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Laidlaw Scholar Programme Research Attachment Report

Using a tamoxifen inducible fate reporter to investigate the effect of CTLA-4 blockade on $CD8^+$ T cell homeostasis

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Abstract

Cytotoxic T-lymphocyte associated protein 4 (CTLA-4) is an inhibitory receptor primarily expressed by Tregs and activated T cells. CTLA4 competes with the costimulatory protein CD28 to bind CD80/86 and is vital for maintaining immunological tolerance and homeostasis. Increasingly, CTLA-4 pathways are targeted as part of immune modulatory strategies to treat cancers, known as immune checkpoint blockade, and conversely to manage autoimmunity and CTLA-4 deficiency.

In this study, we used a CD4-CreERT RosaR26mTOM fate reporter system to investigate the developmental kinetics of $CD8^+$ T cells and how CTLA-4 blockade impacts this development on $CD8^+$ T cell homeostasis, a population of T cells which is essential for immune defence against intracellular pathogens and tumours. Utilizing the CD4-CreERT RosaR26mTOM fate reporter system, we successfully tracked the developmental trajectory of double-positive (DP) thymocytes, CD8 single positive (CD8SP) T cells, naïve and other memory subsets. We suggest that CTLA4 blockade does not impact the developmental kinetics of naïve $CD8^+$ T cells but exhibits a delay in memory subset development.

These findings suggest that anti-CTLA-4 antibodies may influence the development of $CD8^+$ T cells. Further investigation is required to fully appreciate the role of CTLA4 in the control of $CD8^+$ T cell homeostasis.

Introduction

CD8$^+$ T cell development in the thymus

The development of CD8$^+$ T cells begins when CD34$^+$ haematopoietic stem cells (HSC), termed early lymphoid progenitors (ETPs), are released from the bone marrow and enter the thymus (see Figure 1) (Awong et al., 2011). Once they have reached the thymus, lymphoid progenitors, now termed thymocytes, progress through stages of maturation which can be characterised by the expression of CD4 and CD8 (Yang, Jeremiah Bell and Bhandoola, 2010). Thymocytes are first double-negative (DN) for CD8 and CD4, which is followed by duel expression of these markers on double-positive (DP) thymocytes, before differentiating into CD8$^+$ or CD4$^+$ single-positive (SP) thymocytes (see Figure 1) (Yang, Jeremiah Bell and Bhandoola, 2010). During CD8$^+$ T cell specification, the DP cells undergo positive selection in the thymic cortex through interaction with the peptide MHC class I complexes, leading to the generation of CD8$^+$ SP cells (see Figure 1).

Following this, the SP cells are observed to move from the cortex to the medulla, where negative clonal selection occurs to remove T cells that exhibit high-affinity interactions with self-antigens (Klein et al., 2014). In the process of negative selection, thymocytes expressing T cell receptors (TCRs) with a strong affinity for self-peptide-MHC complexes are prompted to undergo apoptosis, a process supported by the intense signalling that takes place when their TCRs pose a strong binding with self-antigens (Klein et al., 2014). According to the affinity model of selection, a robust signalling cascade is triggered by TCRs that identify self-antigens with high affinity, resulting in cell death and effectively eliminating potentially autoreactive T cells from the immune repertoire (Klein et al., 2014).

Conversely, TCRs with low affinity for self-antigens are not adequately signalled to trigger anti-apoptotic pathways in thymocytes (Kurd and Robey, 2016). Frequently, these cells find themselves in a neglected state, lacking crucial survival signals (Kurd and Robey, 2016). As a result of the absence of these signals, their demise occurs through mechanisms such as anergy or passive neglect (Kurd and Robey, 2016). The resulting population is released into the periphery as naïve $CD8^+$ T cells, where they survey the host organism for foreign antigens (see Figure 1) (Yang, Jeremiah Bell and Bhandoola, 2010).

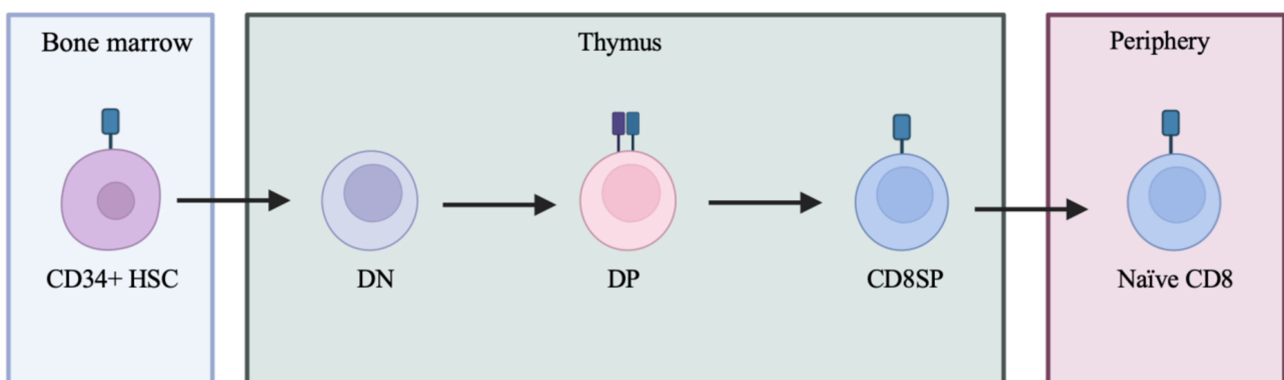

Figure 1 Developmental stages of CD8+ T cells. CD34+ HSCs are generated in the bone marrow and migrate to the thymus as DN thymocytes. These DN thymocytes will differentiate into DP thymocytes and undergo positive selection to become CD8SP cells. Followed by negative selection, they will then migrate into the periphery as naïve $CD8^+$ T cells.

