Disorganisation of the splenic microanatomy in ageing mice

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Abbreviations: Marginal zone macrophages, MZM; marginal metallophilic macrophages, MMM; trinitrophenyl, TNP; keyhole limpet haemacyanin, KLH; fibroblastic reticular cells, FRC; periarterial lymphatic sheaths, PALS; follicular dendritic cells, FDC; Lymphoid tissue inducer, LTi; transmissible spongiform encephalopathy, TSE.

Summary

The precise mechanisms responsible for immunosenescence still remain to be determined, however considering the evidence that disruption of the organisation of primary and secondary lymphoid organs results in immunodeficiency, we propose this could be involved in the decline of immune responses with age. Therefore, we investigated the integrity of the splenic microarchitecture in mice of increasing age and its reorganisation following immune challenge in young and old mice. Several differences in the anatomy of the spleen with age in both the immune and stromal cells were observed. There is an age-related increase in the overall size of the white pulp, which occurs primarily within the T cell zone and mirrored by the enlargement of the T cell stromal area, concurrent to the distinct boundary between T cells and B cells becoming less defined in older mice. In conjunction, there appears to be a loss of marginal zone macrophages, which is accompanied by an accumulation of fibroblasts in the spleens from older animals. Furthermore, whereas the reorganisation of the white pulp is resolved after several days following antigenic challenge in young animals, it remains perturbed in older subjects. All these age-related changes within the spleen could potentially contribute to the age-dependent deficiencies in functional immunity.

Introduction

It is now recognised that the activity and efficiency of the immune system declines with age; a phenomenon referred to as immunosenescence.[1-3] In older individuals this leads to an increased susceptibility to and severity of infections, a decline in response to vaccinations and a higher prevalence of cancer and autoimmune diseases.[1, 4, 5] These all contribute to the increased rates of mortality and morbidity that are observed in the elderly.[6]

Whilst the exact mechanisms underlying immunosenescence are still unclear, several studies have identified intrinsic defects within many of the cells of the immune system. For instance, various reports have revealed a reduction of receptor diversity in ageing T and B cells, together with alterations in their phenotype and function.[2, 7-10] While others have observed, reduced phagocytic ability and reactive oxygen species production in aged macrophages and neutrophils;[11, 12] as well as alteration in natural killer cell subsets and impaired cytotoxicity.[13, 14] However, given the essential role the microenvironment plays in the development and activation of many immune cell types, [15, 16] changes in the tissue architecture may also contribute towards immunosenescence.[17] Indeed, we and others have observed that regression of the thymus is accompanied by significant alterations of the thymic microenvironment,[18-21] and such changes are believed to contribute towards the reduced thymic function that is observed in the aged.[22-24]

The spleen is a secondary lymphoid organ that is responsible for initiating immune responses to blood-borne antigens.[25, 26] It is composed of two morphologically and functionally distinct compartments, the red pulp and the white pulp which have different roles.[25, 26] The red pulp is responsible for

the removal of old and damaged platelets, old erythrocytes and apoptotic cells mediated by splenic macrophages[26, 27] and are separated from the white pulp by the marginal zone. This contains many resident cells that maintain its integrity including, two specific subsets of macrophages; marginal zone macrophages (MZM) and marginal metallophilic macrophages (MMM), as wells as marginal zone B cells and dendritic cells. [26, 28] These are supported by a network of specialised cells that line the marginal-zone called fibroblastic reticular cells (FRC).[29]

The white pulp consists of lymphoid tissue and is further subdivided into a periarterial lymphatic sheaths (PALS), where T cells encircle the central arteriole, and B cell follicles.[25] Embedded within this compartmentalised structure are stromal cells which are uniquely designed to allow effective filtration of the blood as it passes through the zones containing T and B cells, enabling capturing of pathogens, facilitating exchanges between APC and pathogen-specific lymphocytes, and providing necessary signals for survival and differentiation for leukocytes.[16] Therefore, the intact microarchitecture of the spleen supports productive lymphocyte-APC interactions and is critical for the ability of an organism to produce an efficient and robust immune response.[16] Indeed, animals with disordered splenic microanatomy, such as alymphoplasia (aly/aly) mice or those deficient for lymphotoxin, exhibit reduced humoral and cell-mediate immune responses.[16, 30] Moreover, the immune response of these mice is similar to those seen in aged animals; suggesting that disruption of the splenic microanatomy may contribute towards peripheral immunosenescence. However, a detailed analysis of the splenic microarchitecture in mice at varying ages has not been performed. In

this study, we examine the splenic microarchitecture in young and old mice together with analysing splenic remodeling in these animals following immunisation with a T-independent and T-dependent antigen.

Material and methods

Ethics statement

Animal experiments were conducted in accordance with our project licence (PPL 70/7243), which was approved by the Home Office under the Animal Scientific Procedures Act (1986). The project was approved by the local Ethical Review Committee at the Royal Veterinary College.

Mice

Male C57BL/6 were purchased from Charles Rivers Laboratory (Kent, UK) and maintained at the Royal Veterinary College (RVC), London. Animals killed by schedule 1 method and tissues were harvested from one month, six month, 12 month, and 18 month old mice

Antibodies & Reagents

Monoclonal antibodies (mAb) isotype-Fluorescein (FITC), isotype-biotin, anti-CD3-biotin, anti-B220-FITC, IgM-biotin, anti-CD157 and isotype controls were all purchased from eBioscience (Hatfield, UK). Rabbit anti-rat-biotin was purchased from Jackson ImmunoResearch Laboratories (PA, USA). MOMA-1-FITC was obtained from Serotec (Oxfordshire, UK). Alexa 594 was from Invitrogen Molecular Probes (Paisley, UK). Anti-Gp38 was obtained from Hybridoma Studies Bank (Lowa, USA). ERTR7 was a generous gift from Professor Graham Anderson (University of Birmingham), ERTR9 was a kind gift from Professor van Ewijk and anti-FDC-M2 generous gift from Professor David Gray (Edinburgh University) and Dr Marie Kosco-Vilbois (Novimmune). Trinitrophenyl (TNP)–FICOLL, TNP- Keyhole Limpet Haemacyanin (KLH) and

TNP-BSA were obtained from Biosearch Technologies Inc (California, USA). Anti-mouse IgM-HRP and anti-mouse IgG-HRP were purchased from Southern Biotech (AL, USA).

Immunohistological studies

Seven µm think tissue sections were cut, air dried overnight, fixed in acetone and stored at -20°C. Sections were stained using a standard protocol.[18] Briefly, double staining was performed by sequentially incubating primary antibodies, which after 45minute incubations and three washes in PBS were revealed with the appropriate secondary antibody and Streptavidin-Alexa594. Sections were then probed with a second primary antibody that if not directly conjugated to FITC, was recognised by a FITC-conjugated secondary. These were then mounted with VectaShield mounting medium and viewed on Leica SP5 confocal microscope (Leica Microsystems Ltd, Milton Keynes, UK). Multiple photographs of each sample were taken and analysed.

Quantification of Mean Fluorescence Intensity

Fluorescent images were analysed using *ImageJ* (http://rsb.info.nih.gov/ij/) as previously described.[18]

Immunisation Protocol

Mice (either 1 month or 18 months of age) were immunised via an intraperitoneal injection with either TNP-Ficoll, 500µg (at day 0) or TNP–KLH, 100µg (at day 0 & day 28).[31] Blood was obtained from all mice by cardiac puncture or from the saphenous vein. Tissues were harvested from the young

and old mice at time points of day (d) 0, d3 and d28 for TNP-FICOLL, and d0, d14 and d35 for TNP-KLH.

Enzyme-Linked Immunosorbent Assay (ELISA)

Specific antibody for TNP-Ficoll and TNP-KLH, detected by an enzyme-linked was immunosorbent assay (ELISA) was developed.[31] Flat bottom 96-well plates were coated with 5µg of TNP-BSA in carbonate buffer (pH9.6) and incubated overnight at 4°C. After washing, blocking buffer was added and incubated at room temperature for two hours. After further washing, individual serum diluted in PBS 0.5% Tween-20 was added, incubated for one hour and washed. The secondary antibody, either IgM-HRP or IgG-HRP was added and incubated for one hour. Finally, 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Sigma) was added to each well. The optical density was read at 405nm.

