Targeting macrophages rescues age-related immune deficiencies in C57BL/6J geriatric mice

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Summary

Changes to innate cells, such as macrophages and myeloid-derived suppressor cells (MDSCs), during aging in healthy or tumor-bearing hosts are not well understood. We compared macrophage subpopulations and MDSCs from healthy young (6 - 8 weeks) C57BL/6J mice to those from healthy geriatric (24 - 28 months) mice. Spleens, lymph nodes and bone marrow of geriatric hosts contained significantly more M2-macrophages and MDSCs than their younger counterparts. Peritoneal macrophages from geriatric, but not young, mice co-expressed CD40 and CX3CR1 that are usually mutually exclusively expressed by M1 or M2 macrophages. Nonetheless macrophages from geriatric mice responded to M1 or M2 stimuli similarly to macrophages from young mice, although they secreted higher levels of TGF-β in response to IL-4. We mimicked conditions that may occur within tumors by exposing macrophages from young versus geriatric mice to mesothelioma or lung carcinoma tumor cell-derived supernatants. Whilst both supernatants skewed macrophages towards the M2-phenotype regardless of age, only geriatric-derived macrophages produced IL-4, suggesting a more immunosuppressive tumor microenvironment will be established in the elderly. Both geriatric- and young-derived macrophages induced allogeneic T cell proliferation, regardless of the stimuli used, including tumor supernatant.
However, only macrophages from young mice induced T cell IFN-γ production. We examined the potential of an IL-2/agonist anti-CD40 antibody immunotherapy that eradicates large tumors in young hosts to activate macrophages from geriatric mice. IL-2/CD40 activated macrophages rescued T cell production of IFN-γ in geriatric mice. Therefore, targeting macrophages with IL-2/anti-CD40 antibody may improve innate and T cell immunity in aging hosts.

Introduction

Currently, long-term eradication of many cancers including mesothelioma and lung cancer in humans is not possible using standard therapies such as chemotherapy and surgery. Most of these cancer patients are members of an aging population and recent studies have shown that specific components of immune function decline with age (Fulop et al. 2010). These observations are concerning as effective chemotherapy can require a fully functioning immune system (Zitvogel et al. 2008; Jackaman et al. 2012b). Alternative treatment strategies, such as immunotherapy have also shown promise (Fraser et al. 2010). However, most preclinical analyzes of anti-cancer therapies are inevitably performed in young adult mice and therefore are not representative of human cancers that occur in aging populations. Furthermore, the majority of immunotherapeutic strategies focus on inducing or enhancing T cell activation, yet T cell number and function decline with age meaning that they require a rescue strategy (Haynes & Maue 2009).

The mechanisms behind age-related T cell dysfunction are not yet clear, although declining naïve T cell output due to thymic involution as well as reduced antigen presenting cell capacity may be contributing factors (Haynes & Maue 2009; Fulop et al. 2010). Furthermore,
with age, there is an increase in regulatory T cells (T\textsubscript{reg}) which have been shown to suppress CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell responses via direct cell-cell contact and/or secretion of suppressive factors such as TGF-\beta (Gregg \textit{et al.} 2005).

There is evidence that appropriate activation of elderly-derived dendritic cells can restore T cell function (Sharma \textit{et al.} 2006). However, the majority of T cells in aged populations are memory T cells (Haynes & Maue 2009; Fulop \textit{et al.} 2010) and these cells can be activated or suppressed by macrophages and/or myeloid-derived suppressor cells (MDSCs), in tissue sites and tumors (Mantovani \textit{et al.} 2009), yet the function and role of the latter two cell types is not yet well understood during aging.

Macrophages can be subdivided into pro-inflammatory M1 macrophages, or anti-inflammatory M2 macrophages (Mantovani \textit{et al.} 2004); importantly, they are capable of switching phenotypes based on the stimuli present (Arnold \textit{et al.} 2007). Classically-activated M1 macrophages secrete pro-inflammatory cytokines, such as tumor necrosis factor (TNF-\alpha) and interleukin-12 (IL-12) and exert anti-tumoricidal activity (Mantovani \textit{et al.} 2004). Alternatively-activated M2 macrophages secrete anti-inflammatory cytokines such as IL-4, IL-10 and transforming growth factor-beta (TGF-\beta) which promote angiogenesis, fibrosis and tumor growth (Mantovani \textit{et al.} 2004). MDSCs are macrophage-like immature cells that are often increased in cancer and have similarities to M2 macrophages (Sinha \textit{et al.} 2007).

The phenotype and function of macrophages may differ in young versus old mice (Dace & Apte 2008). For example, aging has been associated with an inability to respond to M1 stimuli (Mahbub \textit{et al.} 2012). However, aging has also been associated with enhanced M1
macrophage function through the production of reactive oxygen species (Smallwood et al. 2011). One possible reason for the contradictory results is different definitions of elderly mice; some studies define elderly mice as 12 to 15 months old (Ly et al. 2010; Smallwood et al. 2011) whilst others define 18 to 24 months old as elderly (Mahbub et al. 2012). Different anatomical locations may also contribute to the contradictory results. For example (Ly et al. 2010) showed an increase in M2-macrophage numbers in the eye during aging. The eye is considered to be an immunologically privileged and profoundly immunosuppressive site that protects damage to vision by dampening local inflammatory responses. In contrast (Smallwood et al. 2011) demonstrated increased M1 peritoneal macrophage function with age. Few studies have defined and examined geriatric mice. In this study we used the Jackson laboratory definition of age (Yuan et al. 2009) which complements our recently published data for old (24 months of age) and geriatric mice (greater than 24 months), where sarcopenia (age-related loss of muscle mass) was evident at 24 months and more pronounced by 27/29 months in female C57Bl/6J mice (Shavlakadze et al. 2010), with these ages generally corresponding to elderly (around 70 years old) and geriatric (80+ year old) humans.

