





CD8⁺ cytotoxic T cell responses to dominant tumor-associated antigens are profoundly weakened by aging yet subdominant responses retain functionality and expand in response to chemotherapy

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ABSTRACT

Increasing life expectancy is associated with increased cancer incidence, yet the effect of cancer and anti-cancer treatment on elderly patients and their immune systems is not well understood. Declining T cell function with aging in response to infection and vaccination is well documented, however little is known about aged T cell responses to tumor antigens during cancer progression or how these responses are modulated by standard chemotherapy. We examined T cell responses to cancer in aged mice using AE17sOVA mesothelioma in which ovalbumin (OVA) becomes a 'spy' tumor antigen containing one dominant (SIINFEKL) and two subdominant (KVVRFDKL and NAIIVFKGL) epitopes. Faster progressing tumors in elderly (22–24 months, cf. 60–70 human years) relative to young (2–3 months, human 15–18 years) mice were associated with increased pro-inflammatory cytokines and worsened cancer cachexia. Pentamer staining and an *in-vivo* cytotoxic T lymphocyte (CTL) assay showed that whilst elderly mice generated a greater number of CD8⁺ T cells recognizing all epitopes, they exhibited a profound loss of function in their ability to lyse targets expressing the dominant, but not subdominant, epitopes compared to young mice. Chemotherapy was less effective and more toxic in elderly mice however, similar to young mice, chemotherapy expanded CTLs recognizing at least one subdominant epitope in tumors and draining lymph nodes, yet treatment efficacy still required CD8⁺ T cells. Given the significant dysfunction associated with elderly CTLs recognizing dominant epitopes, our data suggest that responses to subdominant tumor epitopes may become important when elderly hosts with cancer are treated with chemotherapy.

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Introduction

Over the last 50 years, the number of people aged greater than 65 years old has tripled worldwide, and it is estimated that in 2020 716 millions people will be greater than 65 years old.¹ This dramatic increase in life expectancy has led to an increased incidence of age-related cancers. Yet aging and cancer represent an understudied field and preclinical studies of anti-cancer therapies are mostly conducted in young animals with intact immunity that may not reflect the aged environment. Mesothelioma, a cancer caused by asbestos, emerges in the elderly, with the majority of patients being greater than 60 years old, and its incidence is growing to global epidemic proportions.^{2,3} There is a long latency period (greater than 30 years) between asbestos exposure and development of diagnosable disease⁴ with survival being inversely related to age^{5,6} which may at least partly be explained by declining age-related immunity, termed immunosenescence.⁷ Until now, no studies had examined the effects of aging on T cell immunity in mesothelioma. Indeed, there is very little research into the effects of aging on cancer progression and cancer

therapy in general. Similar to most other cancers, standard chemotherapy extends life expectancy for people with mesothelioma by only a few months, thus there is an urgent need for new treatment options for elderly cancer patients. A deeper understanding of immune function in aging hosts with cancer is required to provide a stronger knowledge base for the design of effective immunotherapeutic interventions in elderly patients.

CD8⁺ cytotoxic lymphocytes (CTLs) are key anti-tumor effector cells that mediate tumor regression. Numerous studies have shown that T cell responses in 6–8 week old mice, equivalent to 13–18 year old humans, can be enhanced to levels that mediate tumor regression. The few studies that have tested T cell-targeting therapies in elderly 18–24 month old mice, equivalent to 56–69 year old humans,⁸ showed poorer outcomes.^{9–12} This may be because T cell function declines with age on account of declining naïve T cell output due to: thymic involution; expanded populations of memory CD8⁺ T cells that are near the end stage of replicative senescence; and defects in TCR signaling responses.^{13,14} These factors might induce changes to CTL immunodominance patterns. CTLs mount strong responses to one or two epitopes

from a given protein antigen, i.e. dominant epitopes, meaning that the immune response focuses on these epitopes. However, proteins contain multiple epitopes, and of the few epitopes that express characteristics enabling CTL recognition, a further two or three may elicit weaker responses, termed subdominant.^{15,16} The mechanisms governing CTL dominance remain unclear, but likely start when proteins are degraded into peptides via the proteasome, with abundant proteins governing dominant responses.¹⁷ Selection continues upon the translocation of proteasomally degraded peptides into the endoplasmic reticulum (ER) via transporters associated with antigen processing (TAP) 1 and 2 that have a preference for 8–40 amino acid peptides. Peptides in the ER compete for binding to MHC class I molecules within the peptide loading complex, comprising TAP and chaperone molecules, such as tapasin, calreticulin and ERp57 which determine the relative proportion of peptides that bind MHC class I molecules¹⁸ meaning only a few peptides could become antigenic epitopes; the latter is driven by the TCR.

The TCR plays a key role in defining dominance hierarchies, with the frequency of antigen specific T cell precursors and high affinity TCRs capable of rapid activation and proliferation responses determining numerical and functional clonal dominance over other CTLs.¹⁹ Under inflammatory conditions such as viral infection the temporal appearance of peptides may also be a factor.²⁰ Dominant CTLs may maintain their hierarchical position by outcompeting lower affinity CTLs for cytokines and other factors. However, these hierarchies are relative, as weaker or subdominant CTLs may assume dominance under circumstances where dominant CTLs experience loss of function, such as those anergized or deleted during tolerance processes. Moreover, under pathological conditions involving inflammation and tissue damage, such as infection or autoimmunity, epitopes recognized by lower affinity TCRs may be released in high concentrations thereby promoting subdominant CTLs. One or more of these processes may be altered by aging, cancer and/or chemotherapy such that they modify the hierarchy of CTL responses to epitopes from tumor antigens.

We have shown, in young tumor-bearing mice, that cisplatin and gemcitabine (two current chemotherapy options for thoracic cancers) slow tumor growth and reveal weaker tumor antigens to the immune system resulting in amplified CTL responses to subdominant epitopes.¹⁵ Others, have shown that responses to subdominant tumor epitopes can be biologically significant.^{21,22} Until now, it was unknown if functional responses to dominant and/or subdominant tumor-derived epitopes are generated in the elderly during tumor growth, and if they are modulated during chemotherapy. As there are no tumor-specific antigens with a known hierarchy of CTL epitopes for mesothelioma, we used a murine mesothelioma model that expresses a neo tumor antigen, ovalbumin (OVA), which contains a known hierarchy of epitopes for MHC class I molecules¹⁵ to evaluate in vivo CTL responses to dominant and subdominant epitopes during tumor growth with or without chemotherapy treatment in elderly versus young mice.

We show that whilst elderly tumor-bearing mice generated a quantitatively stronger CD8⁺ T cell response to dominant epitopes during tumor progression, their lytic capacity was profoundly compromised relative to young tumor-bearing mice. In contrast, elderly mice generated a greater CD8⁺ T cell response to subdominant tumor epitopes that demonstrated at least equal CTL activity to young tumor-bearing controls. Chemotherapy enhanced CTL responses to at least one of the subdominant tumor epitopes in both age groups, however in elderly mice this means their contribution to tumor cell lysis may become more important on account of increasingly dysfunctional dominant-specific CTLs. These data imply an opportunity for inclusion of immunotherapies that target subdominant-specific CTLs in the elderly.

Results

Faster growing tumors are associated with increased cancer cachexia in elderly mice

AE17 mesothelioma tumor cells were inoculated into young and elderly mice and tumor growth rate monitored (Figure 1A). Tumors progressed faster in elderly mice (Figure 1B). Tumor growth was associated with reduced body weight in both age groups relative to healthy, non-tumor-bearing, age-matched controls (Figure 1C), implying development of cancer cachexia. However, healthy elderly mice weighed more than their younger counterparts (Figure 1C) therefore elderly tumor-bearing mice lost a greater percentage of body weight relative to their healthy controls (Figure 1C), implying worsened cachexia.

Healthy aging has been associated with increased circulating pro-inflammatory cytokines,²³ and cancer cachexia may be mediated by pro-inflammatory cytokines.²⁴ As elevated cytokine concentrations may account for exacerbated cachexia in elderly hosts we measured key inflammatory cytokines in serum from age-matched healthy mice and tumor-bearing mice (measured at endpoint when tumors reached the maximal size allowed by Animal Ethics). IFN- γ , IL-6, MCP-1 and TNF- α levels were significantly elevated in elderly tumor-bearing mice relative to their healthy controls, and to young healthy and tumor-bearing mice (Figure 1D) and likely contribute to the development of cancer cachexia and modulate immune responses in the elderly.

Increased inflammatory cytokines may induce liver and kidney damage, for example elevated IL-6 is associated with iron accumulation leading to oxidative tissue damage.²⁵ Therefore, we also looked for evidence of age-related changes to liver and kidney function. Elderly healthy mice showed pre-existing signs of increased liver damage relative to young mice that did not change when they had progressing tumors. Specifically, healthy elderly mice had significantly higher levels of ALT and total protein (Figure 1E) relative to healthy young adult mice. We did not see evidence of kidney damage (i.e. increased urea or creatinine) with healthy aging or in the presence of a tumor (data not

