb and PBS immunization, while Nb immunization caused significantly greater number and frequency of monocytes as compared to PBS (**Figure 4.6b)**. Ms immunization had significantly higher TN DC and CD11b+ counts than Nb and PBS (**Figure 4.6c**). Ms immunization trended towards higher frequencies of CD11b+ DC than Nb, while both Ms and Nb had significantly higher frequencies of CD11b+ DC compared to PBS. Interestingly, Ms had significantly lower frequencies of TN DC than Nb and PBS, whilst Nb and PBS had similar frequencies (**Figure 4.6c**).

To identify mature DC populations, CD86 and BST2 were used as activation markers known to become upregulated following Nb treatment[17], CD86 as a general activation marker, and BST2 as a marker for type I interferon stimulation. Both Ms and Nb immunization had significantly higher CD86 MFI across all three migratory DC subtypes compared to PBS, with Ms immunization having higher CD86 MFI than Nb (**Figure 4.6d**). Ms trended towards higher BST2 MFI compared to Nb across all

migratory DC subtypes. Both Ms and Nb immunization had significantly higher BST2 MFI compared to PBS across all three DC subtypes (**Figure 4.6d**).

CTLA4-AP staining frequency on CD86+ monocytes was significantly lower than cDC1, CD11b+ DC, and TN DC and CD86+ TN DC had significantly lower frequency of CTLA4-AP staining than cDC1 and CD11b+ DC under Ms and Nb immunization. TN DC showed significantly greater MFI of CTLA4-AP staining than all other cell types only under Ms conditions, with a slight trend towards greater MFI seen under Nb immunization. The trend of significantly lower frequency of staining but significantly higher MFI on TN DC was continued under PBS conditions (**Figure 4.7a** and **b**). BST2+ TN DC had significantly lower CTLA4-AP staining than all other cells under Nb immunization and significantly lower staining than cDC1 under Ms conditions, but trended towards lower staining than all other cells. Conversely, TN DC CTLA4-AP MFI was significantly higher than other cell subtypes under both Ms and Nb conditioning. CTLA4-AP staining on TN DC was significantly lower than CD11b+ and cDC1 cells, but there was no change in MFI (**Figure 4.7c** and **d**).

CD86+ monocytes had significantly lower frequency of Nrp1-AP staining than cDC1 and CD11b+ DC and trended towards lower staining than TN DC, and had significantly higher MFI than all three other cell subtypes under both Ms and Nb immunization. CD86+ CD11b+ DC had significantly higher frequency of Nrp1-AP staining than other cells under all three conditions, but had no change in MFI (**Figure 4.8a** and **b**). BST2+ monocytes had significantly higher frequency of Nrp1-AP staining than the other cell subtypes under Ms immunization, with significantly higher MFI than cDC1 and a trend towards higher MFI than CD11b+ and TN DC. From Nb immunization, BST2+ monocytes had significantly higher frequency of Nrp1-AP staining than TN DC, and trended toward higher staining compared to CD11b+ DC and cDC1. However, there was no significant change in MFI, although a slight trend towards higher MFI on monocytes was observed. Nrp1-AP staining was significantly lower on BST2+ TN DC than other cell subtypes, with a trend towards lower MFI on TN DC observed (**Figure 4.8c** and **d**).

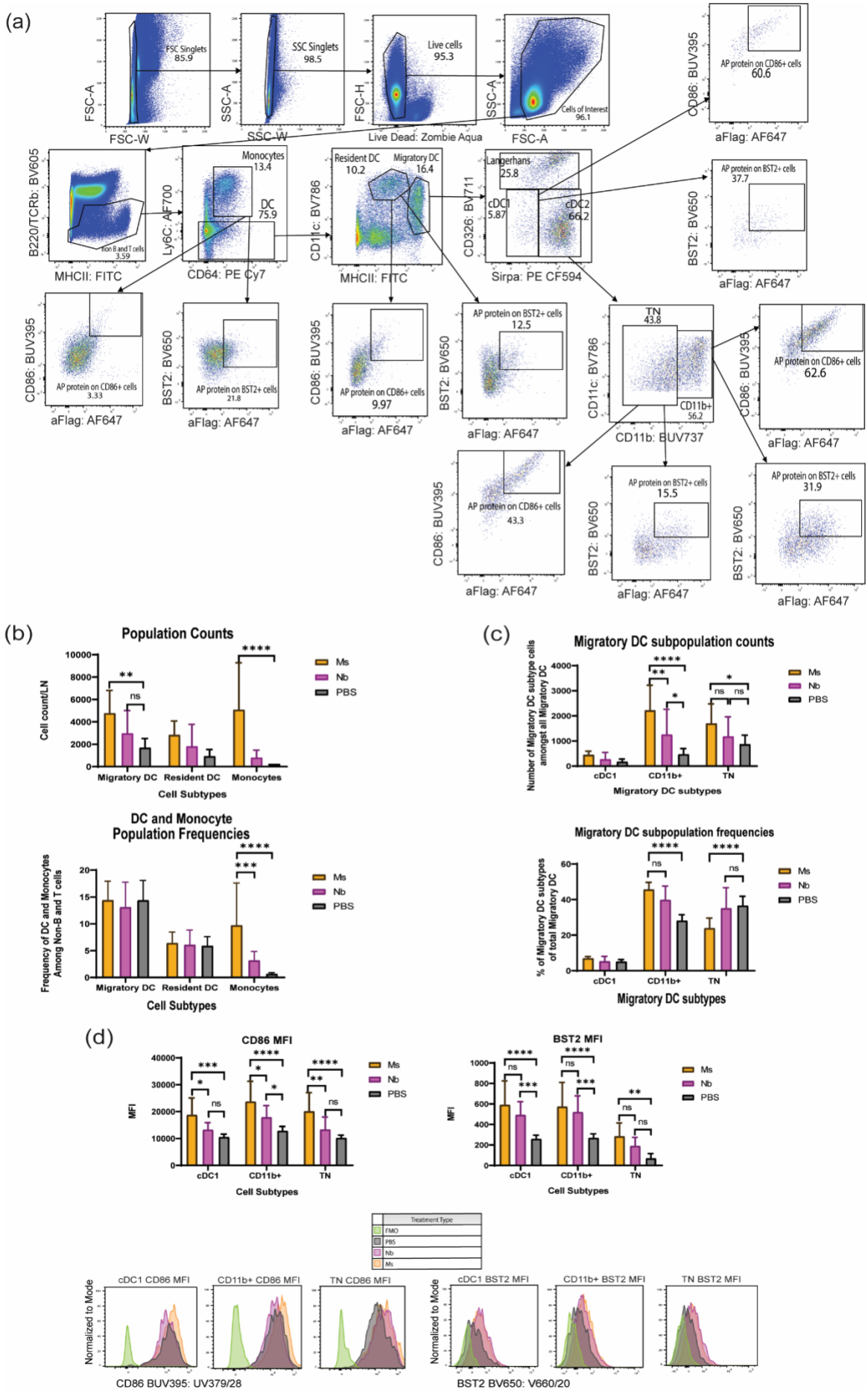
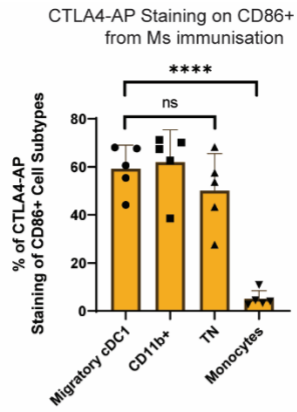
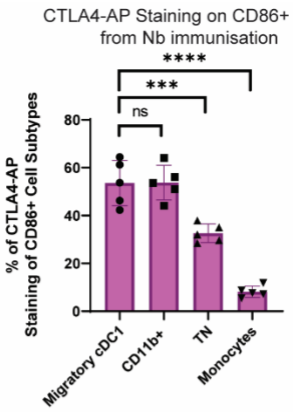
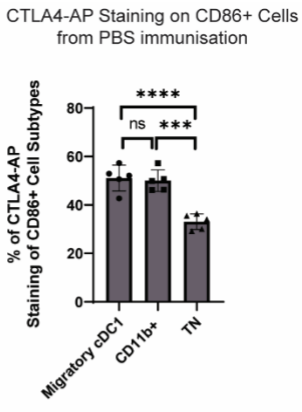

(a)
FSC Singlets
85.9
SSC Singlets
98.5
Live cells
95.3
Cells of Interest
96.1
Live Dead: Zombie Aqua
Non B and T cells
3.59
Monocytes
13.4
DC
75.9
Resident DC
10.2
Migratory DC
16.4
Langerhans
25.8
cDC1
5.87
cDC2
66.2
AP protein on CD86+ cells
60.6
AP protein on BST2+ cells
37.7
AP protein on CD86+ cells
3.33
AP protein on BST2+ cells
21.8
AP protein on CD86+ cells
9.97
AP protein on BST2+ cells
12.5
TN
43.8
CD11b+
56.2
AP protein on CD86+ cells
62.6
AP protein on CD86+ cells
43.3
AP protein on BST2+ cells
15.5
AP protein on BST2+ cells
31.9
(b)
Population Counts
Cell count/LN
Migratory DC
Resident DC
Monocytes
Cell Subtypes
Ms
Nb
PBS
DC and Monocyte
Population Frequencies
Frequency of DC and Monocytes
Among Non-B and T cells
(c)
Migratory DC subpopulation counts
Number of Migratory DC subtype cells
amongst all Migratory DC
cDC1
CD11b+
TN
Migratory DC subtypes
Migratory DC subpopulation frequencies
% of Migratory DC subtypes
of total Migratory DC
(d)
CD86 MFI
BST2 MFI
MFI
Cell Subtypes
Treatment Type
FMO
PBS
Nb
Ms
cDC1 CD86 MFI
CD11b+ CD86 MFI
TN CD86 MFI
cDC1 BST2 MFI
CD11b+ BST2 MFI
TN BST2 MFI
Normalized to Mode
CD86 BUV395: UV379/28
BST2 BV650: V660/20

Figure 4.6 Distinct Cell Population Enrichment and Activation Marker Upregulation Induced by Differing Immune Stimulants. (a) Example Gating Strategy used to identify monocytes, resident DC, migratory cDC1, TN DC, and CD11b+ DC to determine differences in protein binding across cell types. (b) Population counts and frequencies of cDC and monocytes. (c) Population counts and frequencies of migratory DC sub populations. (c) CD86 and BST2 MFI of migratory cDC subpopulations.

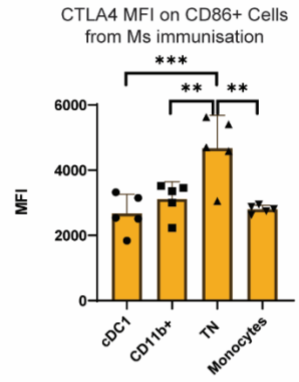
(a)

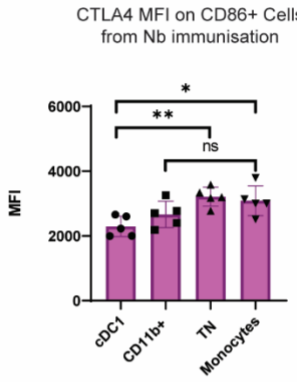

CTLA4-AP Staining on CD86+ Cells from Ms immunisation
% of CTLA4-AP Staining of CD86+ Cell Subtypes
****
ns
Migratory cDC1
CD11b+
TN
Monocytes

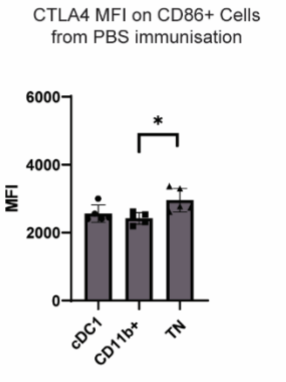

CTLA4-AP Staining on CD86+ Cells from Nb immunisation
% of CTLA4-AP Staining of CD86+ Cell Subtypes
****
***
ns
Migratory cDC1
CD11b+
TN
Monocytes


CTLA4-AP Staining on CD86+ Cells from PBS immunisation
% of CTLA4-AP Staining of CD86+ Cell Subtypes
****
ns
***
Migratory cDC1
CD11b+
TN

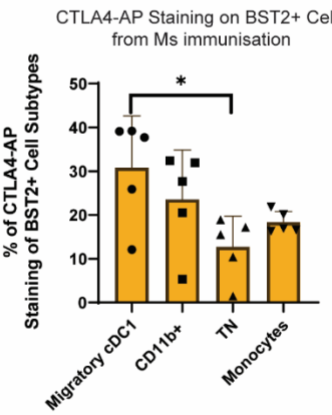
(b)

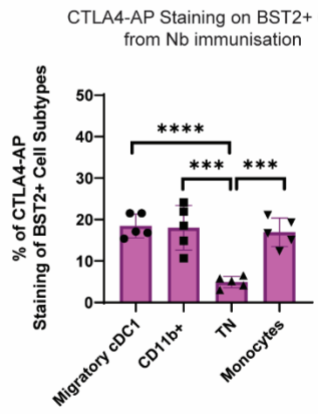

CTLA4 MFI on CD86+ Cells from Ms immunisation
MFI
***
**
**
cDC1
CD11b+
TN
Monocytes

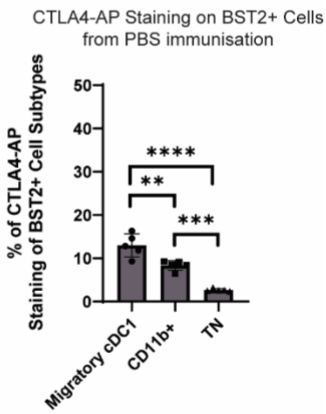

CTLA4 MFI on CD86+ Cells from Nb immunisation
MFI
*
**
ns
cDC1
CD11b+
TN
Monocytes


CTLA4 MFI on CD86+ Cells from PBS immunisation
MFI
*
cDC1
CD11b+
TN

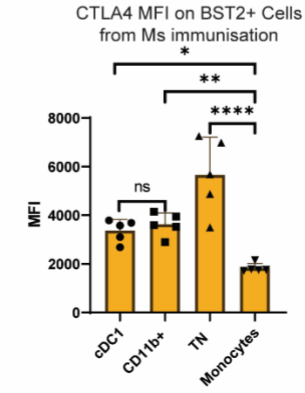
(c)

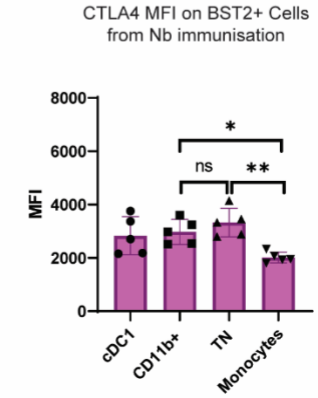

CTLA4-AP Staining on BST2+ Cells from Ms immunisation
% of CTLA4-AP Staining of BST2+ Cell Subtypes
*
Migratory cDC1
CD11b+
TN
Monocytes


CTLA4-AP Staining on BST2+ Cells from Nb immunisation
% of CTLA4-AP Staining of BST2+ Cell Subtypes
****
***
***
Migratory cDC1
CD11b+
TN
Monocytes


CTLA4-AP Staining on BST2+ Cells from PBS immunisation
% of CTLA4-AP Staining of BST2+ Cell Subtypes
****
**
***
Migratory cDC1
CD11b+
TN

(d)


CTLA4 MFI on BST2+ Cells from Ms immunisation
MFI
*
**
****
ns
cDC1
CD11b+
TN
Monocytes


CTLA4 MFI on BST2+ Cells from Nb immunisation
MFI
*
ns
**
cDC1
CD11b+
TN
Monocytes

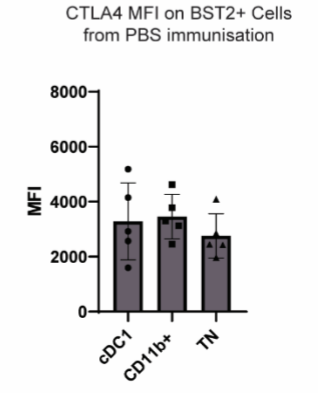
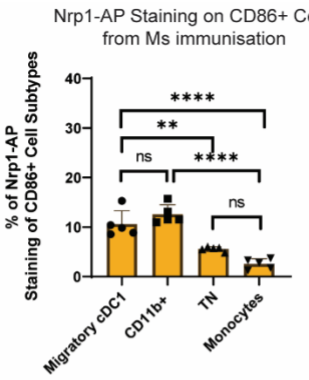
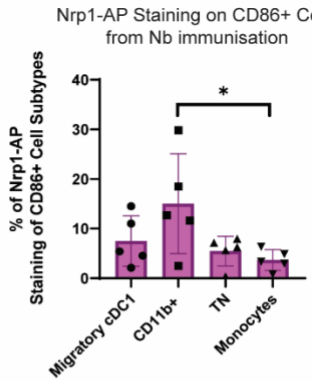
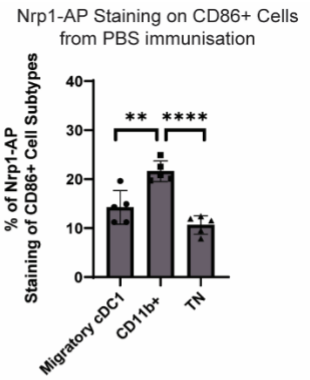

CTLA4 MFI on BST2+ Cells from PBS immunisation
MFI
cDC1
CD11b+
TN

Figure 4.7 CTLA4-AP Staining on Migratory cDC subtypes and monocytes. (a) CTLA4-AP staining frequency on CD86+ migratory cDC subtypes and monocytes. (b) CTLA4-AP MFI on CD86+ migratory cDC subtypes and monocytes (c) CTLA4-AP staining frequency on BST2+ migratory cDC subtypes and monocytes. (d) CTLA4-AP MFI on BST2+ migratory cDC subtypes and monocytes.

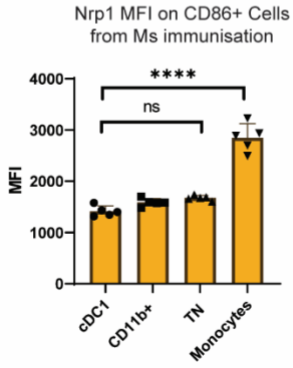
(a)

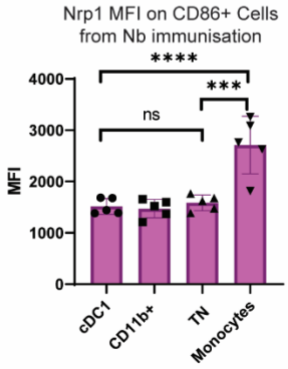

Nrp1-AP Staining on CD86+ Cells from Ms immunisation
% of Nrp1-AP Staining of CD86+ Cell Subtypes
****
**
ns
****
ns
Migratory cDC1
CD11b+
TN
Monocytes

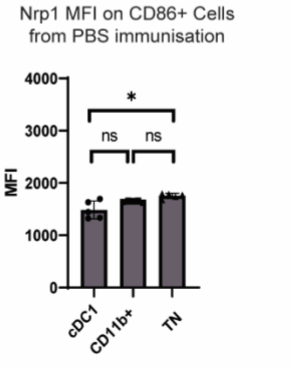

Nrp1-AP Staining on CD86+ Cells from Nb immunisation
% of Nrp1-AP Staining of CD86+ Cell Subtypes
*
Migratory cDC1
CD11b+
TN
Monocytes


Nrp1-AP Staining on CD86+ Cells from PBS immunisation
% of Nrp1-AP Staining of CD86+ Cell Subtypes
**
****
Migratory cDC1
CD11b+
TN

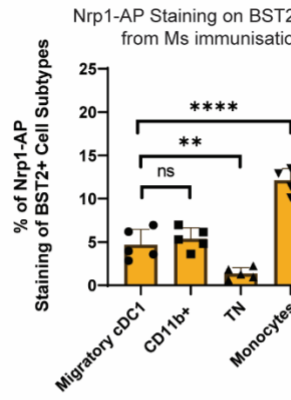
(b)

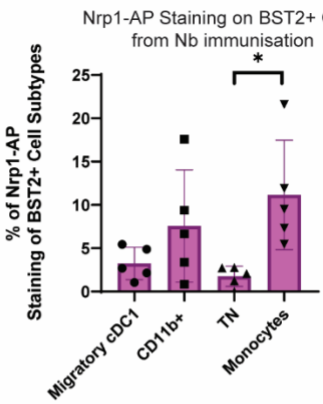

Nrp1 MFI on CD86+ Cells from Ms immunisation
MFI
****
ns
cDC1
CD11b+
TN
Monocytes

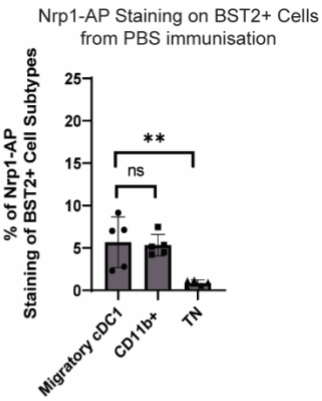

Nrp1 MFI on CD86+ Cells from Nb immunisation
MFI
****
***
ns
cDC1
CD11b+
TN
Monocytes


Nrp1 MFI on CD86+ Cells from PBS immunisation
MFI
*
ns
ns
cDC1
CD11b+
TN

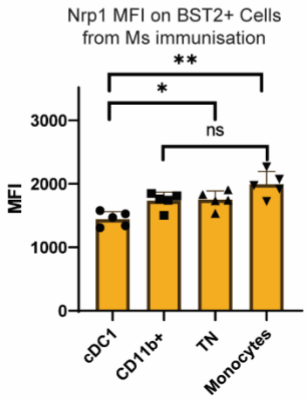
(c)

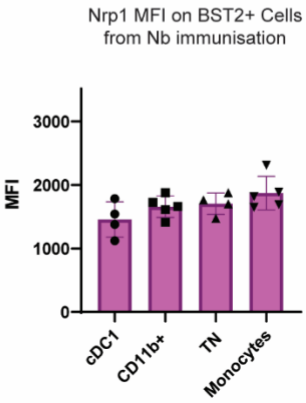

Nrp1-AP Staining on BST2+ Cells from Ms immunisation
% of Nrp1-AP Staining of BST2+ Cell Subtypes
****
**
ns
Migratory cDC1
CD11b+
TN
Monocytes


Nrp1-AP Staining on BST2+ Cells from Nb immunisation
% of Nrp1-AP Staining of BST2+ Cell Subtypes
*
Migratory cDC1
CD11b+
TN
Monocytes


Nrp1-AP Staining on BST2+ Cells from PBS immunisation
% of Nrp1-AP Staining of BST2+ Cell Subtypes
**
ns
Migratory cDC1
CD11b+
TN

(d)


Nrp1 MFI on BST2+ Cells from Ms immunisation
MFI
**
*
ns
cDC1
CD11b+
TN
Monocytes


Nrp1 MFI on BST2+ Cells from Nb immunisation
MFI
cDC1
CD11b+
TN
Monocytes

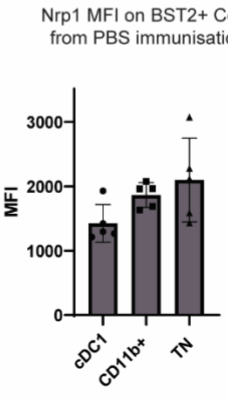

Nrp1 MFI on BST2+ Cells from PBS immunisation
MFI
cDC1
CD11b+
TN

Figure 4.8 Nrp1-AP Staining on Migratory cDC subtypes and monocytes. (a) Nrp1-AP staining frequency on CD86+ migratory cDC subtypes and monocytes. (b) Nrp1-AP MFI on CD86+ migratory cDC subtypes and monocytes (c) Nrp1-AP staining frequency on BST2+ migratory cDC subtypes and monocytes. (d) Nrp1-AP MFI on BST2+ migratory cDC subtypes and monocytes.

