Immune surveillance of senescent cells - biological significance in cancer- and non-cancer pathologies

Lisa Hoenicke[1], Lars Zender[1,2,#,*]

[1]Helmholtz Centre for Infection Research, Inhoffenstrasse 7, 38124 Braunschweig, Germany.

[2]Hannover Medical School, Dept. of Gastroenterology, Hepatology and Endocrinology, Carl-Neuberg-Strasse 1, 30625 Hannover, Germany.

[#]Current address: Division of Molecular Oncology of Solid Tumors, Department of Internal Medicine I, University Hospital Tübingen, Otfried-Mueller-Strasse 10, 72076 Tuebingen, Germany.

*To whom correspondence should be addressed

Contact information:

Lisa Hoenicke
Helmholtz Centre for Infection Research
Inhoffenstraße 7
38124 Braunschweig
Germany
Phone: +49 (0) 531 6181 3132
Email: Lisa.Hoenicke@helmholtz-hzi.de

Lars Zender, M.D.
Helmholtz Centre for Infection Research
Inhoffenstraße 7
38124 Braunschweig
Germany
Phone: +49 (0) 531 6181 3130
Email: Lars.Zender@helmholtz-hzi.de
Lars.Zender@med.uni-tuebingen.de

Abstract:

Cellular senescence, a state of stable growth arrest, can occur in response to various stress stimuli such as telomere shortening, treatment with chemotherapeutic drugs or the aberrant activation of oncogenes. Senescent cells communicate with their environment by secreting various cytokines and growth factors, and it has become clear that this 'secretory phenotype' can have pro- as well as anti-tumorigenic effects. Recent work from our laboratory showed that premalignant, senescent hepatocytes are recognized and cleared through an antigen specific immune response and that this immune response, designated as "senescence surveillance" is crucial for tumor suppression in the liver (1). It is an emerging concept that immune responses against senescent cells have a broader biological significance in cancer- as well as non-cancer pathologies and current data suggest that distinct immune responses are engaged to clear senescent cells in different disease settings. In this review article, we will discuss different examples how immune responses against senescent cells are involved to restrict disease progression in cancer- and non-cancer pathologies.

Types and hallmarks of cellular senescence

Almost 50 years ago, cellular senescence was described as a state of stable proliferation arrest that accompanies the replicative exhaustion of cultured human cells (2). Senescent cells show typical changes in cell morphology, metabolism, chromatin structure and display a characteristic gene expression pattern (3). A hallmark of senescent cells is the upregulation of senescence associated ß-galactosidase (SA-ß-gal) activity (4), a marker, which in combination with other markers allows to identify senescent cells *in vitro* or *in vivo* (5). Cellular senescence has to be distinguished from quiescence, a proliferative arrest of growth factor deprived cells which immediately can be reverted upon provision of mitogenic signals. While cellular senescence was initially defined in cells with exhausted replicative potential upon telomere erosion, it is now well established that the same genetic program can be engaged in "young" cells upon the aberrant activation of oncogenes, exposure to chemotherapeutic drugs, oxidative stress or suboptimal culture conditions (3). For example, mutations rendering the *Ras* oncogene constitutive active can promote uncontrolled cell division, but under some circumstances also induce cellular senescence, which in this context is called oncogene induced senescence (OIS) (6). This program engages signaling through mitogen activated protein kinase (MAPK) pathway and involves the retinoblastoma and p53 tumor suppressor pathways (7).

