HETEROCHRONIC PARABIOSIS STUDIES OF THE AGING IMMUNE SYSTEM

by

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ABBREVIATIONS

ANOVA – Analysis of variance

APC – Antigen presenting cell

β2m – β2-microglobulin

B6 – unspecified C57BL/6 substrain

B6J – C567BL/6J substrain from JAX

B6.SJL – CD45.1 congeneric to B6

CD45/Ly5/PTPRC – alternative names for the pan-hematopoietic lineage phosphatase with alternative alleles (CD45.1 and CD45.2, aka Ptpre<sup>a</sup> and Ptpre<sup>b</sup> or Ly5.1 and Ly5.2)

DAMPs – Danger associated molecular patterns

DC – dendritic cell

GvHD – Graft-versus-host disease

Heterochronic Parabiosis – The surgical union of two animals of disparate ages

Isochronic Parabiosis – The surgical union of two animals of matching ages

HSC – hematopoietic stem cell

ILC – Innate-like lymphocyte

JAX – The Jackson Laboratory, Bar Harbor, ME

LN – Lymph Node

MHC (pMHC) – Major histocompatibility complex (peptide-MHC)

NCI – National Cancer Institute

NIA – National Aging Institute

NK – Natural Killer

Parabiont – One of the conjoined animals paired in parabiosis
**PRR** – Pattern recognition receptors

**SNP** – Single nucleotide polymorphism

**TCR** – T cell receptor

**T_N** – Naïve T cell

**T_eff** – Effector T cell

**T_M** – Memory T cell

**T_CM** – CD8+ Central memory T cell

**T_EM** – CD8+ Effector memory T cell

**T_RM** – Resident memory T cell

**T_VM** – Virtual memory T cell

**Treg** – Regulatory T cell
ABSTRACT

Parabiosis is the surgical union of two organisms resulting in the development of a single, shared circulatory system. When animals of different ages are conjoined (i.e. heterochronic parabiosis), blood-borne factors from the parabionts can affect the physiology of the other parabiont. This is manifested sometimes by beneficial, rejuvenating impact upon the older animal’s tissues and organs (anti-geronic effect), and sometimes by younger animal’s tissues regressing and appearing old-like (pro-geronic effect). These effects, and the ability to identify individual factors that could recapitulate pro- and anti-geronic effects, have made heterochronic parabiosis a very attractive approach to studying biology of aging and rejuvenation.

However, heterochronic parabiosis has not been widely used to investigate the aged immune system. An important question to be answered is whether the cellular defects involved in the aged immune system are due to intrinsic defects or if they can be rescued by extrinsic factors. Heterochronic parabiosis is ideal to test cellular migration patterns, interrogate the mechanisms driving migration defects that occur with aging, establish if these defects can be rejuvenated and identify molecules that are targets for intervention. Here, we provide evidence of the importance of reducing differences in the background genetics of different C57BL/6 substrains prior to parabiosis. This improvement allowed us to improve survival and confirm robust lymphocyte equilibration across secondary, but not primary, lymphoid tissues. We found no evidence for rejuvenation of the old immune cells, whereas results suggested that adult peripheral lymph nodes (pLN) lost mass and cellularity, potentially indicating the presence of a pro-geronic factor(s) in the old circulation that affects pLN function. Adult and old immune cells were present in equal frequencies in both adult and old
secondary lymphoid tissues, indicating that there was no restriction of cellular migration due to the age of the cell or age of the tissue. The propensity of adult immune cells (i.e. large naïve compartment) to occupy lymph nodes and old immune cells (i.e. large memory compartment) to occupy bone marrow was retained following heterochronic parabiosis. Finally, parabiosis separation experiments illuminated the peripheral survival advantage of old T cells over adult T cells. These results highlight the power of heterochronic parabiosis in studying immune aging and provide hypothesis-generating data for future mechanistic studies of peripheral T cell maintenance with aging.
CHAPTER 1 – INTRODUCTION

SECTION 1.1 – Introduction to the Immune System

1.1.1 – The Role of the Immune System

The immune system is a dynamically integrated series of organs, cells and processes within an organism with a primary role to defend against microorganisms. Classical views of the immune system typically describe two subsystems: either 1) humoral (i.e. macromolecules found in extracellular space) and cellular immunity or 2) the innate and adaptive immune system. The majority of the cells of the immune system are derived from hematopoietic stem cells (HSCs); however, it is likely that every cell (or nearly every cell) in the body is integrated into the immune system (1–6). Traditionally non-immune cells participate in at least three ways. First, they express pattern-recognition receptors (PRRs) for danger-associated molecular patterns (DAMPs) that allow them to analyze the local extracellular and intracellular environments (7–9). Second, following the engagement of PRRs, cells secrete both innate effector molecules and/or signaling molecules that inform the immune system of the cell’s status (whether or not the cell is infected, or sensed the infection of neighboring cells) (10–14). Lastly, non-immune cells express or alter expression of molecules [e.g. major histocompatibility complex (MHC) proteins that bind a sampling of peptides for T cells to survey (explained in Section 1.1.3.2), natural killer (NK) receptors, a complex “eat me/don’t eat me” signaling network for phagocytes, etc.] that instruct effector cells of the immune system to mount the appropriate response (3, 15–18). The remainder of the discussion is focused upon specialized cells of the adaptive and innate immune system.
1.1.2 – The Innate Immune System

The hallmark of the innate immune system is immediate protection against pathogens. This feature is highly conserved throughout evolution and is present in all forms of life. The innate immune system is comprised of (i) the massive barrier structures that comprise the skin, lungs and gastrointestinal tracts (19–21), (ii) an ever expanding arsenal of paracrine effector molecules both biological- and chemical-based (22–26), (iii) the system of PRRs that recognize conserved molecular DAMPs (27), (iv) complex cascades that initiate systemic defense responses (*e.g.* the pyrogenic response, complement and coagulatory responses) (28, 29), and (v) *bona fide* innate immune cells that engage in phagocytosis, tissue formation/remodeling capabilities and produce and release molecules mentioned under (ii) (30, 31).

Classical cells of the innate immune system, which primarily develop in the bone marrow from HSCs, include the myeloid-lineage derived phagocytes (neutrophils, macrophages and dendritic cells (DCs)), basophils, eosinophils, mast cells and the lymphoid-lineage derived plasmacytoid DCs, NK cells and a growing class of innate-lymphoid cells (ILCs), the discussion of which is beyond the scope of this thesis (30–33). Phagocytes (‘eating cells’) play a critical role in tissue development and maintenance (34, 35). They are also responsible for the clearance of host cells that have died either by homeostatic (non-inflammatory) forms of death or by pro-inflammatory forms of cellular death (3, 36–39). However, outside of homeostatic conditions, such as in certain disease states, phagocytes can also contribute to tissue destruction and many other pathologies (40–44).

Neutrophils play a vital role in the initial phases of many immune responses as well as in tissue repair and normally represent 50-60% of circulating leukocytes in most mammals
The lifespan of neutrophils is very short, and when activated, neutrophils unleash a vigorous kamikaze response involving reactive oxygen species (ROS) (e.g. hydrogen peroxide), reactive nitrogen species, and even hypochlorite (46). Other neutrophils end their life in a selfless act called netosis in which neutrophils release extracellular fibers, primarily comprised of genomic DNA, which form neutrophil extracellular traps (NETs) in order to trap pathogens (47). This short life of a neutrophils mandates high production, and indeed a healthy human produces over $10^{11}$ neutrophils a day (45, 48).

Monocytes/macrophages are a complicated, heterogeneous lineage containing many tissue resident macrophage cell types that are not likely derived from classical bone marrow HSCs (49). Regardless of the derivation of a given macrophage, these cells reside in both lymphoid and non-lymphoid tissues where they play critical roles in tissue maintenance and repair (35). Macrophages also function as resident sentinel immune cells capable of participating in and instructing an immune response (50). Macrophages are capable of being antigen-presenting cells (APCs) for T cells, albeit less important to initiation of the immune response compared to DCs (35, 51, 52).

DCs are present both in circulation as well as in temporarily tissue resident roles, particularly at barrier sites in contact with the external environment where they position for optimal early access to antigens by phagocytosis (53). Upon obtaining antigen, along with particular PRR-derived cues, DCs drain to the nearest secondary lymphoid organ to either pass off the antigen to resident DCs, present the antigen to T cells, or both (54). DCs represent a critical link between the innate and adaptive immune response as a large component of the adaptive immune response requires the proper activation of $T_N$ cells, and this appears to be an exclusive domain of DCs (53, 55, 56).
The innate cells positioned at the site of infection often eliminate any signs of infection through phagocytosis and the secretion of effector molecules, before there are any symptoms of the disease (30, 57). In the event of failure in this initial control, the innate immune cells play a vital role in orchestrating the adaptive immune response both through the secretion of soluble signaling molecules that recruit more cells and through the migration to the draining lymphoid tissue in order to prime T cells and B cells to mount an adaptive immune response (56). The DCs that drain from the site of infection to the lymphoid tissues either directly present, or pass off antigen to resident DCs to present foreign antigen in the context of MHC, which will serve as “signal 1” for a naïve T cell. Signal 2 is provided in the form of costimulatory receptors, namely CD28-CD80/86 (although there are many others), only if the APC was appropriately activated by the proper milieu of DAMPs. Signal 3 is provided in the form of soluble cytokines (i.e. IL-12), which is also instructed by specific PRR activated by the microbial pathogen within the DC. The precise contexts of signals 1, 2 and 3, likely with other molecules involved, contributes to the type of effector response (or lack thereof) that will be mounted (56, 58–60).

1.1.3 – The Adaptive Immune System

The hallmarks of the adaptive immune system are the ability to mount a long-lived, antigen-specific memory response (i.e. the response is faster and more robust upon rechallenge with antigen), and the ability to rearrange the germline to create a diverse array of antigen-specific receptors. Descriptions here will be limited to jawed vertebrates, specifically mammals, which use Recombination-activating genes (RAGs) and immunoglobulins to accomplish the latter requirement (61). The major reason why reinfection with the same pathogen is either asymptomatic or mild, and why vaccines work, are these highly diverse
antigen-specific receptors and the memory potential conferred in the cells bearing these receptors. Putting aside recently described memory features in cells generally attributed to the innate immune system (62–64), the adaptive immune system is comprised of two main cell-types derived from HSCs, B cells and T cells.

1.1.3.1 – B Cells

‘B’ in B cell stands for ‘bursa of Fabricus’, a specialized organ that is the site of hematopoiesis, as well as B cell production, in birds, where B cells were first discovered. However, it is easier to think of the ‘B’ as denoting ‘bone marrow’ because in mammals, B cells develop in the bone marrow prior to exiting to the periphery for a chance to encounter the cognate antigen, recognized by their newly rearranged B cell receptor (BCR) (65). The most important function attributed to B cells is antibody secretion, the adaptive component of humoral immunity. However, intricate to optimal antibody production and other processes, B cells are also efficient APCs and secrete immunomodulatory cytokines, among other roles (66). Also, antibodies play an important role in providing specificity to the innate immune system as the use of different alternative constant regions (i.e. antibody isotypes), or Fc regions that bind to specific Fc receptors expressed by specialized cells in the innate immune system (and non-immune cells) (67). Like other lymphocytes, B cells are thought to primarily reside in primary and secondary lymphoid organs. One method of preventing antibody mediated autoimmunity involves the necessity of B cells to interact with CD4 T cells, thereby requiring B cell to bind antigen with its BCR, internalize it, process and present to a nearby activated T cell specific for the same antigen (68).
1.1.3.2 – T Cells

T cells are a lymphoid-lineage cells that express rearranged T cell receptors (TCRs). The ‘T’ is short for thymus-derived because the maturation of T cells occurs in the thymus (with few exceptions). The primary role attributed to T cells is cellular immunity, whether it be CD4+ helper T cells or CD8+ cytotoxic T cells capable of killing target cells. However, the integrated role of T cells goes far beyond the influence on B cell antibody production and innate immune cell responses, to new roles including homeostatic tissue maintenance and remodeling (69–75). NKT cells, γδT cells, and other innate-like T cells will not be discussed here. One must understand how T cells develop to truly appreciate how the T cell compartment, with combinatorial potential approaching $10^{15-17}$ distinct TCRs, charged with protecting the organism primarily through the detection of small peptides (~8-13 amino acids) presented on MHC molecules (pMHC); the same T cells must also avoid overt reaction to a vast array of self-peptides:MHC complexes and near ubiquitous expression of MHC molecules (76).

T cell, or thymocyte, development involves a stepwise progression through discrete regions in the thymus that provide the environment facilitating TCR rearrangement and subsequent positive and negative selection processes (77). T cell development and selection involves complex cross-talk between the developing T cell and thymic stromal cells via cytokines, receptor/ligand interactions and fine-tuned TCR signaling events. Early T cell development and positive selection events occur in the cortical thymic microenvironments, selecting for T cells that have formed a proper TCR heterodimer that can recognize pMHC complexes (78). A small fraction of developing T cells (1-5%) will succeed to generate TCR and pass positive selection. These cells then move on to the medullary region of the thymus
where negative selection occurs. The medulla expresses tissue-restricted self-antigens via promiscuous gene expression, which helps remove T cells that are self-reactive, one method of preventing the formation of autoimmunity (79). Thymic regulatory T cell (Tregs) (tTregs, previously referred to as natural Tregs, or nTregs) are derived from thymocytes that escape negative selection yet were of high affinity to self-peptides and were endowed with regulatory properties (80). Intrathymic positive and negative selection events are required for the generation of immunocompetent, self-tolerant and non-self-reactive T cell pool. After positive and negative selection, CD4⁺CD8⁺ double positive (DP) thymocytes subsequently become a MHC-II restricted CD4 T cell or MHC-I restricted CD8 T cell and exit to the periphery as naïve T (Tₜ) cells (81).

1.1.3.2.1 – Peripheral Maintenance of T cells

Tₜ cells recirculate between or reside in secondary lymphoid tissues (i.e. spleen and lymph nodes (LNs)), where they are maintained by homeostatic signals in the form of IL-7 and subactivating, tonic TCR engagement with self-peptide-MHC molecules (82). These signals maintain optimal survival through JAK/STAT, AKT, PLC-γ and MAPK intracellular signaling pathways (83). Ultimately, Tₜ cells are lost from the T cell compartment due to some combination of (i) antigenic stimulation and conversion into effector or memory cells as a consequence of lifelong encounters with microbial invaders; (ii) homeostatic conversion to memory-like cells (virtual memory T (TᵥM) cells); and (iii) physiological turnover and apoptosis, presumably due to insufficient IL-7 and/or pMHC signals in the secondary lymphoid organ microenvironment (83, 84). The primary process countering this loss is production of new Tₜ cells by the thymus.
Effector T cells are short-lived, and similar to long-lived TrM cells, that primarily reside in the appropriate non-lymphoid tissues (31, 85). The tissue of residence and variety of effector response of these T cells were tailored during antigenic stimulation by APCs and likely reinforced by the local microenvironment of the residing tissue (85). Ideally, both effector T cells and TrM cells provide rapid, robust antigen clearance and consequent resolution of pathology in situ (85–88). The molecular requirements for the function and persistence of these cells are outside of the scope of the topic of this thesis as all the data presented here are from primary and secondary lymphoid tissues where there are very few TrM and effector T cells. However, these populations can interact with, and be replenished by effector memory T (T_{EM}) and central memory T (T_{CM}) cells (85, 88).

Both CD4 and CD8 memory T (T_{M}) cells, derived from T_{N} cells, circulate through lymphoid and non-lymphoid tissues, and the bone marrow is particularly important amongst the former (85, 89, 90). T_{EM} are less abundant in lymphoid tissues, compared to T_{N} and T_{CM}, and are dominantly present in non-lymphoid tissues (this is more extreme for TrM cells) (85, 88). However, T_{EM} cells are found alongside T_{CM} cells which primarily recirculate between secondary lymphoid tissues (e.g. spleen and LNs) and bone marrow (85, 91). T_{M} cells use IL-15 as the key maintenance factor and likely need less, if any TCR stimulation, unlike T_{N} cells (83). However, T_{M} cells can also utilize IL-7 for maintenance, which provide a potential point of competition with T_{N} cells (92). The roles and maintenance requirements for T_{N}, T_{M} (CD4 T_{M}, CD8 T_{CM} and CD8 T_{EM}) and T_{VM} cells are summarized in Figure 1 (93).
Peripheral T cell subset development and maintenance requirements. Naïve T cells may participate in an immune response and generate effector and memory T cells. Memory T cells generate effector T cells upon reencounter of antigen. Additionally, virtual memory cells arise from naïve T cells during lymphopenic conditions, in the presence of excess cytokines or by imprecisely defined mechanisms that rely on age.

Figure 1 - T Cell Subset Roles and Maintenance Requirements

Adapted from Jimerson, NR (2002)
SECTION 1.2 – Introduction to Aging

1.2.1 – General Biology of Aging

Aging is the accumulation of uncorrected mutations and/or alterations in macromolecules that result in altered cellular responses and function, DNA damage, oxidative stress and reduced macromolecular biosynthesis, degradation and turnover (94). While there are more than 300 theories of aging (95), the theories accompanied by supporting data can broadly be categorized into two broad groups: disposable soma and antagonistic pleiotropy theories (96–99). Disposable soma theory posits that once the reproductive portion of the lifespan is complete the somatic tissues become dispensable and decline. Essentially, this proposes that aging is programmed and that the post-reproductive lifespan is not selected for (i.e. somatic maintenance and repair are not a priority during selection). Conversely, antagonistic pleiotropy argues that lifespan, like other traits, is selected for in the genome. However, traits beneficial to early survival and reproduction will be favored during selection and some of these traits will be deleterious with age. The disposable soma theory had its most popular specific cases in the free radical and DNA damage theories of aging (100, 101), while antagonistic pleiotropy gave the reproductive-metabolic-cell cycle theory and inflammaging (inflammation of aging) (102, 103). While there is evidence supporting both theories, antagonistic pleiotropy is currently favored as it is the only theory that explains aging on a psychological, evolutionary and molecular level (102, 104). Antagonistic pleiotropy allows for additional explanations for psychological changes with age, the wide ranging diversity of lifespan (even in similar species), as well as some species living prolonged periods of time in a post-reproductive state (102, 105, 106). One of the paramount examples of antagonistic
pleiotropy is also a hallmark of an aged immune system, thymic involution, which is conserved across all animals with a thymus (107).