Statistical Analysis

Statistical significance was calculated with a one-way or two-way ANOVA comparing all variables with Bonferroni's multiple comparison post-test using prism software (GraphPad Prism 5). *P* values of 0.05 or less were considered significant.

Results

Disorganisation of cellular positioning in the spleen of ageing mice

Immune function declines with age and although disorganisation of the splenic architecture can lead to a profound loss of immunocompetence, little is known regarding age-related changes in the spleen. In this study, the microanatomy of the spleen from mice of different ages was examined. The splenic T and B cell compartment was identified using antibodies to CD3 (in green) and B220 (in red) respectively, and was quantified by Image J analysis. Using this approach, the T and B cell zones show a clear demarcation in animals at 1 and 6 months of age, however this becomes increasingly obscured in older mice, demonstrated by an increase in $CD3^{+}B220^{+}$ areas, which was significantly greater in size in both 12 and 18 month old mice compared to younger animals (Fig1a). MZM, identified by the antibody ERTR9 (in red), were clearly observed to be encircling MMM, recognised by MOMA-1 (in green) in younger mice (Fig 1b). However, in splenic sections from 12 month and 18 month old mice, MZM were more difficult to distinguish, whilst the MMM appeared not to fully surround the marginal zone.

Splenic stromal cell organisation is altered with age

As stromal cells provide the foundation for splenic organisation, the stromal cells that contribute to this splenic scaffolding were investigated. The arrangement of the T and B cell zone stroma was ascertained by staining sections for gp38 (podoplanin; in red) and CD157 (BP-3; in green) respectively (Fig.2a). In young mice, the stromal cells in the T cell zone, characterised by

gp38 staining, is centred around the PALS. However, with increasing age the T cell area becomes significantly larger as determined by Image J analysis. In contrast, the B cell zone remained unchanged with age. The ER-TR7 Ab identifies the FRC network which encapsulates the white pulp, but also present within the PALS. [32] This staining pattern can be seen in the spleen of young mice (Fig 2b); however this network becomes denser around the marginal zone region with increasing age implying an accumulation of fibroblast in this area (Fig 2b). We observed that follicular dendritic cells (FDC) as identified by FDC-M2 Ab, are arranged in tight clusters within the white pulp from spleen of young mice while in older mice they become scattered and more diffuse (Fig 2c). Collectively, these observations support the findings that there are age-related changes in the distribution of cells and the cellular network in the spleen of mice.

Splenic remodelling in young and aged mice following an immune response to a T-independent and T-dependent antigen

Changes in the splenic microanatomy are often seen during an immune challenge and are believed to be necessary in order to facilitate an efficient immune response.[16] Given our observations showing disorganisation of the splenic architecture in older mice, we therefore examined the ability of immune responses to alter the microanatomy of this secondary lymphoid organ by challenging young (1 month of age) and older (18 months of age) mice with either a T-independent antigen (TNP-Ficoll) or T-dependent antigen (TNP-KLH) and subsequently examined the splenic structure.

Both young and older mice demonstrated an increase in IgM serum responses during the time course following immunisation with TNP-Ficoll (Fig.3a). However, basal levels of IgM in older mice appeared significantly higher prior to challenge, which continued until d7 when induced-IgM production peaked in both cohorts. Both young and older mice show disorganisation of T and B cells within the white pulp at day 3 after immunisation, which appears to be resolved in the spleen of young mice at day 28, but is still apparent in older animals (Fig. 3b). Examination of the splenic marginal zone microarchitecture, in particular MZM and MMM, reveals alterations in the spleen of immunised young mice, notable being the appearance of enhanced clustering of MMM around the marginal zone, and the infiltration into the white pulp. In contrast, such reorganisation, as judged by the pattern of MZM and MMM staining, appears less prominent within the spleen of immunised old mice (Fig. 3b).

Following challenge with TNP-KLH, IgG increased in both cohorts of mice and similar to the TNP-Ficoll IgM response, older mice exhibited a higher basal level of IgG (Fig. 4). The response peaks at day 7 after immunisation and then plateaus, however after another immunisation at day 28, the IgG level raises further in both age cohorts. Examination of the white pulp area from both age groups 14 days post-immunisation revealed a greater degree of disorganisation of T and B cells in the spleen of old mice, which is also apparent 8 days after secondary immunisation; indicating a failure of the spleen in older mice to undergo resolution following antigenic challenge. Similar the MZM and MMM appeared not to fully encircle the marginal zone in older mice.

Discussion

Immunosenescence is characterised by an age-related decline in immune function and, although the precise mechanism is not known, evidence points to an accumulation of both intrinsic and extrinsic defects.[1-5] The microenvironment plays an essential role in supporting and facilitating the development and activation of immune cells [15, 25] and while many have observed significant alterations of the thymic architecture in the aged,[33] much less is known about the impact of age on the structure and integrity of secondary lymphoid organs (SLO). In this study we aim to address this deficiency and identified clear age-related alterations of the splenic architecture.

We observed that the delineation between the T and B cell zones became less distinct with increasing age, and T cells appear to be present in B cell regions. This loss of the demarcation between the T and B cell area is also seen in the SLO from various mouse models that exhibit altered chemokine expression.[16, 26] Primarily, the chemokine CXCL13 is required for the organisation and maintenance of the B cell area,[34] while CCL19 and CCL21 is needed to perform a similar role in the T cell region.[35] Moreover, these chemokines play an instrumental role in orchestrating immune responses in SLO.[16, 26] Interestingly, recent studies have shown altered patterning of CXCL13[36, 37] and CCL21[37] expression in the aged mouse spleen together with reduced splenic protein content of CCL21 and CCL19.[37] Thus, these reports could offer an explanation for the observed dissolution between the T and B cell boundary. Furthermore, in adoptive transfer experiments, the dysregulation of these chemokines may account for the impaired recruitment of young T [37] and B cells[36] in the spleen

of aged mice. Thus, a consequence of lymphocyte being out of position is the inability to forge key physical interactions[16, 26] with the likely scenario of generating reduced immune responses within the aged SLO. Moreover, these studies highlight that the microenvironment in ageing SLO can potentially contribute towards peripheral immunosenescence.[17]

The stromal network represents key components to the functional activity of SLO and we noted that the stromal network undergoes dramatic changes with age. In particular, the enlargement of the T cell zone stroma which may account for the loss of the distinct boundary between the T and B cell area. Similarly the microarchitecture of the marginal zone region appears to be disrupted in the aged spleen, as judged by the organisation and distribution of MZM and MMM. This could be attributed to the disappearance of MZM and MMM or the loss of the antibody epitope that identify these cell types. The alteration of the marginal zone region could be caused by changes in CCL19, CCL21 or CXCL13 expression since these chemokines have been shown to play a key role in the organisation of this area. [26] Lymphoid tissue inducer (LTi) cells may also regulate the organisation of the MZ as they control chemokine expression level by lymphotoxin-β receptor signalling.[38] Disruption of the marginal zone sinus-lining may also account for the altered distribution of MZM and MMM. Indeed, the marginal zone sinus-lining cells as detected by the expression of MADCAM-1, has been shown to be less intact in the aged spleen.[39, 40] Furthermore, sphingosine-1-phosphate receptor 3 deficient mice display a disorganised marginal zone region and exhibit a reduced T-independent response;[41] highlighting the importance of an intact region. Moreover, this region is not only

important in the entry and exit point of leukocyte trafficking, but also the site where antigens are sequestered. Thus, the age–associated disorganisation of the marginal zone could impact on the ability of the spleen to mount an immune response, as evident by the recent report showing reduced binding of dextran, administered intravenously, within the MZ region of spleen from old mice in comparison to young.[39]

The suggestion that the aged spleen has a reduced ability to capture antigen could also be extended to FDC. Using FDC-M2 Ab, we observed that the FDC in young mice were arranged in tight clusters, whereas in old mice their organisation appeared diffuse and scattered. Interestingly, FDC-M2 labelling has been shown to specifically reflect deposition of immune complexes associated with complement activation.[42] Additionally, the authors observed that old FDC, as assessed by flow cytometry, have lower levels of FcγRII -a key molecule involved in capturing immune complexes and also demonstrated that such cells from aged animals exhibit reduced co-stimulatory activity.[43]

