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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 vs. geriatric mice to mesothelioma or lung carcinoma tumor cell–derived supernatants. While both supernatants skewed macrophages toward 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.

Key words: aging; cancer immunotherapy; CD40; interleukin-2; macrophage.

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 patients with cancer 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_{reg}) that have been shown to suppress $CD4^+$ and $CD8^+$ T-cell responses via direct cell–cell contact and/or secretion of suppressive factors such as TGF-β (Gregg *et al.*, 2005).

There is evidence that appropriate activation of elderly-derived dendritic cells can restore T-cell function (Sharma *et al.*, 2006). However, the majority of T cells in aged populations are memory T cells (Haynes & Maue, 2009; Fulop *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 *et al.*, 2009), yet the function and role of the latter two cell types are not yet well understood during aging.

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

The phenotype and function of macrophages may differ in young vs. old mice (Dace & Apte, 2008). For example, aging has

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been associated with an inability to respond to M1 stimuli (Mahbub *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 12–15 months old (Ly *et al.*, 2010; Smallwood *et al.*, 2011), while others define 18- to 24-month-old mice 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+ years 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 polarization 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) vs. geriatric (24–28 months old) mice were phenotypically compared. Phenotypic, cytokine, and T-cell activation responses of peritoneal macrophages from young vs. 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, 2012a; Jackaman & Nelson, 2011); 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 old) and geriatric C57BL/6J mice (24–28 months old). The cells were stained for M1 macrophages ($CD11b^+$ $F4/80^+$ $Ly6C^{hi}$ $CX3CR1^{lo}$; (Arnold *et al.*, 2007; Movahedi *et al.*, 2010), M2 macrophages ($CD11b^+$ $F4/80^+$ $Ly6C^{lo}$ $CX3CR1^{hi}$; (Arnold *et al.*, 2007; Movahedi *et al.*, 2010), or MDSCs ($CD11b^+$ $F4/80^{lo}$ $Ly6C^+$ $Ly6G^+$; (Sinha *et al.*, 2007) and analyzed by flow cytometry (gating strategy shown in Fig. 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 (Fig. 1B) and bone marrow (Fig. 1D) relative to their younger counterparts (Figs 1B and D). This coincided with a decrease in the M1 macrophage subpopulation (Figs 1C and E) and a significant increase in the M2 macrophage subset in geriatric compared with young mice (Fig. 1C). M2 macrophages appeared to increase in the bone marrow although the difference did not reach statistical significance (Fig. 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 Fig. 1A). Interestingly, we observed a significant age-related increase in other suppressive subsets, that is, MDSCs and M1/M2 (putative suppressive M3 macrophages; (Biswas *et al.*, 2006; Tsai *et al.*, 2007) macrophages in LNs (Supplementary Fig. 1A). As cytokine secretion is an important delineator of M1 (TNF-α) vs. 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 (Fig. 1F), with no change in TNF-α (Supplementary Fig. 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. 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 with young mice in their resting state prior to stimuli (Figs 2A and B). These data imply the presence of M3 macrophages also in the