Recent studies using young adult mice have shown that targeting tumor-associated M2 macrophages such that they skew back to the M1 phenotype may be an effective anti-cancer treatment strategy (Allavena et al. 2008). For example, (Guiducci et al. 2005) used CpG plus an anti-IL-10 receptor antibody (Ab) and demonstrated switching tumor-infiltrating M2 macrophages to M1 cells in mouse models of adenocarcinoma and colon carcinoma. Furthermore, (Watkins et al. 2007) described in situ reprogramming of tumor-associated macrophages using IL-12. Whether this will be an effective treatment strategy in the elderly is yet to be fully determined.
Advanced age has been associated with impaired macrophage responses to polarisation signals, in particular to M1 stimuli (Mahbub et al. 2012). Yet others have shown that deliberately targeting macrophage activation can be effective in generating tumor regression in old, but not young, mice (Leibovici et al. 2009). Again, different definitions of elderly mice may contribute to the different results. Therefore, in this study, spleen, bone marrow, lymph node and peritoneal macrophage subpopulations from young adult (6 - 8 weeks old) versus geriatric (24 - 28 months) mice were phenotypically compared. Phenotypic, cytokine and T cell activation responses of peritoneal macrophages from young versus geriatric mice exposed to tumor supernatant, and classical M1 or M2 stimuli were examined. We have previously shown that an IL-2/agonist anti-CD40 Ab immunotherapy induces curative regression of large mesothelioma and lung carcinoma tumors in young adult hosts (Jackaman et al. 2008; Jackaman & Nelson 2011; Jackaman et al. 2012a) therefore, in the present study, we examined the potential of IL-2/anti-CD40 Ab to activate macrophages from geriatric mice such that they induce/rescue effector T cell function.

**Results**

*M2 macrophages increase in spleen and bone marrow of geriatric mice*

The first experiments examined macrophage subpopulations and MDSCs in lymph node, spleens and bone marrow from young (6 - 8 weeks) and geriatric C57BL/6J mice (24 - 28 months). The cells were stained for M1 macrophages (CD11b+ F4/80+ Ly6Ch Ly6CRLCX3CR1lo; (Arnold et al. 2007; Movahedi et al. 2010), M2 macrophages (CD11b+ F4/80+ Ly6Cloh CX3CR1hi; (Arnold et al. 2007; Movahedi et al. 2010) or MDSCs (CD11b+ F4/80loh Ly6C+h Ly6G+; (Sinha et al. 2007) and analyzed by flow cytometry (gating strategy shown in Figure 1A). Samples were also analyzed for macrophage (CD11b+ F4/80+) expression of IL-10.
(associated with M2 cells) and TNF-α (associated with M1 cells) by intracellular cytokine staining.

Total F4/80+ macrophage and MDSC proportions were significantly increased in geriatric spleens (Figure 1B), and bone marrow (Figure 1D) relative to their younger counterparts (Figures 1B and D). This coincided with a decrease in the M1 macrophage subpopulation (Figures 1C and E) and a significant increase in the M2 macrophage subset in geriatric compared to young mice (Figure 1C). M2 macrophages appeared to increase in the bone marrow although the difference did not reach statistical significance (Figure 1E). A further small population of macrophages expressing a mixed M1/M2 phenotype was observed in all organs examined. Unlike the spleen and bone marrow, M2 macrophage proportions within the lymph nodes (LN) did not significantly increase with age (Supplementary Figure 1A). Interestingly, we observed a significant age-related increase in other suppressive subsets, i.e. MDSCs and M1/M2 (putative suppressive M3 macrophages; (Biswas et al. 2006; Tsai et al. 2007) macrophages in LNs (Supplementary Figure 1A). As cytokine secretion is an important delineator of M1 (TNF-α) versus M2 (IL-10) macrophage function, we also measured intracellular cytokine levels and found that they corresponded with surface phenotype expression as M2-associated IL-10 was significantly increased in macrophages in geriatric spleens and bone marrows (Figure 1F), with no change in TNF-α (Supplementary Figure 1C).

**Macrophages from geriatric mice respond appropriately to M1 and M2 stimuli**

Recent studies have suggested that macrophages from aged mice may be unable to respond to M1 stimuli (Mahbub et al. 2012). Therefore, the next experiments assessed whether macrophages from aged mice responded to polarization stimuli in our model system.

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Peritoneal macrophages from young or geriatric C57BL/6J mice were cultured with IL-4 (M2 stimuli) or IFN-γ/LPS (M1 stimuli) and stained for CX3CR1 (M2 macrophages are CX3CR1$^{hi}$)(Arnold et al. 2007; Movahedi et al. 2010) or CD40 (M1 macrophages are CD40$^{hi}$) expression before analysis by flow cytometry. Interestingly, peritoneal macrophages from geriatric mice co-expressed significantly higher levels of both CD40 and CX3CR1 compared to young mice in their resting state prior to stimuli (Figures 2A and B). These data imply the presence of M3 macrophages also in the peritoneal cavity of elderly populations. Nonetheless, peritoneal macrophages from both young and geriatric mice were capable of responding appropriately to M1 or M2 stimuli, and upregulated CD40 with M1 stimuli (Figures 2A and C) or CX3CR1 with M2 stimuli (Figures 2B and D) respectively (example histograms shown in Supplementary Figure 2). Whilst macrophages from geriatric mice exhibited higher initial expression levels of CD40 and CX3CR1 compared to macrophages from young mice (example histograms shown in Supplementary Figure 2), the fold change relative to the no stimuli controls was similar in both age groups (Figures 2C and D).