1.2.2 – The Age Related Decline of the Immune System

Aging is accompanied by changes that affect many components of the innate and adaptive immune systems, often resulting in a combination of predictable and idiosyncratic state of immune deficiency (108–110). The culmination of aging of the immune system is poor responsiveness to new or evolving pathogens, reduced vaccine efficacy, increased cancer and other immune related pathologies. By contrast, immunological memory established earlier in life is much less affected (111, 112). Currently, 12% of the world population is ≥ 60, and that is projected to rise to 20% by 2030 (108). This trend is occurring everywhere, not just industrialized countries, as it is projected that 75% of the elderly population will be in less developed countries. Therefore, maintaining a healthy aging population is a major public health challenge.

Defects in the aged immune system start at the beginning with alterations in the HSC niche in the bone marrow and concomitant changes in HSC function (113). The decreased production of progenitor cells paradoxically coincides with increased mobility, numbers and self-renewal of aged HSCs (113). However, aged HSCs exhibit altered subset development, including producing many more myeloid-lineage progenitor cells at the expense of lymphoid-lineage progenitor cells (114).

There are substantial gaps in our knowledge and even disagreement in the literature with regards to the defects in innate cells with age (115, 110). Cell-type specific descriptions of the age-related changes of NK cells, DCs, Macrophages and Neutrophils can be found in sections 5.3-5.6, respectively. As described in Section 1.1.2, the ability to mount an adaptive
immune response requires the interaction of phagocytes (namely DCs and macrophages) with the adaptive immune system. Therefore, it is reasonable that defects in these interactions contribute to the reduced responsiveness to vaccines and new pathogens. For example, one defect in the innate immune system that seems to be in agreement is decreased phagocytic activity (110, 115, 116). This is congruent with the well-established biology of phagocytes outside the context of aging, as it is known that phagocytic activity is maximal under homeostatic conditions and decreases upon inflammation, meanwhile aging is accompanied with inflammation (108, 117, 118). The majority of information regarding the aged immune system is about the adaptive immune system (cells derived from the lymphoid-progenitor population) that decreases with age (108, 117, 119, 120).

The decreased lymphoid-progenitor population in the aged bone contributes to the reduced LN cellularity through decreased numbers of naïve B and T cells. B cells, the subject of section 5.2, additionally exhibit some defects in memory expansion, although this is not necessarily consistently associated with decreased efficacy of the memory B cell response (109, 117). However, defects related to the development and function of the other lymphoid-lineage derived adaptive immune cell, T cells, are the best characterized defects of the aged immune system.

As described in Section 1.1.3.2, T cells develop in the thymus. One of the most ubiquitous changes in the aging immune system, thymic involution, precedes the dramatic changes seen in aged HSCs (119, 121, 122). Thymic involution begins at puberty, likely before, marked by disrupted thymic architecture and severely curtailed export of T\textsubscript{N} cells to the periphery. The exact causes of thymic involution, and further deterioration for the remainder of life, remain elusive. However, as aging progresses, it is likely that the reduction of thymic
seeding progenitor cells, due to the defects in aged HSCs, along with the alterations in thymic architecture and disrupted cross-talk between developing thymocytes with the aged, involuted stroma further compounds defects in T<sub>N</sub> production (107, 119, 123). Key molecules that control thymopoiesis checkpoints have been shown to be altered with age, including decreased IL-7, notch ligand DLL4, MHC-II, as well as increased IL-6 family member expression. A systemic undertaking by Ehrlich et al., to define the stromal contribution to thymic involution, primarily found a decrease in cell cycle genes and an increase in proinflammatory genes within various subsets of thymic stroma (123). Regardless of the exact causes, the reduction of T<sub>N</sub> cell output puts a stress upon peripheral T cell homeostasis.

There are established changes in the numbers and distribution of T cells. This consistently includes decreased numbers of total T cells, CD4 and CD8 T<sub>N</sub> cells, and increased frequency, but not numbers, of CD8 T<sub>M</sub> (124). The literature is inconsistent but there seems to be at least a representational increase in CD4 T<sub>M</sub> and an increase in Tregs with age as well (125, 126). Recent evidence suggests that while there may be an increase in tTregs with age, there is decreased ability to generate iTregs from CD4 T<sub>N</sub> cells (80, 127). There have been reports of intrinsically decreased TCR signaling, proliferation, cytokine production, and cytotoxic activity in aged T cells. However, most of these studies were probably confounded with a lack of precision, usually by conducting the experiments using bulk populations, by attributing population effects as cell intrinsic effects or failing to account for other aspects of the aged system (e.g. decreased burst size after infection could be due to lower precursor numbers (population effect) and/or inefficient priming by aged DCs (systemic effect)). The majority of modern, high precision studies indicate very little differences on a cell intrinsic level, but tend to recapitulate the long-held findings of T cell subset redistribution. There was
one study clearly showing intrinsic defects in human aged CD4 T_\text{N} TCR signaling via a microRNA and a phosphatase (128). It is widely reported that T_\text{N} cells undergo homeostatic conversion in the aged mouse environment and convert to what is often called virtual memory T (T_\text{VM}) cells, as it is presumed that many of the T_\text{M} cells found in an aged organism, at least aged lab animals, never encountered their cognate antigen. However, there are numerous other plausible sources of antigenic encounters, that include food antigens, microbiota, altered self-antigens via mutation or the increase of advanced glycation end products with age (84, 129–133). Another unavoidable aspect of an aged T cell compartment, or the aged adaptive immune system, is a lifetime of exposure to antigens that can contribute to the memory expansion observed with age, particularly due to the presence of persistent infections (e.g. CMV or other herpes viruses, etc.) (134–137).

Measuring age-related changes in many vital molecules has proven to be difficult, but when you take into account additional information, it does appear that there is evidence of an age-related decline in IL-7 biology (i.e. lower levels of IL-7 expression, bioavailability and/or insufficient signaling in T cells) in the thymus, bone marrow and lymph nodes (84, 119, 138, 139). There is also evidence of decreases CXCL12 (aka SDF1) with a parallel increase in adipose content and CCL5 levels with age (89, 113, 119, 140). Both of these molecules are known T cell attractants and the age-related changes in these molecules may correlate with the increase of IL-15 and a higher number and proportion of CD8 T_\text{M} cells, which rely on IL-15, in the aged bone (89–91). Whether reduced by aging or not, these molecules, specifically IL-7, offer potential avenues for therapeutic restoration of age-related defects in the T cell compartment, despite the fact that many early attempts with IL-7 have failed (141, 142). Heterochronic parabiosis provides a unique approach to identifying IL-7 niches in vivo, if they
decline with age, if they can be rejuvenated by anti-geronic factors in young blood and whether there are any intrinsic differences with regards to IL-7 responsiveness or consequent lifespan of aged T cells.

1.2.3 – Approaches to Rejuvenate the Aged-Related Disorders

The quest for anti-aging therapies has existed for as long as human culture. In that context, particularly as related to improving health in the last third of lifespan, the understanding of the biology of aging, and how to intervene will have huge ramifications for society. The top 10 causes of disability and death in the United States each have their own unique risk factors, but the largest risk factor for all of them is age. The elucidation of factors contributing to the aging process has the potential to identify a single treatment for disparate pathologies.

The most reproducible and consistent method to increase longevity is calorie restriction/dietary restriction, which has been shown to be effective from single celled yeast all the way through humans (143). However, moderate calorie restriction or even intermittent fasting has shown to be beneficial and are more applicable approaches for society (144, 145). Some of the hallmarks of calorie restriction have been emulated by pharmacological agents such as those that inhibit nutrient sensing, alter metabolism or induce autophagy (143, 146–148). While it is possible that a single molecule may have successful wide-ranging anti-aging effects, it is more likely that a systemic, or at least a targeted combination approach will be necessary to simultaneously repel different defects of aging. This is particularly why heterochronic parabiosis is such a powerful platform for understanding the complexity of aging. The anti-aging effects of heterochronic parabiosis been thoroughly demonstrated in multiple tissues (149–157).
SECTION 1.3 – Introduction to Parabiosis

1.3.1 – History of Parabiosis

Parabiosis, (‘para’ meaning “next to” and ‘bios’ meaning “life”), was first described in the literature in 1864 by a French physiologist Paul Bert (158, 159). Due to the hallmark formation of vascular anastomosis, or fusion of the vasculature, this powerful experimental technique is uniquely suited for studying the trafficking of cells and molecules and the physiological impact of hematogenous factors. The technique has been widely implemented in a variety of organisms to study cell intrinsic and/or cell extrinsic factors involved in different physiological outcomes. These studies included but were not limited to vascular remodeling and the sharing of vascular fluids, the diffusion of hormones, proteins, metabolites or other signaling molecules, nutrition, metabolism, cancer metastasis, cellular ontogeny, cellular migration, and anti-aging (149, 160–176).

The term ‘parabiosis’ was first seen in the literature in 1908 when Sauerbruch and Heyde published the technique (177). The Scopus database allows for robust literature searches, but like most literature databases, publications prior to the digital era are incomplete. Figure 2 shows the results from searching the Scopus database for the indicated terms (parabiosis, aging parabiosis and heterochronic parabiosis). In fact, while the Bert 1864 (J Anatomie Physiologie) citation can be found in multiple publications, there is no evidence of this publication in the Scopus or Google Scholar databases. However, another similarly titled publication was found in Google Scholar (158, 178). The only certain document obtainable in complete form is the thesis of Paul Bert, published in 1866, which had no reference to any 1864 publications (159). Hopefully, as digital archives exponentially expand into the past, this confusion regarding the earliest published history of parabiosis will be clarified.
Numerous important parabiosis publications occurred prior to the first Scopus reference in 1921. In fact, in 1909, the future Nobel Laureate Peyton Rous used the technique to test the hypothesis that there exist “circulating anti-bodies, if such there be,” in rodents immune to transplantable tumors (179). At the time, there was a “mass of evidence against such anti-bodies,” but it was previously demonstrated that the transfer of blood from immune dogs could reverse the progression of established tumors in another animal (180). A publication in 1920 in Scientific American by Heyde, entitled “Artificial Siamese Twins,” reviewed the push made in the prior decade by Heyde and others to extend the use of parabiosis beyond studying vascular anastomosis into other clinically relevant areas of physiology and pathology (181). Heyde notes that parabiosis experiments, conducted across animal species and higher organisms show that these organisms, “represent thoroughly individual biological entities, which refuse to permit any heterogeneous element to remain in their community of cells.” He later refers to this resistance as “athreptic immunity,” or the extreme malnourishment and wasting of one animal, thought to be due to the deficiency of substances required for growth. This concept is now referred to as marasmus, and does not accurately describe the symptoms they observed. Both of these observations by Heyde foreshadowed the discovery of Graft-versus-Host Disease (GvHD) events during parabiosis (182–184), which is the topic of Chapter 3 of this thesis. Heyde further predicted the readiness of the parabiotic technique to address various complex questions in biology. Indeed, following the first entry on parabiosis in the Scopus database in 1921, parabiosis research continued over the next period until the end of World War II despite a very limited number of entries (185, 186) (Fig. 2).
Today, the Scopus database contains over 1700 publications containing the word “parabiosis” in either the title, abstract or keywords (Fig. 2). The majority of publications occurred between 1960-1980, with a newfound interest sparked circa 2005. While the early days of parabiosis were dominated by descriptive physiology, the fields of immunology and biogerontology have leveraged the technique more than other fields.

1.3.2 – Applications of Parabiosis

1.3.2.1 – Non-Aging/Non-Immunological Applications of Parabiosis

The most common use of parabiosis outside of immunology or biogerontology has been to study nutrition/metabolism (187). One of the most cited sets of parabiosis experiments involved leptin receptor deficient mice (db/db), initiated by Coleman in 1969 (188, 189).

Rather than rescue the insatiable appetite of db/db mice, WT mice lost weight due to a lack of appetite. This was because of an increased leptin concentration due to the lack of uptake by mutant cells, which lead to the term “satiety factor” referring to leptin. 25 years later, Friedman cloned the gene responsible for these effects in mouse and humans, naming the molecule leptin (190). Later, Harris showed that the db/db mutations could be balanced by the
ob/ob mutations via parabiosis, because the over-production of leptin in one animal was balanced by the deficiency of leptin in the other, respectively (175). In 2010, Coleman and Friedman shared the Lasker Award for the discovery of leptin (191). Also in 1969, Dahl utilized a parabiosis model to predict that the kidney is the source of a molecule responsible for hypertension (192). This was later confirmed and the molecule was identified as parathyroid hypertensive factor (PHF) (193). These early studies highlighted the power of parabiosis models to study and identify new, physiologically active, circulatory/hematogenous factors.

1.3.2.2 – Immunological Applications of Parabiosis

Prior to 1950, parabiosis was already used to study transplantation biology and anti-cancer immunity (179, 181). A common outcome of parabiosis became a model of tissue damage/complication, variably called “parabiotic intoxication,” “parabiotic disease,” “parabiotic sickness,” or perhaps most accurately, GvHD (182, 184, 194–208). GvHD is the subject of Chapter 3 of this thesis. As early as 1956, White used parabiosis to examine the effects of age and diet on intestinal microbiota (209). Subsequently, immunologists begin to use parabiosis to study the trafficking and ontogeny of lymphoid cells as well as their decay rates. In 1992, Goldschneider and coworkers showed that while the thymus seems to remain a restricted site of chimerism (10-20% rather than ~50%), the organ was still seeded in discrete waves by blood-borne prothymocytes throughout postnatal life, documenting the previously postulated existence of pro-thymocyte niches (210). The niches were then quantified by Kruger’s group, albeit their histoanatomical description is still missing (172). Numerous studies used parabiosis to examine T cell trafficking. This allowed authors to shed light on the residency of Tregs as well as to define a previously unappreciated subset of memory T cells, now referred to as tissue-resident memory T (TRM) cells (169, 171, 211–213, 73). Parabiosis
was further instrumental in elucidating the role of various retention factors, such as VCAM-1 in the case of macrophage retention of HSCs in the spleen or the PZLF transcription in NKT cell integrin biology (214, 215). Similarly, parabiosis experiments were critical in the discovery of a series of disparate developmental pathways, one originating in the primordial embryonic yolk sac and another originating in the bone marrow from adult HSCs (216–221). Other cellular fate-mapping approaches clarified contested differences between certain resident immune cells in the intestines as well as another study demonstrating the role of a dermal lineage cell in wound healing (57, 174). The use of parabiosis in studying cancer immunotherapy also experienced a resurgence, most recently in two studies involving Tregs and innate lymphoid cells (ILCs) (173, 222). The above examples, while by no means exhaustive, illustrate the versatility of this technique.

The importance of parabiosis in immunology should not be overlooked as other methodologies, including adoptive cell transfer, chemical/biochemical supplementation, genetic enrichment techniques, antibody depletion, chemical/biochemical depletion, and/or genetic ablation techniques each carry caveats when trying to attribute, or exclude, with certainty a role for a given blood-borne cell type. While parabiosis cannot replace these techniques, but it provides an attractive complementary approach. Most importantly, parabiosis is the only approach that allows for the assay of every cell capable of moving via hematogenous routes or molecules or stationary cells that affect hematopoietic-lineage cells, providing a strong initial experimental step in studying circulatory factor physiology. Furthermore, the systemic, multi-organ nature of parabiosis makes it particularly suited to true multi-disciplinary investigations, as evident by the study that blended neuroscience, immunology and biogerontology to demonstrate that age-related defects in remyelination in the
central nervous system were restored by microglia derived from the adult mouse conjoined to the old animal (152).

1.3.2.3 – Aging Biology Applications of Parabiosis (Heterochronic Parabiosis)

Heterochronic parabiosis, or the surgical union of animals of disparate ages, was first described by McCay, as a life extension method in 1956 and confirmed subsequently by Lunsford in 1963 (176, 176, 223). The technique at the time was called parabiosis of aging or aging parabiosis and the exact term “heterochronic parabiosis” did not appear until 1980 (224). However, searching the Scopus database for “aging parabiosis” showed that the field blossomed between 1956-1980, was somewhat dormant for 25 years and surged again since 2005 (Fig. 2). McCay and colleagues provided descriptive accounts of increased longevity, appearance and tissue function of the older parabiont (176, 223, 225, 226). They also added a calorie restriction group in one of the first incarnations of heterochronic parabiosis (176). A beneficial effect on lifespan extension of the old parabiont was observed when the adult parabiont was previously severely calorie restricted to the point of growth retardation. Early experiments also involved attaching three adult animals to an old animal to attempt to dilute the old blood even further, and successfully followed up with separation experiments to investigate longevity as well (223). These early studies were underpowered and it was difficult to draw interpretable conclusions from their findings. A decade later another flurry of papers came out describing the how improvement of cholesterol turnover and another describing increased longevity in females with proper unpaired and isochronic (organisms of the same age) paired animals (149, 227). Of note, while the excitement focuses on the rejuvenation capacity of young blood on the aged animal, it is important to mention that in many cases
examined the beneficial effect conferred on the aged animal coincided with a reciprocally negative effect in the young animal.

After a period of relative dormancy, parabiosis reemerged when two high profile studies in 2001 and 2002 by the Weissman group, examining the physiological migration of blood-borne HSCs and the engraftment and transdifferentiation of HSCs during steady-state conditions (228, 229). An even greater surge of interest, including by the popular media, started from the 2005 study where Rando, Conboy and colleagues collaborated with Weissman’s group (150). In these experiments, joining of an adult and an old organism allowed investigators to explore the existence and nature of pro- and anti-geronic circulatory factors that can induce organ remodeling via cellular rejuvenation. Their study demonstrated improved muscle stem cell (i.e. satellite cells) function in aged mice, as well as increased proliferation in the liver progenitor cells following heterochronic parabiosis. These phenomena were attributed to soluble factors in circulation permeating from the young blood, and notch signaling in muscle stem cells was identified as a key component (150). Other groups demonstrated the rejuvenation potential of heterochronic parabiosis in the contexts of increased oligodendrocyte differentiation in the CNS, increased pancreatic β cell proliferation, increased bone repair, vascular and neurogenesis improvements, muscle regeneration and physiology as well as the reversal of age-related cardiac hypertrophy (153–156, 230, 231). Since the β cell proliferation and bone repair studies were largely descriptive in nature and one cannot conclude that changes in them were a result of acellular events.