Senescence as an anti tumor barrier and the senescence associated secretory phenotype

While there had been a long scientific debate whether cellular senescence may solely represent a cell culture phenomenon attributable to high Ras expression levels or unphysiological culture conditions (8), a series of elegant *in vivo* studies ultimately showed that cellular senescence occurs in relevant contexts *in vivo* and plays a key role in counteracting tumor development (5,9-11). For example it was shown that senescence occurs in human melanocytic nevi harboring a mutated form of *BRAF*, a protein kinase and downstream effector of Ras. *In vitro* experiments furthermore showed that even endogenous expression levels of mutated BRAF were sufficient to induce senescence in fibroblasts and melanocytes (10). Along the same lines, Serrano and colleagues showed that senescent cells can be detected in premalignant lesions of the murine lung and pancreas that were triggered by activation of a mutated endogenous *Kras* allele (5). In addition to an altered

expression of genes related to the senescence associated cell cycle arrest, e.g. p53 and the cyclin-dependent kinase (CDK) inhibitors Cdkn1a (p21) and Cdkn2a ($p16^{INK4a}$), senescent cells were also found to overexpress genes encoding for secreted proteins, including a number of growth factors, chemokines and inflammatory cytokines (12-14). Collectively this phenotype was designated as the senescence-associated secretory phenotype (SASP) or the senescence messaging secretome (SMS) (15-17). Via secreted factors senescent cells communicate with their environment and thus influence the tissue microenvironment and induce angiogenesis or impact cellular differentiation and proliferation (18-20). With regard to tumor development it was a paradigm for many years that factors secreted from senescent cells are tumor promoting. More than ten years ago, a study by Campisi and colleagues showed that co-injection of senescent fibroblasts with human mammary tumor cells increased the growth of subcutaneous breast carcinomas (21) and that factors secreted from senescent cells were causative for this effect. However, more recent data suggest that the senescence associated secretory phenotype must be seen as a double edged sword in tumorigenesis. For example a study by Lowe and colleagues took advantage of a mosaic liver cancer mouse model, where endogenous p53 could be reactivated via conditional RNA interference. Interestingly, even brief reactivation of endogenous p53 in Ras driven murine liver carcinomas triggered a cellular senescence response that was associated with a pronounced secretion of various chemo- and cytokines. Factors secreted from senescent cells were shown to attract innate immune cells (macrophages, neutrophils and NK cells) which mediated the clearance of senescent tumor cells. This study showed for the first time an interaction between the cellular senescence program and the immune system and suggested that senescence inducing therapies may be used to trigger immune responses against tumors (22). Further but different examples for antitumorigenic roles of the SASP were provided by the Gil and Peeper laboratories (23,24). Work by Kuilmann *et al*. showed for example that the induction and maintenance of replicative- as well as oncogenic $BRAF^{E600}$- mediated senescence strongly depends on cell-autonomous signaling of interleukin-6 and -8 (IL-6 and IL-8), which was regulated by the transcription factors C/EBPß and NFκB (24). In parallel Acosta *et al.* showed that IL-8 and other CXCR2 ligands secreted from senescent cells can reinforce and stabilize senescence by enhancing the DNA damage response (23). Along the same lines the Bernards and Green laboratories had

shown that other secreted factors such plasminogen activator inhibitor-1 or IGFBP7 also play important roles for senescence induction and maintenance (25,26)

Examples of pro- as well as anti-tumorigenic functions of factors secreted from senescent cells are summarized in table 1.