Again, as cytokine secretion is a powerful indictor of macrophage function, supernatants from macrophages from young and geriatric mice were assayed for cytokine production using cytokine bead arrays (CBAs) and flow cytometry. Peritoneal macrophages from young and geriatric mice responded appropriately to M1 or M2 stimuli, leading to production of M1 cytokines IFN-γ (Figure 2E) and TNF-α (Figure 2F), or M2 cytokines IL-4 (Figure 2G) and TGF–β (Figure 2H) respectively. Interestingly, macrophages from geriatric mice produced higher levels of TGF–β after exposure to the M2 stimuli compared to young mice, which suggests the generation of a profoundly immunosuppressive macrophage.
**Tumor supernatant induces M2 macrophages regardless of age**

The next experiments assessed if murine AE17 mesothelioma or LL carcinoma tumor cell-derived soluble factors polarize macrophages. Peritoneal macrophages from young and geriatric mice cultured with AE17 mesothelioma tumor cell-derived supernatant or LL tumor supernatant were stained for flow cytometry as per Figure 2. Similar to IL-4 stimulated M2 macrophages, macrophages exposed to mesothelioma supernatant or LL tumor supernatant upregulated CX3CR1 regardless of age (Figures 3B and D, example histograms in Supplementary Figure 3). In contrast, CD40 expression did not change after exposure to tumor supernatant (Figures 3A and C), although, it is possible that the higher starting expression levels of CD40 plus the M3-like phenotype in geriatric-associated macrophages masked potential changes in this population. Nonetheless, these data suggest that tumor-derived factors polarize macrophages into M2 macrophages, regardless of age.

To further explore the hypothesis that tumor-derived factors induce M2 macrophages, supernatants from macrophages from young and geriatric mice were assayed for cytokine production using CBA assays. Neither peritoneal macrophages from young or geriatric mice cultured with AE17 mesothelioma supernatant or LL tumor supernatant produced the M1 cytokines, IFN-γ and TNF-α (data not shown). Interestingly, macrophages from geriatric but not young mice exposed to both AE17 and LL tumor supernatants produced IL-4 (Figure 3E). Both AE17 mesothelioma supernatant and LL tumor supernatant induced TGF-β production by macrophages from geriatric and young mice (Figure 3F), which was significantly higher in macrophages from geriatric mice; these data imply tumor-induced skewing into M2 macrophages. Together the data suggests that macrophages from geriatric mice are hyper-responsive to the suppressive effects of tumor-derived stimuli.
Macrophages from young and geriatric mice induce CD4⁺ and CD8⁺ T proliferation

The next experiments assessed whether macrophages induce T cell proliferation, an important functional role for macrophages in viral infection and tumor settings. Peritoneal macrophages from young or geriatric mice were stimulated with M1 or M2 stimuli as described above. Allogeneic T cells from young (6 - 8 week old) Balb/c mice spleens were fluorescently labelled with CFSE and incubated with stimulated macrophages at varying concentrations for 5 days. Samples were stained with CD4 and CD8 and T cell proliferation measured by flow cytometry (as indicated by dilution of CFSE intensity; Figure 4A). Use of stimuli did not improve the ability of macrophages from young or geriatric mice to induce CD4⁺ (Supplementary Figure 4) and CD8⁺ (Figures 4B, C and D) T cell proliferation, including macrophages cultured with AE17 mesothelioma supernatant (Figure 4E) or LL tumor supernatant (Figure 4F). Regardless of stimuli all macrophages at the higher macrophage:T cell ratio induced the proliferation of T cells from young hosts.

Macrophages from young mice induce proliferation of geriatric T cells

To determine whether the defect was at the macrophage level or T cell level the next experiments asked if macrophages from young mice could induce geriatric T cells to proliferate. Peritoneal macrophages from young (6 – 8 weeks) Balb/c mice were stimulated as described and incubated with allogeneic CFSE-labelled C57BL/6J T cells from young or geriatric mice for 5 days. Samples were stained for CD4 and CD8 expression and T cell proliferation measured by flow cytometry. Similar to the experiments performed above, all stimulated macrophages induced proliferation of CD4⁺ (Supplementary Figure 5) and CD8⁺ T cells (Supplementary Figure 6) from geriatric mice. Interestingly, CD4⁺ and CD8⁺ T cells from geriatric mice showed a trend towards higher levels of proliferation at lower macrophage:T cell ratios, compared to T cells from young mice. Furthermore, proliferation of
T cells from geriatric mice did not increase greatly with increasing macrophage:T cells ratios (relative to proliferation of T cells from young mice). This suggests that the T cells from geriatric mice may be memory cells that rapidly reached senescence and were unable to further proliferate with increased macrophage stimuli.

