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CD5 blockade enhances *ex vivo* CD8$^+$ T cell activation and tumour cell cytotoxicity

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Abbreviations:

(SRCR) scavenger receptor cysteine-rich

(TCR) T-cell receptor

(CTL) Cytotoxic T lymphocyte

(MAb) Monoclonal antibody

(Treg) T regulatory

(NK) Natural killer

(ICS) Intracellular cytokine staining

(AICD) Activation-induce cell death

(FasR) Fas receptor

(FasL) Fas ligand

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Abstract

CD5 is expressed on T cells and a subset of B cells (B1a). It can attenuate T-cell receptor (TCR) signalling and impair cytotoxic T lymphocyte (CTL) activation and is a therapeutic targetable tumour antigen expressed on leukemic T and B cells. However, the potential therapeutic effect of functionally blocking CD5 to increase T cell anti-tumour activity against tumours (including solid tumours) has not been explored. CD5 knockout mice show increased anti-tumour immunity: reducing CD5 on CTLs may be therapeutically beneficial to enhance the anti-tumour response. Here we show that *ex vivo* administration of a function-blocking anti-CD5 monoclonal antibody (MAb) to primary mouse CTLs of both tumour-naïve mice and mice bearing murine 4T1 breast tumour homografts enhanced their capacity to respond to activation by treatment with anti-CD3/anti-CD28 MAbs or 4T1 tumour cell lysates. Furthermore, it enhanced TCR signalling (ERK activation) and increased markers of T cell activation, including proliferation, CD69 levels, interferon-γ production, apoptosis, and Fas receptor and Fas ligand levels. Finally, CD5 function-blocking MAb treatment enhanced the capacity of $CD8^+$ T cells to kill 4T1-mouse tumour cells in an *ex vivo* assay. These data support the potential of blockade of CD5 function to enhance T cell-mediated anti-tumour immunity.

MHC-TCR interaction leads to T cell activation, but CD5 expression in the immunological synapse attenuates it. Functional blockade of CD5 will then enhance T cell activation and antitumor function

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Introduction

CD5, also known as Ly-1 (mouse)/T1 or Leu-1 (human), is a 67 kDa type-I transmembrane glycoprotein that maps to chromosome 11q12.2 in humans and chromosome 19 in mice (1). It contains three scavenger receptor cysteine-rich (SRCR) domains, making it a member of the highly conserved group B SRCR superfamily (1). CD5 is expressed on both αβ and γδ T cells, is detectable at early double-negative stages of T cell development (*i.e.*, both $CD4^-$ and $CD8^-$), and increases during T cell development from double-negativity to single-positivity ($CD4^+CD8^-$ or $CD4^-CD8^+$) (2). CD5 is used to fine-tune positive and negative selection for T cells with receptors capable of binding to the MHC molecule, and enhances the development of high-affinity antigen binding (3). CD5 physically interacts and co-localizes with the T cell receptor (TCR)/CD3 complex on T cells and with B cell receptors on B cells in the immunological synapse with antigen-presenting cells, where it acts to reduce their capacity to activate intracellular TCR signalling through IκB, JNK, and ERK1/ERK2 (3). Consequently, CD5 knockout mice lacking attenuation of receptor signalling on T and B cells are hyperresponsive to TCR stimulation, with increased T cell proliferation and phosphorylation of downstream signalling proteins (4).

CD5 has been used as a characteristic surface marker for T and B cell malignancies: the majority of T-cell tumours (T cell acute lymphoblastic leukaemia

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[T-ALL] and T cell lymphoma) and many B-cell malignancies are CD5-positive (5). As a consequence, CD5 has been validated as a target tumour antigen in those tumour types, useful in a variety of passive immunotherapeutic approaches. Anti-CD5 immunoconjugates linked to a variety of cytotoxic molecules have been used in clinical trail to treat $CD5^+$ hematologic malignancies (6). More recently, several studies assessing the application of CAR-T cells engineered to target CD5 cells in treatment of lymphoma have yielded promising results (7, 8). CD5 expression on the surface of hematological tumours appears to be a target for both passive and active immunotherapy.

With respect to CD5 on normal T cells, less is known but data suggest that lack of CD5 activity increases anti-tumour immune surveillance and reduces tumour growth. Mice with germ-line CD5 knockout have increased T cell activation and reduced capacity to support growth of B16F10 syngeneic homograft melanoma tumours compared to mice with CD5 proficiency (9). Furthermore, transgenic mice with increased expression of soluble CD5 (sCD5) displayed slower B16F10 tumour homograft growth than control mice (10), a phenomenon associated with reduced numbers of regulatory T cells (T_{regs}) in lymph node and spleen and increased numbers of natural killer (NK) cells in spleen. Because soluble CD5 is capable of binding cell surface CD5 (11), the authors hypothesized that sCD5 acted to block CD5 from binding within the TCR/CD3 complex and impaired the capacity of cell-surface CD5 to attenuate TCR signalling (10). Moreover, tumour-infiltrating

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lymphocytes isolated from lung cancer patients exhibit differential anti-tumour activity based on CD5 expression, where CD5 levels were negatively correlated with anti-tumour activity (12). Increased activation-induced death has been reported in T cells with undetectable CD5 levels compared to $CD5^{high}$ T cells, suggesting that CD5 could impair activation of anti-tumour T cells (13). Thus, functionally blocking CD5 signalling could lead to increased anti-tumour immunity and enhanced T cell activation.

To further investigate the efficacy of using anti-CD5 MAb to enhance T cell activation and anti-tumour immunity in solid tumours, spleen T cells isolated from both tumour-naïve, non-tumour-bearing mice and mice implanted with syngeneic 4T1 murine breast tumour homograft were activated *ex vivo,* with and without treatment with function-blocking anti-CD5 MAb, and assessed for a marker of TCR signalling (ERK phosphorylation) and markers of T cell activation. They were also co-cultured with 4T1 tumour cells *ex vivo* and their capacity to induce tumour cell death measured. T cells isolated from both groups of mice and treated with antibodies to functionally block CD5 showed increased capacity to respond to activating signals (anti-CD3/anti-CD28 MAb treatment in the case of tumour-naïve mice, or exposure to 4T1 tumour cell lysate in the case of 4T1 tumour-homografted mice) with increased proliferation, CD69-positivity, interferon gamma (IFNγ) levels, apoptosis, and FasR and FasL levels. Furthermore, they had increased functional capacity to kill 4T1 tumour cells in an *ex vivo* cytotoxic T lymphocyte assay. These

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data suggest that functional blockade of CD5 may be a therapeutically advantageous strategy for treatment of solid, non-hematological tumours.