It was recently demonstrated that adult or old serum did not affect skeletal muscle proliferation; however the lymphocyte secretome of adult T cells enhanced skeletal muscle proliferation, while the secretome of old T cells attenuated the proliferation (232). This
provides initial evidence that some of the rejuvenation effects observed in parabiosis may be conferred onto the old organism by the infiltration of, and cytokine secretion by, young immune cells (232). Furthermore, it was recently demonstrated that an age-related defect in local IL-33-dependent accumulation of Tregs causes poor repair of skeletal muscle in aged mice (72). There have been additional calls for the reexamination of the contribution of adult blood-borne cells in the rejuvenation effects of heterochronic parabiosis, and this certainly remains an important and exciting direction in parabiosis studies (233).

The transformative potential, the sometimes controversial nature of parabiosis experiments and the necessity of exceptionally careful controls and measurements were all highlighted by publications on muscle aging that came out of the Wagers group (153, 231, 234). Wagers was amongst the pioneers that reintroduced parabiosis to HSC and aging research, as the primary author on the Weissman group publication that reintroduced parabiosis and coauthor on the Conboy-Rando manuscript that reintroduced heterochronic parabiosis (150, 229). The next important publication, reported in Cell by Loffredo, et al., examined the impact of parabiosis upon age-related cardiac hypertrophy (the counterpart of congestive heart disease in humans), and concluded that an age-related decline in growth differentiation factor 11 (GDF11) serum levels is key to this condition (153). Indeed, in this publication, and a subsequent report, Wagers’ group elegantly demonstrated that heterochronic parabiosis restored GDF11 levels in the old parabiont, and that exogenous administration of GDF11 to an old mouse rejuvenated cardiac hypertrophy, muscle regeneration and muscle physiology (231).

However, a vigorous debate erupted when a Novartis-lead group published an experimental rebuttal demonstrating that GDF11 increased with age and inhibited muscle regeneration (235). This publication claimed that the methods of detection of GDF11 used by
Loffredo, et al. were inconclusive and have accidentally identified the evolutionary paralog of GDF11, myostatin (GDF8), which has opposing functions. Another group, led by Smith and Houser, demonstrated that GDF11 does not rescue age-related cardiac hypertrophy because they failed to reproduce Loffredo et al. results on cardiac remodeling and shrinking caused by exogenous GDF11 administration (236). Then, Poggioli, et al. provided evidence that there could have been dramatic differences in bioactivity between GDF11 protein lots and protein preparation methodologies between the Wagers and Houser groups (153, 231, 236, 237). That publication also submitted a strong biochemical retort to the Novartis group with regards to the homology of GDF11 and myostatin (GDF8), demonstrating that the Novartis detection method cross-reacts with immunoglobulin and that the low levels of GDF11/8 further decline with age in multiple species (235, 237). Therefore, despite intense research, the role of GDF11 at the present await experimental validation. These controversies illustrate both the profound power of, and complications in rigorously interpreting the results of heterochronic parabiosis, including the need for multiple lines of corroborating experimentation.

Another set of experiments by Villeda and Wyss-Coray were notable for both their elegance, careful and thorough approach identified a chemokine, CCL11, increased in the blood of aged mice and caused reduced hippocampal neurogenesis (151). This was proven using parabiosis, heterochronic plasma exchange, administration of CCL11 to young mice as well as anti-CCL11 antibodies to aged mice, all of which led to multiple cross-validating outcomes (neuronal proliferation, cellular density in a particular region in the brain, behavioral tests and neuronal electrical activity). This group followed up the initial report with a state-of-the-art neuroscience approach to age-related decline in cognitive function and synaptic plasticity in another immense undertaking involving serial injections of young and old plasma
Furthermore, using similar approaches described above, Villeda, et al. found that another important immune molecule, $\beta_{2m}$, increases in the serum with age in mice and humans and impairs cognitive function and neurogenesis (157). These reports by Villeda et al. demonstrates not only the amount of work to thoroughly control a multidisciplinary aging study, but also illustrates the complexity of aging as two disparate molecules of the immune system, CCL11 and $\beta_{2m}$, contribute to similar outcomes in the cognitive decline accompanying age.

By contrast to the above explosion of gerontological research, there have been to date a mere four studies using heterochronic parabiosis for the study of the immune system. The first study, which coined the term ‘heterochronic parabiosis’ in 1980, and although the publication is in Russian, the abstract indicated that the antibody response to sheep red blood cells remained reduced after the surgery (224). Kim, et al. published that heterochronic parabiosis fails to restore the age-related decline in thymus function and that this defect is primarily due to the thymic stroma (239). Pishel et al. have published the other two papers, which characterized peripheral T cells following heterochronic parabiosis (240, 241). The first concluded that accelerated aging of the immune system, rather than rejuvenation as determined by the failure to restore thymic mass and the influx of CD44$^+$ memory T cells, is a hallmark of an aged T cell compartment in the young animal (240). Next, in vitro lymphocyte stimulation (phytohemagglutinin, or PHA) studies were done by the same group, who came to a similar conclusion of diminished function of T cells in the young animal upon heterochronic parabiosis (241). However, both of these studies suffered from a lack of discriminatory power. Specifically, neither utilized congeneric markers to discriminate between adult and old T cells in critical experiments, and conducted bulk T cell assays that do not account for different
proportions of T<sub>N</sub> and T<sub>M</sub> cells with aging. This was also the case for the use of “autologous” DCs and macrophages during co-culture PHA stimulation, as there were no attempts to characterize the contribution of adult or old cells to these populations as a potential explanation for the priming defects observed (241).

To circumvent the above pitfalls, this thesis strived to thoroughly optimize heterochronic parabiosis in order to conduct a robust characterization of many different immune cells, with an emphasis on T cells, in primary and secondary lymphoid tissues. These procedures are detailed in the next chapter on methodological and statistical aspects that pertain to our parabiosis experiments.

**SECTION 1.4 – Significance and Goals of this Dissertation**

**1.4.1 – Significance**

Prior studies utilizing heterochronic parabiosis have either disregarded the role of the immune system, failed to properly distinguish adult cells from old cells, and/or may have been influenced by the myriad of GvHD symptoms that occur with parabiosis when the genetic background is not precisely controlled. At the onset of this project, I set out to see if the profound rejuvenation effects of heterochronic parabiosis would be observed in the aged immune system. The heterochronic parabiosis model described here inherently tests the potential rejuvenation of HSC development through surrogate measures of the lymphoid:myeloid ratio as well as the potential for the rejuvenation of the thymus, a vital goal in aging immunology. The model was also set to compete old cells against adult cells for homeostatic reconstitution of adult and old tissues, additionally allowing the influence of the age of the tissue microenvironment to be assessed. Prior to directly testing pointed questions
by utilizing established experimental models of the immune system I sought to characterize the homeostatic redistribution of the immune cells described at the end of Section 2.5. The differences in the cell types present and their distribution in the animal across age, and how these cells interact and redistribute upon heterochronic parabiosis must be known prior to experiments which address specific questions on immune function. This thesis provides a thorough parabiosis protocol as well as a significant amount of background data to set the stage for significant further developments utilizing the model.

1.4.2 – Goals of this Dissertation

My intention of this dissertation is to provide a near complete account of the history of heterochronic parabiosis, along with an emphasis of applicable information regarding aging and the immune system. Additionally, this will ideally prove as a resource for those interested in leveraging the model. This thesis aims to thoroughly characterize the homeostatic redistribution of the main immune cell types in the context of well controlled, congenic heterochronic parabiosis as a platform for dissecting the aged immune system, the contribution of the immune system to aging, and mechanisms of immune rejuvenation.
CHAPTER 2 – MATERIALS AND METHODS, WITH SPECIFIC EMPHASIS UPON
METHODOLOGY AND STATISTICS OF HETEROCHRONIC PARABIOSIS

SECTION 2.1 – Introduction

The intricacies of the specific parabiosis protocol used here are described thoroughly in section 2.2. Animal breeding, tissue processing, flow cytometry and statistical design of the experiments will also be described in this chapter. The subsequent chapters, 3-5, extensively rely on the methodologies and statistical design detailed below.

SECTION 2.2 – Surgical Protocol

2.2.1 – Optimization of the Surgical Protocol

This adaptation of the parabiosis protocol was established at The University of Arizona with the help of University Animal Care personnel, in particular Dr. Paula Johnson. While there is substantial overlap between this protocol and some recently published (242–244), the changes I have applied here stress mouse genetic matching and surgical improvements resulting in increased survival. I wish to emphasize that I did not test other parameters for improvement. I am merely providing a detailed protocol that in my hands provided significant improvements in survival, health and lymphoid organ cellularity.

2.2.2 – Parabiosis and Subsequent Separation Protocol

Prospective parabiotic pairs (parabionts) were temperament matched in their own cage for a week prior to the surgery. On the day of surgery, an intramuscular injection of ketamine, xylazine and acepromazine (45 mg/kg, 9 mg/kg and 1.35 mg/kg, respectively) was administered to each mouse. After the mice were anesthetized, a subcutaneous injection of buprenorphine and meloxicam (0.1 mg/kg and 5 mg/kg, respectively) was administered for palliative care. Anesthetized mice were shaved at the corresponding lateral flanks, and
matching skin incisions were made from below the base of the ear to the hip. Ipsilateral whiskers were trimmed to prevent potential agitation effects of proximal sensing among the parabionts. The subcutaneous fascia was bluntly dissected to create ~2 cm of free skin on either side of the center of the incision. Excess skin was trimmed from both sides of the incision (maximum of ~1 cm of skin trimmed at the center of the incision while tapering off to no trimming at the edges of the incision) to ensure the parabionts were comfortably restrained next to each other, while preventing them from twisting, turning, fighting and ultimately harming each other. Laying the animals on their backs, side-by-side, the dermis of the ventral portion of the lateral incision of one parabiont was pressed against the other and was stapled together with 9 mm surgical staples. With a dorsal view of the animals, the dorsal portions of the lateral incisions were trimmed in the same manner as the ventral aspects of the incisions. Next, the skin was stapled together with 9 mm surgical staples. Each parabiont was then given a subcutaneous injection of 1 mL of saline and allowed to recover on a heated surgical table. Palliative care extended for 3 days after surgery, resulting in six injections of buprenorphine, every 12 hrs, and three injections of meloxicam, every 24 hrs. Two weeks later, each parabiont was anesthetized with ketamine and xylazine (50 mg/kg and 5 mg/kg, respectively) and the staples were removed.

For some experiments we additionally separated the parabionts following 4-5 weeks of parabiosis. Separation surgeries proceed similarly, except the anesthesia used was ketamine and xylazine (75 mg/kg and 7.5 mg/kg, respectively) followed by the same three-day course of palliative care with buprenorphine and meloxicam. The surgical site was shaved and cleaned with three alternating wipes of isopropyl alcohol and betadine, as before. With a dorsal view of the animals, an assistant gently holds the animals apart and an incision is made to separate
the mice. Each mouse is placed on their contralateral flanks, with respect to the surgical site, and the ipsilateral flanks were stapled together with 9 mm surgical staples. Each animal received a subcutaneous injection of 1 mL of saline and allowed to recover on a heated surgical table. The remainder of the procedure was as described above.

After resolving genetic complications (described in Section 2.3 for the breeding protocol and other details), much of the long-term survival failure seemed to be due to excess skin, permitting twisting as well as too much access to the adjacent animal. We developed modifications that emphasized the importance of securing forelimb mobility, restricting the ability to twist, and vital preventative care such as nail trimming. We further introduced two sources of food and water, so that each animal can access them simultaneously and to reduce pre-surgical complications. The key differences in this protocol that allowed us to secure forelimb mobility (compared to the other approaches) was the extension of the flank incision to behind the ear and the trimming away of excess skin (≤ 1 cm) from either side of the incision prior to using 9 mm surgical staples on both the ventral and dorsal flaps. These optimizations allowed us to forego the use of internal suturing, which also decreased the surgery and anesthesia time. Upon dissection, we could ascertain that these animals exhibited no signs of internal irritation, unlike when internal sutures were used to fix either the subscapular spine or quadriceps muscles or both. This technique also avoids unnecessary complications that could arise due to suture removal prior to separation experiments.
SECTION 2.3 – Animal Breeding Protocol

2.3.1 – Animal Sources and Breeding

All mice used in these studies were male, largely due to the dearth of old female animal supply from our main vendor, the National Institute of Aging (NIA). All animal experiments were approved by The University of Arizona’s Institutional Animal Care and Use Committee. These studies utilized the Ptprc (aka, Ly5 or CD45) congenic allele mouse system on a C57BL/6 background. In the text I will be using CD45 nomenclature. Adult C57BL/6J, CD45.2+/+ (JAX Stock # 000664); and B6.SJL-PtprcPepcb/BoyJ, CD45.1+/+ (CD45.1 congenic to B6 background) (JAX Stock # 002014), mice were purchased from The Jackson Laboratory (JAX), Bar Harbor, ME. B6.SJL-PtprcPepcb/BoyNCI, CD45.1+/+ congenic mice were purchased from the National Cancer Institute (these mice are no longer available). Old C57BL/6JNia, CD45.2+/+ mice were procured from the NIA (via the Charles River, Inc., Boston, MA). This colony is rederived every 6-7 years from the C67BL/6J mice from JAX. The C57BL/6J mouse strain (stock # 000664) is under JAX’s patented Genetic Stability Program, which essentially ensures no more than 5 generations of genetic drift. The CD45.1 mice used here were not under formal genetic stability protocols. However, the JAX strain was backcrossed to C57BL/6J three times in 2009-2010. As detailed in Chapter 3, due to poor health and survival outcomes and the cost of aging mice, we decided to control for minor histocompatibility antigens and imprinted genes by robust reciprocal crossings of male and female CD45.1 (JAX #002014) and CD45.2 (JAX #000664) to generate heterozygous mice, which were subsequently crossed to off-spring from the reciprocal parental grouping (Fig. 3). By doing this we generated homozygous CD45.1 or CD45.2 siblings with significantly decreased potential of GvHD upon parabiosis (Fig. 3).
Figure 3 – A Breeding Scheme to Reduce Complications During Parabiosis Due to Strain Background Genetics  A Diagram of the breeding strategy used to interbreed CD45.2 WT (JAX # 000664) with CD45.1 WT (JAX # 002014) in order to reduce potential minor histocompatibility issues.

The most optimal way to reduce genetic differences between mice would be to continuously pair siblings from the N1F1 generation. However, the yield of such strategy would be prohibitively low, with only 12.5% of the offspring being of the proper genotype and matching sex. Furthermore, sibling pairing is not possible in the heterochronic context. We therefore decided to start with n=3 for all mice in the parental cross (6 breeder pairs), and to use 16 breeder pairs of the next two generations to obtain optimally equilibrated background genetics at the N1F2 level (Fig. 3). No breeding protocol can eliminate the substantial linked genomic content transferred along with the CD45 locus during crossover. Relevant to that, a recent study described the amount of SJL DNA crossed over to the B6 background in CD45.1 mice from JAX. Their data indicated that 40-50 Mb of chromosome 1, which contains the
CD45.1 allele (*Ptprc*, 120 Kb of genomic DNA), of SJL origin (245). The authors identified six additional polymorphisms on six separate chromosomes, which would be theoretically corrected for by this breeding scheme if there is no selection (coinherence) bias due to the SJL CD45 locus. The ideal congenic mouse strain would be generated with site directed targeting directly on the genomic background of interest. Since such a strain is not commercially available, we proceeded with classical techniques, which provided a dramatic improvement. To ensure that we did not end up with isochronic old pairs that reject each other, we decided to age CD45.2 and CD45.1 mice generated in the breeding scheme (the latter are not available at advanced ages). As our animal care facilities allow five mice per cage and CD45.2 mice are available from the NIA, we weaned three CD45.1 mice and two CD45.2 mice into a cage at three weeks and left such co-housed animals to age until \( \geq 18 \) months old.

Based upon our experience detailed in section 2.2 above, we suggest that all mice should have their nails trimmed and have skin irritations treated with betadine immediately to ensure the health of the animals. This is particularly true after separation from parabiosis, but was found to be indispensable in the general aging colony as well.
SECTION 2.4 – Statistical Model

2.4.1 – Optimal Three Factor Study Design

<table>
<thead>
<tr>
<th>Age</th>
<th>• Young (~3 mo)</th>
<th>• Old (~18 mo)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Surgery</td>
<td>• Nonsurgical Controls</td>
<td>• Parabiosis</td>
</tr>
<tr>
<td>Strain</td>
<td>• B6J (CD45.2)</td>
<td>• B6.SJL (CD45.1)</td>
</tr>
</tbody>
</table>

**Figure 4 – Diagram Illustrating Factors Controlled for in this Three Factor Study Design**

Heterochronic parabiosis utilizing congenic mouse strains should be optimally controlled for by using this three factor study design controlling for age, surgery and mouse strain.

Ideally, a perfect study utilizing heterochronic parabiosis would require controlling for the three factors: age, surgery and strain (or genotype) of mouse (Fig. 4). One could argue that the fourth is the microbiome, however, our pairing of animals for temperament matching already equalizes that parameter between parabionts. In order to identify age-related defects and/or rejuvenation one needs to compare desired outcomes across disparate ages (age factor). The tissue injury and stress of every surgery needs to be controlled, although there are many options for surgical controls. In the case of heterochronic parabiosis, one needs surgical controls for the age of the conjoined animals: heterochronic (young-old), isochronic (young-young) and isochronic (old-old). Since parabiosis results in the transfer of blood, I was much more concerned with the interaction components between cells and molecules found in young or old blood. I found the most important thing to control for was the composition of the immune system and environment in which it is traversing. I also believe that the isochronic
surgical pairs adequately control for the heterochronic surgical condition and the duration of the experiment allows measuring parameters of interest long after the surgical insult. Therefore, I felt that the isochronic surgical controls were more relevant than sham surgery, compression cuffs (to restrict two mice together without surgery), or other options; all these were simply replaced with nonsurgical control mice. Lastly, any congenic method used to track a cell type of interest can involve strain-specific differences that need to be controlled for as well, such as incomplete congenic marking of all cells (246), different cellular composition between congenic strains (247) and immune responses to the congenic allele (248–251). As mentioned earlier, and to be discussed in detail in Chapter 3, the CD45.1 (B6.SJL) mice have many genetic differences relative to CD45.2 B6 mice, some of which have been demonstrated to alter HSC activity and the development and location of various B cell subsets (245, 247). Indeed, the allele effects are one of the key potential confounders in our parabiosis model.