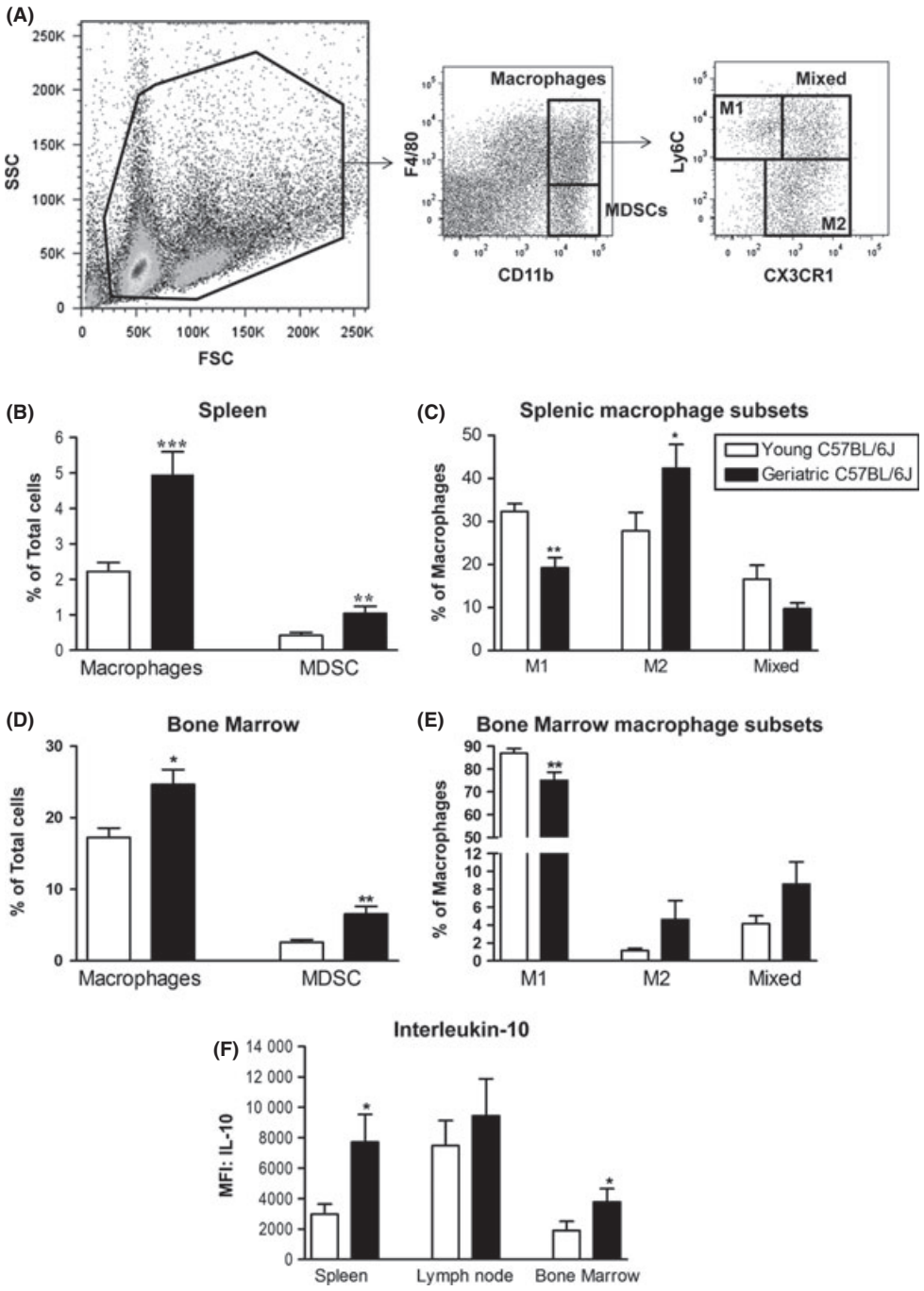

Fig. 1 Suppressive M2 macrophages and MDSCs increase with age. Spleens and bone marrow collected from young or geriatric C57BL/6J mice were stained for markers of macrophages ($CD11b^+$ $F4/80^+$; 1B and 1D), M1 macrophages ($CD11b^+$ $F4/80^+$ $Ly6C^{hi}$ $CX3CR1^{lo}$), M2 macrophages ($CD11b^+$ $F4/80^+$ $Ly6C^{lo}$ $CX3CR1^{hi}$), 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 are 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.

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 (Figs 2A and C) or CX3CR1 with M2 stimuli (Figs 2B and D), respectively (example histograms shown in Supplementary Fig. 2). While macrophages from geriatric mice exhibited higher initial expression levels of CD40 and CX3CR1 compared with macrophages from young mice (example histograms shown in Supplementary Fig. 2), the fold change relative to the no-stimuli controls was similar in both age groups (Figs 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 (Fig. 2E) and TNF-α (Fig. 2F), or M2 cytokines IL-4 (Fig. 2G) and TGF-β (Fig. 2H), respectively. Interestingly, macrophages from geriatric mice produced higher levels of TGF-β after exposure to the M2 stimuli compared with young mice, which suggests the generation of a profoundly immunosuppressive macrophage.

