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Cell renewal: terms and conditions may apply

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Cell renewal: terms and conditions may apply

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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“It is an abstract change without object. Am I the one who has changed?” – Jean Paul Sartre (Nausea)

ABSTRACT

The longevity and turnover of the different constituent cells of an organism define its development, size, health, and biological age. The present thesis discusses the renewal rates and functions of cells in several organs and tissues: peripheral blood, bone marrow, intestine and central nervous system.

By applying a recently developed method to assess average cell age to peripheral T cell subsets, we provide evidence that in humans the thymus remains active beyond the age of 30 and that naïve T cell renewal rates slowly decrease throughout life. We further examined how turnover rates can impact the total naïve T cell population, by applying a variety of additional mathematical and computational models as well as functional assays. Using these approaches we propose a model by which clonal diversity can be sustained regardless of the spatial location of the constituent cells. Furthermore, we identify a subset of individuals above the age of 65 who exhibit a dramatic increase in turnover despite having no overt pathological conditions. Given recent evidence that aging is associated with immunological dysfunction, this observation highlights a subset of the population that may be more susceptible to new infections and less receptive to vaccinations. **(PAPER I)**.

We determined that plasma cells have different renewal rates depending on the subpopulation and, more importantly, depending on the niche. Both the bone marrow and the intestine harbor plasma cell responders to antigens from childhood exposures. However, bone marrow plasma cells **(PAPER II)** renew much faster than their intestinal counterparts **(PAPER III)**. In the small intestine, some plasma cells can persist for decades without being replaced or undergoing further divisions **(PAPER III)**. The differences described suggest different mechanisms of immune memory maintenance possibly due to different pressures in the niches. These results also demonstrate that plasma cells in the bone marrow and in the intestine are likely to be differentially impacted by treatments that target fast renewing cells **(PAPER II and III)**.

In mammals, the central nervous system (CNS) governs many of the body's vital functions. Disturbance to cell homeostasis such as microglia depletion or spinal cord injury have tremendous consequences. Our data reveals that the vast majority of the microglia population in the human cortex undergoes constant renewal albeit at a slow rate. Microglia are key regulators of the CNS; the

progressive turnover of this cell population ensures the constant presence of a pool of young microglia **(PAPER IV)**. Another example of a self-sustaining cell population in the CNS are ependymal cells. We showed that these resident stem cells are capable of fast proliferation and differentiation in response to spinal cord injury. The progeny of ependymal cells play a crucial rule in scar formation helping preventing secondary enlargement of the wound and consequently preventing further deterioration. Interestingly, ependymal cells are incapable of responding to injury and originate progeny if cell division is blocked, demonstrating how the processes of cell-renewal and differentiation can be interconnected **(PAPER V)**.

LIST OF SCIENTIFIC PAPERS

I. **Pedro Réu***, Jeff E. Mold*, Axel Olin, Samuel Bernard, Jakob Michaëlsson, Azadeh Khosravi, Mehran Salehpour, Göran Possnert, Petter Brodin and Jonas Frisén
Dynamics of naïve T cell homeostasis in adult humans
Manuscript

II. Jeff E. Mold*, **Pedro Réu***, Carl Jorns, Paola Martinez Murillo, Ann-Christin Croon, Maria Söderström, Øystein Jynge, Mehran Salehpour, Göran Possnert, Gunilla Karlsson Hedestam and Jonas Frisén
Continuous renewal of plasma cells in the human bone marrow
Manuscript

III. Ole J. B. Landsverk, Omri Snir, Raquel Bartolomé Casado*, Lisa Richter*, Jeff E. Mold, **Pedro Réu**, Rune Horneland, Vemund Paulsen, Sheraz Yaqub, Einar Martin Aandahl, Ole M. Øyen, Hildur Sif Thorarensen, Mehran Salehpour, Göran Possnert, Jonas Frisén, Ludvig M. Sollid**, Espen S. Baekkevold** and Frode L. Jahnsen**
Antibody secreting plasma cells persist for decades in the human intestine
J Exp Med. 2017 Feb;214(2):309-317.

IV. **Pedro Réu**, Azadeh Khosravi, Samuel Bernard, Jeff E. Mold, Mehran Salehpour, Kanar Alkass, Shira Perl, John Tisdale, Göran Possnert, Henrik Druid and Jonas Frisén
The lifespan and turnover of microglia in the human brain
Manuscript

V. Hanna Sabelström, Moa Stenudd, **Pedro Réu**, David O. Dias, Marta Elfineh, Sofia Zdunek, Peter Damberg, Christian Göritz, Jonas Frisén
Resident neural stem cells restrict tissue damage and neuronal loss after spinal cord injury in mice
Science. 2013 Nov 1;342(6158):637-40

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** These authors contributed equally to this work.

CONTENTS

LIST OF ABBREVIATIONS

CNS	Central nervous system
Fas-L	Fas ligand
TNF	Tumor necrosis factor
IL-2	Interleukin 2
Treg	Regulatory T cell
APC	Antigen presenting cell
TREC	T cell receptor excision circle
TCR	T cell receptor
PE-CAM-1	Platelet endothelial cell adhesion molecule 1
IL-7	Interleukin 7
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells

1 AIMS

The overall aim of the present thesis is to discuss cell proliferation and renewal, to explore different examples of this phenomenon, and to make a case for its relevance. It is not straightforward to determine when or why a cell divides. The rate of turnover can differ significantly between cell types depending on a variety of variables such as their origin, function, and location. Nevertheless, the instructions for when and why a cell initiates the process is many times scripted in detail in the “fine print”. The research in this field aims to investigate the inheritable and environmental instructions that govern cell renewal.

