Project Proposal for a PhD position
granted by the International Graduate School in Molecular Medicine Ulm

1. Institute Address

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2. Project Title

Towards Understanding Impaired Clearance of Senescent Fibroblasts from Aging Skin - Therapeutical Implications

3. Keywords

Immunosenescence, natural killer cells, macrophages, clearance of senescent fibroblasts

4. Research Training Group

Aging and Degeneration

5. Project Description

Background

Fibroblasts constitute the principal component of the connective tissue. The capacity of fibroblasts to synthesize and organize the extracellular matrix and to communicate with adjacent cells and tissues of distinct histogenetic origin makes them a central component in organ homoeostasis and aging. During aging fibroblasts accumulate higher concentrations of reactive oxygen species (ROS) (Treiber et al., 2011) which contribute to damage of DNA, proteins and other macromolecules. High ROS levels result in an aging stress response which may promote aging and organ atrophy. Senescent fibroblasts adopt i.) an irreversibly growth arrested state by the induction of $p16^{INK4a}$, a cyclin kinase inhibitor and *in vivo* marker for skin and organ aging (Ressler et al., 2006; Krishnamurthy et al., 2004; He and Sharpless, 2017), ii.) a senescence-associated secretory phenotype (SASP) with the release of matrix-degrading enzymes (Waldera-Lupa et al., 2015) and pro-inflammatory chemo- and cytokines (Tchkonia et al., 2013), iii.) resistance to apoptosis and, iv.) possibly impaired removal from the tissue by cells of the innate immune system which in combination with the above mentioned mechanisms might lead to the accumulation of senescent fibroblasts in organs. Senescent fibroblasts may even - by SASP factors - spread senescence to adjacent young fibroblasts, adipocytes, epidermal and other cells (Nelson et al., 2012). During aging senescent $p16^{INK4a}$-positive fibroblasts accumulate in the skin and other organs (Krishnamurthy et al. 2004, Ressler et al., 2006). Genetic depletion of $p16^{INK4a}$-positive senescent cells - though not proven for senescent fibroblasts - leads to a partial rejuvenation of different organs (Baker et al., 2016) supporting the notion that cellular senescence determines organ and organismal aging and related aging disorders. Of note, senescent cells are reliably removed in conditions of wound healing, during embryonic development and in amphibian vertebrates with high regeneration potential by cells of the adaptive immunity like natural killer cells and macrophages (for review see Lujambio, 2016; Demaria et al. 2014; Munoz-Espin et al., 2013; Yun et al., 2015). However, it is largely unknown whether aging of hematopoietic stem cells and the lineages derived thereof like natural killer (NK) cells and macrophages are affected in terms of their clearance capacity of senescent cells from tissues. Macrophages reveal impaired recruitment, but for other functions like phagocytosis and response to LPS/IFNγ the data are controversial (Jackaman et al., 2017). Interestingly, Hall et al. (2016) showed that senescent human fibroblasts spread senescence to a subpopulation of $p16^{INK4a}$- and senescence associated β-galactosidase positive macrophages. Currently it is unclear whether these senescent macrophages (Prattichizzo et al., 2016) loose their potential to remove senescent fibroblasts from the skin and other organs. A hint for this possibility comes from the notion that senescent macrophages are critically involved in the accelerated development of arteriosclerosis, an aging associated vascular disease, in low-density receptor-deficient mice (Childs et al., 2016).

Scientific objectives

The overall objective is to dissect whether the accumulation of senescent fibroblasts in the connective tissue of skin is due to reduced clearance capacity of senescent macrophages and NK cells for $p16^{INK4a}$ senescent fibroblasts *in vitro* and *in vivo*. Based thereon, we will try to enhance the removal capacity of senescent fibroblasts by cells of the innate immune system.

Preliminary work

Employing a senescent fibroblast specific deletion approach, we could show that murine skin can be substantially rejuvenated. These data imply that removal of senescent fibroblasts is of utmost interest for tissue homeostasis and even bears preventive and therapeutic potential to attenuate aging. Similar to Sagiv et al. (2016) we showed that human senescent fibroblasts (replicative senescent and etoposide-induced DNA damaged fibroblasts) but not quiescent and proliferating young fibroblasts are killed by $CD56^{bright}$ CD3-NK cells, and this is due to the interaction of the activating NK cell receptor NKG2D and the corresponding ligands MICA and ULBP2, polypeptides both related to MHC class I expressed on senescent fibroblasts. Also, we have preliminary data that young macrophages are able to remove apoptotic and senescent fibroblasts *in vitro.*

Working programme

The role of natural killer cells and macrophages in the removal of senescent fibroblasts *in vitro*.