Introduction to CD8$^+$ T cells

Cytotoxic T lymphocytes (CTLs), also known as CD8+ T cells, are one of the important pieces in the immune system. These cells are crucial in defending intracellular pathogens, including viruses and bacteria, as well as the surveillance of tumors (Raskov et al., 2021). Unlike CD4$^+$ T cells, CD8$^+$ T cells recognize antigens presented by MHC Class I molecules, where the molecules are expressed on the surface of all nucleated cells (Koh et al., 2023). There are two signals required to activate CD8$^+$ T cells. The first signal is the binding of the T cell receptor (TCR) to a specific peptide presented on major histocompatibility complex (MHC) class I molecules by antigen-presenting cells (APCs) (Alberts et al., 2002). The second is a costimulatory signal, which occurs between the interaction of CD28 on the T cell with its ligands, CD80 and CD86, located on the surface of the APC (Beyersdorf, Kerkau, and Hünig, 2015). This co-stimulation mediated by the CD28 ligand can foster a strong immune response and for preventing T cell anergy (Beyersdorf, Kerkau, and Hünig, 2015).

Upon its activation, CD8$^+$ T cells execute various strategies to eliminate infected or cancerous cells. One of the key mechanisms is the release of cytotoxic granules. Activated CD8+ T cells discharge cytotoxic granules which contain perforin and granzymes (see Figure 2) (Chen et al., 2024). Perforin is a protein that forms pores in the membrane of the targeted cell, enabling granzymes to penetrate the cell (Chen et al., 2024). Once the targeted cells are penetrated, granzymes will trigger apoptosis to kill infected or malignant cells (Chen et al., 2024). This cytotoxic mechanism can eliminate targeted cells displaying foreign peptides upon the presence of MHC Class I molecules directly.

Aside from the release of cytotoxic granules, CD8+ T cells induce apoptosis of target cells through the Fas ligand pathway. Upon its activation, $CD8^+$ T cells will express FasL on its surface, which interacts with Fas receptor on the targeted cell (see Figure 2) (Raskov et al., 2021). This interaction initiates a signaling cascade which eventually results in programmed cell death of the target cell (Raskov et al., 2021). This FasL pathway is particularly important in eliminating cells that may evade detection by other means, providing additional immune surveillance and control over potentially harmful cells (Raskov et al., 2021).

Furthermore, activated CD8+ T lymphocytes will release cytokines including tumor necrosis factor (TNF) and interferon-gamma (IFN-γ). Both signaling proteins can enhance the immune response (Hoekstra, Vijver and Schumacher, 2021). They not only promote inflammation but also activate other immune cells, thus recruiting additional effector cells to the site of infection or tumor (Hoekstra, Vijver and Schumacher, 2021). This cytokine-mediated response creates a more robust immune environment and facilitates the effective targeting and elimination of infected or cancerous cells (Hoekstra, Vijver and Schumacher, 2021).

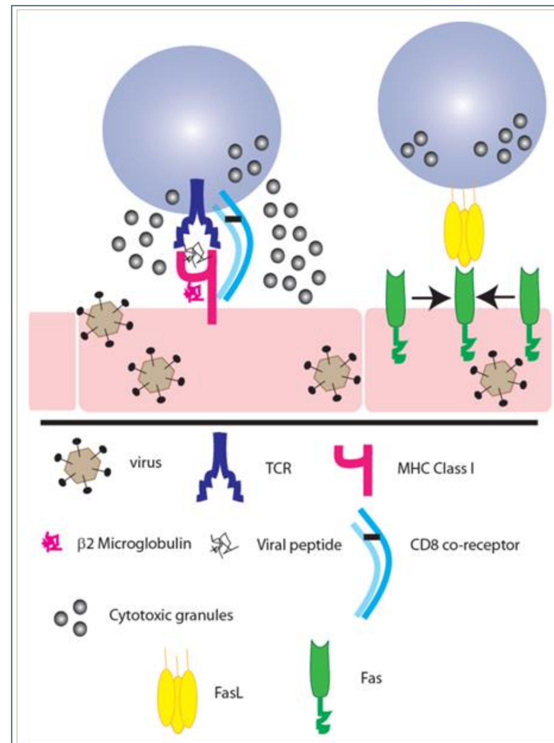

Figure 2 Interaction between a CD8+ lymphocyte and an APC (Wissinger, n.d.). Viral peptides presented by MHC Class I molecules on antigen-presenting cells (APC) are recognized by CD8+ T cells. CD8+ T cells will express the co-receptor CD8 which binds to the α3 domain of MHC Class I (with β2 macroglobulin being a part of it). Cytotoxic granules will then be released at the immune synapse, creating pores on the membrane and triggering apoptosis of targeted cell (left panel). Activated CD8+ T cells will then express FasL on their surface, which interacts with Fas receptor on the target cell's surface (right panel). This binding leads to the trimerization of Fas molecules, thus inducing apoptosis in the target antigen-presenting cell.