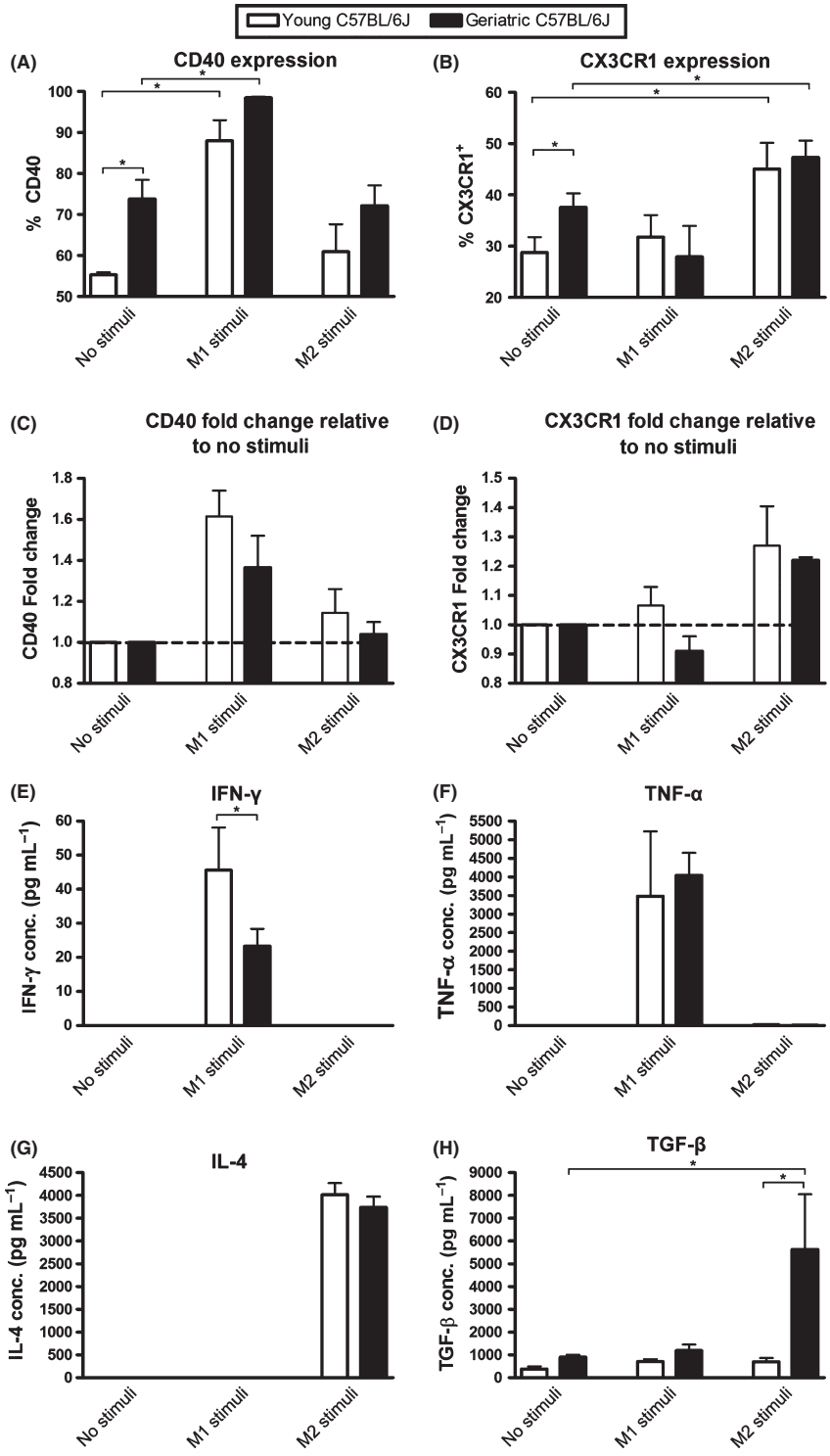

Fig. 2 Macrophages from geriatric mice are capable of responding to M1 or M2 stimuli. Peritoneal macrophages harvested from young or geriatric mice were cultured overnight with IFN-γ/LPS (M1 stimuli) or IL-4 (M2 stimuli). Macrophages ($CD11b^+$ $F4/80^+$) were identified by flow cytometry for surface 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, are shown as mean ± SEM. * = $P < 0.05$.

Tumor supernatant induces M2 macrophages regardless of age

The next experiments assessed whether 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 Fig. 2. Similar to IL-4-stimulated M2 macrophages, macrophages exposed to mesothelioma supernatant or LL tumor supernatant upregulated CX3CR1 regardless of age (Figs 3B and D, example histograms in Supplementary Fig. 3). In contrast, CD40 expression did not change after exposure to tumor supernatant (Figs 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. Peritoneal macrophages from neither young nor 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 (Fig. 3E). Both AE17 mesothelioma supernatant and LL tumor supernatant induced TGF-β production by macrophages from geriatric and young mice (Fig. 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.