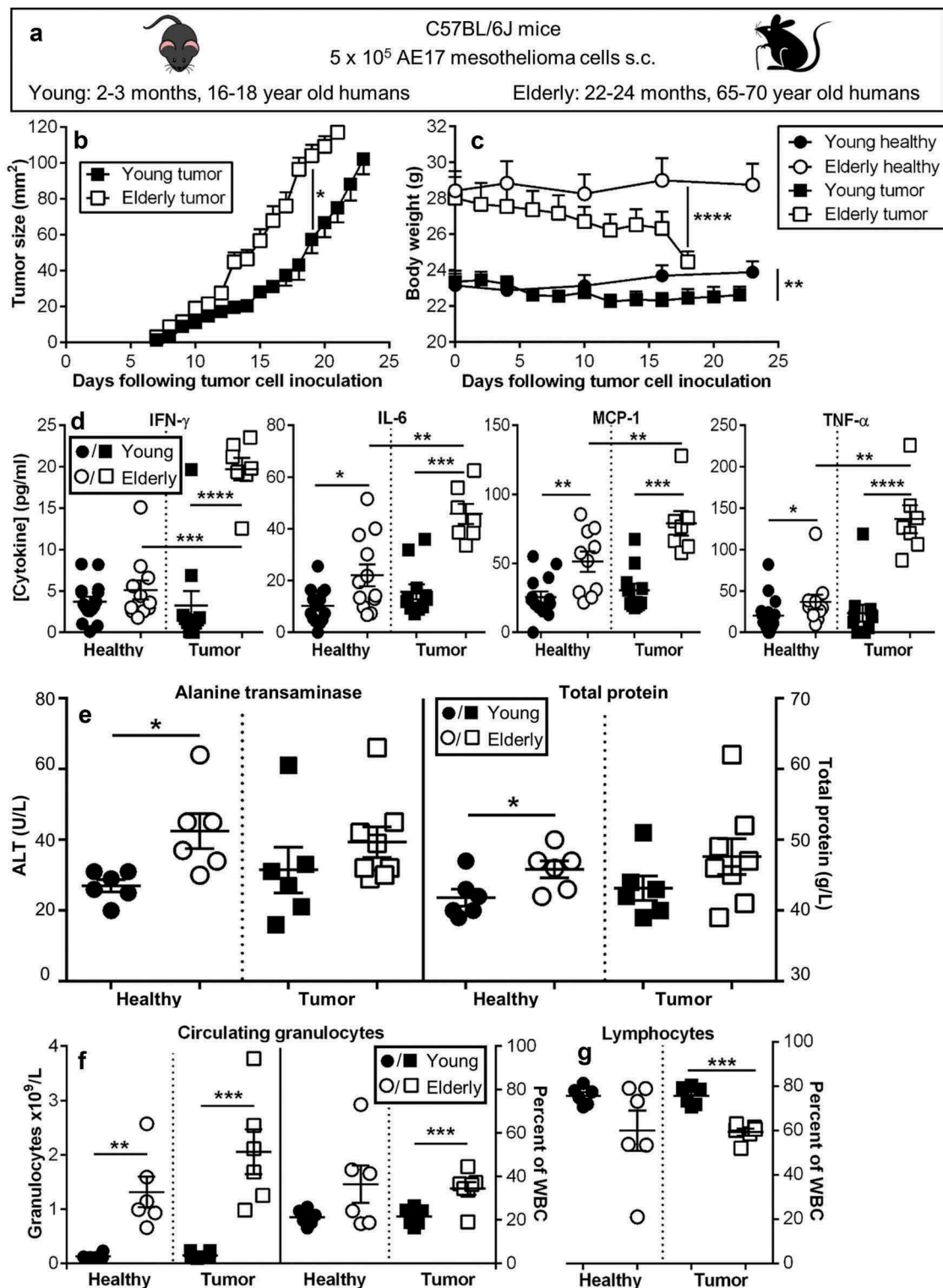


Figure 1. Faster growing tumors are associated with increased cancer cachexia in elderly mice. Young ($n = 11$) or elderly ($n = 13$) C57BL/6J mice (A) were inoculated with 5×10^5 AE17 cells s.c. and monitored daily for tumor growth (B) and body weight (C). Blood collection was performed at endpoint (based on tumor size of 120mm^2 , as per AEC conditions) and serum analyzed for cytokine expression ($n = 10$ healthy young, $n = 11$ AE17-bearing young mice; $n = 5$ healthy old; $n = 7$ old AE17-bearing mice (D), and alanine transaminase and total protein ($n = 6$ healthy young or old mice; $n = 6$ young AE17-bearing mice; $n = 8$ old AE17-bearing mice) (E), whilst $13 \mu\text{l}$ whole blood per mouse ($n = 6$ mice per group) was analyzed for circulating granulocytes (F) and lymphocytes (G) in blood. Data is represented as mean \pm SEM from young or elderly mice in B and C, individual mice shown in D-G. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, analyzed by unpaired t-tests for 1B-G.

shown). These data show that tumor-driven cytokine elevation does not worsen liver or kidney damage in the elderly.

Examination of circulating WBCs revealed that the numbers and percentage of granulocytes increased with healthy aging, this

remained unchanged in the presence of tumor (Figure 1F). In contrast, whilst the numbers (data not shown) and percentages of circulating lymphocytes did not change with healthy aging, the percentage of lymphocytes significantly reduced in elderly relative

to young tumor-bearing mice (Figure 1G); this may be due to increased granulocytes suggesting inflammation. Increased granulocytes could be a source of inflammatory cytokines. Taken together, these data show that elderly mice experience more inflammation and cancer cachexia than their younger counterparts.

Elderly mice generate a quantitatively stronger CD8⁺ T cell response to dominant and subdominant tumor epitopes

We next used our AE17sOVA mesothelioma model to examine CD8⁺ T cell responses in young versus elderly tumor-bearing mice. Similar to AE17, AE17sOVA tumor growth was faster in elderly mice compared to young mice (Supplementary Figure 1). AE17sOVA cells express ovalbumin (OVA) so that in AE17-sOVA tumor-bearing C57BL/6J mice OVA becomes a 'neo' or spy tumor antigen. Protein antigens, including ovalbumin, contain several MHC class I epitopes, with one or two being strongly recognized, or dominant, a few being weakly recognized or subdominant, and the remainder being cryptic. OVA contains a known hierarchy of epitopes that bind MHC class I H-2K^b molecules expressed by mice on the C57BL/6J background, i.e. the dominant peptide OVA_{257–264} (SIINFEKL), and two subdominant peptides OVA_{55–62} (KVVRFDKL) and OVA_{176–183} (NAIVFKGL).²⁶ As CTL responses to dominant tumor epitopes may be tolerized or modified by the aging process and/or tumors, we examined CD8⁺ T cell responses to dominant (SIINFEKL) and one of the subdominant epitopes (KVVRFDKL) in elderly AE17-sOVA-bearing mice at day 21 of tumor growth using pentamers (representative flow cytometry plots in Figure 2A and B).

CD8⁺ T cell proportions (of total cells) were lower in elderly mice in AE17-sOVA tumor-draining LNs (DLNs) and spleens (Figure 2C), yet the proportion of CD8⁺ T cells that were specific for the dominant epitope, SIINFEKL, were significantly higher in elderly DLN, relative to young mice (Figure 2D). Interestingly, the proportion of CD8⁺ T cells specific for the subdominant epitope, KVVRFDK (KV), were also significantly elevated in elderly DLN relative to their younger counterparts (Figure 2E).

Elderly mice generate a qualitatively weaker CTL response to dominant tumor epitopes

Pentamer staining shows that tumor-antigen specific CTL are present but does not measure whether they are functional. Thus, the next series of experiments examined the in vivo CTL response generated to all three epitopes in young and elderly mice during AE17sOVA tumor progression, with analysis starting 7 days after tumor cell inoculation. We modified an in vivo CTL assay to enable simultaneous visualization in vivo lysis of target cells carrying the 3 epitopes plus 2 control peptides. This assay involves target cells that can only be recognized by CTLs specific to each peptide; i.e. target cells carrying the peptides on surface MHC class I molecules. Their disappearance, due to in vivo killing by functional effector CTLs, can be seen when analyzed by flow cytometry. In this modified assay, pooled target cells from the LNs and spleens of naïve C57BL/6J mice were divided into five populations, as described in the methods, and injected i.v. into mice. FACS

analysis of disaggregated organs was conducted 18 hours later (representative plots shown in Figure 3A).

All young AE17-sOVA-bearing mice developed a strong CTL response to SIINFEKL in DLNs and spleens 7 days after tumor cell inoculation, this response significantly increased by day 14, and significantly diminished at 21 days of tumor growth in DLNs (Figure 3B and C) likely due to tolerizing mechanisms. In contrast, only one of three elderly AE17-sOVA-bearing mice demonstrated a strong CTL response to SIINFEKL at day 7, this increased to four of five mice showing detectable responses at day 14. By day 21 all five elderly mice had weak but detectable CTL responses to SIINFEKL, however the differences in CTL lysis did not reach statistical significance. An even weaker and slower response was detected in elderly AE17-sOVA-bearing spleens (Figure 3B and C), and again the differences were not statistically significant.

Elderly mice generate a qualitatively equal or enhanced CTL response to subdominant tumor epitopes

CTL responses to the KVVRFDKL (KV) subdominant epitope were varied yet detectable in the majority of DLNs from young AE17-sOVA-bearing mice, whilst responses to KV in DLNs from elderly AE17-sOVA-bearing mice were not seen at day 7, emerged at day 14 and persisted to day 21 (Figure 3D). Splenic KV CTLs responses were detected by day 14 in young and elderly mice, with three of five elderly mice demonstrating persisting responses (Figure 3E).

CTL responses to the NAIVFKGL (NAIV) subdominant epitope were absent in most young and elderly AE17-sOVA-bearing DLNs at day 14 (day 7 was not analyzed), however stronger responses were detectable in both age groups by day 21 of tumor growth (Figure 3F). No NAIV responses were seen in young AE17-sOVA-bearing spleens at day 14, with two mice showing weak responses at day 21. In contrast, three of five elderly AE17-sOVA-bearing spleens showed weak but detectable responses at days 14 and 21 (Figure 3G).

Taken together, the data show that AE17sOVA is an immunogenic tumor, and that functional CTL responses to dominant and subdominant epitopes can be detected in young and elderly tumor-bearing mice. Moreover, there was evidence for at least equal, and in some cases, enhanced responses to subdominant epitopes in elderly mice, particularly at day 21 in elderly spleens. Nonetheless, tumor growth rates outpace CTL responses in both age groups, and faster tumor growth implies this is more pronounced in elderly mice.