Immune responses are often accompanied by remodeling of secondary lymphoid tissue, which subsequently resolved.[16] Our results of immunising young and old mice revealed that whilst the spleen from young animals showed resolution after immunisation, the spleen from the older cohort remained disorganised. The higher basal level of antibody reactivity that was observed in old may be due to the age-associated increase in autoantibody production.[44] Interestingly, studies have shown that some parasitic[45, 46] and viral[47, 48] infections can directly cause significant disruption of the splenic architecture

resulting in the inability of the host to mount a robust immune response, suggesting this may be a potential mechanism utilised by pathogens in order to reduce immune efficacy and increase pathogenesis. Thus, the age-associated disruption of the splenic architecture may reflect the accumulation of remodelling due to the continual antigenic challenge of the host, which over time might impact on the structural integrity of SLO. A consequence of such continual remodelling is the likely increase in fibrosis that may also account for the altered patterning of FRC, as judged by ERTR7 staining, in the aged spleen. These cells have been showed to be a target for viral infections altering their functions[49] and in some instances displaying immunosuppressive properties. [48] Furthermore, aged stromal cells may also contribute to the 'inflamm-ageing' process itself, [1] as a recent study showed that splenic stromal cells from ageing mice produce higher amounts of IL-6 in comparison to young animals. [50] Interestingly, the production of pro-inflammatory cytokines along with the secretion of growth factors, chemokines and proteases represent important characteristic of senescent cells. Such cells are referred to having a senescence-associated secretory phenotype (SASP) [51] and their accumulation, which occur with age, have been postulated to cause alteration in tissue architecture, impair tissue homeostasis; contributing towards pathophysiology.[51, 52] Furthermore, the expression of p16, which promote cellular senescence, has been shown to be upregulated in the spleen of aging mice. [53] Thus the aging process itself may contribute towards the alteration of tissue architecture.

Corroboratively, the studies highlighting splenic remodeling and the efficacy of the immune response, [46-48] indicate a correlation between the structural integrity of the splenic microenvironment and immune competency.[16] As previously mentioned adoptive transfer experiments demonstrated altered trafficking of young lymphocytes within the aged spleen. [36, 37] Moreover, adoptive transfer experiments of young anti-virus specific transgenic CD8 T cells fail to clonally expand in aged spleen independently of antigen presenting activity. [54] In contrast, it was recently demonstrated that the inability of aged mice to develop transmissible spongiform encephalopathy (TSE) was due to disruption of the splenic marginal zone microarchitecture which led to reduced accumulation of the TSE agent in the spleen; thereby limiting disease progression.[40]

In summary, our study shows that the splenic architecture undergoes age-associated changes, which is consistent with similar observations that have been reported in rats[55, 56] and human, and suggests that such alterations might impact on immune activity and therefore contribute towards peripheral immunosensecence and may also offer a potential target for rejuvenating the immune system.

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Author contributions

DA, LH, YN, ETC and RL performed the experiments. DA, RL and DBP designed the experiments. DA, LH, RL and DBP wrote the paper.

Disclosures

The authors have no financial or commercial conflicts of interest.

References

1. Aw D, Silva AB, Palmer DB. Immunosenescence: emerging challenges for an ageing population. *Immunology* 2007; **120**:435-46.
2. Haynes L, Swain SL. Why aging T cells fail: implications for vaccination. *Immunity* 2006; **24**:663-6.
3. Pawelec G, Akbar A, Caruso C, Solana R, Grubeck-Loebenstein B, Wikby A. Human immunosenescence: is it infectious? *Immunological reviews* 2005; **205**:257-68.
4. Chen WH, Kozlovsky BF, Effros RB, Grubeck-Loebenstein B, Edelman R, Sztein MB. Vaccination in the elderly: an immunological perspective. *Trends in immunology* 2009; **30**:351-9.
5. Gardner ID. The effect of aging on susceptibility to infection. *Reviews of infectious diseases* 1980; **2**:801-10.
6. McElhaney JE, Zhou X, Talbot HK, Soethout E, Bleackley RC, Granville DJ, Pawelec G. The unmet need in the elderly: how immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines. *Vaccine* 2012; **30**:2060-7.
7. Akbar AN, Henson SM. Are senescence and exhaustion intertwined or unrelated processes that compromise immunity? *Nature reviews Immunology* 2011; **11**:289-95.
8. Goronzy JJ, Weyand CM. T cell development and receptor diversity during aging. *Current opinion in immunology* 2005; **17**:468-75.
9. Cancro MP, Hao Y, Scholz JL, Riley RL, Frasca D, Dunn-Walters DK, Blomberg BB. B cells and aging: molecules and mechanisms. *Trends in immunology* 2009; **30**:313-8.
10. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: a quantitative review. *Vaccine* 2006; **24**:1159-69.
11. Kovacs EJ, Palmer JL, Fortin CF, Fulop T, Jr., Goldstein DR, Linton PJ. Aging and innate immunity in the mouse: impact of intrinsic and extrinsic factors. *Trends in immunology* 2009; **30**:319-24.
12. Shaw AC, Joshi S, Greenwood H, Panda A, Lord JM. Aging of the innate immune system. *Current opinion in immunology* 2010; **22**:507-13.
13. Hayhoe RP, Henson SM, Akbar AN, Palmer DB. Variation of human natural killer cell phenotypes with age: identification of a unique KLRG1-negative subset. *Human immunology* 2010; **71**:676-81.
14. Gayoso I, Sanchez-Correa B, Campos C, *et al.* Immunosenescence of human natural killer cells. *Journal of innate immunity* 2011; **3**:337-43.
15. Anderson G, Jenkinson EJ. Lymphostromal interactions in thymic development and function. *Nature reviews Immunology* 2001; **1**:31-40.
16. Junt T, Scandella E, Ludewig B. Form follows function: lymphoid tissue microarchitecture in antimicrobial immune defence. *Nature reviews Immunology* 2008; **8**:764-75.
17. Su DM, Aw D, Palmer DB. Immunosenescence: a product of the environment? *Current opinion in immunology* 2013; **25**:498-503.
18. Aw D, Silva AB, Maddick M, von Zglinicki T, Palmer DB. Architectural changes in the thymus of aging mice. *Aging cell* 2008; **7**:158-67.
19. Aw D, Taylor-Brown F, Cooper K, Palmer DB. Phenotypical and morphological changes in the thymic microenvironment from ageing mice. *Biogerontology* 2009; **10**:311-22.

20. Gray DH, Seach N, Ueno T, Milton MK, Liston A, Lew AM, Goodnow CC, Boyd RL. Developmental kinetics, turnover, and stimulatory capacity of thymic epithelial cells. *Blood* 2006; **108**:3777-85.
21. Steinmann GG, Klaus B, Muller-Hermelink HK. The involution of the ageing human thymic epithelium is independent of puberty. A morphometric study. *Scandinavian journal of immunology* 1985; **22**:563-75.
22. Andrew D, Aspinall R. Age-associated thymic atrophy is linked to a decline in IL-7 production. *Experimental gerontology* 2002; **37**:455-63.
23. Zhu X, Gui J, Dohkan J, Cheng L, Barnes PF, Su DM. Lymphohematopoietic progenitors do not have a synchronized defect with age-related thymic involution. *Aging cell* 2007; **6**:663-72.
24. Gui J, Zhu X, Dohkan J, Cheng L, Barnes PF, Su DM. The aged thymus shows normal recruitment of lymphohematopoietic progenitors but has defects in thymic epithelial cells. *International immunology* 2007; **19**:1201-11.
25. Cesta MF. Normal structure, function, and histology of mucosa-associated lymphoid tissue. *Toxicologic pathology* 2006; **34**:599-608.
26. Mebius RE, Kraal G. Structure and function of the spleen. *Nature reviews Immunology* 2005; **5**:606-16.
27. Brendolan A, Rosado MM, Carsetti R, Selleri L, Dear TN. Development and function of the mammalian spleen. *BioEssays : news and reviews in molecular, cellular and developmental biology* 2007; **29**:166-77.
28. Martin F, Kearney JF. Marginal-zone B cells. *Nature reviews Immunology* 2002; **2**:323-35.
29. Balogh P, Horvath G, Szakal AK. Immunoarchitecture of distinct reticular fibroblastic domains in the white pulp of mouse spleen. *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society* 2004; **52**:1287-98.
30. Karrer U, Althage A, Odermatt B, Hengartner H, Zinkernagel RM. Immunodeficiency of alymphoplasia mice (aly/aly) in vivo: structural defect of secondary lymphoid organs and functional B cell defect. *European journal of immunology* 2000; **30**:2799-807.
31. Gaspal FM, McConnell FM, Kim MY, Gray D, Kosco-Vilbois MH, Raykundalia CR, Botto M, Lane PJ. The generation of thymus-independent germinal centers depends on CD40 but not on CD154, the T cell-derived CD40-ligand. *European journal of immunology* 2006; **36**:1665-73.
32. Bajenoff M, Glaichenhaus N, Germain RN. Fibroblastic reticular cells guide T lymphocyte entry into and migration within the splenic T cell zone. *J Immunol* 2008; **181**:3947-54.
33. Palmer DB. The effect of age on thymic function. *Frontiers in immunology* 2013; **4**:316.
34. Ansel KM, Ngo VN, Hyman PL, *et al.* A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000; **406**:309-14.
35. Gunn MD, Kyuwa S, Tam C, Kakiuchi T, Matsuzawa A, Williams LT, Nakano H. Mice lacking expression of secondary lymphoid organ chemokine have defects in lymphocyte homing and dendritic cell localization. *The Journal of experimental medicine* 1999; **189**:451-60.
36. Wols HA, Johnson KM, Ippolito JA, Birjandi SZ, Su Y, Le PT, Witte PL. Migration of immature and mature B cells in the aged microenvironment. *Immunology* 2010; **129**:278-90.