2 CELL RENEWAL

2.1 CELL RENEWAL

Cell renewal is the process by which cells in a given system are replaced. Typically in a mammalian tissue, the two sides of this coin are mitotic cell division and cell death. In homeostasis, the maintenance of a tissue's function and morphology is achieved by the equilibrium between the two. Cell renewal rates vary substantially between tissues and frequently reflect the tissue's function. For instance, due to persistent aggression by the digestion process, the intestinal epithelium is constantly renewed. In such a system, cells are replaced by morphologically and functionally similar ones on regular intervals (Barker 2014). The deregulation of the balance between cell death and mitotic cell division can lead to either tissue degeneration or neoplasia. Indeed, tissues with higher cell turnover have a higher probability of developing tumors (Tomasetti and Vogelstein 2015).

Mitotic cell division is the process by which one cell gives origin to two cells with identical DNA content. As a result, every pair of daughter cells comes from a previously existing one. Cell death is the event that terminates the cell's physiological functions. Two communally described forms of cell death are apoptosis and necrosis (Nikoletopoulou, Markaki et al. 2013). The former is programmed and well coordinated, with little impact to the surrounding tissue (Nikoletopoulou, Markaki et al. 2013). The latter is chaotic, often caused by external factors and usually followed by inflammation (Los, Mozoluk et al. 2002). Apoptosis is characterized by the activation of caspases, permeabilization of the mitochondrial membrane, DNA fragmentation and chromatin condensation (Green 2005). In a necrotic cell it is common to see swollen organelles and disruption of the plasma membrane (Schweichel and Merker 1973, Leist and Jaattela 2001).

Apoptosis is involved in the selection of T cell precursors in the thymus (Stritesky, Jameson et al. 2012). In a process know as "death by neglect", T cell precursors with low affinity T cell receptors (TCRs) do not receive the necessary signals for positive selection and thus enter apoptosis (Stritesky, Jameson et al. 2012). In addition, if these cells show high affinity for self-antigens, they also undergo apoptosis in a process designated "clonal deletion" (Stritesky, Jameson et al. 2012).

2.2 WHEN TO STOP?

One long lasting question in biology relates to the rules that govern the stop of growth during development and the maintenance of cell number in an adult tissue. A possible explanation relates to the existence of a limited quantity of growth factors and survival factors in a system and the inevitable competition for those resources (Raff 1996). The loss of cells leads to a wider availability of these factors per cell, hence promoting cell division. The increase of cell number decreases the availability of these factors per cell, hence suppressing cell division. Another possibility is the existence of a negative feedback loop created within the system. In other words, the cells of a given tissue produce molecules that inhibit their own cell proliferation. When the system reaches the right cell number it is producing enough inhibitors to stop its own grown (Raff 1996, McPherron, Lawler et al. 1997, Huang, Ma et al. 2006).

2.3 RELEVANCE

Determining the rates of cell renewal in different tissues and systems is crucial for a better understating of the homeostatic processes of tissue maintenance, the response to insults, and abnormal proliferation. Furthermore, investigating the mechanisms responsible for the induction and repression of cell expansion and renewal can help in the design of therapies for disorders associated with degeneration such as Alzheimer's disease, for diseases associated with uncontrolled proliferation such as neoplasias, and for lesions such as spinal cord injuries.

3 MEASURING CELL TURNOVER IN HUMANS

The wide availability of labeling strategies for experimental animals and for *in vitro* experiments has made cell proliferation measurements common. However, assessing cell turnover in humans is still far from trivial and the techniques available are limited.

3.1 KI-67

Ki-67 is a protein absent in G_0 but expressed throughout the different phases of the cell cycle: G_1, S phase, G_2 and mitosis (Scholzen and Gerdes 2000). Ki-67 is commonly used as a marker of cell division in immunocytochemistry, immunohistochemistry, as well as in flow cytometry. However, this method is not a direct measurement of turnover and its use requires certain assumptions to be made concerning cell cycle length. Furthermore, it is prone to overestimate cell proliferation as cells with G_1/S blockage will continue to express Ki-67 although they are stopped in G1 or are destined to enter apoptosis (Combadere, Blanc et al. 2000). It is worth mentioning that, naturally other cell cycle markers can be used to assess DNA replication (Williams and Stoeber 2007).

3.2 THYMIDINE ANALOGS

Thymidine analogs are incorporate into the genomic DNA during S phase. Data from Ki-67 and thymidine analogs are not directly comparable and the length of the cell cycle should be take into account, since the S phase represents approximately half of the cell cycle (Cameron and Greulich 1963). To a certain extent, data heterogeneity from thymidine analogs experiments can be explained by the unpredictable incorporation of these extracellular compounds through the salvage pathway (Busch, Neese et al. 2007). Finally, it is important to point out that if labeled cells continue to proliferate after the labeling period, the newly generated cells will also be labeled, leading to an over estimation of cell division (Busch, Neese et al. 2007).