Table 1 – Optimal Three Factor Study Design Grouping for Utilizing Heterochronic Parabiosis

<table>
<thead>
<tr>
<th>SURGERY</th>
<th>Non-surgical Control</th>
<th>Isochronic</th>
<th>Heterochronic</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGE</td>
<td>Young</td>
<td>Old</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CD45.1 (1)</td>
<td>CD45.2 (4)</td>
<td>CD45.1 (10)</td>
</tr>
<tr>
<td></td>
<td>CD45.1 (2)</td>
<td>CD45.2 (5)</td>
<td>CD45.1 (11)</td>
</tr>
<tr>
<td></td>
<td>CD45.1 (3)</td>
<td>CD45.2 (6)</td>
<td>CD45.1 (12)</td>
</tr>
</tbody>
</table>

Therefore, this kind of study design requires 12 groups (Table 1). The overall main effect of surgery is readily controlled by utilizing contrast statistics across rows. Each row is comprised of a fixed age and mouse strain thereby controlling for every surgical condition. The mouse strain is controlled for, using contrast statistics, both within and across ages (e.g. groups 1 vs 10, 4 vs 7, 2 vs 11, etc.). Thus far, every comparison described involved independent variables, however, the fact that parabionts share a common circulatory system
has to be accounted for by utilizing pairwise statistics. Every measured outcome from two
different animals is linked after parabiosis, this is denoted in Table 1 by the solid shading
indicating matched-pairs for isochronic groups and checkered shading indicating matched-
pairs for heterochronic groups. This increases the likelihood of identifying potential
differences when there is a high degree of variability and decreases the chances of identifying a
difference when the outcome being measured doesn’t change consistently across all pairs of a
group. For example, a difference in outcome X at the group level contrast statistics may
indicate a significant difference, but if individual data points in each group are variable, then
when one controls matched-paired values by using pairwise statistics this difference could end
up not significant (i.e. not attributable to factors tested). This can also reduce the number of
mice that is necessary since the experimental design controls for variation.

The sheer number of mice and groups necessary to carry out a study design makes
much of this very difficult, if not impossible to do. However, if done properly with
simultaneous measurements of many discreet and/or continuous (e.g. frequency and/or count)
variables, one can obtain a very robust dataset for thorough statistical modeling that could
identify complex interactions between multiple outcomes in a complete system in a manner
that would be very difficult to measure otherwise. The potential power to provide substantial
evidence for or against a given hypothesis is probably overshadowed by the power to provide
hypothesis generating observations that might have otherwise not been considered in such a
complex system.
2.4.2 – Practical Study Design and Statistics

The complexity of biological systems is further muddled by issues involving the practicality of executing a proper study design, namely the lack of resources and funding. For example, CD45.1 (B6.SJL) mice are not available at advanced ages from any vendor. Therefore, in order to obtain CD45.1 mice at $\geq 18$ mos. one has to age the mice at their own risks and cost. This is not commonly required from investigators, therefore often controls for the congenic allele effects and the isochronic old group are entirely missing from the published data. Typically, when congenic tracking is necessary, heterochronic pairs are controlled for by utilizing isochronic young pairs. While it is difficult to interpret data without such controls, it is typically necessary to rely on accompanying data using parabiosis-independent methodologies.

### Table 2 – Practical Alterations to the Three Factor Study Design for Utilizing Heterochronic Parabiosis

<table>
<thead>
<tr>
<th>AGE</th>
<th>Surgery</th>
<th>Strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Young</td>
<td>Nonsurgical Control</td>
<td>CD45.1 (1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.1 (2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.1 (3)</td>
</tr>
<tr>
<td></td>
<td>Isochronic</td>
<td>CD45.2 (4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.2 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.2 (6)</td>
</tr>
<tr>
<td>Old</td>
<td>Heterochronic</td>
<td>CD45.2 (7)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.2 (8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.2 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.1 (10)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.1 (11)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CD45.1 (12)</td>
</tr>
</tbody>
</table>

Having this in mind, the study described herein, designed to optimize and characterize heterochronic parabiosis for the study of the aged immune system, found a reasonable middle ground between the optimal design described in Section 2.4.1 and the general lack of congenic controls and comparisons to nonsurgical baselines. The time and costs involved to routinely age mice were the most prohibitive, so we conceded on the necessity of the reciprocal heterochronic pairing of congenic mice (i.e. CD45.2 (B6J) adult mouse paired a with CD45.1 (B6.SJL) old mouse), or group 6 and 12 described above (Table 1). Additionally, compelling
data emerged from an incomplete dataset (prior to waiting until Spring 2016 for our colony to age) and we decided to present data analysis utilizing a less powerful but well controlled alteration to the model described here in Section 2.4.2. Here we are missing groups 6, 8, 10, 11 and 12 (Table 2). However, this model should be altered to a two factor model where age and strain are combined to a single variable. In this case, the CD45.2 adult mouse is used in contrast tests between nonsurgical control CD45.1 adult mice, CD45.2 old mice and CD45.2 adult isochronic mice.

Table 3 – Nested Statistical Design of Altered Two Factor Study Design for Utilizing Heterochronic Parabiosis without Old Congenic Mice

<table>
<thead>
<tr>
<th>Age/Strain x Surgery</th>
<th>Nonsurgical Control</th>
<th>Isochronic Adult</th>
<th>Heterochronic Pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45.1-Adult</td>
<td>Group 1</td>
<td>Group 2</td>
<td>Group 3</td>
</tr>
<tr>
<td>CD45.2-Adult</td>
<td>Group 4</td>
<td>Group 5</td>
<td></td>
</tr>
<tr>
<td>CD45.2-Old</td>
<td>Group 6</td>
<td></td>
<td>Group 7</td>
</tr>
</tbody>
</table>

This model is still incomplete, and statistical validity was based on a nested set of both contrast and pairwise statistics illustrated in Table 3. Like before, the effect of surgery is controlled for across the row, while the effect of the Age/Strain combined factor is controlled down the column. The solid arrows spanning across the rows as well as the Nonsurgical Control column indicate where two-way analysis of variance (ANOVA) would be used to test the influence of the both independent variables (Age/Strain and Surgery) on the dependent variables (being measured outcomes, in this case many different cell types of the immune system in multiple tissues), correcting for multiple comparisons using Sidak’s posttest. The open arrows (between Groups 2-5 and 3-7) represent the same statistical test, however, in a pairwise manner with repeated measures to account for outcomes observed in conjoined parabions. Lastly, we only claim that findings were affected by age if the statistical differences in the data are unique to the heterochronic condition (i.e. a given comparison has a
statistical difference between groups 3 and 7 (heterochronic pairs) but not groups 2 and 5 (isochronic adult pairs), represented by the dashed line in Table 3). We also made sure to include the outcomes for all relevant tissues and marked the outcomes from different tissues of the same mouse as repeated measures to account for the redistribution of cells of the immune system across all of these tissues. However, the effect the CD45.1 allele on aging, the effect of parabiosis surgery in a strictly old environment, and the effect of an influx of a youthful CD45.2 compartment vs. a youthful CD45.1 compartment cannot be assessed.

The baseline characterization of this model involved the analysis of primary (bone marrow and thymus) and secondary (spleen and LNs) lymphoid tissues and blood. Initial characterization involved frequency and number of DCs, NK cells, neutrophils, macrophages, various B and T cell phenotypes as well as many different stages of thymopoiesis. The model can be refined as the data from the missing groups become available, maybe one day allowing for a comprehensive integrated model across tissues, cell types and age described in Section 2.5.

SECTION 2.5 – Cell Isolation and Flow Cytometry Methods

2.5.1 – Tissue Harvest and Cell Isolation

The animals were euthanized via isoflurane overdose and the tissues were harvested in the following order. First, the blood was collected via cardiac puncture and placed into vials containing heparin rings (Sarstedt, Numbrecht, Germany). Next the thymus, spleen and LNs (brachial, inguinal and popliteal separated by ipsilateral or contralateral location with respect to the surgical site) were harvested and placed into 500 µL of RPMI and put on ice. After approximately 3 minutes of warm-up time, 1.5 mL of room temperature 1x accutase (BD
Biosciences, Mountain View, CA or eBioscience, San Diego, CA) was added to each sample and they were placed in a 37° C water bath for 30 min. After 5 minutes of cool-down time, 1 mL of ice cold complete RPMI (cRPMI; 10% FBS, pen/strep, L-glutamine and HEPES) was used to quench the accutase and to provide serum to the cells. The tissues were press filtered through 40 µm mesh filters and washed with cRPMI and placed on ice. Both femurs were harvested and placed, together, into 5 mL of cRPMI and put on ice. The bone marrow was flushed out with cRPMI, filtered through a 40 µm filter, and washed with cRPMI. All of the samples were counted using a Hemavet 950FS (Drew Scientific, Oxford, CT). Next, the blood was lysed via hypotonic lysis (prior to this the blood was at room temperature throughout). Approximately 10⁶ lymphocytes were plated into 96-well round bottom plates to be stained for flow cytometry analysis. For longitudinal experiments following separation blood was collected via retroorbital puncture and placed into the vials containing heparin rings (Sarstedt, Numbrecht, Germany). The tissue harvests following separation were focused on T cells, therefore accutase was not used to digest the tissues. Furthermore, in attempt to increase the number of stained cells, and reduce harvest time, both brachial and inguinal LNs (without popliteal LNs) were pooled together. An anatomical diagram of the tissues harvested is provided in Figure 5.
The blood, thymus spleen, LNs and bone marrow were harvested for parabiosis experiments. The brachial, inguinal and popliteal LNs were pooled together and separated to ipsilateral and contralateral pools, with respect to the surgical site.

2.5.2 – Flow Cytometry Method

2.5.2.1 – General Flow Method

The entire protocol was done either on ice or incubating at 2-8°C. Single cell suspensions of approximately $10^6$ lymphocytes were washed with FACS buffer (PBS + 2% FBS + 0.01% azide) and then resuspended in 50 µL of FACS buffer containing 0.5 µg of anti-mouse CD16/CD32 (clone 93, aka Fc block, eBioscience Cat # 14-0161-86) for 15 min. This was followed by the addition of 50 µL of antibody cocktail for each panel (see panels below). All samples were stained for viability using Live/Dead Fixable Aqua or Live/Dead Fixable Yellow, where appropriate, and lightly fixed (approx. 10 min) in BD Cytofix/Cytoperm followed by collection on a custom BD Fortessa flow cytometer.

I obtained approximately $10^6$ lymphocytes in 100 µL by calculating the average total number of lymphocytes for each tissue in adult and old animals both during and without parabiosis and subtracted one standard deviation from that to ensure that 75% of my samples
would have $10^6$ lymphocytes per well. LNs did not contain enough cells to ensure that number of cells, but that did not affect the outcome of the staining. The resuspension volumes determined for each tissue can be seen in table 4.

**Table 4 – Tissue Resuspension Volumes to Obtain $10^6$ Lymphocytes per 100 µL**

<table>
<thead>
<tr>
<th></th>
<th>Blood*</th>
<th>Thymus</th>
<th>Spleen</th>
<th>LNs</th>
<th>Bone Marrow</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>120 µL</td>
<td>7 mL</td>
<td>6 mL</td>
<td>500 µL</td>
<td>2.5 mL</td>
</tr>
<tr>
<td>Old</td>
<td>120 µL</td>
<td>1.5 mL</td>
<td>6 mL</td>
<td>500 µL</td>
<td>2.5 mL</td>
</tr>
</tbody>
</table>

* This volume of whole blood was lysed and stained

Additionally, I found that for single stain controls I needed to spike in cells from both strain and age for the panel voltages to be optimal. If I did not use both strains, I could not properly test CD45.1 and CD45.2 antibodies and if I did not use old animals, certain antigens such as CD44 were more difficult to correct for. For the T cell panels, the spleen was sufficient for compensation controls. However, for the other hematopoietic-lineage panel I would use 80% spleen and 20% bone marrow as the bone marrow contains populations of cells that make it much better single stains (e.g. there are not many neutrophils in the spleen compared to the bone marrow). Lastly, the first three gates of every sample were the same in order to eliminate collection errors (gating by time), doublet exclusion (FSC-A x FSC-H) as well as a viability gate (viability x scatter) prior to panel specific gating (Fig. 6).
Figure 6 – Initial Gating Strategy for All Samples to Obtain a Live Single-Cell Population
All flow cytometry derived data was enriched for quality live single cell data by gating by time first. Next single cells were isolated with a singlet gate using forward scatter height and area parameters. Dead cells were excluded using fixable viability dyes.

2.5.2.2 – T cell Panel

The following T cell panel was used across all tissues and experiments, including the separation experiments (Table 5). The fluorophores, clones and vendors were consistent throughout the experiments and the cytometer settings were remarkably consistent, with the exception of needing to alter forward scatter and side scatter parameters between some experiments.

Table 5 – Parabiosis T Cell Flow Cytometry Panel

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Target</th>
<th>Fluorophore</th>
<th>Vol.</th>
<th>Vendor</th>
<th>Cat #</th>
<th>Clone</th>
</tr>
</thead>
<tbody>
<tr>
<td>405</td>
<td>450/50</td>
<td>CD8a</td>
<td>e450</td>
<td>1 µL / 10⁶</td>
<td>eBioscience</td>
<td>14-0081-82</td>
<td>53-6.7</td>
</tr>
<tr>
<td></td>
<td>545/30 or 585/42</td>
<td>Viability</td>
<td>Live/Dead Yellow</td>
<td>1-2 µL / mL</td>
<td>Invitrogen</td>
<td>L34959</td>
<td>N/A</td>
</tr>
<tr>
<td>488</td>
<td>525/50</td>
<td>CD45.1</td>
<td>FITC</td>
<td>1 µL / 10⁶</td>
<td>BioLegend</td>
<td>110706</td>
<td>A20</td>
</tr>
<tr>
<td></td>
<td>710/50</td>
<td>CD3</td>
<td>PerCP-Cy5.5</td>
<td>1 µL / 10⁶</td>
<td>BioLegend</td>
<td>100218</td>
<td>17A2</td>
</tr>
<tr>
<td>561</td>
<td>582/15</td>
<td>CD62L</td>
<td>PE</td>
<td>1 µL / 10⁶</td>
<td>eBioscience</td>
<td>12-0621-85</td>
<td>MEL-14</td>
</tr>
<tr>
<td>640</td>
<td>670/14</td>
<td>CD45.2</td>
<td>APC</td>
<td>1 µL / 10⁶</td>
<td>eBioscience</td>
<td>17-0454-82</td>
<td>104</td>
</tr>
</tbody>
</table>
The gating strategy for the T cell panel focused on obtaining a clearly distinguishable lymphocyte profile by forward and side scatter parameters (Fig. 7, Population Level 1). Next, I gated through the congeneric markers and all subsequent gates were in parallel in order to normalize by the age/strain compartment variable (Fig. 7, Population Level 2). T cells were electronically isolated by scatter and CD3 fluorescent properties, followed by the identification of CD4 and CD8 T cells (Fig. 7, Population Levels 3 and 4). Lastly, T_N cells were defined as CD44^{lo}CD62L^{hi}, while CD4 T_M were all CD44^{mid-to-hi} cells and CD8 T_CM followed the classical definition of CD44^{hi}CD62L^{hi} while CD8 T_EM were identified as CD44^{mid-to-hi}CD62L^{lo} (Fig. 7, Population Level 5).

T cell decay experiments following separation were gated similarly with the only difference being that the distinction of congeneric marker was not made until after the final CD4 and CD8 T cell subset gate in order to analyze how adult and old (or CD45.1 and CD45.2) cells competed at this subset level, directly.
The gating strategy for T cell panels started by gating on lymphocytes, then through the congenic markers CD45.1 or CD45.2. T cells were isolated as a subset of the parental congenic CD45 gate by identifying CD3+ cells. CD4 and CD8 T cells were analyzed separately to identify naïve and memory populations that were defined using CD44 and CD62L.

2.5.2.3 – Other Hematopoietic-lineage Cell Panel

The following panel was used across all tissues and experiments to quantify additional hematopoietic-lineage cells to complement the T cell panel (Table 6). The fluorophores, clones and vendors were consistent throughout the experiments and the cytometer settings were remarkably consistent, with the exception of needing to alter forward scatter and side scatter parameters between some experiments.
Table 6 – Parabiosis Other Hematopoietic-lineage Cell Flow Cytometry Panel

<table>
<thead>
<tr>
<th>Laser</th>
<th>Detector</th>
<th>Target</th>
<th>Fluorophore</th>
<th>Vol.</th>
<th>Vendor</th>
<th>Cat #</th>
<th>Clone</th>
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<td></td>
<td>545/30 or</td>
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The gating strategy for the other hematopoietic-lineage panel focused on obtaining a prototypical forward- and side-scatter profile for blood to ensure that I am capturing the granulocytes that would be off the axis using the lymphocyte optimized settings from before (Fig. 8, Population Level 1). The next gate was through the congeneric markers and all subsequent gates were in parallel in order to normalize by the age/strain compartment variable (Fig. 8, Population Level 2). T cells were excluded from the analysis by excluding all CD3 positive cells (Fig. 8, Population Level 3). Values reported for the subsequent identified populations were multiplied by the frequency of this T cell dump gate to obtain the value of the cell type per total CD45+ population (Population Level 2). B cells were identified as all CD19+ cells while NK cells were identified as those only expressing DX5. Macrophages were identified as CD11b^{hi}F4/80+ and neutrophils (or more accurately polymorphonuclear cells...
(PMNs)) were identified as CD11b<sup>hi</sup>Gr1<sup>hi</sup>. The DCs were identified through additional gates to ensure the exclusion of all the cells in the macrophage or neutrophil populations from satisfying the criteria of a DC. First, the canonical CD11c<sup>+</sup> cells were gated while excluding Gr1<sup>hi</sup> cells, then F4/80<sup>+</sup> cells were excluded to obtain what we are defining as DCs. This final DC population identified in level 5 was multiplied by the frequency obtained for the parental DC population in level 4 in order to be comparable at the total CD45<sup>+</sup> level with the other quantified cell types. The overall gating strategy for the other hematopoietic-lineage panel can be seen in Figure 8.