Unlike T cells in young mice, T cells in elderly mice cannot restrain tumor growth

We next asked if T cells infiltrate tumors in young and elderly mice. No age-related differences were seen between CD8⁺, SIINFEKL and KV-specific CD8⁺ T cell proportions in tumors, the latter measured using pentamers (Figure 4A). However, analysis of in vivo DLN SIINFEKL CTL activity as a function of tumor size revealed that whilst CTL responses declined in both age groups with increasing tumor size, elderly mice demonstrated weaker functional responses, even when their tumors were small (Figure 4B). Finally, depletion studies confirmed that even though T cells in young mice could not prevent tumor development, they

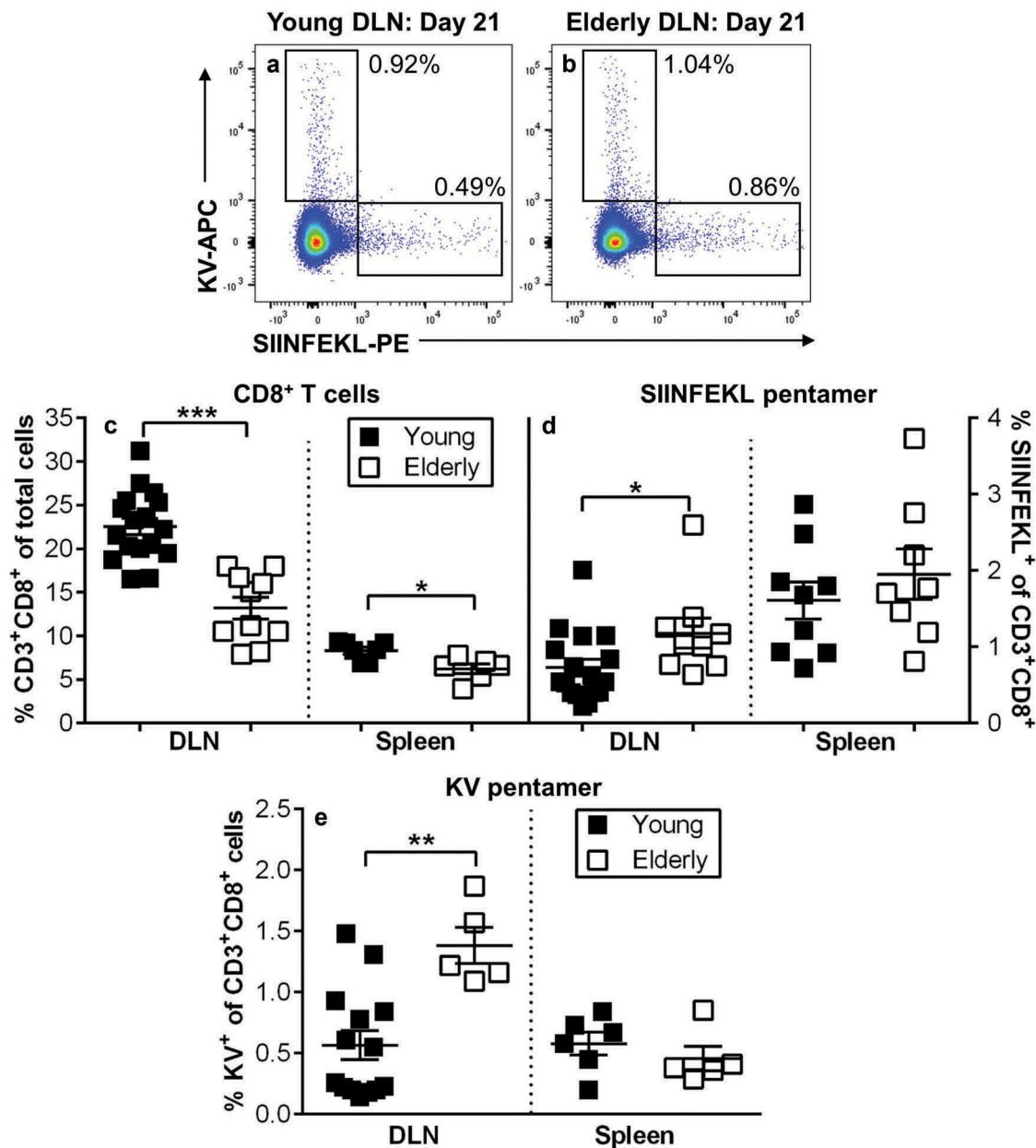


Figure 2. Elderly mice generate a quantitatively stronger CD8⁺ T cell response to dominant and subdominant tumor epitopes. Spleens and draining lymph nodes (DLN) were collected from young or elderly AE17-sOVA-bearing mice 21 days post tumor cell inoculation. Samples were stained for CD3, CD8, SIINF EKL and KV pentamers and analyzed by flow cytometry. Representative plots from young or elderly DLN gated on CD3⁺CD8⁺ are shown in A and B. Individual mice and mean \pm SEM are shown for total CD8⁺ T cells (DLN n = 17 and 10, spleens n = 7 and 6, in young and old AE17-bearing mice respectively) (C), SIINF EKL pentamer (DLN n = 11 and 6, spleens n = 9 and 8, in young and old AE17-bearing mice respectively) (D) and KV pentamer (DLN n = 14 and 5, spleens n = 6 and 5, in young and old AE17-bearing mice respectively) (E). * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Mann-Whitney U-test.

could restrain tumor growth, as in the absence of CD4⁺ or CD8⁺ T cells tumors grew faster (Figure 4C). In contrast, T cell depletion studies in elderly mice showed that CD4⁺ T cells were ineffective, as their absence did not perturb tumor growth rates (Figure 4D). Interestingly, depletion of CD8⁺ T cells in elderly mice significantly slowed tumor growth (Figure 4D), suggesting that these cells become immunosuppressive with aging.

Chemotherapy is not as effective and more toxic in elderly mice

We then examined the effect of two chemotherapeutic agents on young versus elderly tumor-bearing mice. Cisplatin and

gemcitabine display clinical activity against a range of solid tumors including mesothelioma. Cisplatin, a platinum-containing drug, uses transporter molecules to enter cells, bind DNA and interfere with transcription and/or DNA replication, triggering cytotoxic activity and cell death. Gemcitabine, a prodrug activated by deoxycytidine kinase and other kinases into gemcitabine triphosphate, has multiple intracellular targets and can be incorporated into RNA and DNA by competing with deoxycytidine triphosphate to induce cell death. We have previously shown that both chemotherapies enhance CTL activity against subdominant epitopes in young tumor-bearing mice.¹⁵

Gemcitabine and cisplatin were both significantly more effective at restraining tumor growth in young mice relative

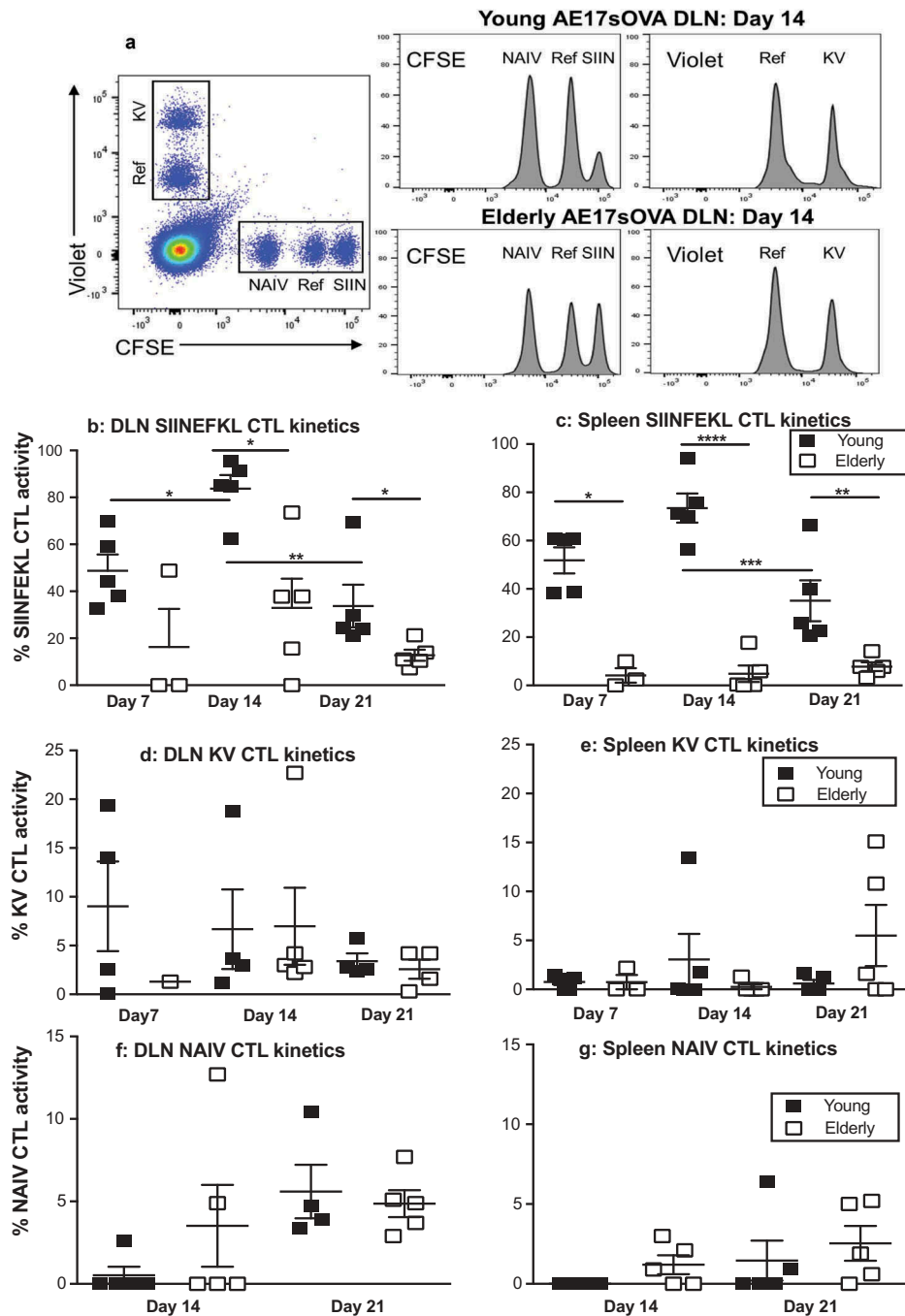


Figure 3. Elderly mice generate a qualitatively weaker CTL response to dominant and an equal response to subdominant epitopes. AE17-sOVA-bearing young or elderly mice were sampled at weekly intervals (day 7, day 14 and day 21) post tumor cell inoculation and analyzed for in vivo CTL activity in DLNs and spleens. Target cells were pooled LN and spleen cells from naïve C57BL/5J divided into five populations. The SIINFVKL-pulsed population was labeled with a high concentration of CFSE, NAIV-pulsed population labeled with a low concentration of CFSE and KV-pulsed population labeled with a high concentration of Violet dye. No peptide control cells were labeled with an intermediate concentration of CFSE or low concentration of Violet dye. The five populations were pooled equally and injected i.v. into mice for flow cytometry analysis 16–18 hours later. Representative flow cytometry plots displaying the five populations in a control mouse are shown in A, along with representative histograms for young or elderly AE17-sOVA-bearing mice in DLNs at day 14. Data from individual mice ($n = 5$ mice/group for all groups except day 7 elderly mice in which there were 3 mice/group) and mean \pm SEM is shown for SIINFVKL (B and C), KV (D and E) and NAIV (F and G). * $p < 0.05$, ** $p < 0.01$ analyzed by Mann-Whitney *U*-test.