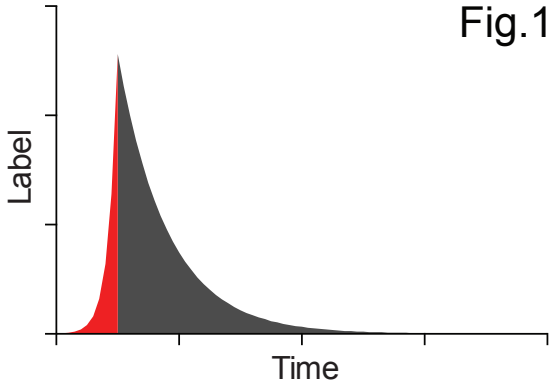


Fig.1

3.3 STABLE ISOTOPE LABELING

Stable isotope labeling experiments similarly to any pulse-chase experiment,

require a period of labeling (red) and a period of de-labeling (grey) (Fig. 1). In broad terms, the labeling period provides information on cell proliferation and the de-labeling period provides information on cell death (Asquith, Debacq et al. 2002). An estimation of cell turnover can be achieved from this type of DNA-labeling assays, once a mathematical model that recapitulates the data is constructed (Asquith, Debacq et al. 2002). Typically, many models with different proliferation and death rates would be tested on the data in order to find the one that best fits (Asquith, Debacq et al. 2002). As a simple example, one begins with an educated guess of the model (Fig. 2A) and then increases or decreases the proliferation and death rates until the model fits the data (Fig. 2B) (Asquith, Debacq et al. 2002).

Deuterated water is a reliable DNA-labeling strategy and it labels newly synthetized nucleotides with the hydrogen isotope deuterium (^{2}H). In the organism, ^{2}H is incorporated into nucleotides through *de novo* synthesis making its incorporation into DNA more consistent and predictable then the previously mentioned thymidine analogs (Busch, Neese et al. 2007). In addition, deuterium has not been reported as significantly toxic despite decades of studies in humans and experimental animal models (Busch, Neese et al. 2007).

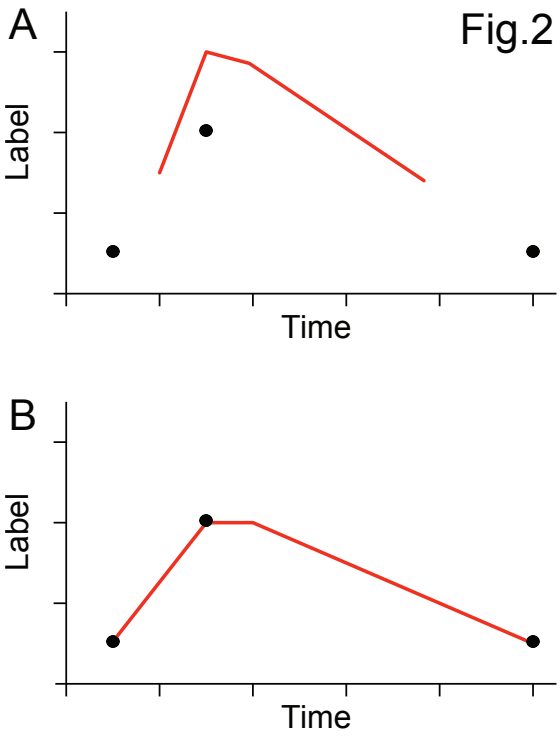


Fig.2

Nonetheless, heavy water studies have a limited window of time to detect cell exchange, just like any other short term labeling assay. Varying the length of the labeling period can dramatically change the number of labeled cells (Fig. 3). In populations with slow exchange rates, if the labeling period is too short (period 1), nearly no cells will be detected, thereby making the interpretation of the results difficult (Fig. 3). This is particularly problematic in

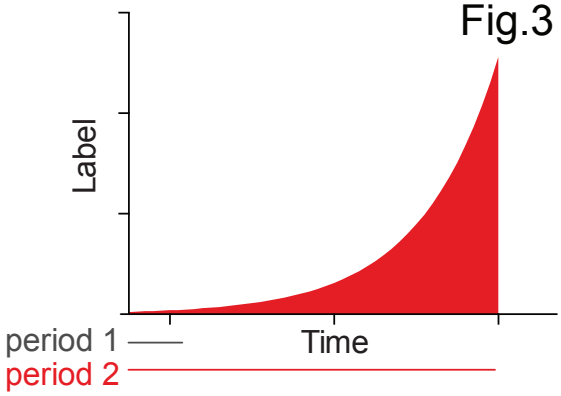


Fig.3

heterogeneous populations where the fast dividing subpopulations will mask the contribution of the slower ones (Asquith, Debacq et al. 2002). In addition, if newly produced cells (detected within the labeling period) do not leave the pool during the chase period it becomes challenging to determine the dynamics of the population (Fig. 4). In these situations one should extend the chase period when possible.