To compare fusion protein staining on migratory cells across conditions, a variation of the gating strategy was used (**Figure 4.9a**). Before the migratory DC cell subtypes were defined, all fusion protein positive migratory DC were gated on. Therefore, cDC1 were determined as fusion protein^{+} Ly6C^{-} CD64^{-} MHCII^{hi} CD11c^{int} Sirpα^{-} CD326^{-}, CD11b+ DC were determined as fusion protein^{+} Ly6C^{-} CD64^{-} MHCII^{hi} CD11c^{int} Sirpα^{+} CD326^{-} CD11b^{+}, and TN DC were determined as fusion protein^{+} Ly6C^{-} CD64^{-} MHCII^{hi} CD11c^{int} Sirpα^{+} CD326^{-} CD11b^{lo}.

Migratory DC had significantly higher counts of CTLA4-AP staining and MFI under Ms conditions as compared to Nb and PBS conditions. Frequency of Nrp1-AP staining on migratory DC was significantly higher under PBS conditions than Ms and Nb conditions, but there was no difference in number and MFI (**Figure 4.9b**). CD11b+ DC had significantly higher counts of CTLA4 staining under Ms conditions compared to Nb and PBS. Additionally, CD11b+ DC had significantly higher frequencies of CTLA4-AP staining under Ms and Nb conditions compared to PBS. Curiously, only TN DC under Ms conditions showed a significant increase in CTLA4-AP MFI compared to Nb and PBS conditions (**Figure 4.9c**). CD11b+ DC had significantly higher counts of Nrp1-AP staining under Ms conditions compared to both Nb and PBS conditions. CD11b+ DC had significantly higher frequencies of Nrp1-AP staining under Ms and Nb conditions compared to PBS. TN DC had significantly higher frequency of Nrp1-AP staining under PBS conditions compared to Ms conditions. Nrp1-AP MFI on CD11b+ DC under PBS conditions was significantly higher than Nb conditions (**Figure 4.9d**).

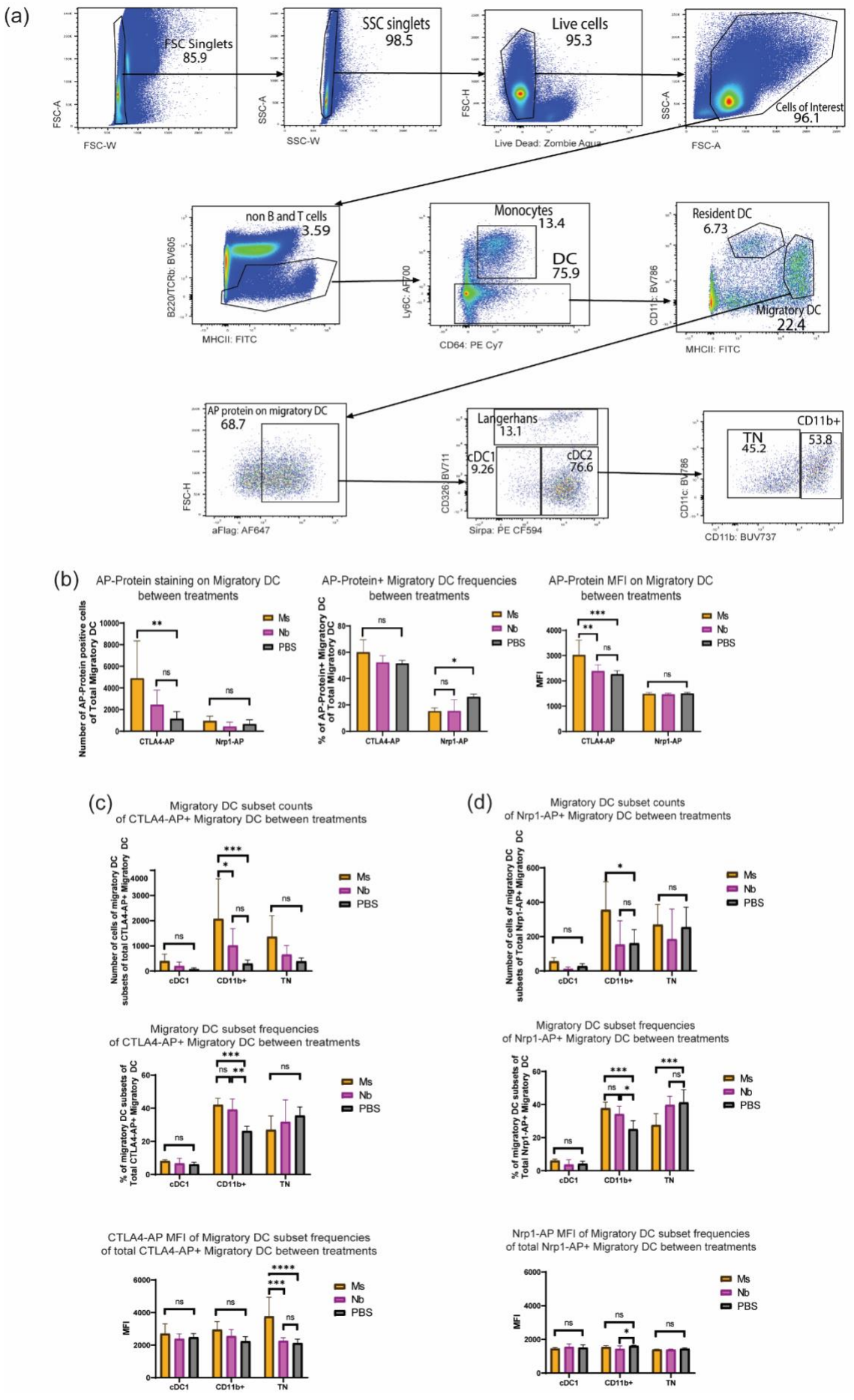

(a)
FSC Singlets
85.9
FSC-A
FSC-W
SSC singlets
98.5
SSC-A
SSC-W
Live cells
95.3
FSC-H
Live Dead: Zombie Aqua
SSC-A
Cells of Interest
96.1
FSC-A
non B and T cells
3.59
B220/TCRb: BV605
MHCII: FITC
Monocytes
13.4
DC
75.9
Ly6C: AF700
CD64: PE Cy7
Resident DC
6.73
CD11c: BV786
Migratory DC
22.4
MHCII: FITC
AP protein on migratory DC
68.7
FSC-H
aFlag: AF647
Langerhans
13.1
cDC1
9.26
cDC2
76.6
CD326: BV711
Sirpa: PE CF594
CD11b+
TN
45.2
53.8
CD11c: BV786
CD11b: BUV737
(b)
AP-Protein staining on Migratory DC between treatments
Number of AP-Protein positive cells of Total Migratory DC
Ms
Nb
PBS
CTLA4-AP
Nrp1-AP
AP-Protein+ Migratory DC frequencies between treatments
% of AP-Protein+ Migratory DC of Total Migratory DC
AP-Protein MFI on Migratory DC between treatments
MFI
(c)
Migratory DC subset counts of CTLA4-AP+ Migratory DC between treatments
Number of cells of migratory DC subsets of total CTLA4-AP+ Migratory DC
cDC1
CD11b+
TN
Migratory DC subset frequencies of CTLA4-AP+ Migratory DC between treatments
% of migratory DC subsets of Total CTLA4-AP+ Migratory DC
CTLA4-AP MFI of Migratory DC subset frequencies of total CTLA4-AP+ Migratory DC between treatments
(d)
Migratory DC subset counts of Nrp1-AP+ Migratory DC between treatments
Number of cells of migratory DC subsets of Total Nrp1-AP+ Migratory DC
Migratory DC subset frequencies of Nrp1-AP+ Migratory DC between treatments
% of migratory DC subsets of Total Nrp1-AP+ Migratory DC
Nrp1-AP MFI of Migratory DC subset frequencies of total Nrp1-AP+ Migratory DC between treatments

Figure 4.9 Distinct binding profiles of CTLA4-AP and Nrp1-AP on migratory DC subpopulations between treatment types. (a) Example Gating strategy to find migratory DC subpopulations amongst AP-protein+ DC. (b) CTLA4-AP and BST2-AP staining count, frequency, and MFI on migratory cDC between treatments. (c) CTLA4-AP staining count, frequency, and MFI on migratory cDC subtypes. (d) Nrp1-AP staining count, frequency, and MFI on migratory cDC subtypes.

4.4 Discussion

Armingol et al (2021), state that “multicellular life relies on the coordination of cellular activities, which depend on cell-cell interactions across an organism’s diverse cell types and tissues”[(140)]. This is especially true for T cells, which rely on surface protein signalling from dendritic cells to initiate adaptive immune responses. T_H2 cells are essential in the induction of allergic responses, yet current research lacks a definitive answer for what proteins cause T_H2 cells to differentiate from T_H0 cells. T_H0 cell to dendritic cell interactions involve intracellular pathways in the T_H0 cell to cause T_H2 differentiation, so it follows that investigating the pathways used by T_H0 and T_H2 surface molecules will likely lead to discovering new proteins important to T_H2 induction. In this chapter I have showed that analysis of genes involved in immunologically relevant pathways can create a protein interaction network that showcases all predicted interactions between proteins. These interactions can be ranked in order of biological significance, of which high biological significance is likely indicative of interactions important within the T_H2 response.

Armingol et al (2021), review the use of various ways to assign biological significance, termed a communication score, including the generation of the expression product[(140)]. The expression product is a continuous type communication score, which allows for more precise quantification of interactions than binary scores. The main assumptions of this method of assigning biological importance is that gene expression reflects protein abundance, and that protein abundance is proportional to the interaction strength. Despite these large assumptions, the expression product has found important interactions previously[(55 and 60 of 140)]. However, it is even more limited with my dataset as my data is bulk-RNA sequencing data, not the recommended single cell-RNA sequencing data. Additionally, as the T_H0 genes were found from proteomic data, it is not the same type of data as the rest of the genes used. This puts even more strain on the assumptions, as I am attempting to use protein expression with gene expression to determine the biological significance of interactions. Despite the limitations of the expression product, it is simple to generate, and I believe shows a good representation of the significance of interactions, as many interactions with high expression products are known to be involved in immune reactions.

Nrp1 had an expression product of just below four with its predicted partners, Plxna1 and Plxna3. These partners, being a part of the Plexin family, are assumed to be involved in immune reactions given that their typical binding partner, the semaphorins, have been shown to be involved in immune responses[55-57]. By investigating the binding profile of Nrp1 to various immune call populations under different stimulatory conditions, I could determine if it was likely to be a good candidate for importance in T_H2 responses, and also test if its binding reflected what is suggested from the RNA-sequencing data and the expression product.

Cytometric analysis of migratory DC, resident DC, and monocytes showed clear accumulation of monocytes by Ms and Nb immunization, which follows current understandings of monocyte kinetics under these immune conditions[174]. Interestingly, there was the lack of TN DC accumulation after Nb immunization, which has been well documented to occur[17,18,174]. This could indicate that the Nb immunizations were not administered well, as the frequency of TN DC in Nb immunizations was similar to PBS. Ms immunization cause increased accumulation of CD11b+ DC, seemingly at the expense of TN DC, indicating that CD11b+ DC are potentially involved during the initial T_H1 response. Increased CD86 MFI on migratory DC in Ms and Nb stimulation shows that they are activated, although Ms is higher than Nb, which makes sense as Ms is a much more inflammatory pathogen than Nb. BST2 MFI increase on cells in both Ms and Nb is also expected, as both pathogens induce a type 1 interferon response. This shows that the models are inducing immune responses as expected, aside from the lack of TN DC increase in Nb immunization.

CTLA4-AP, as a positive control protein, showed binding to ~60% of CD86+ cells, aside from CD86+ monocytes that showed very low frequency of CTLA4-AP staining in both Ms and Nb immunization. BST2+ monocytes however showed similar frequency of CTLA4-AP staining as the dendritic cell subsets. This indicates that monocytes could have lower CD86 expression than the dendritic cell subsets, or the CTLA4-AP protein is interfering with CD86 antibody staining only on monocytes for some reason. CD86+ and BST2+ TN DC show the highest MFI of CTLA4-AP compared to the other cell subsets in Ms treatment, perhaps indicating that TN DC are highly activated by Ms immunization. This trend of CD86+ and BST2+ TN DC is seen in Nb treatment, although it is to a much smaller extent, often just a trend of TN

DC having greater CTLA4-AP MFI than other cell subtypes. Given that both Ms and Nb immunization causes a type I interferon response, it could be that TN DC are more sensitive to type I interferons than cDC1, CD11b+ DC, or monocytes.

Monocytes again show low co-staining frequencies with CD86 and Nrp1-AP, so it is likely that monocytes have much lower CD86 expression than the DC subsets. Interestingly, although CD86+ monocytes have very low staining of Nrp1-AP, they show the highest Nrp1-AP MFI by a large margin in both Ms and Nb immunization. This trend is not seen in BST2+ monocytes despite BST2+ monocytes having the largest frequency of Nrp1-AP staining, indicating that there may be a positive correlation between Nrp1 receptor expression and CD86 expression on monocytes. TN DC show the lowest frequency of Nrp1-AP staining of both CD86+ and BST2+ cells in PBS conditions, yet have a trend to having a higher MFI of Nrp1-AP compared to cDC1 and CD11b+ DC, indicating that the receptor for Nrp1 is expressed more on TN DC than cDC1 and CD11b+ DC in naïve conditions.

CTLA4-AP has increased binding on migratory DC in Ms conditions as compared to Nb and PBS conditions, but Nrp1-AP only shows an increase in frequency on migratory DC in PBS conditions. This could mean that there are more migratory DC in PBS conditions that express the Nrp1 receptor compared to migratory DC in Ms and Nb conditions, but there is no change in the expression of Nrp1 receptor on cells between conditions as indicated by the MFI. CTLA4-AP staining on migratory cell subsets showed no change in cDC1, which was unsurprising as they are not involved in the immune response to Ms and Nb. CD11b+ DC showed an increase in count and proportion of CTLA4-AP staining in Ms and Nb conditions compared to PBS, but there was no change in MFI, suggesting that CD80/86+ CD11b+ DC become enriched when exposed to Ms or Nb, but they do not upregulate CD80/86. TN DC show a reversed trend to CD11b+ DC, with no significant change in CTLA4-AP staining count or frequency, but an increase in the MFI, suggesting that there is no specific enrichment of CD80/86+ TN DC in Ms and Nb conditions, but they do upregulate CD80/86, perhaps indicative of activation. Nrp1-AP staining on migratory DC subsets again showed no change on cDC1 in Ms and Nb conditions compared to PBS. There was an increase in the count and frequency of Nrp1-AP staining of CD11b+ DC in Ms conditions, and an increase in frequency in Nb conditions, but a decrease in MFI in Ms conditions, suggesting that CD11b+ DC actually downregulate

the Nrp1 receptor in inflammatory conditions. TN DC had a trend towards lower frequency and count of cells staining for Nrp1-AP in Nb compared to PBS, and a decrease in frequency in Ms conditions. There however was no change in MFI, indicating TN do not downregulate the Nrp1 receptor, but perhaps are decreasing in Nrp1-AP staining due to the influx of other cell types that do bind to Nrp1-AP in inflammatory conditions, monocytes in particular. All together these results suggest that there in an upregulation of Nrp1 receptor on monocytes in Ms and Nb immunization compared to PBS, perhaps in response to type I interferon signalling, and there is a downregulation of Nrp1 receptor on CD11b+ DC in Nb immunization, suggesting that Nrp1 receptor may be detrimental to CD11b+ DC involvement in T_H2 responses. There was no observable change in TN for Nrp1-AP staining, suggesting that the Nrp1 receptor does not affect the ability of TN DC in T_H2 responses.

This is in contrast to my communication score results, as there is an upregulation of Plxna1 and Plxna3 in Nb positive TN DC. I therefore believe that Nrp1 is not a suitable protein to explore the importance on in T_H2 priming. In order to explore the predicted interactions for their involvement in T_H2 responses, another measure of protein interaction would need to be used alongside the communication score to improve accuracy.

4.5 Conclusions

In this chapter I have identified T_H0 and T_H2 surface genes that are likely to be important in T_H2 priming based upon the intracellular pathways they are mapped to by the DAVID database. I have shown that these genes are predicted to have 336 interactions with Nb positive TN and CD11b+ DC surface and secreted genes at a high degree of confidence, and that these interactions can be ordered into biological importance based upon their calculated expression product that is somewhat comparable to known immunological interactions. Lastly, I have explored Nrp1-AP protein binding profile across multiple cell types using multiple immunizations of dead pathogens and concluded that Nrp1 shows little or no relevance in T_H2 immune responses.

5. General Discussion

5.1 Summary of Findings

DCs are central to the development of T cell immunity and play a critical role in determining the fate of a T cell. Importantly, the signals received by DCs during the initiation of a T_H2 immune response alter the DC transcriptional profile, which translate to molecular messages that instruct naïve T cells to differentiate into T_H2 cells. The interaction between a DC and a T cell largely occurs at the cell surface. Thus, I hypothesised that interrogation of the cell surface and secreted transcriptome of DCs that had acquired antigens derived from the potent T_H2 inducer, Nb, and the cell surface protein and transcriptional repertoire of naïve (T_H0) and T_H2 cells, respectively, could provide insight into the mechanisms or messages involved in T_H2 induction.

Analysis of the cell surface and secreted transcriptional profile of T_H2-associated DC subsets TN and CD11b+ DC DC2 that had been directly primed by T_H2 inducer Nb (Nb positive DC) are distinct from each other for both surface and secreted genes[16,17,25,26]. TN DC expressed significantly more regulated genes compared to CD11b+ DC after exposure to Nb. Furthermore, there was a clear distinction in the functional pathways associated with each subset. TN DC upregulate signal transduction genes for a wide variety of pathways, cell adhesion molecules focusing on the Semaphorin/Plexin pathway, and proteases, including protease inhibitors in the Serpin family. Whereas CD11b+ DCs upregulate a larger proportion of cell adhesion molecules alongside secreted immune response proteins with diverse functions. Among genes that were shared between the DC subsets, most associate with functions required for general activity of a cell (migration, survival etc.). Through the specific analysis of protein superfamily functions, I identified potential protein partners on DC in a T cell:DC interaction. These proteins of interest (which were only selected when expression values reached a level considered biologically relevant (VSTpk > 2)) were then further investigated by literature searches to determine whether there was a relationship with T cell responses (*Ltb-Cd40*, *Cd6*, *Alcam*, *Pvr*, *Adam8*, *Plxna1*, *Plxna3*, *Ramp3*, *Serpina3f*, *Serpinc1*, *Serpine1*, *Sema7a*, *S1pr3*, *Cxcr5*, *Adora2b*, *Cd200*, *Serpinb6b*, *Timp1*) .

This thesis also identified T_H0 and T_H2 surface genes that are likely to be important in T_H2 priming based upon the intracellular pathways they are mapped to by the DAVID database. I have shown that these genes are predicted to have 336

interactions with Nb positive TN and CD11b+ DC surface and secreted genes at a high degree of confidence (**Figure 4.2** and **Supplementary Figure 4**), and that these interactions can be ordered into biological importance based upon their calculated expression product that is comparable to known immunological interactions (*Sirpα-Cd47*, *Ptprc-Cd22*, *Pvr-Cd226*, *Alcam-Cd6*, *Ccl22-Ccr4*).

Nrp1 was predicted to bind to Plxna1 and Plxna3 (**Supplementary Figure 4a**), all of which are involved in the semaphorin/plexin system of proteins. This system was highlighted during cluster analysis of DC genes (**Table 3.1**), indicating potential importance in T_H2 induction. This thesis explored Nrp1-AP protein binding profile across cDC1, CD11b+ DC, TN DC, and monocytes using immunizations of Ms and Nb and determined that Nrp1 appears to bind in greater amounts to monocytes during an inflammatory response to Ms but shows little or no relevance in T_H2 immune responses.

5.2 Do Nb positive TN and CD11b+ DC exhibit transcriptional distinction?

T_H2 responses are responsible for the induction of allergic reactions and are thought to be dependent on TN and CD11b+ cDC2[16,25,26]. The dependency of T_H2 responses on these cDC2 lineages is apparent in the depletion of KLF4 DC in mice, as these mice have impaired T_H2 responses. Ochiai et al (2014), identified TN DC as a major contributor of T_H2 responses during DBP-FITC induced contact hypersensitivity. Kumamoto et al (2013) identified CD301b+ DC, a lineage that CD11b+ DC belong to, as essential in the induction of T_H2 adjuvant induced T_H2 responses. Whilst both cDC2 contribute to T_H2 responses, Connor et al (2017), have shown that they exhibit large transcriptional distinction following Nb immunization. This research examines total TN and CD11b+ populations, but does not examine an enriched antigen positive population, therefore this transcriptional distinction is not known to extend to DC that have directly taken up antigen. Current understanding of T_H2 priming extends to distinct molecules in addition to DC subsets. OX40L and TSLP in particular are heavily implicated in the induction of T_H2 responses[42,45,47,48,253]. However, current research has not fully elucidated the role of these proteins in T_H2 priming, given that suboptimal T_H2 responses can still form in the absence of OX40L[253], and certain helminth infections bypass TSLP to act directly on DC[46]. These proteins implicate a role for surface and secreted factors in

the induction of T_H2 responses, similar to other T_H responses. However, current research has been unable to determine a wholly reliable T_H2 priming protein.

Antigen positive TN and CD11b+ DC are highly functionally distinct, showing large differences in the number of DEG that they express. The distinction between TN and CD11b+ DC remains apparent when examining the variance between the transcriptional data using PCA, clustermaps, and volcano plots, as TN and CD11b+ DEG datasets display high levels of variation from each other. This variation seen between TN and CD11b+ DC could be solely due to intracellular DEG. However, when intracellular DEG were removed to further examine surface and secreted DEG, the variation between TN and CD11b+ DC remains, indicating that at least part of the transcriptional distinction is due to surface and secreted DEG.

TN and CD11b+ DC have observed functional differences in vivo, however current research has not fully distinguished the specific functions that each DC subset possesses. Similar trends of functional distinction were seen by examining the protein superfamilies and general functions of DEG. TN DC upregulated a significantly large proportion of surface signal transduction DEG compared to CD11b+ DC. Some signal transduction DEG such as *Cbl*, *Plcg2*, and *Bin1* are associated with positive regulation of Ca^{2+} signalling. Increase of Ca^{2+} concentration in DC is associated with general activation and maturation, and is even targeted by some microbes such as *Mycobacterium tuberculosis* to prevent this maturation[260,261]. Ca^{2+} signalling also regulates the generation of the secondary messenger sphingosine-1-phosphate (S1P)[260], which is capable of acting extracellularly on its receptors. A gene of one of these receptors, *S1pr2*, is upregulated by Nb positive TN DC and is associated with cell proliferation and survival.

Other TN signal transduction DEG, such as *Rasa3*, *Prkar2b*, and *Rasa2*, are associated with negative regulation of cAMP signalling, which is associated with many different kinds of cellular responses. There is evidence indicating that a lowered concentration of intracellular cAMP in DC skews T cell responses to favour T_H2 responses[262]. These signal transduction DEG in favour of increasing Ca^{2+} concentration and decreasing cAMP concentration specifically in antigen positive TN could explain why TN DC are capable of priming T_H2 responses when transferred to

naïve mice[18]. Additionally, given that these signal transduction DEG do not occur in antigen positive CD11b+ DC, this could also explain why CD11b+ DC are incapable of producing optimal T_H2 responses by themselves, as depletion of TN DC via a CD11c-Cre KLF4-Flox system caused a large reduction in T cell responses from T_H2 antigen immunization[26]. In addition to examining individual genes, cluster analysis also showed large numbers of clusters related to signal transduction pathways largely involved in cellular activation, including the semaphorin/plexin protein family, which has been implicated in immune responses including T_H2 responses. These DEG alongside cluster analysis, as well as a deficit in secretory DEG, suggest a largely surface interaction role of TN DC in T_H2 responses.