Results

CD5 is associated with increased $CD8^+$ T cell activation

CD5 can regulate T cell receptor function and is considered to be an activation marker for B cells (14). To determine whether CD5 can be considered an activation marker for $CD8^+$ T cells, we isolated splenocytes from naïve mice and cultured them with anti-CD3/anti-CD28 MAb to activate $CD8^+$ T cells. After 24 hours, $CD8^+$ T cells were stained with anti-mouse PE-Cy7 CD8 MAb and anti-mouse FITC-CD5 Mab, and cells were analyzed by flow cytometry to determine the intensity of CD5 on the surface of $CD8^+$ T cell (MFI, or mean fluorescence intensity). When cells were stimulated with anti-CD3/anti-CD28 MAb for 24 hours, the MFI increased dramatically compared to unstimulated cells with a peak after 48 hours (*Figure 1).* To further assess the correlation between CD5 and $CD8^+$ T cell activation, anti-mouse PE-CD69 MAb was used to identify the fraction of activated $CD8^+$ T cells, also expressing elevated CD5. Cells were stimulated for 24 hours with anti-CD3/anti-CD28 MAb *in vitro* and then stained with anti-mouse PE-Cy7-CD8, FITC-CD5 and PE-CD69 MAbs. $CD69^+/CD8^+/CD5^{high}$ and $CD69^+/CD8^+/CD5^{low}$ T cells were determined by flow cytometry. After 24 hours activation, the fraction of

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$CD69^+/CD8^+/CD5^{high}$ T cells was higher than the fraction of $CD69^+/CD8^+/CD5^{low}$ T cells (87.3% vs 50% respectively, $p<0.05$) and CD69 MFI was higher in $CD8^+/CD5^{high}$ T cells compared to $CD8^+/CD5^{low}$ T cells ($16X10^3$ vs $6X10^3$, respectively, $p<0.05$)(*Supplementary Figure 1*).

CD5 blockade enhances $CD8^+$ T cell receptor signalling

Increased phosphorylation of ERK is a downstream effect of activating the TCR signalling pathway (15). ERK2 regulates $CD8^+$ T cell proliferation and survival (16). ERK phosphorylation can be elevated in the absence of CD5-CK2 signalling (17). To determine if administration of anti-CD5 MAb could lead to impaired CD5 signalling and increased ERK activation, splenocytes isolated from tumour-naïve mice and treated with anti-CD3 plus anti-CD5 MAbs showed increased phosphorylation of ERK1/2, but not cells receiving anti-CD3 MAb alone *(Figure 2).* Furthermore, treatment with anti-CD5 MAb alone did not induce ERK phosphorylation (*Supplementary Figure 2*).

CD5 blockade enhances $CD8^+$ T cell activation

$CD5^{-/-}$ T cells exhibit increased levels of T cell activation markers (CD25, CD69) after treatment with anti-CD3 MAb (18). To determine whether functionally blocking CD5 using an anti-CD5 MAb, in combination with activation by anti-CD3 MAb/anti-CD28 MAb, would enhance $CD8^+$ T cell activation, we treated primary splenocytes isolated from tumour-naïve mice with anti-CD3/anti-CD28 MAb and anti-

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CD5 MAb for 24 hours *in vitro* and then assessed the level of the activation marker CD69 on $CD8^+$ T cells. Cells treated with anti-CD3/anti-CD28 MAb in addition to anti-CD5 MAb showed an increased fraction of $CD69^+CD8^+$ T cells compared to cells receiving anti-CD3/anti-CD28 MAb and isotype control MAb (82% vs 73.5% respectively, $p < 0.05$)(*Figure 3A*; *Supplementary Figure 3 for the gating strategy*). To further assess $CD8^+$ T cell activation upon treatment with anti-CD5 MAb, proliferation of $CD8^+$ T cells was examined. Splenocytes from either tumour-naïve mice or 4T1 tumour-bearing mice were stained with CFSE cell tracking dye and treated with anti-CD3/anti-CD28 MAb or 4T1 tumour lysate, in addition to anti-CD5 MAb or isotype control MAb. $CD8^+$ T cells treated with anti-CD5 MAb exhibited increased proliferation compared to those treated with isotype control MAb for up to 5 divisions. The increased percentage of cells in each division was different for cells treated with anti-CD5, in divisions 3, 4 and 5 ($p<0.05$) *(Figure 3B, C; Supplementary Figure 4A and B for the flow cytometry gating strategy).*

Increased fraction of $CD8^+$ $IFN\gamma^+$ T cells after treatment with anti-CD5 MAb

IFNγ is an effector cytokine produced by activated $CD8^+$ T cells. To determine whether treatment with anti-CD5 MAb enhances production of IFNγ in $CD8^+$ T cells, we stimulated the splenocytes from tumour-naïve mice, for 24 hours *in vitro,* with anti-CD3/anti-CD28 MAb; and splenocytes from 4T1 tumour-bearing mice with 4T1 tumour lysate. In addition, anti-CD5 MAb or isotype control MAb was added to cells during stimulation. The fraction of cells positive for IFNγ after treatment with both

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anti-CD3/anti-CD28 MAb and anti-CD5 MAb was greater than the fraction after treatment with anti-CD3/anti-CD28 MAb and isotype control MAb, 21% vs 18%, respectively ($p<0.05$)(*Figure 4A*). The MFI was higher in cells treated with both anti-CD3/anti-CD28 MAb and anti-CD5 MAb treatment: $5X10^3$ vs $3X10^3$, respectively ($p<0.05$), indicating that not only was the fraction of $IFN\gamma^+$ cells increased, but the level of IFNγ in those cells was also higher (*Figure 4A*).The fraction of $CD8^+$ T cells from 4T1 tumour-bearing mice treated *ex vivo* with tumour lysate in addition to anti-CD5 MAb also had an increased fraction of $IFN\gamma^+$ T cells (1.5% vs 0.6%, $p<0.05$)(*Figure 4B*, *Supplementary Figure 5A and B for the flow cytometry gating strategy*). Furthermore, to assess whether functionally blocking CD5 has a direct effect on $CD8^+$ T cells, $CD8^+$ T cells were purified from total splenocytes and treated with anti-CD3/anti-CD28 MAb, with or without anti-CD5 MAb. After 24 hours, cells treated with anti-CD3/anti-CD28 MAb and anti-CD5 MAb had elevated levels of IFNγ compared to cells that did not receive anti-CD5 MAb (36.6% vs 22%, respectively) ($p < 0.05$) (*Supplementary Figure 8).*

CD5 blockade increases FasR- and FasL-dependent death of $CD8^+$ T cells.

CD5 may decrease TCR tumour antigen recognition, and CD5 blockade could enhance antigen recognition and intracellular TCR signalling. A consequence of repeated antigen stimulation of the CD3/TCR complex is activation-induced cell death (AICD)(19). To assess whether anti-CD5 MAb could increase $CD8^+$ T cell death after activation as a consequence of enhanced TCR sensitivity to antigen, we

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treated splenocytes from tumour-naïve mice with anti-CD3/anti-CD28 MAb, with or without anti-CD5 MAb. Cells were cultured as previously described, with media refreshed every 48 hours. After 7 days, cells were stained with anti-CD8 MAb followed by staining with FITC-annexin V and PI (*Figure 5A; see Supplementary Figure 6A for gating strategy*). The fraction of $CD8^+$ T cells undergoing AICD (Annexin V^+/PI^+) was increased among those activated with anti-CD3/anti-CD28 MAb and anti-CD5 MAb, compared to those treated with anti-CD3/anti-CD28 MAb and isotype control Mab (76% vs 61%, respectively)($p<0.05$). For splenocytes from 4T1 tumour-bearing mice, treatment with anti-CD5 MAb enhanced AICD compared to treatment with isotype control MAb (57% vs 40%, respectively, $p<0.05$) (*Figure 5B; see Supplementary Figure 6B for gating strategy*). AICD depends on FasR/FasL interaction (20): we therefore assessed the fraction of $FasR^+/CD8^+$ and $FasL^+/CD8^+$ T cells. Splenocytes from tumour-naïve mice and murine 4T1 tumour-bearing mice were isolated and treated with anti-CD3/anti-CD28 MAb or 4T1 lysate, respectively, with or without anti-CD5 MAb. Cells were stained every 24 hours with fluorescent antibodies to detect FasR and FasL on $CD8^+$ T cells. Splenocytes from tumour-naïve mice treated with anti-CD3/anti-CD28 MAb in addition to anti-CD5 MAb had an elevated fraction of $FasR^+/CD8^+$ T cells compared to the isotype control MAb-treated group at 48, 72, and 96 hours after activation (43% vs 28%, 48% vs 35%, and 74% vs 47%, respectively)($p<0.05$)(*Figure 6A*). In addition, the level of FasR in $FasR^+$ cells was increased after anti-CD5 MAb treatment compared to isotype control MAb-

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treated group at 48 hours (MFI of $1.3X10^3$ vs MFI of $1.1X10^3$) and at 96 hours (MFI of 1.6×10^3 vs MFI of 1.2×10^3)($p<0.05$)(*Figure 6A*).