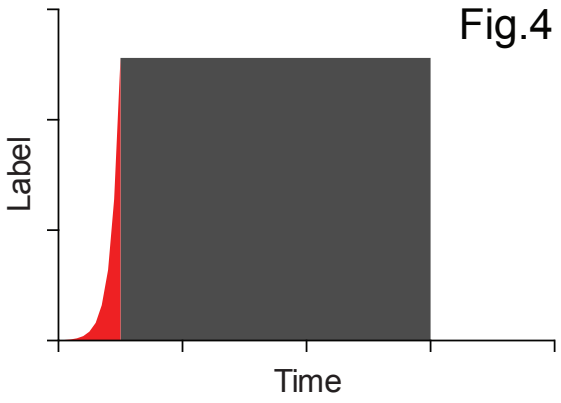


Fig.4

3.4 CARBON-14

Carbon-14 (^{14}C) is a rare but naturally occurring isotope of carbon. The atmospheric level of ^{14}C has been low and constant for thousands of years (Levin, Naegler et al. 2010). During the cold war, due to nuclear bomb testing the level of environmental ^{14}C dramatically increased (Levin, Naegler et al. 2010). The Partial Test Ban Treaty was signed in 1963, and ever since most nuclear tests have not been performed above ground. As a result, the ^{14}C level peaked in the atmosphere in 1964 and has been progressively decreasing (Fig.5) (Levin, Naegler et al. 2010). This decline is not due to radioactive decay (half-life 5730 years) but mainly due to diffusion in the oceans and incorporation into the biotope (Levin and Kromer 2004). Through photosynthesis, $^{14}CO_2$ enters the food chain, hence the ^{14}C content in biomolecules reflects the atmospheric ^{14}C level at the moment of synthesis. Carbon in genomic DNA is almost exclusively exchanged by cell division and the contribution of DNA methylation and repair is negligible (Spalding, Bhardwaj et al. 2005, Bergmann, Liebl et al. 2012, Ernst, Alkass et al. 2014) even in the case of stroke when DNA repair is increased (Huttner, Bergmann et al. 2014). By measuring the ^{14}C content in the DNA of a cell population (Fig. 5, y-axis) and determining its intersection with the atmospheric ^{14}C level, it is possible to assess the average time of birth (Fig.5, x-axis) of that population (Spalding, Bhardwaj et al. 2005). In contrast to short-term labeling

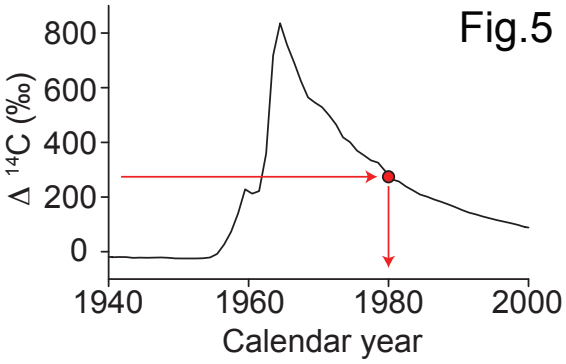


Fig.5

experiments, ^{14}C assessment of turnover reflects the cumulative history of cell division of the population of interest (Spalding, Bhardwaj et al. 2005). Carbon dating has proven to be a valuable tool for detecting the presence of sub-populations of non-dividing cells within a potentially heterogeneous population (PAPER I) since these cells conserve the ^{14}C level from the date of birth of the individual. Cell populations can also contain sub-populations of fast dividing cells. However, assessing the presence of these cells is more challenging since their ^{14}C profile is not unique, and can be the result of many different possible combinations of ^{14}C “dilution” throughout the years. Furthermore, due to the slow pace of change of the environmental levels of ^{14}C, it is hard to precisely measure the turnover rate of cell populations that renew fast.

Presently, to obtain the most complete picture of cell dynamics in human cell populations one should probably use a combination of carbon dating and heavy water labeling. This would provide data on the population as a whole, on the presence of non-dividing sub-populations, as well as detailed information on subpopulations of fast proliferating cells.

4 REGULATION AND FUNCTION OF CELL PROLIFERATION

4.1 MAINTENANCE OF CLONAL DIVERSITY

A group of cells that share a common ancestor in their recent division history can be broadly referred to as a clone. Clonality and the maintenance of diversity is likely to play an important role in various tissues. However, it is notably hard to retrospectively reconstruct phylogenetic trees in humans (Salipante and Horwitz 2006). Both T cell and B cell clones, though, share unique genetically encoded antigen receptors, making these cells ideal candidates for studying how clonality is affected by population level changes in cellular turnover. Akin to the species level, diversity at the cellular level might be evolutionary advantageous, since there are individuals different enough within a population to withstand a variety of environmental changes. In the case of T cells and B cells this is particularly true, as their unique antigen receptors impart specificity for foreign organisms and proteins and thus clonal diversity is inextricably linked to the immunological potential of the populations as a whole.

4.1.1 The thymus

The ancient Greeks referred to the thymus as “the seat of the soul” (Nishino, Ashiku et al. 2006). Probably, the first observation of thymic involution was made by Galen of Pergamum (130-200 AD) who referred to it as an “organ of mystery” when describing its shrinkage throughout life (Peltier 2003). The thymus is a primary lymphoid organ responsible for the maturation of bone marrow-derived T cell precursors into circulating naïve T cells (Miller 2011). For many years the thymus was considered a vestigial organ, since it did not react to infections in the same fashion as other lymphoid organs and since thymectomized adult mice were not immunodeficient (Miller 2011). This sentiment was summed up well by the Nobel Laureate Sir Peter Medawar who in 1963 wrote: “We shall come to regard the presence of lymphocytes in the thymus as an evolutionary accident of no very great significance” (Medawar 1963). Nonetheless, later studies on mice thymectomized at birth, revealed that these animals did in fact have lower lymphocyte counts and were more prone to certain viral infections and tumors (Miller 1961, Miller 1962, Burnet 1971).

4.1.2 Thymus size: a cautionary tale

The first written references to what is in known as ‘sudden infant death syndrome’ date back to 480-406 BC (Russell-Jones 1985). In the late 1800s, several victims

of sudden infant death were autopsied and diagnosed with *Status* thymicolymphaticus, a condition characterized by an enlarged thymus that pressures the trachea and suffocates the child (Russell-Jones 1985). Between 1926 and 1957 thousands of infants were subjected to ionizing radiation as a treatment for this condition (Adams, Shore et al. 2010). Before the 1930s most bodies in anatomy classes came from the poor and it was unknown at the time that a life of poverty, chronic stress, malnutrition, and infection can all cause thymic involution, thus anatomists were incorrect in their measurements of the size of a healthy human thymus (Sapolsky 1997). In 1993 it was shown that individuals that went through the previously mentioned ionizing treatment were 24.3 times more likely to develop thyroid cancer than the average New Yorker of the same age and sex (Shore, Hildreth et al. 1993).