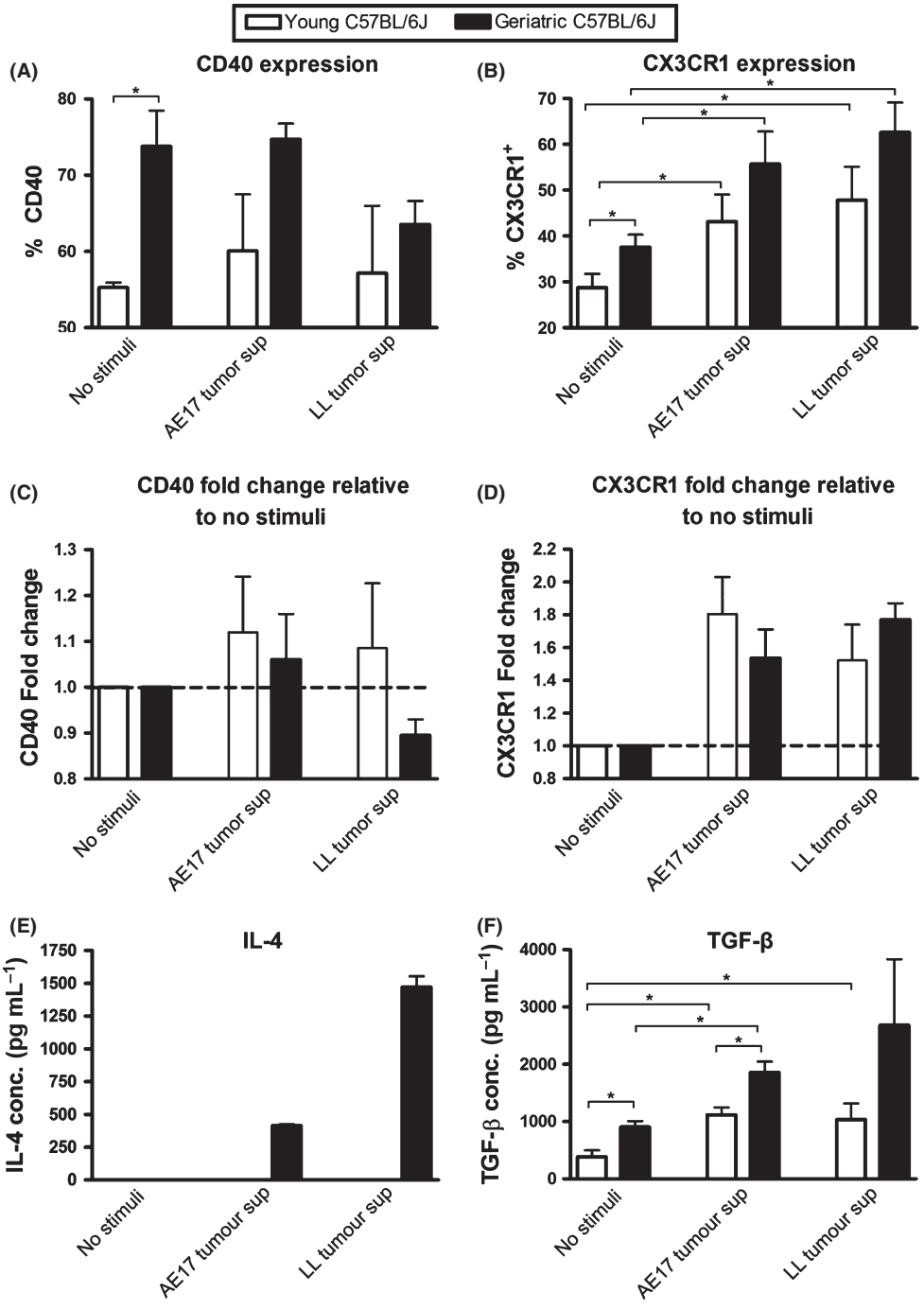

Fig. 3 Tumor supernatant induces M2 macrophages regardless of age. Peritoneal macrophages harvested from young or geriatric mice were cultured overnight with 50% AE17 mesothelioma tumor supernatant or 50% LL carcinoma tumor supernatant and analyzed by flow cytometry for surface expression of CD40 (3A 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 were normalized to tumor supernatants alone. Pooled data from four experiments are shown as mean ± SEM. * = $P < 0.05$.