*Macrophages from young but not geriatric mice induce the production of IFN-γ by T cells*

The previous assays showed that macrophages could induce T cell proliferation however, the functional quality of activated T cells is not measured by this assay. An important measure of T cell function is cytokine secretion, in particular IFN-γ secretion. As described in Figure 4, M1 and M2 macrophages from young and geriatric mice induced similar levels of T cell proliferation. Also, M1 and M2 macrophages from young mice were capable of inducing T cell proliferation from geriatric mice (Supplementary Figure 5 and 6). Therefore, to determine whether this was functional proliferation, we next assayed for T cell production of IL-2, IFN-γ and IL-10 by CBA and intracellular cytokine staining. Regardless of stimuli, macrophages from both young and geriatric mice induced production of IL-2 by T cells (data not shown). Interestingly, macrophages from geriatric mice were impaired in their ability to induce T cell to secrete IFN-γ compared to M1 and M2 stimulated macrophages from young mice (Figure 5A). In contrast, M1 stimulated macrophages from young mice were capable of inducing IFN-γ production by T cells from geriatric mice (Figure 5B). Unexpectedly, M1 and M2 macrophages from young mice drove increased production of IL-10 by CD4+ T cells from geriatric mice relative to the no stimuli controls, whilst CD4+ T cells from young mice showed reduced IL-10 production (Figure 5C). This suggests that there are functional deficiencies within both macrophages and T cells from geriatric mice that impair T cell proliferation. Interestingly, regardless of age macrophages stimulated with tumor supernatant
did not induce T cell derived IFN-γ (Figure 5A and B), and no change in CD4+ T cell IL-10 secretion was observed (Figure 5C).

**IL-2/anti-CD40 antibody immunotherapy rescues T cell production of IFN-γ in geriatric mice**

We have previously shown that targeting large mesothelioma and LL tumors with IL-2/agonist anti-CD40 Ab induces permanent and curative regression (Jackaman et al. 2008; Jackaman & Nelson 2011; Jackaman et al. 2012a). T cells (Jackaman et al. 2008), neutrophils (Jackaman et al. 2008) and macrophages (manuscript submitted) contributed to this response; the latter may be crucial for the elderly. Therefore, we next assessed whether IL-2/anti-CD40 Ab stimulated macrophages from geriatric mice could be sufficiently activated to restore T cell proliferation and IFN-γ secretion.

Peritoneal macrophages from young or geriatric mice were stimulated with IL-2/anti-CD40 Ab and supernatants collected for CBA. Regardless of age, IL-2/CD40 activated macrophages secreted IFN-γ, TNF-α and IL-12 (Supplementary Figure 7), indicating polarisation to an M1-like phenotype. Macrophages were then co-cultured with varying numbers of CFSE-labelled allogeneic splenic T cells from young Balb/c mice for 5 days and CD8+ T cell proliferation measured, as described above. Macrophages from young and geriatric mice stimulated with IL-2/anti-CD40 Ab induced CD4+ (Figure 6A) and CD8+ T cell proliferation (Figure 6B). Again, to better understand the T cell compartment in geriatric mice we stimulated Balb/c macrophages from young mice with IL-2/anti-CD40 Ab and co-cultured them with varying numbers of CFSE-labelled allogeneic splenic T cells from young or geriatric C57BL/6J mice. The IL-2/CD40 Ab activated macrophages induced a better
CD4⁺ (Figure 6C) and CD8⁺ T cell proliferative response at the lower ratios of macrophages:T cells (Figure 6D). Interestingly, IL-2/CD40 Ab stimulated macrophages increased T cell proliferation and resulted in more divisions compared to all other stimuli (Supplementary Figure 8 and Supplementary Figure 9). These data imply the potential for T cell rescue from senescence using IL-2/CD40 Ab.

Importantly, IL-2/anti-CD40 Ab stimulated macrophages from both young and geriatric mice induced T cells to produce high levels of IFN-γ (Figure 6E). Furthermore, IL-2/anti-CD40 Ab stimulated macrophages from young mice rescued IFN-γ production by T cells from geriatric mice (Figure 6F) and reduced CD4⁺ T cell production of IL-10 compared to no stimuli macrophages (Figure 6G). These data suggest that IL-2/anti-CD40 Ab may restore anti-tumor T cell immunity in geriatric mice via activated macrophages.

Discussion
Macrophages play a key role in innate immunity. They represent a numerically dominant tissue-resident immune cell type that is highly sensitive to its microenvironment. Thus, macrophages are strategically placed to function as sensors for, and eliminators of, potentially dangerous disturbances to this microenvironment. However, when located in solid tumors macrophages may respond to tumor cell-derived signals to promote rather than interfere with tumor development (Mantovani et al. 2009). There is still controversy in regard to the effect of the aging process on macrophage function in healthy hosts, and even less is known about their role in tumorigenesis. One possible factor contributing to the confusion is the use of differing ages for older mice, with many parameters of aging not being evident until about 24 months. In these studies we used a previously published definition of geriatric mice (24⁺...
months) based upon levels of muscle wasting that correspond to geriatric 80+ year old humans (Shavlakadze et al. 2010). Our studies were performed in a controlled specific pathogen free environment to ensure the majority of mice would reach an advanced age. In agreement with others, we found that total macrophage and MDSC proportions were significantly increased in geriatric spleens and bone marrow relative to their much younger counterparts, and that most of these cells were anti-inflammatory M2 cells (Dace & Apte 2008; Ly et al. 2010).

Studies have reported that aging affects macrophages in different tissues differently (Stout et al. 2005). This was highlighted in a recent study showing that LPS-stimulated adherent splenocytes, but not bone marrow-derived macrophages, from aging mice demonstrated an aberrant response (Mahbub et al. 2012). In agreement, we observed different macrophage populations in the peritoneal cavity and LN compared to the spleen and bone marrow. Despite M2 macrophages being the dominating subset in the spleens and bone marrow of geriatric mice, LN and peritoneal macrophages appeared to represent an intermediate (M3) macrophage (Pelegrin & Surprenant 2009) or transitioning cell that co-expressed high levels of M1 and M2 markers prior to stimuli. CD40 and CX3CR1 may be genes that are modulated with age, as there are reports that some immune genes may be particularly prone to age-related changes in DNA methylation (Bjornsson et al. 2008). It is also possible that peritoneal macrophages are located in an environment that is frequently exposed to exogenous factors or concurrently exposed to pro- and anti-inflammatory cytokines leading to continual activation and an M3-like phenotype. Interestingly, there is an increase in serum pro-inflammatory cytokines such as IL-1-β, IL-6 and TNF-α during aging (Alvarez-Rodriguez et al. 2012), which is part of a process known as “inflammaging”. However, anti-inflammatory cytokines such as TGF-β (Doyle et al. 2010) and IL-10 (Alvarez-Rodriguez et al. 2012) also increase in
the serum with age. M3 macrophages have been shown to produce both pro- and anti-inflammatory cytokine such as TNF-α, IL-12, TGF-β and IL-10 (Pelegrin & Surprenant 2009). Yet, in vitro experiments have shown that M3 cells generally exhibit a more suppressive phenotype (Biswas et al. 2006). This is further supported by another study which demonstrated that M3 cells promote tumor growth (Tsai et al. 2007).