37. Lefebvre JS, Maue AC, Eaton SM, Lanthier PA, Tighe M, Haynes L. The aged microenvironment contributes to the age-related functional defects of CD4 T cells in mice. *Aging cell* 2012; **11**:732-40.
38. Withers DR, Kim MY, Bekiaris V, *et al.* The role of lymphoid tissue inducer cells in splenic white pulp development. *European journal of immunology* 2007; **37**:3240-5.
39. Birjandi SZ, Ippolito JA, Ramadorai AK, Witte PL. Alterations in marginal zone macrophages and marginal zone B cells in old mice. *J Immunol* 2011; **186**:3441-51.
40. Brown KL, Gossner A, Mok S, Mabbott NA. The effects of host age on the transport of complement-bound complexes to the spleen and the pathogenesis of intravenous scrapie infection. *Journal of virology* 2012; **86**:25-35.
41. Girkontaite I, Sakk V, Wagner M, Borggrefe T, Tedford K, Chun J, Fischer KD. The sphingosine-1-phosphate (S1P) lysophospholipid receptor S1P3 regulates MAdCAM-1+ endothelial cells in splenic marginal sinus organization. *The Journal of experimental medicine* 2004; **200**:1491-501.
42. Taylor PR, Pickering MC, Kosco-Vilbois MH, Walport MJ, Botto M, Gordon S, Martinez-Pomares L. The follicular dendritic cell restricted epitope, FDC-M2, is complement C4; localization of immune complexes in mouse tissues. *European journal of immunology* 2002; **32**:1888-96.
43. Aydar Y, Balogh P, Tew JG, Szakal AK. Follicular dendritic cells in aging, a "bottle-neck" in the humoral immune response. *Ageing research reviews* 2004; **3**:15-29.
44. Zhao KS, Wang YF, Gueret R, Weksler ME. Dysregulation of the humoral immune response in old mice. *International immunology* 1995; **7**:929-34.
45. Cadman ET, Abdallah AY, Voisine C, *et al.* Alterations of splenic architecture in malaria are induced independently of Toll-like receptors 2, 4, and 9 or MyD88 and may affect antibody affinity. *Infection and immunity* 2008; **76**:3924-31.
46. Martin-Jaular L, Ferrer M, Calvo M, *et al.* Strain-specific spleen remodelling in Plasmodium yoelii infections in Balb/c mice facilitates adherence and spleen macrophage-clearance escape. *Cellular microbiology* 2011; **13**:109-22.
47. Benedict CA, De Trez C, Schneider K, Ha S, Patterson G, Ware CF. Specific remodeling of splenic architecture by cytomegalovirus. *PLoS pathogens* 2006; **2**:e16.
48. Mueller SN, Matloubian M, Clemens DM, Sharpe AH, Freeman GJ, Gangappa S, Larsen CP, Ahmed R. Viral targeting of fibroblastic reticular cells contributes to immunosuppression and persistence during chronic infection. *Proceedings of the National Academy of Sciences of the United States of America* 2007; **104**:15430-5.
49. Fletcher AL, Acton SE, Knoblich K. Lymph node fibroblastic reticular cells in health and disease. *Nature reviews Immunology* 2015.
50. Park J, Miyakawa T, Shiokawa A, Nakajima-Adachi H, Tanokura M, Hachimura S. Splenic stromal cells from aged mice produce higher levels of IL-6 compared to young mice. *Mediators of inflammation* 2014; **2014**:826987.
51. Campisi J, Andersen JK, Kapahi P, Melov S. Cellular senescence: a link between cancer and age-related degenerative disease? *Seminars in cancer biology* 2011; **21**:354-9.
52. van Deursen JM. The role of senescent cells in ageing. *Nature* 2014; **509**:439-46.

53. Krishnamurthy J, Torrice C, Ramsey MR, Kovalev GI, Al-Regaiey K, Su L, Sharpless NE. Ink4a/Arf expression is a biomarker of aging. *The Journal of clinical investigation* 2004; **114**:1299-307.
54. Jiang J, Bennett AJ, Fisher E, Williams-Bey Y, Shen H, Murasko DM. Limited expansion of virus-specific CD8 T cells in the aged environment. *Mechanisms of ageing and development* 2009; **130**:713-21.
55. Cheung HT, Nadakavukaren MJ. Age-dependent changes in the cellularity and ultrastructure of the spleen of Fischer F344 rats. *Mechanisms of ageing and development* 1983; **22**:23-33.
56. Pahlavani MA, Richardson A, Cheung HT. Age-dependent changes of the mesenteric lymph node of Fischer F344 rats: morphological and histometric analysis. *Mechanisms of ageing and development* 1987; **39**:137-46.

Figure legends

Figure 1. Changes of the splenic microenvironment with increasing age. (a) Splenic sections were stained with antibodies (anti-CD3, anti-B220) which recognised T cells (shown in green) and B cells (shown in red) respectively and the level of staining was assessed using *ImageJ* and portrayed as a percentage of the total area measured ($FITC^+$ Area refers to CD3, Alexa 594^+ Area refers to B220 and $FITC^+$ Alexa 594^+ Area refers to CD3 & B220). The data, displayed as graphs are shown below the sections. (b) Splenic sections were stained with antibodies ERTR9 and anti-MOMA-1 which recognised MZM (in red) and MMM (in green) respectively. The distinct demarcation of T and B cells within the splenic white pulp is lost with increasing age, which coincides with alterations in the marginal zone. Magnification x100. Data representative of four experiments. * P <0.05; ** P <0.01.

Figure 2. Stromal changes within spleen of young and old mice. (a) Sections were stained with the anti-gp38 (podoplanin; in red) which detects the T cell stroma and anti-CD157 (BP-3) which detects the B-cell stroma (shown in green). The level of staining was assessed by Image J analysis (graphs on the right hand side) revealed an increase in the T cell stromal area. Magnification x100. (b) Staining for the fibroblastic reticular cell (FRC) with ERTR7 Ab revealed an increased deposition of FDC around the marginal zone in the splenic sections from older mice. Magnification x100. (c) Staining for follicular dendritic cells (FDC) using anti-FDC-M2 revealed the network of FDC appears more dispersed in the splenic sections from older mice

Magnification x200. Isotype controls revealed no staining (data not shown). Data representative of four experiments. * P <0.05; ** P <0.01.

