Final Report

International Leibniz Research Cluster "ImmunoMemory"

Leibniz-Institute: Deutsches Rheuma-Forschungszentrum Berlin,
Ein Institut der Leibniz-Gemeinschaft

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Executive summary

The project “ImmunoMemory” aimed at understanding the molecular and cellular mechanisms of the organization of protective and pathogenic immunological memory. To carry out this challenge, the DRFZ established an international research network with 6 national and international partners and a new Junior Research Group, headed by Koji Tokoyoda, an expert in the field of mesenchymal stromal cell biology, at the DRFZ. We have already suggested that stromal cells organize immunological memory. In collaboration with the network partners and external cooperators, we here determined how protective and pathogenic memory cells are generated and maintained. The network coordinator, Koji Tokoyoda has so far shown that memory T helper cells reside and rest in their survival niches composed by interleukin (IL)-7-expressing stromal cells in the bone marrow. Here we focused on the generation and maintenance of memory T helper cells. We identified two activation markers, CD69 and CD49b (also a receptor of collagen types I, II and XI), which are functionally required for the generation of memory T helper cells in the bone marrow. The precursors of memory T helper cells express CD49b and the transcription factor T-bet and comprise 10-20% of activated CD4 T cells in the spleen. Furthermore, we could demonstrate that these precursors transmigrate through collagen type I-expressing bone sinusoid endothelial cells into the marrow using CD49b. In the bone marrow, they first stay on collagen type II-expressing stromal cells and later move and reside in collagen type XI- and IL-7-co-expressing stromal cells in the memory phase. These results suggest that at least one endothelial cell population and two stromal cell populations organize the generation and maintenance of memory T helper cells. In collaboration projects, we also described that the bone marrow maintains memory CD8 T cell, resting in terms of transcription and proliferation. Comparable to CD4 memory T cells, these cells colocalize with IL-7 expressing stromal cells. Moreover, the analyses of CD4 T cells from human bone marrow revealed that also in humans, bone marrow CD4 memory T cells are not activated and rest in terms of proliferation, transcription, and mobility, rather than being pre-activated as assumed before. These comprehensive studies re-defined the understanding of immunological memory, i.e. bone marrow stromal cells organize the migration, residency and survival of memory cells. The results obtained in this international and collaborative project significantly add on the understanding of the regulation of immunological memory. This is the prerequisite for the improvement of current vaccination strategies, and the development of novel ones as well as for the development of selective and curative personalized therapies for inflammatory diseases.

1 Initial question and goals

Immunological memory is a key feature of immunity, mediating protection against pathogens encountered before, but also maintaining chronic inflammation. In recent years, there has been tremendous progress in the understanding of immunological memory. While it was assumed that immunological memory would be maintained by circulating effector cells and chronic immune reactions, we have challenged this paradigm and demonstrated that memory cells are long-lived, like memory plasma cells and resting memory T helper cells. Moreover, we identified their pathogenic counterparts, i.e. autoantibody-secreting memory plasma cells and pathogenic memory T cells. Both cell types are highly refractory to conventional therapies and thus are the main drivers of chronicity of inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis.

Memory plasma and memory T helper cells are maintained in the tissue of the bone marrow by stromal cells, providing signals and soluble factors essential for their survival (Tokoyoda K. et al., Nat. Rev. Immunol. 2010). However, especially for memory T cells, little was known about the mechanisms mediating their establishment and survival. Also, the role of stromal cells for other memory cells of the adaptive immune system, such as memory CD8 T cells or memory B cells, was hardly described. To identify the critical steps and the cells and molecules involved in the generation and maintenance of immunological memory, the German Rheumatism Research Center Berlin, an Institute of the Leibniz-Association, has initiated the International Leibniz Research Cluster "ImmunoMemory" in 2012.

The research cluster brought together experts working on various aspects of immunological memory: **Koji Tokoyoda** originally from Chiba University in Japan was the first who described that stromal cells of the bone marrow organize immunological memory. He has shown that a population of VCAM-1 (a stromal cell marker) positive, non-lymphoid stromal cells producing a chemokine CXCL12 is essential to maintain memory plasma cells in the bone marrow (Tokoyoda K. et al., Immunity 2004). Addressing the role of bone marrow for memory T cells, he identified another population of VCAM-1 positive, IL-7-producing stromal cells that maintain memory CD4 T helper cells (Tokoyoda K. et al., Immunity 2009). In the bone marrow, these memory T helper cells express the surface markers Ly-6C and CD44. They are resting in terms of proliferation and transcription and depend on the presence of the cytokine IL-7. However, the molecular mechanisms involved in the establishment and maintenance of bone marrow memory T helper cells remained unclear. Which molecules are involved in the immigration of memory T helper cells into the bone marrow? And how are memory T helper cells retained in the tissue? These were major questions to be addressed by the research cluster. Moreover, a critical step in the generation of memory T cells is their activation. What are the precursors of bone marrow memory T helper cells? Are B cells required for the generation of these memory cells? To address these questions, we cooperated with **Thomas Winkler** form the University of Erlangen, an expert on B cell biology.

The bone marrow also harbors memory CD8 T cells (Mazo I.B. et al., Immunity 2005). These cells were believed to be maintained as activated cells by homeostatic proliferation (Parretta E. et al., J. Immunol. 2008). The identification of memory T helper cells in the bone marrow, maintained as resting cells by IL-7-producing stromal cells, raised the question if this concept also applies for memory CD8 T cells, or at least for a subpopulation of them? In cooperation with Koji Tokoyoda, the lab of **Andreas Radbruch** at the DRFZ addressed this question. Here, the team got support by the lab of the network partner **Dirk Busch** from the Technical University in Munich. His lab established a technique allowing for tracking specific CD8 T cells through all phases of an immune response into the memory phase.