Senescence surveillance and early tumor prevention

Regarding tumor prevention, cellular senescence has been regarded as a solely cell intrinsic mechanism of tumor suppression, i.e. suppressing tumor development through the induction of a stable cell cycle arrest (27). Against the background of the discussed data on the interaction of senescent tumor cells with the innate immune response, we recently set out to explore whether immune responses against pre-cancerous senescent cells occur and whether such immune responses would have significance for tumor suppression (1). We found that expression of oncogenic $Nras^{G12V}$ triggers oncogene induced senescence in otherwise normal mouse hepatocytes *in vivo*. Chemo- and cytokines secreted from premalignant senescent hepatocytes led to the attraction of innate as well as adaptive immune cells, which were found in close proximity to senescent hepatocytes, suggesting that precancerous senescent hepatocytes could be subjected to immune clearance. Indeed, time course analyses revealed a rapid turnover of precancerous senescent hepatocytes, which was designated senescence surveillance. Genetic experiments revealed that functional senescence surveillance was dependent on an intact adaptive immune response and that in severe combined immunodeficient (SCID) mice and also in CD4 knockout mice the immune clearance of $Nras^{G12V}$ expressing senescent hepatocytes was abrogated. Interestingly, the missing immune clearance of $Nras^{G12V}$ expressing pre-cancerous cells in these mice resulted in the development of hepatocellular carcinomas at later time points. In accordance with the genetic data suggesting a key role of the adaptive immune system in senescence surveillance, mutant Nras-specific CD4 T-cells were found in mice harboring senescent $Nras^{G12V}$ expressing hepatocytes but not in $Nras^{G12V}$ expressing $p19^{Arf}$ knockout mice, in which senescence induction is blunted. Further mechanistic studies excluded an involvement of direct CD4 T-cell cytotoxicity but rather classified the observed CD4 T-cell response as a Th1 type response. CD4 T-cells depended on monocytes and freshly

replenished macrophages to efficiently kill senescent hepatocytes (Figure 1), whereas liver resident macrophages (Kupffer cells) were found not to be involved in the killing of $Nras^{G12V}$ expressing senescent hepatocytes. Collectively, our data showed that oncogene induced senescence (OIS) plays an important role in the induction of specific immune responses against antigens expressed in pre-cancerous cells and that a continuous, CD4 T cell mediated immune clearance of pre-malignant senescent cells is crucial to suppress liver cancer development. These data establish a new cell extrinsic tumor suppressive role of the cellular senescence program.

The discussed data are also particularly interesting against the background of an ongoing scientific debate regarding the stability of the senescence associated cell cycle arrest. While early *in vitro* studies suggested the senescence associated proliferative arrest to be irreversible (28,29), subsequent studies provided evidence that under certain circumstances a subfraction of cells may escape the senescence arrest and re-enter the cell cycle (15,24,30,31). Obviously, the escape of even a small subfraction of precancerous cells can have far-reaching consequences *in vivo*, as there will be strong selection for such cells with subsequent tumor development. Along these lines the evolvement of an immune surveillance mechanism against pre-cancerous cells appears logical and necessary to prevent tumorigenesis. Nevertheless, it should be noted here that a direct demonstration of a senescence escape in a relevant context *in vivo* has not been provided yet.

The identified mechanism of senescence surveillance opens a new research direction in the senescence field and raises many questions that need to be answered in the next couple of years. For example, it will be crucial to test the significance of senescence surveillance for tumor suppression in tissues other than the liver and to address the individual role of particular chemo- and cytokines for the process. While unpublished data from our laboratory suggest that extrahepatic pre-cancerous lesions are also subject to senescence surveillance (D. Dauch and L. Zender, unpublished data), it seems that there are also prominent examples of tissues without an immune surveillance of senescent cells. Melanocytic nevi for example contain large numbers of senescent melanocytes (10), however, in general these nevi are not subject to any kind of immune clearance. Only in a particular, less frequent type of nevi, so called “halo nevi”, an inflammatory infiltrate develops. The inflammatory infiltrate which is characteristically encircling halo nevi is mainly composed of leukocytes and results in a zone of

depigmentation surrounding the nevus (32), however, it is yet unclear against which components of the halo nevus the immune reaction is directed (33).

It will also be crucial to investigate the role of senescence surveillance for tumor suppression in humans. First data from our group suggest that senescence surveillance is operative and involved in the clearance of senescent hepatocytes in human livers. Quantifying the numbers of senescent hepatocytes in patients under immunosuppressive therapy (due to organ transplantation) or in patients with HIV infection (both conditions that impact CD4 T-cell function) we found an accumulation of senescent hepatocytes in both cohorts, thus supporting that immune surveillance of senescent cells also occurs in humans (1).