Figure 3. Architectural changes in the splenic microenvironment following immunisation with a T-independent antigen. (a) Blood was taken from young (1 month) and old (18 months) mice at days; 0, 3, 7, 14, 21 and 28 post immunisation with TNP-Ficoll and the specific anti-IgM TNP-Ficoll response was determined by ELISA. Both young and old mice show a similar increase in the IgM response after immunisation, although the level of the anti-IgM TNP-Ficoll antibody is initially higher at day 0 and 3 in older animals. Six mice were used for each time point. * P <0.05; *** P <0.001. Data representative of three experiments. (b) Splenic sections of young and old mice were stained to detect T (red) and B cells (green) and MZM (red) and MMM (green). Following immunisation, the distinct demarcation of the T and B cell zone of both age groups is disrupted (d3), which is resolved by day 28 in young, but is still disorganised in the spleen of old mice. Magnification x100. Insert of an area highlighted by a white box is shown representing a higher magnification (x200). In the spleen of young mice MMM and MZM encircles the marginal zone which is incomplete, despite the appearance of MZM, in old animals. Magnification x100. Isotype controls revealed no staining (data not shown). Data representative of four experiments.

Figure 4. Alterations in the splenic microenvironment following immunisation with a T-dependent antigen. (a) Young (1 month) and old (18 months) mice were immunised with TNP-KLH at day 0 and day 28, and blood was taken from

young (1 month) and old (18 months) mice at days; 0, 7, 14, 21, 28 and 35 post immunisation. Graph shows the level of anti-IgG TNP-KLH antibody increases after immunisation in both age groups; young mice show a higher level at day 21. Although older mice showed a higher level prior to immunisation. Six mice were used for each time point. * P <0.05; ** P <0.01. Data representative of three experiments. (b) Splenic sections of young and old mice were stained with anti-CD3 (green) and anti-B220 (red) and revealed disruption of the T and B cell zone in both age groups, although it was more apparent in the sections from older mice. Sections stained with MZM (red) and MMM (green) reveals spleen of young mice MMM and MZM encircles the marginal zone which is incomplete, despite the appearance of MZM, in old animals. Magnification x100. Isotype controls revealed no staining (data not shown). Data representative of four experiments.

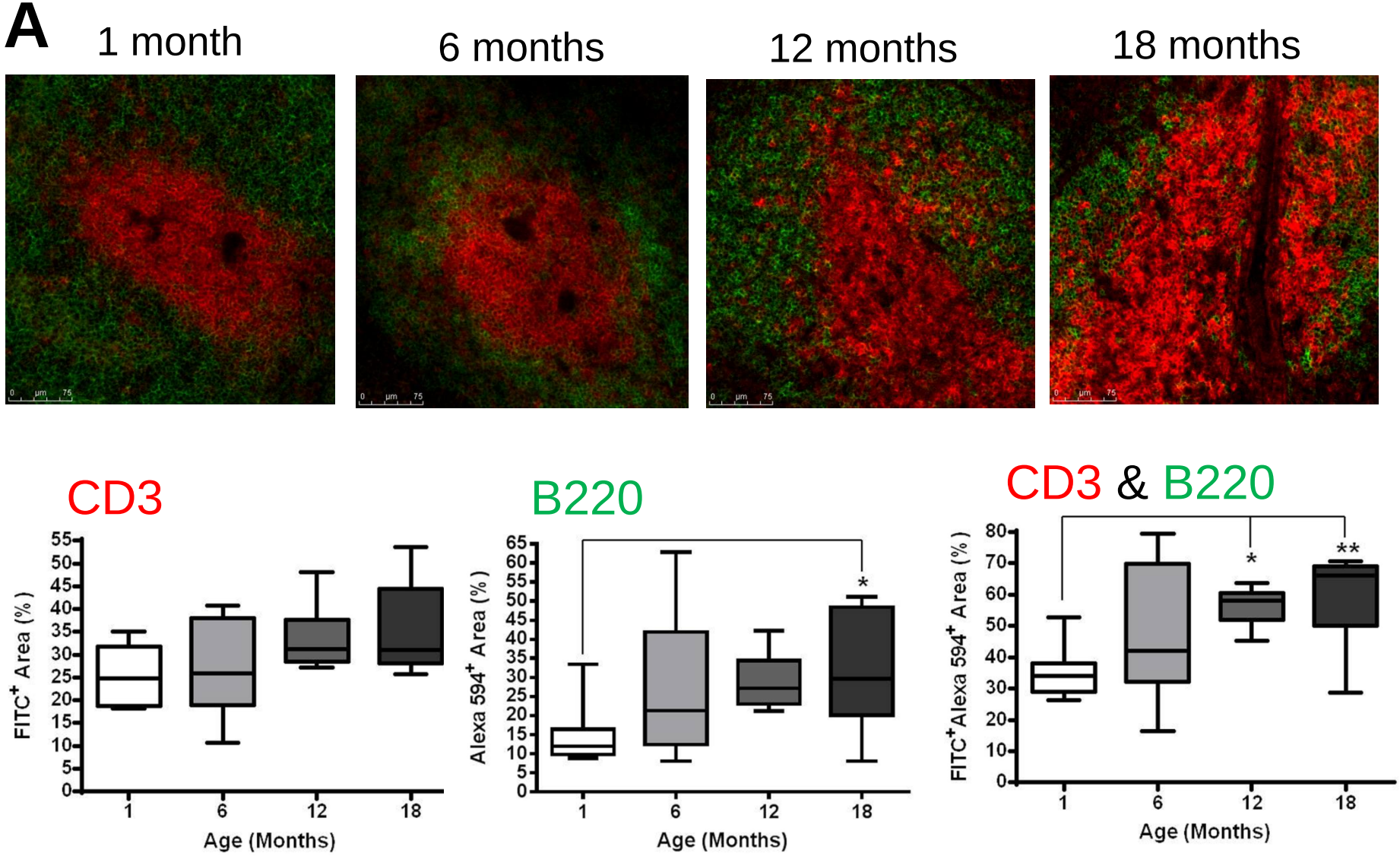

A
1 month
6 months
12 months
18 months
CD3
FITC+ Area (%)
Age (Months)
B220
Alexa 594+ Area (%)
Age (Months)
CD3 & B220
FITC+Alexa 594+ Area (%)
Age (Months)