CD4 and CD8 T cells, B cells and plasma cells are cells of the adaptive immune system, characterized by the expression of specific antigen receptors and the property to establish an antigen-specific memory. In contrast, innate lymphoid cells (ILCs) lack the expression of antigen-specific receptors. ILCs play an important role in tissue homeostasis and in the defense against pathogens and furthermore contribute to inflammatory disorders. In the last

years, ILCs have emerged as a new relevant field in immunology. Based on the transcription factors that govern their differentiation, as well as the cytokines they produce, ILCs are currently categorized into three groups, sharing similarities with T cell subsets. It has also been discussed that cells of the innate immune system can develop into memory-like cells. To understand the regulation of the development of ILCs on the molecular level, we included **Lorenzo Moretta**, University of Genova and Instituto Gianna Gaslini Genova, Italy, in the Leibniz research cluster.

To create the best conditions to conduct the research, one main goal of the project was to strengthen the DRFZ´s expertise on the biology of mesenchymal stromal cells by establishing a new junior research group. Koji Tokoyoda came from Japan to the DRFZ to head the newly installed group "Osteoimmunology". With the focus on the biology of mesenchymal stromal cells and their role in supporting memory lymphocytes, this group perfectly complemented the existing immunological expertise of the DRFZ to investigate the interactions between stromal cells and memory lymphocytes. Koji Tokoyoda already collaborated with the lab of Andreas Radbruch for the studies identifying the stromal cells maintaining memory plasma cells and memory T helper cells. Within the Leibniz Research Cluster "ImmunoMemory", Koji Tokoyoda also cooperated with the network partner **Thomas Pap** from the University of Münster. He was focusing on the biology of stromal cells of the synovial tissue. These sites are severely inflamed in rheumatic diseases, with infiltrates of several cell types. Koji Tokoyoda coordinated the Leibniz Research Cluster.

2 Project results

2.1 Bone marrow memory T helper cells

In previous studies Koji Tokoyoda described that, after activation in secondary lymphoid organs, antigen-specific CD4 T cells relocate to the bone marrow. Here, these cells rest in terms of proliferation and gene transcription next to IL-7-expressing stromal cells (Tokoyoda K. et al., Immunity 2009; Tokoyoda K. et al., Nat. Rev. Immunol. 2010). In the network project, we analyzed the generation and maintenance of memory T helper cells at a molecular level.

2.1.1 CD49b and CD69 are essential for the generation of memory T helper cells in the bone marrow

During an immune reaction, a fraction of antigen-specific CD4 T cells migrates into the bone marrow and differentiates into resting memory T helper cells. The molecules regulating the formation of bone marrow memory T helper cells were unknown. Comparing the transcriptome of $CD44^{high}$ CD4 T cells from the spleen and bone marrow, we found that memory T helper cells in the bone marrow expressed significantly upregulated levels of CD69 as well as CD49b (Tokoyoda K. et al., Immunity 2009). We then demonstrated that CD69, a member of the C-type lectin family, is required for the generation of memory T helper cells in the bone marrow (Shinoda K. et al., Proc. Natl. Acad. Sci. USA 2012): CD69-deficient activated CD4 T cells failed to relocate to the bone marrow and therefore to differentiate into memory T helper cells. Moreover, CD69 is also required for the persistence of memory T helper cells in their survival niches: Activated, CD69-blocked CD4 T cells could not contact stromal cells in the bone marrow. Injection of the Fab fragment of anti-CD69 antibodies inhibited the relocation of activated CD4 T cells to the bone marrow, suggesting that CD69 is a homing receptor of CD4 T cells into the bone marrow. Besides the central role of CD69, the adhesion molecule CD49b was identified as a crucial factor for the generation and maintenance of memory T helper cells in the bone marrow. CD49b, also known as integrin α2, is a receptor of collagen types I, II and XI. CD49b-deficient or -blocked activated CD4 T cells failed to relocate to the bone marrow (Figure 1) (Hanazawa A. et al., Immunol.

Cell. Biol. 2013). Interestingly, these cells could not transmigrate through collagen type I-expressing sinusoidal endothelial cells of the bone marrow.

The data suggests that activated CD4 T cells egress from secondary lymphoid organs and migrate via the peripheral blood onto the sinusoids of the bone (Figure 1). In the bone sinusoids, they contact sinusoidal endothelial cells by using CD69 and then transmigrate into the marrow by using CD49b (Hanazawa A. et al., Front. Immunol. 2013). Although CD69 and CD49b had been known before as activation markers, we could show that these molecules are functional adhesion molecules for memory T helper cells. The high impact of these findings has been commented by Dr. Schoenberger (Schoenberger S.P., Proc. Natl. Acad. Sci. USA 2012).