Role of CTLA4 in controlling CD8⁺ T cells

CTLA-4, termed Cytotoxic T-Lymphocyte Antigen 4, is a critical immune checkpoint co-receptor primarily expressed on Treg and activated T cells, including CD8⁺ T cells. It plays a significant role in regulating T-cell activation and maintaining immune homeostasis (Rowshanravan et al., 2018).CTLA-4 regulates immune responses by binding to the B7 proteins (CD80/CD86) on antigen-presenting cells (APCs), which compromises the ability of CD28 to receive co-stimulation (see Figure 3) (Rowshanravan et al., 2018). In the steady state, CTLA-4 keeps T cells inactivated by binding to either B7-1 or B7-2, maintaining levels of co-stimulation below those required for activation (see Figure 3) (Rowshanravan et al., 2018). Blocking the interaction between B7-1/B7-2 and CTLA-4 using anti-CTLA-4 antibodies enhances CD28 co-stimulation due to increased ligand availability. In certain contexts, such as solid tumors, increased CD28 co-stimulation can lead to T cell activation and targeting of tumor cells, resulting in tumor destruction.

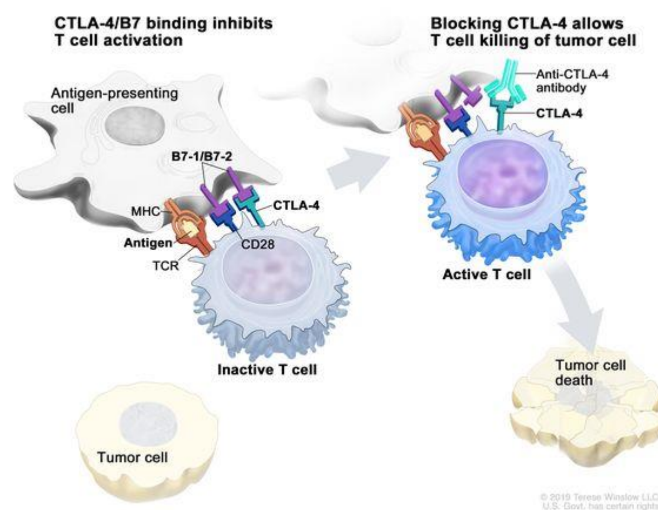

Figure 3 Relationship of CTLA-4 on T cell activation (National Cancer Institute, n.d.). In a steady state, apart from T-cell receptor (TCR) binding to antigens presented by major histocompatibility complex (MHC) molecules, CTLA-4 binds to B7-1/B7-2 to keep T cells inactivated. In the presence of anti-CTLA-4 antibodies that bind to CTLA-4, the CD28 co-stimulatory signal will be strengthened, which can activate T cells and lead to tumor cell death.

CTLA-4 plays an essential role in preventing excess immune activation, which might potentially result in autoimmune disorders or tissues damage. The optimization of CTLA-4 blockade is the focus of ongoing research, especially in its use in combination therapies, which aims to maximize antitumor effects while addressing the possible adverse reactions. Agents like ipilimumab have been developed to bolster T-cell responses against tumors (Vanneman and Dranoff, 2012).

The activation of $CD8^+$ T cells is critically suppressed by CTLA-4. It is believed that CTLA-4 functions as an essential regulator, greatly influencing the development and formation of these cytotoxic T cells. For example, research conducted by Pedicord et al. (2011), aimed to explore how CTLA-4 blockade influences CD8+ T-cell memory responses by utilizing Listeria monocytogenes infection in mice to create antigen-specific CD8+ T-cell memory. During the memory phase of T cell development in response to this overt challenge, a single administration of anti-CTLA-4 antibody was given. Results show that CTLA-4 blockade during the memory response promoted the expansion and cytokine production of antigen-specific CD8+ memory T cells, resulting in a larger pool of functional memory CD8+ T cells that provide enhanced protection against subsequent Listeria infections (Pedicord et al., 2011). Moreover, during the initial $CD8^+$ T-cell response, administration of anti-CTLA-4 led to an increase in the frequency and effector function of antigen-specific memory CD8+ T cells, without negatively impacting the overall T-cell repertoire (Pedicord et al., 2011). The improved memory CD8+ T-cell responses triggered by CTLA-4 blockade necessitated cell-extrinsic blockade, rather than merely relying on cell-intrinsic CTLA-4 deficiency in the antigen-specific $CD8^+$ T cells (Pedicord et al., 2011). The enhanced memory $CD8^+$ T-cell responses and improved bacterial clearance following CTLA-4 blockade indicate promising applications in vaccination and cancer immunotherapy (Pedicord et al., 2011). To summarize, the research shows that temporary blockade of CTLA-4 can boost the formation and functionality of antigen-specific CD8+ T-cell memory, with potential implications for vaccination and cancer immunotherapy.

Aim

This research aims to investigate the developmental trajectory of CD8+ T cells utilizing the CD4-CreERT2 Rosa26R-MTOM fate reporter system and to assess the impact of CTLA-4 blockade on $CD8^+$ T cell development.

Materials and Methods

CD4-Cre ERT2 Rosa26R-mTOM mice

All procedures on animals were performed in accordance with institutional guidelines and approved animal protocols. CD4-Cre ERT2 Rosa26R-mTOM mice were generated on a C57BL6/J background. In cells expressing CD4, inactive Cre recombinase will also be expressed. Cre recombinase will be activated following oral administration of tamoxifen, which results in the excision of a loxp-flanked STOP codon downstream of the Rosa26 locus. With the absence of STOP codon, expression of Rosa26 will also result in the expression of the fluorescent protein, mTOM. As Rosa26 is a constitutively active gene, the fluorescent mTOM protein will also be continuously expressed at a constant level, regardless of cell division or differentiation. After tamoxifen is metabolised, newly developed T cells will not be exposed to them during and therefore will not express the reporter. Thus, this system can be used to track the developmental trajectory of a small population of $CD8^+$ T cells as mTOM will be induced in DP thymocytes that will eventually mature into CD8SP before being released into the periphery as CD8+ naïve T cells, with unlabelled cells following thereafter.