The fraction of $FasL^+/CD8^+$ T cells from tumour-naïve mice was also increased in cells treated with anti-CD5 MAb at 72 hours (63% vs 33%) and 96 hours (80% vs 64%) ($p<0.05$) (*Figure 6A*). Similarly, the fraction of $FasR^+/CD8^+$ T cells among mouse 4T1 tumour-bearing splenocytes treated with anti-CD5 MAb was increased compared to cells treated with isotype control MAb, at 48 and 72 hours after activation (45% vs 33% and 39% vs 33%, respectively) ($p < 0.05$). The level of FasR on $FasR^+/CD8^+$ T cells was higher at 96 hours after anti-CD5 treatment compared to isotype control MAb (MFI of $3X10^3$ vs MFI of $2X10^3$)(*Figure 6B*). The fraction of $FasL^+/CD8^+$ T cells treated with anti-CD5 MAb was increased compared to cells treated with isotype control MAb at 96 hours (66% vs 61%, $p<0.05$) (*Figure 6B*; see *Supplementary Figure 7A and B for the flow cytometry gating strategy*).

CD5 blockade enhances tumour killing by $CD8^+$ T cells

To determine whether treatment of $CD8^+$ T cells with anti-CD5 MAb could enhance T cell-mediated cytotoxicity against tumour cells, we assessed the ability of primary $CD8^+$ T cells isolated from mice and treated with function-blocking anti-CD5 MAb to kill mouse 4T1 tumour cells *ex vivo*. Mice were challenged with 4T1 tumour cells for 21 days, and $CD8^+$ T cells were isolated and co-cultured with 4T1 cells *in vitro* in combination with *ex vivo* treatment with anti-CD5 MAb. Two-dimensional cell

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cultures containing T cells and 4T1 cells were maintained in an IncuCyte live-cell analysis system to capture live images to detect the ability of $CD8^+$ T cells to kill and lyse tumour cells. $CD8^+$ T cells treated with anti-CD5 were more capable of killing tumour cells compared to cells that received isotype control or no treatment. At 6 and 10 hours post-treatment, there were more than 30 dead cells/mm^3 when cells received anti-CD5 blockade, compared to fewer than 20 dead cells/mm^3 when cells received isotype control MAb ($p<0.05$) (*Figure 7*).

Discussion

CD5 is highly expressed on human leukemic cells, including leukemias arising in T and B cell populations (5). As an antigen preferentially and selectively expressed in leukemia, it has been therapeutically targeted in an antibody-drug conjugate approach using anti-CD5 MAb conjugated to toxin (6). More recently, in addition to molecule-based anti-CD5 cancer immunotherapy, cell-based anti-CD5 therapy has been introduced in the form of CAR-T cells engineered to target CD5 on human T and B cell leukemias (8). A clinical trial has been launched targeting CD5 in $CD5^+$ cancer cells using CD5 CAR-T cells (NCT03081910) (21). In addition, CAR-T cells engineered to lack CD5 have been generated to prevent fratricide (*i.e.*, CAR-T cell-mediated ablation of CD5-expressing effector CAR-T cells) and enhance the potential value of anti-CD5 CAR T cell approaches (22). Thus, CD5 expression on non-solid tumours has been the focus of therapy.

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The first report of anti-CD5 MAb-based therapy of solid tumours involved the administration of anti-CD5 against mouse $CD5^+$ leukemias grown as solid tumour homografts (23). Mice injected subcutaneously with $CD5^+$ mouse leukemia tumours or $CD5^-$ mouse lung cancer cells had increased overall survival when treated with anti-CD5 MAb. This effect was abolished in thymectomized mice, suggesting that the therapeutic effect of anti-CD5 MAb was mediated, not by direct effects on $CD5^+$ tumour cells, but on thymus-derived $CD5^+$ cells including non-tumour host mouse T cells. Further studies have shown that homografted mouse tumours grown in CD5 knockout ($CD5^{-/-}$) mice grow more slowly than in wild type $CD5^{+/+}$ mice (9). This suggests that reducing CD5 signalling could lead to enhanced anti-tumour immunity. Assessment of the effect of direct administration of anti-CD5 MAb to block CD5 signalling on $CD8^+$ T cells will lead to increased understanding of the effect of CD5 blockade in tumour immunity and facilitate the development of CD5-targeted anti-cancer immunotherapy. In this study, we investigated the direct effect of anti-CD5 MAb on $CD8^+$ T cell activation and function.

CD5 has been used as a marker for activated human B cells (14) and is upregulated during human T cell activation (24). We investigated CD5 levels on mouse $CD8^+$ T cells during *ex vivo* activation to determine and confirm that it would be available as a target for antibody-mediated inactivation during tumour growth. Primary $CD8^+$ T cells isolated from mice, activated *ex vivo* by exposure to anti-CD3 and anti-CD28 MAbs, had an increased level of CD5 compared to non-activated

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$CD8^+$ T cells (*Figure 1*). This is consistent with reports linking increased CD5 expression with the strength of T cell receptor signalling and function: a possible strategy to fine-tune TCR signalling (3). We also observed that the level of the activation marker CD69 was correlated with increased levels of CD5 on $CD8^+$ T cells (*Supplementary Figure 1).* Thus, $CD8^+$ T cell CD5 levels correlated with the degree of $CD8^+$ T cell activation.

To investigate the effect of anti-CD5 MAb on $CD8^+$ T cell activation and function, we treated primary $CD8^+$ T cells isolated from homografted (tumour-harbouring) and tumour-naïve (non-tumour bearing) mice with anti-CD5 MAb *ex vivo*. Using flow cytometry, we observed that *ex vivo* activation accompanied by treatment with an antagonistic anti-CD5 MAb increased the fraction of $CD8^+$ T cells expressing markers of activation (CD69 and IFNγ) and increased $CD8^+$ T cell proliferation, indicating that anti-CD5 MAb enhances $CD8^+$ T cell activation (25, 26). *Ex vivo* treatment with anti-CD5 MAb alone did not induce $CD8^+$ T cell activation (*Supplementary Figure 2; Figures 3A and 4A)*, suggesting that anti-CD5 treatment mediates enhanced activation by influencing CD5 effects on TCR signalling. In further support of this concept, ERK phosphorylation (indicative of increased TCR signalling) was increased in cells activated by treatment with anti-CD3, combined with treatment with anti-CD5 MAb, compared to activation with anti-CD3 alone *(Figure 2).* Treatment with anti-CD5 alone did not increase ERK phosphorylation (*Supplementary Figure 2*). This is consistent with a report that activation of ERK was

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greater in thymic cells from mice that express unfunctional CD5 challenged with OVA323-339-peptide to induce T cell activation compared to thymic cells from CD5 wild-type mice (27).