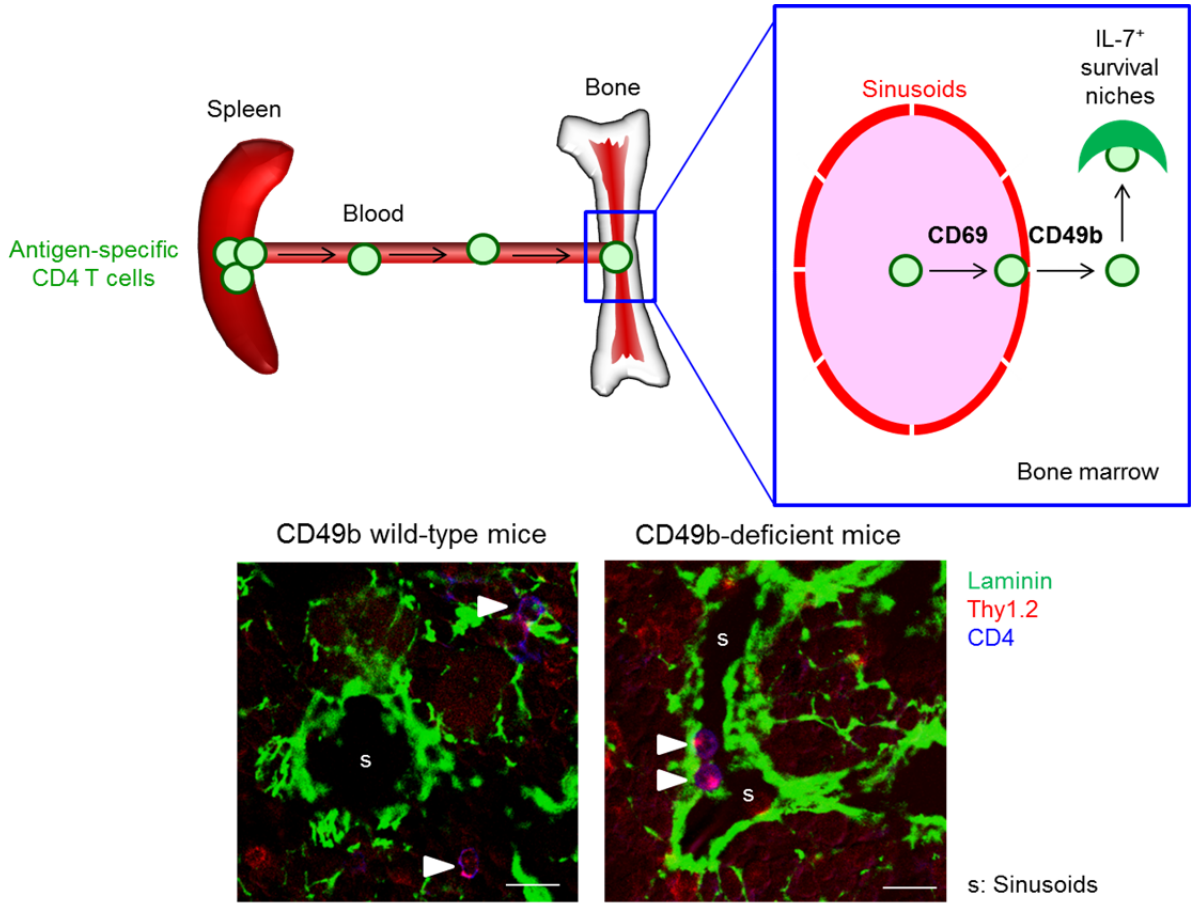


Figure 1. CD49b and CD69 are essential for the establishment of memory T helper cells in the bone marrow. In a primary immune response, a fraction of activated antigen-specific CD4 T cells migrate into the bone marrow via blood. In the bone sinusoids, migrated CD4 T cells contact sinusoidal endothelial cells by using CD69 and then transmigrate into the marrow by using CD49b. Two photos in lower panel show representative localization of wild-type (left) or CD49b-deficient (right) CD4 T cells in the bone sinusoids (s). Frozen sections of bone marrow of wild-type or CD49b-deficient mice were stained with antibodies to laminin (green, a marker of sinusoids), Thy1.2 (red, a marker of T cells) and CD4 (blue). CD4 T cells (Thy1.2$^+$CD4$^+$) are shown in purple and by white arrows. Scale bar: 10 µm.

2.1.2 *Memory T helper cells are essential for the establishment of bone marrow plasma cells*

Studying the role of CD69 for bone marrow memory T helper cells, we have analyzed the immune response in mice adoptively transferred with CD69-deficient CD4 T cells. As described in section 2.1.1, CD69-deficient CD4 T cells did not migrate into the bone marrow and did not differentiate into memory cells. Moreover, CD69-deficient CD4 T cells failed to facilitate the production of high-affinity antibodies, although effector T helper cells (T follicular helper cells) and germinal centre B cells in the spleen were normally generated (Shinoda K. et al., Proc. Natl. Acad. Sci. USA 2012). In fact, an immune response in mice adoptively transferred with CD69-deficient CD4 T cells reduced the generation of long-lived plasma cells in the bone marrow by about 70%, although normal numbers of plasma cells were generated in the spleen. Moreover, transferred normal plasma cells failed to migrate into the bone marrow of CD69-deficient mice. Thus, we suggest that bone marrow memory T helper cells are required for the migration and/or persistence of plasma cells in(to) the bone marrow (Figure 2). How CD4 T cells help plasma cells in the bone marrow at the molecular level will be investigated in the future.

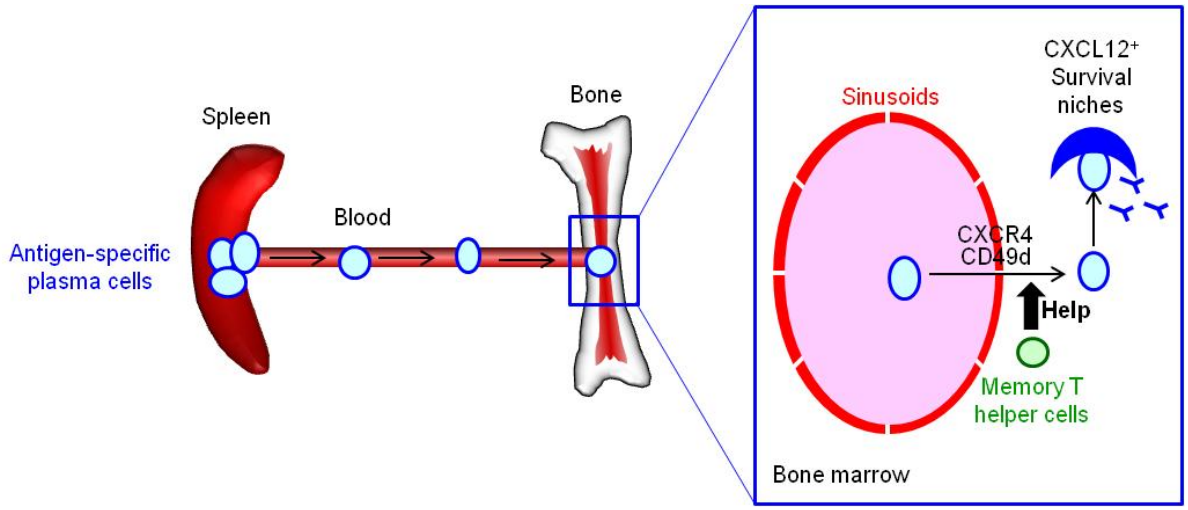


Figure 2. Memory T helper cells help the establishment of bone marrow plasma cells. In the absence of bone marrow memory T helper cells, long-lived bone marrow plasma cells were not generated and high-affinity antibodies were not provided.