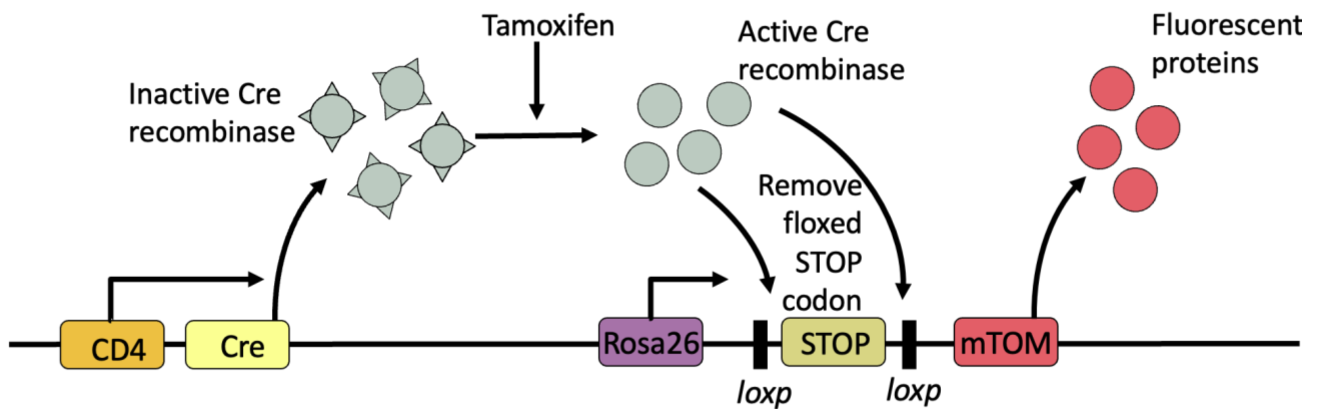

Figure 4 Schematic of the CD4-CreERT2 Rosa26RmTOM strain. In this strain, the CD4 gene expresses a Cre recombinase that is initially inactive. In the absence of tamoxifen treatment, the Rosa26 locus expresses a STOP codon that prevents transcription, thereby inhibiting the expression of the fluorescent mTOM protein. Upon administration of tamoxifen on day 0, the site-targeted Cre recombinase is activated. This activated Cre recombinase recognizes and excises the loxP-flanked STOP codon, allowing the Rosa26 locus to express mTOM, resulting in the production of the fluorescent protein.

Treatment

Male and female mice of 8 weeks of age were treated with an oral administration of 2mg tamoxifen reconstituted in corn oil and administered an intraperitoneal injection of 0.25mg anti-CTLA4 or PBS, the latter of which was thereafter performed weekly. Mice were then culled by cervical dislocation at various time points, and tissues were harvested in Roswell Park Memorial Institute (RPMI) media supplemented with 5% fetal calf serum (FCS).

Preparation of single-cell suspensions from lymphoid tissues Peyer's Patches, lymph nodes and spleen

Peyer's patches were excised from the small intestine and placed into Eppendorf's containing RPMI supplemented with FCS. Peyer's patches were placed in a petri dish between two pieces of gauze and mashed until the tissue became homogenous. Cells were transferred through a 30µm filter into 15ml falcon tubes. This was also performed for the lymph nodes and spleen. Samples were washed in PBS and 5x10(6) cells were taken for staining.

Isolation of Intraepithelial Lymphocytes (IEL) and Lamina Propria lymphocytes (LPL)

After Peyer's Patches were excised from the tissue, the small intestine was cut to expose the contents. Contents were removed by gently scraping the tissue with forceps. Tissue was placed in an extraction buffer containing 30ml RPMI, 500µl FCS, 60µl EDTA, and 30µl DTT. The tissue was then placed in a shaking incubator for 25 minutes at 200rpm and 37 degrees. IEL was collected by filtering the tissue through a 70µm strainer. the remaining tissue was placed onto a petri dish and 1 ml of digestion buffer, consisting of 24.5ml RPMI, 300µl FCS, and 250µl collagenase 8, was added. Tissue was cut until homogenous, placed into the digestion buffer and incubated in a shaking incubator for 15 minutes at 200rpm and 37 degrees. After 15 minutes, a stripette was used to re-suspend the tissue and once again the tissue was placed back in the shaking incubator for 15 minutes at the same conditions. After incubation, the tissue was filtered through a 70µm strainer to collect the LPL. Samples were centrifuged at 1500rpm, 4 degrees for 10 minutes. IELs and LPLs, were passed through a 30µm filter and washed in PBS before staining for flow cytometry.

Surface and intracellular staining

All tissues were transferred into 5 ml FACS tubes. For lymph nodes (LN) and spleen cells, 5 million cells were taken for staining (Measurement done using CASY Cell Counter & Analyzer), for all other samples, the entire cell suspension was stained. Cells were centrifuged and re-suspended in 100µl of antibody master mix, prepared in PBS. Samples were incubated for 30 minutes on ice and washed with FACS buffer before being fixed in 300 µl of fixation buffer (prepared as 1 part concentrate to 3 parts dilute). Samples were incubated for 20 minutes on ice, after which 1 ml of permeabilization buffer (made as 1 part concentrate to 9 parts PBS) was added to each tube. Cells were centrifuged and re-suspended in FACS buffer for overnight storage on ice.