**Figure 8 – Parabiosis Other Hematopoietic-Lineage Cell Panel Gating Strategy** The gating strategy for the other hematopoietic-lineage cell panel started by gating on white blood cells, then through the congenic markers CD45.1 or CD45.2. T cells were excluded from analysis by gating on CD3<sup>+</sup> cells. NK cells were defined as DX5<sup>+</sup> (as well as CD3<sup>−</sup> and CD19<sup>−</sup>). B cells were defined as CD19<sup>+</sup>. Macrophages were defined as CD11b<sup>++</sup>F4/80<sup>+</sup>. Neutrophils were defined as CD11b<sup>+</sup>Gr1<sup>+</sup>. DCs were defined as CD11c<sup>+</sup>F4/80<sup>−</sup>.
SECTION 2.6 – Experimental Design

Figure 9 – CD45 Congenic Parabiosis and/or Separation Chimerism Model A schematic diagram illustrating CD45 congenic parabiosis and separation model. Chimerism is depicted by denoting CD45.1 mice and cells darker than CD45.2 mice and cells.

Both isochronic and heterochronic parabiosis experiments were conducted with the animals conjoined for ~4-5 weeks prior to tissue harvest or separation surgery (Fig. 9). This provides sufficient time to allow for extensive chimerism and the rejuvenation effects that have been reported. At this point, animals were either sacrificed for tissue harvest (explained in Section 2.5.1) or surgically separated and continually followed (explained in Section 2.2.2). Nonsurgical control mice were matched to parabionts by both age and mouse strain.

Normalizing to the known cellular differences between adult and old (or CD45.1 and CD45.1) mice prior to further gating is important for the two-factor statistical model (described in Section 2.4.2, Table 3). This allows for the model to account for both age/strain and surgery at the same time while allowing for the analysis of redistribution and migration patterns of each system. Furthermore, this model allows for statistical questions regarding the intrinsic effects of the age of the cell as well as extrinsic effects of the age of the tissue.

At the outset of the experiments the primary hypotheses were that heterochronic parabiosis would rejuvenate the aged thymus and aged HSC defects. We additionally sought to utilize the model to investigate age-related changes in the dynamics of the T cell compartment distribution and maintenance in primary and secondary lymphoid tissues.
SECTION 2.7 – Conclusions

This chapter provides a detailed protocol and study design for a congenic heterochronic parabiosis model emphasizing the analysis of hematopoietic-lineage derived cells. This is the first report integrating simultaneous measurements of over 10 different cell types, across primary and secondary lymphoid tissues and across age in the competitive context of parabiosis. Furthermore, this is the first study integrating the homeostatic competition between five different T cell subsets in the entire (i.e. whole body) T cell compartment both in the adult and old environments.

Lastly, I would like to emphasize the importance of transparency and trust that investigators should have with their animal care personnel. This is of course vital for the well-being of the animals, but also for the integrity of the research.
CHAPTER 3 – MOUSE GENETIC HETEROGENEITY CONSIDERATIONS FOR
OPTIMAL SURVIVAL AND HEALTH OF MICE IN (HETEROCHRONIC)
PARABIOSIS STUDIES

SECTION 3.1 – Introduction to Parabiotic Intoxication (Graft-versus-Host
Disease)

3.1.1 – The History of Parabiotic Intoxication and the use of Parabiosis to
Study GvHD

The earliest publications utilizing parabiosis note the common and frequent post-
surgical mortality. In 1910, “A Note on the Parabiosis of Rats and Mice” described a study
that included 50 surgeries and reported that 30 pairs lived ~3+ days and only 1 pair lived 28
days with the majority dying between 7-14 days (252). A 1920 review highlighted the power
of parabiosis but more importantly provided guidelines for more consistent results by urging
investigators to “make use as far as possible of animals of the same sex and the same litter,”
and minimize tissue injury during and after surgery (181). This phenomenon of tissue injury
and death was originally referred to as “parabiosis intoxication” or “parabiotic disease” (181–
183, 196–198). Finally, in 1960, a study by Nakic et al. defined the phenomenon underlying
“parabiotic disease” as GvHD and leveraged parabiosis to study GvHD (184). Parabiosis was
thoroughly used to study GvHD and tissue rejection biology until about 1980 (184, 200–204,
206, 207, 253). However, most of this research was subsequently ignored, despite a 1990
study utilizing parabiosis for the very purpose to study GvHD effects (208). Indeed, as
discussed below, the immunogenetics of the animals undergoing surgery is critical, yet often
ignored and this may be the most important consideration prior to conduction parabiosis.
SECTION 3.2 – Immunogenetics of the CD45.1 Congenic Mice

3.2.1 – Inherent Risks of Genetic Drift in Utilizing Congenic Mouse Models

Figure 10 – Large Linked Locus of SJL Origin in Chromosome 1 of CD45.1 (B6.SJL) Mice A map was obtained from Waterstrat et al. (2010).
The widespread utilization of congenic mouse models, including the most popular naturally occurring allelic variants (*e.g.* CD45.1/CD45.2, and to a lesser extent, CD90.1 and CD90.2) and the more recent engineered models with ectopic expression of fluorescent proteins or other distinguishable markers, has tremendously advanced biological, and in particular immunological studies. However, one has to remain alert to the possibility that the animals in question still are genetically distinct and that these differences could provide basis for unwanted immune reactions. Indeed, studies dating nearly two decades ago demonstrated that disparate alleles and traceable markers pose a legitimate potential concern for GvHD. Specifically, T cell responses against both CD45.1 and CD45.2 alleles and against green fluorescent protein (GFP), used as a genetically engineered tracer, were clearly documented (248–251). Moreover, there is considerable evidence for genetic drift amongst C57BL/6 WT strains, as well as congenic substrains, including publications noting the immunological impact of such drift (245, 254–257). While it is unknown which C57BL/6 lineage was originally used in the creation of the B6.SJL mice, additional genetic differences with C57BL/6J likely exist as the diversity among C57BL/6 substrains has been described (256–258). Most importantly, a recent study demonstrated 277 single nucleotide polymorphisms (SNPs) between C57BL/6J and C57BL/6NJ substrains, including 10 nonsynonymous mutations (258).

CD45.1 mice were originally submitted to repositories (*e.g.* JAX and NCI) by Boyse and coworkers at the Sloan Kettering Institute, after backcrossing SJL mice to C57BL/6 mice and selecting for CD45.1 expressing mice for 22 generations. The CD45.1 (B6.SJL, JAX Stock # 002014) utilized here contain a large ≥ 42 Mb linked locus containing a substantial segment of SJL genomic DNA flanking the CD45.1 congenic allele (Fig. 10). A medium density linkage mapping panel of 1449 SNPs was used to identify the known genetic
differences across the genome between a broad panel of mouse strains (illustrated in Figure 10) (245). Bioinformatic analysis of this region using a mouse SNP database revealed 124 SNPs between B6J and SJL, 45 of which fall within gene encoding regions. A selection of genes important to the immune system and aging in this locus include Bcl2, Il-10, mir181a as well as mutations in Cxcr4 and the pan-hematopoietic lineage phosphatase Ptprc (a.k.a. CD45, the molecule whose allelic variation provides congenic markers used widely for cell transfer studies as well as in parabiosis and in this thesis). This study discovered that there are at least 6 other SNPs on 6 other chromosomes of SJL origin, however it is unclear how much SJL DNA is linked to these loci or whether these loci are coinherited with the CD45.1 locus. This raises issues relative to the biological impact of such (immuno)genetic differences. Indeed, congruent with the prior study’s findings on HSC differences between B6J and B6.SJL mice, we have demonstrated basal differences in the lymphoid/myeloid ratio in these mice (Fig. 11). We found that 3 month old B6J mice exhibit a significantly lower lymphoid/myeloid ratio compared to than B6.SJL mice by t-test (#, Fig. 11). However, this difference was not maintained upon adding the aged, 18 mo B6J group and conducting a one-way ANOVA, nor was a difference found between adult B6.SJL and old B6J mice. It is possible that the analysis of old B6.SJL mice could reveal age-related sensitivity in this trait that could still allow interpretation of the impact of parabiosis upon the lymphoid/myeloid ratios, but due to the lack of availability of old B6.SJL mice this will have to await future experimentation. The age-related decline of the lymphoid/myeloid ratio was one age-sensitive parameter heterochronic parabiosis might have rejuvenated. However, following the results from Fig. 11, it was clear that statistical and discriminatory power to examine and interpret this issue was drastically reduced.
Despite these caveats, the use of congenic mice has remained popular and has been instrumental in many discoveries. We found that some of these differences become more pronounced upon parabiosis, urging caution in experimental design. Indeed, while even the most careful breeding schemes cannot eliminate linked or coinherited mutations after segregation of congenic alleles, a deliberate interbreeding practice can significantly improve long-term survival during parabiosis. Furthermore, properly controlled, these caveats can be entirely mitigated or minimized, especially because most outcomes measured did not exhibit an allelic biases.

Figure 11 – Myeloid Bias in Adult CD45.1\(^+\) B6.SJL Mice Resemble Changes in WT B6J with Aging  Lymphoid to myeloid ratio was calculated from values obtained from blood using a Hemavet 940 FS veterinary hematology device. There was n=4 adult mice and n=5 old mice. Significance was obtained (p < 0.05) by comparing the adult groups using a two-tailed students T test (#). Significance of the old group was significantly different from B6J adults by T test, but also by ANOVA when all three groups were present (p < 0.05, *).
3.1.3 – Controlling Mouse Genetics to Reduce GvHD

**Figure 12 – Differential Survival Rates of Parabiosis Due to Strain Background**  Survival rates 28 days following parabiosis when CD45.2\(^*\) mice were conjoined to the indicated parabiont. NCI and JAX indicate the source of wild-type B6.SJL, CD45.1\(^*\) mice.

After practicing the surgery, we utilized the CD45.1/CD45.2 congenic model whereby we would pair C57BL/6J adult mice from The Jackson Laboratory or aged mice from the National Institutes of Aging with the B6.SJL-PtpArePepc\(^b\)/BoyJ (CD45.1) from the National Cancer Institutes. CD45.1 mice obtained from the NCI displayed dramatically lower survival rates after parabiosis compared to CD45.1 mice obtained from JAX (Fig. 12, red and pink bars). This is important because survival here is reported as long-term post surgery (~28 days) rather than acute (~72 hr) survival, the latter often being a reported time for survival rates following parabiosis surgery (225, 227, 244). In our hands, GvHD-like symptoms did not tend
to manifest until 7-14 days, consistent with the classical GvHD literature, therefore reporting 72 hr survival rates would ignore GvHD entirely (184, 225, 259). In these experiments we ruled out obvious concerns related to technical failures and the sources of strains of the parabionts. For example, the first six surgeries utilized OT-I mice where we had recently generated CD45.1.OT-I and CD45.2.OT-I lines via intercrossing of CD45.1/CD45.2.OT-I siblings, thereby controlling for as many minor histocompatibility antigens as possible. In this case, the survival rate was excellent (5/6) (Fig. 12, purple bar). The next 12 pairs included spare mice from our colony that were never interbred, but were C57BL/6 mice congenic at the CD45 locus (CD45.1 mice were originally obtained from the NCI and the CD45.2 mice from JAX), and their survival rates were poor (Fig. 12, orange bar). It is important to note that when genetics was properly controlled, the vast majority of unintended deaths were due to animal behavior (typically twisting into knots due to excess skin) or to unintentional overdose of isoflurane.

The NCI has ended its mouse breeding services, and the CD45.1 strain at JAX (stock #002014) became our main source. According to JAX nomenclature, this strain was subsequently backcrossed to the background strain (C57BL/6J, stock #000664) three times without enhanced breeding protocols (e.g. speed congenics). Regardless, this CD45.1 strain had good survival and no discernible GvHD-like symptoms when paired with the WT-CD45.2 B6J mice obtained from JAX and the NIA (Fig. 12, blue and red bars, respectively). It is also interesting to note that there does not appear to be any survival disadvantage in heterochronic pairs when the parabiont was immunodeficient Rag1-KO. RAG1-deficient mice (obtained from JAX) (Fig. 12, green bars), illustrate that minor histocompatibility antigens other than the three amino acid difference between the CD45 congenic alleles, contributed significantly, as
both CD45.1 and CD45.2 mice had similar survival rates when a Rag1-KO mouse was the common parabiont. If Rag1-KO mice had zero genetic differences with CD45.2-WT mice and the only difference with CD45.1-WT mice being the congenic allele, one would expect a higher rate of death in Rag1-KO:CD45.1-WT pairs due to the anti-CD45.2 response. However, the rates of survival were not different from CD45.2-WT pairings. Due to these results, and the cost of aging mice, we decided to control for genetic factors using the breeding strategies outlined in Section 2.3.1.

Figure 13 – Thymic Cellularity Issues in Groups with a High Incidence of GvHD  CD45.2 mice from JAX (45.2 NS, 45.2 Iso, 2 GvHD) or from NIA (via JAX; Old NS, Old Het, O GvHD) were parabiosed to CD45.1 mice from JAX (45.1 NS, Adult Het, 45.1 Iso) or from NCI (A GvHD, 1 GvHD) and thymic cellularity was determined on day 28. Mouse groups were n=3-13. Data combined from 2-5 experiments.
The detrimental effects on mice surviving from groups where GvHD-like symptoms were observed was also evident when we compared thymic cellularity data obtained from CD45.1 (NCI) and CD45.2 congenic parabionts with CD45.1 (JAX or the mice we generated explained in Section 2.3.1) and CD45.2 (JAX/NIA) parabionts directly (Fig. 13). These thymus cellularity data contrast with results obtained after we controlled for genetic background, where both isochronic and heterochronic parabiotic thymi retain similar cellularity to their non-surgical control animals (Fig. 13). Furthermore, routine thymic cellularity data from our lab indicates that the expected thymic cellularity of an adult (12-16 wk old) B6 mouse is ~90-110x10^6 cells while for an 18 month old B6 mouse it is ~16-26x10^6 cells, and these data demonstrate that one should expect the same 28 days following parabiosis.

Figure 14 – Tissue Mapping of Total Hematopoietic Lineage Chimerism in Isochronic Parabiosis Groups Exhibiting GvHD (CD45.1 NCI Parabionts) Data represents the frequency of CD45.1 or CD45.2 cells among all alive single-cells obtained from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1 or CD45.2 CD45^+ cells. (B) CD45.2 tissue level analysis of CD45.1 or CD45.2 CD45^+ cells. (C) CD45.1 CD45^+ level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 CD45^+ level analysis in CD45.1 or CD45.2 tissues. All panels include data from 3-4 experiments, n=4-5. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Since GvHD-like symptoms were not expected or intended, the only new datapoints available from these groups were from the pairs that were the healthiest pairs and timepoints
were not adjusted due to failed long-term survival. Therefore, our data could very well underrepresent thymic cellularity issues under GvHD, especially at earlier time points when survival would be much better. Of note, we also observed increased variability and skewing in CD45-level chimerism across all tissues, including the thymus when B6J mice were paired with CD45.1 mice from the NCI (Fig. 14 and 15, isochronic and heterochronic, respectively), especially compared when B6J mice were paired with CD45.1 mice from JAX (Fig. 16 and 17, isochronic and heterochronic, respectively). This could be consistent with different intensities of GvHD in surviving mice.

Figure 15 – Tissue Mapping of Total Hematopoietic Lineage Chimerism in Heterochronic Parabiosis Groups Exhibiting GvHD (CD45.1 NCI Parabionts) Data represents the frequency of A (CD45.1) or O (CD45.2) cells among all alive single-cells obtained from the indicated tissue. (A) Adult tissue level analysis of A or O CD45+ cells. (B) Old tissue level analysis of A or O CD45+ cells. (C) Adult CD45+ level analysis in A or O tissues. (D) Old CD45+ level analysis in A or O tissues. All panels include data from 2-3 experiments, n=2-3. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
3.2.4 – Conclusions

From the data shown above, we conclude that the most important consideration prior to conducting parabiosis is the genetic background of parabionts. When next-generation genomic editing tools get used to create a more optimal congenic mouse model, many of these concerns will be abrogated. However, until then it is important for investigators to attempt to control for minor histocompatibility antigens by interbreeding. Furthermore, it may be wise to conduct a long-term (~28 days) survival pilot study including a longitudinal evaluation of tissue integrity and leukocyte infiltration even if such lengths are not followed in the experimental design to ensure that there are not subclinical or preclinical GvHD events altering the outcomes under observation.
CHAPTER 4 – AGE-RELATED CHANGES IN MIGRATORY PROPERTIES AND MAINTENANCE OF T CELL SUBSETS

SECTION 4.1 – Introduction

4.1.1 – Aging T Cell Biology

T cell numbers and distribution are known to change with age. An old animal has a dramatic drop in T_N cell numbers, within both the CD4 and CD8 pool. This shift in the T cell compartment follows thymic involution and the consequent severe reduction in T_N production. Evidence suggests that production and/or bioavailability of trophic factors (e.g. IL-7) and homing molecules (e.g. CCL19) required for T_N maintenance and migration decrease with age in the LNs, the primary tissue of T_N residence (84, 119, 138, 139). IL-7 has also been reported to decrease in the aged thymus and bone marrow, while IL-15, a T_M trophic factor, has been shown to increase in the latter (89–91, 119, 260), although both cytokines are very difficult to measure accurately in vivo and we still lack a comprehensive picture of their quantitative fluctuations. Regardless of age, T cells were made in the thymus where they exit to the periphery and migrate via blood and lymphatics to lymphoid tissues (e.g. spleen, LNs and bone marrow) and peripheral non-lymphoid tissues (e.g. skin, gut and other tissue parenchyma) where some T cells, not described here, form residency (T_RM cells) (85, 88). Evidence suggests that there is an integration of T cell differentiation and function with the migratory capacity of the cell (85). T_N cells spend the majority of their time in the LNs where they are most likely to encounter both the necessary trophic factors to survive as well as encounter a cognate antigen in order to engage in an immune response (261). Since T_N cells decrease with age, the occupancy of T_N niches could be depleted and LN cellularity decreases as well, possibly because the cross-talk between T cells and LN stroma gradually gets
disrupted. Conversely, T<sub>M</sub> cells proportionally increase with age and even in the adult environment these cells exhibit homing biases to the bone marrow, presumably in part due to the IL-15 rich environment (91, 262). This saturation of T<sub>M</sub> cells in the aged animal is accompanied by larger number of T cells in the old bone marrow compared to the adult bone marrow, and this has been suggested as a factor in the reduced long-lived plasma cell formation with age via competition for this space (108). Evidence to date suggests that in heterochronic parabiosis there is no rejuvenation of the aged thymus (as thymic cellularity remains diminished) and no rejuvenation of the aged T cell compartment because T<sub>M</sub> cells were found in a higher than expected proportion the spleen and blood of both the adult and old parabionts (239–241). Here we utilize heterochronic parabiosis and subsequent separation to directly assess the age-related changes in migratory properties, persistence and competition of many different T cell subsets to elucidate cell intrinsic and cell extrinsic (i.e. tissue microenvironment) influences on T cell behavior.