4.1.3 Of mice and men

Microorganisms keep the immune system under an enormous evolutionary pressure due to their short life spans and high mutation rates (McDade and Worthman 1999). After 65 million years of divergent evolution, humans and mice are quite distinct: mice have much shorter lifespans than humans, have much smaller body sizes and occupy different ecological niches (Mestas and Hughes 2004). There is a large number of variables that might explain differences in cell turnover rate across species, therefore it is important to assess it in humans whenever possible. This is particularly important for immune cells, since the majority of the laboratory animals, live in pathogen free barrier facilities (Beura, Hamilton et al. 2016).

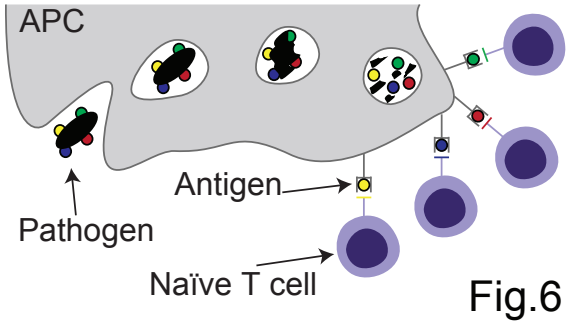


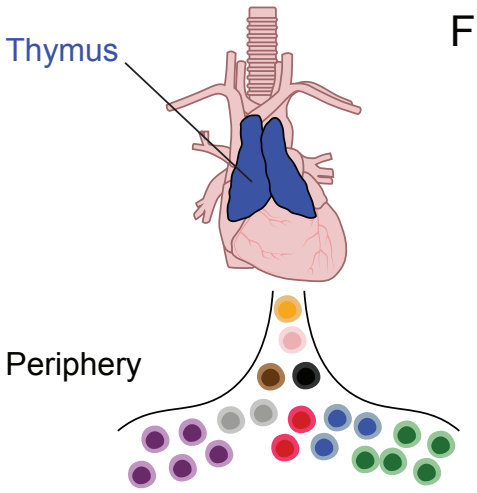
Fig.6

Maintaining a diverse T cell repertoire for many years is not a trivial task (PAPER I), in mice it has been shown that the naïve T cell population is maintained throughout life primarily by thymic production (den Braber, Mugwagwa et al. 2012), thus new and diverse T cells are constantly joining the pool. In adult humans however, our data supports the view that the major source of new cells is the peripheral division of existing naïve T cells (PAPER I). This raises the question of how the repertoire is being maintained.

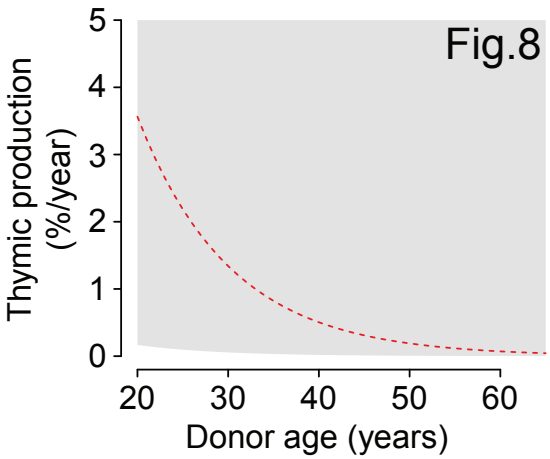
4.1.4 T cell repertoire

A diverse naïve T cell repertoire is crucial for proper primary immune responses since different T cells recognize different antigens from a single pathogen (Fig. 6). The proliferative capacity of lymphocytes has been known for decades, in the wise words of Lord Florey (1954): “Nothing of importance is known regarding the potentialities of lymphocytes other than that they move and that they reproduce themselves” (Florey 1954). New naïve T cells are added to the peripheral pool either by thymic production or by division of existing ones (Fig. 7). Thymic production increases diversity, since each new cell expresses a unique T cell receptor (Fig. 7). Peripheral division decreases the relative diversity of the pool by diluting the frequency of smaller clones with each division of a larger one (Fig. 7). Previous studies have reported naïve T cell turnover estimates, varying from months to years (Michie, McLean et al. 1992, Neese, Misell et al. 2002, Macallan, Wallace et al. 2004, Vrisekoop, den Braber et al. 2008). Understanding the turnover dynamics of these cells throughout life allows the prediction of how diverse the repertoire is (PAPER I). In other words, by determining the relative production of each source of new cells and by evaluating production changes throughout life, it is possible to estimate the average size and the maximum size of clones in individuals of different ages (PAPER I). In order to dissect thymic production from peripheral production, two main data sets are necessary: *(1)* T-cell receptor excision circles (TREC) measurements (Douek, Betts et al. 2001) and *(2)* data on cell age (PAPER I). *(1)* In order to form unique TCRs, T cells in thymus must undergo TCR rearrangement, a process where the portions of genomic DNA that code for the receptor (V, D and J gene segments) are recombined generating a functional receptor (Hazenberg, Verschuren et al. 2001). The excised regions of genomic DNA generated during this process, termed TRECs, form circular bits of DNA that persist in cells for extended periods of time and that get diluted out in the pool as the cells divide (Hazenberg, Borghans et al. 2003). This makes TRECs good makers of cell division history at the population level (Hazenberg, Verschuren et al. 2001). *(2)* As previously

Fig.7



mentioned, ^{14}C dating allows for the determination of cell age and offers a comprehensive view of the turnover history of a cell population, making it an ideal tool for these types of studies.