Fig 1a

B

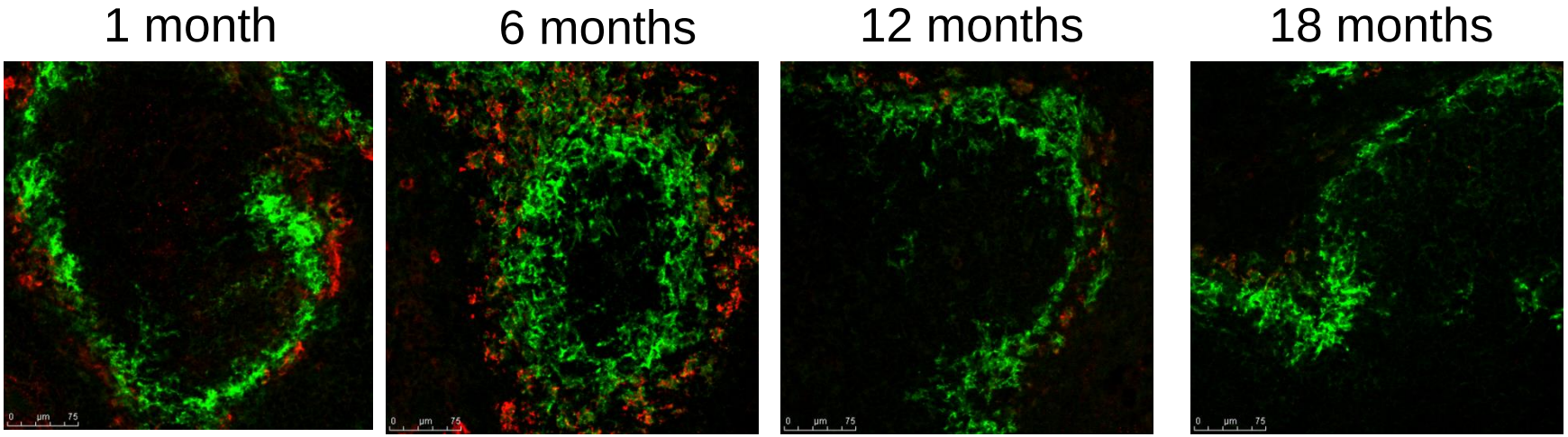
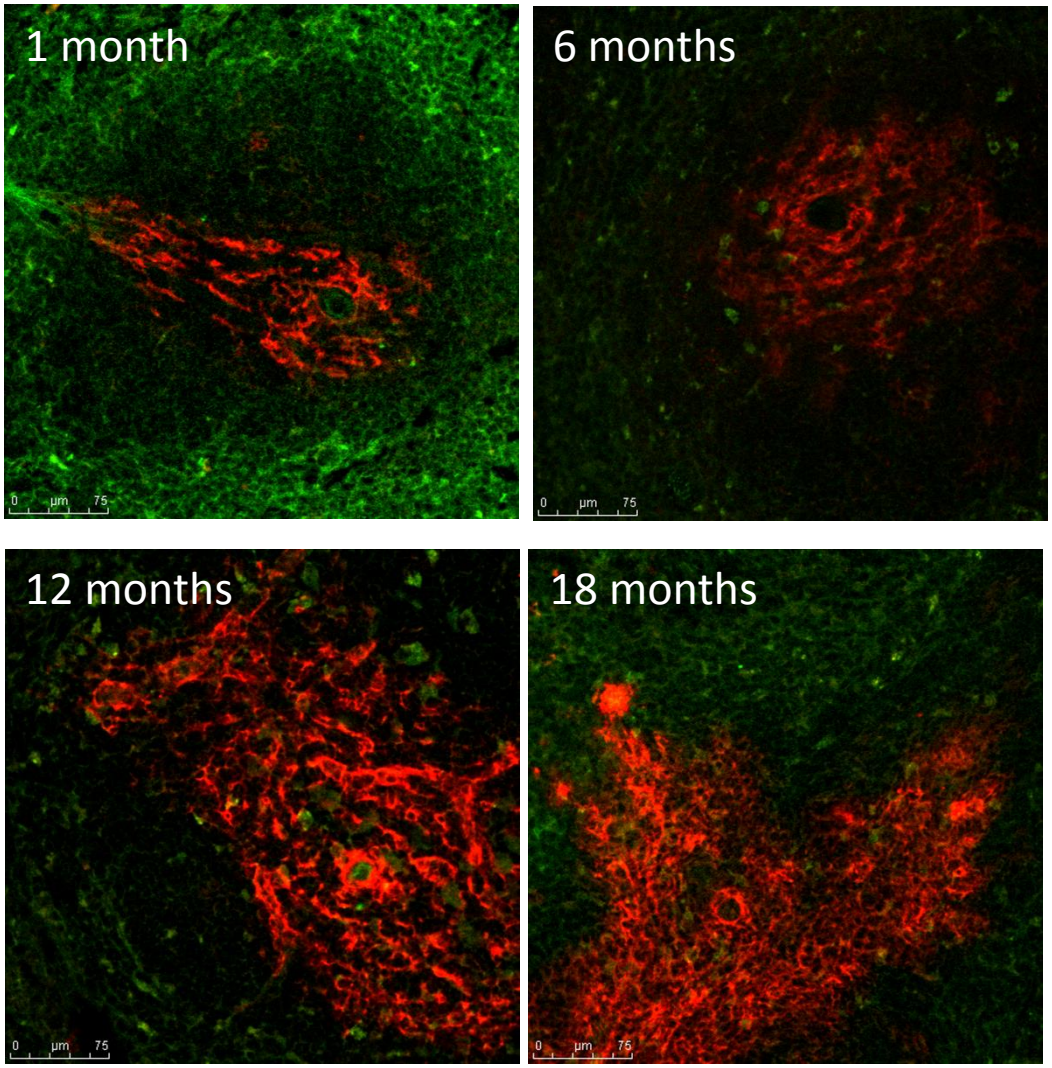
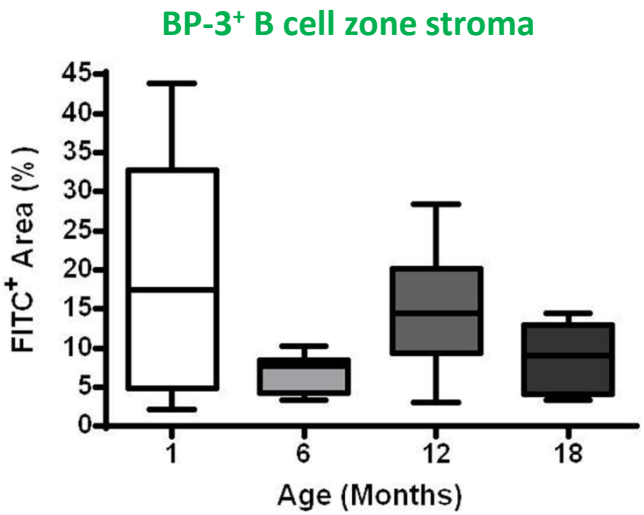


Fig. 1b

A


1 month
6 months
12 months
18 months


BP-3+ B cell zone stroma
FITC+ Area (%)
45
40
35
30
25
20
15
10
5
0
1
6
12
18
Age (Months)

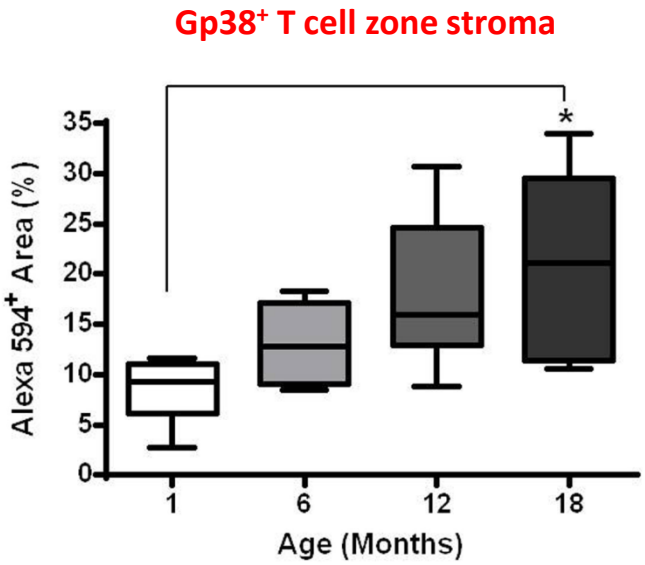
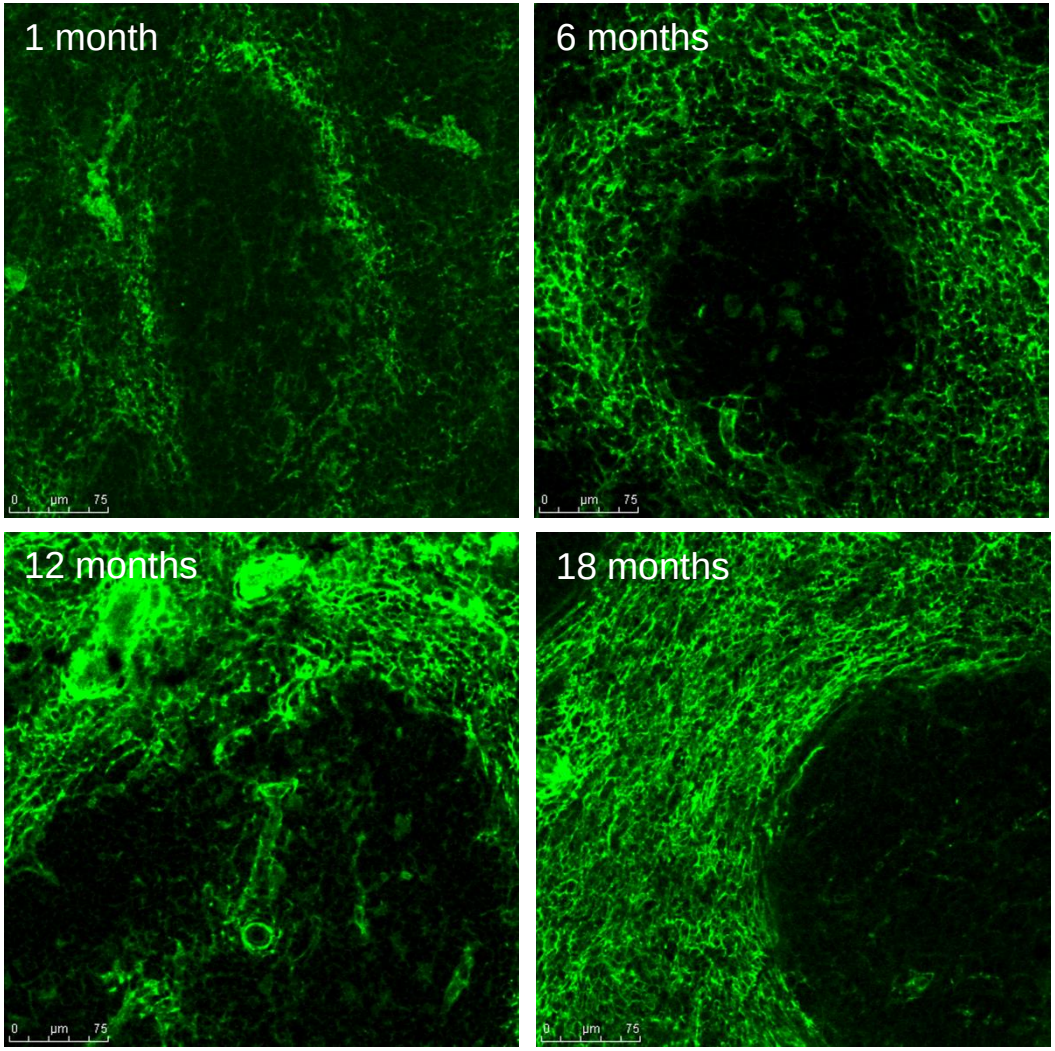