**SECTION 4.2 – Primary and Secondary Lymphoid Tissues Show Different Permissiveness to Heterochronic Engraftment**

Prior to characterization of specific hematopoietic-lineage subsets, we sought to confirm expected chimerism in five target tissues: blood, spleen, LNs (separated into contralateral and ipsilateral LNs (contLNs and ipsiLNs, respectively), thymus and bone marrow. The separation of the LNs with respect to the surgical site was done to ensure that there was no effect on LN cellularity due to surgery or due to the distance that short-lived cells has to migrate.
4.2.1 – Isochronic Engraftment in Primary and Secondary Lymphoid Tissues

**Figure 16** – Tissue Mapping of Total Hematopoietic Lineage Chimerism in Isochronic Parabionts

Data represents the frequency of CD45.1 or CD45.2 cells among all alive single-cells obtained from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1+ or CD45.2+ cells. (B) CD45.2 tissue level analysis of CD45.1+ or CD45.2+ cells. (C) CD45.1 level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 level analysis in CD45.1 or CD45.2 tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 16 represents the proportion of cells from each indicated tissue derived from the CD45.1 adult mouse or the CD45.2 adult mouse. CD45.1 derived data points are dark and CD45.2 derived data points are light colored, and the data points from either group, when found in the opposing animal are of a lighter shade (i.e. black represents CD45.1 cells found in CD45.1 tissues, while dark grey represents CD45.1 cells found in CD45.2 tissues). There are
clear differences in chimerism between secondary lymphoid tissues (blood, spleen and LN) and primary lymphoid tissues (thymus and bone marrow), but the chimerism is relatively even within secondary lymphoid tissues (±10%).

4.2.2 – Heterochronic Engraftment in Primary and Secondary Lymphoid Tissues

**Figure 17** – Tissue Mapping of Total Hematopoietic Lineage Chimerism in Heterochronic Parabionts Data represents the frequency of A (CD45.1) or O (CD45.2) cells among all alive single-cells obtained from the indicated tissue. (A) Adult tissue level analysis of A or O CD45+ cells. (B) Old tissue level analysis of A or O CD45+ cells. (C) Adult CD45+ level analysis in A or O tissues. (D) Old CD45+ level analysis in A or O tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

Figure 17 represents the proportion of cells from indicated tissues, derived from the CD45.1 adult mouse or the CD45.2 old mouse. Adult-derived data points are blue and old derived data points are red, and the data points from either group, when found in the opposing
animal are of a lighter shade (i.e. blue represents adult cells found in adult tissues, while light blue represents adult cells found in old tissues). There are clear differences in chimerism between secondary lymphoid tissues (blood, spleen and LNs) and primary lymphoid tissues (thymus and bone marrow).

### 4.2.3 – Conclusions

As has been shown in the literature, we observed roughly equal chimerism in secondary lymphoid tissues and ~10% chimerism in primary lymphoid tissues in isochronic adult parabionts. However, for the first time, we demonstrate that this ratio also holds in heterochronic parabiosis. The low degree of chimerism in the thymus has been attributed to periodic importation of prothymocytes in a coordinated, gated phenomenon (263, 264). Also, there is low engraftment of HSCs into unconditioned bone marrow, and this is true in parabiosis as well (228). The separation of contLNs and ipsiLNs, relative to the site of surgery, failed to reveal substantial variation amongst any cell type analyzed between the two sites. This suggests that neither surgery nor the distance a cell must travel affects leukocyte chimerism during parabiosis. In both control isochronic adult and heterochronic parabionts we observed similar extents of chimerism. We therefore conclude that aging does not impart defects in gross cellular migration and/or tissue retention. Unless otherwise stated, the remaining data presented herein will highlight unique findings where heterochronic parabionts differ from isochronic controls.
SECTION 4.3 – Neither the Age of the T Cell nor the Age of the LN Environment Restrict T Cell Migration and Subset Distribution

4.3.1 – Differences in T Cell Subset Distribution and Cellularity Between Adult and Old T Cell Compartments Before but Not After Parabiosis

There are about \(2 \times 10^5\) T\(_M\) cells in combined bilateral brachial, inguinal and popliteal (six total) LNs, regardless of age, therefore, the T cell contribution to the reduction of LN cellularity with age is due to T\(_N\) numerical deficiencies with age (Fig. 18A,B). In fact, roughly \(12.0 \times 10^5\) T\(_N\) cells exist in the six adult LNs and this number dwindles to \(2.6 \times 10^5\) T\(_N\) cells with age. Upon parabiosis, the number of adult T cells in the LNs equaled that of pre-parabiotic old T cell compartment, whereas the number of old T cells was reduced to roughly half of the number of pre-parabiotic old T cells (Fig. 18C). Overall, the gross overall T cell numbers were similar in adult and old LNs following parabiosis, and these were marginally higher and not significantly different from old control LNs. Pooled T cells from LN of control adult + old mice (~2.04 \(\times 10^6\)) exceeded that from parabiosed adult + old mice (~1.52 \(\times 10^6\)) and we are still investigating the significance of this observation. Because we found an increased proportion of adult T cells in old LNs, we tested if which adult T cell subsets were responsible for this observation and whether adult and old LNs harbored similar and proportional fractions of T cell subsets.
Figure 18 – Increased LN Infiltration by Adult T Cells Reflects Large Pre-Parabiotic $T_N$ Compartment in Adult Mice  
Numbers of CD4 and CD8 T cells and the proportion of their subsets in non-surgical control LNS from (A) adult mice and (B) old mice. (C) Total adult and old CD4 and CD8 T cell LN numbers compared in non-surgical control and heterochronic parabiosis settings. Mouse groups were n=5-6, 2-3 experiments.
Figure 19 – Adult and Old Parabiotic LNs Contain Remarkably Similar Proportions of T Cell Subsets Regardless of the Age of the T cell

Total CD4 and CD8 T cell numbers in adult and old LNs 4-5 weeks following heterochronic parabiosis. (A) Adult LN content of A and O T cell subsets in heterochronic parabiosis. (B) Old LN content of A and O T cell subsets in heterochronic parabiosis. Mouse groups were n=6.

Table 7 – The Relative Representation of Adult or Old T Cell Subsets Among All Cells in Heterochronic Parabiotic LNs

<table>
<thead>
<tr>
<th>% of ALL</th>
<th>CD4</th>
<th>CD8</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Naive</td>
<td>Memory</td>
</tr>
<tr>
<td>A in Adult</td>
<td>9.2</td>
<td>2.4</td>
</tr>
<tr>
<td>A in Old</td>
<td>9.2</td>
<td>2.5</td>
</tr>
<tr>
<td>O in Adult</td>
<td>2.3</td>
<td>1.9</td>
</tr>
<tr>
<td>O in Old</td>
<td>2.3</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Upon parabiosis, adult and old LNs equilibrated in T cell subset distribution (Fig. 18C, 19, Table 7). This suggests that, at least in the context of heterochronic parabiosis, there is no difference between the adult and old LN environments with respect to either permissiveness or
retention of T cell subset or age of the cell. The distribution of subsets within the adult T cell compartment was similar to that seen in young isochronic parabiotic control mice (data not shown). The most abundant cells in the parabiosed LNs were the adult CD4 T_N, making up 9.2 ± 1.1% and 9.2 ± 0.6% of all cells (58% and 56% of CD4 T cells), in adult and old LNs, respectively, followed by adult CD8 T_N, which made up 6.6 ± 0.9% and 6.8 ± 0.6% of all cells (41% and 40% of CD8 T cells), in adult and old LNs, respectively (Fig. 19, Table 7). Adult CD4 T_N cells were found to be fourfold more abundant than old CD4 T_N cells, and made up 2.3 ± 0.3% of all cells (14% of CD4 T cells) in adult and old LNs (Fig. 19, Table 7). Furthermore, adult CD4 T_N were threefold more abundant than the largest subset in the old T cell compartment, the old CD8 T_CM cells, which made up 2.9 ± 0.4% and 3.3 ± 0.4% of all cells (18% and 20% of CD8 T cells), in adult and old LNs, respectively (Fig. 19, Table 7). Similarly, adult CD8 T_N were 2.6-fold more numerous than old CD8 T_N in both adult and old LNs. Conversely, the pre-parabiotic differences in T_CM composition remained after parabiosis, and we found >2-fold more old T_CM than adult T_CM cells in both adult and old LNs. By contrast, the numbers and representation of CD4 T_M and CD8 T_EM were similar between adult and old T cell compartments (data not shown, Table 7).

4.3.2 – Conclusions

Overall, the pre-parabiotic dominance of T_N and T_CM in adult and old T cell compartments, respectively, was retained after parabiosis and equilibrated with the opposing parabiont’s tissues. There was no evidence that the age of the cell or the age of the tissue impacted T cell migration and retention on LNs. One could speculate that there is a designated amount of space in the system for each subset, and while the numbers and proportions of each T cell subset certainly change with age, the amount of space available for each subset does not
(or is dynamically tuned to the numbers and proportions of T cells in the system).

Alternatively, there may be a pro-geronic factor in old blood that has the ability to alter the amount of space available for $T_N$ cells in LNs, which could explain the failure of the adult $T_N$ compartment from fully rescuing LN cellularity.
SECTION 4.4 – Differential T Cell Subset Homing to Bone Marrow is Compounded by Age

4.4.1 – Increased Proportion of Old T Cells in Adult and Old Bone Marrow is Due to their Increased Proportion in the Old Parabiont

Figure 20 – Numbers of Major T Cell Subsets in the Bone Marrow of Nonsurgical Control, Heterochronic Parabiosis and Isochronic Adult Parabiosis Mice  (A) Numbers of total T cells in the bone marrow of adult CD45.1, adult CD45.2 or old CD45.2 non-surgical control animals. (B) Chimerism of A or O T cells in heterochronic parabiosis bone marrow. (C) Chimerism of adult CD45.1 or adult CD45.2 T cells in isochronic parabiosis bone marrow. (D) Numbers of T cell subsets in the bone marrow of adult CD45.1, adult CD45.2 or old CD45.2 non-surgical control animals. (E) Chimerism of A or O T cell subsets in heterochronic parabiosis bone marrow. (F) Chimerism of adult CD45.1 or adult CD45.2 T cells in isochronic parabiosis bone marrow. Mouse groups were n=5-6.
Along with the thymus, bone marrow was a restricted tissue, exhibiting reduced levels of overall chimerism (Fig. 17). Despite similar gross CD45+ chimerism profiles, the old bone marrow numerically harbored more T cells in nonsurgical control mice (Fig. 20A). Furthermore, old T cells outnumbered adult T cells in both adult and old bone marrow following heterochronic parabiosis (especially CD8 T cells, Fig. 20B).

A more detailed evaluation of old T cell trafficking to, and retention in, bone marrow indicated that the increased T cell cellularity was primarily due to the increase in all old T\textsubscript{M} subset numbers and frequencies in the adult and even more prominently, the old T compartment in the bone marrow (Fig. 20D,E). Despite an increased number of old CD4 T\textsubscript{M} and CD8 T\textsubscript{EM} cells in the adult bone marrow (reminder of phenotypic definitions in Section 2.5.3.2, Figure 7), such cells were still significantly more abundant in the old bone marrow. This suggests that these subsets either do not recirculate out of their resident bone marrow or that they have a longer average dwell time in bone marrow than 4-5 weeks (the duration of parabiosis in our experiments) (Fig. 20D,E). CD4 T\textsubscript{M} and CD8 T\textsubscript{EM} displayed similar retention properties in isochronic controls, with some weak allele-specific trends (Fig. 20F). Old CD8 T\textsubscript{CM} cells also outnumbered their adult counterparts in both adult and old bone marrow, and showed no difference in isochronic hosts (Fig. 20D-F). While CD4 and CD8 T\textsubscript{N} cells were proportionately different between adult and old T cell compartments, they did not different numerically, which suggests that there is a maximal T\textsubscript{N} niche capacity which is reached in both adult and old mice (Fig 20D-E).

Similar to the redistribution of the T cell compartment relative to nonsurgical control T cell compartment proportions seen in the LNs, the redistribution of CD8 T cells in the bone marrow followed the same trend (Fig. 21A,B). While there were no significant differences in
CD8 T<sub>N</sub> numbers residing in adult and old bone marrow, comparing the chimerism in the heterochronic versus the isochronic condition suggested that the increased systemic numbers of adult T<sub>N</sub> cells lead to increased proportional seeding of old bone marrow (Fig. 21B, data not shown). Additional evidence for this was found when adult CD8 T<sub>N</sub> cells were found in higher proportions in old bone marrow relative to the influx of other adult CD8 T cells (Fig. 21B). Similar analysis of the old cell influx revealed that old T<sub>CM</sub> cells also exhibit preferential representation in adult marrow (Fig. 21B), whereas CD8 T<sub>EM</sub> cells showed strong preference for self marrow. This data further corroborates that T<sub>EM</sub> cells, regardless of age, appear to have
a longer average dwell time than 4-5 weeks in the bone marrow (Fig. 21B).

Figure 21 – Pre-Parabiotic and Post-Parabiotic Bone Marrow T Cell Compartments Reveal Age-Dependent and -Independent Bone Marrow Homing Properties

Numbers of CD4 and CD8 T cells and the proportion of their subsets in non-surgical control bone marrow from (A) adult mice and old mice. (B) The degree of preference of self or opposing bone marrow in heterochronic parabiosis, calculated for each subset by the frequency of the subset in self BM subtracted by the frequency of subset in heterochronic bone marrow. In the legend, * indicates the subset significantly retained in the opposing bone marrow and # indicates the subset significantly retained in the bone marrow from which it was derived. Mouse groups were n=6.
4.4.2 – Conclusions

The numerical and proportional abundance of T\textsubscript{M} cells in the old T cell compartment resulted in another saturated niche in the bone marrow. However, CD4 T\textsubscript{M} and CD8 T\textsubscript{EM} cells, which represented a large proportion of the old bone marrow compartment, were preferentially retained in the bone marrow of which they were derived, regardless of age-related cellularity differences. While it was known that the bone marrow represents a niche for T\textsubscript{CM} in the adult animal, with age the bone becomes numerically and proportionately dominated by all T\textsubscript{M} cells, CD4 T\textsubscript{M} and CD8 T\textsubscript{EM} cells included. Furthermore, CD8 T\textsubscript{CM} cells redistribute freely while CD4 T\textsubscript{M} and CD8 T\textsubscript{EM} cells do not. Another important finding is that the apparent size of the T\textsubscript{N} niche in the bone marrow remains constant in size across time and surgery. This implies that this niche is important, or at least unaffected by age, warranting future investigations.

SECTION 4.5 – Aging Selects for T cells with a Peripheral Maintenance Advantage Over Adult T Cells

4.5.1 – The Old Environment is Not Hospitable for Adult T Cells

To evaluate how age-specific environmental factors contribute to T cell maintenance, we examined how well different T cell subsets persisted in adult or old hosts following separation of parabiotic partners and re-establishment of adult or old circulating environment. In these experiments, mice underwent parabiosis surgery and remained connected for 4-5 weeks, after which they were surgically separated and longitudinally bled for the following 10-12 weeks to assess T cell subset decay rates. To make direct comparisons at the T cell subset level, we first gated through the subset of choice and subsequently through CD45.1 or CD45.2, performing regression analysis to describe the starting population and slopes of
loss/persistence of different adult or old T cell subsets. In their own setting, adult and old T cells survived and were maintained very well (Fig. 22A,D). However, linear regression analysis demonstrated that old T cells were, on average, 1.5-fold better at persisting in adult environment (Fig. 22C) compared to adult T cells in the old environment (Fig. 22B). This persistence of old T cells in the adult circulation was evident despite the presence of robust thymic output of new T cells in the adult animal. By contrast, all adult T cell subsets precipitously declined over the 12 weeks in the old animal (Fig. 22B). The average decline of adult T cell subsets was 21.2% over the 12 weeks. The loss was the highest for the CD4 T_N cells at 53.5% decline and the lowest for CD4 T_M cells at 5.4% loss. This decline occurred despite the fact that thymic output was considerably lower in old hosts, and that the cells that failed to persist were younger. These results were surprising at the face value, and call for reexamination of peripheral factors that govern mature T cell maintenance with aging.
Figure 22 – Adult and Old T Cell Persistence Kinetics in Adult or Old Environments  Data represent the frequency of A (CD45.1) or O (CD45.2) cells among the indicated T cell subset in blood, measured longitudinally after parabiont separation. (A) Adult T cell subsets in the blood of the A animal. (B) Adult T cell subsets in the blood of the O animal. (C) Old T cell subsets in the blood of the A animal. (D) Old T cell subsets in the blood of the O animal. Statistical values depict difference in linear regressions, where each slope is tested whether it is significantly different from 0 (filled diamonds) or not (open diamonds). Additionally, within each graph the slopes of all lines were tested for significance as a group. * p < 0.05, ** p < 0.01, *** p < 0.001, **** p > 0.0001. Mouse groups were n=7-9.