Our data show increased AICD (a consequence correlated with increased T cell activation (19)) in activated $CD8^+$ T cells treated with anti-CD5 MAb *(Figure 5A and B)*. In addition, and consistent with increased AICD, treatment of spleen cells increased the fraction of $FasR^+$ and $FasL^+$ T cells *(Figure 6A and B)*: TILs isolated from tumour-bearing mice with CD5 deficiency are similarly susceptible to AICD via increased FasR expression (9). AICD can occur as a result of over-stimulation of TCR (19). CD5 acts to fine-tune TCR responsiveness and, therefore, blockade of CD5 could lead to enhanced TCR sensitivity to antigen and increased AICD. Based on our data showing that functionally blocking CD5 on activated T cells by treatment with anti-CD5 function-blocking MAb enhances $CD8^+$ T activation (as shown by elevated CD69 and enhanced production of IFNγ *ex vivo*) and that activation is associated with increased proliferation (as shown by altered CFSE signal), we propose that administration of function-blocking anti-CD5 MAb could enhance anti-tumour immunity. In a cytotoxic T cell lysis (CTL) assay, we observed that $CD8^+$ T cells treated with anti-CD5 MAb were more capable of targeting and killing mouse 4T1 tumour cells *ex vivo* than cells treated with control MAb *(Figure 7).* These data strongly suggest that treatment with anti-CD5 MAb can increase the ability of $CD8^+$ T cells to target and eliminate tumour cells and supports the concept that functionally

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blocking CD5 to enhance anti-tumour $CD8^+$ T cell function has potential as an *in vivo* anti-cancer immunotherapy.

Considering that CD5 is expressed on all T cells, treatment with anti-CD5 MAb could have multiple effects, including adverse consequences; such possible consequences require further investigation. It has been reported that B6 polyclonal T cells with no or low CD5 expression are less capable of generating induced T_{regs} (iT_{regs}) that, under certain conditions, play a role in suppressing immune cells (28). Anti-CD5 MAb administration could, therefore, result in adverse autoimmune effects. In contrast, generation of T_{regs} is not affected by the absence of the CK2 binding domain in CD5, suggesting that reduced CD5 activity has little or no T_{reg} effects (17). It is possible that the presence of the whole CD5 receptor could affect generation of T_{regs} but that deletion of part of the CD5 molecule functional in TCR signalling, or temporary blockade of the CD5 signalling pathway, will have little effect on generation of T_{regs} and reduced danger of autoimmune effects. Nevertheless, multiple immune cell types (B cells, dendritic cells, and others) express CD5 (29), and the consequences of systemic administration of anti-CD5 capable of blocking CD5 function on both anti-tumour $CD8^+$ T cells (with potential to enhance anti-tumour immunity) and other immune cells (where blockade may have adverse effects) requires further study, including *in vivo* administration of anti-CD5 MAb in both naïve and tumour-bearing mice. Functionally blocking CD5 on $CD8^+$ T cells could lead to hyper-activation that, although potentially desirable in increasing anti-tumour action

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in the short term, could increase inhibitory receptors that, over time, act to suppress $CD8^+$ T cell function. Therefore, targeting additional T cell molecules in combination with CD5 blockade (for example, immune checkpoint molecules and/or Fas R) may be necessary to prevent $CD8^+$ T cell exhaustion and sustain $CD8^+$ T cell function. With due consideration of additional effects, our data nevertheless show that blockade of CD5 on T cells has promise as an anti-tumour immunotherapy.

Materials and Methods

Mice and cells

Female BALB/c mice were purchased from The Jackson Laboratories (Jackson Laboratories, Bar Harbor, ME). All animals were between 8 and 12 weeks of age and housed in the Animal Care and Veterinary Services Facility at the Victoria Research Building, Lawson Health Research Institute, according to guidelines of the Canadian Council for Animal Care and under the supervision of the Animal Use Subcommittee of the University of Western Ontario. 4T1 mouse breast mouse tumour cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA), and cultured in Dulbecco modified Eagle medium supplemented with10% fetal bovine serum (FBS)(Invitrogen). All cells were kept at 37°C in 5% CO_2. Tumour cells ($5X10^4$) were counted by Coulter counter and injected subcutaneously into the right flanks of 2 month-old female BALB/c mice and allowed to grow for 21 days before animals were euthanized by CO_2 inhalation. Single cell suspensions of

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lymphocytes were obtained from mice by pressing spleens through a 70 µm Falcon Cell Strainer (VWR, Mississauga, ON) into RPMI 1640 medium (GIBCO). Cells were then centrifuged (300xg, 10 mins, 4°C), and erythrocytes were lysed using Ammonium-Chloride-Potassium (ACK) red cell lysis buffer. The resulting live (trypan blue-negative) splenocytes were counted manually (microscope slide) and cultured for further assessment.

Western immunoblotting

Splenocytes were stimulated for 30 min with 2 µg/ml anti-CD3 MAb (Clone: 145-2C11. BD Biosciences) or 2 µg/ml anti-CD3 + 5 µg/ml anti-CD5 MAbs (Clone: 53-7.3. BioLegend), a stimulation protocol dependent on CD28-mediated co-stimulation by spleen antigen-presenting cells (30). After stimulation, cells were washed twice with cold PBS and lysed with RIPA lysis buffer (10 mM Tris pH 7.2, 2mM EDTA, 0.1% SDS, 1% sodium deoxycholate, 1% Triton 100), supplemented with protease inhibitors, including 10 µg/ml aprotinin, 10 µg/ml leupeptin, 100 µM sodium orthovanadate, 100 µM phenylmethylsulfonyl fluoride, and sonicated at 4ºC using a Vibra CellTM ultrasonic processor (Sonics & Materials Inc., Danbury, CT) to disrupt membranes. Lysates were centrifuged at 13,000 × g for 15 min at 4ºC. Protein concentration was measured using the Bradford assay. For SDS PAGE, 30 µg protein per lane was electrophoresed on a 7% polyacrylamide gel and proteins were then transferred to a nitrocellulose membrane using a semi-dry transfer apparatus (Bio-Rad) and blocked with 5% skim milk in TBST for 1 hour. Membranes

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were incubated overnight with the primary antibody at 4ºC. After washing three times in TBST, membranes were incubated with the appropriate secondary antibody-horse radish peroxidase conjugate for 1 hour at 21^{o} C and proteins were then detected by using enhanced chemiluminescence reagents (Western Lightning Plus – ECL [Perkin Elmer, Inc Products]). Primary antibodies were used to detect anti-phospho-ERK, (Cell SignalingTechnology/New England Biolabs, Whitby, ON) and anti-actin (Sigma Aldrich Canada, Oakville, ON). Anti-mouse or anti-rabbit horse radish peroxidase antibody conjugates were purchased from Cell Signaling.