Simply based on TREC content and cell number it is not possible to properly estimate thymic production (Fig.8, grey area); indeed, information on cell age is needed to obtain a more reliable measurement (Fig.8, red dashed line). In more detail, Fig. 8 shows in grey, predictions of thymic output based on cell age assumptions varying from 1 to 13 years and in red predictions of thymic output using known cell age (our model).

We predicted that the average clone size is maintained remarkably stable throughout life (PAPER I), which helps explain the capacity of humans to efficiently mount primary immune responses throughout adulthood despite a large decrease in thymic production. This observation suggests the existence of mechanisms that favor the proliferation of less expanded clones. Otherwise, should any cell have an equal chance of dividing, regardless its clonal origin a paradigm similar to "patrimonial capitalism" (Piketty and Goldhammer 2014) would arise. This economic concept describes inheritance as a large contributor for the maintenance and expansion of family wealth (Piketty and Goldhammer 2014). Similarly, we believe that in the absence of a regulatory mechanism, early settlers would have more time to expand their clones, and if all cells were equally competent to respond to homeostatic division cues, large clones would outcompete small clones for resources and become very large later in life (PAPER I). The mechanism proposed in PAPER I suggests that the probability of a naïve T cell to divide decreases with the expansion of its clone, offering one potential mechanism to prevent this from occurring.

4.1.5 Restrictions to Clonal Expansion

CD31 (PE-CAM1) is considered a marker of recent thymic emigrants. Evidence for this comes from several observations: (1) $CD4^+$ T cells in the thymus are almost uniformly $CD31^+$, (2) levels of CD31 expression decrease within the naïve $CD4^+$ T cell compartment throughout life, (3) and $CD31^-$ cells have lower TREC content

than their CD31$^+$ counterparts (Kimmig, Przybylski et al. 2002, Kohler and Thiel 2009).

CD31$^-$ naïve CD4$^+$ cells are less capable of expanding in response to IL-7 than CD31$^+$ cells (PAPER I) (Azevedo, Soares et al. 2009). Importantly, this homeostatic expansion is happening without activation and consequently without differentiation into effector and memory cells (PAPER I). Nonetheless, in the presence of tumor necrosis factor alpha (TNFα) both CD31$^+$ and CD31$^-$ promptly proliferate, indicating that CD31$^-$ are not exhausted but simply quiescent cells (PAPER I). Additionally, both cell types are capable of differentiating into effector and memory phenotypes in response to activation signals (anti-CD3/anti-CD28), reinforcing the idea that both cell types are competent. Also, we show that the response to proliferative signals (IL-7, anti-CD3/IL-7 or TNFα/IL-7) in both the CD31$^-$ and CD31$^+$ compartment is dependent on the NF-κB pathway. Interestingly we also observed that basal NF-κB phosphorylation levels were positively correlated with the expression of CD31 in CD4$^+$ naïve T cells providing a mechanism for the increased proliferative capacity of CD4$^+$CD31$^+$ cells in response to homeostatic cues (Figure 9) (PAPER I). Finally, the expression of CD31 is not binary but more of a continuum where CD4$^+$ naïve T cells have a spectrum of expression levels (PAPER I). In summary, these results are consistent with a mechanism where naïve T cell expansion in the periphery leads to a progressive loss of CD31 expression coincident with a reduction in basal NF-κB activation. Together, these changes lead to a reduced capacity to proliferate in response to homeostatic survival signals such as IL-7.

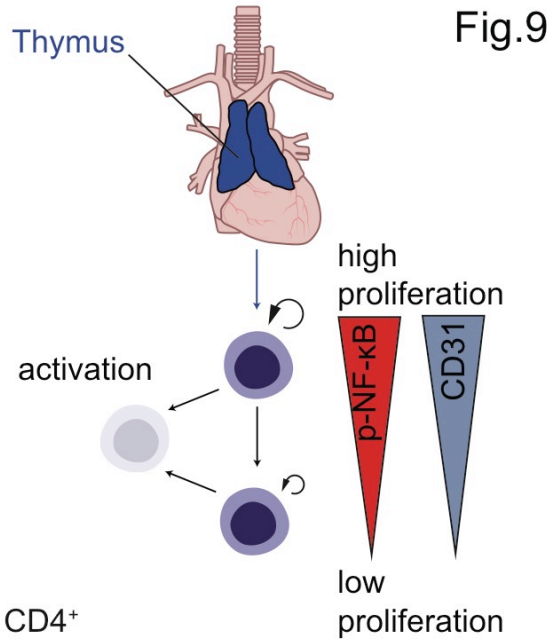


Fig.9

It can be inferred that the size of individual clones may be limited by an intrinsic regulatory mechanism that favors the division of non-expanded clones over highly expanded clones, which would be expected to have reduced CD31 expression (Figure 9) (PAPER I). While it remains unknown the precise factors that lead to the reduction in CD31 expression, this model is consistent with data on naïve T cell

turnover rates (PAPER I), TREC content (Douek, Betts et al. 2001), as well as current estimates of total naïve T cell repertoire diversity (Qi, Liu et al. 2014).