For intracellular staining, 1 ml of permeabilization buffer to the samples, followed by centrifugation. 100 µl of antibody master mix in permeabilization buffer was added to each tube and cells were incubated for 1 hour on ice. Cells were washed twice with perm buffer and re-suspended the samples in 400 µl of FACS for analysis by flow cytometry.

Analysis

Cells were analyzed by flow cytometry using BD LSRFortessa. For PP, IEL and LP, 10,000 counting beads were added immediately before acquisition. Data was analyzed using FlowJo.

Results

Developmental trajectories of double-positive (DP) thymocytes and CD8 single-positive (CD8SP) thymocytes in thymus

At day 3 post-tamoxifen treatment, approximately 55% of $mTOM^+$ double-positive (DP) thymocytes were detected (see Figure 5A). The frequency of $mTOM^+$ DP thymocytes declined rapidly over time, reaching an undetectable level by day 10 (see Figure 5B). This decline is attributable to the maturation of DP thymocytes into either $CD4^+$ or $CD8^+$ single-positive (SP) cells, coupled with the generation of newly developed unlabelled DP cells. Importantly, previous laboratory data shows an induction efficiency of approximately 90% 1-day post-tamoxifen treatment (data not shown). Thus, we assume that the 45% unlabelled DP cells are a consequence of having waited 3 days post-tamoxifen and not due to insufficient labelling.

Upon analysis of $CD8^+$ SP cells, a maximum of approximately 22% were found to be $mTOM^+$ at day 3 (see Figure 5B). Given the use of a CD4-CreERT RosaR26mTOM fate reporter system, the $mTOM^+$ $CD8^+$ SP cells are derived from mTOM-labelled DP thymocytes, as they express both CD4 and CD8 before maturing into $CD8^+$ SP cells. Subsequently, the $CD8^+$ SP population decreased to below 5% by day 17 (see Figure 5B), a reduction that is due to the migration of $CD8^+$ SP T cells to peripheral organs, such as lymph nodes and their replacement by unlabelled cells.

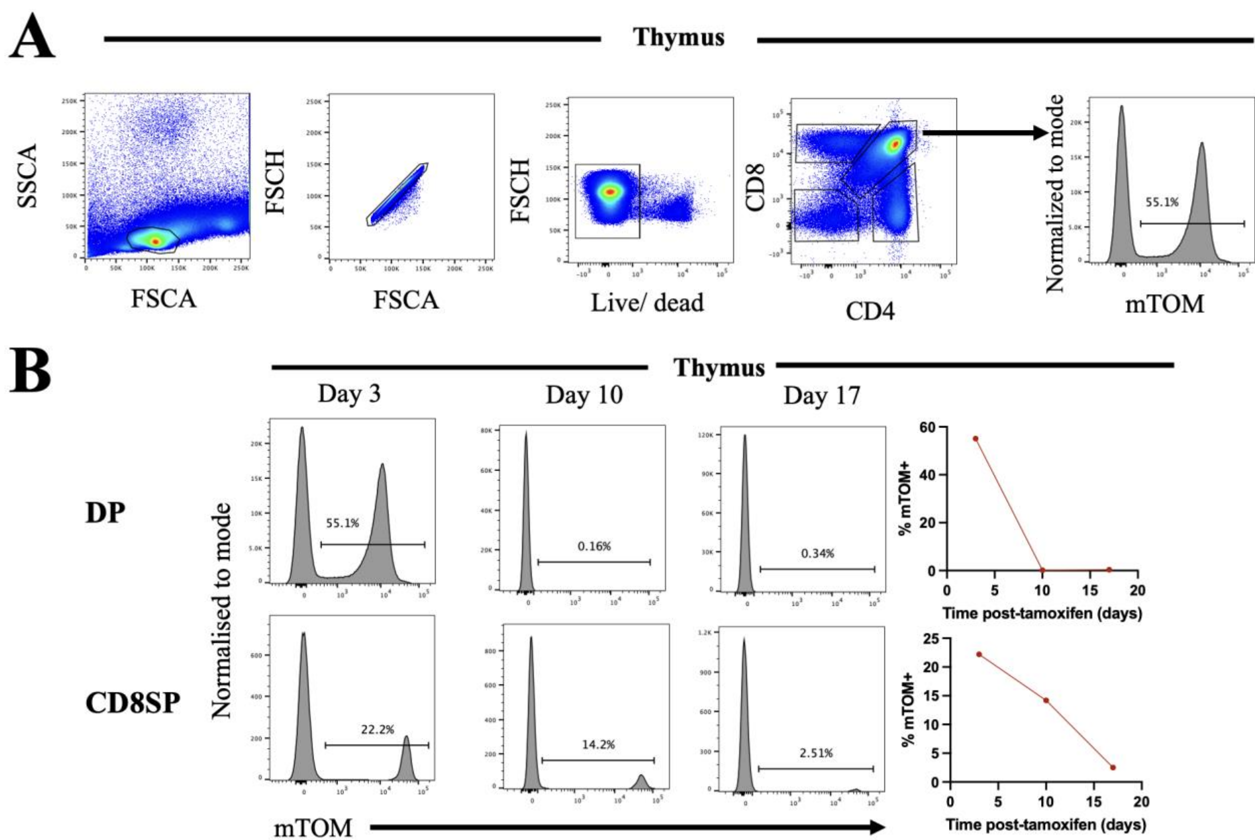

Figure 5 Developmental trajectories thymocytes in thymus. A) FACS plots showing the gating hierarchy for thymus to identify mTOM+ thymocyte populations. B) Histograms showing percentages of DP thymocytes and CD8SP cells expressing mTOM over time.