2.1.3 *B cells suppress the establishment of memory T helper cells in the bone marrow*

Most recently, we showed how the precursors of bone marrow memory T helper cells are generated in the secondary lymphoid organs in the primary immune response (Hojyo S. et al., Front. Immunol. 2016). Several studies have so far shown, that B cell depletion reduces the persistence of memory T helper cells in the spleen (Linton P.J. et al., J. Immunol. 2000; van Essen D. et al., J. Immunol. 2000; Misumi I. et al., J. Immunol. 2014). To analyze the role of B cells for the generation of bone marrow memory T helper cells, we studied an immune response induced in mice pre-treated with rat anti-IgD and then mouse anti-rat IgG to deplete B cells. Surprisingly, the number of specific memory T helper cells was significantly higher in the bone marrow of B cell-depleted mice, although similar numbers were detected in the spleen. On the other hand, the co-transfer of B cells suppressed the establishment of bone marrow memory T helper cells in mice lacking B and T cells. We have also identified the precursors of bone marrow memory T helper cells. These cells co-express CD49b and T-bet (a transcription factor regulating interferon-γ) and constitute 10-20% of activated CD4 T cells in

the spleen (Figure 3A). The precursors preferentially localized in the red pulp area of the spleen (Figure 3B) and then migrate into the bone marrow in a CD49b-dependent and T-bet-independent manner. The result suggests that B cells negatively control the generation of memory T helper cells and may play a role in the bifurcation of activated ”effector” and resting ”memory” T helper cell lineages. We are currently investigating where CD4 T cells finally differentiate into memory cells and how they survive in the bone marrow.

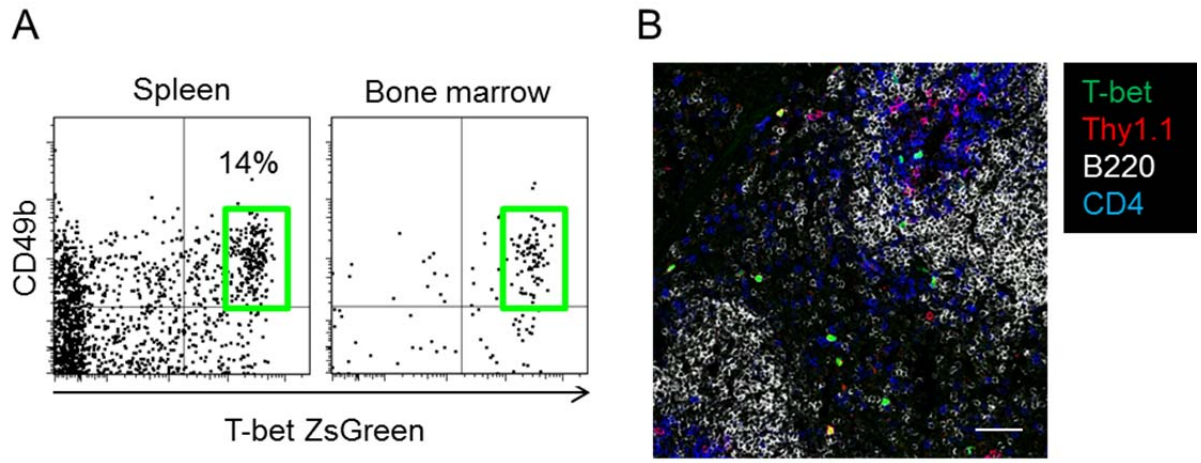


Figure 3. B cells suppress the establishment of memory T helper cells in the bone marrow. (A) CD49b^{+}T-bet^{+} antigen-specific CD4 T cells are the precursors of bone marrow memory T helper cells. Purified Thy1.1^{+} T-bet-ZsGreen reporter lymphocytic choriomeningitis virus (LCMV)-TCR transgenic CD4 T cells were transferred into C57BL/6 mice followed by immunization with LCMV peptide plus lipopolysaccharide. On day 6, CD49b^{+}T-bet^{+} (ZsGreen^{+}) population in Thy1.1^{+} CD4 T cells in the spleen and bone marrow was analyzed by flow cytometry. CD49b^{+}T-bet^{+} antigen-specific CD4 T cells were enriched at the highest percentage in the bone marrow. (B) T-bet-expressing antigen-specific CD4 T cells preferentially localize in the red pulp of the spleen. Frozen section prepared from the spleen of a host mouse from Figure 3A were stained with anti-Thy1.1 (red), anti-B220 (gray, a marker of B cells), and anti-CD4 (blue) antibodies. T-bet^{+}Thy1.1^{+} cells preferentially localize in the B220^{-}CD4^{-} red pulp area compared with T-bet^{-}Thy1.1^{+} cells.

2.1.4 The number of bone marrow memory T helper cells is independent of the magnitude and duration of the immune reaction

We have shown that more than 95% of memory T helper cells in the bone marrow co-localize with IL-7-expressing stromal cells, which comprise about 2% of the total bone marrow cell population (Tokoyoda K. et al., Immunity 2009). These IL-7-expressing stromal cells are defined as the survival niches for memory T helper cells in the bone marrow. Some studies show that newly-generated memory T helper cells compete with older ones (Hataye J. et al., Science 2006; Blair D.A. and Lefrancois L., Proc. Natl. Acad. Sci. USA 2007), suggesting that the number of memory cells may be limited. We compared immune responses to an LCMV peptide with responses to a natural LCMV infection and found that they substantially differ in terms of magnitude and duration. On day 117 post-immunization with LCMV peptide, more than 90% of LCMV-specific CD4 T cells reside in the bone marrow. On day 257 post-infection, 45% of the specific CD4 T cells reside in the spleen and 55% in the bone marrow. However, the absolute numbers of specific memory T helper cells in the bone marrow were comparable, irrespective of the adjuvant (Hanazawa A. et al., Front. Immunol. 2013). In addition, we detected similar absolute numbers of antigen-specific memory T helper cells in

the bone marrow in response to ovalbumin plus lipopolysaccharide or in response to ovalbumin in the precipitated form (aluminum hydroxide; soluble lipopolysaccharide induces transient stimulation, while aluminum hydroxide induces persistent stimulation). The threshold of the immune response reflects the number of long-lasting effector cells in the secondary lymphoid organs, whereas the number of resting T helper memory cells is strictly controlled by their survival niches. These results provide the basis for easier and safer vaccination strategies by using vaccination antigens and adjuvants inducing only weak effector responses, but yet generating solid memory T helper cell numbers.