Gp38+ T cell zone stroma
Alexa 594+ Area (%)
35
30
25
20
15
10
5
0
*
1
6
12
18
Age (Months)

Fig 2a

B


1 month
6 months
12 months
18 months
0 µm 75

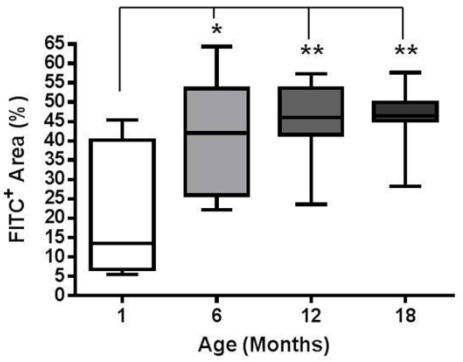

FITC+ Area (%)
65
60
55
50
45
40
35
30
25
20
15
10
5
0
*
**
**
1
6
12
18
Age (Months)

Fig 2b

C

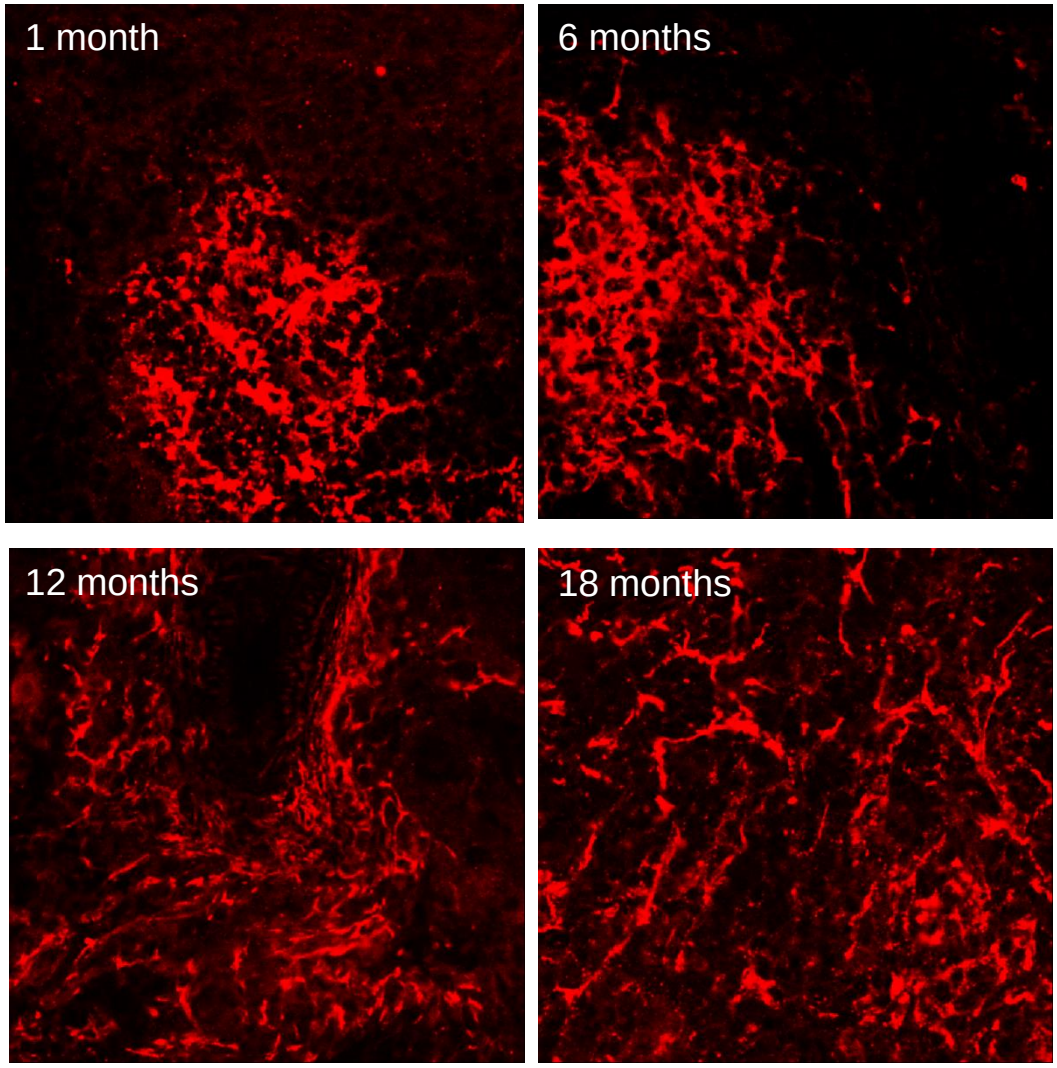
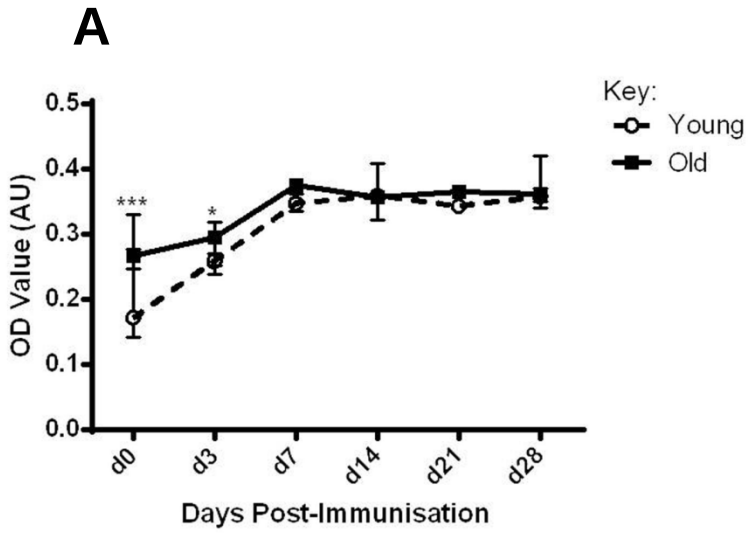


Fig 2c


A
Key:
Young
Old
OD Value (AU)
0.5
0.4
0.3
0.2
0.1
0.0
***
*
d0
d3
d7
d14
d21
d28
Days Post-Immunisation