Antigen positive CD11b+ DC upregulate a large proportion of secreted immune response DEG as compared to TN DC. Some immune response DEG, including *Il1a* and *Il27* are involved in lymphocyte activation and inflammation. Others, such as *C1qa*, *C1qb*, and *C1qc*, are involved in the activation of the complement system. This implicates the complement system as having a role in T_H2 priming. Additionally, cluster analysis reveals a large number of clusters involved in complement activation, cellular response to proinflammatory cytokines, and chemokine mediate signalling pathway, further reinforcing a secretory role for CD11b+ DC.

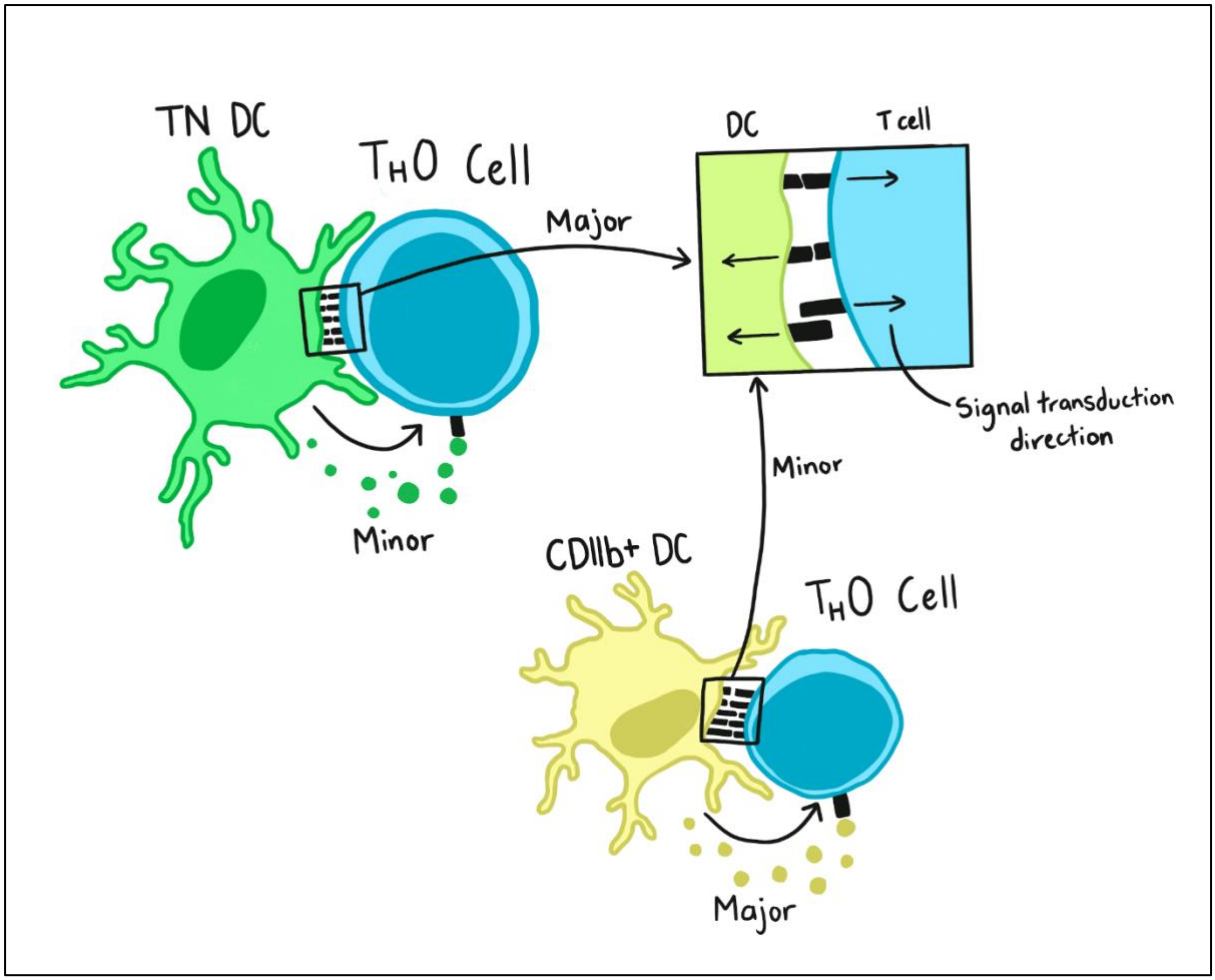


Figure 5.1. Proposed roles of TN and CD11b+ DC in T_H2 priming. Antigen positive TN DC are hypothesised to have a major surface interaction role with a minor secretory role. Antigen positive CD11b+ DC are hypothesised to have a major secretory role with a minor surface interaction role. Surface interactions can induce signal transduction in one direction, dither acting on the DC or T cell, or bidirectionally.

5.3 T cell pathway analysis and predicted interactors flow cytometry analysis

T_H0 cells use intracellular pathways after surface protein interactions to become activated and mature into specialised lineages such as T_H2 cells. Examination of the intracellular pathways associated with T_H0 and T_H2 surface genes shows that there are 35 pathways common to both cell types, many of which are involved in immunity such as cytokine-cytokine receptor interactions, cell adhesion molecules, and integrin-cell surface interactions. These pathways may represent activation pathways widely used by T cells to perform optimally, whereas pathways used only by T_H0 or T_H2 cells are likely involved in functions unique to each cell. Pathways used only by T_H0 cells, such as co-stimulatory signal during T-cell activation, Lck and Fyn tyrosine kinases in initiation of TCR activation, and generation of second messenger molecules, are related to T cell activation, whereas pathways used only by T_H2 cells, such as interleukin-2 signalling family, are involved in interleukin signalling and functions.

Genes of selected T_H0 and T_H2 genes were shown to have 336 predicted interactions with the surface of secreted genes of antigen positive TN and CD11b+ DC. However, only 9% of these predicted interactions are documented receptor-ligand pairs. The remaining predicted interactions are unknown to occur in vivo, and represent potential targets for further exploration into if they do occur and what their role is in T cell activation. Ranking interactions by expression product, and working under the assumption that high expression product is equivalent to high biological significance, provided a smaller number of potential targets that are more likely to have significance during T cell activation. Two such predicted interactions is Nrp1-Plxna1 and Nrp1-Plxna3. As mentioned previously, Nrp1 is a member of the plexin/semaphorin protein family and is involved in binding with both plexins and semaphorins, which have been implicated in driving airway hyperreactivity and contact hypersensitivity, conditions primarily driven by T_H2 responses[241,242]. Upon examination of Nrp1-AP binding across multiple cell types under multiple immune stimulatory conditions, it appeared that Nrp1 preferentially bound to monocytes, however, the proportion of Nrp1-AP binding was similar between different immune stimulatory conditions Ms (T_H1-like) and Nb (T_H2-like). In contrast, Nrp1-AP bound to a higher frequency of CD11b+ DC from mice treated with PBS, compared to primed CD11b+ DC, suggesting that the binding partners of Nrp1, Plxna1 and Plxna3, are

upregulated on monocytes and downregulated (marginally) on CD11b DC+ under inflammatory conditions. There was no change in binding observed on TN DC across conditions, which did not correlate with the transcriptional data. It is possible that there is no change in Plxna1 and Plxna3 translation under T_H2 conditions despite an upregulation of mRNA being observed. There also may be additional binding partners of Nrp1 that are not well documented, which could influence the binding profile of Nrp1-AP across cells types. Additionally, the amount of protein used in the experiments was not known. With the large number of monocytes that accumulated, particularly under Ms conditions, there may have not been enough protein to achieve saturation across all cell types, potentially limiting the observable binding profile. It would be interesting to attempt this experiment with differing concentrations of purified protein to see if similar binding profiles are observed. In a similar viewpoint, purifying TN and CD11b+ DC before staining with Nrp1-AP would also be interesting to see if the binding profile changes with the removal of the monocyte 'sink'. However, based on this experiment, it appears that the Nrp1-Plxna1 and Nrp1-Plxna3 are not good candidates for exploration into their potential role in T_H2 priming.

5.4 Limitations and Future Directions

There are important factors within this thesis that need to be considered before further analysis of protein partners between T_H0/T_H2 and TN and CD11b+ DC is explored. Firstly, this thesis primarily analysed the transcriptional data of the DC and T cell subsets. While there is some correlation between the amount and fold change of a gene with the abundance of its protein, there are additional factors controlling the abundance of a protein aside from the amount of its transcript, such as post-transcriptional silencing[189]. A micro-RNA screen for silencing RNA would be useful in tandem with messenger RNA sequencing, to determine what, if any, genes are being silenced[190].

Conversely, a lowly differentially expressed gene may have high translation under T_H2 conditions, resulting in greater abundance of the protein. There are several ways to determine protein abundance, such as detection of MFI shifts of markers by flow cytometry, a surface protein mass spectrometry screen, or ribosomal profiling to determine translation efficiency[191,192]. Flow cytometry and mass spectrometry would be the most accurate, as they determine physical protein amount, but are time consuming and costly, especially with the number of genes detected by mRNA-sequencing. Ribosomal profiling offers an intermediate, as it is detecting the mRNA fragments bound to ribosomes, rather than the actual protein, but is less time consuming and costly. Future work would benefit from ribosomal profiling to determine genes that are likely increased in protein expression.

During my analysis of the RNA-seq data of Nb+ TN and CD11b+ DC, and T_H2 cells, I removed over 50% of genes from each RNA-seq data set to focus my analysis pipeline on particular genes I deemed important. Analysis of these genes using String would be interesting, as there may be interactions important for T_H2 priming that could be explored through protein binding and functional assays. The interactions found through my analysis are only predicted interactions, meaning that they may not occur between DC and T cells during T_H2 priming at all. Protein binding assays to determine which interactions occur would be useful and interesting to perform, as it would highlight interactions to explore further, and test my pipeline in its ability to detect real interactions. This work is currently progressing using an ELISA-based protein screening method but is not yet complete[193].

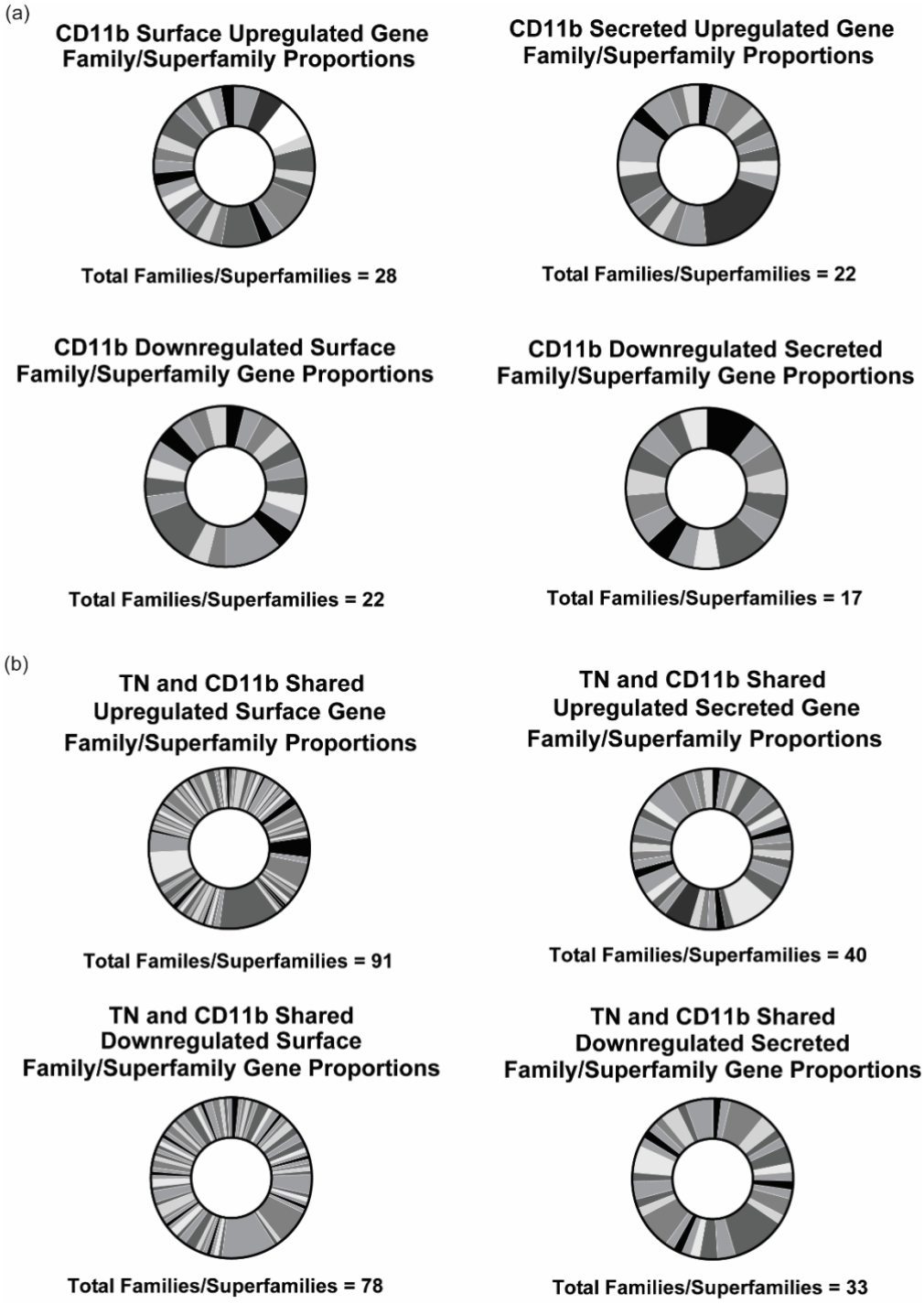
5.5 Final Conclusions

The analyses performed in this thesis outlines the transcriptional and implicated functional distinction of Nb positive TN and CD11b DC, as well as exhibiting the hundreds of predicted interactions between T_H0/T_H2 and Nb positive TN and CD11b+ DC that may be important for T_H2 priming. This is of importance as the priming events, and the proteins within, of T_H2 responses are not well understood by current research, despite the rising incidence of allergy driven by T_H2 responses.

In Summary:

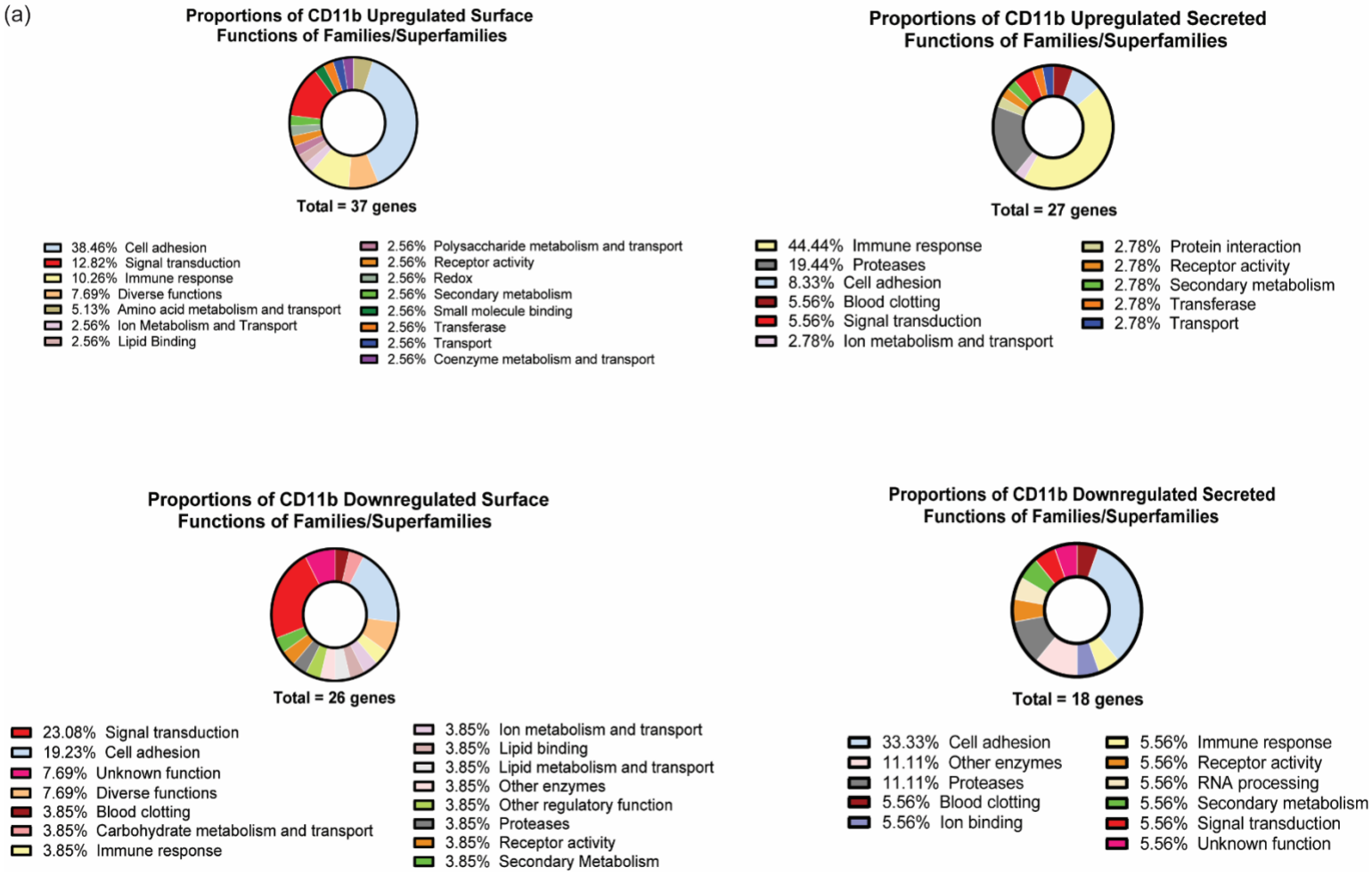
1) Nb positive TN and CD11b+ DC are transcriptionally different from each other in their surface and secreted gene compartments.
2) This transcriptional difference implies a difference in function based upon the general function of superfamilies that genes belong to
3) There are at least 336 predicted interactions between Nb positive TN and CD11b+ DC and T_H0/T_H2 cells, a small portion of which are documented interactions.

Supplementary Figures



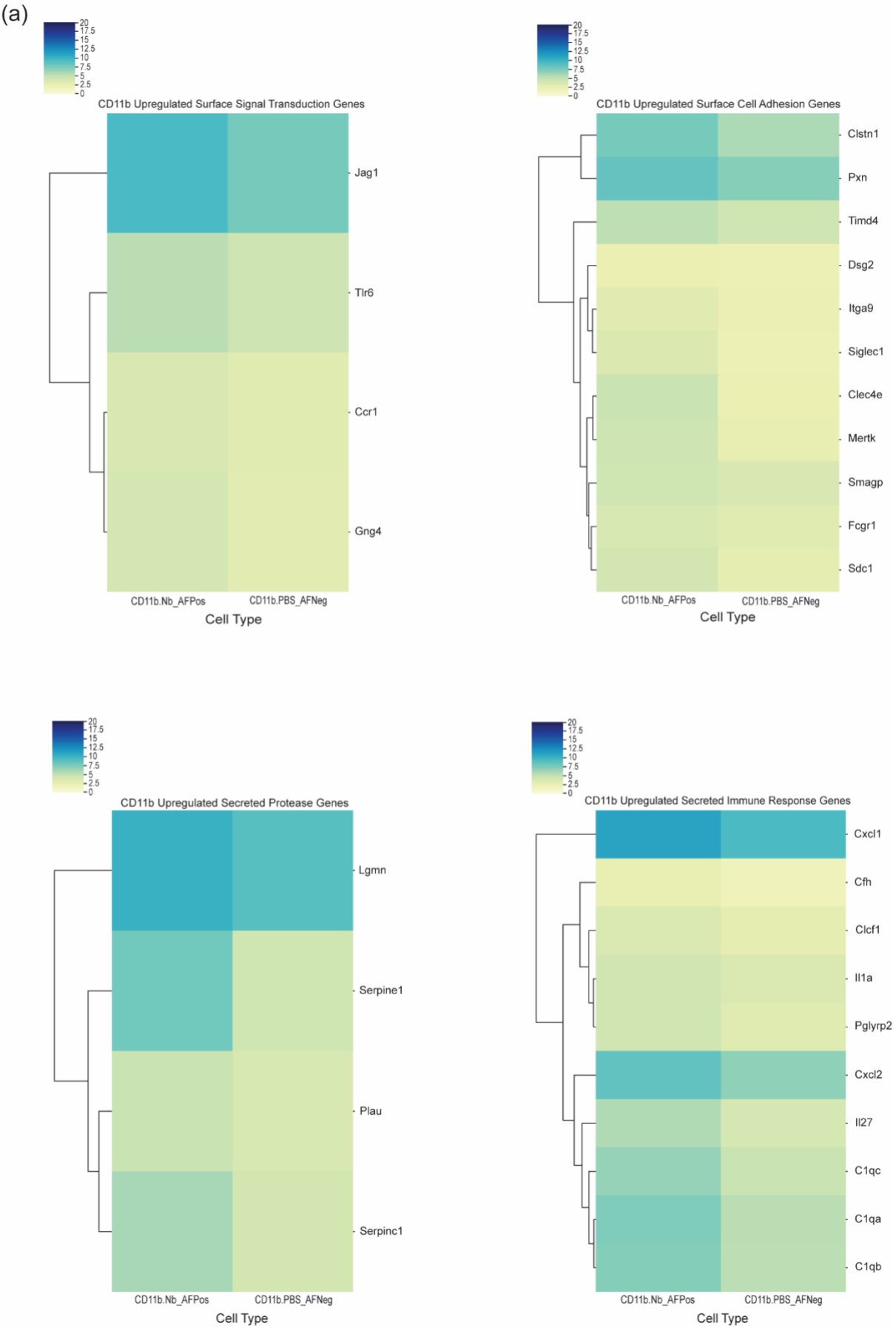
Supplementary Figure 1. Superfamily proportions of (a) surface and secreted genes that only Nb positive CD11b+ DC differentially express, and (b) surface and secreted differentially expressed genes shared by Nb positive TN and CD11b+ DC.