to elderly mice (Figure 5A to D). Gemcitabine and cisplatin induced liver damage in young and elderly mice relative to untreated tumor-bearing controls, indicated by elevated Alk Phos, this was particularly evident in elderly mice (Figure 5E). Whilst we examined several pro-inflammatory cytokines are per Figure 1D, the only cytokine that was elevated in elderly

but not young mice, and only by cisplatin, was MCP-1 (Figure 5F). Moreover, elderly tumor-bearing mice lost significantly more body weight during treatment with both chemotherapeutic agents (Figure 5G). Taken together, these data show that chemotherapy is not as effective and is more toxic in elderly mice.

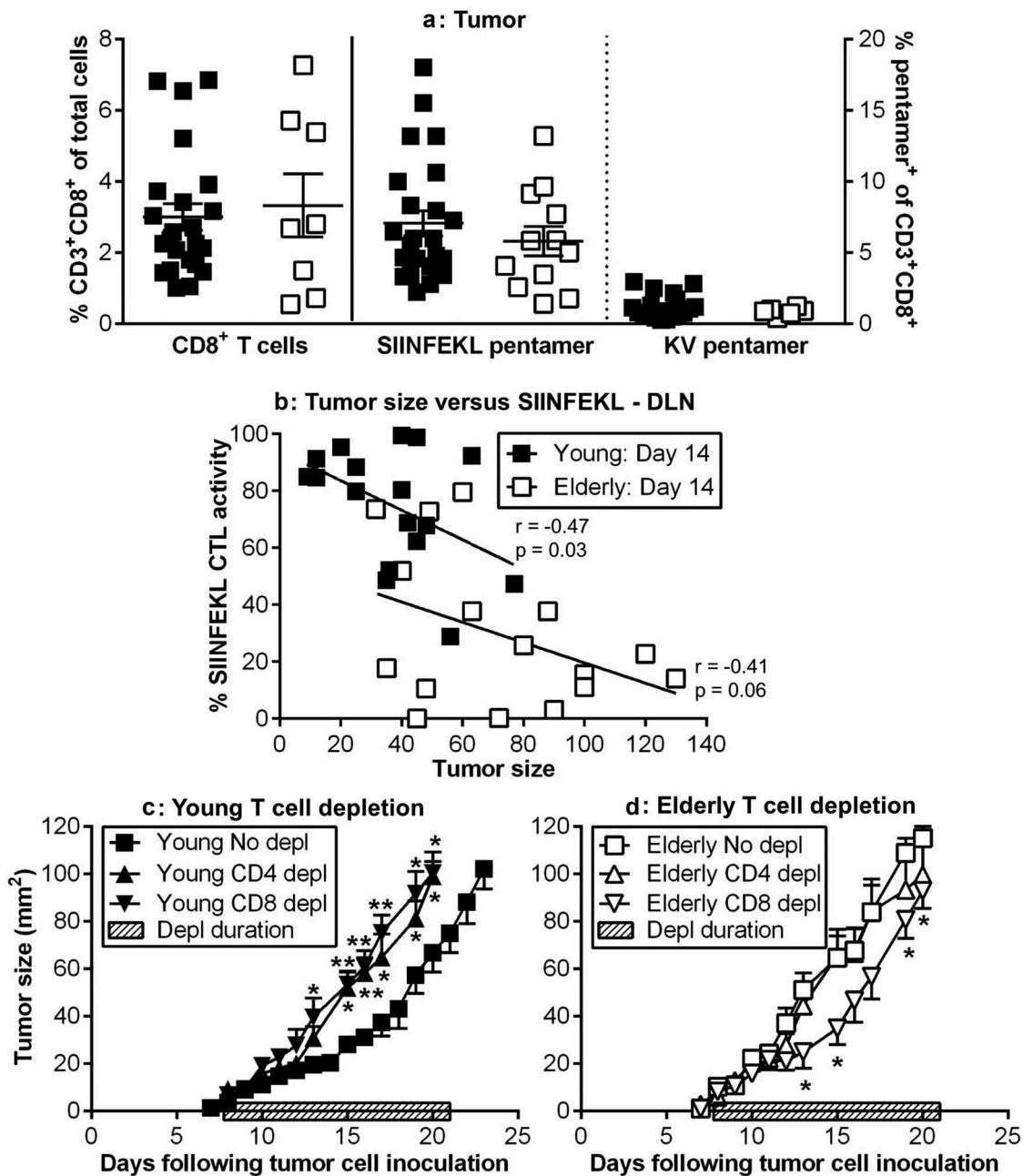


Figure 4. T cells in elderly mice cannot restrain tumor growth. Tumors were collected from young or elderly AE17-sOVA-bearing mice 21 days post tumor cell inoculation, stained for CD3, CD8 and SIINFEKL or KV pentamers and analyzed by flow cytometry (A) as per Figure 2. CD8⁺ T cell data from $n = 24$ young and 8 old mice, SIINFEKL pentamer data from $n = 24$ young and 12 old mice, and KV pentamer data from $n = 18$ young and 6 old mice; shown as individual mice and mean \pm SEM, no age-related differences were seen, analyzed by Mann-Whitney U -test. In vivo CTL activity in DLNs at day 14 was correlated by linear regression analysis with tumor size from AE17-sOVA-bearing young ($n = 17$) or elderly ($n = 16$) mice (B), data shown as individual mice. In a separate experiment, young (C) or old (D) AE17-bearing mice were depleted of CD4⁺ ($n = 7$ young and $n = 8$ old CD4 depleted mice, $n = 13$ young and $n = 11$ old no depletion control mice) or CD8⁺ T cells ($n = 7$ young and $n = 8$ old CD4 depleted mice, $n = 8$ young and old no depletion control mice) during tumor growth from day 7 to day 21; data shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ analyzed by Mann-Whitney U -test.

Chemotherapy efficacy in elderly mice still requires T cells

Whilst the numbers of circulating lymphocytes were not significantly affected by chemotherapy in elderly tumor-bearing mice compared to their age-matched untreated controls, elderly mice still had lower numbers of lymphocytes relative to young mice (Figure 6A). Despite lower lymphocyte numbers, T cell depletion studies showed that the efficacy of both chemotherapeutic agents required the presence of CD4⁺ and CD8⁺ T cells in elderly mice, as in the absence of T cells tumors grew faster in young and elderly mice (Figure 6C to F).

Gemcitabine increases the proportion of subdominant CD8⁺ T cells in tumors

Gemcitabine reduced the proportion of CD8⁺ T cells that recognized the dominant SIINFEKL epitope (measured using pentamers) in DLNs mid-way through treatment in both age groups; cisplatin had little effect (Figure 7A). Examination of subdominant pentamer KV-specific CD8⁺ T cells revealed that gemcitabine exerted little effect in either age group, yet cisplatin reduced the proportion of KV-specific CD8⁺ T cells in DLNs in elderly mice