Importantly, the M3-like geriatric peritoneal cells responded appropriately to M1 and M2 stimuli in terms of skewing towards CD40 or CX3CR1 expression and secreting the appropriate cytokines. In fact, macrophages from geriatric mice not only maintained their ability to respond to stimuli, but appeared to be more responsive than macrophages from young mice to M1/M2 stimuli; i.e. they expressed higher levels of the surface markers and produced more TGF-β in response to M2 stimuli. Recent studies using mouse models of Alzheimer’s disease have shown that TGF-β is increased in macrophages within the brain during aging (Doyle et al. 2010). This was associated with an increase in TGF-β signalling via increased SMAD 2/3 (Doyle et al. 2010) and may again reflect age-related changes in DNA methylation, leading to dysregulated immune function (Bjornsson et al. 2008). However, others have reported decreased cytokine secretion from macrophages from elderly animal models (Boehmer et al. 2004). This discrepancy may reflect use of a different model (e.g. rat), different housing conditions or different mouse strains and/or macrophages from a different tissue site; we addressed the latter by examining four different tissue sites.

Our previous studies examined rescue of geriatric muscle regeneration by the young immune response (Shavlakadze et al. 2010). In geriatric mice, whilst the onset of macrophage-driven inflammation in vivo is delayed in response to muscle necrosis in whole muscle grafts
transplanted between young and geriatric mice, there is still an effective response which results in muscle regeneration (Shavlakadze et al. 2010). A recent parabiotic study (Ruckh et al. 2012) joined the circulatory system of an old wild-type mouse with that of a young mouse to uncouple elderly oligodendroglial cell intrinsic factors from systemic factors in blood that may affect remyelination. That study showed that macrophages from young mice reversed inefficient central nervous system remyelination in elderly mice, correcting elderly-derived intrinsic signals. These studies suggest that intrinsic defects during aging can be reversed if given the correct stimuli.

Interestingly, in our studies, regardless of M1 or M2 stimulation, macrophages from both young and geriatric mice induced CD8$^+$ and CD4$^+$ T cell proliferation. However, whilst these activated T cells secreted IL-2, macrophages from geriatric animals led to impaired CD8$^+$ T cell production of IFN-γ. Furthermore, macrophages from geriatric mice led to increased T cell production of IL-10. These data imply that only macrophages from young mice present antigen in association with the correct co-stimulation required to induce fully functional T cells. These data are in agreement with others who have shown reduced antigen presentation capacity in macrophages from elderly hosts (Seth et al. 1990).

Here, we observed increased IFN-γ production by T cells from geriatric mice cultured with M1 stimulated or IL-2/CD40 stimulated macrophages implying rescued T cell function in aging mice. One possibility is that the IL-12 secreted by activated macrophages (Supplementary Figure 7C) rescues T cell responses in particular CD8$^+$ CTL responses, as has been shown in aged mice with influenza (Zhang et al. 2000). That study showed that the IL-12 p70/p40 subunit ratio was reduced in old mice, suggesting a relative deficiency in
functional IL-12 activity. Addition of exogenous IL-12 restored T cell IFN-γ production. Other studies have also suggested that IL-12 may play a role in enhancing elderly immunity (Warrington et al. 2003).

Studies suggest that T cells from geriatric mice are memory T cells that are close to reaching the end stage of replicative senescence (Effros et al. 2005), which may account for the weaker proliferative response to M1 macrophages. Yet IL-2/CD40 activated macrophages appeared to also rescue the proliferative responses of T cells from geriatric mice. These data imply the presence of an as yet unidentified mechanism that enables appropriately activated macrophages to improve the function of geriatric T cells possibly by overriding signals associated with senescence. Interestingly, IL-12 has been shown to recover CD28 expression by aged CD4+ T cells, leading to rescue of senescence (Warrington et al. 2003). It may be that IL-12 secreted by IL-2/CD40 activated macrophages overrides senescence, however further studies are required to address this issue.

Similar to normal healthy tissue, tumors, including lung carcinomas and mesotheliomas, also contain large numbers of macrophages (Jackaman et al. 2009). When we turned our focus onto the potential role of macrophages in tumor development we found that lung cancer and mesothelioma derived soluble factors polarized macrophages into M2 macrophages, regardless of age. In the healthy state, M2 macrophages can be induced by exposure to cytokines such as IL-4, IL-10, IL-13, TGF-β and macrophage colony stimulating factor (M-CSF) produced by activated T lymphocytes, B lymphocytes, basophils and/or mast cells, often at healing tissue sites (Mantovani et al. 2004). Tumor cells can also polarise macrophages into the M2 phenotype by secreting IL-10 and/or TGF-β (Mantovani et al. 2004).
Others have shown that human and murine mesothelioma tumor cells (including AE17 cells (Fitzpatrick et al. 1994) and LL cells (Walsh & Young 2010) secrete TGF-β. Thus, TGF-β signaling could be responsible for the induction of M2 cells in our model system as has been shown by others (Mantovani et al. 2009); further studies are required to confirm this hypothesis.