(a)

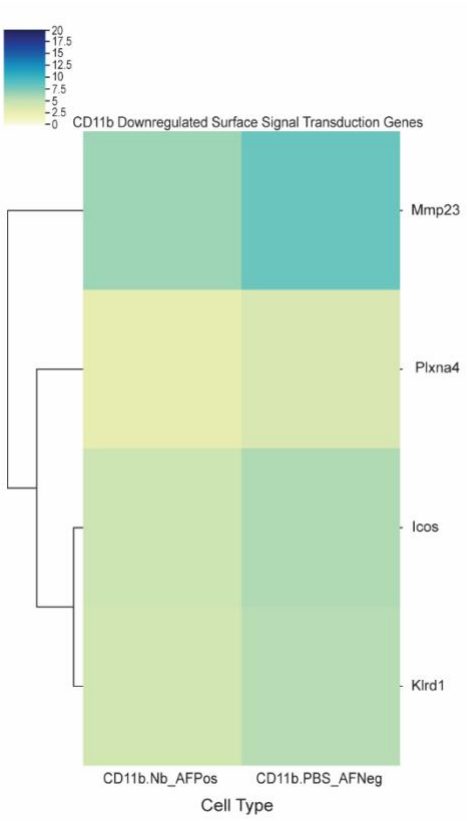

Proportions of CD11b Upregulated Surface Functions of Families/Superfamilies
Total = 37 genes
38.46% Cell adhesion
12.82% Signal transduction
10.26% Immune response
7.69% Diverse functions
5.13% Amino acid metabolism and transport
2.56% Ion Metabolism and Transport
2.56% Lipid Binding
2.56% Polysaccharide metabolism and transport
2.56% Receptor activity
2.56% Redox
2.56% Secondary metabolism
2.56% Small molecule binding
2.56% Transferase
2.56% Transport
2.56% Coenzyme metabolism and transport
Proportions of CD11b Upregulated Secreted Functions of Families/Superfamilies
Total = 27 genes
44.44% Immune response
19.44% Proteases
8.33% Cell adhesion
5.56% Blood clotting
5.56% Signal transduction
2.78% Ion metabolism and transport
2.78% Protein interaction
2.78% Receptor activity
2.78% Secondary metabolism
2.78% Transferase
2.78% Transport
Proportions of CD11b Downregulated Surface Functions of Families/Superfamilies
Total = 26 genes
23.08% Signal transduction
19.23% Cell adhesion
7.69% Unknown function
7.69% Diverse functions
3.85% Blood clotting
3.85% Carbohydrate metabolism and transport
3.85% Immune response
3.85% Ion metabolism and transport
3.85% Lipid binding
3.85% Lipid metabolism and transport
3.85% Other enzymes
3.85% Other regulatory function
3.85% Proteases
3.85% Receptor activity
3.85% Secondary Metabolism
Proportions of CD11b Downregulated Secreted Functions of Families/Superfamilies
Total = 18 genes
33.33% Cell adhesion
11.11% Other enzymes
11.11% Proteases
5.56% Blood clotting
5.56% Ion binding
5.56% Immune response
5.56% Receptor activity
5.56% RNA processing
5.56% Secondary metabolism
5.56% Signal transduction
5.56% Unknown function

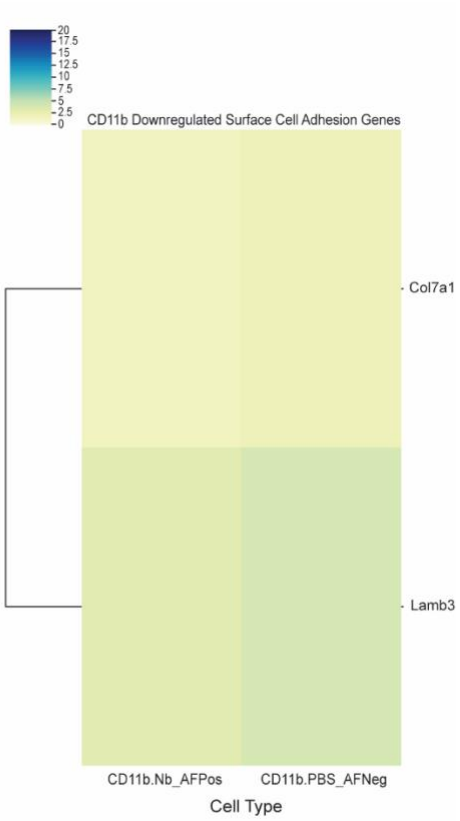
Supplementary Figure 2. Proportions of general functions of upregulated and downregulated surface and secreted superfamilies of (a) CD11b+ upregulated surface and secreted genes and (b) TN and CD11b+ shared surface and secreted genes.

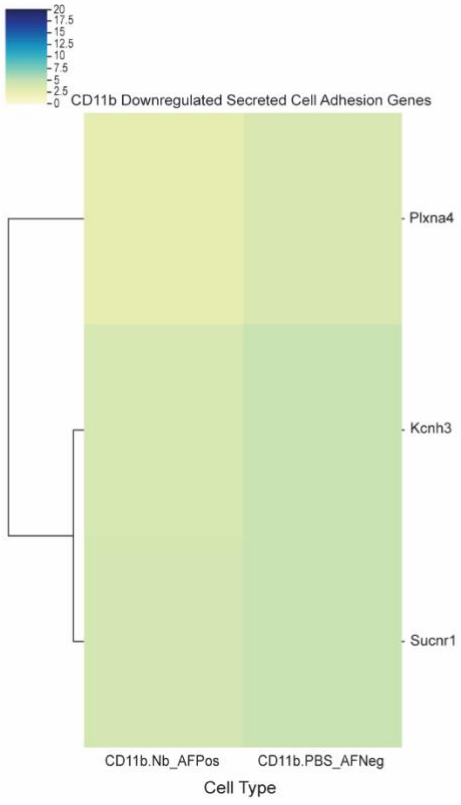
(a)


CD11b Upregulated Surface Signal Transduction Genes
Jag1
Tlr6
Ccr1
Gng4
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type
CD11b Upregulated Surface Cell Adhesion Genes
Clstn1
Pxn
Timd4
Dsg2
Itga9
Siglec1
Clec4e
Mertk
Smagp
Fcgr1
Sdc1
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type
CD11b Upregulated Secreted Protease Genes
Lgmn
Serpine1
Plau
Serpinc1
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type
CD11b Upregulated Secreted Immune Response Genes
Cxcl1
Cfh
Clcf1
Il1a
Pglyrp2
Cxcl2
Il27
C1qc
C1qa
C1qb
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type

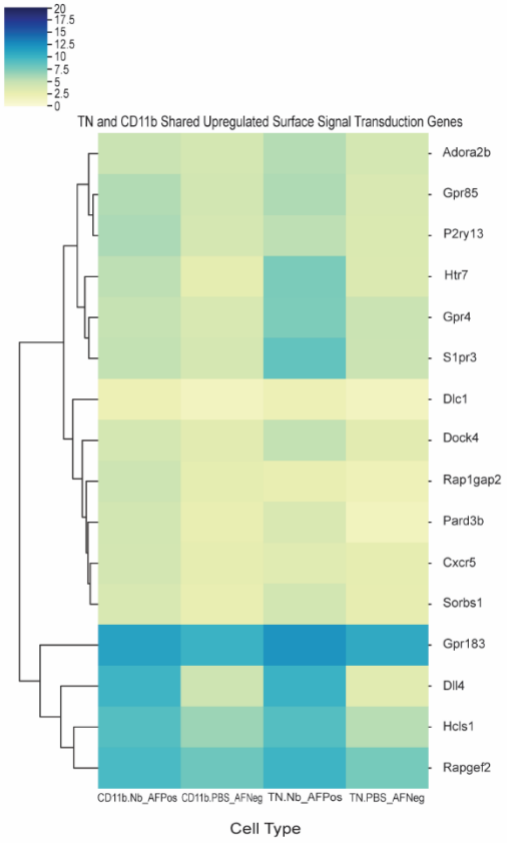
(b)

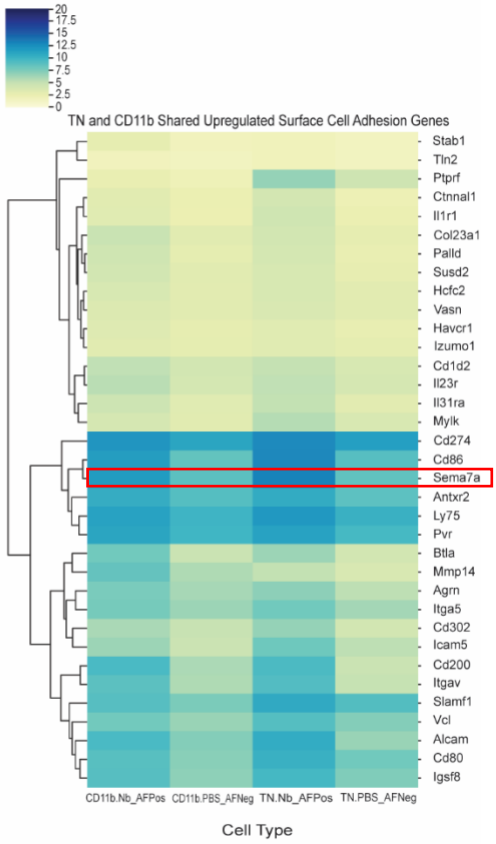

20
17.5
15
12.5
10
7.5
5
2.5
0
CD11b Downregulated Surface Signal Transduction Genes
Mmp23
Plxna4
Icos
Klrd1
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type

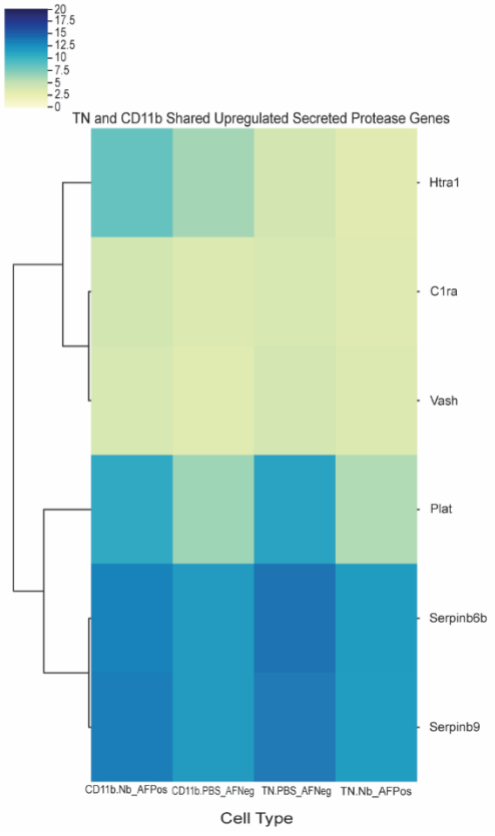

20
17.5
15
12.5
10
7.5
5
2.5
0
CD11b Downregulated Surface Cell Adhesion Genes
Col7a1
Lamb3
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type

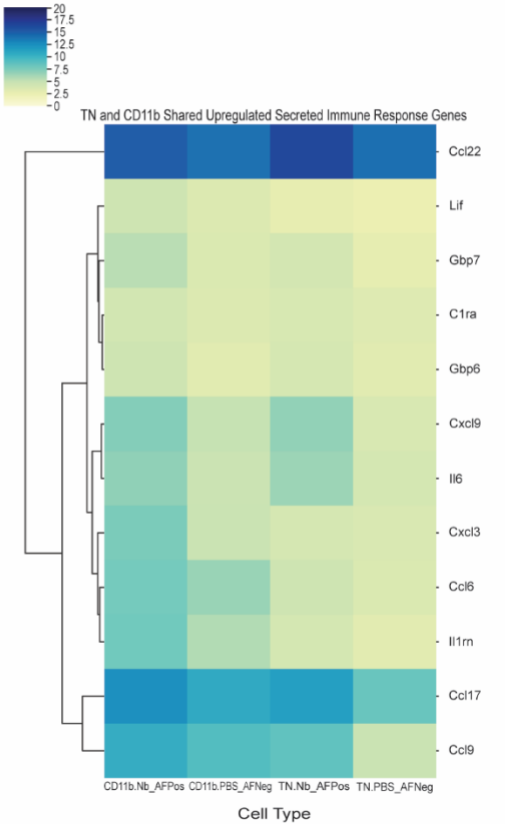

20
17.5
15
12.5
10
7.5
5
2.5
0
CD11b Downregulated Secreted Cell Adhesion Genes
Plxna4
Kcnh3
Sucnr1
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
Cell Type

(c)

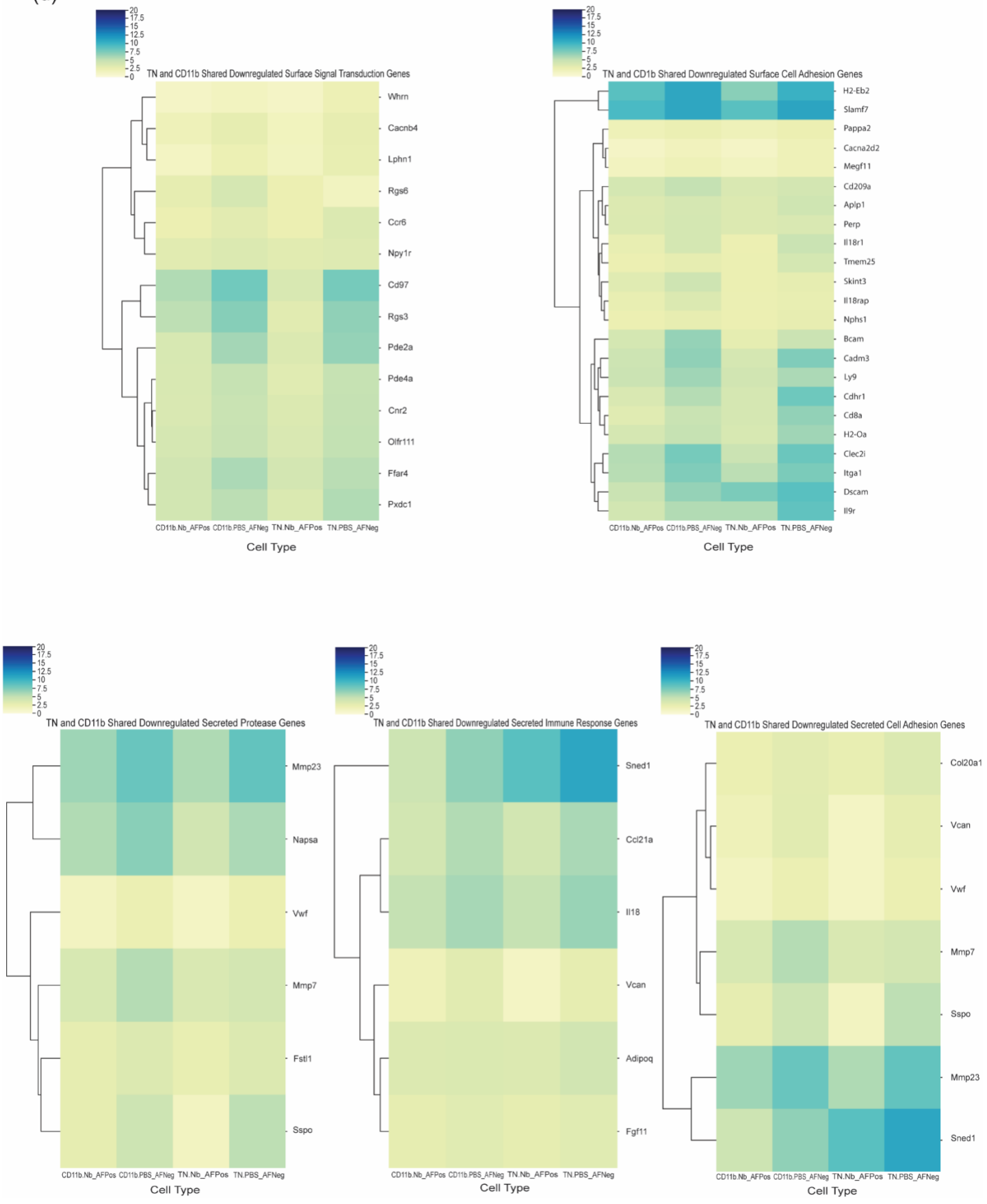

TN and CD11b Shared Upregulated Surface Signal Transduction Genes
20
17.5
15
12.5
10
7.5
5
2.5
0
Adora2b
Gpr85
P2ry13
Htr7
Gpr4
S1pr3
Dlc1
Dock4
Rap1gap2
Pard3b
Cxcr5
Sorbs1
Gpr183
Dll4
Hcls1
Rapgef2
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type


TN and CD11b Shared Upregulated Surface Cell Adhesion Genes
20
17.5
15
12.5
10
7.5
5
2.5
0
Stab1
Tln2
Ptprf
Ctnnal1
Il1r1
Col23a1
Palld
Susd2
Hcfc2
Vasn
Havcr1
Izumo1
Cd1d2
Il23r
Il31ra
Mylk
Cd274
Cd86
Sema7a
Antxr2
Ly75
Pvr
Btla
Mmp14
Agrn
Itga5
Cd302
Icam5
Cd200
Itgav
Slamf1
Vcl
Alcam
Cd80
Igsf8
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type


TN and CD11b Shared Upregulated Secreted Protease Genes
20
17.5
15
12.5
10
7.5
5
2.5
0
Htra1
C1ra
Vash
Plat
Serpinb6b
Serpinb9
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
TN.PBS_AFNeg
TN.Nb_AFPos
Cell Type

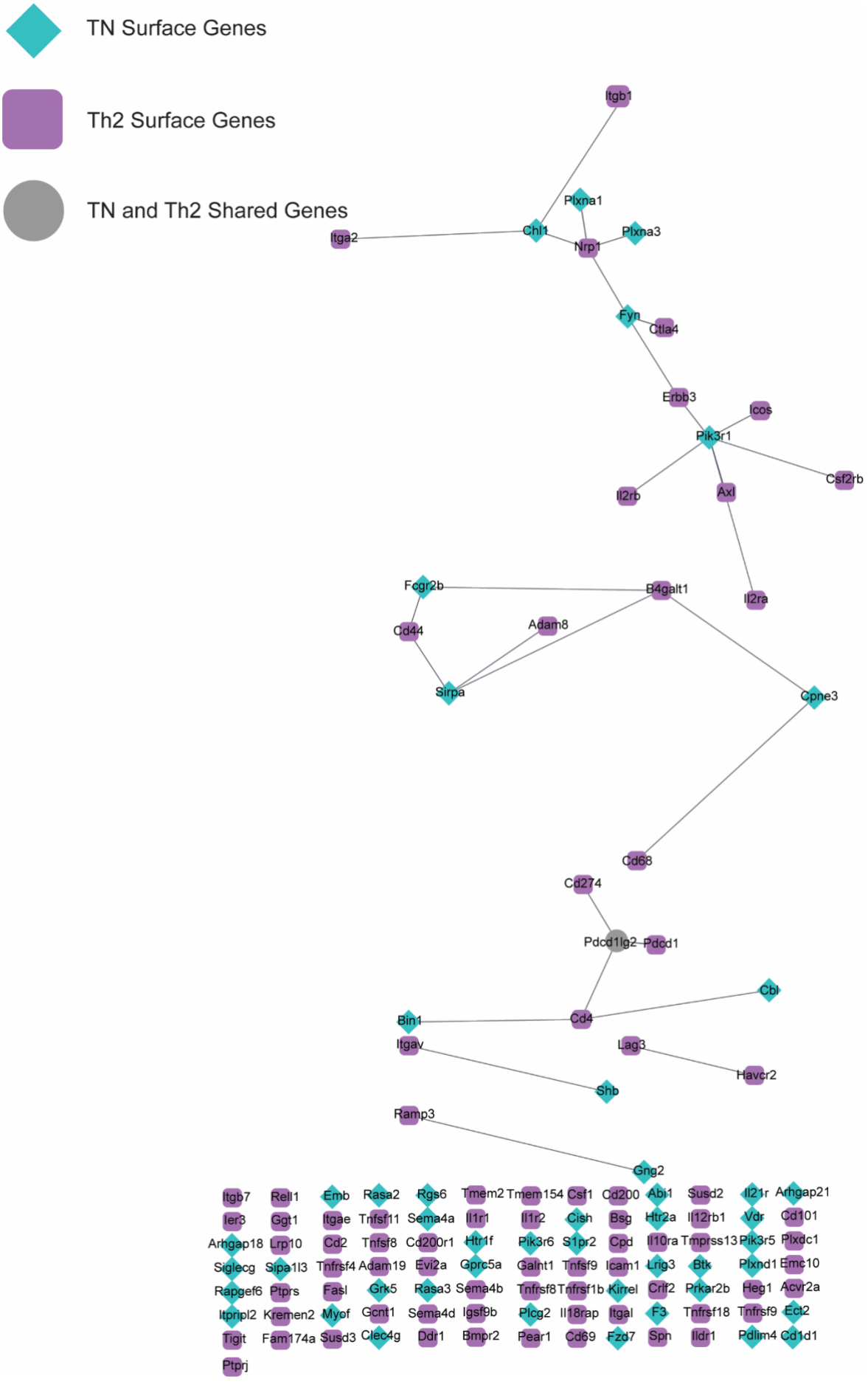

TN and CD11b Shared Upregulated Secreted Immune Response Genes
20
17.5
15
12.5
10
7.5
5
2.5
0
Ccl22
Lif
Gbp7
C1ra
Gbp6
Cxcl9
Il6
Cxcl3
Ccl6
Il1rn
Ccl17
Ccl9
CD11b.Nb_AFPos
CD11b.PBS_AFNeg
TN.Nb_AFPos
TN.PBS_AFNeg
Cell Type

(d)

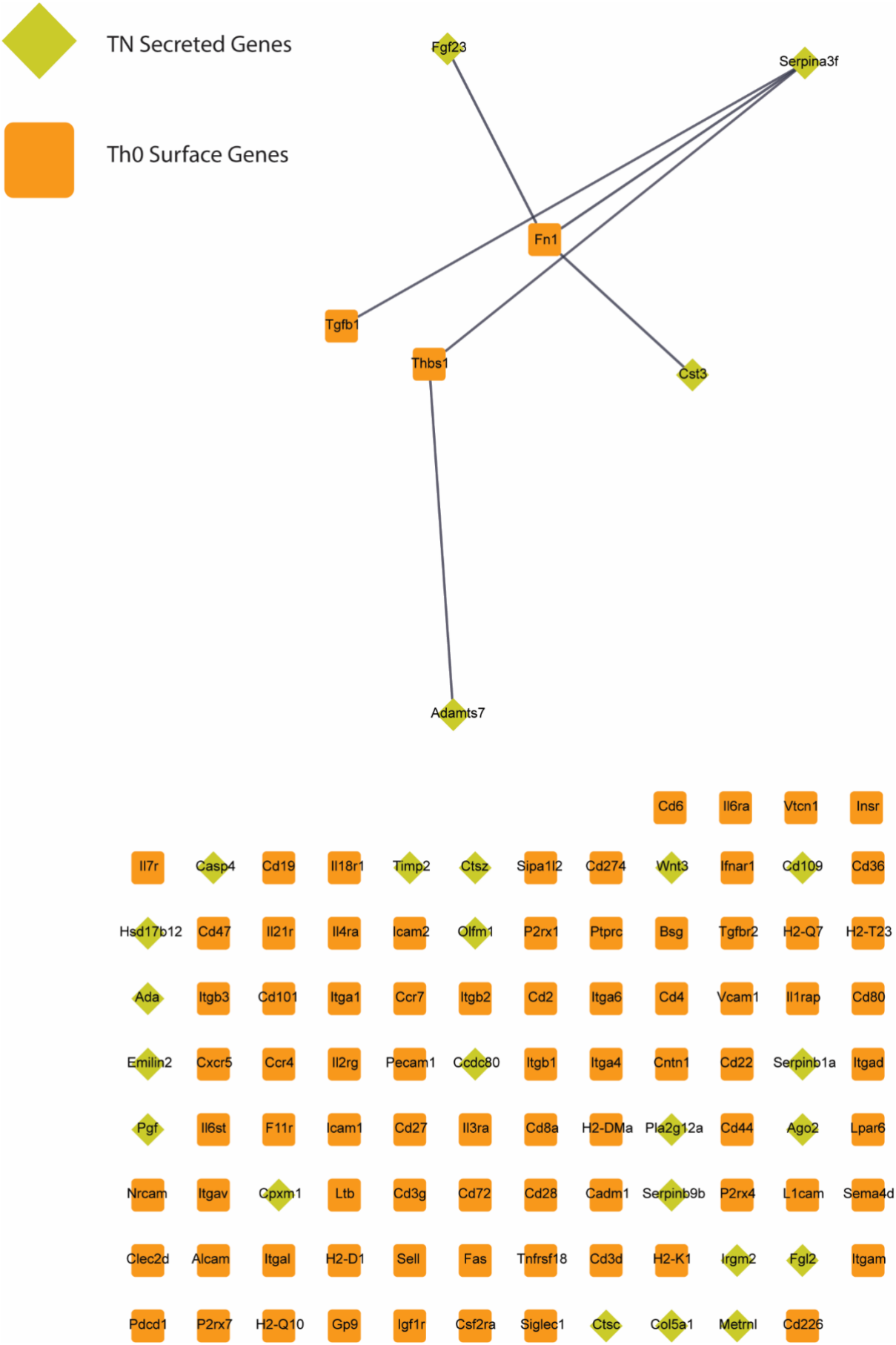

TN and CD11b Shared Downregulated Surface Signal Transduction Genes
Whrn
Cacnb4
Lphn1
Rgs6
Ccr6
Npy1r
Cd97
Rgs3
Pde2a
Pde4a
Cnr2
Olfr111
Ffar4
Pxdc1
CD11b.Nb_AFPos CD11b.PBS_AFNeg TN.Nb_AFPos TN.PBS_AFNeg
Cell Type
TN and CD1b Shared Downregulated Surface Cell Adhesion Genes
H2-Eb2
Slamf7
Pappa2
Cacna2d2
Megf11
Cd209a
Aplp1
Perp
Il18r1
Tmem25
Skint3
Il18rap
Nphs1
Bcam
Cadm3
Ly9
Cdhr1
Cd8a
H2-Oa
Clec2i
Itga1
Dscam
Il9r
CD11b.Nb_AFPos CD11b.PBS_AFNeg TN.Nb_AFPos TN.PBS_AFNeg
Cell Type
TN and CD11b Shared Downregulated Secreted Protease Genes
Mmp23
Napsa
Vwf
Mmp7
Fstl1
Sspo
CD11b.Nb_AFPos CD11b.PBS_AFNeg TN.Nb_AFPos TN.PBS_AFNeg
Cell Type
TN and CD11b Shared Downregulated Secreted Immune Response Genes
Sned1
Ccl21a
Il18
Vcan
Adipoq
Fgf11
CD11b.Nb_AFPos CD11b.PBS_AFNeg TN.Nb_AFPos TN.PBS_AFNeg
Cell Type
TN and CD11b Shared Downregulated Secreted Cell Adhesion Genes
Col20a1
Vcan
Vwf
Mmp7
Sspo
Mmp23
Sned1
CD11b.Nb_AFPos CD11b.PBS_AFNeg TN.Nb_AFPos TN.PBS_AFNeg
Cell Type

Supplementary Figure 3. VSTpk heatmaps of genes from (a) CD11b+ upregulated surface signal transduction, surface cell adhesion, and secreted immune response genes (b) downregulated signal transduction, surface cell adhesion, and secreted cell adhesion genes (c) TN and CD11b+ shared upregulated surface signal transduction, surface cell adhesion, secreted immune response, and secreted protease genes (d) downregulated surface signal transduction, surface cell adhesion, secreted cell adhesion, secreted immune response, and secreted cell adhesion genes.