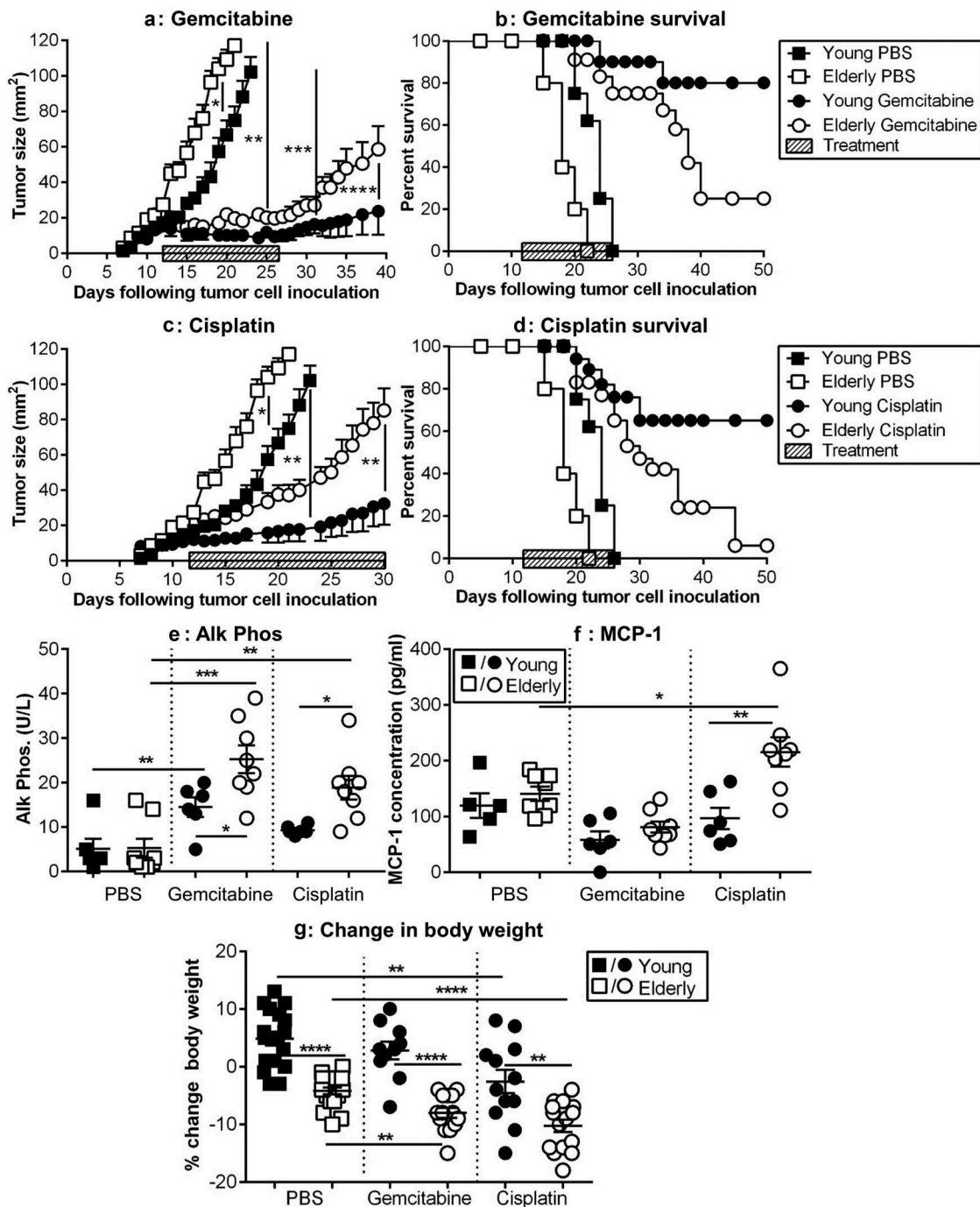


Figure 5. Chemotherapy is not as effective and more toxic in elderly mice. AE17-bearing young or elderly mice were treated with the PBS diluent, gemcitabine (120 $\mu\text{g/g/dose}$ every three days for two weeks; $n = 12$ mice/group) or cisplatin (6 $\mu\text{g/g/dose}$ weekly for three weeks; $n = 17$ mice/group). Tumor growth (A and C), survival (B and D) and body weight (G) were monitored daily. Serum was analyzed for Alk Phos (E; $n = 6$ for all young and the healthy old group, $n = 8$ for other old groups) and MCP-1 (F; $n = 5$ for AE17-bearing young mice, $n = 6$ for healthy young AE17-bearing mice treated with either chemotherapy, and healthy old mice, $n = 8$ for all other old groups) at day 21. Individual mice and mean \pm SEM are shown. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ analyzed by Mann-Whitney U -test.

(Figure 7B). Comparing the relative proportions of SIINFEKL and KV pentamer⁺ T cells in DLNs in each treatment group (Figure 7C) confirms that the elderly response is more skewed away from SIINFEKL and towards KV responses. Figure 7C also reveals that gemcitabine expands the KV response such that it adopts a more prominent position in both age groups, with the elderly demonstrating slightly more KV⁺ T cells. The opposite happens after cisplatin treatment, with elderly

DLN SIINFEKL⁺ T cells becoming dominant and looking remarkably similar to young mice.

SIINFEKL-specific CD8⁺ T cell proportions in tumors from young and elderly mice were also significantly reduced with gemcitabine; cisplatin showed minimal effects (Figure 7D). In contrast, gemcitabine expanded KV-specific CD8⁺ T cells in young and elderly tumors (Figure 7E). Note tumors were collected at day 21 (mid-way through treatment) to enable comparisons to the

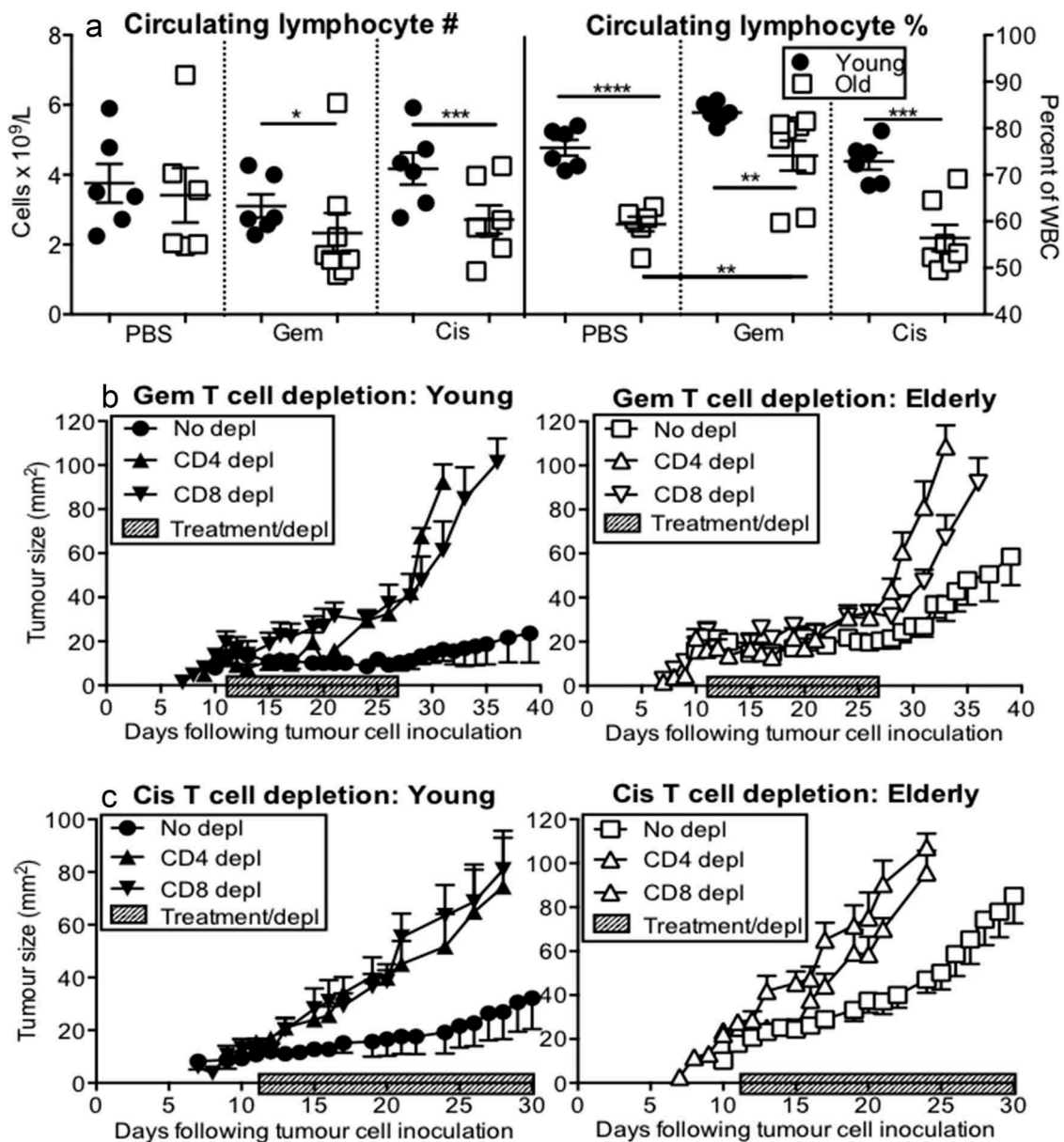


Figure 6. Chemotherapy efficacy in elderly mice still requires T cells. AE17-bearing young or elderly mice were treated with gemcitabine or cisplatin and blood analyzed for circulating lymphocytes at day 21 (A and B, individual mice and mean \pm SEM shown; $n = 6$ mice/group for young and old healthy mice, AE17-bearing mice given the PBS diluent control, and young AE17-bearing treated with either chemotherapy, $n = 7$ and 8 for old AE17-bearing mice treated with cisplatin or gemcitabine respectively). In a separate experiment, AE17-bearing mice were depleted of CD4⁺ ($n = 12$ for young and old no depletion controls, $n = 8$ old CD4 depleted and $n = 7$ young CD4 depleted) or CD8⁺ T cells ($n = 8$ for young and old no depletion controls, $n = 8$ old CD8 depleted and $n = 6$ young CD8 depleted) using monoclonal antibodies two days before the start of either gemcitabine (C and D) or cisplatin (E and F) chemotherapy; antibody depletions were continued for 14 days; data shown as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$ analyzed using unpaired t-tests for 6A and B.

untreated controls shown in Figure 2. Comparing the relative proportions of SIINFEKL and KV pentamer⁺ T cells in tumors (Figure 7E) shows that although the SIINFEKL response remains dominant, the elderly response has a higher level of KV responses, this KV response is significantly amplified by gemcitabine in both age groups. Unexpectedly, cisplatin refocused elderly tumor-infiltrating CD8⁺ T cells back onto SIINFEKL.

Taken together, these data show that gemcitabine increases the proportion of KV-specific subdominant-specific CD8⁺ T cells, however the magnitude of the response in DLNs is different to that seen in tumors.

Chemotherapy maintains or enhances functional (qualitative) CTL responses to subdominant tumor epitopes in elderly mice

Gemcitabine reduced SIINFEKL-specific CTL activity in DLNs in young mice, but not elderly mice (Figure 8A). Cisplatin had a minimal effect on SIINFEKL-specific CTLs in DLNs in young and elderly mice.