Developmental trajectories of CD8+ Naïve, CM, VM and EM cells in lymph nodes

In lymph nodes, no CD8+ Naïve T cells were detected at day 3 but by day 10, the population of mTOM+ Naïve CD8+ T cells increased to approximately 6% (Figure 6C). This is broadly consistent with estimates of the time it takes DP thymocytes to mature into naïve T cells. It is noteworthy that at 17 days post tamoxifen the frequency of mTOM+ Naïve CD8+ T cells had marginally declined. This could be attributed to the loss of the mTOM+ Naïve population either through the mechanism of cell death or differentiation into further CD8 subsets.

We analysed the mTOM content in different subsets of memory CD8+ subsets during this timeframe. While mTOM+ Naïve CD8 were observed by day 10, mTOM+ VM and CM could only be seen by day 17 indicating a delay in the generation of these subsets which is consistent with CM being derived from Naïve precursors (Figure 6c). Interestingly, at day 17 a very large fraction of both memory populations were identified as mTOM+. This high frequency could be explained by a rapid differentiation of naïve CD8 T cells into CM CD8 T cells. As only a small fraction of mTOM+ Naïve CD8 T cells have differentiated, the high frequency of mTOM within CM would also presumably have to be accompanied by a short half-life of the cells within this compartment i.e. a loss of unlabeled cells that were present prior to tamoxifen treatment. Thus, the percentage of mTOM+ in CM CD8 T cells is likely the result of a rapid differentiation of precursor and a short half-life of the pre-existing cells. Further time points measuring the loss of mTOM+ CM would be required to confirm this.

Similar to VM/ CM, there was a delay in the generation of mTOM+ EM (Figure 6c). However, unlike CM, EM only showed a modest increase in mTOM content over time, suggesting that the generation of EM is slower than that of CM, which is consistent with the literature. It would be interesting to extend the time course to see if EM also shows a high peak mTOM content at later intervals.

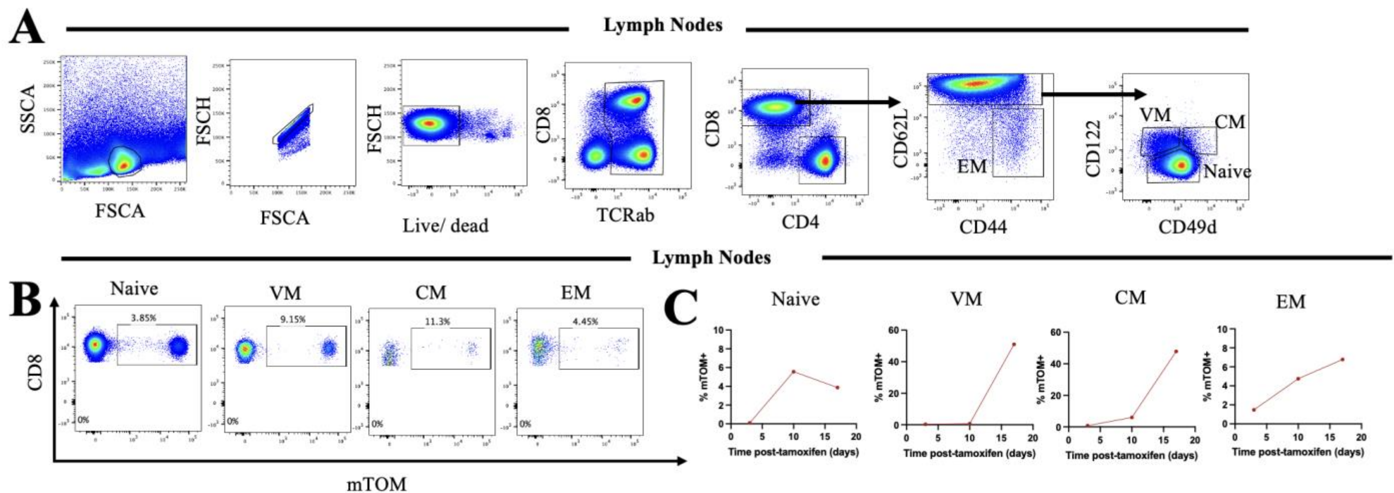

Figure 6. Developmental trajectories of CD8$^+$ Naïve, VM, CM and EM cells in lymph nodes. A) FACS plots showing the gating hierarchy for lymph nodes to identify mTOM$^+$ CD8$^+$ Naïve, CM, VM and EM cells in the periphery. B) Flow cytometry plots show %mTOM$^+$ CD8$^+$ T cell subsets in lymph nodes and C) shows %mTOM$^+$ CD8$^+$ T cell subsets over time.

CTLA-4 Blockade does not influence the development of DP or CD8SP in thymus

Having observed the developmental trajectory of our cells of interest in the steady state, we wanted to see if CTLA4 blockade altered this. We first investigated DP and CD8SP mTOM frequencies in PBS and Anti-CTLA4 treated mice. At all time points, mTOM content appeared comparable between groups in both DP and CD8SP populations (see Figures 7a & 7b). The only difference was observed in DP thymocytes at day 3, however, given the limited sample size, it is possible that this was simply due to experimental variation. Thus we suggest that CTLA4 blockade does not impact DP or CD8SP development and therefore, any changes that we observe in the periphery are not a consequence of altered thymic output.