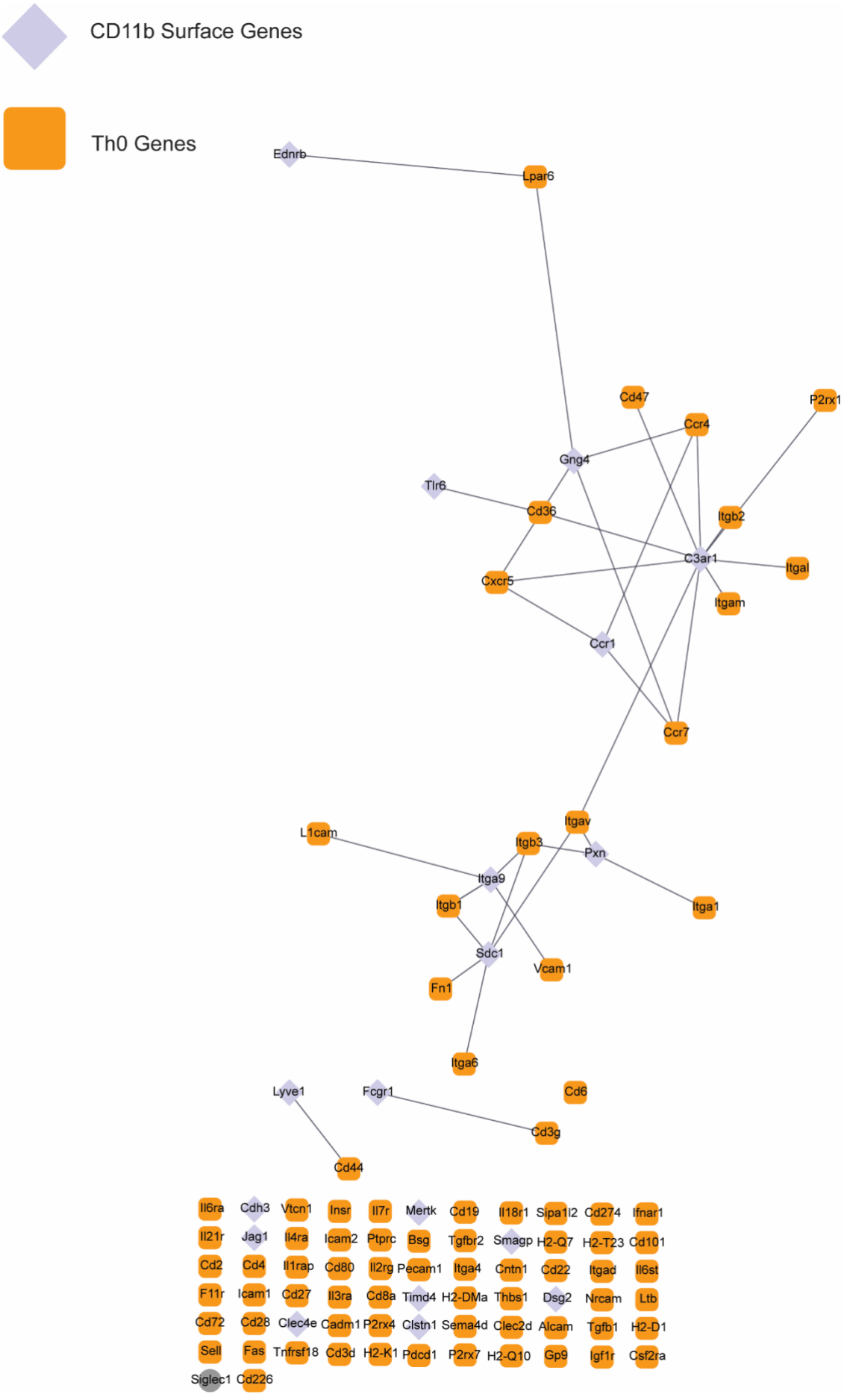
(a)


TN Surface Genes
Th2 Surface Genes
TN and Th2 Shared Genes
Itgb1
Plxna1
Itga2
Chl1
Plxna3
Nrp1
Fyn
Ctla4
Erbb3
Icos
Pik3r1
Csf2rb
Il2rb
Axl
Il2ra
Fcgr2b
B4galt1
Cd44
Adam8
Sirpa
Cpne3
Cd68
Cd274
Pdcd1lg2
Pdcd1
Cbl
Bin1
Cd4
Itgav
Lag3
Havcr2
Shb
Ramp3
Gng2
Itgb7
Rell1
Emb
Rasa2
Rgs6
Tmem2
Tmem154
Csf1
Cd200
Abi1
Susd2
Il21r
Arhgap21
Ier3
Ggt1
Itgae
Tnfsf11
Sema4a
Il1r1
Il1r2
Cish
Bsg
Htr2a
Il12rb1
Vdr
Cd101
Arhgap18
Lrp10
Cd2
Tnfsf8
Cd200r1
Htr1f
Pik3r6
S1pr2
Cpd
Il10ra
Tmprss13
Pik3r5
Plxdc1
Siglecg
Sipa1l3
Tnfrsf4
Adam19
Evi2a
Gprc5a
Galnt1
Tnfsf9
Icam1
Lrig3
Btk
Plxnd1
Emc10
Rapgef6
Ptprs
Fasl
Grk5
Rasa3
Sema4b
Tnfrsf8
Tnfrsf1b
Kirrel
Crlf2
Prkar2b
Heg1
Acvr2a
Itpripl2
Kremen2
Myof
Gcnt1
Sema4d
Igsf9b
Plcg2
Il18rap
Itgal
F3
Tnfrsf18
Tnfrsf9
Ect2
Tigit
Fam174a
Susd3
Clec4g
Ddr1
Bmpr2
Pear1
Cd69
Fzd7
Spn
Ildr1
Pdlim4
Cd1d1
Ptprj

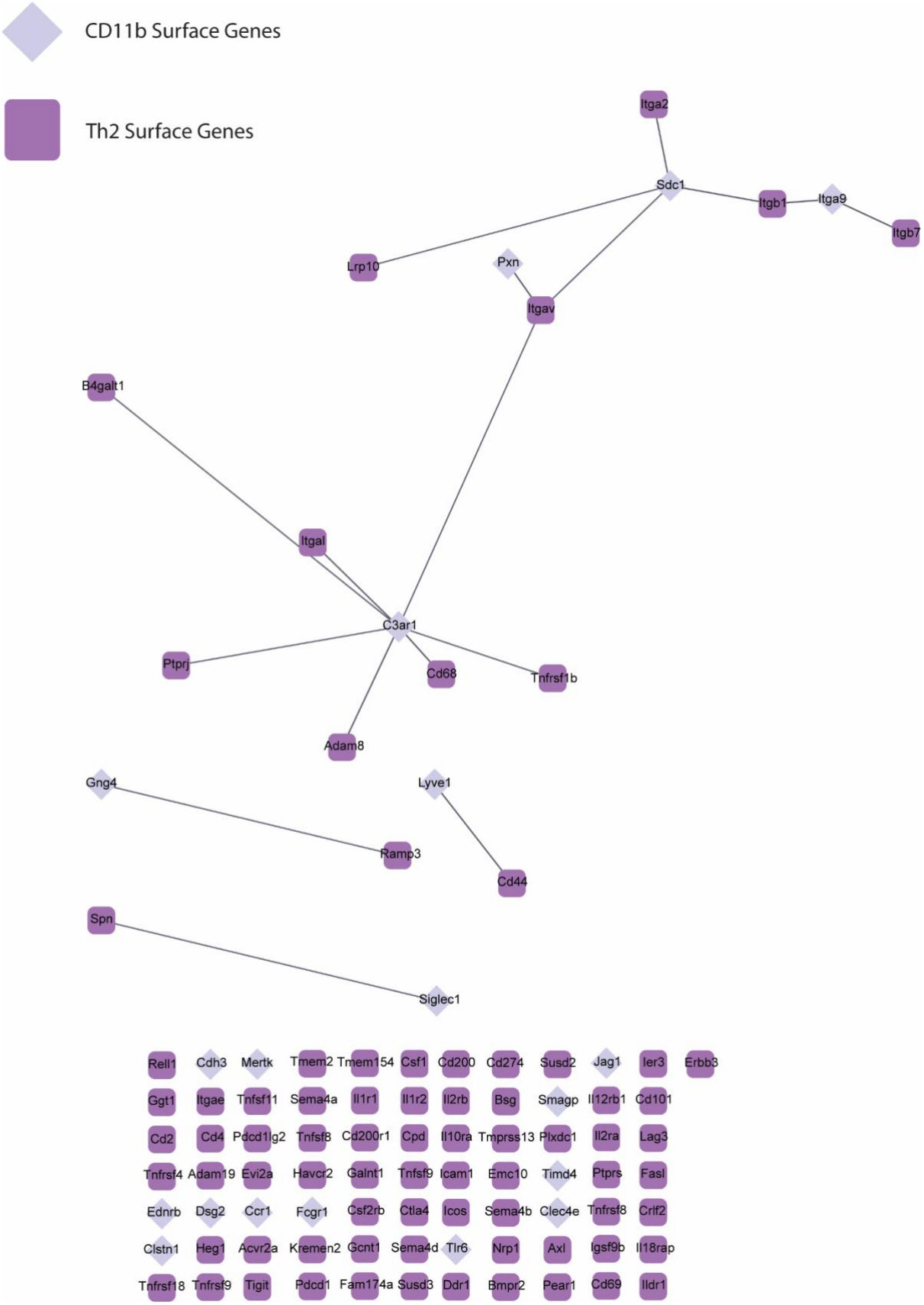
(b)


TN Secreted Genes
Th0 Surface Genes
Fgf23
Serpina3f
Fn1
Tgfb1
Thbs1
Cst3
Adamts7
Cd6
Il6ra
Vtcn1
Insr
Il7r
Casp4
Cd19
Il18r1
Timp2
Ctsz
Sipa1l2
Cd274
Wnt3
Ifnar1
Cd109
Cd36
Hsd17b12
Cd47
Il21r
Il4ra
Icam2
Olfm1
P2rx1
Ptprc
Bsg
Tgfbr2
H2-Q7
H2-T23
Ada
Itgb3
Cd101
Itga1
Ccr7
Itgb2
Cd2
Itga6
Cd4
Vcam1
Il1rap
Cd80
Emilin2
Cxcr5
Ccr4
Il2rg
Pecam1
Ccdc80
Itgb1
Itga4
Cntn1
Cd22
Serpinb1a
Itgad
Pgf
Il6st
F11r
Icam1
Cd27
Il3ra
Cd8a
H2-DMa
Pla2g12a
Cd44
Ago2
Lpar6
Nrcam
Itgav
Cpxm1
Ltb
Cd3g
Cd72
Cd28
Cadm1
Serpinb9b
P2rx4
L1cam
Sema4d
Clec2d
Alcam
Itgal
H2-D1
Sell
Fas
Tnfrsf18
Cd3d
H2-K1
Irgm2
Fgl2
Itgam
Pdcd1
P2rx7
H2-Q10
Gp9
Igf1r
Csf2ra
Siglec1
Ctsc
Col5a1
Metrnl
Cd226

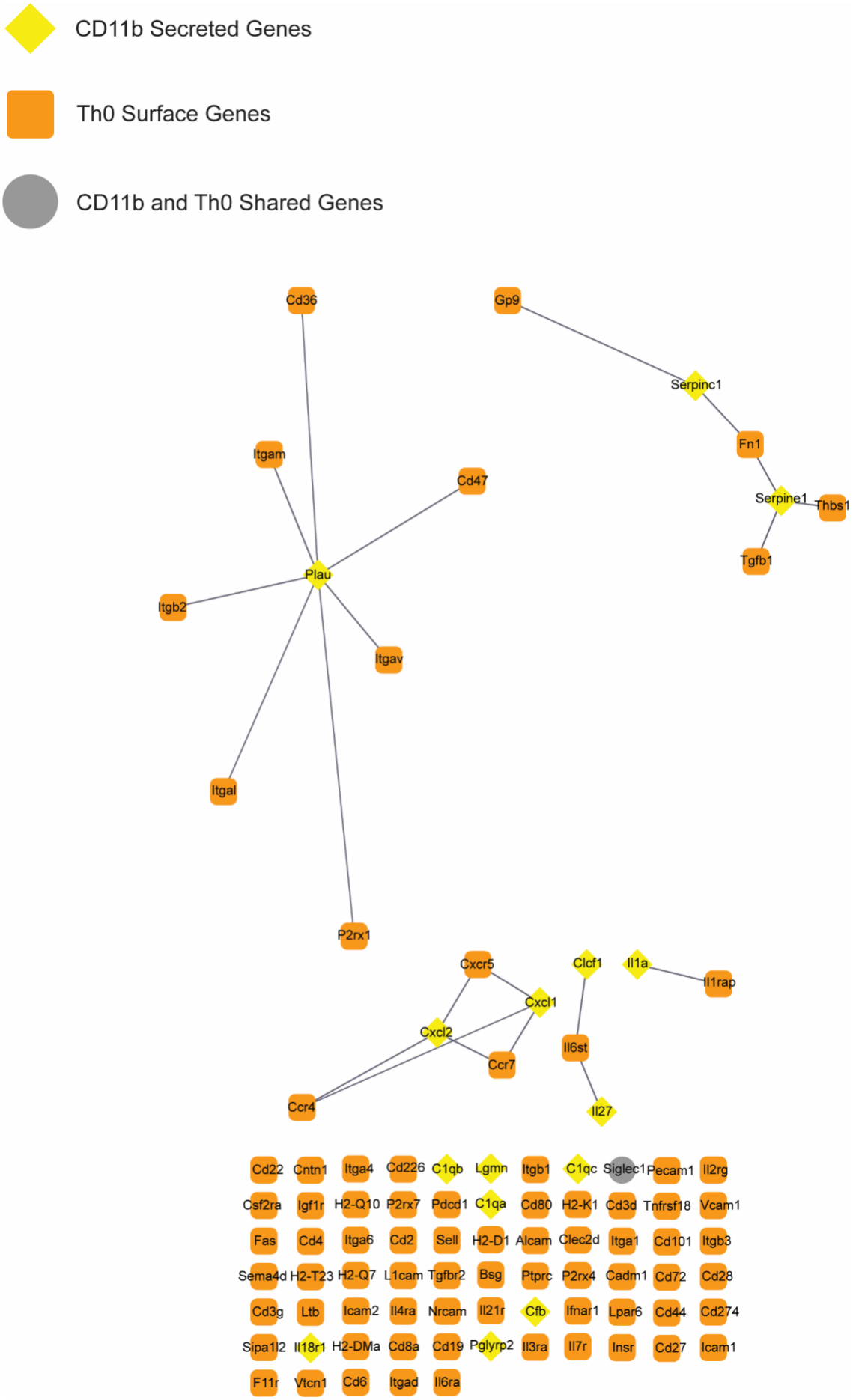
(c)


CD11b Surface Genes
Th0 Genes
Ednrb
Lpar6
Cd47
P2rx1
Ccr4
Gng4
Tlr6
Cd36
Itgb2
C3ar1
Itgal
Cxcr5
Itgam
Ccr1
Ccr7
Itgav
L1cam
Itgb3
Pxn
Itga9
Itgb1
Itga1
Sdc1
Vcam1
Fn1
Itga6
Lyve1
Fcgr1
Cd6
Cd3g
Cd44
Il6ra
Cdh3
Vtcn1
Insr
Il7r
Mertk
Cd19
Il18r1
Sipa1l2
Cd274
Ifnar1
Il21r
Jag1
Il4ra
Icam2
Ptprc
Bsg
Tgfbr2
Smagp
H2-Q7
H2-T23
Cd101
Cd2
Cd4
Il1rap
Cd80
Il2rg
Pecam1
Itga4
Cntn1
Cd22
Itgad
Il6st
F11r
Icam1
Cd27
Il3ra
Cd8a
Timd4
H2-DMa
Thbs1
Dsg2
Nrcam
Ltb
Cd72
Cd28
Clec4e
Cadm1
P2rx4
Clstn1
Sema4d
Clec2d
Alcam
Tgfb1
H2-D1
Sell
Fas
Tnfrsf18
Cd3d
H2-K1
Pdcd1
P2rx7
H2-Q10
Gp9
Igf1r
Csf2ra
Siglec1
Cd226

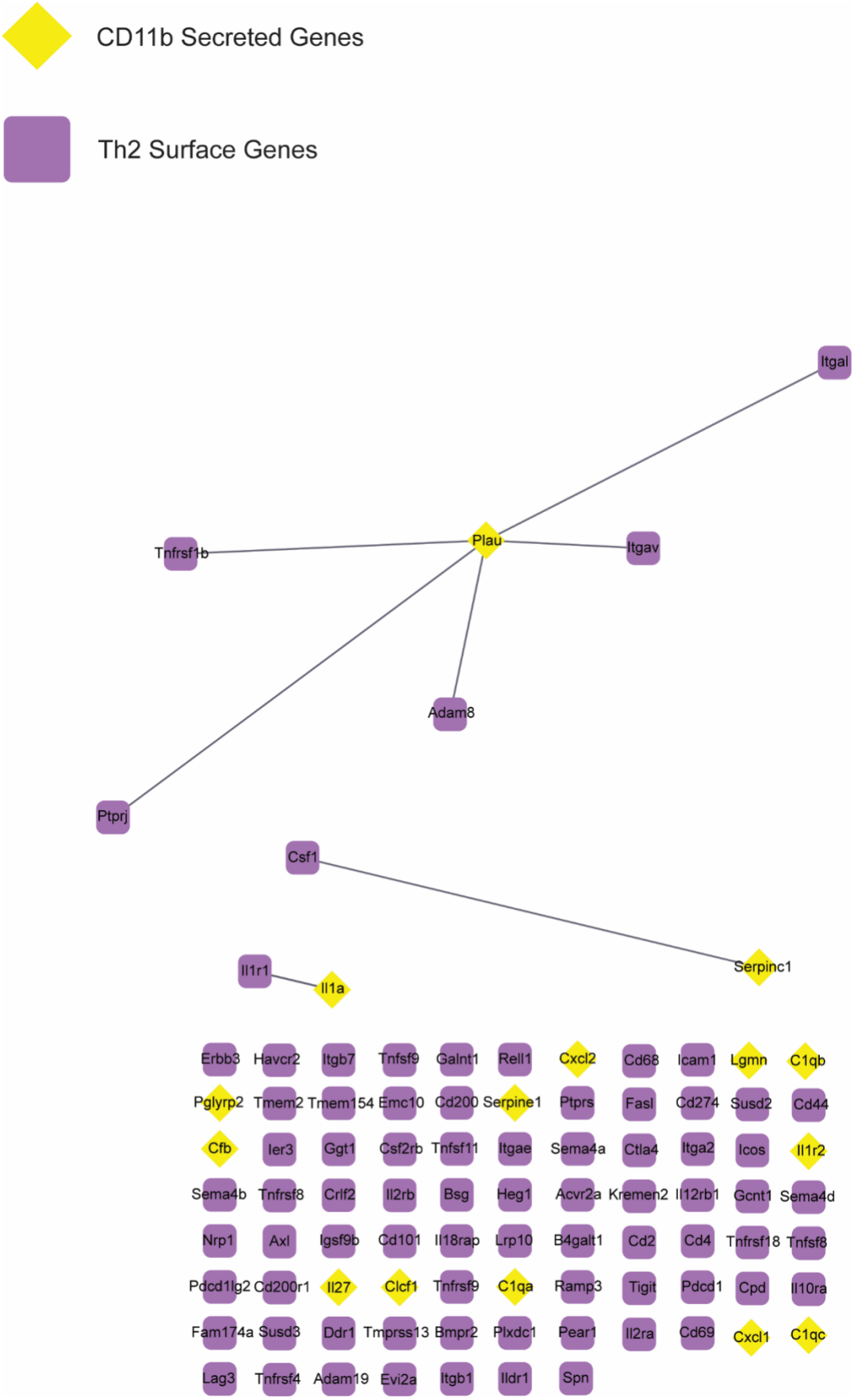
(d)


CD11b Surface Genes
Th2 Surface Genes
Itga2
Sdc1
Itgb1
Itga9
Itgb7
Lrp10
Pxn
Itgav
B4galt1
Itgal
C3ar1
Ptprj
Cd68
Tnfrsf1b
Adam8
Gng4
Lyve1
Ramp3
Cd44
Spn
Siglec1
Rell1
Cdh3
Mertk
Tmem2
Tmem154
Csf1
Cd200
Cd274
Susd2
Jag1
Ier3
Erbb3
Ggt1
Itgae
Tnfsf11
Sema4a
Il1r1
Il1r2
Il2rb
Bsg
Smagp
Il12rb1
Cd101
Cd2
Cd4
Pdcd1lg2
Tnfsf8
Cd200r1
Cpd
Il10ra
Tmprss13
Plxdc1
Il2ra
Lag3
Tnfrsf4
Adam19
Evi2a
Havcr2
Galnt1
Tnfsf9
Icam1
Emc10
Timd4
Ptprs
Fasl
Ednrb
Dsg2
Ccr1
Fcgr1
Csf2rb
Ctla4
Icos
Sema4b
Clec4e
Tnfrsf8
Crlf2
Clstn1
Heg1
Acvr2a
Kremen2
Gcnt1
Sema4d
Tlr6
Nrp1
Axl
Igsf9b
Il18rap
Tnfrsf18
Tnfrsf9
Tigit
Pdcd1
Fam174a
Susd3
Ddr1
Bmpr2
Pear1
Cd69
Ildr1