CD8^{+} T cell apoptosis and proliferation

Single cell suspensions of splenocytes were generated from 4T1 tumour-bearing mice or tumour-naïve mice using the described protocol and in accord with MIATA guidelines. On day 0, cells (2X10^{5}) were treated with or without purified anti-mouse anti-CD5 MAb at 5 µg/ml (Clone: 53-7.3. BioLegend) for 15 min before seeding into U-bottom 96-well plates pre-coated with 5 µg/ml anti-CD3 antibody, in RPMI media containing 10% FBS, IL-2 (50 IU/ml)(PeproTech Canada Inc), and soluble anti-CD28 antibody (Clone: 37.51. BD Biosciences, 2 µg/ml). As an alternative to activation with anti-CD3/anti-CD28 MAb, cells were activated by addition of 4T1 tumour cell lysate (200 µg/ml). 4T1 tumour lysate was prepared by detaching confluent cultures of 4T1 tumour cells with 0.01% EDTA for 10 min, washing the cells twice with PBS, resuspending cells in serum-free medium (5X10^{6} cells/ml), and lysis by 5 freeze/thaw cycles (-80 to 37°C). Cultured splenocytes,

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activated and unactivated, were kept at 37°C in 5% CO_2 and the media refreshed every 48 hours. On day 7, apoptotic and dead cell fractions were determined using a FITC-labeled Annexin-V and propidium iodide (PI) kit (Biolegend, San Diego, CA) according to the manufacturer's protocol. Briefly, cells were stained with anti-mouse PE-Cy7-labeled anti-CD8 MAb at 4°C for 30 min, washed twice with cold FACS buffer, and resuspended in Annexin V staining buffer (Biolegend, San Diego, CA). FITC-Annexin V (1 µg/ml) and PI (10 µg/ml) were added to the cell suspension and incubated at 21°C for 15 min in the dark. Annexin V staining buffer (400 µl) was added and cells were analyzed using a Cytomics FC 500 (Beckman Coulter, Inc). For CFSE labelling, isolated splenocytes were stained with CFSE (1 µg/ml; Biolegend, San Diego, CA) for 30 min at 37°C and washed twice with FBS-containing buffer to stop the reaction. Cells were then cultured as previously described for 7 days at 37°C in 5% CO_2, followed by flow cytometry using a Cytomics FC 500 (Beckman Coulter, Inc). Data were analyzed using Flowjo software (BD Bioscience).

Flow cytometry

To assess the levels of CD69, CD5, FasR, and FasL in $CD8^+$ T cells, the following antibodies were used for flow cytometry: anti-mouse FITC-CD8 (clone 53-5.8), PE-Cy7-CD8 (clone 53-5.8), FITC-CD5 (clone 53-7.3), PE-Fas (clone DX2), PE-Cy7-FasL (clone MFL3), and PE-CD69 (clone H1.2F3)(Biolegend, San Diego, CA). All flow cytometric analyses were performed using appropriate isotype controls

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(Biolegend, San Diego, CA). Flow cytometry was performed using a Cytomics FC 500 (Beckman Coulter, Inc.) and data analyzed using Flowjo software (BD Bioscience). To assess the level of the indicated markers, spleens were collected from either tumour-naïve mice or tumour-bearing mice 21 days after tumour cell injection. Splenocytes were prepared as previously described, and $2X10^5$ splenocytes were treated as described above. At desired time points, cells were treated with purified anti-mouse CD16/32 antibody (Clone 93)(Biolegend, San Diego, CA) for 15 min at 21°C in the dark to block CD16/CD32 interactions with the Fc domain of immunoglobulins. Cells were then stained with appropriate antibodies for 25 mins on ice in the dark, washed twice with FACS staining buffer, suspended in 0.5 ml FACS staining buffer, and analyzed by flow cytometry. The *European Journal of Immunology Guidelines for the use of flow cytometry and cell sorting in immunological studies (31)* were followed.

Intracellular cytokine staining (ICS)

To measure IFNγ in $CD8^+$ T cells, ICS was restricted to detect IFNγ (a cytokine produced by $CD8^+$ cells upon activation). Splenocytes from either tumour-naïve or 4T1 tumour-bearing BALB/c mice were isolated and single cell suspensions prepared as described. Splenocytes from tumour-naïve mice ($2X10^5$) were treated as described above. For splenocytes obtained from tumour-bearing mice, 4T1 tumour lysate was added to re-stimulate the cells, with or without addition of function-blocking anti-CD5 MAb. Cells were incubated at 37°C overnight. Brefeldin A (10

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μg/ml) was added to retain secretion of IFNγ in the Golgi apparatus. After 3 hours cells were stained with anti-mouse FITC-CD8a (clone 53-6.7) MAb (Biolegend, San Diego, CA)(1 μg/ml in 50 μl FACS buffer) on ice and incubated in the dark for 30 min. The samples were washed twice and fixed in 2% paraformaldehyde (50 μl). To detect $CD8^+$ T cell activation, samples were stained for 30 min with PE-conjugated anti-mouse IFNγ (1 μg/ml in PBS/0.1% saponin)(Biolegend, San Diego, CA). Samples were then washed and harvested in FACS buffer for flow cytometry. Flow cytometric data were analyzed using Flowjo software (BD Bioscience).

Ex vivo cytotoxic T lymphocyte (CTL) assay

4T1 cells were stained with CFSE cell tracking dye (green) and plated in glass-bottom, 96-well plates ($5X10^3$ cells/well) in triplicate. After cells adhered to the plate for 2 hours, $CD8^+$ T cells isolated from spleens of tumour-bearing mice using a MojoSort™ Mouse CD8 T Cell Isolation Kit (Biolegend, San Diego, CA) were treated with isotype control or anti-CD5 MAb and added into each well at a 1:1 ratio with a final well volume of 200 μl. Propidium iodide was added to wells to detect dead cells. Images were taken every 2 hours, and the cytotoxic capacity of $CD8^+$ T cells was measured using an IncuCyte Zoom live cell imaging system (Essen BioScience, Ann Arbor, MI) by counting yellow objects using metric phase object confluence (propidium iodide [red] binding to dead $CFSE^+$ 4T1 cells [green]).

Statistical Analysis

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Statistical differences were assessed using a Student's unpaired one-tailed *t*-test (GraphPad Prism 8.2.1). Data points indicate means of *n* values ± standard deviation (SD). Differences between data sets where $p \leq 0.05$ were considered to be significant. Asterisks represent statistical significance.

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Conflicts of interest

The authors declare no commercial or financial conflict of interest.

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Figure 1.