Senescence surveillance in the general context of cancer immune surveillance

Our results show that senescence induction in pre-cancerous cells is important for an efficient immune surveillance and tumor suppression in the liver. While our current experimental data is restricted to the liver, it is tempting to speculate that senescence may play a general role in the "elimination phase" of tumor immune surveillance, as referred to by Schreiber and Smyth (34). The elimination phase of tumor immune surveillance describes an early phase, where incipient tumors are detected by interacting innate and adaptive immune cells (34). Nevertheless, it should be noted here that there has been a long scientific debate regarding the significance of a T-cell dependent immune surveillance mechanism of pre-cancerous cells and in a recent study, for example, it was shown that intrahepatic activation of the Simian virus 40 (SV40) large T antigen directly results in HCC development instead of induction of an immune response against the hepatocytes (35). In the used model, tumor-specific T-cells were found to produce TGF-β rather than IFN-γ. Our data may help to resolve some of the controversy, as it suggest that the induction of a specific immune response against pre-cancerous cells is dependent on an intact cellular senescence response. Along these lines, tumor immune surveillance may be restricted to those lesions containing senescent pre-cancerous cells. However, if the induction of the cellular senescence program is affected (e.g. due to impaired p53 function or reduced levels of $p14^{Arf}$ ($p19^{Arf}$ in mice)), a specific immune response against

such tumor prone cells may not occur. The fact that SV40 is known to primarily disable senescence induction may explain the lack of immune surveillance of SV40 expressing hepatocytes (35).

Control of senescent cell numbers and senescence surveillance in non-cancer pathologies

A recent study suggested that immune responses against senescent cells are not restricted to cancer- or pre-cancerous cells but also play important roles in non-cancer pathologies. It was shown that during chronic liver damage and fibrosis development, hepatic stellate cells, the major matrix producing and therefore fibrogenic cell type in the liver, undergo senescence and thus restrict fibrosis progression. Interestingly, restriction of fibrosis progression was dependent on a natural killer (NK) cell dependent clearance of senescent stellate cells (36). Furthermore, a different study showed that myofibroblast senescence is a programmed wound healing response that functions as a self-limiting mechanism for fibrogenesis. Myofibroblasts that initially proliferate and produce extracellular matrix (ECM) eventually were shown to undergo senescence, thus blocking further proliferation and converting them into matrix-degrading cells (37). Similarly, it was shown by Pitiyage *et al.* that senescent mesenchymal cells accumulate in human oral submucous fibrosis (OSMF) by a telomere-independent mechanism and ameliorate fibrosis through secreted matrix metalloproteinases (38). Though not formally shown in the latter studies, it is an interesting and obvious concept that also in these settings senescent cells need to be eliminated at some point, for example to restrict the secretion of matrix metalloproteinases.

A recent study also tried to further clarify the role of senescence in aging. As senescent cells are known to accumulate in aging tissues (39), Baker *et al.* generated a transgenic mouse line, wherein senescent, $p16^{Ink4a}$-expressing cells can be specifically eliminated via the administration of a particular drug. When this system was used to eliminate senescent cells in the BubR1 progeroid mouse background, age-related pathologies could be prevented in organs such as adipose tissue, skeletal muscle and eye. Most strikingly, late-life clearance of senescent cells still attenuated progression of already established age-related disorders (40). These data indicate that cellular senescence is causally implicated in generating age-related phenotypes and that removal of senescent cells can prevent or improve tissue dysfunction and extend healthspan. While in the

discussed study, an experimental transgenic system was used to eliminate senescent cells from aging tissues, future studies will have to address to what extent the immune system is involved in regulating the number of senescent cells in aging tissues and if aging of the immune system itself contributes to the accumulation of senescent cells in aging organisms.