4.5.2 – Old T_N Cells Exhibit a Peripheral Maintenance Advantage Over Adult T_N Cells
As we found with tissue homing and retention, different T cell subsets exhibited different decay kinetics, and both the age of the T cell subset and the age of the environment impacted the maintenance process (Fig. 23). At the time of separation, the differences in T cell...
subset representation were apparent, with ~60.7% of the T cells being adult T cells in both adult and old animals. Despite being outnumbered, old total CD3+ and CD3+CD4+ T cells decayed slower (-0.19%/day and -0.25%/day, respectively) compared to their adult counterparts (-0.48%/day; and -0.62%/day, respectively) (data not shown). Total CD8 cells, by contrast, were roughly equal at the moment of separation (~51.8% vs ~48.2%, adult vs old) and the difference in decay, while threefold, did not reach statistical significance (-0.35%/day vs -0.12%/day, adult vs old). Overall, we found that CD4 T cells decay roughly twice as fast as CD8 T cells, regardless of age, providing further basis for their gradual disappearance with aging. Subset differences were particularly striking when analyzing naïve T cells. Again, despite being outnumbered, old CD4 and CD8 T_N cells decayed slower (-0.21% and -0.20%/day, respectively) compared to their adult counterparts (-0.87% and -0.37%/day respectively) (Fig. 23C,D). Furthermore, while adult CD4 T_N cells decayed more than twice as fast as adult CD8 T_N cells, this difference was lost with aging and old CD4 T_N cells exhibited lower decay rates, indistinguishable from old CD8 T_N cells (Fig. 23A,B). Adult CD4 and CD8 T_N cells did not decay differently in the old environment than they did in the adult CD45.2 environment (-0.59% and -0.44%/day, respectively) (Fig. 24A,B). The difference of adult CD4 T_N decay in the old (-0.87%/day) and the adult (-0.59%/day) environments did not reach statistical significance. Moreover, the persistence of adult CD4 T_M cells seems to be due to the old environment as old CD4 T_M cells decayed at a similar rate as adult CD4 T_M cells in the adult environment (Fig. 23C, 24C).
Figure 24 – Similar Decay Kinetics Among CD45.1 and CD45.2 Adult T Cell Subsets in Isochronic Adult Separated Environments  Data represented the frequency of adult CD45.1 or adult CD45.2 cells among the indicated T cell subset in blood, measured longitudinally after separation. (A) CD8 T_{CM} subset kinetics in the blood. (B) CD8 T_{EM} subset kinetics in the blood. (C) CD4 T_{N} subset kinetics in the blood. (D) CD8 T_{N} subset kinetics in the blood. (G) CD4 memory subset kinetics in the blood. Statistical values depict difference in linear regressions, where each slope is tested whether it is significantly different from 0 (filled diamonds) or not (open diamonds). Additionally, within each graph the slopes of all lines were tested for significance as a group. Mouse groups were n=3. There were no statistically significant differences between any of the decay slopes.
4.5.3 – Conclusions

Taken together, these data indicate that rules for maintenance of T cell subsets change significantly with age due to extrinsic increased CD4 T_M cell maintenance in the old environment and an intrinsic peripheral survival advantage enriched in old CD4 and CD8 T_N cells.

SECTION 4.6 – Conclusions

Data exists to supporting the notion that, under infection conditions, there are age-related defects in the migration of B cells and CD4 T cells in the draining LN following West Nile Virus infection (265). However, so far, no study has examined migration of multiple cell types and subsets simultaneously in steady-state conditions in adult and old mice. We found that the T cell composition of the immune system of each parabiont seems to influence the T cell cellularity in tissues, with T_N heavily influencing LN cellularity and T_M cells impacting bone marrow T cell cellularity. We are currently entertaining the possibility that a pro-geronic factor in old mice may drive the deterioration of LN function (and possibly structure) was responsible for the failure of the adult thymus and T cell compartment to restore LN cellularity. Alternatively, it is possible that the combined T cell output from the adult and old thymi is insufficient for peripheral reconstitution of T_N cellularity during heterochronic parabiosis. We also found evidence for a potential cell extrinsic factor(s) in the old environment that help maintain CD4 T_M persistence as the adult CD4 T_M population was maintained better in the old environment than in adult isochronic controls or compared to old CD4 T_M cells in the adult environment (I must note that the CD4 T_M defined here may contain Tregs as well). Lastly, we note that the decay rates of adult CD4 T_N were faster than adult
CD8 T<sub>N</sub>, however, this difference was lost with age. Furthermore, a 2-fold peripheral survival advantage was conferred upon aged T<sub>N</sub> cells, likely due to long-term peripheral selection in the absence of a functional thymus. We strongly favor the explanation that peripheral selection yielded old T<sub>N</sub> cells that exhibit a competitive advantage for self pMHC, IL-7, and/or other trophic factors. This is consistent with decay and modeling data of Den Braber et al. as well as with corroborating independent studies from Hogan et al. and Tsukamoto et al. that indicate a peripheral survival advantage of old T cells (266–268). More recently, evidence for peripheral selection of old T cells, including T<sub>N</sub> cells, suggesting that CD8 T<sub>N</sub> cells may be selected for increased self-reactivity (269–271), whereas CD4 T<sub>N</sub> cells appear more cross-reactive (272). This increased self-reactivity could be investigated by measuring CD5 levels on the T cells (272–275), and remains to be fully elucidated by future experiments. Results shown here uniquely demonstrate that old T<sub>N</sub> cells are intrinsically superior in their ability to compete for trophic factors, regardless of old or adult environment, and regardless of the plethora of other, chronologically younger T cells. We suggest that age-related LN defects play a previously underappreciated role in this peripheral selection and immune aging.
CHAPTER 5 – DISTRIBUTION AND TRAFFICKING OF NON-T CELLS IN HETEROCHRONIC PARABIOSIS

SECTION 5.1 – Introduction and Statistical Considerations

To complete a true systemic model of the immune cell subset aging and parabiosis we sought to comprehensively measure the numbers and ratios of various myeloid and lymphoid cells across primary and secondary lymphoid tissues. Furthermore, this systemic analysis could potentially reveal important interactions between disparate cell types, that would otherwise have been ignored. Therefore, I measured DCs, macrophages, neutrophils, NK cells, B cells and T cells throughout the bone marrow, thymus, spleen, LNs and blood. Not shown in this chapter are the additional data on thymic T cell subsets, and B cell subsets.

In the results presented below, some of the data may visually appear as statistically significant but is not labelled as such, or vice versa. To that effect, it is important to remember the statistical model described in Section 2.4. I have chosen to follow strict statistical rules of specific and applicable tests and with a small sample size, multiple comparisons between groups can often diminish visually apparent significance. Furthermore, in the parabiosis data, the matched-paired nature of the study can provide statistical significance when there are subtle differences and high between-the-pairs variation if the data within each pairs changes in a consistent manner. In another example, numerical B cell data included match-pairing across tissues as well as many multiple comparisons and the differences analyzed were not statistically significant.

The dataset presented below could prove valuable as the background data that could allow investigators to propose hypotheses and ask specific questions in a statistical framework optimized to answer such questions.
SECTION 5.2 – B Cells

5.2.1 – Introduction to B Cells and Aging

It is clear that in aging antibodies generated to vaccines or novel pathogens in old individuals are generally less protective because of lower titer and affinity, and the formation of auto-antibodies increases with age (109, 117). The difficulty, however, comes in answering to what extent B cells themselves may experience age-related defects, and to what extent alterations in other components of the immune system that are necessary for optimal B cell function. Not only do aged HSCs exhibit altered development (122), but the aged bone marrow also contains a smaller niche for plasma cells (terminally differentiated B cells) and this niche was occupied with plasma cells representative of previous antigen encounters (276). Additionally, non-B cell contributions to defective antibody production in aging include the age-related reduction in CD4 T cells, especially T_N cells, along with defective T follicular helper cell function and increased representation of T follicular regulatory cells (109, 277). In mice, there seems to be a reduction of naïve B cells and an increase in memory B cells with age, however, in humans there have been reports corroborating this finding as well as those finding the opposite results (109, 117, 278, 279). Defects in CD19+ B cell migration in the draining LN, along with delayed germinal center formation and reduced antibody production was observed in response to West Nile Virus infection in old mice (265). Furthermore, the mouse model has elucidated reduced Activation-Induced Deaminase (AID, necessary for somatic hypermutation and further affinity maturation of antibodies), decreased E47 transcription factor necessary for these functions, and an overall reduction in differentiation and function of B cells (280–282). The results of decreased AID and E47 were recently
discovered in a human cohort (283). Additionally, new human data demonstrates that while the generation of memory B cells is maintained in the elderly, the differentiation of memory B cells to plasma cells as well as the antibody response is impaired (284). The age-related increase in incidence of B cell lymphomas has recently been demonstrated to be due to aging-associated inflammation leading to the selection of cells with oncogenic mutations (285). Furthermore, reversing inflammation in the aged environment prevented oncogenesis, even in the presence of cells harboring oncogenic mutations. Considering these findings, the heterochronic parabiosis model could be a powerful tool for further insights into the complex nature of the failure of the aged B cell response.

5.2.2 – Nonsurgical Control Distribution of B Cells in Primary and Secondary Lymphoid Tissues of Adult and Old Mice

In control, surgically unmanipulated adult and old mice, we found no significant numerical differences in total B cell numbers in any of the tissues analyzed (Fig. 25). The age-related decrease in the frequency of B cells in the bone marrow is related to the loss of lymphoid differentiation with age.
Figure 25 – Numbers and Frequency of B Cells in Nonsurgical Control Tissues  Data represents the (A) numbers and (B) frequency of A (CD45.1), A (CD45.2) or O (CD45.2) CD19+ B cells within the CD45+ compartment from the indicated tissue. Adult CD45.1 and CD45.2 groups were n=4 while the old CD45.2 group was n=5. Significance was determined by a two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
5.2.3 – Isochronic Engraftment of B Cells in Primary and Secondary Lymphoid Tissues

Differences in the proportion of B cell subsets inherent to CD45.1 (proportionally more marginal zone B cells) compared to CD45.2 (proportionally more T1 and T2 transitional B cells) mice are known (247). However, utilizing the model proposed here could control for these differences. CD45.2 B cells are represented in higher numbers compared to CD45.1 B cells in most tissues following parabiosis (Fig. 26 A,B). B cells otherwise seem to move freely across the thymus, spleen and LNs (Fig. 26 D,E).

Figure 26 – Chimerism and Engraftment of B Cells in Isochronic Parabiosis Tissues Data represents the frequency of CD45.1 or CD45.2 B cells within the CD45.1 or CD45.2 compartment, respectively, from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1 or CD45.2 B cells. (B) CD45.2 tissue level analysis of CD45.1 or CD45.2 B cells. (C) CD45.1 B cell level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 B cell level analysis in CD45.1 or CD45.2 tissues. All panels include data from 3-4 experiments, n=6. Significance
was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.2.4 – Heterochronic Engraftment of B Cells in Primary and Secondary Lymphoid Tissues

While CD19+ is one of the cardinal B cell markers, it may not be sufficient to define B cells by itself, particularly if one seeks to evaluate the precise effect of aging compared to the effects of genetics/mouse strain. However, even with this limited discrimination, we found that overall B cell chimerism and engraftment patterns were similar to isochronic pairs, albeit with more dramatic alterations in the thymus and bone marrow (Fig. 26, 27).

![Figure 27 - Chimerism and Engraftment of B Cells in Heterochronic Parabiosis Tissues](image)

Data represents the frequency of A (CD45.1) or O (CD45.2) B cells within the A or O compartment, respectively, from the indicated tissue. (A) Adult tissue level analysis of A or O B cells. (B) Old tissue level analysis of A or O B cells. (C) Adult B cell level analysis in A or O tissues. (D) Old B cell level analysis in A or O tissues. All panels include data from 3-4
experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.2.5 – Conclusions

While old B cells tend to be more numerous than adult B cells, it is very likely that this finding is an allelic effect (247). However, there were some differences in the bone marrow that mirrored what was observed in T cells (Section 4.4), and these results merited further investigation. Ultimately, results were inconclusive because of a lack of precision in defining B cell subsets across tissues in the data. Therefore, one of the first experiments to do would be to precisely subdivide B cells into distinct phenotypic subsets and to examine their behavior similar to the studies on T cells (Chapter 4).

SECTION 5.3 – NK Cells

5.3.1 – Introduction to NK Cells and Aging

Age-related defects of important NK cell functions, such as cytotoxicity and cytokine production have been documented (110). There seems to be a decrease in the immature, cytokine producing NK cells and a proportional increase in the mature, cytotoxic NK cells, however, both of their functions are diminished with age (110). Lastly, there is evidence of the inability of aged NK cells to migrate to the inflamed sites during infection and reports of altered NK cell repertoire (110, 286–289). Overall, the vital role of NK cells in preventing oncogenesis and viral pathogenesis seem to deteriorate with age.

5.3.2 – Nonsurgical Control Distribution of NK Cells in Primary and Secondary Lymphoid Tissues of Adult and Old Mice

The data from the nonsurgical control mice illustrate an age-related increase in NK cell numbers in the blood as well as a massive increase in NK cells in the bone marrow of CD45.1
mice, suggesting an allelic effect (Fig. 28). While there were no numerical differences of NK cells in the LNs, they were proportionally overrepresented with age, likely due to the decrease in T\textsubscript{N} cellularity. Overall, we found nothing remarkably different about total NK cell numbers and frequencies with age.
Figure 28 – Numbers and Frequency of NK Cells in Nonsurgical Control Tissues Data represents the (A) numbers and (B) frequency of A (CD45.1), A (CD45.2) or O (CD45.2) NK cells within the CD45+ compartment from the indicated tissue. Adult CD45.1 and CD45.2 groups were n=4 while the old CD45.2 group was n=5. Significance was determined by a two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
5.3.3 – Isochronic Engraftment of NK Cells in Primary and Secondary Lymphoid Tissues

Despite a marked increase in NK cell numbers and proportion in the bone marrow of CD45.1 adult mice, there were no remarkable differences during isochronic parabiosis (Fig. 29). The increased NK cells in host bone marrow are no surprise, as this is the tissue where these cells are made, and the trend for increased host NK cells in the blood is likely a result of the same as the exodus of new NK cells to the periphery happens via the blood.

![Figure 29](image)

**Figure 29 – Chimerism and Engraftment of NK Cells in Isochronic Parabiosis Tissues**

Data represents the frequency of CD45.1 or CD45.2 NK cells within the CD45.1 or CD45.2 compartment, respectively, from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1 or CD45.2 NK cells. (B) CD45.2 tissue level analysis of CD45.1 or CD45.2 NK cells. (C) CD45.1 NK cell level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 NK cell level analysis in CD45.1 or CD45.2 tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
5.3.4 – Heterochronic Engraftment of NK Cells in Primary and Secondary Lymphoid Tissues

Similar to isochronic pairs, we found even chimerism and/or engraftment of NK cells during heterochronic parabiosis (Fig. 30). The same trends of increased host NK cells in the blood and bone marrow remained.

Figure 30 – Chimerism and Engraftment of NK Cells in Heterochronic Parabiosis Tissues
Data represents the frequency of A (CD45.1) or O (CD45.2) NK cells within the A or O compartment, respectively, from the indicated tissue. (A) Adult tissue level analysis of A or O NK cells. (B) Old tissue level analysis of A or O NK cells. (C) Adult NK cell level analysis in A or O tissues. (D) Old NK cell level analysis in A or O tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.3.5 – Conclusions

While we had concerns that adult CD45.1 mice have many more bone marrow NK cells prior to parabiosis, this, however, did not seem to influence the data. If it did, one would
expect to see a higher proportion of CD45.1+ NK cells in the bone marrow. NK cells seem to move freely throughout adult and old tissues and are enriched in the blood and bone marrow, the latter is where they are formed.

SECTION 5.4 – Dendritic Cells

5.4.1 – Introduction to Dendritic Cells and Aging

Dendritic cells (DCs) represent a heterogeneous population of cells that are distributed throughout the body with a primary task to serve as sentinels that sense, ingest, process and present microbial antigens. The literature regarding the precise definition of DC subsets and their respective functions has only recently begun to be resolved in the adult mouse and human immune systems. The literature regarding aged DCs is even more controversial, with just about every discovery other than migratory defects being contested (117, 290–292). One of the reasons for conflicting results lies in the fact that many studies utilized in vitro differentiated DCs, a process that potentially favors outgrowth and selection of the best proliferating cells, that in turn may no longer exhibit age-related defects (293). However, there is evidence of DC subset alterations with age, together with reduction in immunity and chronic inflammation (293–295). In vivo alterations of antigen uptake, pathogen sensing and/or antigen presentation of CD8α+ DCs contribute to impaired adaptive immune responses to microbial pathogens with age (296).
5.4.2 – Nonsurgical Control Dendritic Cell Distribution in Primary and Secondary Lymphoid Tissues of Adult and Old Mice

The only differences found in DCs (CD11c+Gr1$^{\text{mid/lo}}$F4/80$^{-}$) prior to parabiosis is a decrease in numbers in the spleen accompanying aging (Fig. 31). Otherwise, I found no numerical or proportional differences in DCs in any tissue interrogated.
Figure 31 – Numbers and Frequency of DCs in Nonsurgical Control Tissues  Data represents the (A) numbers and (B) frequency of A (CD45.1), A (CD45.2) or O (CD45.2) dendritic cells within the CD45^+ compartment from the indicated tissue. Adult CD45.1 and CD45.2 groups were n=4 while the old CD45.2 group was n=5. Significance was determined by a two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
5.4.3 – Isochronic Engraftment of Dendritic Cells in Primary and Secondary Lymphoid Tissues

Isochronic control data indicated that DCs do not form equal chimerism in the spleen and LNs, with the host component dominating its own tissues (Fig. 32). Moreover, CD45.1 DCs seemed to more efficiently home to the CD45.2 thymus than the CD45.1 thymus, even more prominently than CD45.2 DCs homed to the CD45.2 thymus. It is unclear at present what factors may govern DC homing to the thymic medulla, and therefore the resolution of mechanisms behind this phenomenon will have to be provided at a later date.

Figure 32 – Chimerism and Engraftment of DCs in Isochronic Parabiosis Tissues Data represents the frequency of CD45.1 or CD45.2 DCs within the CD45.1 or CD45.2 compartment, respectively, from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1 or CD45.2 DCs. (B) CD45.2 tissue level analysis of CD45.1 or CD45.2 DCs. (C) CD45.1 DC level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 DC level analysis in CD45.1 or CD45.2 tissues. All panels include data from 3-4 experiments, n=6. Significance
was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.4.4 – Heterochronic Engraftment of Dendritic Cells in Primary and Secondary Lymphoid Tissues

The bias of host DCs for its own spleen and LNs seen in isochronic pairs was not observed during heterochronic parabiosis (Fig. 33). There does appear to be a relative increase of DCs in old blood compared adult blood, however, additional experiments would be necessary to confirm this. Notably, the difference between adult DCs in old blood seems to be effected by one mouse (Fig. 33 D,E). There were no remarkable differences in migration and retention of adult and old DCs in adult and old tissues.