To a certain extent, the coordinated behavior of naïve T cell clones resembles that of an ant colony. In the context of emergence of complexity, Jad Abumrad, (funder and host of Radiolab) wondered: “Where does organization come from? […] How do you get the complexity of an ant colony if there is no leader and everyone in town is stupid?”. Similarly to ants, cells are capable of completing complex tasks without top down orders, but instead using a vast number of individuals that follow a simple set of rules. It is easy to imagine that there is an incentive at the cellular level to utilize as many resources as possible and to keep dividing. However, from an evolutionary point of view, the fittest organism is probably one with mechanisms for containing uncontrollable cell proliferation. In the case of naïve T cells, permanently turning off cell division is not an option. Hence, a self-regulatory mechanism that progressively tunes proliferation down at the clonal level regardless of anatomic or temporal factors is a simple and yet highly effective solution (PAPER I).

4.2 CELL LONGEVITY: ILLUSION OF CERTAINTY

Circulating naïve B cells are the progeny of B cell precursors in the bone marrow (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015). Naïve B cells can mature into a variety of B cell subsets found in the spleen, lymph nodes, or in the peritoneal and pleural cavities (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015). Once activated by their cognate antigen, B cells proliferate quickly and can differentiate into plasmoblasts and short-lived plasma cells, both capable of producing antibodies (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015). Some B cells enter germinal center reactions and become memory B cells or long-lived plasma cells. In the germinal center, B cells proliferate and undergo somatic hypermutation in the antibody encoding genes (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015). Additionally, they are selected based on antibody affinity and can undergo class switch to alter the functionality of the antibody produced by each cell (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015). The result is B cells with refined antigen recognition. Memory B cells can also give rise to long-lived plasma cells and the latter can be found in different niches such as the bone marrow, secondary lymphoid organs, and inflamed tissue (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015).

Some studies have suggested that plasma cells are long-lived, based on the fact that 60% of the bone marrow plasma cells in mice can live longer than 90 days (Manz, Thiel et al. 1997) and that antigen specific antibody titers have been shown to be maintained for over 25 years in humans (Amanna, Carlson et al. 2007). On the other hand, in inflamed tissue when the inflammation is cleared, the survival niches disappear and the plasma cells die as a consequence (Radbruch, Muehlinghaus et al. 2006).

This is a relevant but still controversial subject. Experimental animals are not ideal to assess the longevity of a population that responds to pathogens. In the wild, new bugs are constantly stimulating the immune system and newly produced plasma cells are likely to compete for survival niches with existing ones. In a pathogen free barrier facility, following the initial response induced by the experimental vaccination or infection, and in the absence of secondary infections, it is possible that no turnover will occur as a result of a lack of competition driven by new B cell activation events. Long lasting immune memory (mediated by B cells or T cells) is unquestionable, but if it is maintained by long-lived cells or by continuously renewing cells, remains generally unknown. The bone marrow has historically been considered the main residence for long-lived plasma cells (Radbruch, Muehlinghaus et al. 2006, Nutt, Hodgkin et al. 2015), but our results contradict this hypothesis (PAPER II). Although, isolated plasma cells produced antibodies against antigens from childhood vaccines, our data is inconsistent with the presence of a large population of long-lived cells (PAPER II). Additionally, preliminary results on peripheral memory B cells suggest that these cells might be in average older than bone marrow plasma cells, offering an alternative explanation for immune memory preservation where plasma cells in the bone marrow are constantly replaced by slow-dividing memory B cells following an amplification step (PAPER II).

As mentioned above, plasma cells can be found in different survival niches, and we showed that the human small intestine houses large numbers of truly long-lived plasma cells (PAPER III). Here, plasma cells were defined as $CD27^{+}/CD38^{+}$ and further sub-divided into three populations: $CD45^{+}/CD19^{+}$ (double positives), $CD45^{+}/CD19^{-}$ (single positives) and $CD45^{-}/CD19^{-}$ (double negatives) (PAPER III). In pancreatic transplants, part of the donor's small intestine was transplanted into the recipient. One year after the transplant, biopsies of small intestine were collected and the percentage of replacement of donor cells by recipient cells was:

32% in the double positive, 2.3% in the single positives and only 0.01% in the double negatives (PAPER III). By measuring the average age of each population using ^{14}C analysis, we determined that double positive cells are continually renewed, single positives are on average 8 years old and double negatives are on average 18 years old (truly long-lived plasma cells) (PAPER III). In addition, we sequenced the heavy-chain variable region of the three cell populations. The results of this experiment revealed that in terms of shared clones, the single positive plasma cells are situated in between the double positive and the double negative ones (PAPER III). The intermediate phenotype, replacement rate, cell age, and clone overlap of the single positive population suggests that it might be a developmental stage between the other two (PAPER III).

These results provide strong evidence for an important contribution by long-lived plasma cells to the maintenance of humoral immunity at least in the gastrointestinal niche (PAPER III). The higher renewal rate in bone marrow plasma cells (PAPER II) could be the result of higher competition for survival factors in this niche in comparison with the intestine and is the topic of ongoing investigation.