2.2 Bone marrow plasma cells disappear in Salmonella infection

Salmonella enterica are gram-negative bacteria and causes illnesses such as (para) typhoid fever and food poisoning. Salmonella is an intracellular pathogen in macrophages and can escape from the host immune systems, resulting in the establishment of systemic infection. It remained unclear why Salmonella can survive in the body for a while and how Salmonella escapes from humoral immunity. Together with an expert of microbiology, Prof. Yamamoto from Chiba University, Dr. Tokoyoda analyzed the maintenance of bone marrow plasma cells upon Salmonella infection. Surprisingly, Salmonella infection specifically and dramatically reduced the number of IgG-secreting plasma cells in the bone marrow (Figure 4), but not bone marrow IgM-secreting and splenic IgM- or IgG-secreting plasma cells. We could further show that the reduction was also induced by culture supernatant of Salmonella and was independent of Toll-like receptor ligands, liposaccharide and flagellin. Infection studies with several mutant Salmonella lines and chemical studies with chromatography and mass spectrometry revealed that this effect seems to be mediated by a protein which is secreted from Salmonella. The study is ongoing and we are currently performing experiments to decipher this effect on the molecular level. The findings will directly contribute to the improvement of vaccine against *Salmonella* by using a mutant strain lacking the protein.

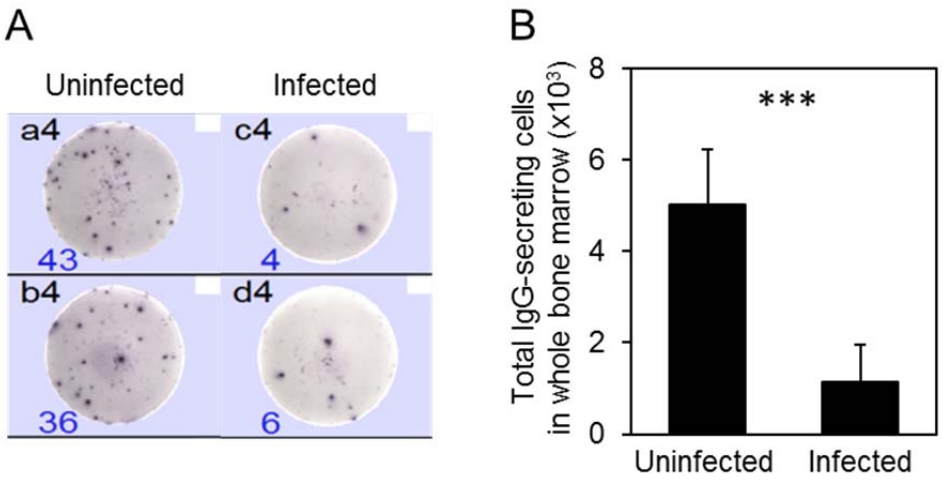


Figure 4. Salmonella reduces IgG-secreting plasma cells in the bone marrow. BALB/c mice were infected intraperitoneally with 10^4 colony forming units of attenuated Salmonella enterica serovar Typhimurium and 4 days later analyzed for IgG-secreting plasma cells in the bone marrow by ELISpot assay. Photos of spots for IgG-secreting plasma cells in the bone marrow (A, duplicate) and a bar graph of the number of the plasma cells (B) are shown.

2.3 The role of bone marrow for the organization of immunological memory

Our data strongly suggests that the organization of memory T helper cells follows the same rules as those of memory plasma cells, which are maintained in a stroma-dependent manner (Tokoyoda K. et al., Nat. Rev. Immunol. 2010). So far, we have demonstrated that in the bone marrow a fraction of activated CD4 T cells first contact collagen type II-expressing stromal cells in the early phase of an immune response and then move to collagen type XI- and IL-7-co-expressing stromal cells (Hanazawa A. et al., Immunol. Cell. Biol. 2013). Thus, at least two stromal cells seem to organize the generation of memory T helper cells in the bone marrow. We have also demonstrated that CD49b, a receptor of collagen types II and XI, is essential for the interaction with both stromal cell types. We have shown earlier that resting memory T helper cells in the bone marrow express the anti-apoptotic protein bcl-2 (Tokoyoda K. and Radbruch A., Cell. Mol. Life Sci. 2012). Other groups have reported that IL-7 is required for survival of memory T helper cells and that IL-7 induces the expression of bcl-2 (Kondrack R.M. et al., J. Exp. Med. 2003). Thus, it is likely that the collagen type XI- and IL-7-co-expressing stromal cells are the cells constituting the final survival niche of memory T helper cells in the bone marrow, inducing the expression of bcl-2 in the memory T helper cells.

Taking all these results into account, we suggest a multistep model in which immigrating T cell moves from one stromal cell to another stromal cell, receiving distinct signals and finally attach to the stromal cell maintaining the resting memory T cell (Figure 5). Thus, different stromal cells would regulate the migration, the differentiation and the maintenance of memory T helper cells in the bone marrow. Further studies are needed to investigate why and how the generation is regulated by distinct stromal cell.