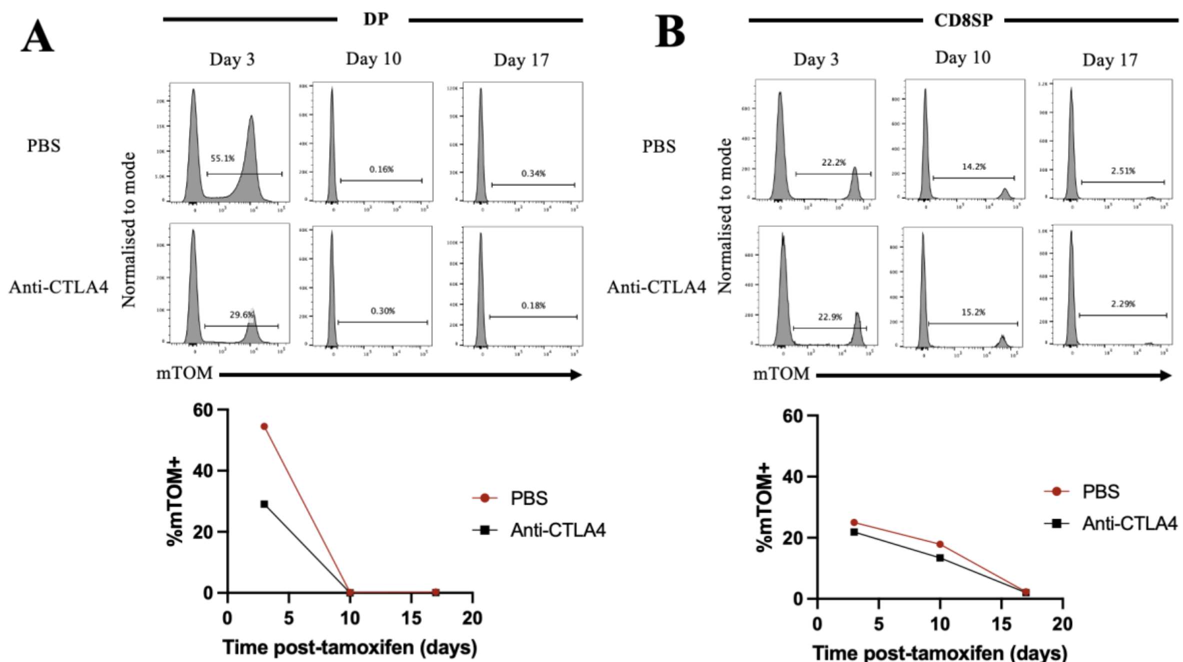

Figure 7. Effect of CTLA-4 Blockade on developmental trajectories of DP thymocytes and CD8 SP cells. A) Histograms showing percentage of DP thymocytes expressing mTOM over time in PBS and Anti-CTLA4 treated mice B) Histograms showing percentage of CD8SP cells expressing mTOM over time.

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CTLA-4 Blockade impedes development of Memory CD8$^+$ T cell subsets in lymph nodes

Given our suggestion that CTLA-4 blockade does not affect the development of double-positive (DP) thymocytes or CD8 single-positive (CD8SP) cells in the thymus, we aimed to investigate whether similar findings could be observed in naïve and memory CD8$^+$ T cell subsets within peripheral organs, such as lymph nodes..

We assessed the frequencies of mTOM+ naïve, CM, virtual memory VM, and effector memory EM CD8+ T cells in mice treated with PBS and Anti-CTLA-4. Surprisingly, the percentage of mTOM$^+$ memory CD8$^+$ T cell subsets by day 17 was lower in mice treated with anti-CTLA-4 compared to the PBS control, suggesting that the generation of CD8+ T cell memory may be impaired in the presence of CTLA4 blocking antibodies. In contrast, mTOM content in naïve CD8 T cells was similar at days 3 and 17, with a difference of approximately 3% observed at day 10. Given the limited sample size, this 3% variation in mTOM$^+$ naïve CD8 T cells is likely due to experimental variability, suggesting that CTLA-4 blockade does not influence the development of naïve CD8 T cells.