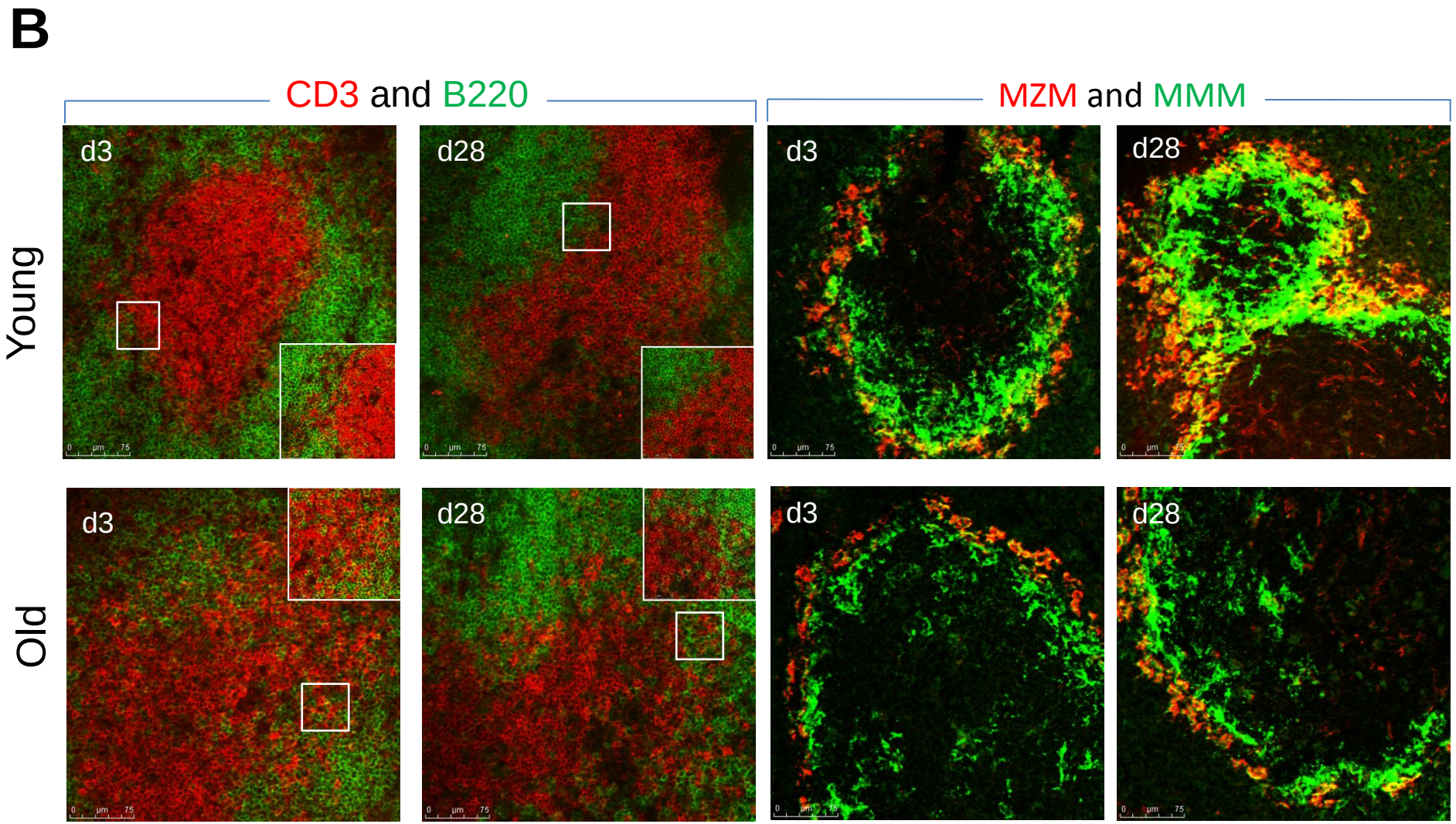
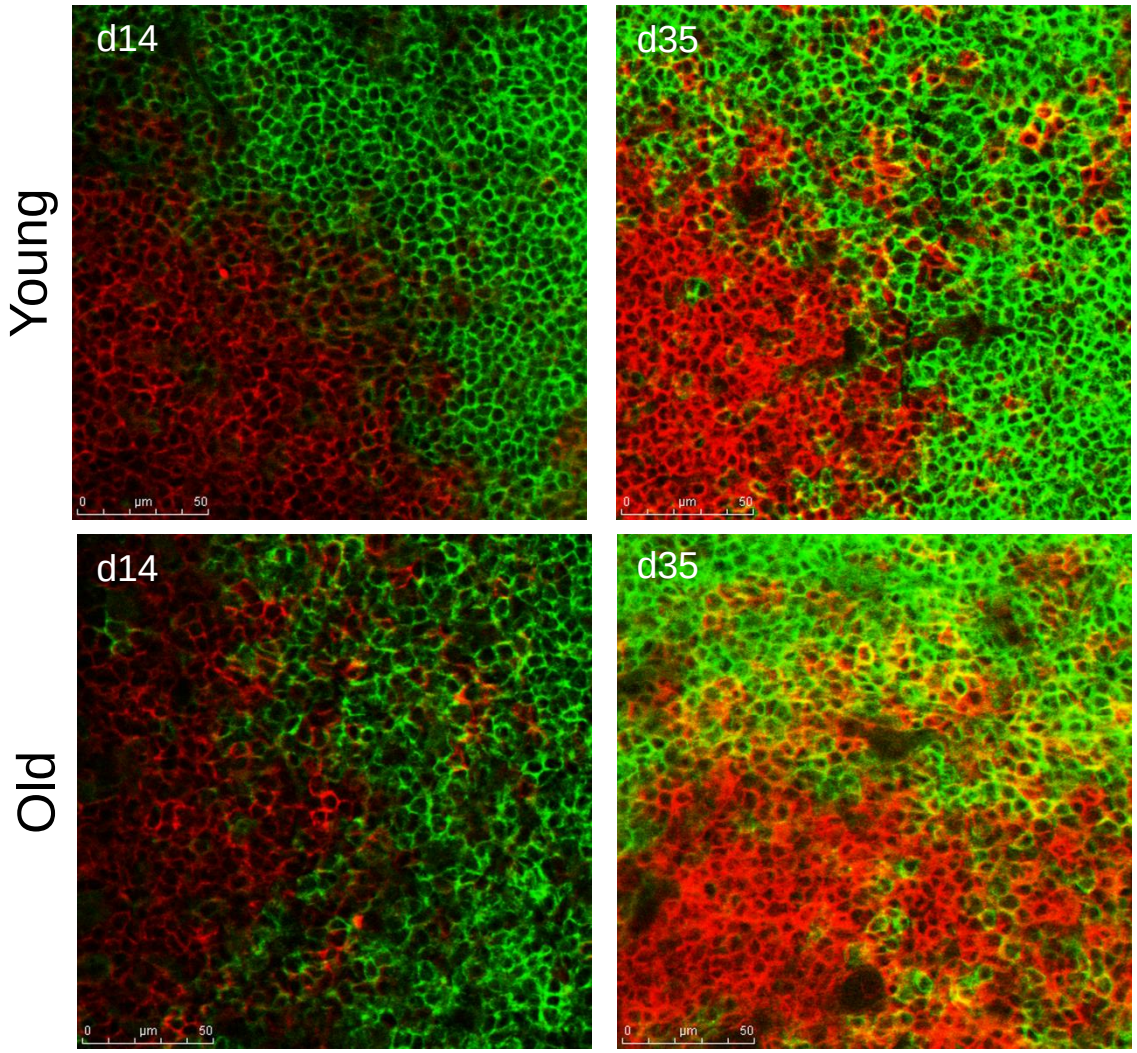
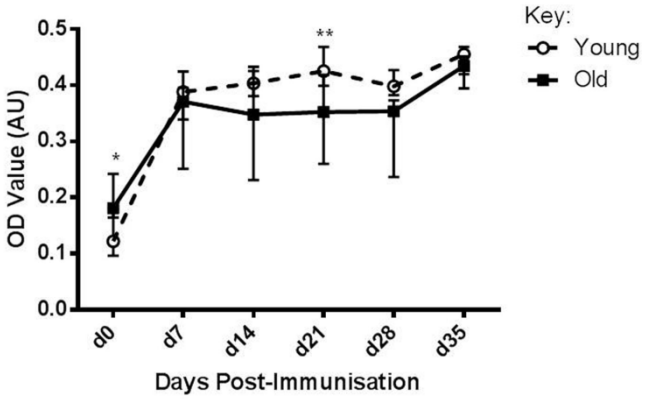


Fig 3b


Young
Old
d14
d35
d14
d35
0
µm
50


OD Value (AU)
0.0
0.1
0.2
0.3
0.4
0.5
*
**
d0
d7
d14
d21
d28
d35
Days Post-Immunisation
Key:
Young
Old