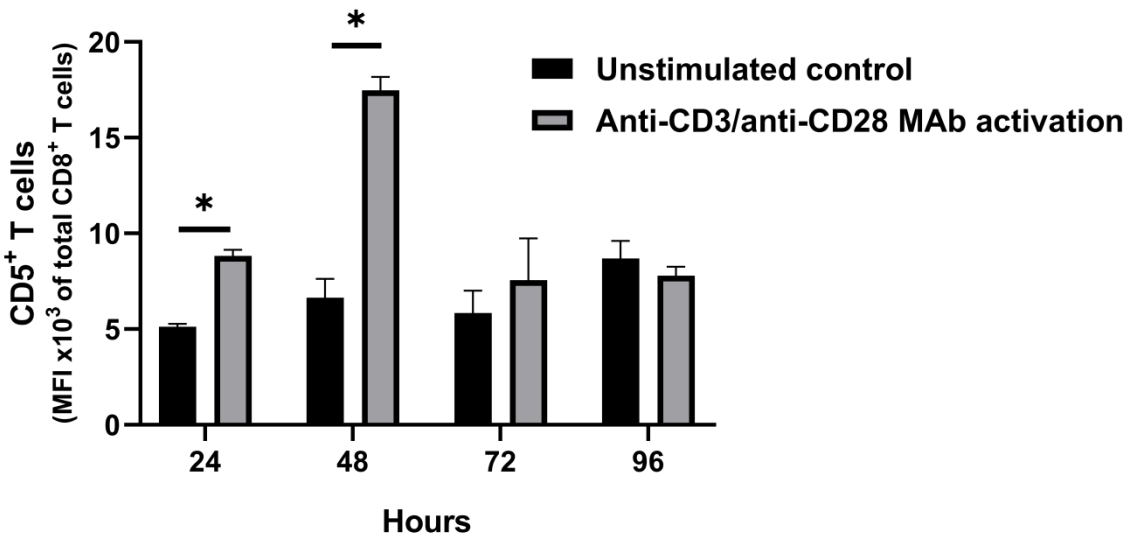

Figure 1. **CD5 levels in CD8$^+$ T cells following activation**. Splenocytes isolated from naïve BALB/c mice were treated with anti-CD3/anti-CD28 MAb on day 0 and cells were quantified by flow cytometry every 24 hours for CD5 level on CD8$^+$ T cells. Data are mean ± SD (n=3 mice), one representative experiment of three. *$p < 0.05$ (Student's unpaired one-tailed t-test).

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Figure 2.

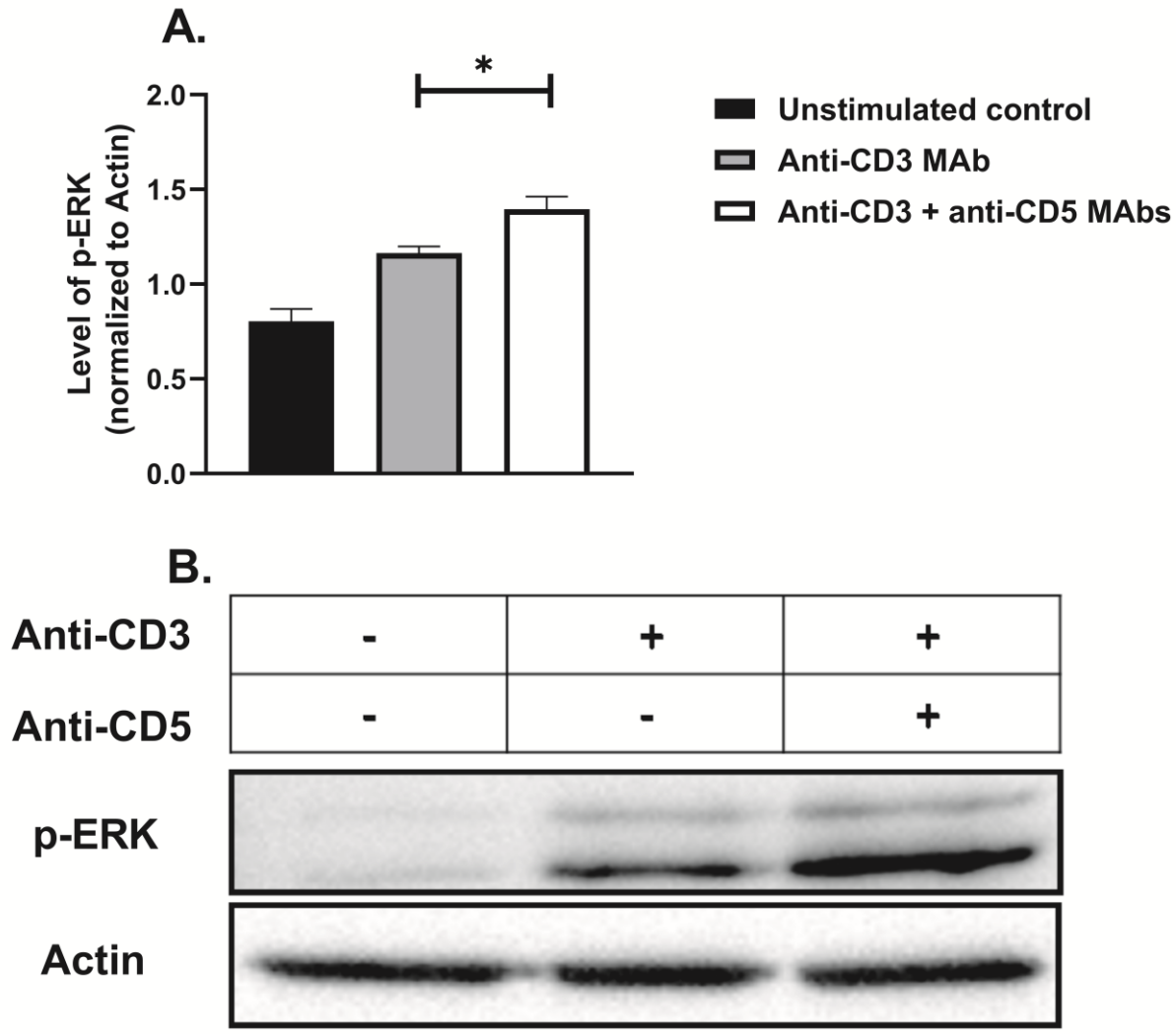

Figure 2. Increased ERK phosphorylation in anti-CD3/anti-CD5 MAb-treated cells. Splenocytes isolated from naïve BALB/c mice were treated with anti-CD3 MAb or with anti-CD3 and anti-CD5 MAbs for 30 mins and then cells were lysed and phosphorylation of Erk was detected by western blot. **(A)** The level of ERK phosphorylation was normalized to loading control actin. **(B)** Membrane immunoblotted for p-ERK and actin. Data are mean ± SD (n=3 mice), one representative experiment of three. *$p < 0.05$ (Student's unpaired one-tailed t-test).

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Figure 3.

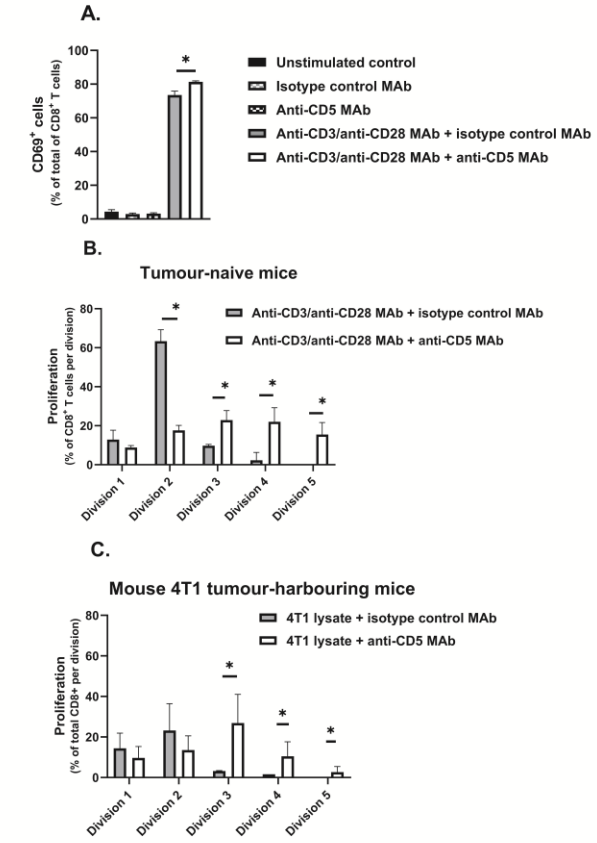

Figure 3. CD8$^+$ T cell activation and proliferation after treatment with anti-CD5 MAb blockade. **(A)** Splenocytes isolated from naïve BALB/c mice were treated with anti-CD3/anti-CD28 MAb with or without anti-CD5 MAb (day 0), CD69$^+$/CD8$^+$ cells were quantified by flow cytometry 24 hours later using PE anti-mouse CD69. **(B)** Splenocytes from naïve, non-tumour bearing BALB/c mice were stained with cell tracking dye (CFSE) and then activated with anti-CD3/anti-CD28 MAb, with or without anti-CD5 MAb (day 0). **(C)** Splenocytes from mouse 4T1 breast tumour-harbouring BALB/c mice were treated with 4T1 lysate with or without anti-CD5 MAb (day 0). Cells were incubated for 7 days and media were replaced every 48 hours. CFSE dye levels were quantified by flow cytometry: increased dilution of dye indicated increased CD8$^+$ T cell proliferation **(B and C)**. The gating strategy is shown