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 weeks old) Balb/c mice spleens were fluorescently labeled 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; Fig. 4A). Use of stimuli did not improve the ability of macrophages from young or geriatric mice to induce CD4$^+$ (Supplementary Fig. 4) and CD8$^+$ (Figs 4B, C, and D) T-cell proliferation, including macrophages cultured with AE17 mesothelioma supernatant (Fig. 4E) or LL tumor supernatant (Fig. 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 whether 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-labeled 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 Fig. 5) and CD8$^+$ T cells (Supplementary Fig. 6) from geriatric mice. Interestingly, CD4$^+$ and CD8$^+$ T cells from geriatric mice showed a trend toward higher levels of proliferation at lower macrophage/T cell ratios, compared with 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 Fig. 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 Figs 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 with M1 and M2 stimulated macrophages from young mice (Fig. 5A). In contrast, M1 stimulated macrophages from young mice were capable of inducing IFN-γ production by T cells from geriatric mice (Fig. 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, while CD4$^+$ T cells from young mice showed reduced IL-10 production (Fig. 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 (Figs 5A and B), and no change in CD4$^+$ T-cell IL-10 secretion was observed (Fig. 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, 2012a; Jackaman & Nelson, 2011). 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 Fig. 7), indicating polarization to an M1-like phenotype. Macrophages were then cocultured with varying numbers of CFSE-labeled 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$^+$ (Fig. 6A) and CD8$^+$ T-cell proliferation (Fig. 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 cocultured them with varying numbers of CFSE-labeled allogeneic splenic T cells from young or geriatric C57BL/6J mice. The IL-2-/CD40 Ab-activated macrophages induced a better CD4$^+$ (Fig. 6C) and CD8$^+$ T-cell proliferative response at the lower ratios of macrophages/T cells (Fig. 6D). Interestingly, IL-2-/CD40 Ab-stimulated macrophages increased T-cell proliferation and resulted in more divisions compared with all other stimuli (Supplementary Fig. 8 and Supplementary Fig. 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 (Fig. 6E). Furthermore, IL-2-/anti-CD40 Ab-stimulated