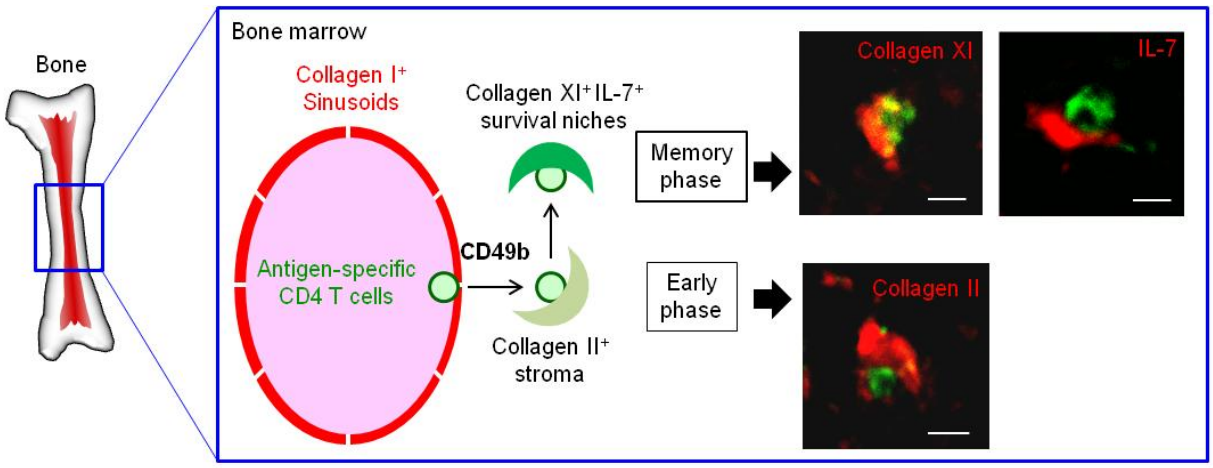


Figure 5. A multistep migration model for the settlement of memory T helper cells in the bone marrow. A fraction of activated CD4 T cells transmigrate through collagen type I-expressing sinusoidal endothelial cells, contact collagen type II-expressing stromal cells and then move to collagen type XI- and IL-7-co-expressing stromal cells. In the early phase of an immune reaction (on day 12 after immunization), antigen-specific CD4 T cells localize with collagen type II-expressing stromal cells that do not express collagen type XI- or IL-7 (lower). In the memory phase of the immune reaction (on day 117 after immunization), antigen-specific CD4 T cells localized on collagen type XI- and IL-7-co-expressing stromal cells (upper). The images show the representative localization of Thy1.1-stained T cells (green) versus collagen type II, XI or IL-7-stained cells (red) in the bone marrow of mice transferred with Thy1.1$^+$ LCMV-TCR transgenic CD4 T cells on day 12 or 117 after immunization with LCMV plus lipopolysaccharide. Scale bar: 5 µm (upper) and 10 µm (lower).

2.4 Contributions of cooperation partners

2.4.1 Memory CD8 T cells in the murine bone marrow

Having demonstrated that the bone marrow harbors a resting plasma cell memory as well as a resting CD4 T cell memory, the question arose if this also applies for CD8 T cells. CD8 T cells play an essential role in the defense against intracellular pathogens, but also in graft rejection. Until recently it was believed that the CD8 T cell memory is maintained by slowly proliferating memory CD8 T cells in secondary lymphoid organs, peripheral tissues and the bone marrow. Now, the lab of Andreas Radbruch from the DRFZ, together with Koji Tokoyoda, described a population of resting memory CD8 T cells in the murine bone marrow. Comparably to resting memory CD4 T helper cells, these cells individually colocalize with stromal cells producing the cytokine IL-7 (Sercan Alp Ö. et al., Eur. J. Immunol. 2015). The regulation of the maintenance of the CD8 T cell memory is also addressed by the lab of our network partner Dirk Busch. By serial adoptive transfers of individual T cells from different T cell populations and infection-driven re-expansions *in vivo*, they now showed that central memory T cells have the capacity to give rise to a diverse CD8 T cell progeny, including T effector memory and T effector cells. These cells thus have the potential to fully reconstitute immunocompetence and demonstrate stem cell behavior (Graef P. et al., Immunity 2014). At present, the relation between these cells and the resting bone marrow memory CD8 T cells is unclear and needs to be addressed in future studies.

2.4.2 Memory CD4 T cells in the human bone marrow

The description of a resting CD4 T cell memory in the bone marrow of mice raised the question whether a similar memory population exists also in the bone marrow of humans. This was recently addressed by the lab of Andreas Radbruch from the DRFZ, together with Koji Tokoyoda. They could show, that compared to memory T cells isolated from blood, memory CD4 T cells isolated from the bone marrow were resting in terms of proliferation, transcription and mobility. Moreover, comparable to murine bone marrow memory T cells, these cells expressed CD69 on their surfaces. Repertoire analyses demonstrated that the memory CD4 T cell population of the bone marrow was mainly specific for systemic antigens, such as tetanus toxoid, rubella and measles (Okhrimenko A. et al., Proc. Natl. Acad. Sci. USA 2014; Dong J. et al., Z. Rheumatol. 2015).

2.4.3 The molecular regulation of tissue-resident group 3 ILCs

Another type of cells having emerged as a new relevant field in immunology is the group of ILCs. At present, ILCs are categorized into three groups, based on the transcription factors directing their functions and the cytokines they produce. Little is known about the factors and signals driving their differentiation. In a joint cooperation with Lorenzo Moretta, the DRFZ analyzed the molecular requirements inducing acquisition of effector programs and differentiation of a defined subset of human ILCs, namely ILC3. ILC3 represent a tissue-resident radioresistant lymphocyte population, which regulates epithelium and commensal homeostasis, while also displaying a dual role in the pathogenesis of chronic inflammatory disorders. It could be shown that ILC3 effector signatures are imprinted early during differentiation, and that this process takes place in the tissue from resident hematopoietic progenitors (Montaldo E. et al., Immunity 2014; Montaldo E. et al., Eur. J. Immunol. 2015).

2.4.4 Memory CD4 T cells in allergy and autoimmune diseases

The labs of Toshinori Nakayama and Hiroshi Nakajima from Chiba University in Japan study the role of pathogenic memory T cells in allergy and autoimmune diseases, respectively. They recently demonstrated that Ezh2 is regulating the differentiation and plasticity of T helper cells. In collaboration with Koji Tokoyoda, they studied the role of Ezh2 in allergy-induced mice. They could show that loss of Ezh2 aggravated allergy, with increased

eosinophilic inflammation and production of Th2 cytokines. The number of memory CD4 T cells in the spleen of Ezh2 deficient CD4 T cells transferred mice significantly increased over time, indicating that Ezh2 is a negative regulator of the generation and maintenance of pathogenic memory T helper cells (Tumes D.J. et al., Immunity 2013). Koji Tokoyoda was also involved in studies describing the role of IL-25 in allergy-induced mice (Kawashima S. et al., Int. Arch. Allergy Immunol. 2013) and the role of mast cells in autoimmune-induced mice (Yokota M. et al., Arthritis Res. Ther. 2014).