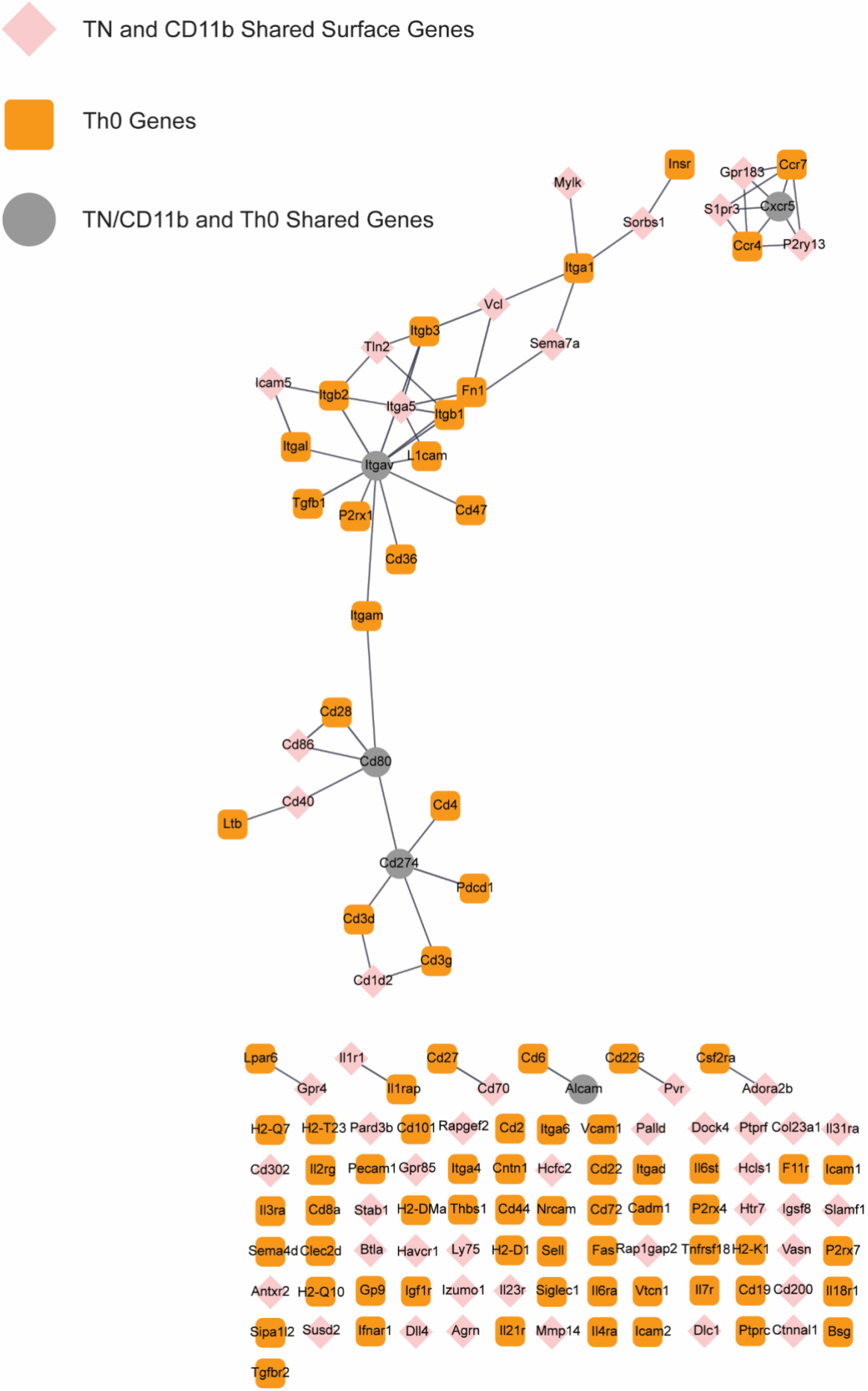
(e)


CD11b Secreted Genes
Th0 Surface Genes
CD11b and Th0 Shared Genes
Cd36
Gp9
Serpinc1
Fn1
Itgam
Cd47
Serpine1
Thbs1
Tgfb1
Plau
Itgb2
Itgav
Itgal
P2rx1
Cxcr5
Clcf1
Il1a
Il1rap
Cxcl1
Cxcl2
Il6st
Ccr7
Ccr4
Il27
Cd22 Cntn1 Itga4 Cd226 C1qb Lgmn Itgb1 C1qc Siglec1 Pecam1 Il2rg
Csf2ra Igf1r H2-Q10 P2rx7 Pdcd1 C1qa Cd80 H2-K1 Cd3d Tnfrsf18 Vcam1
Fas Cd4 Itga6 Cd2 Sell H2-D1 Alcam Clec2d Itga1 Cd101 Itgb3
Sema4d H2-T23 H2-Q7 L1cam Tgfbr2 Bsg Ptprc P2rx4 Cadm1 Cd72 Cd28
Cd3g Ltb Icam2 Il4ra Nrcam Il21r Cfb Ifnar1 Lpar6 Cd44 Cd274
Sipa1l2 Il18r1 H2-DMa Cd8a Cd19 Pglyrp2 Il3ra Il7r Insr Cd27 Icam1
F11r Vtcn1 Cd6 Itgad Il6ra

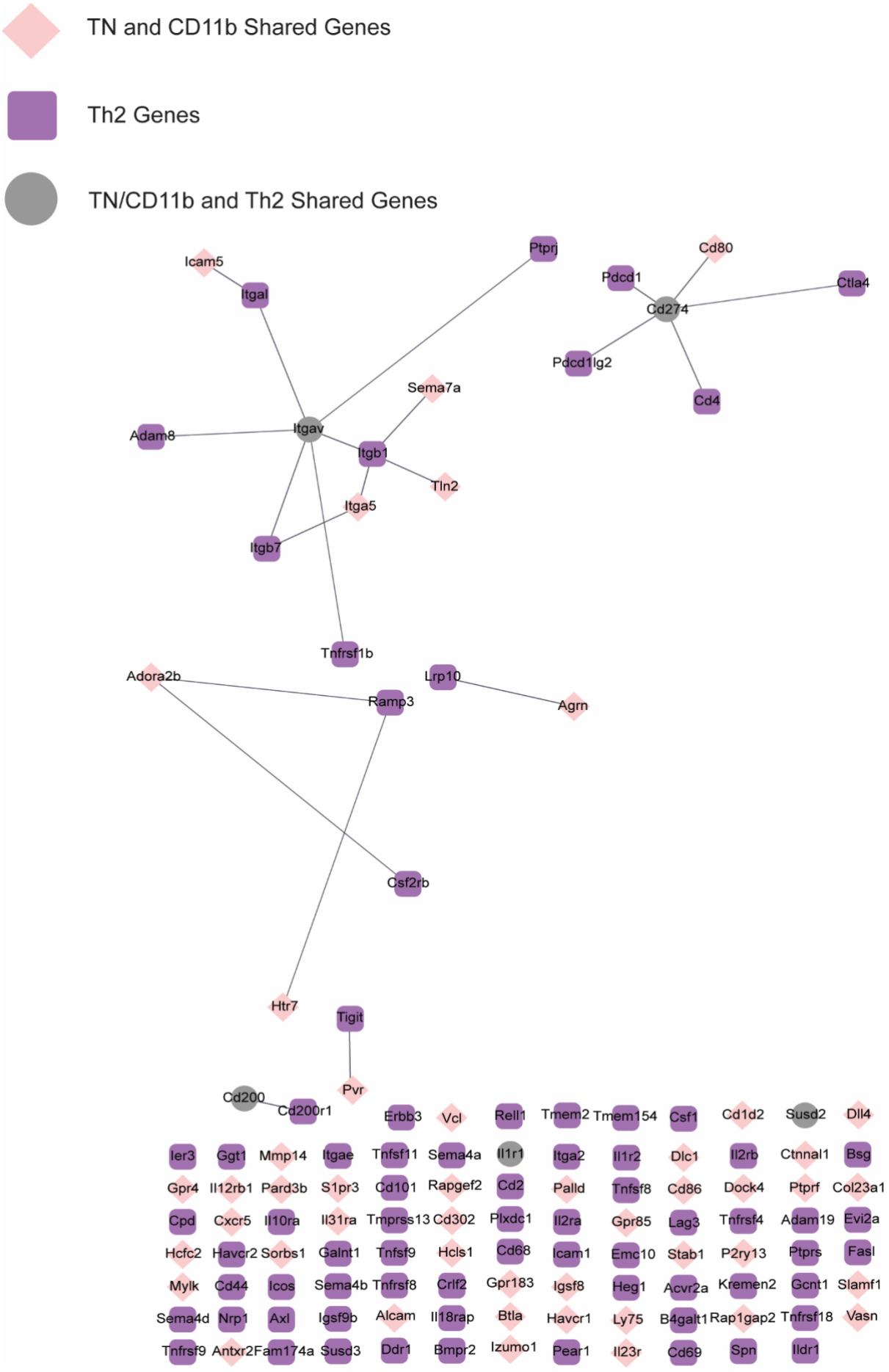
(f)


CD11b Secreted Genes
Th2 Surface Genes
Itgal
Tnfrsf1b
Plau
Itgav
Adam8
Ptprj
Csf1
Serpinc1
Il1r1
Il1a
Erbb3 Havcr2 Itgb7 Tnfsf9 Galnt1 Rell1 Cxcl2 Cd68 Icam1 Lgmn C1qb
Pglyrp2 Tmem2 Tmem154 Emc10 Cd200 Serpine1 Ptprs Fasl Cd274 Susd2 Cd44
Cfb Ier3 Ggt1 Csf2rb Tnfsf11 Itgae Sema4a Ctla4 Itga2 Icos Il1r2
Sema4b Tnfrsf8 Crlf2 Il2rb Bsg Heg1 Acvr2a Kremen2 Il12rb1 Gcnt1 Sema4d
Nrp1 Axl Igsf9b Cd101 Il18rap Lrp10 B4galt1 Cd2 Cd4 Tnfrsf18 Tnfsf8
Pdcd1lg2 Cd200r1 Il27 Clcf1 Tnfrsf9 C1qa Ramp3 Tigit Pdcd1 Cpd Il10ra
Fam174a Susd3 Ddr1 Tmprss13 Bmpr2 Plxdc1 Pear1 Il2ra Cd69 Cxcl1 C1qc
Lag3 Tnfrsf4 Adam19 Evi2a Itgb1 Ildr1 Spn

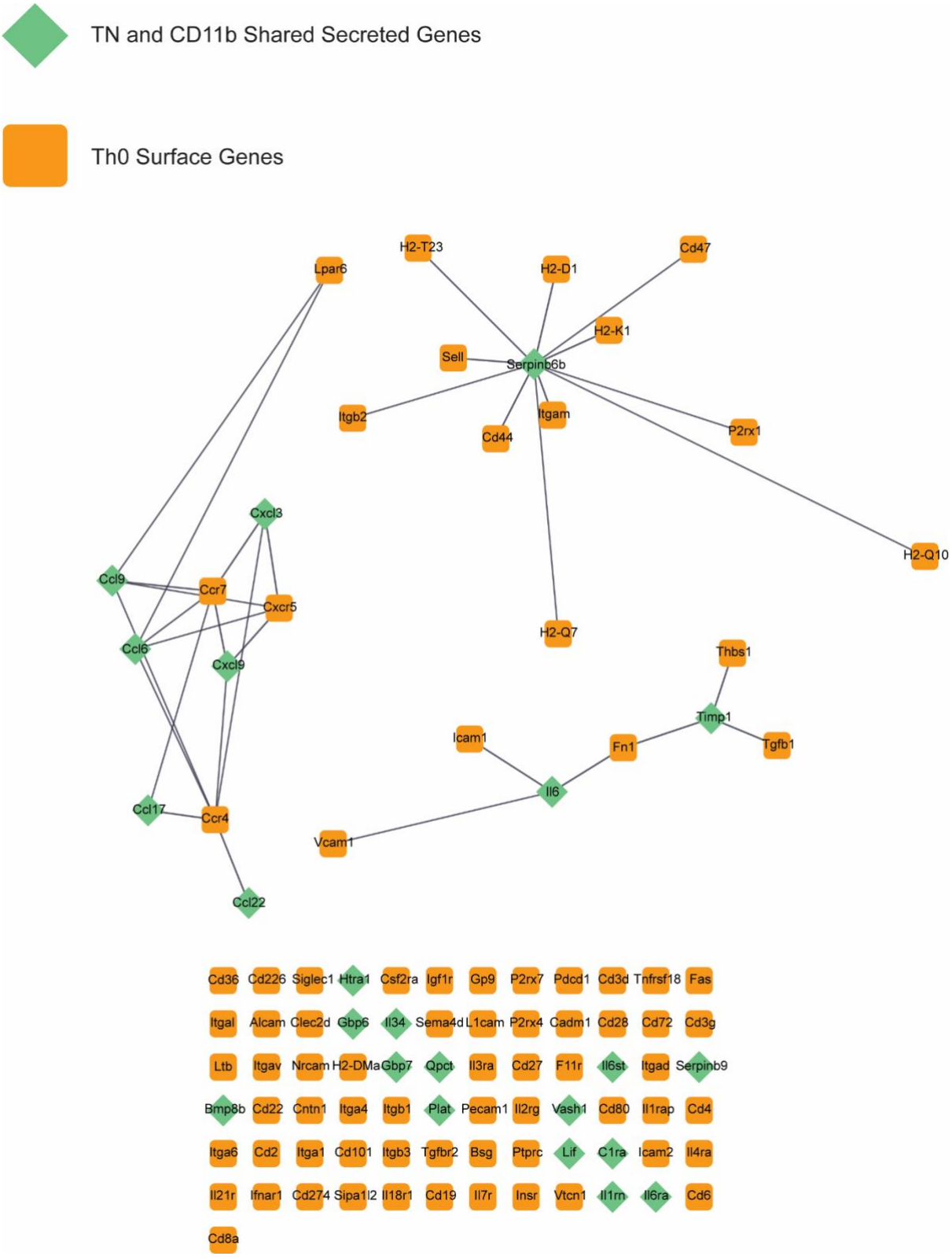
(g)


TN and CD11b Shared Surface Genes
Th0 Genes
TN/CD11b and Th0 Shared Genes
Insr
Mylk
Gpr183
Ccr7
S1pr3
Cxcr5
Sorbs1
Ccr4
P2ry13
Itga1
Vcl
Itgb3
Tln2
Sema7a
Icam5
Itgb2
Fn1
Itga5
Itgb1
Itgal
Itgav
L1cam
Tgfb1
P2rx1
Cd47
Cd36
Itgam
Cd28
Cd86
Cd80
Cd40
Ltb
Cd4
Cd274
Pdcd1
Cd3d
Cd3g
Cd1d2
Lpar6
Gpr4
Il1r1
Il1rap
Cd27
Cd70
Cd6
Alcam
Cd226
Pvr
Csf2ra
Adora2b
H2-Q7
H2-T23
Pard3b
Cd101
Rapgef2
Cd2
Itga6
Vcam1
Palld
Dock4
Ptprf
Col23a1
Il31ra
Cd302
Il2rg
Pecam1
Gpr85
Itga4
Cntn1
Hcfc2
Cd22
Itgad
Il6st
Hcls1
F11r
Icam1
Il3ra
Cd8a
Stab1
H2-DMa
Thbs1
Cd44
Nrcam
Cd72
Cadm1
P2rx4
Htr7
Igsf8
Slamf1
Sema4d
Clec2d
Btla
Havcr1
Ly75
H2-D1
Sell
Fas
Rap1gap2
Tnfrsf18
H2-K1
Vasn
P2rx7
Antxr2
H2-Q10
Gp9
Igf1r
Izumo1
Il23r
Siglec1
Il6ra
Vtcn1
Il7r
Cd19
Cd200
Il18r1
Sipa1l2
Susd2
Ifnar1
Dll4
Agrn
Il21r
Mmp14
Il4ra
Icam2
Dlc1
Ptprc
Ctnnal1
Bsg
Tgfbr2

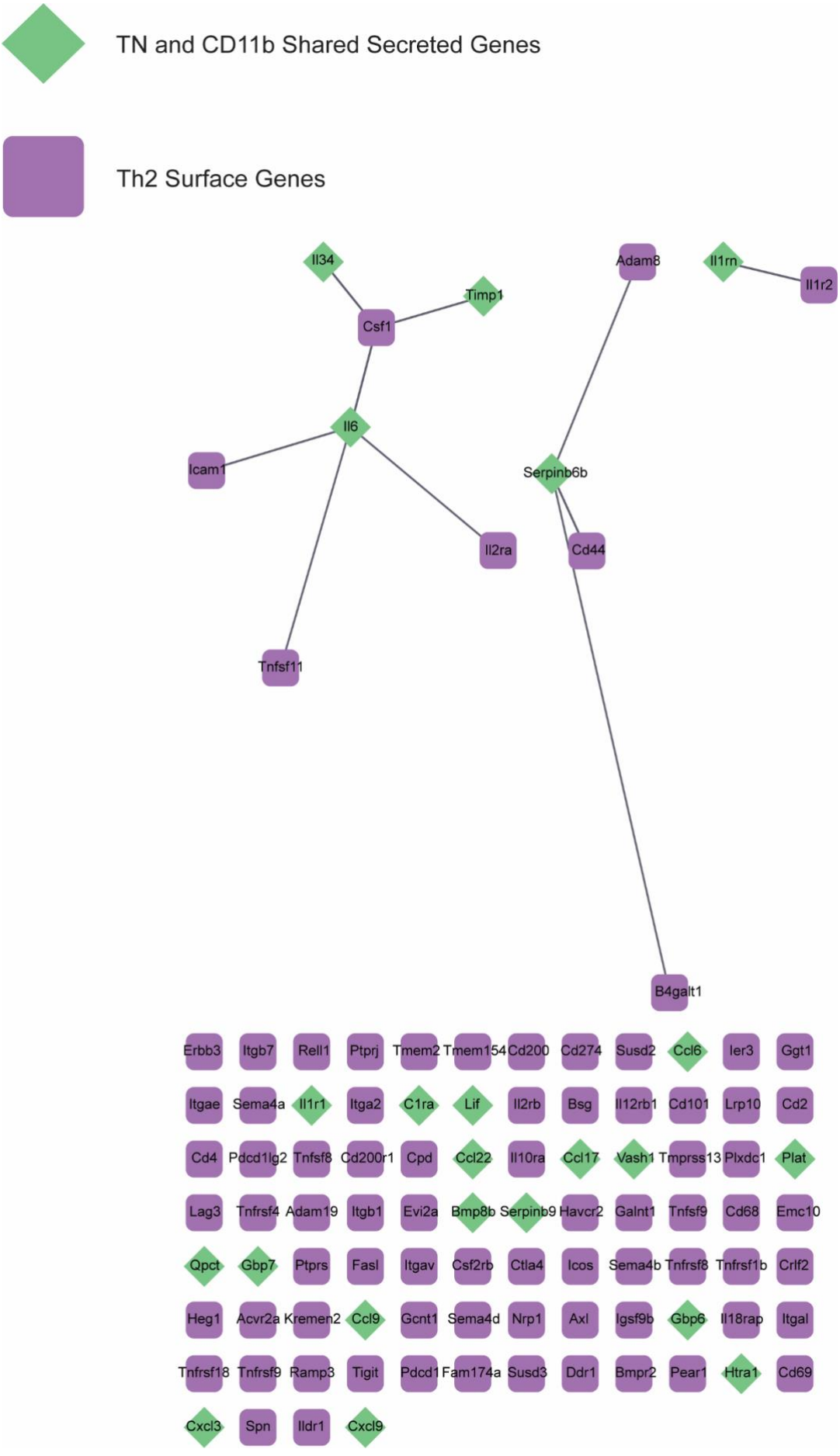
(h)


TN and CD11b Shared Genes
Th2 Genes
TN/CD11b and Th2 Shared Genes
Icam5
Itgal
Ptprj
Pdcd1
Cd80
Ctla4
Cd274
Pdcd1lg2
Cd4
Sema7a
Adam8
Itgav
Itgb1
Tln2
Itga5
Itgb7
Tnfrsf1b
Adora2b
Lrp10
Ramp3
Agrn
Csf2rb
Htr7
Tigit
Pvr
Cd200
Cd200r1
Erbb3
Vcl
Rell1
Tmem2
Tmem154
Csf1
Cd1d2
Susd2
Dll4
Ier3
Ggt1
Mmp14
Itgae
Tnfsf11
Sema4a
Il1r1
Itga2
Il1r2
Dlc1
Il2rb
Ctnnal1
Bsg
Gpr4
Il12rb1
Pard3b
S1pr3
Cd101
Rapgef2
Cd2
Palld
Tnfsf8
Cd86
Dock4
Ptprf
Col23a1
Cpd
Cxcr5
Il10ra
Il31ra
Tmprss13
Cd302
Plxdc1
Il2ra
Gpr85
Lag3
Tnfrsf4
Adam19
Evi2a
Hcfc2
Havcr2
Sorbs1
Galnt1
Tnfsf9
Hcls1
Cd68
Icam1
Emc10
Stab1
P2ry13
Ptprs
Fasl
Mylk
Cd44
Icos
Sema4b
Tnfrsf8
Crlf2
Gpr183
Igsf8
Heg1
Acvr2a
Kremen2
Gcnt1
Slamf1
Sema4d
Nrp1
Axl
Igsf9b
Alcam
Il18rap
Btla
Havcr1
Ly75
B4galt1
Rap1gap2
Tnfrsf18
Vasn
Tnfrsf9
Antxr2
Fam174a
Susd3
Ddr1
Bmpr2
Izumo1
Pear1
Il23r
Cd69
Spn
Ildr1