Figure 33 – Chimerism and Engraftment of DCs in Heterochronic Parabiosis Tissues  Data represents the frequency of A (CD45.1) or O (CD45.2) DCs within the A or O compartment, respectively, from the indicated tissue.  (A) Adult tissue level analysis of A or O DCs.  (B) Old tissue level analysis of A or O DCs.  (C) Adult DC level analysis in A or O tissues.  (D) Old DC
level analysis in A or O tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak's post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.4.5 – Conclusions

Prior to parabiosis, there was not a difference of between adult congeneric mice on DCs numbers or percentages across all lymphoid organs. However, isochronic parabiosis revealed there there was a bias for host DCs in the spleen and LNs. This would suggest that DCs are either short lived, do not travel long distances in the blood prior to seeding tissues, and/or are regenerated in situ. However, this bias for host DCs in these tissues was not observed during heterochronic parabiosis. Ultimately, it appears that the chimerism and engraftment of DCs is quite comparable across tissues with regards to age and surgery, especially in the heterochronic condition.

SECTION 5.5 – Macrophages

5.5.1 – Introduction to Macrophages and Aging

Like DCs, the heterogeneity amongst macrophages is plentiful and difficult to discern (219, 221). Attempts to determine age-related changes in macrophage function have yielded inconsistent results with respect to cytokine production, phagocytosis and migration (297). While some phagocytic activity is retained with age, the ability of macrophages to phagocytose apoptotic cells is reduced in mice and humans alike with age (116, 298). TLR signaling and cytokine production defects occur in macrophages with age across species (110).

5.5.2 – Nonsurgical Control Distribution of Macrophages in Primary and Secondary Lymphoid Tissues in Adult and Old Mice

There were no numerical differences in macrophages (CD11b++F4/80++) across any of the tissues surveyed (Fig. 34). The higher percentage of macrophages in the blood of aged
animals corresponds to a trend of higher numbers as well, although the difference was not statistically significant. Overall, there were no remarkable differences in macrophages across strains or age of mice when analyzed at this level.

![Figure 34 – Numbers and Frequency of Macrophages in Nonsurgical Control Tissues](image)

Data represents the (A) numbers and (B) frequency of A (CD45.1), A (CD45.2) or O (CD45.2) macrophages within the CD45+ compartment from the indicated tissue. Adult CD45.1 and CD45.2 groups were n=4 while the old CD45.2 group was n=5. Significance was determined by a two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
5.5.3 – Isochronic Engraftment of Macrophages in Primary and Secondary Lymphoid Tissues

Similar to other innate cells, the major finding of macrophages during parabiosis is an abundance of host cells in the bone marrow and blood (Fig. 35). Like DCs in section 5.4.3, CD45.1 macrophages seemed to be more abundant in the CD45.2 thymus than CD45.2 macrophages, and more abundant itself in the CD45.1 thymus, a finding whose implications are unclear at the moment. There seem to be no limitations in the chimerism formed in the spleen and LNs (Fig. 35).

**Figure 35 – Chimerism and Engraftment of Macrophages in Isochronic Parabiosis Tissues** Data represents the frequency of CD45.1 or CD45.2 macrophages within the CD45.1 or CD45.2 compartment, respectively, from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1 or CD45.2 macrophages. (B) CD45.2 tissue level analysis of CD45.1 or CD45.2 macrophages. (C) CD45.1 macrophage level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 macrophage level analysis in CD45.1 or CD45.2 tissues. All panels include data
from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.5.4 – Heterochronic Engraftment of Macrophages in Primary and Secondary Lymphoid Tissues

Similar to isochronic pairs, there were no remarkable finding with regards to macrophage chimerism or engraftment during heterochronic parabiosis (Fig. 36). The differences noted in the thymus, and to a lesser extent the blood, were not observed during heterochronic parabiosis. There seemed to be no restriction in tissue residence due to age of the macrophage or age of the tissue (Fig. 36).

![Figure 36 – Chimerism and Engraftment of Macrophages in Heterochronic Tissues](image)

Data represents the frequency of A (CD45.1) or O (CD45.2) macrophages within the A or O compartment, respectively, from the indicated tissue. (A) Adult tissue level analysis of A or O macrophages. (B) Old tissue level analysis of A or O macrophages. (C) Adult macrophage level analysis in A or O tissues. (D) Old macrophage level analysis in A or O tissues. All panels
include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak's post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

### 5.5.5 – Conclusions

We found no numerical defects in macrophages across strains or age, with proportional differences exhibited in the blood and bone marrow. While there were differences in the blood and bone marrow after parabiosis, these differences followed a pattern of favoring the host component, as is seen in other innate cells. The thymus (other than a CD45.1 preference for CD45.2 thymus), spleen and LNs show equal chimerism in both isochronic and heterochronic parabiosis.

### SECTION 5.6 – Neutrophils

#### 5.6.1 – Introduction to Neutrophils and Aging

The heterogeneity and lack of precise identification of various subsets of myeloid cells that many would identify as neutrophils complicate the study of neutrophils, even outside the context of aging (299, 45). This is in part due to the fact that neutrophils are produced up to $2 \times 10^{11}$ per day and were thought to live 1.5-8 hours but have now been shown to live up to 5.4 days. With aging neutrophils have been implicated in the pathogenesis of Alzheimer’s disease, atherosclerosis, cancer and autoimmune diseases (117). While GM-CSF promotes the chemotaxis of neutrophils in an adult animal, this is not the case in old neutrophils because of defects in the Jak/STAT pathway (300, 301). Furthermore, diminished neutrophil chemotaxis and infiltration was found to be involved with delayed wound healing in aged mice (302). Aging also reduces the phagocytic and bactericidal activity of neutrophils in numerous models (110, 303, 304). Defects in neutrophils with age contribute to increased pathogenesis, delayed wound healing and impaired tissue homeostasis.
5.6.2 – Nonsurgical Control Distribution of Neutrophils in Primary and Secondary Lymphoid Tissues in Adult and Old Mice

**Figure 37 – Numbers and Frequency of Neutrophils in Nonsurgical Control Tissues**

Data represents the numbers and frequency of A (CD45.1), A (CD45.2) or O (CD45.2) CD11b++Gr1++ neutrophils within the CD45+ compartment from the indicated tissue. Adult CD45.1 and CD45.2 groups were n=4 while the old CD45.2 group was n=5. Significance was determined by a two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.
There was a trend of an accumulation of CD11b\(^{++}\)Gr1\(^{++}\) neutrophils with aging in every tissue besides the thymus, however the data were only significant in the bone marrow (Fig. 37A). This trend extended to a proportional increase of neutrophils in all aged tissues, including the thymus, achieving significance in the blood and bone marrow (Fig. 37B). There was no apparent effect on the neutrophil population due to the genetic background of congenic strains (Fig. 37).

5.6.3 – Isochronic Engraftment of Neutrophils in Primary and Secondary Lymphoid Tissues

There were trends of a host preference of neutrophils in every tissue besides the thymus after isochronic parabiosis (Fig. 38). However, the only significant tissues, blood and bone marrow, followed the same trends of all other innate cells. Again, this may not be surprising because the bone marrow is the site of neutrophil formation and they disseminate into the periphery via blood.
Figure 38 – Chimerism and Engraftment of Neutrophils in Isochronic Parabiosis  Data represents the frequency of CD45.1 or CD45.2 neutrophils within the CD45.1 or CD45.2 compartment, respectively, from the indicated tissue. (A) CD45.1 tissue level analysis of CD45.1 or CD45.2 neutrophils. (B) CD45.2 tissue level analysis of CD45.1 or CD45.2 neutrophils. (C) CD45.1 neutrophil level analysis in CD45.1 or CD45.2 tissues. (D) CD45.2 neutrophil level analysis in CD45.1 or CD45.2 tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.6.4 – Heterochronic Engraftment of Neutrophils in Primary and Secondary Lymphoid Tissues

Neutrophils, similar to some innate cells, formed much better chimerism during heterochronic parabiosis than during isochronic parabiosis. The propensity of neutrophils to prefer the host component in the blood was not observed in the adult animal during heterochronic parabiosis (Fig. 39). This could be because of the increased number and proportion of neutrophils in the aged animal (Fig. 37B).
Figure 39 – Chimerism and Engraftment of Neutrophils in Heterochronic Parabiosis Tissues Data represents the frequency of A (CD45.1) or O (CD45.2) CD11b++Gr1++ neutrophils within the A or O compartment, respectively, from the indicated tissue. (A) Adult tissue level analysis of A or O neutrophils. (B) Old tissue level analysis of A or O neutrophils. (C) Adult neutrophil level analysis in A or O tissues. (D) Old neutrophil level analysis in A or O tissues. All panels include data from 3-4 experiments, n=6. Significance was determined by repeated measures two-way ANOVA with Sidak’s post-test. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001.

5.6.5 – Conclusions

The proportion and distribution of neutrophils did not change much, if at all, in the course of either iso- or heterochronic parabiosis. Like other cells, there was no observed defect due to the age of cell or age of tissue on migration and retention. Neutrophil chimerism was ~50:50 during heterochronic parabiosis, which should allow for investigation of neutrophil-intrinsic or extrinsic defects in aging.
CHAPTER 6 – DISCUSSION AND CONCLUSIONS

SECTION 6.1 – Considerations for Heterochronic Parabiosis

- Control for Mouse Genetics by Backcrossing/Interbreeding
- Congenic Models Come with Inherent Risks and Benefits

The robust chimerism of hematopoietic-lineage derived cells during parabiosis, following the surgical insult, creates an environment that is susceptible to GvHD. As a result, it is imperative to control for genetic drift that occurs between colonies that are never interbred. The impact of the genetic drift among different C57BL/6 strains maintained at The Jackson Laboratory (JAX) can be dramatic, and even more prominent when comparing different colonies maintained around the world (256–258, 305–307). The issue of drift is not just true for C57BL/6 mice, the most widely used mouse strain in research, but in all mouse strains (308, 309). In fact, to reduce the problem of genetic drift, JAX implements a patented genetic stability program (GSP) ensuring limited genetic drift by rederiving 12 different foundation strains (including C57BL/6J and C57BL/6NJ) every five generations from cryopreserved, pedigreed embryos (308). They also have a program that periodically backcrosses mutant colonies maintained on these backgrounds to reduce drift from the parental inbred strain. While this latter program is significantly less robust, it likely contributed to the survival benefits observed compared to CD45.1 mice obtained from the NCI, as the JAX stock of CD45.1 mice were backcrossed to C57BL/6J at least three times by JAX. Nonetheless, if one cannot ascertain the precise background of the strains they wish to use prior to parabiosis, we recommend a breeding strategy is described in Figure 3 of section 2.3.1.

No congenic model to date is perfect for all experimental questions, however, the use of congenics is an indispensable tools necessary to provide complimentary evidence to answer
difficult questions in biology (310). A recent study demonstrated that nearly all of the congenic mutant mice made utilizing a genetically modified mouse protocol that make use of 129 and B6 chimeric embryos contain passenger mutations that potentially influence phenotypic outcomes (311). While linked mutations cannot be fixed by breeding protocols, these issues have lead researchers to recommend that interbreeding mice and using the desired genotype obtained after crossing heterozygous mice is ideal for reducing complications, as described in Figure 3 of section 2.3.1 (308, 310). Here, we chose to use CD45 congenic mice because we reasoned that there would be less antigenic mutations compared to an entirely foreign GFP congenic mouse. Other congenic models are either not as simple to combine with flow cytometry and microscopy techniques or they are insufficient at marking all hematopoietic-lineage cells (246). The utilization of properly controlled congenic parabiosis models is an immensely powerful tool for biologists.

SECTION 6.2 – Factors That Govern T Cell Maintenance and Migration Need to be Reexamined with Age

- Pre-parabiotic Adult T_N-Rich Environment Favors T Cell LN Cellularity, While Old T_M-Rich Environment Favors T Cell Bone Marrow Cellularity
- Old CD4 and CD8 T_N Cells Exhibit a Peripheral Maintenance Advantage Over Adult T_N Cells

Overall, the pre-parabiotic differences between adult and old T cell compartments was retained and reached homeostasis with the opposing parabiont’s tissues. There was no evidence that the age of the cell or age of the tissue impacted T cell migration or retention in these tissues. The T_N-rich adult T cell compartment favors LN seeding, while the T_M-rich old
T cell compartment favors increased numbers of T cells in the bone. Interestingly, the number of T<sub>M</sub> cells in the LN and T<sub>N</sub> cells in the bone marrow seem to remain static across age (Fig. 18, 20). This implies that these T cell subsets occupy static niches in these tissues that are unaffected by age, meriting future studies.

Due to remarkable equilibration of T cells, the data here indicate that there is a designated amount of space in the system for each T cell subset, or alternatively, T cell niches are dynamically tuned to the numbers and proportion of T cell subsets present in the system. The one exception to this was the age-independent behavior of some CD4 T<sub>M</sub> and CD8 T<sub>EM</sub> cells, which were retained in their own bone marrow throughout the duration of the experiment. The failure of adult T<sub>N</sub> cells to fully rescue LN cellularity could be explained by the inability of the combined naïve T cell output of the adult and old thymus from filling the space of two organisms. However, the adult thymus can export ~10<sup>6</sup> naïve T cells per day, a number thought to be in excess (266). Alternatively, there may be a pro-geronic factor in old blood that has the ability to shrink the amount of space available to sustain T<sub>N</sub> cells in LNs.

The most compelling data came from separation experiments which allowed the measurement of the decay kinetics of adult and old T cell subsets in the old or adult environment, respectively. These data demonstrated that the old environment is better at maintaining CD4 T<sub>M</sub> cells than the adult environment. Furthermore, they demonstrate that old CD4 and CD8 T<sub>N</sub> cells maintain an intrinsic peripheral survival advantage over adult T<sub>N</sub> cells. Additionally, while adult CD4 T<sub>N</sub> cells decay twice as fast as adult CD8 T<sub>N</sub> cells, this difference was not observed among old CD4 and CD8 T<sub>N</sub> cells. While this experiment was not properly statistically powered, data suggested that the old environment resulted in accelerated adult CD4 T<sub>N</sub> decay when compared to isochronic adult controls. These data provide evidence
that the decrease in the CD4:CD8 ratio in the aged T cell compartment may be imposed by selection in the old environment. Therefore, while the old environment may contain an extrinsic factor(s) that accelerate adult CD4 T<sub>N</sub> decay, CD8 T<sub>N</sub> decay does not seem to be subject to similar extrinsic factors. Therefore, the peripheral survival advantage of old CD8 T<sub>N</sub> cells is likely an intrinsic benefit conferred over time. However, it is known that the biology of recent thymic emigrants (RTEs) is different than that of mature peripheral T<sub>N</sub> cells, and as the adult T cell compartment contains a larger proportion of RTEs, this too could be contributing to these findings (81). We favor the explanation that peripheral selection resulted in old T<sub>N</sub> cells with a competitive advantage over adult T<sub>N</sub> cells. Evidence for peripheral selection of old T cells, including T<sub>N</sub> cells, resulting in higher CD5 levels and increased cross-reactivity has been demonstrated (269–272). This avenue represents a valuable hypothesis-driven experimental opportunity for discovering the definitive factor(s) that govern the peripheral survival advantage in aged T cells. Additionally, we suggest that age-related LN defects may play a role in the peripheral selection of T cells exhibiting a peripheral survival advantage in old T cells.

The data here provide promising evidence that the old environment is permissive to engraftment of new T cells, thereby supporting the rationale of thymic rejuvenation approaches to restoration of T cell function with age. Future investigations into the stromal populations that support T cells in tissues, and how they change with age, are warranted. While there appeared to be no difference in adult or old LNs in adult or old T cell composition in this homeostatic model, this may not be true during inflammatory conditions and this model is ideal for addressing such questions. Furthermore, the alterations of T cell subset maintenance
dynamics with age could have profound implications during immune defense and for the design of vaccines and the utilization of these cells in immunotherapy.

SECTION 6.3 – Robust Dataset of a Broad Analysis of Vital Cells of the Immune System Across Strain, Age, Isochronic Adult and Heterochronic Parabiosis

- Large Dataset of the Numbers and Proportion of Many Different Cell Types Across Primary and Secondary Lymphoid Tissues
- Well Defined Model of Heterochronic Parabiosis for Future Studies

Our data demonstrate that there were no gross differences in B cells, NK cells, DCs, macrophages or neutrophils in relative proportion during parabiosis that can be attributed to age. The caveat to this dataset is the lack of cell-type specific subset analysis, many of which are known to change with age. Another confounder that hampered us from drawing conclusions was the impact of the congenic allele, which would need to be controlled for by age as well. Nonetheless, this dataset provides a gross snapshot of the dynamics during heterochronic parabiosis of several broad lineages of cells in the immune system. However, this is the first dataset of its kind that integrates simultaneous measurements of over 10 different cell types across primary and secondary lymphoid tissues and across age as well as in the competitive context of parabiosis. These data not only provide a significant amount of information for researchers to be able to consider prior to conducting experiments, but also represent a dataset that can be mined to search for differential outcomes on tissue cellularity and composition resulting from the interactions of different cells of the immune system. It also provides a foundation and framework for future studies. For example, when old isochronic parabiosis data are obtained and the dataset is complete, robust statistical analyses can be
conducted. Such experiments could create much more complex models and utilize the wild-type and homeostatic datasets for statistical modeling. The dataset can be obtained using the following Google Fusion table:

https://drive.google.com/open?id=1L_tppjZ2UJ_CrEkftvfsXKTRRyrOrFtlqhppkJGc

This is provided in order to facilitate the ease of use of the data presented herein.

SECTION 6.4 – Summary and Future Directions

This model of heterochronic parabiosis is powerful despite the failure to rejuvenate the aged thymus and lymphoid/myeloid ratio. There appeared to be no defect due to the age of the cell or the age of the tissue with regards to migration and retention. Furthermore, when the additional groups of Isochronic Old, Nonsurgical Control CD45.1 Old, and possibly the reciprocal heterochronic parabionts would complete a robust statistical model.

The findings from these studies form the basis of new projects designed to explore T cell-stromal cell dynamics in aging. Many interesting findings with regards to T cell production and peripheral migration and maintenance merit further investigation. Additionally, the robust characterization of the homeostatic migration and maintenance of over 10 different cell types during parabiosis provides a thorough platform for targeted investigations of specific questions regarding defects in the immune system.
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