Macrophages from young and geriatric mice secreted TGF-β in response to the tumor-derived factors however, only macrophages from geriatric mice also produced IL-4. This may further drive M2 differentiation in an autocrine manner as IL-4 induces upregulation of JMJD3, which then decreases H3K27 methylation at the promoters of those M2-associated genes to activate transcription (Mantovani et al. 2009). Furthermore, JMJD3 inhibits the transcription of typical M1-associated genes (Mantovani et al. 2009). These data imply that macrophages from geriatric mice are particularly sensitive to signals that promote their development into highly immunosuppressive M2 macrophages that would promote tumor development.

Tumor-exposed macrophages from both young and geriatric mice induced CD8⁺ T cells to proliferate and secrete IL-2 but not IFN-γ, and led to increased CD4⁺ T cell production of IL-10. These data suggest that CD8⁺ T cells activated by tumor-exposed macrophages, i.e. macrophages in the tumor microenvironment, are unlikely to be fully functional and would not execute tumor cell killing. Taken together, these data suggest that, regardless of age, local macrophages will rapidly respond to tumor cell derived-signals by adopting an immunosuppressive phenotype that renders the tissue microenvironment permissive to tumor cell proliferation and angiogenesis. However, macrophages from aged mice may provide an even more suppressed environment that readily promotes tumor development. Any CD8⁺ T

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cell that penetrates this microenvironment will be thwarted by these macrophages and lose its capacity to kill target tumor cells.

We have shown that targeting large mesothelioma and LL tumors with IL-2/anti-CD40 Ab induces a permanent cure (Jackaman et al. 2008; Jackaman & Nelson 2011; Jackaman et al. 2012a) mediated by a complex, and as yet poorly understood, collaboration between T cells, neutrophils (Jackaman et al. 2008), NK cells (Jackaman et al. 2012a) and macrophages (manuscript submitted). Both cancers manifest in elderly populations at a time when T cell immunity is declining (Haynes & Maue 2009). Here we show that IL-2/anti-CD40 Ab stimulated macrophages from both young and geriatric mice rescued T cell function by restoring CD8$^+$ T cell proliferation and IFN-$\gamma$ secretion. These results are promising, particularly for the elderly, and are similar to others who have shown that monocytes from elderly healthy people maintain their ability to differentiate into dendritic cells that activate effector function in senescent T cells (Lung et al. 2000). These data are also in agreement with those who have suggested that use of cytokines may improve the anti-tumor function of macrophages in the elderly (Stout et al. 2005). Our data suggest that use of an IL-2/anti-CD40 Ab-based immunotherapy could be effective in geriatric cancer-bearing hosts. Future in vivo studies are required to delineate the direct and indirect (via CD8$^+$ T cells and other effector cells) anti-tumor role of IL-2/anti-CD40 activated macrophages in geriatric hosts.

Taken together our data show that macrophages from geriatric hosts are hypersensitive to environmental M1, M2 and tumor-derived stimuli. However, they are exquisitely sensitive to immunosuppressive stimuli that are likely to provide a highly tumorigenic microenvironment. Nonetheless, macrophages from geriatric mice maintain their capacity to transition into M1
or M2 cells and if appropriately activated, for example via IL-2/CD40, they can rescue tumor-induced and/or age-related T cell dysfunction. These proof-of-principle studies provide the basis for further in depth studies of activating innate immunity in the elderly.

**Experimental Procedures**

**Mice**

C57BL/6J mice aged 6 - 8 weeks (young) or 24 - 28 months (defined geriatric based Jackson Laboratory definitions (Yuan et al. 2009) were obtained from Animal Resources Centre (ARC) and maintained under specific pathogen free conditions at Curtin University and the University of Western Australia animal facilities. Any mice with a palpable mass, enlarged lymph nodes, enlarged spleen or enlarged liver were excluded from this study so that only healthy non-tumor bearing mice were examined. All experiments were performed according to the Australian Code of Practice for the care and use of animals for scientific purposes as per Curtin University Animal Ethics Committee.

**Tumor cell lines and tumor cell-conditioned media (supernatant)**

AE17 is a malignant mesothelioma cell line derived from the peritoneal cavity of C57BL/6J mice injected with asbestos fibres and has been previously described (Jackaman et al. 2003). The Lewis lung carcinoma (LL) cell line developed in C57BL/6J mice was obtained from the American Tissue Culture Collection (Manassas, VA, USA). The tumor cell lines were maintained in complete medium, consisting of RPMI 1640 (Invitrogen, California, USA) supplemented with 10% fetal calf serum (FCS; ThermoScientific, Victoria, Australia), 50 mg/L gentamicin (ThermoScientific), 60 mg/L benzylpenicillin (CSL Ltd, Pennsylvania, USA), 2 mM L-glutamax (Invitrogen) and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, USA).
Missouri, USA). Cells were cultured at 37°C in a 5% CO₂ atmosphere. For the generation of tumor cell-conditioned media, tumor cells were cultured in serum-free medium (Invitrogen). After 48 - 72 hours, conditioned media from tumor cell cultures were centrifuged and cell-free supernatants collected and stored at -80°C until use.