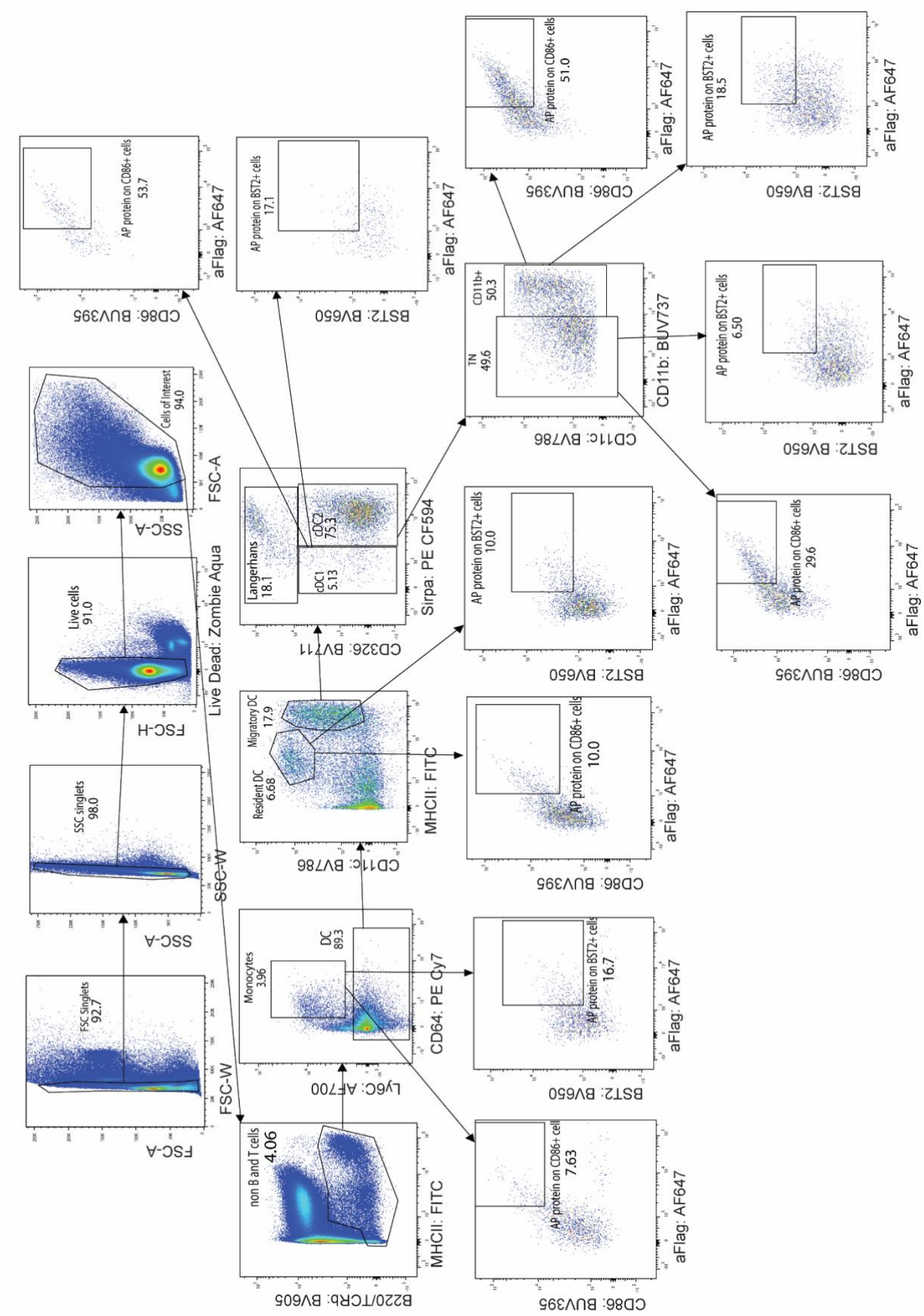
(i)

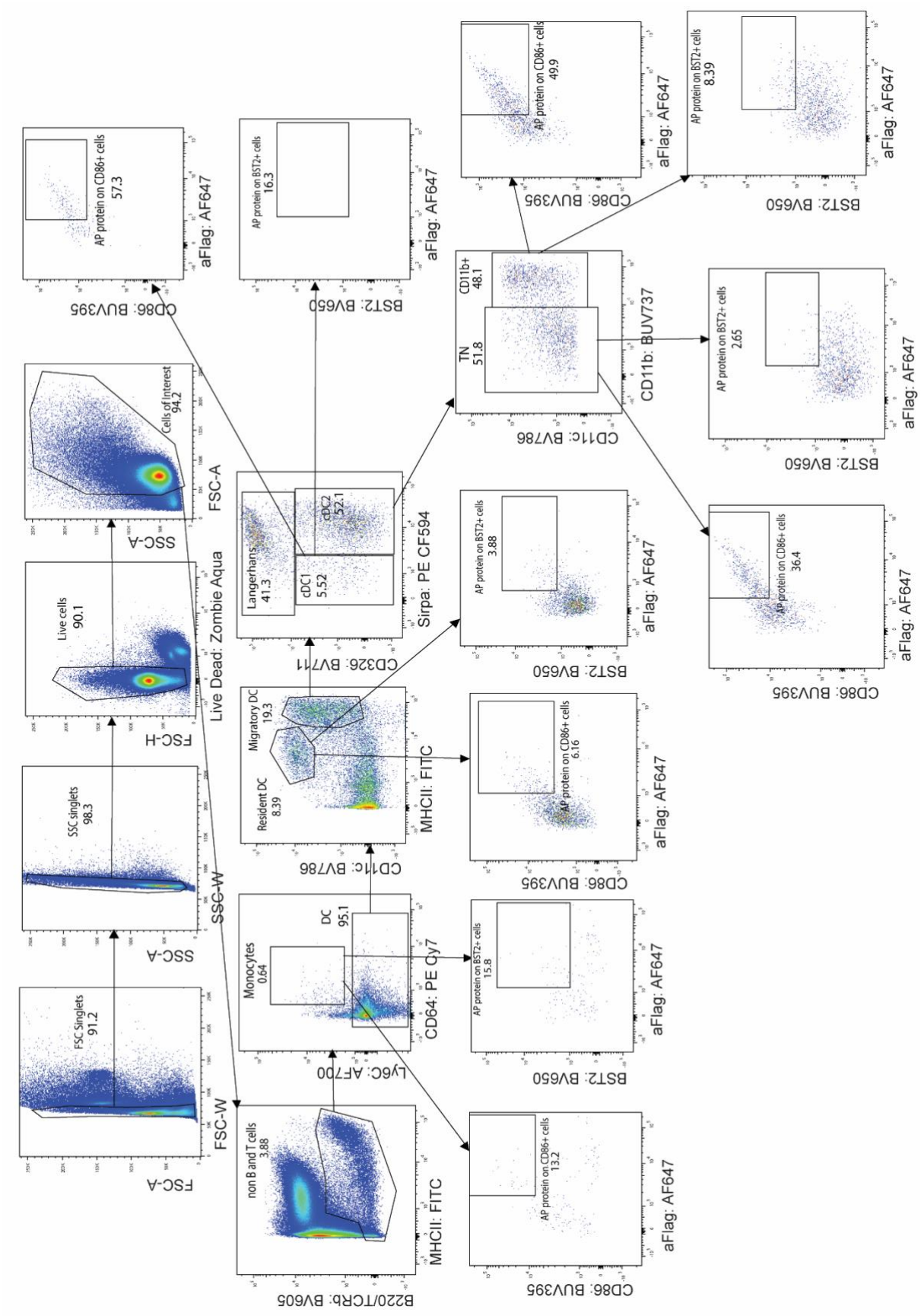

TN and CD11b Shared Secreted Genes
Th0 Surface Genes
H2-T23
Cd47
Lpar6
H2-D1
H2-K1
Sell
Serpinb6b
Itgb2
Itgam
P2rx1
Cd44
H2-Q10
Cxcl3
Ccl9
Ccr7
Cxcr5
H2-Q7
Ccl6
Cxcl9
Thbs1
Timp1
Icam1
Fn1
Tgfb1
Il6
Ccl17
Ccr4
Vcam1
Ccl22
Cd36 Cd226 Siglec1 Htra1 Csf2ra Igf1r Gp9 P2rx7 Pdcd1 Cd3d Tnfrsf18 Fas
Itgal Alcam Clec2d Gbp6 Il34 Sema4d L1cam P2rx4 Cadm1 Cd28 Cd72 Cd3g
Ltb Itgav Nrcam H2-DMa Gbp7 Qpct Il3ra Cd27 F11r Il6st Itgad Serpinb9
Bmp8b Cd22 Cntn1 Itga4 Itgb1 Plat Pecam1 Il2rg Vash1 Cd80 Il1rap Cd4
Itga6 Cd2 Itga1 Cd101 Itgb3 Tgfbr2 Bsg Ptprc Lif C1ra Icam2 Il4ra
Il21r Ifnar1 Cd274 Sipa1l2 Il18r1 Cd19 Il7r Insr Vtcn1 Il1rn Il6ra Cd6
Cd8a

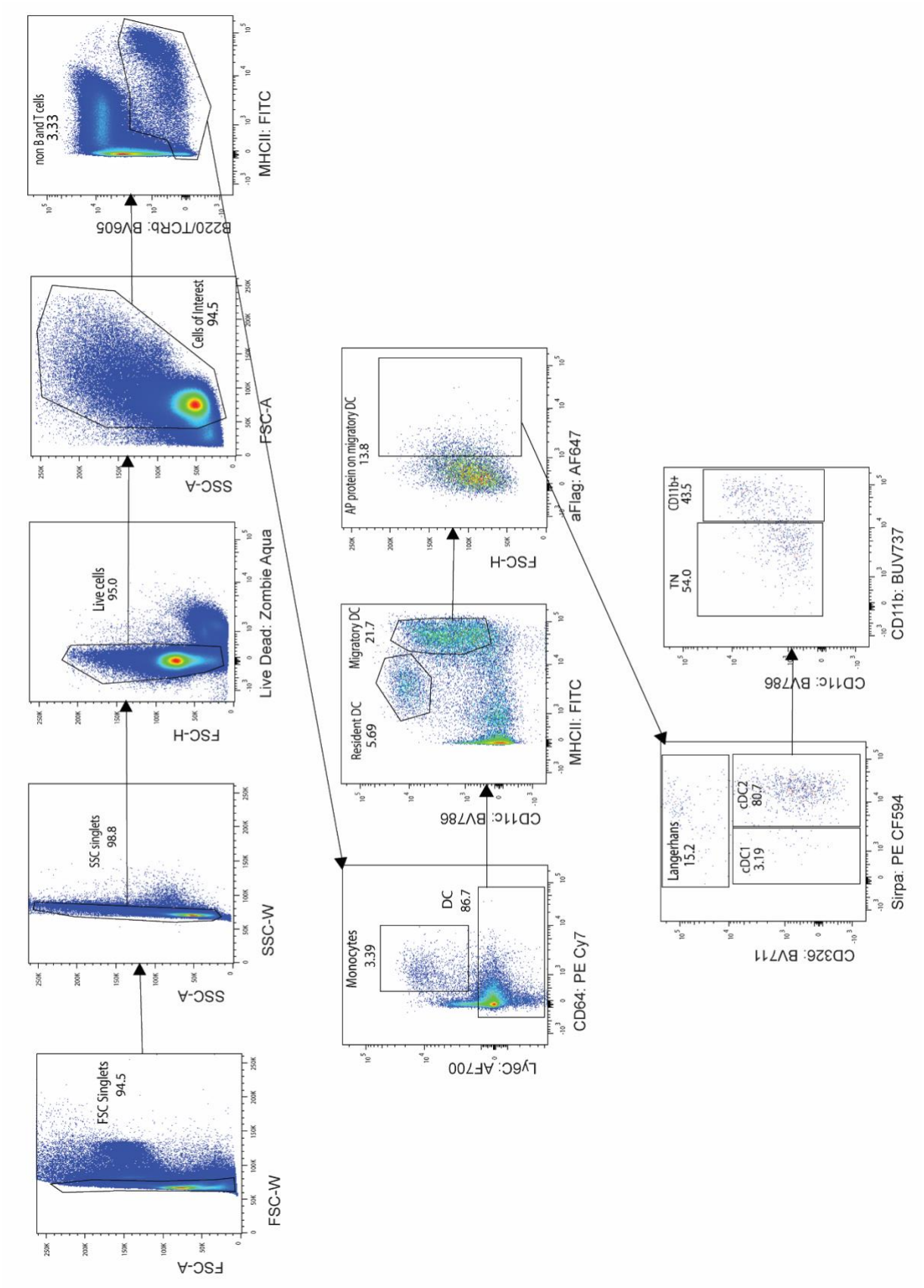
(j)

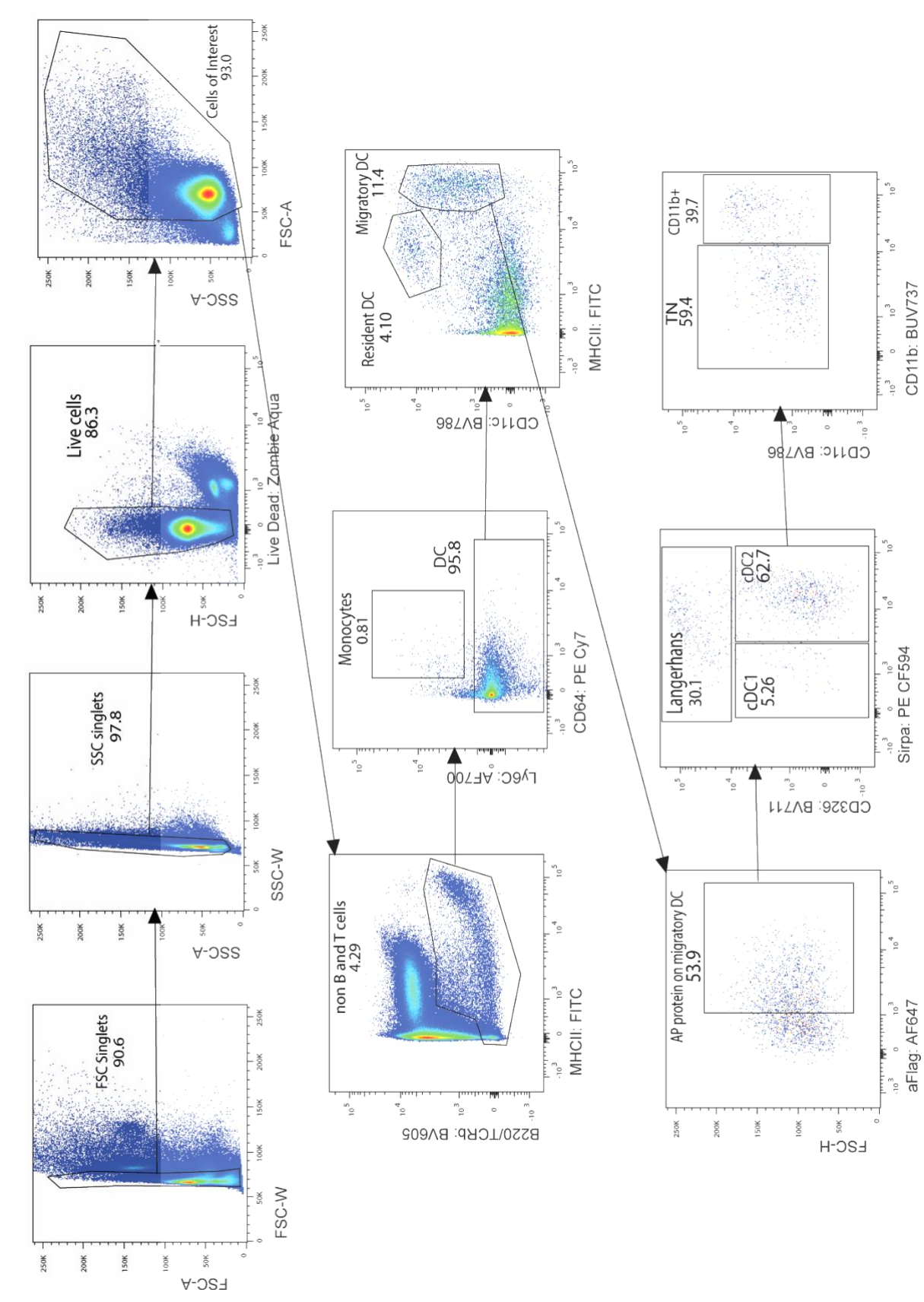

TN and CD11b Shared Secreted Genes
Th2 Surface Genes
Il34
Timp1
Csf1
Il6
Icam1
Il2ra
Tnfsf11
Adam8
Il1rn
Il1r2
Serpinb6b
Cd44
B4galt1
Erbb3 Itgb7 Rell1 Ptprj Tmem2 Tmem154 Cd200 Cd274 Susd2 Ccl6 Ier3 Ggt1
Itgae Sema4a Il1r1 Itga2 C1ra Lif Il2rb Bsg Il12rb1 Cd101 Lrp10 Cd2
Cd4 Pdcd1lg2 Tnfsf8 Cd200r1 Cpd Ccl22 Il10ra Ccl17 Vash1 Tmprss13 Plxdc1 Plat
Lag3 Tnfrsf4 Adam19 Itgb1 Evi2a Bmp8b Serpinb9 Havcr2 Galnt1 Tnfsf9 Cd68 Emc10
Qpct Gbp7 Ptprs Fasl Itgav Csf2rb Ctla4 Icos Sema4b Tnfrsf8 Tnfrsf1b Crlf2
Heg1 Acvr2a Kremen2 Ccl9 Gcnt1 Sema4d Nrp1 Axl Igsf9b Gbp6 Il18rap Itgal
Tnfrsf18 Tnfrsf9 Ramp3 Tigit Pdcd1 Fam174a Susd3 Ddr1 Bmpr2 Pear1 Htra1 Cd69
Cxcl3 Spn Ildr1 Cxcl9

Supplementary Figure 4. Protein interaction networks of (a) surface TN and surface T_H2 genes (b) secreted TN and surface T_H2 genes (c) surface CD11b+ and T_H0 surface genes (d) surface CD11b+ and T_H2 surface genes (e) secreted CD11b+ and T_H0 surface genes (f) secreted CD11b+ and T_H2 surface genes (g) shared surface TN and CD11b+ and T_H0 genes (h) shared surface TN and CD11b+ and T_H2 surface genes (i) shared secreted TN and CD11b+ and T_H0 surface genes (j) shared secreted TN and CD11b+ and T_H2 surface genes.


FSC-A
FSC Singlets
92.7
FSC-W
SSC-A
SSC singlets
98.0
SSC-W
FSC-H
Live cells
91.0
Live Dead: Zombie Aqua
SSC-A
Cells of Interest
94.0
FSC-A
B220/TCRb: BV605
non B and T cells
4.06
MHCII: FITC
Ly6C: AF700
Monocytes
3.96
DC
89.3
CD64: PE Cy7
CD11c: BV786
Resident DC
6.68
Migratory DC
17.9
MHCII: FITC
CD326: BV711
Langerhans
18.1
cDC1
5.13
cDC2
75.3
Sirpa: PE CF594
CD11c: BV786
TN
49.6
CD11b+
50.3
CD11b: BUV737
CD86: BUV395
AP protein on CD86+ cells
7.63
aFlag: AF647
BST2: BV650
AP protein on BST2+ cells
16.7
aFlag: AF647
CD86: BUV395
AP protein on CD86+ cells
10.0
aFlag: AF647
BST2: BV650
AP protein on BST2+ cells
10.0
aFlag: AF647
CD86: BUV395
AP protein on CD86+ cells
29.6
aFlag: AF647
BST2: BV650
AP protein on BST2+ cells
6.50
aFlag: AF647
CD86: BUV395
AP protein on CD86+ cells
51.0
aFlag: AF647
BST2: BV650
AP protein on BST2+ cells
18.5
aFlag: AF647
BST2: BV650
AP protein on BST2+ cells
17.1
aFlag: AF647
CD86: BUV395
AP protein on CD86+ cells
53.7
aFlag: AF647


FSC-A
FSC-W
FSC Singlets
91.2
SSC-A
SSC-W
SSC singlets
98.3
FSC-H
Live cells
90.1
FSC-A
SSC-A
Cells of interest
94.2
Live Dead: Zombie Aqua
B220/TCRb: BV605
MHCII: FITC
non B and T cells
3.88
Ly6C: AF700
CD64: PE Cy7
Monocytes
0.64
DC
95.1
CD11c: BV786
MHCII: FITC
Migratory DC
19.3
Resident DC
8.39
CD326: BV711
Sirpa: PE CF594
Langerhans
41.3
cDC1
5.52
cDC2
52.1
CD11c: BV786
CD11b: BUV737
TN
51.8
CD11b+
48.1
CD86: BUV395
aFlag: AF647
AP protein on CD86+ cells
13.2
BST2: BV650
aFlag: AF647
AP protein on BST2+ cells
15.8
CD86: BUV395
aFlag: AF647
AP protein on CD86+ cells
6.16
BST2: BV650
aFlag: AF647
AP protein on BST2+ cells
3.88
CD86: BUV395
aFlag: AF647
AP protein on CD86+ cells
36.4
BST2: BV650
aFlag: AF647
AP protein on BST2+ cells
2.65
CD86: BUV395
aFlag: AF647
AP protein on CD86+ cells
49.9
BST2: BV650
aFlag: AF647
AP protein on BST2+ cells
8.39
BST2: BV650
aFlag: AF647
AP protein on BST2+ cells
16.3
CD86: BUV395
aFlag: AF647
AP protein on CD86+ cells
57.3


FSC-A
FSC-W
FSC Singlets
94.5
SSC-A
SSC-W
SSC singlets
98.8
FSC-H
Live Dead: Zombie Aqua
Live cells
95.0
SSC-A
FSC-A
Cells of Interest
94.5
B220/TCRb: BV605
MHCII: FITC
non B and T cells
3.33
Ly6C: AF700
CD64: PE Cy7
Monocytes
3.39
DC
86.7
CD11c: BV786
MHCII: FITC
Resident DC
5.69
Migratory DC
21.7
FSC-H
aFlag: AF647
AP protein on migratory DC
13.8
CD326: BV711
Sirpa: PE CF594
Langerhans
15.2
cDC1
3.19
cDC2
80.7
CD11c: BV786
CD11b: BUV737
TN
54.0
CD11b+
43.5


FSC-A
FSC-W
FSC Singlets
90.6
SSC-A
SSC-W
SSC singlets
97.8
FSC-H
Live cells
86.3
Live Dead: Zombie Aqua
SSC-A
FSC-A
Cells of Interest
93.0
B220/TCRb: BV605
MHCII: FITC
non B and T cells
4.29
Ly6C: AF700
CD64: PE Cy7
Monocytes
0.81
DC
95.8
CD11c: BV786
MHCII: FITC
Resident DC
4.10
Migratory DC
11.4
FSC-H
aFlag: AF647
AP protein on migratory DC
53.9
CD326: BV711
Sirpa: PE CF594
Langerhans
30.1
cDC1
5.26
cDC2
62.7
CD11c: BV786
CD11b: BUV737
TN
59.4
CD11b+
39.7

Supplementary Figure 5. Example gating strategies of (a) AP-protein binding on resident DC, migratory DC, and monocytes under Nb immunization (b) migratory DC subpopulations of AP-protein+ migratory DC under Nb immunization (c) AP-protein binding on resident DC, migratory DC, and monocytes under PBS immunization (d) migratory DC subpopulations of AP-protein+ migratory DC under PBS immunization.