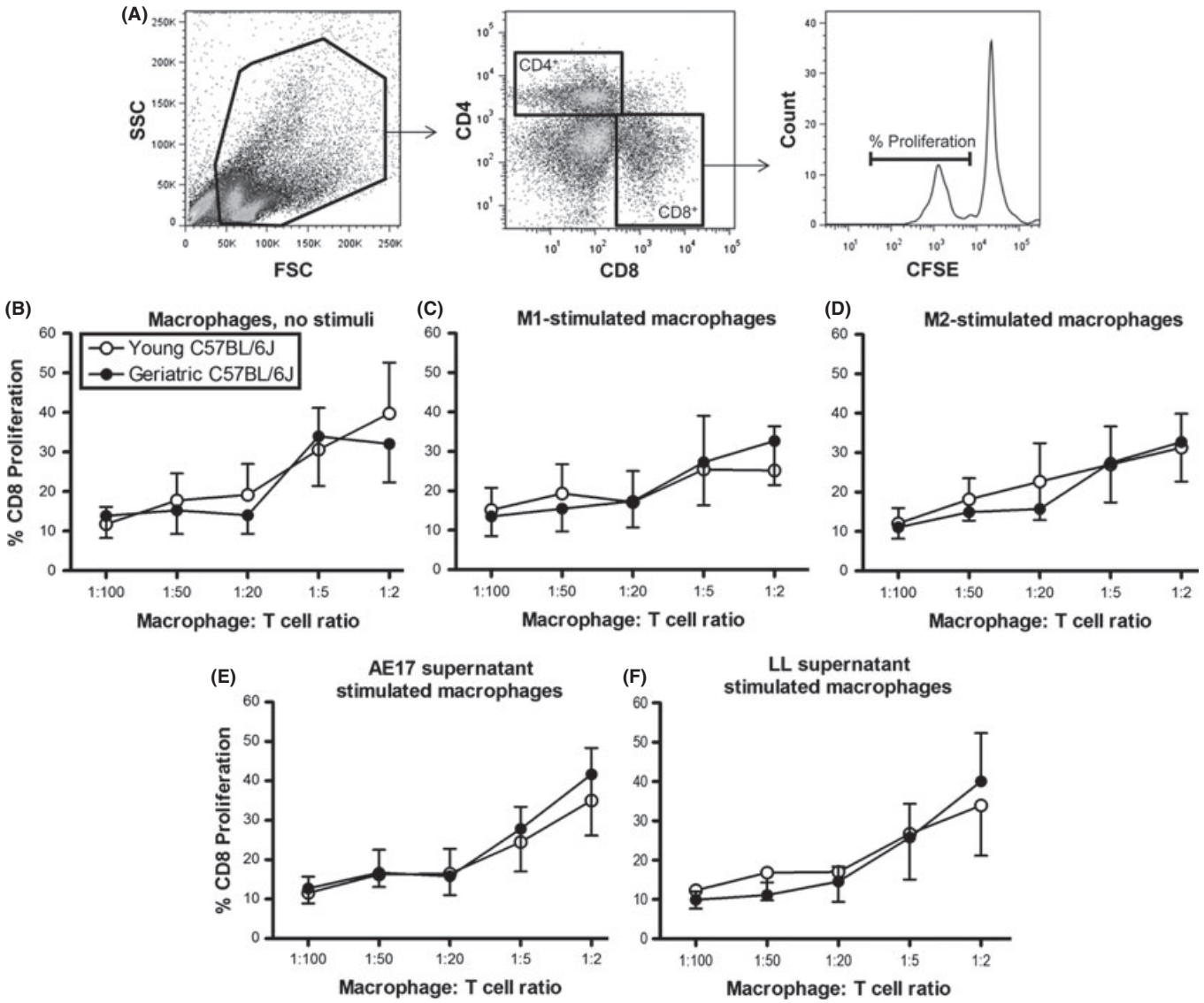

Fig. 4 Geriatric-derived macrophages can induce CD8$^+$ T-cell proliferation. Peritoneal macrophages from young or geriatric mice were cultured overnight with IFN-γ/LPS (M1 stimuli; 4C), IL-4 (M2 stimuli; 4D), AE17 mesothelioma supernatant (4E), or LL tumor supernatant (4F). CFSE-labeled Balb/c splenocytes 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 are from four experiments represented as mean ± SEM.

macrophages from young mice rescued IFN-γ production by T cells from geriatric mice (Fig. 6F) and reduced CD4$^+$ T-cell production of IL-10 compared with no-stimuli macrophages (Fig. 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