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in *Supplementary Figure 3 and 4*. Data are mean ± SD (n=3 mice), one representative experiment of two. *p < 0.05 (Student's unpaired one-tailed t-test).

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Figure 4.

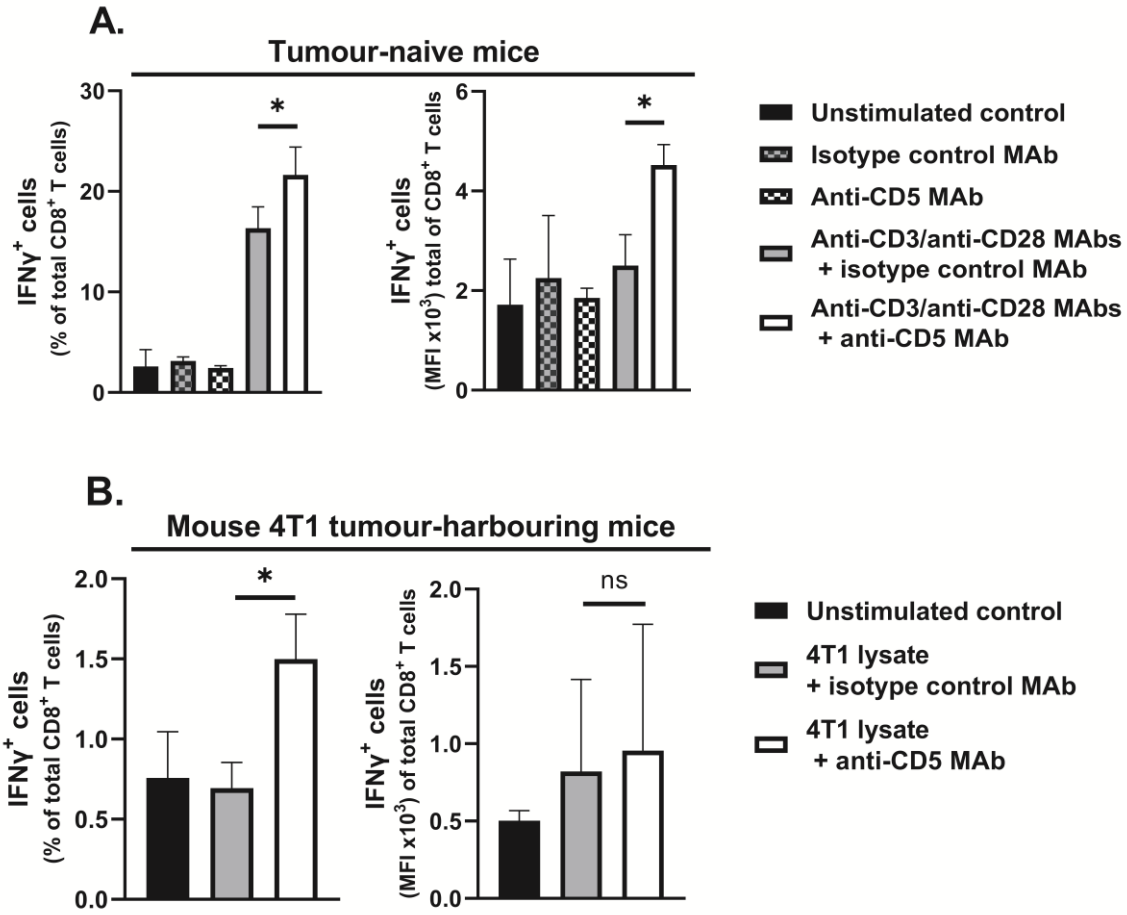

Figure 4. Increased fraction of CD8$^+$ IFNγ$^+$ T cell after treatment with anti-CD5 MAb. (A) Splenocytes isolated from naïve BALB/c mice were treated with anti-CD3/anti-CD28 MAb with or without anti-CD5 MAb (day 0). **(B)** Splenocytes from mouse 4T1 breast tumour-harbouring BALB/c mice were treated with 4T1 lysate with or without anti-CD5 MAb (day 0). CD8$^+$/ IFNγ$^+$ cells were quantified using PE anti-mouse IFNγ MAb in flow cytometry. The gating strategy is shown in *Supplementary*

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Figure 5. Data are mean ± SD (n=3 mice), one representative experiment of three. *p < 0.05, ns = not significant (Student's unpaired one-tailed t-test).

Figure 5.