4.3 PROLIFERATION IN THE CNS

A general theme of the work presented in this thesis is that cells in different systems proliferate at much different rates. As an example, cells of the innate immune system such as monocytes and neutrophils renew very often (Busch, Neese et al. 2007) while many cells of central nervous system (CNS) are exchanged slowly (Spalding, Bergmann et al. 2013, Ernst, Alkass et al. 2014, Yeung, Zdunek et al. 2014) or not at all (Bhardwaj, Curtis et al. 2006). Microglia are the resident macrophages of the CNS and serve a variety of functions, such as immune surveillance, affect neurogenesis, synaptic pruning and apoptotic clearance (Casano and Peri 2015). Having a healthy microglia population is likely to be important for CNS homeostasis, since low numbers of microglia cells can result in abnormal behavior and learning disabilities (Parkhurst, Yang et al. 2013). We have provided evidence that human microglia renew much faster than other cell types in the CNS (Spalding, Bhardwaj et al. 2005, Spalding, Bergmann et al. 2013, Yeung, Zdunek et al. 2014) at a median rate of 28% a year (PAPER IV). With an average age of 4.2 years, not all microglia cells divide simultaneously, hence some are newly generated and a few may be over 20 years old (PAPER IV). Nonetheless, we found no evidence for the existence of a large population of

non-dividing microglia, meaning that the majority of microglia (>96%) in the human cortex will eventually be exchanged throughout life (PAPER IV), similarly to what has been observed in mice (Askew, Li et al. 2017).

Lining the ventricles in the brain and the central canal in the spinal cord are a group of ciliated cells designated ependymal cells (Del Bigio 2010). In homeostasis, these cells are capable of slow self-renewal in the spinal cord (PAPER V) (Horner, Power et al. 2000, Meletis, Barnabe-Heider et al. 2008). Much like turnover, regeneration involves the substitution of a given number of cells in a tissue by new ones. Regeneration however, implies the occurrence of an injury and this often leads to scar formation. Spinal cord scarring has both beneficial and detrimental effects. On one hand it physically restricts the injury to avoid its spread and limits inflammation by sealing the area (Pekny, Johansson et al. 1999, Okada, Nakamura et al. 2006, Herrmann, Imura et al. 2008). On the other hand it restricts the regeneration of the severed axons (Silver and Miller 2004, Fawcett 2006, Okada, Nakamura et al. 2006, Sofroniew 2009). Following spinal cord injury, ependymal cells proliferate extensively and give rise to progeny that leave the central canal (PAPER V). This progeny is mainly constituted of astrocytes, which play a crucial role in scar formation (PAPER V). The scar restricts secondary enlargement of the lesion, which could otherwise cause further impairment (PAPER V). Interestingly, if proliferation is blocked in ependymal cells these became unable to differentiate, as both processes seem to be linked (PAPER V). Importantly, we also show that in the regions adjacent to the injury ependymal cell progeny exert neurotrophic effects on local neurons, promoting their survival. Consequently, if ependymal cell proliferation and migration is blocked there is an increased neuronal loss (PAPER V).

The spinal cord contains illustrative examples of the proliferative capacities of different cell types and of how differently cells are programmed to respond to environmental challenges. Like ependymal cells (PAPER V), astrocytes and oligodendrocytes are slowly maintained by cells of their own lineage, in homeostasis (Barnabe-Heider, Goritz et al. 2010). However in response to injury, ependymal cells are capable of giving rise to different lineages (PAPER V) (Barnabe-Heider, Goritz et al. 2010). Another cell type that contributes to the formation of the scar is type A pericytes (Goritz, Dias et al. 2011). Upon spinal cord injury, these cells proliferate, migrate to the lesion site, and their progeny forms the stromal component of the scar (Goritz, Dias et al. 2011). In summary, the scar

formed after spinal cord injury is constituted by an external layer of astrocytes originated from duplication of pre-existing astrocytes, followed by a layer of ependymal cell-derived astrocytes, and finally by a central stromal region of type A pericyte progeny (Sabelstrom, Stenudd et al. 2014).

5 FUTURE PERSPECTIVES

Further research into clonal diversity in different stem cell systems will be of great help for a better understanding of how cells regulate proliferation and differentiation and how these functions are affected by disease. The knowledge that a significant number of new naïve T cells are being produced by the thymus throughout the first three decades of life and that diversity slowly decreases in adulthood can lead to a variety of treatments. For instance: cell cryopreservation early in adulthood for autologous transplant later in life; *ex vivo* expansion of naïve or memory populations following fresh collection or cryopreservation; and *in vivo* expansion by manipulation of the NF-κB pathway. This might be particularly relevant for elderly individuals with increased naïve T cell proliferation that might be associated to lower diversity and consequently to lower responsiveness to new pathogens.

An important aspect of our studies into the population dynamics of plasma cells is the difference between the bone marrow and the intestinal niches. Contrary to intestinal plasma cells, bone marrow plasma cells are more likely to be affected by drugs targeting fast renewing cells due their short lifespans. This knowledge, can contribute to the design of re-vaccination protocols for patients undergoing chemotherapy. In addition, this data may lead to a better understanding of the different efficacies of injectable versus oral vaccinations. Investigating the repertoire overlap between niches will determine if plasma cell clones have a preference for a given niche depending on their receptor. If so, this will help developing vaccination protocols that target specific niches.

Determining the homeostatic turnover rate of microglia provides a standard that can help us to understand the diseased brain, where this rate is potentially altered. Furthermore, it provides clues about how other tissue resident macrophages are likely to behave. Also in the CNS, we investigated the function of ependymal cells following spinal cord injury. We found that this population of neural stem cells gives origin to a group of astrocytes, crucial for scar formation. Our results suggest that further investigation into therapies that promote acute scar formation is needed, since it can prevent secondary enlargement of the lesion.

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