Elderly AE17-sOVA-bearing untreated mice demonstrated a broader KV-specific CTL response ranging from a readily detectable response to little CTL activity, whilst young mice demonstrated weak KV-CTL activity (Figure 8B). Cisplatin increased the KV-specific CTL response in young mice and

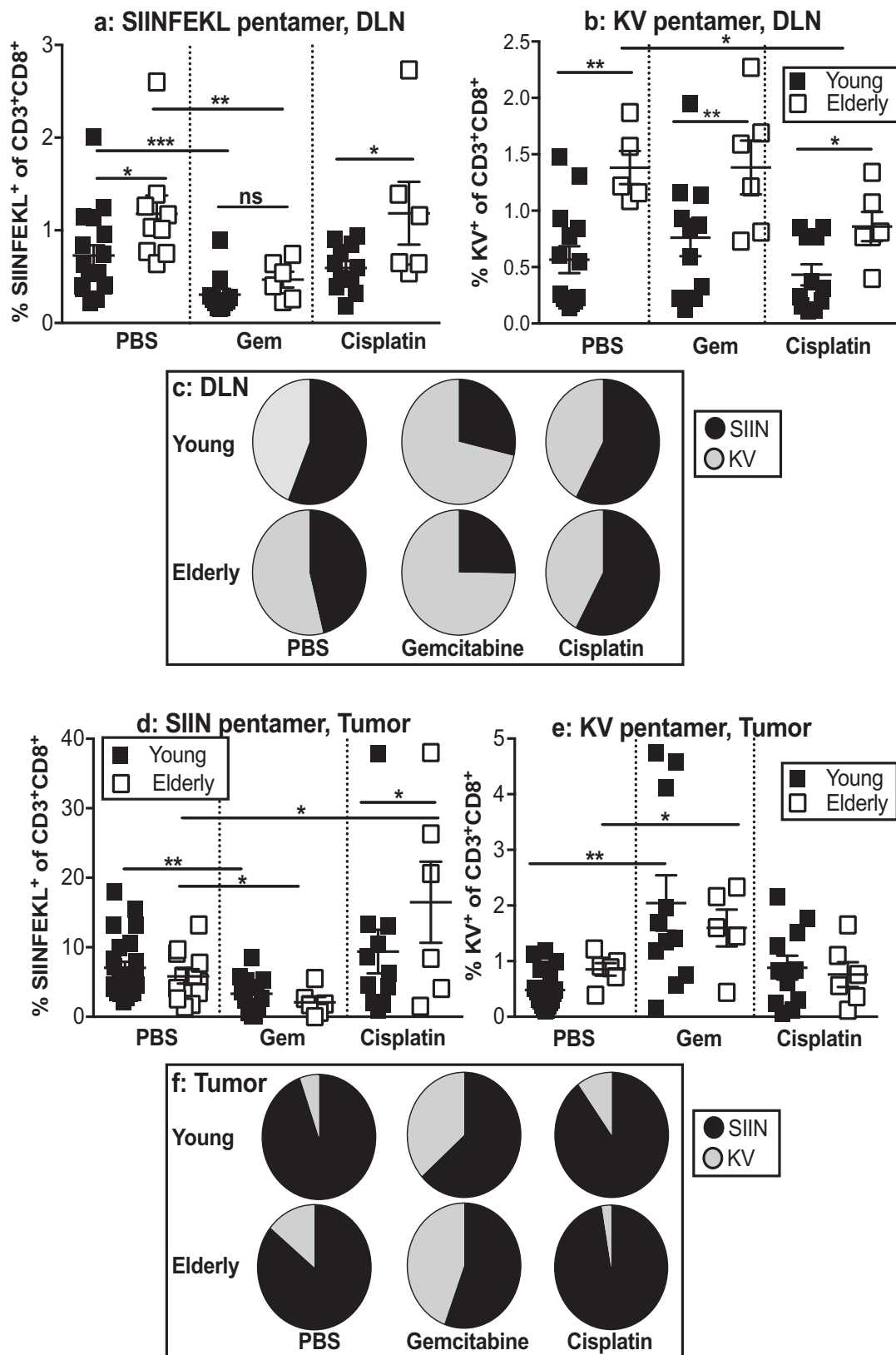


Figure 7. Gemcitabine increases subdominant CD8⁺ T cells in elderly tumors. DLNs (A and B) and tumors (D and E) from AE17-sOVA-bearing young or elderly mice treated with gemcitabine or cisplatin were collected at day 21, stained for CD3, CD8, SIINFEKL (A and D) and KV pentamers (B and E) and analyzed by flow cytometry (as per Figure 2). Individual mice and mean \pm SEM are shown for SIINFEKL pentamer (A; n = 17 and 9 young and elderly untreated mice respectively, n = 11 young, n = 6 elderly chemotherapy treated mice, B; n = 9 and 8 young and elderly untreated mice respectively, n = 6 young and elderly chemotherapy treated mice, and F; n = 24 and 12 young and elderly untreated mice respectively, n = 11–12 young and n = 6 elderly chemotherapy treated mice), KV pentamer (C; n = 14 and 5 young and elderly untreated mice respectively, n = 11 young, n = 5 elderly chemotherapy treated mice, D; n = 6 and 5 young and elderly untreated mice respectively, n = 6 young and elderly chemotherapy treated mice, and G; n = 18 and 6 young and elderly untreated mice, n = 11–12 young and n = 6 elderly chemotherapy treated mice) and total CD8⁺ T cells (E; n = 23 and 8 untreated young and elderly untreated mice, n = 11–12 young and n = 6–8 elderly chemotherapy treated mice). * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Mann-Whitney U-test. Figures C (in DLN) and F (in tumors) show the proportional relationship between SIINFEKL and KV pentamer positive T cells as parts of a whole.

neither chemotherapy changed the breadth of KV-specific CTL responses in elderly mice.

NAIV-specific CTL activity in DLNs significantly increased in young and elderly mice in response to gemcitabine (Figure 8C), a similar trend was seen with cisplatin for elderly but not young mice.

Comparing the relative proportions of SIINFEKL, KV and NAIV CTLs in DLNs (Figure 8D) shows again that the SIINFEKL response remains dominant and that the elderly response has a higher level of responses to both subdominant epitopes, with the latter clearly enhanced by gemcitabine in both age groups. However, the overall accumulated (total) CTL activity is profoundly weaker in elderly mice relative to young mice and this is accounted for by significantly weaker dominant epitope-specific CTLs.

Taken together these data suggest that SIINFEKL-specific CTLs are reduced by chemotherapy. Interestingly, chemotherapy-induced changes to subdominant epitope-specific CTL activity were seen in DLNs and tumors with responses to the NAIV subdominant epitope being particularly robust in elderly mice. Our data demonstrate an increased contribution to overall CTL activity by subdominant epitopes-specific CTLs in elderly mice, mainly due to the declining competency of CTL responses to dominant epitopes.

Discussion

Here, we examined differences in hierarchical CD8⁺ T cell responses to dominant and subdominant tumor-derived epitopes in young and elderly mice with progressing tumors and

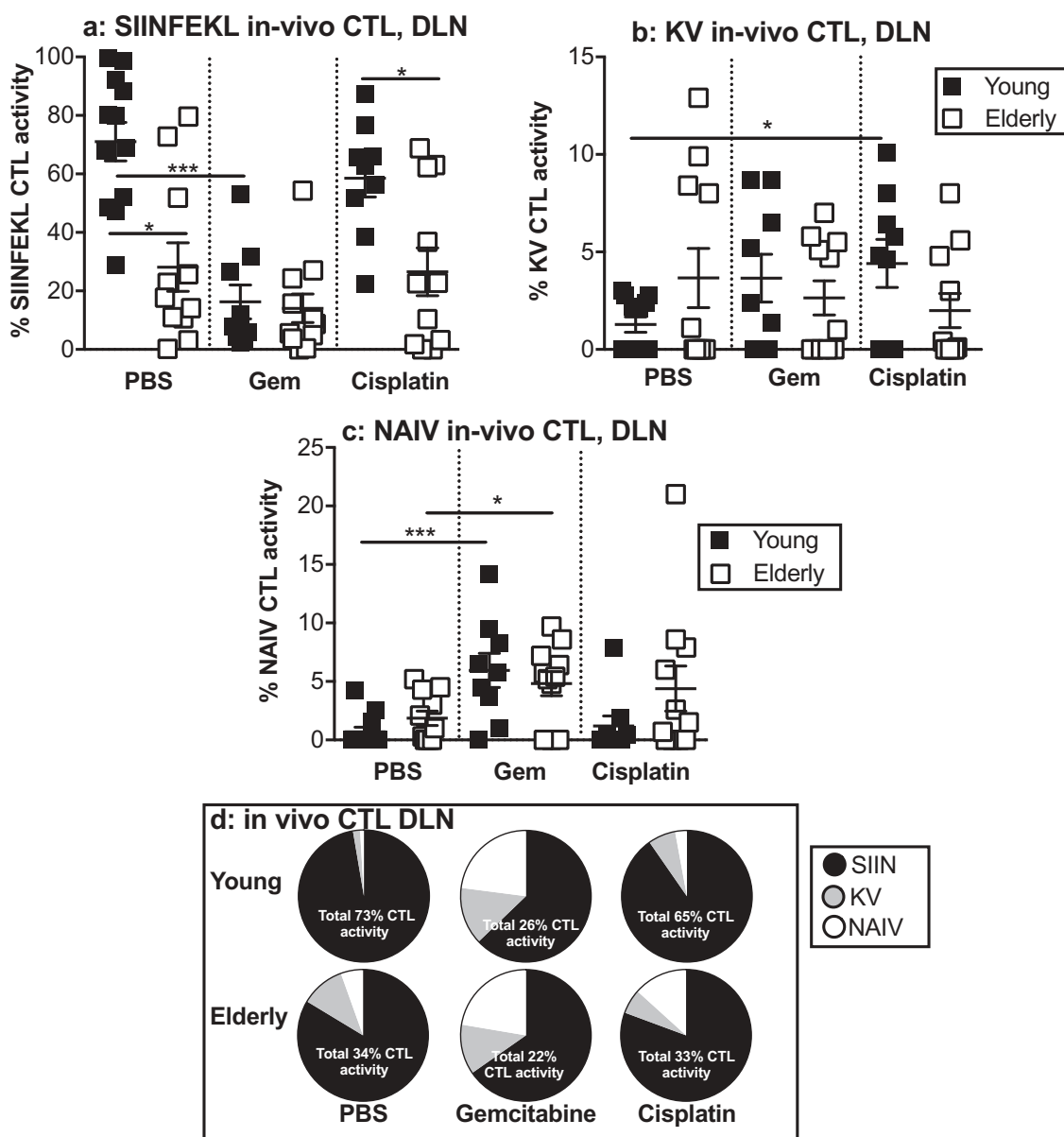


Figure 8. Gemcitabine enhances CTL responses to the NAIVFKGL subdominant epitope in elderly mice DLN. AE17-sOVA-bearing mice treated with PBS (n = 12 young and 11 elderly mice), gemcitabine (n = 9 young and 11 elderly mice), or cisplatin (n = 9 young and 11 elderly mice), were analyzed for in vivo CTL activity (as per Figure 3) in DLNs and spleens at day 21. Individual mice and mean \pm SEM are shown for SIINFEKL activity (A and B), KV activity (C and D) and NAIV activity (E and F). * p < 0.05, ** p < 0.01, *** p < 0.001 analyzed by Mann-Whitney U-test. Figure D shows the proportional relationship between SIINFEKL, KV and NAIV CTL activity as parts of a whole. Total CTL activity refers to the sum of SIINFEKL, KV and NAIV CTL activity.