**Collection and in vitro stimulation of peritoneal macrophages**

Cells obtained from the peritoneal cavity of mice by washing with ice cold PBS were incubated for 2 - 4 hours at 37°C, after which non-adherent cells were removed; the remaining adherent population was > 95% F4/80⁺ macrophages (Jackaman & Nelson 2010). Where specified, macrophages were cultured overnight with 50% AE17 mesothelioma supernatant, 50% LL supernatant, M2 stimuli (20 ng/ml IL-4; Shenandoah Biotechnology, Pennsylvania, USA), M1 stimuli (20 ng/ml IFN-γ, Shenandoah Biotechnology; and 1 µg/ml LPS, Sigma Aldrich) or IL-2/anti-CD40 Ab (10 µg/ml of each, IL-2 obtained from Cetus Corporation, California, USA; anti-CD40 Ab, or FGK45 obtained from AbSolutions, Western Australia).

**Flow cytometry**

Samples for intracellular staining were collected in PBS/2% FCS solution containing Brefeldin A (Biolegend, California, USA). Tissue samples were disaggregated into single cell suspensions by gentle dispersion between two frosted glass slides and stained for flow cytometric analysis. The following anti-mouse primary antibodies were incubated for 1 hour at 4°C in the dark: anti-CD11b-PE-Cy7 (Biolegend), anti-F4/80-APC-Cy7 (Biolegend), anti-Ly6C biotin (Biolegend), anti-Ly6G-PerCP-Cy5.5 (Biolegend), anti-CD40-PE and rabbit anti-CX3CR1 (Abcam, Massachusetts, USA). Following 3 washes in PBS/2% FCS, secondary Ab streptavidin-V500 (Becton Dickinson, California, USA) and Alexafluor® 488-
conjugated anti-rabbit Ab (Biolegend) were incubated for 30 minutes at 4°C in the dark. For intracellular staining, cells were fixed in 1 % paraformaldehyde on ice for 15 minutes, followed by permeabilization with PBS/2% FCS solution containing 0.1% saponin. Cells were then stained with anti-TNF-α-APC and anti-IL10-Brilliant Violet 421 (both from Biolegend). Cells were washed and resuspended in PBS/2% FCS for analysis on a FACSCanto II using FACSDiva software (Becton Dickinson) or FlowJo software (TreeStar, Oregon, USA).

**Allogeneic Mixed Lymphocyte Reaction (MLR) using CFSE-labelled T cells**

Splenocytes collected from either Balb/c mice or C57BL/6J mice were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen), a fluorescent dye that binds to cell membranes (Lyons & Parish 1994). Balb/c splenocytes were used as allogeneic responder T cells for C57BL/6J macrophages and C57BL/6J splenocytes were used as allogeneic responder T cells for Balb/c macrophages. The cells were washed in PBS, resuspended at 2 x 10^7 cells/ml in RPMI containing 3.5 μM CFSE, incubated at room temperature for 10 min, then washed three times with RPMI. Labelled splenocytes were cultured for 2 - 4 hours to remove adherent cells. Non-adherent cells (containing T and B cells) were co-cultured for 3-5 days with varying macrophage:T-cell ratios (1:2 to 1:100). Splenocytes cultured with 1 μg/mL Concanavalin A, (ConA; Sigma-Aldrich) were used as a positive control (data not shown). After 3 - 5 days, cells were cultured with Brefeldin A for 4 hours before being stained with anti-mouse antibodies: anti-CD4-APC-Cy7 and anti-CD8-PerCP-Cy5.5 (Becton Dickinson). Cells were then fixed with paraformaldehyde, permeabilized with saponin and stained with IL-10-Brilliant Violet 421 (Biolegend) and analyzed by flow cytometry. As T cells proliferate, CFSE segregates equally between each daughter population and in flow cytometric analysis each round of proliferation is seen as
sequential halving of CFSE staining intensity. The percentage of T cell proliferation was calculated based on loss of staining intensity of the parent peak.

**Cytokine Bead Array (CBA)**

Concentrations of the cytokines TNF-α, IL-2, IL-4, IFN-γ and TGF-β in stimulated macrophage supernatants and MLR supernatants were measured using BD cytokine bead arrays (CBA, BD Biosciences, California, USA). TNF-α, IL-2, IL-4 and IFN-γ were optimised so that each test could be performed using 1/10 of the manufacturer’s recommended volumes for samples and reagents. The TGF-β CBA and all other aspects of the mouse CBAs were carried out in accordance with the manufacturer’s instructions. Analysis was performed on a FACSCanto II using FACSDiva software (BD Biosciences).

**Data analysis**

Statistical significance was calculated using GraphPad PRISM 4 (California, USA). Student’s t-test and Mann–Whitney U-test were used to determine differences between two populations. P-values of < 0.05 were considered statistically significant.

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Author contributions

Conceived and designed the experiments: CJ DJN. Performed the experiments: CJ. Contributed reagents and in the design of some experiments: HRC ZS TS MDG. Analyzed the data and wrote the manuscript: CJ DJN.

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Figure legends

Figure 1: Suppressive M2 macrophages and MDSCs increase with age

Spleens and bone marrow collected from young or geriatric C57BL/6J mice were stained for markers of macrophages (CD11b^+ F4/80^+; 1B and 1D), M1 macrophages (CD11b^+ F4/80^+ Ly6C^hi CX3CR1^lo), M2 macrophages (CD11b^+ F4/80^+ Ly6C^lo CX3CR1^hi), or MDSC (CD11b^+ F4/80^lo Ly6C^+ Ly6G^+) and analyzed by flow cytometry (1C and 1E). Gating strategy shown in A. Macrophages were confirmed as Ly6G negative (data not shown). Spleens, lymph node and bone marrow were also stained for macrophages (CD11b^+ F4/80^+) and intracellular IL-10 (expressed as mean fluorescent intensity; MFI, shown in F). Data is represented as mean ± SEM from young C57BL/6J mice; n = 6, or geriatric C57BL/6J mice; n = 10. * = p < 0.05, ** = p < 0.01, *** = p < 0.001 comparing young to geriatric mice.