(a)

TN Gene name	TN L2FC	Th2 Gene Name	Th2 L2FC	Expression Product
Sirpa	1.49	Adam8	6.258236	9.324771224
Chl1	1.56	Itgb1	5.057121	7.889108243
Fyn	1.38	Ctla4	4.710659	6.500709926
Pik3r1	1.02	Axl	4.659509	4.752698773
Pik3r1	1.02	Icos	4.617477	4.709826952
Gng2	1.05	Ramp3	4.006815	4.20715528
Plxna1	1.45	Nrp1	2.73313	3.963038466
Plxna3	1.41	Nrp1	2.73313	3.853713267
Fyn	1.38	Nrp1	2.73313	3.771719367
Pik3r1	1.02	Csf2rb	3.192644	3.256496939
Pdcd1lg2	1.26	Pdcd1	2.410074	3.036693799
Pik3r1	1.02	Il2ra	2.971166	3.030589363
Pik3r1	1.02	Il2rb	2.867135	2.924477637
Cbl	1.7	Cd4	1.39525	2.371925352
Fcgr2b	1.79	Cd44	1.268913	2.271354314
Cpne3	1.27	Cd68	1.661831	2.110525016
Fcgr2b	1.79	B4galt1	1.142027	2.044227566
Sirpa	1.49	Cd44	1.268913	1.890680407
Pdcd1lg2	1.26	Cd4	1.39525	1.758015261
Sirpa	1.49	B4galt1	1.142027	1.701619594
Bin1	1.15	Cd4	1.39525	1.604537738
Fyn	1.38	Erbb3	1.104697	1.524481902
Shb	1.2	Itgav	1.228028	1.47363324
Cpne3	1.27	B4galt1	1.142027	1.450373748
Pdcd1lg2	1.26	Cd274	1.118851	1.40975185
Pik3r1	1.02	Erbb3	1.104697	1.126790971

(b)

Gene Name	TN VSTPk	Gene Name	Th0 L2Ex	Expression Product
Cst3	14.13	Fn1	13.7803	194.7152151
Adamts7	2.06	Thbs1	16.511	34.0127012
Serpina3f	0	Tgfb1	15.8513	0
Fgf23	0	Fn1	13.7803	0
Serpina3f	0	Fn1	13.7803	0
Serpina3f	0	Thbs1	16.511	0

(c)

CD11b Gene Name	CD11b VSTPk	Th0 Gene Name	Th0 L2Ex	Expression Product
Pxn	8.523333333	Itgav	16.1417	137.5811749
Pxn	8.523333333	Itga1	15.1791	129.3768699
Pxn	8.523333333	Itgb3	13.4357	114.5167792
Tlr6	5.513333333	Cd36	16.3531	90.15992593
Fcgr1	3.353333333	Cd3g	23.7375	79.59983941
Sdc1	4.216666667	Itgav	16.1417	68.06421051
Ccr1	3.763333333	Ccr4	18.0842	68.05694793
Sdc1	4.216666667	Itga6	15.5924	65.74785495
Ccr1	3.763333333	Cxcr5	17.4289	65.59091086
Ccr1	3.763333333	Ccr7	15.999	60.20965781
Sdc1	4.216666667	Fn1	13.7803	58.10680517
Gng4	3.183333333	Ccr4	18.0842	57.56810033
Sdc1	4.216666667	Itgb1	13.5849	57.28306528
Sdc1	4.216666667	Itgb3	13.4357	56.653784
Gng4	3.183333333	Cxcr5	17.4289	55.48212566
Gng4	3.183333333	Ccr7	15.999	50.93022427
Gng4	3.183333333	Lpar6	14.8441	47.253591
Itga9	3.276666667	L1cam	14.0915	46.17301727
Itga9	3.276666667	Vcam1	13.7486	45.04964487
Itga9	3.276666667	Itgb1	13.5849	44.51324362
Itga9	3.276666667	Itgb3	13.4357	44.0242448
C3ar1	0	P2rx1	14.4444	0
C3ar1	0	Itgb2	17.2906	0
C3ar1	0	Cxcr5	17.4289	0
C3ar1	0	Cd36	16.3531	0
C3ar1	0	Itgal	17.4049	0
C3ar1	0	Cd47	18.9311	0
C3ar1	0	Ccr7	15.999	0
C3ar1	0	Itgam	14.6063	0
C3ar1	0	Ccr4	18.0842	0
Lyve1	0	Cd44	12.4889	0
Ednrb	0	Lpar6	14.8441	0
C3ar1	0	Itgav	16.1417	0

(d)

CD11b Gene Name	CD11b L2FC	Th2 Gene Name	Th2 L2FC	Expression Product
Gng4	2.47	Ramp3	4.00681455	9.896831944
Sdc1	1.72	Itgb1	5.05712067	8.69824755
Itga9	1.42	Itgb1	5.05712067	7.18111135
Sdc1	1.72	Itga2	2.16188698	3.718445611
Sdc1	1.72	Lrp10	1.92786991	3.315936241
Itga9	1.42	Itgb7	2.00080828	2.84114776
Sdc1	1.72	Itgav	1.2280277	2.112207644
Siglec1	1.87	Spn	1.08973182	2.037798507
Pxn	1.05	Itgav	1.2280277	1.289429085

(e)

CD11b Gene Name	CD11b VSTPk	Th0 Gene Name	Th0 L2Ex	Expression Product
Cxcl1	11.27333333	Ccr4	18.0842	203.8694401
Cxcl1	11.27333333	Cxcr5	17.4289	196.4822502
Cxcl1	11.27333333	Ccr7	15.999	180.362323
Cxcl2	8.723333333	Ccr4	18.0842	157.7546791
Cxcl2	8.723333333	Cxcr5	17.4289	152.0384533
Cxcl2	8.723333333	Ccr7	15.999	139.5648135
Serpine1	8.086666667	Thbs1	16.511	133.5191151
Serpine1	8.086666667	Tgfb1	15.8513	128.183775
Serpinc1	6.176666667	Gp9	18.8319	116.3184258
Serpine1	8.086666667	Fn1	13.7803	111.4364501
Il27	5.93	Il6st	17.0792	101.2795078
Plau	4.78	Cd47	18.9311	90.49057833
Serpinc1	6.176666667	Fn1	13.7803	85.11613437
Plau	4.78	Itgal	17.4049	83.1953264
Plau	4.78	Itgb2	17.2906	82.64898833
Plau	4.78	Cd36	16.3531	78.1676746
Plau	4.78	Itgav	16.1417	77.1573738
Plau	4.78	Itgam	14.6063	69.81833707
Plau	4.78	P2rx1	14.4444	69.0441364
Clcf1	3.676666667	Il6st	17.0792	62.79443342
Il1a	4.333333333	Il1rap	13.1297	56.89515

(f)

CD11b Gene name	CD11b L2FC	Th2 Gene Name	Th2 L2FC	Expression Product
Serpinc1	2.28	Csf1	8.263268	18.84025149
Plau	1.37	Adam8	6.258236	8.573782937
Il1a	1.36	Il1r1	4.831201	6.570432693
Plau	1.37	Ptprj	3.301989	4.52372459
Plau	1.37	Tnfrsf1b	1.953383	2.676135393
Plau	1.37	Itgav	1.228028	1.682397949
Plau	1.37	Itgal	1.125337	1.541711136

(g)

TN Gene Name	TN VSTPk	Th0 Gene Name	Th0 L2Ex	TN Expression Product
Cd274	12.84	Cd3g	23.7375267	304.7898424
Cd274	12.84	Cd3d	21.8549067	280.6170016
Cd86	12.9166667	Cd28	18.8633367	243.651432
Cd274	12.84	Cd4	18.2864833	234.798446
Cd274	12.84	Pdcd1	17.76249	228.0703716
Gpr183	12.34	Ccr4	18.08422	223.1592748
Gpr183	12.34	Cxcr5	17.42894	215.0731196
Pvr	11.3366667	Cd226	17.8109433	201.9167276
Alcam	10.67	Cd6	18.6847167	199.3659268
Sema7a	13.1266667	Itga1	15.17914	199.2515111
Gpr183	12.34	Ccr7	15.9990233	197.4279479
Cd80	10.46	Cd28	18.8633367	197.3105015
Cd40	12.4866667	Ltb	15.30937	191.1630001
Sema7a	13.1266667	Itgb1	13.5849167	178.3246728
Itgav	9.37333333	Cd47	18.9310833	177.4473544
Itgav	9.37333333	Itgal	17.40488	163.1417419
Itgav	9.37333333	Itgb2	17.2905833	162.0704011
Cd86	12.9166667	Cd80	12.223	157.8804167
Cd274	12.84	Cd80	12.223	156.94332
S1pr3	8.62	Ccr4	18.08422	155.8859764
Itgav	9.37333333	Cd36	16.35307	153.2827761
Cd80	10.46	Itgam	14.6063467	152.7823861
Cd40	12.4866667	Cd80	12.223	152.6245267
S1pr3	8.62	Cxcr5	17.42894	150.2374628
Icam5	8.12666667	Itgal	17.40488	141.4436581
Icam5	8.12666667	Itgb2	17.2905833	140.5148072
Itga5	8.11666667	Itgb2	17.2905833	140.3419014
Vcl	9.21666667	Itga1	15.17914	139.9010737
S1pr3	8.62	Ccr7	15.9990233	137.9115811
Itgav	9.37333333	Itgam	14.6063467	136.9101561
Itgav	9.37333333	P2rx1	14.44438	135.3919885
Itgav	9.37333333	L1cam	14.09146	132.0839517
Itgav	9.37333333	Fn1	13.78027	129.1670641
Itgav	9.37333333	Itgb1	13.5849167	127.3359522
Vcl	9.21666667	Fn1	13.78027	127.0081552
Itgav	9.37333333	Itgb3	13.43568	125.9371072
Vcl	9.21666667	Itgb3	13.43568	123.832184
Cd1d2	5.12666667	Cd3g	23.7375267	121.6943867
Itga5	8.11666667	L1cam	14.09146	114.3756837
Cd1d2	5.12666667	Cd3d	21.8549067	112.0428215
Itga5	8.11666667	Fn1	13.78027	111.8498582
Gpr4	7.5	Lpar6	14.84406	111.33045
Itga5	8.11666667	Itgb1	13.5849167	110.2642403
Itga5	8.11666667	Itgb3	13.43568	109.052936
Cd70	6.79	Cd27	15.79269	107.2323651
P2ry13	5.31666667	Ccr4	18.08422	96.14776967
P2ry13	5.31666667	Cxcr5	17.42894	92.66386434
Mylk	5.7	Itga1	15.17914	86.521098
P2ry13	5.31666667	Ccr7	15.9990233	85.06147406
Adora2b	5.63666667	Csf2ra	14.86541	83.79136104
Tgfb1	4.79666667	Itgav	16.14171	77.42640231
Sorbs1	4.27666667	Itga1	15.17914	64.91612207
Sorbs1	4.27666667	Insr	14.67559	62.76260657
Cxcr5	3.39333333	Ccr4	18.08422	61.36578653
Il1r1	4.42333333	Il1rap	13.12965	58.0768185
Cxcr5	3.39333333	Ccr7	15.9990233	54.29001917
Tln2	2.01666667	Itgb2	17.2905833	34.86934306
Tln2	2.01666667	Itgb1	13.5849167	27.39624862
Tln2	2.01666667	Itgb3	13.43568	27.095288

CD11b Gene Name	CD11b VSTPk	Th0 Gene Name	Th0 L2Ex	CD11b Expression Product
Cd274	12.20333333	Cd3g	23.7375267	289.6769503
Cd274	12.20333333	Cd3d	21.8549067	266.7027109
Cd274	12.20333333	Cd4	18.2864833	223.1560516
Cd86	11.61333333	Cd28	18.8633367	219.0662164
Cd274	12.20333333	Pdcd1	17.76249	216.7615862
Gpr183	11.36666667	Ccr4	18.08422	205.5573007
Pvr	11.15333333	Cd226	17.8109433	198.6513879
Gpr183	11.36666667	Cxcr5	17.42894	198.1089514
Cd40	12.23333333	Ltb	15.30937	187.2846263
Gpr183	11.36666667	Ccr7	15.9990233	181.8555653
Alcam	9.65	Cd6	18.6847167	180.3075158
Sema7a	11.77666667	Itga1	15.17914	178.7596721
Cd80	9.026666667	Cd28	18.8633367	170.2730523
Itgav	8.84	Cd47	18.9310833	167.3507767
Sema7a	11.77666667	Itgb1	13.5849167	159.9850353
Itgav	8.84	Itgal	17.40488	153.8591392
Itgav	8.84	Itgb2	17.2905833	152.8487567
Cd40	12.23333333	Cd80	12.223	149.5280333
Cd274	12.20333333	Cd80	12.223	149.1613433
Itgav	8.84	Cd36	16.35307	144.5611388
Cd86	11.61333333	Cd80	12.223	141.9497733
Itga5	7.88	Itgb2	17.2905833	136.2497967
Cd80	9.026666667	Itgam	14.6063467	131.8466226
Itgav	8.84	Itgam	14.6063467	129.1201045
Itgav	8.84	P2rx1	14.44438	127.6883192
Cd1d2	5.273333333	Cd3g	23.7375267	125.1758906
Itgav	8.84	L1cam	14.09146	124.5685064
Itgav	8.84	Fn1	13.78027	121.8175868
Itgav	8.84	Itgb1	13.5849167	120.0906633
Itgav	8.84	Itgb3	13.43568	118.7714112
Cd1d2	5.273333333	Cd3d	21.8549067	115.2482078
Icam5	6.49	Itgal	17.40488	112.9576712
Icam5	6.49	Itgb2	17.2905833	112.2158858
Itga5	7.88	L1cam	14.09146	111.0407048
Vcl	7.3	Itga1	15.17914	110.807722
Itga5	7.88	Fn1	13.78027	108.5885276
P2ry13	5.976666667	Ccr4	18.08422	108.0833549
Itga5	7.88	Itgb1	13.5849167	107.0491433
Itga5	7.88	Itgb3	13.43568	105.8731584
P2ry13	5.976666667	Cxcr5	17.42894	104.1669647
Vcl	7.3	Fn1	13.78027	100.595971
Vcl	7.3	Itgb3	13.43568	98.080464
P2ry13	5.976666667	Ccr7	15.9990233	95.62082946
S1pr3	5.16	Ccr4	18.08422	93.3145752
Cd70	5.813333333	Cd27	15.79269	91.80817119
S1pr3	5.16	Cxcr5	17.42894	89.9333304
S1pr3	5.16	Ccr7	15.9990233	82.5549604
Cxcr5	4.193333333	Ccr4	18.08422	75.83316253
Gpr4	5.063333333	Lpar6	14.84406	75.1604238
Adora2b	4.786666667	Csf2ra	14.86541	71.15576254
Tgfb1	4.393333333	Itgav	16.14171	70.91591259
Cxcr5	4.193333333	Ccr7	15.9990233	67.08923784
Mylk	4.01	Itga1	15.17914	60.8683514
Sorbs1	3.886666667	Itga1	15.17914	58.99625747
Sorbs1	3.886666667	Insr	14.67559	57.03912647
Il1r1	3.386666667	Il1rap	13.12965	44.465748
Tln2	1.803333333	Itgb2	17.2905833	31.18068527
Tln2	1.803333333	Itgb1	13.5849167	24.49813305
Tln2	1.803333333	Itgb3	13.43568	24.2290096

(h)

TN Gene Name	TN L2FC	Th2 Gene Name	Th2 L2FC	TN Expression Product
Itgav	3.43	Adam8	6.25823572	21.46574852
Sema7a	3.69	Itgb1	5.05712067	18.66077527
Itgav	3.43	Itgb1	5.05712067	17.34592389
Htr7	3.14	Ramp3	4.00681455	12.58139769
Itgav	3.43	Ptprj	3.30198875	11.32582142
Pvr	1.48	Tigit	6.16649942	9.126419141
Adora2b	2.09	Ramp3	4.00681455	8.374242414
Itga5	1.44	Itgb1	5.05712067	7.282253763
Tln2	1.36	Itgb1	5.05712067	6.877684109
Itgav	3.43	Itgb7	2.00080828	6.862772406
Itgav	3.43	Tnfrsf1b	1.9533835	6.700105401
Adora2b	2.09	Csf2rb	3.19264406	6.672626081
Cd200	3.83	Cd200r1	1.70095559	6.514659898
Cd274	1.3	Ctla4	4.71065937	6.123857177
Cd274	1.3	Pdcd1lg2	3.92133527	5.097735853
Itgav	3.43	Itgal	1.1253366	3.859904522
Cd274	1.3	Pdcd1	2.41007444	3.133096776
Itga5	1.44	Itgb7	2.00080828	2.881163926
Icam5	2.36	Itgal	1.1253366	2.655794365
Agrn	1.36	Lrp10	1.92786991	2.621903074
Cd80	1.82	Cd274	1.11885067	2.036308228
Cd274	1.3	Cd4	1.39525021	1.81382527

CD11b Gene Name	CD11b L2FC	Th2 Gene Name	Th2 L2FC	CD11b Expression Product
Itgav	2.31	Adam8	6.25823572	14.45652452
Itgav	2.31	Itgb1	5.05712067	11.68194874
Sema7a	2.29	Itgb1	5.05712067	11.58080633
Htr7	2.04	Ramp3	4.00681455	8.173901686
Itgav	2.31	Ptprj	3.30198875	7.627594017
Pvr	1.13	Tigit	6.16649942	6.968144344
Tln2	1.08	Itgb1	5.05712067	5.461690322
Itga5	1.05	Itgb1	5.05712067	5.309976702
Cd200	2.85	Cd200r1	1.70095559	4.847723423
Cd274	1.01	Ctla4	4.71065937	4.757765961
Itgav	2.31	Itgb7	2.00080828	4.621867131
Itgav	2.31	Tnfrsf1b	1.9533835	4.512315882
Adora2b	1.05	Ramp3	4.00681455	4.20715528
Cd274	1.01	Pdcd1lg2	3.92133527	3.960548624
Adora2b	1.05	Csf2rb	3.19264406	3.352276261
Itgav	2.31	Itgal	1.1253366	2.599527535
Cd274	1.01	Pdcd1	2.41007444	2.434175188
Agrn	1.14	Lrp10	1.92786991	2.197771695
Itga5	1.05	Itgb7	2.00080828	2.100848696
Icam5	1.71	Itgal	1.1253366	1.924325578
Cd80	1.42	Cd274	1.11885067	1.588767958
Cd274	1.01	Cd4	1.39525021	1.409202709

(i)

TN Gene Name	TN VSTPk	Th0 Gene Name	Th0 L2Ex	TN Expression Product
Serpinb6b	13.93	H2-K1	23.42695667	326.3375064
Ccl22	16.42	Ccr4	18.08422	296.9428924
Serpinb6b	13.93	H2-Q10	20.49152333	285.44692
Serpinb6b	13.93	Cd47	18.93108333	263.7099908
Serpinb6b	13.93	H2-T23	18.76508	261.3975644
Serpinb6b	13.93	Sell	17.79839	247.9315727
Serpinb6b	13.93	Itgb2	17.29058333	240.8578258
Serpinb6b	13.93	H2-Q7	17.19818	239.5706474
Serpinb6b	13.93	H2-D1	15.12411	210.6788523
Ccl17	11.55	Ccr4	18.08422	208.872741
Serpinb6b	13.93	Itgam	14.60634667	203.4664091
Serpinb6b	13.93	P2rx1	14.44438	201.2102134
Ccl17	11.55	Ccr7	15.99902333	184.7887195
Serpinb6b	13.93	Cd44	12.48885	173.9696805
Ccl9	8.693333333	Ccr4	18.08422	157.2121525
Ccl9	8.693333333	Cxcr5	17.42894	151.5155851
Ccl9	8.693333333	Ccr7	15.99902333	139.0848428
Ccl9	8.693333333	Lpar6	14.84406	129.0443616
Cxcl9	6.863333333	Ccr4	18.08422	124.1180299
Cxcl9	6.863333333	Cxcr5	17.42894	119.6206249
Cxcl9	6.863333333	Ccr7	15.99902333	109.8066301
Il6	6.49	Fn1	13.78027	89.4339523
Il6	6.49	Vcam1	13.74862	89.2285438
Il6	6.49	Icam1	12.88695333	83.63632713
Ccl6	4.496666667	Ccr4	18.08422	81.31870927
Ccl6	4.496666667	Cxcr5	17.42894	78.37213354
Cxcl3	4.036666667	Ccr4	18.08422	72.99996807
Ccl6	4.496666667	Ccr7	15.99902333	71.94227493
Cxcl3	4.036666667	Cxcr5	17.42894	70.35482114
Ccl6	4.496666667	Lpar6	14.84406	66.7487898
Cxcl3	4.036666667	Ccr7	15.99902333	64.58272419
Timp1	0	Fn1	13.78027	0
Timp1	0	Thbs1	16.51102	0
Timp1	0	Tgfb1	15.85125	0

CD11b Gene Name	CD11b VSTPk	Th0 Gene Name	Th0 L2Ex	CD11b Expression Product
Serpinb6b	13.17333333	H2-K1	23.42695667	308.6111091
Ccl22	15.15	Ccr4	18.08422	273.975933
Serpinb6b	13.17333333	H2-Q10	20.49152333	269.9416673
Serpinb6b	13.17333333	Cd47	18.93108333	249.385471
Serpinb6b	13.17333333	H2-T23	18.76508	247.1986538
Serpinb6b	13.17333333	Sell	17.79839	234.4641242
Serpinb6b	13.17333333	Itgb2	17.29058333	227.7746177
Serpinb6b	13.17333333	H2-Q7	17.19818	226.5573578
Ccl17	12.51	Ccr4	18.08422	226.2335922
Ccl17	12.51	Ccr7	15.99902333	200.1477819
Serpinb6b	13.17333333	H2-D1	15.12411	199.2349423
Serpinb6b	13.17333333	Itgam	14.60634667	192.4142734
Ccl9	10.58	Ccr4	18.08422	191.3310476
Serpinb6b	13.17333333	P2rx1	14.44438	190.2806325
Ccl9	10.58	Cxcr5	17.42894	184.3981852
Ccl9	10.58	Ccr7	15.99902333	169.2696669
Serpinb6b	13.17333333	Cd44	12.48885	164.519784
Ccl9	10.58	Lpar6	14.84406	157.0501548
Ccl6	7.966666667	Ccr4	18.08422	144.0709527
Ccl6	7.966666667	Cxcr5	17.42894	138.8505553
Cxcl3	7.626666667	Ccr4	18.08422	137.9223179
Cxcl3	7.626666667	Cxcr5	17.42894	132.9247157
Cxcl9	7.343333333	Ccr4	18.08422	132.7984555
Cxcl9	7.343333333	Cxcr5	17.42894	127.9865161
Ccl6	7.966666667	Ccr7	15.99902333	127.4588859
Cxcl3	7.626666667	Ccr7	15.99902333	122.019218
Ccl6	7.966666667	Lpar6	14.84406	118.257678
Cxcl9	7.343333333	Ccr7	15.99902333	117.4861613
Il6	6.946666667	Fn1	13.78027	95.72694227
Il6	6.946666667	Vcam1	13.74862	95.50708027
Il6	6.946666667	Icam1	12.88695333	89.52136916
Timp1	0	Fn1	13.78027	0
Timp1	0	Thbs1	16.51102	0
Timp1	0	Tgfb1	15.85125	0

(j)

TN Gene Name	TN L2FC	Th2 Gene Name	Th2 L2FC	TN Expression Product
Il6	2.71	Csf1	8.2632682	22.39345681
Il6	2.71	Tnfsf11	6.34469778	17.19413098
Serpinb6b	2.18	Adam8	6.25823572	13.64295387
Il1rn	1.33	Il1r2	6.56884152	8.736559222
Il6	2.71	Il2ra	2.97116604	8.051859975
Il6	2.71	Icam1	1.66886349	4.522620068
Serpinb6b	2.18	Cd44	1.26891302	2.766230394
Serpinb6b	2.18	B4galt1	1.14202657	2.48961793

CD11b Gene Name	CD11b L2FC	Th2 Gene Name	Th2 L2FC	CD11b Expression Product
Il6	1.95	Csf1	8.2632682	16.11337298
Il6	1.95	Tnfsf11	6.34469778	12.37216067
Il1rn	1.76	Il1r2	6.56884152	11.56116108
Serpinb6b	1.23	Adam8	6.25823572	7.697629937
Il6	1.95	Il2ra	2.97116604	5.793773782
Il6	1.95	Icam1	1.66886349	3.254283813
Serpinb6b	1.23	Cd44	1.26891302	1.56076302
Serpinb6b	1.23	B4galt1	1.14202657	1.404692685

Supplementary Figure 6. Predicted Interaction Expression Products for surface and secreted genes of TN /CD11b+ DC and surface genes of T_H0/T_H2 cells. (a) TN surface and T_H2 predicted interactions expression products. (b) TN secreted and T_H0 predicted interactions expression products. (c) CD11b+ surface and T_H0 predicted interactions expression products. (d) CD11b+ surface and T_H2 predicted interactions expression products. (e) CD11b+ secreted and T_H0 predicted interactions expression products. (f) CD11b+ secreted and T_H2 predicted interactions expression products. (g) TN and CD11b+ shared surface and T_H0 predicted interactions expression products. (h) TN and CD11b+ shared surface genes and T_H2 predicted interactions expression products. (i) TN and CD11b + shared secreted and T_H0 predicted interactions expression products. (j) TN and CD11b+ shared secreted and T_H2 predicted interactions expression products. Highlighted are known receptor-ligand pairs.

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