determined how these responses were changed by treatment with chemotherapy. We found that whilst responses to the dominant epitope (SIINFEKL) are functionally blunted in the elderly, responses to the weaker epitopes remain functionally intact and that some chemotherapies can expand this response numerically, as seen in the case of the KV epitope in response to gemcitabine, and functionally as seen for the NAIIV epitope in response to both chemotherapies.

Our studies showed that the proportion of CD8⁺ T cells specific for the dominant and subdominant tumor epitopes (SIINFEKL and KVVRFDKL respectively, measured using pentamers) were higher in elderly than in young tumor-DLNs. These data suggest that antigen presenting cells in elderly mice processed and presented mesothelioma-associated tumor antigens at sufficient levels to generate CD8⁺ T cell responses to dominant and subdominant tumor epitopes. This age-related ability to respond to dominant and subdominant epitopes is similar to findings showing that elderly, but not young, influenza-infected mice mounted an expanded T cell repertoire response that covered dominant and subdominant epitopes.²⁷ In our studies, elderly dominant SIINFEKL-specific CTLs demonstrated lytic paralysis *in-vivo*, as their ability to lyse transferred target cells was significantly diminished relative to their younger counterparts. However, there was evidence of a delayed functional SIINFEKL-specific CTL response, implying that elderly hosts may eventually be able to mount a stronger T cell response. The problem being that this delay provides tumors with a greater chance of outpacing the anti-cancer immune response. The slower, impaired SIINFEKL-specific CTL response implies significant T cell dysfunction, and this is supported by our depletion studies showing that unlike young T cells, elderly T cells could not restrain tumor growth. These results were expected, as loss of function in elderly T cells is well known.

The potential mechanisms behind this loss of function are multiple, complex and incompletely understood. However, we also found, in agreement with others, evidence of age-associated inflammation.²³ Our healthy elderly mice demonstrated an increased potential for liver damage concomitant with increased circulating granulocytes, and a trend towards elevated pro-inflammatory cytokines in serum relative to young healthy controls. The presence of mesothelioma did not further increase markers of liver damage, yet IFN- γ , IL-6, MCP-1 and TNF- α levels were significantly elevated, coinciding with greater body weight loss in elderly mice relative to young mice. These data imply that older hosts experience exacerbated cancer cachexia. This may be because tumors grow at a faster rate, meaning elderly mice had larger tumors leading to higher levels of factors associated with cancer cachexia, such as IL-6.²⁸ Therefore, distorted cytokine secretion on account of inflammaging that is further amplified by tumors in the elderly alongside increased expression of exhaustion/checkpoint molecules on T cells such as PD-1 and CTLA-4^{29,30} and dendritic cells such as PDL-1/2³¹⁻³³ may account for the loss of functional responses to dominant epitopes. Indeed, we have data showing that elderly CD8⁺ T cells from tumor draining LNs express higher levels of all enzymes and receptors involved in the adenosine pathway (CD39, CD73, A2AR and A2BR), as well as higher PD-1,

ICOS, CTLA-4, LAG3, IL-10 and TGF β relative to their younger counterparts; this difference was amplified in elderly mice treated with both chemotherapies (manuscript under review). Those studies were on global T cell populations and future studies will compare dominant and subdominant-specific T cells for intracellular cytokine expression and checkpoint molecule expression.

The results for elderly subdominant-epitope specific CTLs were unexpected as the proportion of CD8⁺ T cells specific for the subdominant epitopes was elevated in elderly DLNs and their killing ability was similar to, or better than, that seen in young mice. Similar results have been reported in the same infection model discussed above in which elderly but not young mice immunized against influenza mounted a stronger response to subdominant relative to dominant influenza epitopes.²⁷ These data demonstrate an age-related modification of hierarchical T cell responses, regardless of the source of the antigen, and imply that responses to subdominant epitopes retain functionality with aging. Nonetheless, subdominant T cells responses were inadequate and did not significantly slow tumor growth in elderly mice.

Gemcitabine and cisplatin chemotherapy proved to be less effective and more toxic in elderly mice with mesothelioma. In humans, there is evidence that chemotherapy is less effective in elderly mesothelioma patients, with being aged over 75 years representing a prognostic indicator associated with shorter overall survival.³⁴ However, age-related chemotherapy efficacy appears to vary according tumor type. For example, chemotherapy remained effective for men and women with Stage III colon until the age of 89.³⁵ There appears to be a greater consensus that human elderly cancer patients are more susceptible to toxic side effects in general, and in mesothelioma in particular. A recent study analyzed mesothelioma treatment trends by age group and found that younger patients are more likely to benefit from chemotherapy, whilst older patients are more likely to die from treatment-related complications.³⁶ These data imply that our studies are representative of the human disease and likely to have translational potential.

Each chemotherapeutic agent exerted a distinctive effect on hierarchical T cell responses. For example, gemcitabine but not cisplatin reduced the numbers and lytic activity of SIINFEKL-specific CTL in DLNs in young and elderly mice. Moreover, gemcitabine exerted little effect on the proportions of subdominant pentamer KV-specific CD8⁺ T cells in DLNs in either age group, yet cisplatin reduced KV-specific CD8⁺ T cells in DLNs in elderly mice. Other differences were seen when examining tumor-infiltrating T cells, demonstrating that events occurring in DLNs are not necessarily being replicated in tumors. Gemcitabine significantly expanded the numbers and killing ability of tumor-associated subdominant-specific (KV-specific) CD8⁺ T cells in tumors in both age groups, similar results were seen for NAIIV-specific CTL. In contrast cisplatin reduced KV-specific numbers in elderly DLNs and did not change their proportions in tumors in either age group. In fact, cisplatin appeared to numerically refocus the tumor-infiltrating response back onto the dominant SIINFEKL epitope. However, in terms of contribution to total CTL activity cisplatin preserved the contribution of

subdominant-specific CTLs in tumors. Given the weakened response to the dominant epitope in the elderly, these elderly subdominant-specific T cells may have been sufficiently functional to impact tumors, as our depletion studies showed that the efficacy of both chemotherapeutic agents required CD4⁺ and CD8⁺ T cells in elderly tumor-bearing mice.

Our data show that chemotherapy, gemcitabine in particular, can broaden the functional CTL response to subdominant epitopes in young and elderly hosts with cancer, with a greater functional subdominant CTL proportion contributing to overall lysis in the elderly. This chemotherapeutically-driven amplification of subdominant CTL responses may be capitalized upon. Indeed, the potential of therapeutically targeting the ability of elderly hosts to mount functional responses to subdominant tumor epitopes is attractive, given the likelihood of potent age-related and tumor-induced tolerizing and escape mechanisms that prevent responses to dominant tumor epitopes. Importantly, there is evidence that subdominant epitope-specific CD8⁺ CTLs can eradicate tumors. For example, adoptive T cell transfer studies eradicated large, established tumors in mice when targeting the subdominant but not dominant epitope.³⁷ Similarly, autologous whole-tumor-cell vaccines amplified subdominant tumor antigen responses that protected against the Sp6 mouse plasmacytoma model.³⁸ However, a murine study using a breast cancer model revealed reduced CD8⁺ T-cell responses to Mage-b vaccination that could not protect against metastatic spread in elderly compared with young mice,¹¹ this might be because Mage-b represents a dominant antigen.

Crucially, similar results are emerging in human cancer studies. For example, 38 patients with HER-2/neu-overexpressing breast, ovarian, or non-small-cell lung cancers immunized with HER-2/neu subdominant peptide epitopes developed T-cell immunity to HER-2/neu peptides that, in some cases, persisted for more than a year.³⁹ The same investigators extended their studies by infusing autologous T cells expanded from previously vaccinated patients with HER-2⁺ breast and ovarian cancer. This vaccine induces diverse T cell populations including responses to lower avidity subdominant epitopes. These T cell responses were associated with partial clinical responses in 43% of patients.⁴⁰ The involvement of lower avidity T cells may enhance the persistence and function of T cells in vivo as higher avidity T-cells are more likely to be tolerized and lose function in the tumor environment.^{41,42} Importantly, others have shown in metastatic melanoma that recognition of multiple epitopes, particularly those with lower binding affinity to HLA molecules, is associated with tumor regression after adoptive T cell transfer.⁴³ In contrast, many cancer vaccines have not induced tumor regression and this might be because they failed to induce T cells responses to subdominant epitopes. Moreover, most studies do not investigate the impact of age-related modification of T cell hierarchical responses, or the contribution of subdominant-specific T cell to clinical responses in detail. Nonetheless, others have shown in elderly melanoma patients that whilst their CD8⁺ T cell responses to Flu antigen were compromised, their responses to a melanoma antigen, the Mart-127-35 epitope (likely a lower affinity epitope), were comparable, suggesting that elderly

cancer patients can respond to tumor antigens.⁴⁴ Taken together, the data suggest a potentially crucial role for subdominant epitopes in tumor eradication, and this may be particularly important for the elderly.