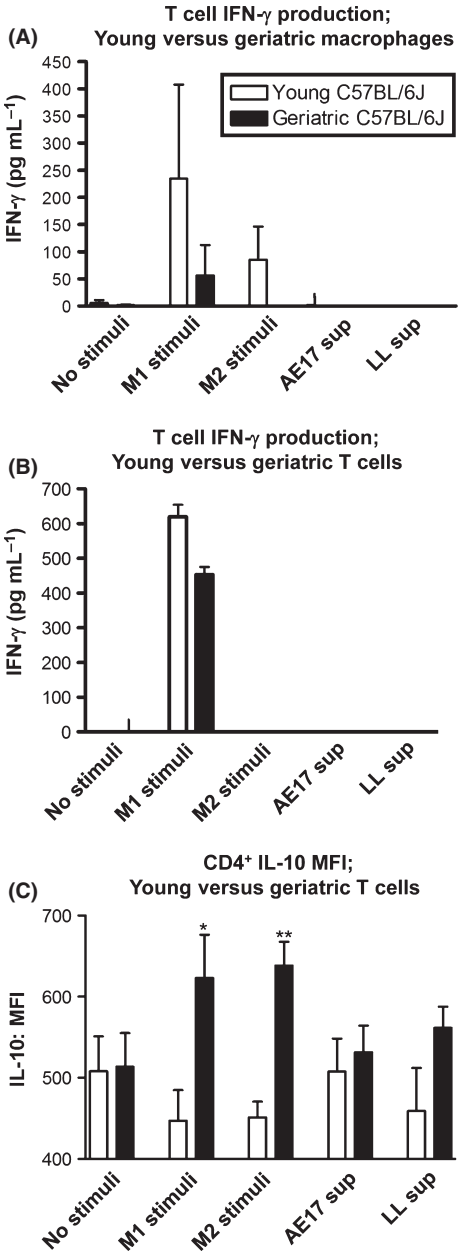

Fig. 5 Macrophages from geriatric mice show impaired ability to stimulate T-cell production of IFN-γ. Supernatants were collected from the macrophage/T cell ratio of 1:2 MLR assay described above (Fig. 4, Supplementary Figs 4, 5, and 6) and assayed for IFN-γ by cytokine bead array (Figs 5A and B). The data from four experiments were normalized to macrophages alone and are 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, Fig. 5C). Data are from two experiments and are shown as mean ± SEM. * = $P < 0.05$, ** = $P < 0.01$, comparing M1 and M2 stimuli to no-stimuli macrophages from geriatric mice.

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 with 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 that 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 toward 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; that is, 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 signaling 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, while 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 that results in muscle regeneration (Shavlakadze *et al.*, 2010). A recent parabiotic study