In summary, there is increasing evidence that control of senescent cell numbers determines cancer onset, cancer progression, tissue damage and aging and it has been shown that immune responses against senescent cells play important roles in cancer as well as non-cancer pathologies. Against the background of an emerging broader biological significance of immune surveillance of senescent cells, it is interesting that very distinct immune responses seem to be involved in different disease settings (Fig. 1). Senescence induction in already established liver carcinomas results in an innate immune response, comprising neutrophils, NK cells and macrophages and this immune response was shown to be sufficient for the clearance of senescent tumor cells (22) (Fig.1). In contrast, an adaptive immune response depending on the interaction of antigen specific CD4 T-cells and monocytes/macrophages was found to mediate the immune clearance of pre-cancerous senescent hepatocytes (1) (Fig. 1). In turn, an NK cell response against senescent hepatic stellate cells was found to be crucial to restrict liver cirrhosis progression (36) (Fig.1). Future studies are needed to characterize which factors that are secreted from senescent cells or overexpressed on senescent cells determine the nature of the resulting immune response. Along the same lines it will be crucial to find out why in some settings (e.g. most melanocytic nevi) no immune responses against senescent cells are induced.

Figure Legends:

Figure 1: Schematic representation of immune responses against senescent cells in different disease settings. Upon senescence induction in established liver carcinomas, an innate immune response is triggered and senescent cells are cleared by macrophages, neutrophils and natural killer cells (NK cells) (first column). In contrast, premalignant senescent hepatocytes, induced by aberrant activation of oncogenic Nras, are subject to an antigen specific CD4 T cell mediated immune response, which also involves monocytes/macrophages (second column). NK cell mediated clearance of senescent hepatic stellate cells was shown to be crucial to restrict the progression of liver fibrosis in chronically damaged livers (third column). Future work is needed to address whether immune responses against senescent cells in aging tissues occur and which components of the immune system are involved.

Reference List

1. Kang, T.W.*et al*.(2011) Senescence surveillance of pre-malignant hepatocytes limits liver cancer development. *Nature*, **479**, 547-551.

2. Hayflick, L.*et al*.(1965) The limited in vitro lifetime of human diploid cell strains. *Exp.Cell Res.*, **37**, 614-636.

3. Kuilman, T.*et al*.(2010) The essence of senescence. *Genes Dev.*, **24**, 2463-2479.

4. Dimri, G.P.*et al*.(1995) A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proc.Natl.Acad.Sci.U.S.A*, **92**, 9363-9367.

5. Collado, M.*et al*.(2005) Tumour biology: senescence in premalignant tumours. *Nature*, **436**, 642-

6. Serrano, M.*et al*.(1997) Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, **88**, 593-602.

7. Serrano, M.*et al*.(2001) Putting the stress on senescence. *Curr.Opin.Cell Biol.*, **13**, 748-753.

8. Narita, M.*et al*.(2005) Senescence comes of age. *Nat.Med.*, **11**, 920-922.

9. Chen, Z.*et al*.(2005) Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*, **436**, 725-730.

10. Michaloglou, C.*et al*.(2005) BRAFE600-associated senescence-like cell cycle arrest of human naevi. *Nature*, **436**, 720-724.

11. Braig, M.*et al*.(2005) Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*, **436**, 660-665.

12. Mason, D.X.*et al*.(2006) Defined genetic events associated with the spontaneous in vitro transformation of ElA/Ras-expressing human IMR90 fibroblasts. *Carcinogenesis*, **27**, 350-359.

13. Freund, A.*et al*.(2010) Inflammatory networks during cellular senescence: causes and consequences. *Trends Mol.Med.*, **16**, 238-246.

14. Shelton, D.N.*et al*.(1999) Microarray analysis of replicative senescence. *Curr.Biol.*, **9**, 939-945.

15. Coppe, J.P.*et al*.(2008) Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS.Biol.*, **6**, 2853-2868.

16. Kuilman, T.*et al*.(2009) Senescence-messaging secretome: SMS-ing cellular stress. *Nat.Rev.Cancer*, **9**, 81-94.

17. Rodier, F.*et al*.(2009) Persistent DNA damage signalling triggers senescence-

associated inflammatory cytokine secretion. *Nat.Cell Biol.*, **11**, 973-979.