Our data suggest that dominant tumor epitope-specific CTLs may be reduced by some chemotherapies in young and elderly hosts, likely on account of proliferating in response to APCs activated by endogenous danger signals and increased tumor cell death. Interestingly, chemotherapy-induced differential changes to subdominant epitope-specific CTL activity in DLNs and tumors. Responses to the subdominant epitopes appeared robust in elderly mice, as they were at least as effective, and often superior, to their younger counterparts. These data suggest that responses to subdominant epitopes may be better preserved than those to dominant epitopes with aging. It is possible the responses to subdominant epitopes become more important in the elderly due to the declining competency of exhausted CTL responses to dominant epitopes. However, whilst chemotherapy might enhance CTL responses to subdominant tumor epitopes in elderly mice, these CTL are still unable to restrain tumor growth in meaningful manner. Nonetheless, their presence reveals an opportunity for inclusion of immunotherapies that could target subdominant-specific CTLs, for example, vaccines, use of IL-7 to expand elderly T cells and/or combination with checkpoint inhibitors to further facilitate their lytic capacity.

Materials and methods

Mice

Female C57BL/6J mice from the Animal Resources Centre (Perth, Western Australia) were housed under pathogen-free conditions at the Curtin University animal facility. Eighteen to 24 month old mice are viewed as equivalent to 56 to 70 year old humans and define an age when senescent changes are detected.⁴⁵ Sarcopenia (age-related loss of muscle mass) seen in 70 years old humans also becomes evident in 24 month old mice.⁸ The mice we used were young (2–3 months; equivalent to approximately 16–18 human years) and elderly (22–24 months; equivalent to approximately 65–70 human years). Any mice with a palpable mass, enlarged spleen, kidney, gastrointestinal tract or liver were excluded. 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 (AEC#2012_21).

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

As we have previously described, AE17 is a mesothelioma cell line derived from the peritoneal cavity of C57BL/6J mice after injection with asbestos (crocidolite) fibres.⁴⁶ This is one of the few cancer cells lines developed using the correct carcinogen in vivo. Moreover, AE17 cells were generated from excised orthotopic tumor deposits that emerged in elderly mice, making the model relevant for the aged setting. Injection of AE17 cells

into naïve C57BL/6J mice results in MM tumors histologically similar to human MM.⁴⁶ AE17sOVA was developed by transfecting the AE17 parental cell line with cDNA coding for secretory ovalbumin (sOVA).⁴⁶ 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 (Pharmacia and Upjohn, Western Australia, Australia), 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% CO₂ atmosphere.

In vivo tumor growth

One hundred percent confluent tumor cells were harvested using trypsin and prepared for injection by washing three times in PBS. Viability was always greater than 95%. Mice were injected subcutaneously (s.c.) in the left flank at day 0 with 5×10^5 tumor cells per mouse in 100 µl PBS and tumor growth monitored using microcallipers. Mice were regularly checked and sacrificed when tumor dimension reached 120 mm² as per Curtin Animal Ethics Committee (AEC) approval number 2012_21. Bodyweight was recorded at regular intervals, including endpoint.

Blood collection

Whole blood (13 µl per mouse) was collected into citrate buffer and analyzed using an automated Hematology blood analyzer (Mindray BC-2800Vet) specifically designed for veterinary purposes and has a pre-determined mouse setting.

Chemotherapeutic agents

Gemcitabine (Eli Lilly, Indianapolis, USA) is an anti-metabolite that prevents cells from making or repairing DNA, and cisplatin (Bristol Meyers-Squibb, USA) is a platinum compound that cross links DNA preventing cell division. Mice receiving gemcitabine were given the previously reported maximum tolerated dose of 120 µg/g body weight/dose every three days for five doses.^{15,47} Cisplatin was given at 6 µg/g body weight/dose once a week for three doses.^{15,47} Each mouse received 300 µl of gemcitabine or cisplatin diluted in PBS via intraperitoneal (i.p.) injection.

CD4⁺ and CD8⁺ in-vivo depletion

Two doses (100–150 µg/dose) of the anti-mouse CD8 antibody (YTS-169, Absolutions, Perth, WA) and the anti-mouse CD4 antibody (GK1.5, Absolutions) were injected i.p. daily before chemotherapy treatment, and continued for three doses/week (100–150 µg/dose) for two weeks. Flow cytometric analysis showed that CD4⁺ and CD8⁺ cell depletion was 95–99% effective as we have previously reported,⁴⁶ including in elderly mice.

Peptides

The dominant peptide OVA_{257–264} (SIINFEKL) and subdominant peptide OVA_{55–62} (KVVRFDKL)²⁶ were purchased from ProImmune (Sarasota, USA). The subdominant peptide OVA_{176–183} (NAIVFKGL),²⁶ was manufactured by the Centre for Cell and Molecular Biology (University of Western Australia, Perth) at a purity of >89%.

Flow cytometry

Spleen, lymph node and tumor samples were disaggregated into single cell suspensions by gentle dispersion between two frosted glass slides and stained for flow cytometric analysis. Samples were blocked with CD16/32 (Biolegend) for 30 minutes at 4°C in the dark and the following anti-mouse primary antibodies and pentamers incubated for 1 hour at 4°C in the dark: anti-CD3-APC-Cy7 (clone 1452C11, BD), anti-CD8α-PerCP-Cy5 (clone 53–6.7, BD), SIINFEKL-PE (ProImmune), KVVRFDKL-APC (ProImmune) and B220-PE-Cy7 (RA3-6B2, Biolegend). Cells were washed twice and resuspended in PBS/2% FCS for analysis on a FACSCanto II or LSR Fortessa using FACSDiva software (Becton Dickinson) or FlowJo software (TreeStar, Oregon, USA).

In vivo analysis of CTL function

In this study we developed a '5 peak' in vivo CTL assay based on our previously described '3 peak' assay.²⁶ Briefly, C57BL/6J pooled spleen and lymph node cell suspensions were divided into five populations. Three populations were pulsed with 5 µg/ml of one of SIINFEKL, KVVRFDKL or NAIVFKGL peptide for 90 minutes at 37°C in complete medium. Two populations were left uncoated as control cells and also incubated for 90 minutes at 37°C in complete medium. Following incubation, the cells were washed in complete medium and labeled with either a high concentration of CFSE (5 µM, SIINFEKL population), low CFSE (0.05 µM, NAIVFKGL population) or low CellTrace Violet Cell Proliferation dye (0.5 µM, KVVRFDKL population). The other two control uncoated target cells were labeled with either an intermediate concentration of CFSE (0.5 µM) or high concentration of Violet dye (5 µM). For i.v. injection 1×10^7 cells of each population were mixed in 400 µl PBS per recipient mouse. Specific in vivo cytotoxicity was determined by collecting the draining LNs (DLN), non-draining LNs (non-DLN) and spleens from recipient mice 16–18 hours post i.v. injection and detecting differentially labeled fluorescent target cell populations by flow cytometry.

Further controls included naïve healthy mice, AE17 tumor-bearing mice (with or without chemotherapy treatment) and untreated recipient mice. To normalize data, allowing inter-experimental comparisons, the data was expressed relative to the no tumor naïve control mice that were included in every experiment. That is, relative killing was calculated by determining the ratios between the percentages of peptide-coated targets in no tumor control mice versus tumor-bearing mice and multiplying by 100 to obtain a percentage value.

Analysis of liver and kidney damage

Alanine aminotransferase (ALT), alkaline phosphatase (Alk Phos) and gamma glutamyl transpeptidase (GGT) are enzymes mostly found in the liver. Elevated serum or plasma levels of ALT are indicative of liver damage, whilst increases in Alk Phos and GGT suggest damage involving the bile ducts. Urea is a waste product formed from the breakdown of proteins and creatinine is a waste product made by muscles, elevated plasma levels of either indicate that the kidneys may not be functioning properly. Individual plasma samples from mice were measured by Vetpath Laboratory Services (Perth, Western Australia).

Cytokine bead array

Concentrations of TNF- α , IL-6, IL-10, IFN- γ , IL-12 and MCP-1 in plasma samples were measured using BD cytokine bead arrays (BD Biosciences) as per manufacturer's instructions. Data was collected on either an Attune[®] NxT Acoustic Focusing Cytometer using Attune[®] Cytometer software or BD FACS Canto II and analyzed using Flowjo[™] version 7.0 software. Standard curve values were used to calculate cytokine concentrations using linear regression and GraphPad Prism 6[™] software.

Data analysis

Statistical significance was calculated using GraphPad PRISM 6[™] (San Diego, CA, USA). Unpaired student's *t*-test and Mann-Whitney *U*-test were used to determine differences between two populations. One-way analysis of variance (ANOVA) with post-hoc Bonferroni test was used to determine differences between more than two populations. *P*-values of < 0.05 were considered statistically significant. Data was also analysed using the General Linear Model procedure in SPSS Version 25, to assess the main effects of age, chemotherapy treatment and their interaction using 2-way ANOVA.

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Disclosure of potential conflicts of interest

In accordance with Taylor & Francis policy and my ethical obligation as a researcher, I am reporting that I act as a non-salaried Chief Scientific Officer for a cancer immunotherapy start-up company, Selvax. This company may be affected by the research reported in the enclosed paper. I have disclosed those interests fully to Taylor & Francis and to Selvax and have in place an approved plan for managing any potential conflicts arising.

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Author contributions

CJ: Performed experiments, data collection and analysis and contributed to design of the study. JKG, FT, JS, HG, DED, SF, SP, PM: Performed specific experiments, contributed to experimental data collection and analysis. DJN: Funded and designed the study, analyzed the data and drafted the manuscript.

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