2.4.5 Stromal cells supporting hematopoiesis in the fetal liver

Lymphocyte development is known to be highly dependent on the environment during hematopoiesis. In the bone marrow, several stromal cells supporting different developmental stages of early B cell have been described. The lab of Fritz Melchers from the Max-Plank Institute for Infection Biology, Berlin, is aiming at the understanding of the regulation of early B cell development in the fetal liver. Koji Tokoyoda was involved in a study identifying two stromal cell populations in the fetal liver with different functions for hematopoiesis, especially with residency of common lymphoid progenitors and B cell precursors (Tsuneto M. et al., Stem Cells 2013; Tsuneto M. et al., Immunol. Lett. 2014).

2.4.6 The regulation of bone- and cartilage cells in inflamed joints

Non-lymphoid cells are essential regulators of several aspects of lymphocyte biology. Stromal cells control the early development during hematopoiesis, their differentiation as well as their maintenance. These processes take place in the bone marrow and the fetal liver, and the lymphoid organs. Upon activation, lymphocytes migrate to sites of infection and inflamed tissues, locally help to control the antigen and finally clear the infection. Lymphocytes also accumulate in chronically inflamed tissues, e.g. in the inflamed joints of patients suffering from rheumatoid arthritis. This results in a progressive destruction of the cartilage and the bone. The lab of Thomas Pap is aiming at a comprehensive understanding of the molecular regulation of synovial tissue cells, bone-forming osteoblasts and bone-resorbing osteoclasts, as well as the interaction of these cells with lymphocytes. They now described that myostatin is upregulated in the synovial tissue of patients suffering from rheumatoid arthritis, which increases osteoclast formation and thus might accelerates bone loss (Dankbar B. et al., Nat. Med. 2015).

3 Impact of the findings and future perspectives

Within the Leibniz Research Network “Immunomemory”, we further highlighted and emphasized the central role of the bone marrow for the maintenance of long-term memory lymphocytes. The results of our studies revealed further evidence that the generation and establishment of memory lymphocytes in the bone marrow are tightly controlled at several levels. We have identified critical steps and factors for the development of different bone marrow memory lymphocytes such as CD69-dependent migration and adhesion, CD49b-dependent transmigration and residency and IL-7- and collagen type XI-dependent maintenance of memory T helper cells or their precursors.

Understanding the biology and regulation of memory lymphocytes is the prerequisite to improve current vaccination strategies and to develop novel ones in order to achieve effective, long-lasting protection. Besides the central role of memory lymphocytes in providing protective immunity against recurrent pathogens or vaccination antigens, they are also essential players in several diseases, such as autoimmune disorders (e.g. rheumatoid arthritis, systemic lupus erythematosus and multiple sclerosis), as well as in diseases resulting from persistent pathogens and allergies. The researchers at the DRFZ have already

identified pathogenic memory plasma cells and pathogenic memory T lymphocytes in chronic inflammations. There is several evidence that, due to their lifestyles (i.e. longevity and protection in the survival niches) pathogenic memory lymphocytes are refractory to physiological regulation and state-of-the-art therapeutic immunosuppression, and by this induce and maintain immunopathology and chronic inflammation (Hiepe F. et al., Nat. Rev. Rheumatol. 2011; Chang H.D. et al., Ann. Rheum. Dis. 2011). Thus, these cells are a major challenge for the treatment of patients. Understanding the biology of the immunological memory is essential to improve current and develop novel, curative strategies for the treatment of chronic immune-mediated diseases.

Follow-up projects

In our studies we have shown that the survival niches for memory T helper cells are composed by collagen type XI^+ $IL\text{-}7^+$ stromal cells of the bone marrow. However, the molecules mediating the survival and residency of memory T helper cells in the bone marrow are still unknown. In future studies, we will aim at the identification of cytokines, chemokines and adhesion molecules involved in the maintenance of memory T helper cells in the bone marrow. In particular, we will further clarify the roles of CD49b and its ligand collagen type XI, and of IL-7 and its receptor. If the survival and residency of memory T helper cells in the bone marrow cannot be explained by only collagen type XI and IL-7, we will further search for factors involved in the maintenance of memory T helper cells. This will aim at a molecular understanding of how the memory T helper cells are kept in quiescence and survive in their survival niches.

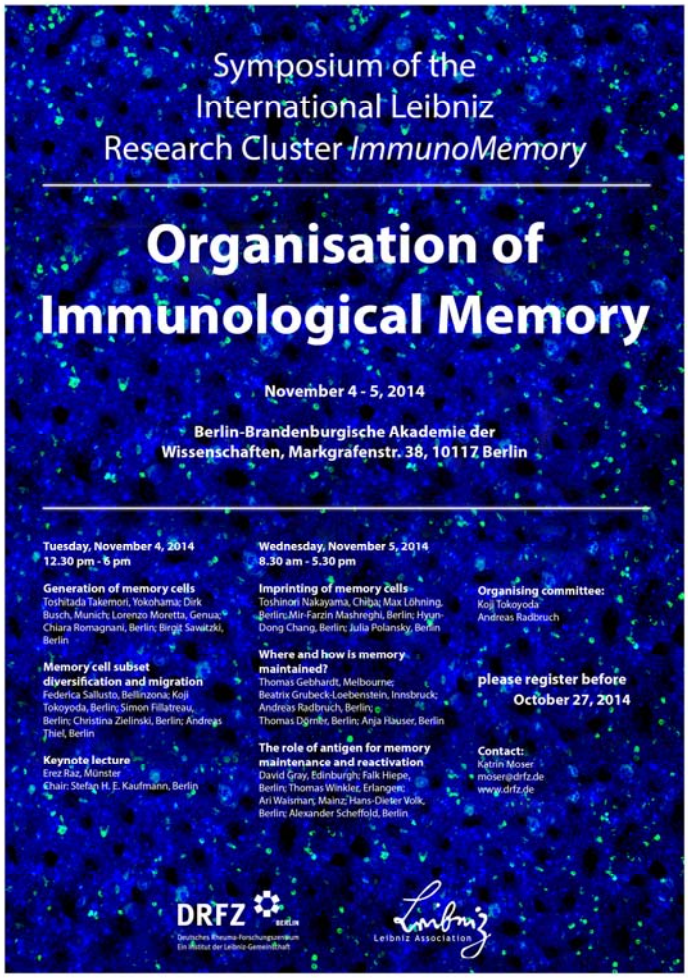
Furthermore, we will determine how memory T helper cells are reactivated in recall responses. Immunological memory provides recall response when previously-encountered pathogens are invaded again. Recall response is an intimate collaboration between memory B and memory T helper cells, resulting in long-lasting humoral protection. However, it remains unclear how and where memory T helper cells reactivate memory B cells. Do the memory T cells encounter the recall antigen in the bone marrow? If so, how does the antigen reach the bone marrow, and what are the antigen-presenting cells activating memory T helper cells in the bone marrow? Or, are the memory T helper cells in the bone marrow activated by non-specific signals, resulting in their relocation from the bone marrow to secondary lymphoid organs where they meet memory B cells? We will track memory T helper cells during recall response and determine how memory T helper cells are reactivated and mobilized in the bone marrow. In particular, we want to determine which cells reactivate memory T helper cells, how memory T helper cells are mobilized in the bone marrow during recall response.