18. Parrinello, S.*et al*.(2005) Stromal-epithelial interactions in aging and cancer: senescent fibroblasts alter epithelial cell differentiation. *J.Cell Sci.*, **118**, 485-496.

19. Coppe, J.P.*et al*.(2006) Secretion of vascular endothelial growth factor by primary human fibroblasts at senescence. *J.Biol.Chem.*, **281**, 29568-29574.

20. Bavik, C.*et al*.(2006) The gene expression program of prostate fibroblast senescence modulates neoplastic epithelial cell proliferation through paracrine mechanisms. *Cancer Res.*, **66**, 794-802.

21. Krtolica, A.*et al*.(2001) Senescent fibroblasts promote epithelial cell growth and tumorigenesis: a link between cancer and aging. *Proc.Natl.Acad.Sci.U.S.A*, **98**, 12072-12077.

22. Xue, W.*et al*.(2007) Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature*, **445**, 656-660.

23. Acosta, J.C.*et al*.(2008) Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*, **133**, 1006-1018.

24. Kuilman, T.*et al*.(2008) Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*, **133**, 1019-1031.

25. Kortlever, R.M.*et al*.(2006) Plasminogen activator inhibitor-1 is a critical downstream target of p53 in the induction of replicative senescence. *Nat.Cell Biol.*, **8**, 877-884.

26. Wajapeyee, N.*et al*.(2008) Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell*, **132**, 363-374.

27. Lowe, S.W.*et al*.(2004) Intrinsic tumour suppression. *Nature*, **432**, 307-315.

28. Kato, D.*et al*.(1998) Features of replicative senescence induced by direct addition of antennapedia-p16INK4A fusion protein to human diploid fibroblasts. *FEBS Lett.*, **427**, 203-208.

29. Zhu, J.*et al*.(1998) Senescence of human fibroblasts induced by oncogenic Raf. *Genes Dev.*, **12**, 2997-3007.

30. Beausejour, C.M.*et al*.(2003) Reversal of human cellular senescence: roles of the p53 and p16 pathways. *EMBO J.*, **22**, 4212-4222.

31. Dirac, A.M.*et al*.(2003) Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J.Biol.Chem.*, **278**, 11731-11734.

32. Sutton, R.L.*et al*.(1916) An unusual variety of vitiligo (leukoderma acquisitum centrifugum). A report of two cases. *J.Cutan.Dis.*, **34**, 797-

33. Speeckaert, R.*et al*.(2011) Immune reactions in benign and malignant melanocytic lesions: lessons for immunotherapy. *Pigment Cell Melanoma Res.*, **24**, 334-344.

34. Schreiber, R.D.*et al*.(2011) Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science*, **331**, 1565-1570.

35. Willimsky, G.*et al*.(2005) Sporadic immunogenic tumours avoid destruction by inducing T-cell tolerance. *Nature*, **437**, 141-146.

36. Krizhanovsky, V.*et al*.(2008) Senescence of activated stellate cells limits liver fibrosis. *Cell*, **134**, 657-667.

37. Jun, J.I.*et al*.(2010) The matricellular protein CCN1 induces fibroblast senescence and restricts fibrosis in cutaneous wound healing. *Nat.Cell Biol.*, **12**, 676-685.

38. Pitiyage, G.N.*et al*.(2011) Senescent mesenchymal cells accumulate in human fibrosis by a telomere-independent mechanism and ameliorate fibrosis through matrix metalloproteinases. *J.Pathol.*, **223**, 604-617.

39. Campisi, J.*et al*.(2005) Senescent cells, tumor suppression, and organismal aging: good citizens, bad neighbors. *Cell*, **120**, 513-522.

40. Baker, D.J.*et al*.(2011) Clearance of p16Ink4a-positive senescent cells delays ageing-associated disorders. *Nature*, **479**, 232-236.