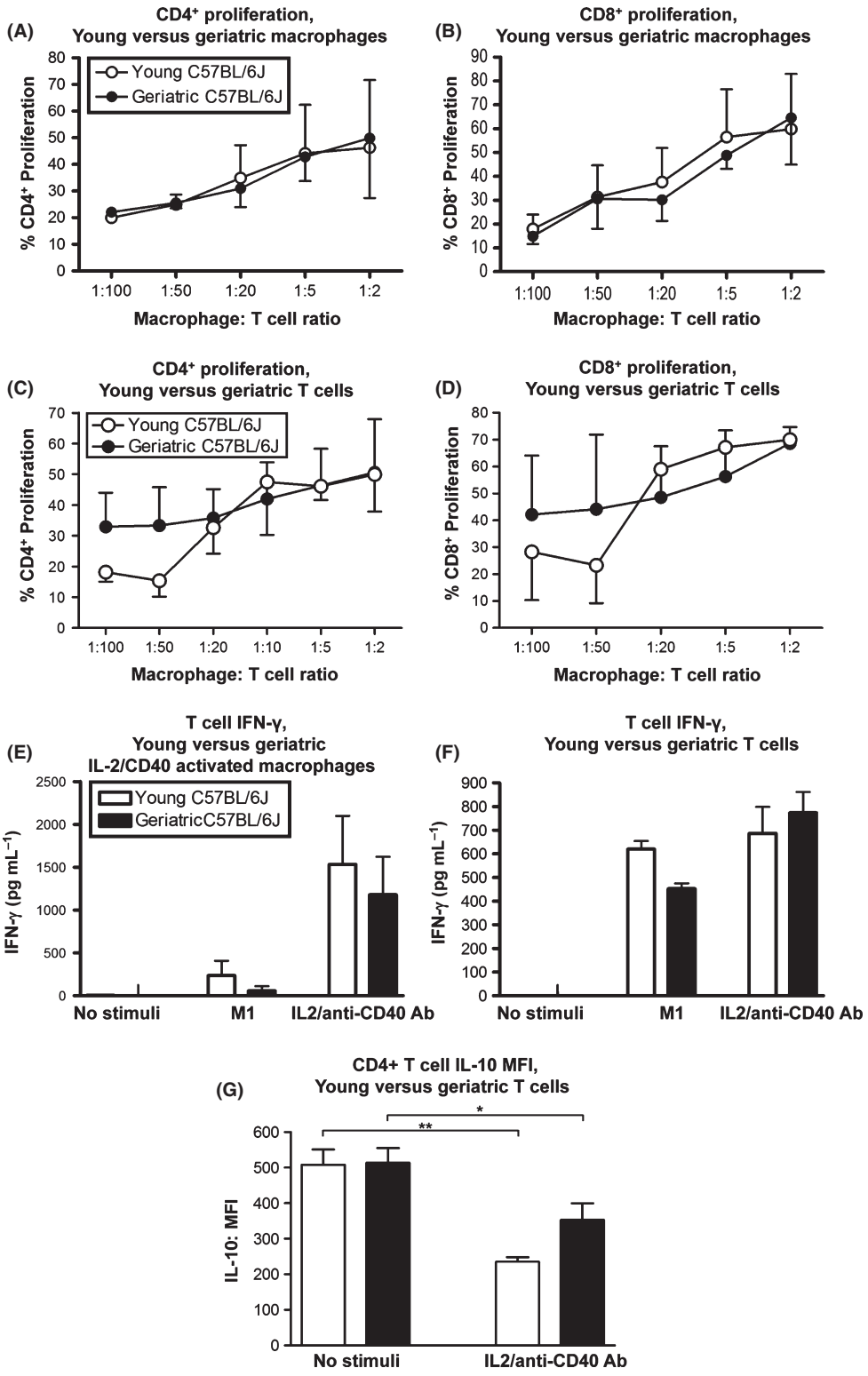

Fig. 6 IL-2/anti-CD40 Ab rescues T-cell production of IFN-γ in geriatric mice. Peritoneal macrophages harvested from young or geriatric mice were cultured overnight with IL-2/anti-CD40 Ab. CFSE-labeled splenocytes were then added to macrophages at varying ratios. After 5 days, cultures were stained with CD4-APC-Cy7 (Figs 6A and 6C) and CD8-PerCP-Cy5.5 (Figs 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 are 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 (Figs 6E and 6F). The data from three experiments were normalized to macrophages alone and are 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, Fig. 6G). Data are from two experiments and are shown as mean ± SEM. * = $P < 0.05$, ** = $P < 0.01$, comparing IL-2-/CD40-stimulated macrophages to no-stimuli macrophages from geriatric mice.

(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, while 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 costimulation 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 Fig. 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 polarize macrophages into the M2 phenotype by secreting IL-10 and/or TGF-β (Mantovani *et al.*, 2009). 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, that is, 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 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, 2012a; Jackaman & Nelson, 2011) 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-γ 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 nontumor-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 fibers 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^{-1} gentamicin (ThermoScientific), 60 mg/L benzylpenicillin (CSL Ltd, Pennsylvania, USA), 2 mM L-glutamax (Invitrogen), and 0.05 mM 2-mercaptoethanol (Sigma-Aldrich, Missouri, USA). Cells were cultured at 37 °C in a 5% CO_2 atmosphere. For the generation of tumor cell-conditioned media, tumor cells were cultured in serum-free medium (Invitrogen). After 48–72 h, 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 h at 37 °C, after which nonadherent 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^{-1} IL-4; Shenandoah Biotechnology, Pennsylvania, USA), M1 stimuli (20 ng mL^{-1} IFN-γ, Shenandoah Biotechnology; and 1 μg mL^{-1} LPS, Sigma-Aldrich), or IL-2/anti-CD40 Ab (10 μg mL^{-1} 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 h 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 three 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 min at 4 °C in the dark. For intracellular staining, cells were fixed in 1% paraformaldehyde on ice for 15 min, 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-labeled T cells

Splenocytes collected from either Balb/c mice or C57BL/6J mice were labeled 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 2×10^7 cells mL^{-1} in RPMI containing 3.5 μM CFSE, incubated at room temperature for 10 min, and then washed three times with RPMI. Labeled splenocytes were cultured for 2–4 h to remove adherent cells. Nonadherent cells (containing T and B cells) were cocultured for 3–5 days with varying macrophage/T cell ratios (1:2–1:100). Splenocytes cultured with 1 μg mL^{-1} 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 h 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 optimized so that

each test could be performed using one-tenth 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

CJ and DJN conceived and designed the experiments. CJ performed the experiments. HRC, ZS, TS, and MDG contributed reagents and in the design of some experiments. CJ and DJN analyzed the data and wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Fig. S1 Suppressive MDSCs increase with age in lymph nodes.

Fig. S2 Macrophages from geriatric mice are capable of responding to M1 or M2 stimuli.

Fig. S3 Tumor supernatant induces M2 macrophages regardless of age.

Fig. S4 Macrophages from geriatric mice can induce CD4+ T cell Proliferation.

Fig. S5 Macrophages from young mice can induce proliferation of CD4+ T cells from geriatric mice.

Fig. S6 Macrophages from young mice can induce proliferation of CD8+ T cells from geriatric mice.

Fig. S7 IL-2/CD40 activated macrophages from young and geriatric mice secrete TNF-α, IFN-γ and IL-12.

Fig. S8 IL-2/CD40 activated macrophages from young and geriatric mice induce greater T cell divisions.

Fig. S9 IL-2/CD40 activated macrophages from young mice induce greater cell division of both young and geriatric T cells.