In section 2.2, we have shown that Salmonella infection selectively reduces IgG-secreting plasma cells in the bone marrow. We next want to determine, on the molecular level, how the protein specifically reduces IgG-secreting plasma cells in the bone marrow. Moreover, antibodies of the IgG class play a central role in the pathogenesis of some diseases, e.g. systemic lupus erythematosus. In future studies we will test whether the identified protein also reduces autoantibody-secreting plasma cells in such disease models and whether this improves the pathology. These analyses will add on our understanding how memory plasma cells are maintained in the bone marrow.

4 Reports, public relations, and knowledge transfer

4.1 Scientific Meetings

The International Leibniz Research Cluster “ImmunoMemory” started in April 2012 with a kick-off meeting where the principal investigators met to discuss the actions of the network project in detail. Particularly, by the **Symposium of the International Leibniz Research Cluster *ImmunoMemory* “Organisation of Immunological Memory”** which took place on November 4 - 5, 2014 in Berlin, the Leibniz Cluster obtained international attention. The partners of the research network and 20 other internationally renowned speakers from Japan, Italy, Australia, Great Britain, Austria and Germany came to Berlin to discuss their results and concepts on immunological memory.



The two days were sub-divided into five sessions, covering different aspects of immunological memory. A session *Generation of memory cells* addressed the formation of CD4 memory T cells and CD8 memory T cells. In addition, two speakers presented their latest results on memory-like NK cells. The impact of the antigen and the cytokines milieu during cell activation in an immune response against pathogens and during vaccination was discussed in the session *Memory cell subset diversification and migration*. In a session *Imprinting of memory cells*, the molecular regulation of memory cells by epigenetic modifications, microRNAs and transcription factors was discussed. The session *Where and how is memory maintained* focused on the environment of memory cells, on the cells and molecules involved in the regulation of the lifestyle of different types of memory cells in health and disease. The session *The role of antigen for memory maintenance and reactivation* addressed the role of the antigenic stimulus on the memory formation, with a special focus on autoantigens triggering autoimmune disorders, as well as alloantigens in graft rejection and tolerance. The keynote lecture was given by Erez Raz from the University of Münster who shared his knowledge on cell motility and chemokine - guided migration in the zebrafish.

The meeting attracted nearly 200 scientists from several research institutions in Germany and all over the world, reflecting the high relevance of the research area of the Leibniz Research Cluster. About 50% of the participants were young scientists (PhD students and postdocs). Due to the fast scientific progress and the high impact of the new concepts on the development of vaccines, as well on the treatment of patients, the project members decided to organize a follow up meeting. The Symposium “What´s Immunological Memory?”, taking place in Berlin on October 30 - 31, 2015, was organized together with the *International Immunological Memory and Vaccine Forum (IIMVF),* an initiative of the Graduate School of Medicine, Chiba University, Japan.

4.2 Guests scientists

05/2013 Dr. Shintaro Hojyo (Laboratory for Homeostatic Network, RIKEN Center for Integrative Medical Sciences, Japan); Seminar “The zinc transporter SLC39A10/ZIP10 controls B cell receptor signaling for mature B cell maintenance and humoral immune response”. Shintaro Hojyo became postdoc in the lab of Koji Tokoyoda in 2014.

08/2013 Kaori Shimoyama (Ehime University School of Medicine); Internship student. Kaori Shimoyama contributed to a screening work to find markers of memory T helper cell precursors in Tokoyoda’s lab.

09/2013 Prof. Tomoko Yamamoto and Dr. Akiko Takaya (Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Japan); the lab of Tomoko Yamamoto is studying the host response in bacterial infection (see 2.2).

11/2013 Elisa Montaldo, student from the lab of Prof. Lorenzo Moretta, University of Genova; 2-weeks internship in the lab of Chiara Romagnani (see section 2.4.3).

01/2014 Dr. Kengo Saito (Department of Virology, school of Medicine, Chiba University); Seminar “Oncolytic sindbis virus for cancer therapy”.

04/2014 Kosuke Hino, a diploma student from the lab of Prof. Tomoko Yamamoto (Department of Microbiology and Molecular Genetics, Graduate School of Pharmaceutical Sciences, Chiba University, Japan); 4-weeks internship in Tokoyoda’s lab. He has established a chronic Salmonella infection model.

06/2014 Dr. Naoto Kawakami (Institute of Clinical Neuroimmunology, Ludwig-Maximilians-Universität München); Seminar “Visualizing migration and activation of autoreactive T cells in the CNS”.

09/2014 Prof. Masato Kubo (Laboratory for Cytokine Regulation, RCAI, RIKEN Center for Integrative Medical Sciences); Seminar “Influenza virus vaccination effectively induces the protective antibody response independent on germinal centers”.

12/2014 Dr. Yosuke Endo (Department of Immunology, Graduate School of Medicine, Chiba University); Seminar “Pathogenic memory type Th2 cells in allergic inflammation”.

03/2015 Prof. Takeshi Tsubata (Laboratory of Immunology, School of Biomedical Science, Tokyo Medical and Dental University); Seminar “CD22-binding synthetic sialosides as a tool to elucidate the role of CD22 cis-ligand and as a novel immunostimulant”.

4.3 List of publications resulted from the project

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