Figure 2: Macrophages from geriatric mice are capable of responding to M1 or M2 stimuli

Peritoneal macrophages harvested from young or geriatric mice were cultured overnight with IFN-γ/LPS (M1 stimuli) or IL-4 (M2 stimuli). Macrophages (CD11b^+ F4/80^+) were identified by flow cytometry for surface expression of CD40 (M1 cells; 2A and C) and CX3CR1 (M2 cells: 2B and D). Supernatants were analyzed for IFN-γ (2E), TNF-α (2F), IL-4 (2G) and TGF-β (2H) by cytokine bead array. Pooled data from four experiments, each with duplicates is shown as mean ± SEM. * = p < 0.05.

Figure 3: Tumor supernatant induces M2 macrophages regardless of age

Peritoneal macrophages harvested from young or geriatric mice were cultured overnight with 50% AE17 mesothelioma tumor supernatant or 50% LL carcinoma tumor supernatant and

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analyzed by flow cytometry for surface expression of CD40 (3A and C) and CX3CR1 (3B and D). Supernatants were analyzed for IFN-γ (data not shown), TNF-α (data not shown), IL-4 (3E) and TGF-β (3F) by cytokine bead array. Data was normalised to tumor supernatants alone. Pooled data from four experiments is shown as mean ± SEM. * = p < 0.05.

**Figure 4: Geriatric-derived macrophages can induce CD8$^+$ T cell proliferation**

Peritoneal macrophages from young or geriatric mice were cultured overnight with IFN-γ/LPS (M1 stimuli; 4C), IL-4 (M2 stimuli; 4D), AE17 mesothelioma supernatant (4E) or LL tumor supernatant (4F). CFSE-labelled Balb/c splenocytes were then added to the macrophages at varying ratios. After 5 days, cells were stained with CD8-PerCP-Cy5.5 and analyzed by flow cytometry. The percentage of CD8$^+$ proliferation was calculated based on loss of staining intensity of the parent peak (gating strategy shown in A). Data is from four experiments represented as mean ± SEM.

**Figure 5: Macrophages from geriatric mice show impaired ability to stimulate T cell production of IFN-γ**

Supernatants were collected from the macrophage:T cell ratio of 1:2 MLR assay described above (Figure 4, Supplementary Figure 4, 5 and 6) and assayed for IFN-γ by cytokine bead array (Figure 5A and B). The data from four experiments was normalised to macrophages alone and is shown as mean ± SEM. Expression of intracellular IL-10 by proliferating CD4$^+$ T cells was analyzed by flow cytometry (expressed as mean fluorescent intensity; MFI, Figure 5C). Data is from two experiments and is shown as mean ± SEM. * = p < 0.05, ** = p < 0.01, comparing M1 and M2 stimuli to no stimuli macrophages from geriatric mice.
Figure 6: IL-2/anti-CD40 Ab rescues T cell production of IFN-γ in geriatric mice

Peritoneal macrophages harvested from young or geriatric mice were cultured overnight with IL-2/anti-CD40 Ab. CFSE-labelled splenocytes were then added to macrophages at varying ratios. After 5 days, cultures were stained with CD4-APC-Cy7 (Figure 6A and 6C) and CD8-PerCP-Cy5.5 (Figure 6B and 6D) and analyzed by flow cytometry. The percentage of CD4^+ and CD8^+ proliferation was calculated based on loss of staining intensity of the parent peak. Data is from three experiments shown as mean ± SEM. Supernatants collected from MLRs from a macrophage: T cell ratio of 1:2 were assayed for IFN-γ by cytokine bead array (Figure 6E and 6F). The data from three experiments was normalised to macrophages alone and is shown as mean ± SEM. Expression of intracellular IL-10 by proliferating CD4^+ T cells was analyzed by flow cytometry (expressed as mean fluorescent intensity; MFI, Figure 6G). Data is from two experiments and is shown as mean ± SEM. * = p < 0.05, ** = p < 0.01, comparing IL-2/CD40 stimulated macrophages to no stimuli macrophages from geriatric mice.
Figure 1

A

SSC vs. FSC plot showing the distribution of cells.

B: Spleen

Bar graph showing the percentage of total cells in Macrophages and MDSCs for Young C57BL/6J and Geriatric C57BL/6J.

C: Splenic macrophage subsets

Bar graph showing the percentage of Macrophages, M1, M2, and Mixed subsets for Young C57BL/6J and Geriatric C57BL/6J.

D: Bone Marrow

Bar graph showing the percentage of total cells in Macrophages and MDSCs for Young C57BL/6J and Geriatric C57BL/6J.

E: Bone Marrow macrophage subsets

Bar graph showing the percentage of Macrophages, M1, M2, and Mixed subsets for Young C57BL/6J and Geriatric C57BL/6J.

F: Interleukin-10

Bar graph showing the MFI of IL-10 in Spleen, Lymph node, and Bone Marrow for Young C57BL/6J and Geriatric C57BL/6J.
Figure 4

A: SPC and CD4+ cell distribution

B: Macrophages, no stimuli
- Young C57BL/6J
- Geriatric C57BL/6J

C: M1-stimulated macrophages

D: M2-stimulated macrophages

E: AE17 supernatant-stimulated macrophages

F: LL supernatant-stimulated macrophages

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Figure 5

A: T cell IFN-γ production; Young versus geriatric macrophages

B: T cell IFN-γ production; Young versus geriatric T cells

C: CD4+ IL-10 MFI; Young versus geriatric T cells

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