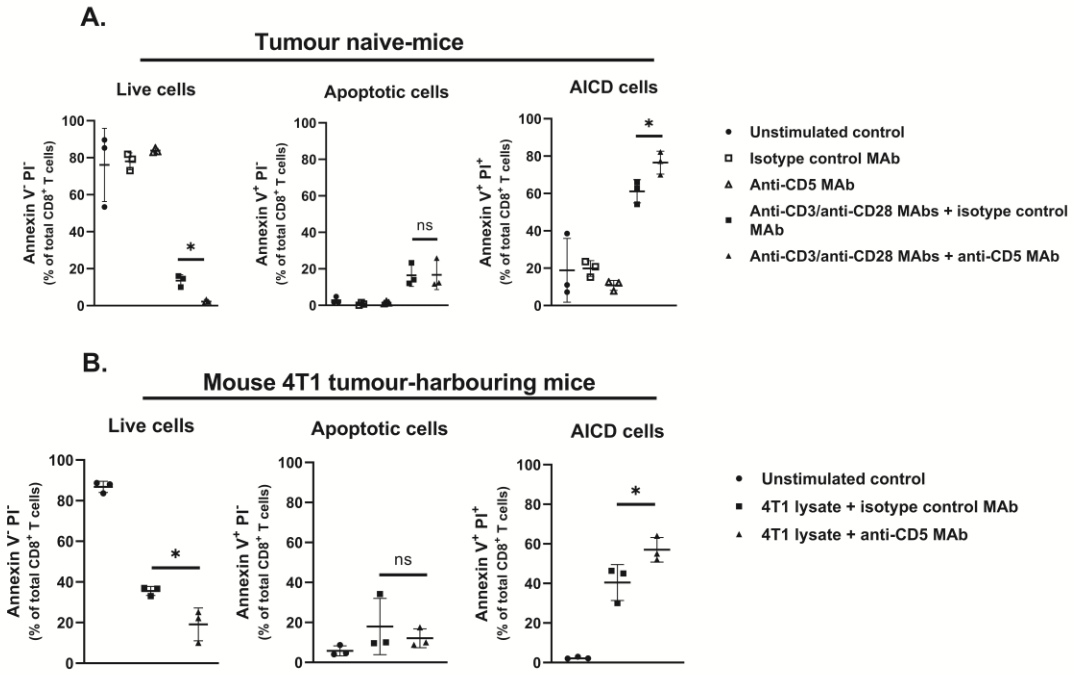

Figure 5. Increased CD8$^+$ T cell apoptosis 7 days after treatment with anti-CD5 MAb. (A) Splenocytes from naïve, non-tumour bearing BALB/c mice were activated with anti-CD3/anti-CD28 MAb, with or without anti-CD5 MAb. **(B)** Splenocytes from mice harbouring 4T1 breast tumours were treated with 4T1 lysate, with or without anti-CD5 MAb. Cell viability was assessed by flow cytometry on day 7 (cells positive for Annexin V only were deemed to be undergoing apoptosis; for both Annexin V and propidium iodide labelled as Activation-induce-cell death [AICD]). The gating strategy is shown in *Supplementary Figure 6*. Data are mean ± SD (n=3 mice), one representative experiment of two. *p < 0.05, ns = not significant (Student's unpaired one-tailed t-test).

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Figure 6.

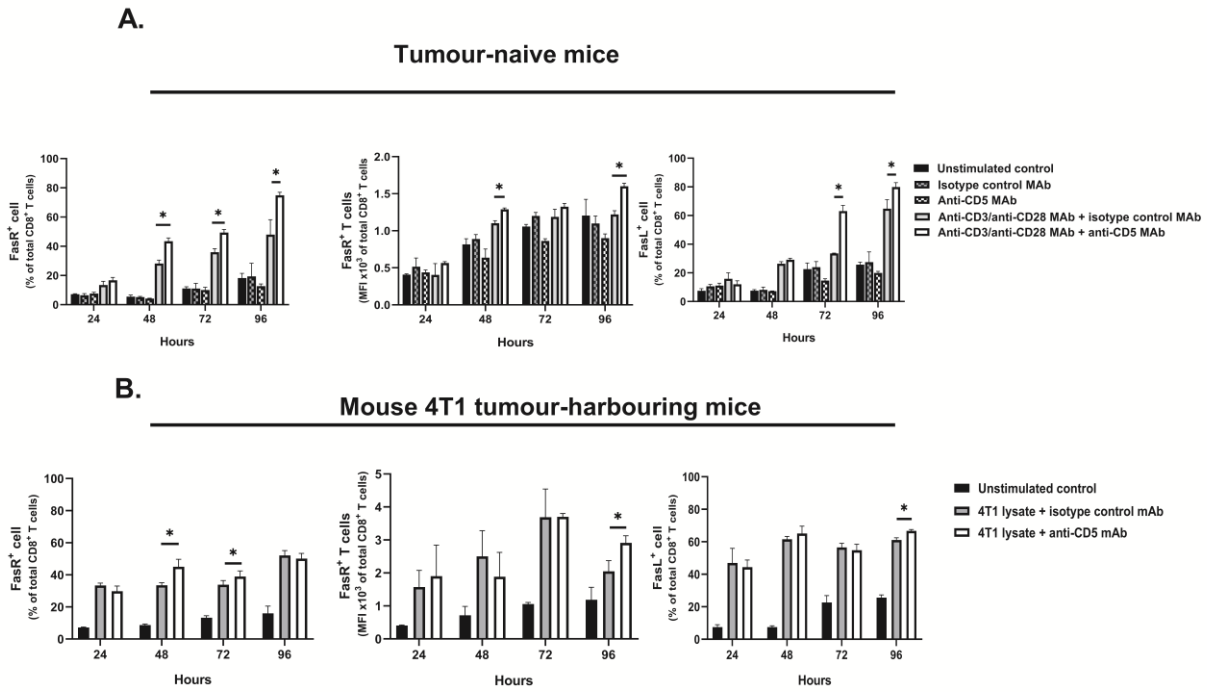

Figure 6. Fraction of FasR$^+$/CD8$^+$ and FasL$^+$/CD8$^+$ T cells after treatment with anti-CD5 MAb. **(A)** Splenocytes from naïve, non-tumour bearing BALB/c mice were treated on day 0 with anti-CD3/anti-CD28 MAb with or without anti-CD5 MAb. **(B)** Splenocytes from BALB/c mice harbouring 4T1 breast tumours were treated with 4T1 lysate, with or without anti-CD5 MAb (day 0). CD8$^+$ T cells were quantified by flow cytometry every 24 hours for FasR and FasL positivity. The gating strategy is shown in *Supplementary Figure 7.* Data are mean ± SD (n=3 mice), one representative experiment of three. *p < 0.05 (Student's unpaired one-tailed t-test).

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Figure 7.

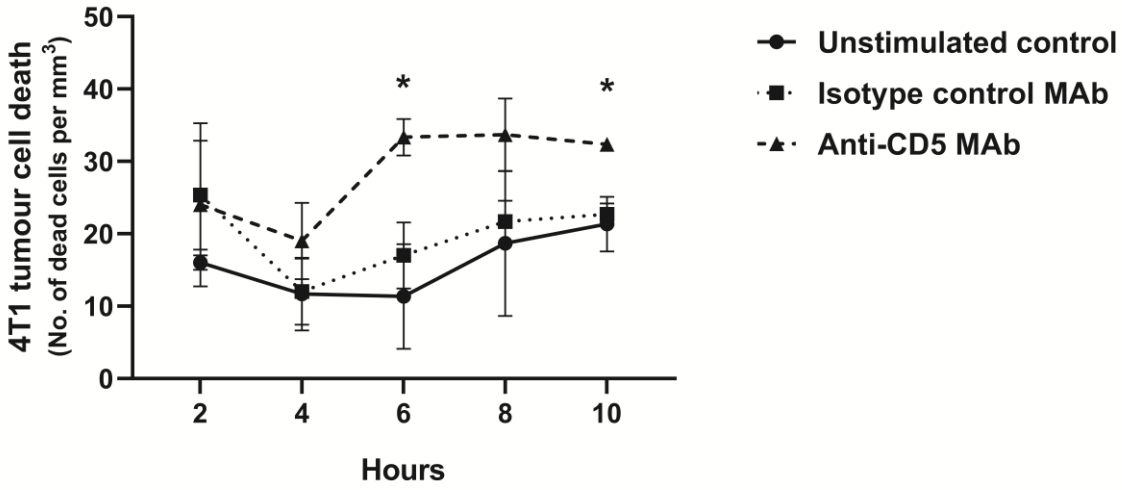

Figure 7. Anti-CD5 MAb enhances *ex vivo* CD8$^+$ T cell-mediated killing of mouse 4T1 breast tumour cells. Cytotoxic CD8$^+$ T cells were isolated from BALB/c mice bearing mouse 4T1 breast tumours, treated with the isotype control MAb or anti-CD5 MAb or control (nothing), and mixed with CFSE-stained 4T1 cells *in vitro* at a 1:1 ratio. Propidium iodide was added to measure cell death and an IncuCyte® S3 Live-Cell Analysis System was used to image the cells every 2 hours. Yellow objects which represent CFSE-stained 4T1 (green) binding to propidium iodide (red) were counted as a measure of 4T1 tumour cell death. Data are mean ± SD (n=3 mice), one representative experiment of two. *$p < 0.05$ (Student's unpaired one-tailed t-test).

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