The question will be addressed whether NK cells of elderly individuals (>75 years) as opposed to young individuals will display reduced killing activity towards senescent fibroblasts *in vitro* (approved by the ethical committee). Activation markers like CD69, CD107 and phosphorylation of AKT (Lannier et al., 2008) reliably correlating with the killing activity of NK cells and perforin, the effector molecule which initiates target cell lysis, will be studied using flow cytometry and confocal microscopy. The killing activity will be assessed by the cell index indicating the presence of live cells before and after adding NK cells using Real Time Cell Analysis. Similar co-culture experiments will be performed with monocyte/macrophages activated towards a M1 pro-inflammatory phenotype (Sindrilaru et al., 2011) prior to being co-cultured with senescent and young human fibroblasts. Phagocytosis will be analyzed using confocal microscopy and quantified using mass cytometry (Kapellos et al., 2016) **[Milestone M1]**.

Expected results: These *in vitro* data will give insight into the consequences of aging of the adaptive immune system and whether this affects the killing ability of NK cells and phagocytosis of senescent connective tissue resident fibroblasts by macrophages.

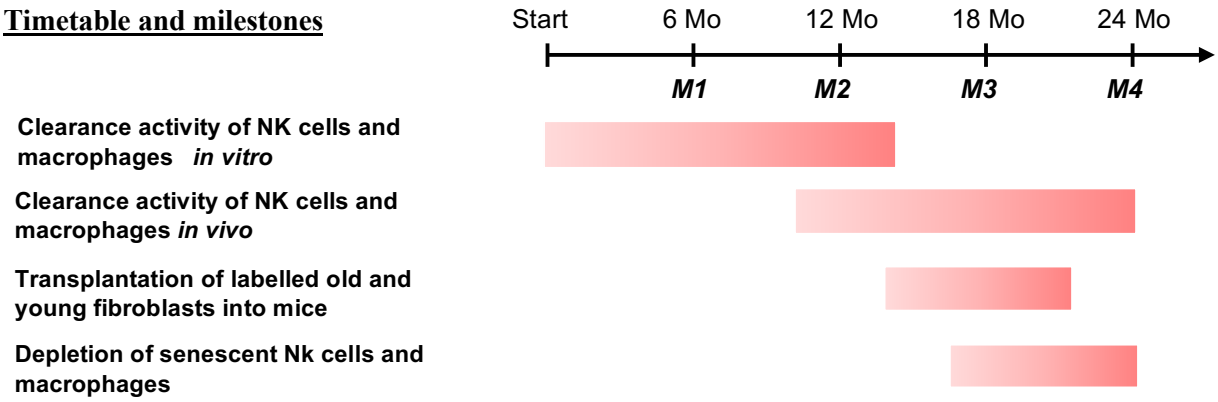
The role of natural killer cells and macrophages for the removal of senescent fibroblasts *in vivo*.

To further explore whether cells of the innate immunity are found in close neighborhood of senescent fibroblasts, immunostaining of skin sections derived from old and young mice and humans will be assessed for co-localization of senescent fibroblasts and activated NK cells (CD56) as well as macrophages, respectively. Since the SASP of fibroblasts contains chemoattractants both for NK cells and macrophages like IL-1β, IL-6, IL-15 and CCL1 (own unpublished data), we expect that NK cells and macrophage numbers will be increased in aging skin. To further study the functional removal of senescent fibroblasts by cells of the innate immune system, senescent murine fibroblasts expressing a mCherry fluorescent protein tag together with GFP expressing young fibroblasts at a 1 : 1 ratio subcutaneously will be transplanted in young and in aged wild type mice and the ratio of young and old fibroblasts at different time points by counting cells in serial sections or by bioluminescence detection of labelled cells employing *in vivo* imaging as reported by Xu et al. (2017). Alternatively, murine young and old ear fibroblasts will be isolated for transplantation from GFP- and RFP-mice, respectively **[Milestone M2 and M3]**. These data will answer the question whether senescent fibroblasts are less removed in old as opposed to young tissue. We will also assess fibroblasts engulfed from macrophages by confocal microscopy in a time kinetic. As a control for phagocytotic activity, we will inject apoptotic neutrophils labelled with a life tracker (CMRA) to demonstrate that macrophages are phagocytotically functional. Antibody depletion of NK cells and macrophage depletion by liposome-encapsulated clodronate, which specifically removes macrophages (Sindrilaru et al., 2011), will be used to assess whether under conditions of NK-cells/macrophages depletion senescent fibroblasts are less removed as compared to NK and macrophage competent controls. Senescent cells will be quantified by flow cytometry for SA-β-Gal in combination with high content image analysis (Biran et al., 2017). Complementary to clodronate treatment, inducible Lys-Cre macrophage and NCR1-Cre NK cells diphtheria toxin depleting murine models will be employed **[Milestone M4]**.

Expected results: The project will generate a clear understanding whether native immune cells from aged mice and humans due to their reduced removal capacity are in part responsible for the accumulation of senescent fibroblasts in aging skin *in vivo*. Skin and later also bone functionality will be assessed by the "Geropanel", functional assessments of skin and bone. This approach will hold substantial promise

for novel strategies to improve the removal and clearance capacity of senescent fibroblasts and in perspective osteoblasts by cells of the innate immune system.

Timetable and milestones



Cooperation partner (if existing)

Hassan Jumaa for the innate immune system, and Anita Ignatius for bone analysis, both Ulm University

Funding of the project

Consumables, core facilities, and animal costs are paid from the budget of the Department.

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