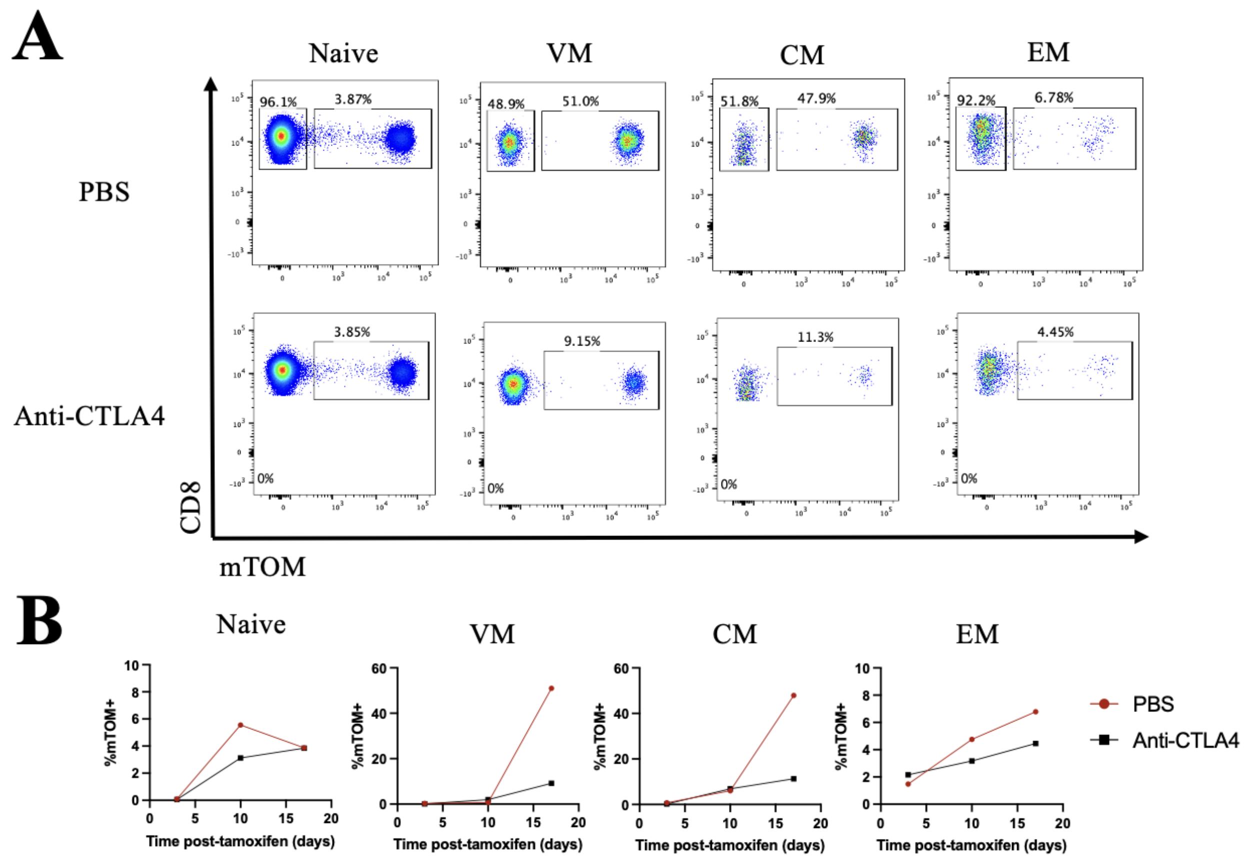

Figure 8 Effect of CTLA-4 Blockade on developmental trajectories of CD8^{+} Naïve, CM and EM cells in lymph nodes with anti-CTLA4 treatment. A) Flow cytometry plots showing %mTOM^{+} CD8^{+} T cell subsets in lymph nodes in PBS and anti-CTLA4 treated mice at day 17 and B) shows the respective %mTOM^{+} CD8^{+} T cell subsets over time.

Discussion

Our findings suggest that CTLA-4 blockade does not significantly influence the development of double-positive (DP) or CD8 single-positive (CD8SP) T cells in the thymus. CTLA-4 blockade even appears to impede the development of memory CD8+ T cell subsets in the lymph nodes, which was surprising considering that enhanced CD28 co-stimulation is typically associated with enhanced activation and differentiation.

We expect CTLA-4 blockade to increase co-stimulatory signals of CD28 ligands, leading to enhanced T cell activation and differentiation as CD28 is typically associated with t cell activation and differentiation. This reduction in mTOM+ CD8+ memory T cells did not appear to be a downstream consequence of alters thymic development as mTOM content in DP, CD8SP and naïve cd8+ t cells was comparable to PBS controls. Further investigation is necessary to confirm these observations and understand the role of CD28/ CTLA4 for the control of CD8 T cell differentiation.

One of the limitation of our study was the low sample size, which may have introduced outlier data in mTOM expression due to experimental variation. Increasing the sample size would provide more robust and reliable results to support the hypothesis and provide more persuasive evidence with minimised variability. Additionally, including additional time points would also aid future studies. In our studies, we only measured up to day 17 post treatment, extending this time course could reveal more long-term effects of Anti-CTLA4 on the development of CD8 T cells and therefore perhaps more obvious phenotypes. It is also valuable to explore other models that inhibit CTLA-4 without using antibodies, like utilising CTLA-4 knockout mice which would further strengthen the study by providing external validation using a completely different system to block CTLA-4. These improvements would allow for a more comprehensive understanding of the effects of CTLA-4 blockade on the development of memory CD8+ T cell subsets.

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Reference

1. Awong, G., Herer, E., La Motte-Mohs, R.N. and Zúñiga-Pflücker, J.C., 2011. Human CD8 T cells generated in vitro from hematopoietic stem cells are functionally mature. BMC immunology, 12, pp.1-9.
2. Yang, Q., Jeremiah Bell, J. and Bhandoola, A., 2010. T-cell lineage determination. Immunological reviews, 238(1), pp.12-22.
3. Klein, L., Kyewski, B., Allen, P.M. and Hogquist, K.A., 2014. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nature Reviews Immunology, 14(6), pp.377-391.
4. Kurd, N. and Robey, E.A., 2016. T-cell selection in the thymus: a spatial and temporal perspective. Immunological reviews, 271(1), pp.114-126.
5. Koh, C.H., Lee, S., Kwak, M., Kim, B.S. and Chung, Y., 2023. CD8 T-cell subsets: heterogeneity, functions, and therapeutic potential. Experimental & Molecular Medicine, 55(11), pp.2287-2299.
6. Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K. and Walter, P., 2002. T cells and MHC proteins. In Molecular Biology of the Cell. 4th edition. Garland Science.
7. Beyersdorf, N., Kerkau, T. and Hünig, T., 2015. CD28 co-stimulation in T-cell homeostasis: a recent perspective. ImmunoTargets and therapy, pp.111-122.
8. Chen, Y., Yu, D., Qian, H., Shi, Y. and Tao, Z., 2024. CD8+ T cell-based cancer immunotherapy. *Journal of Translational Medicine*, *22*(1), p.394.
9. Raskov, H., Orhan, A., Christensen, J.P. and Gögenur, I., 2021. Cytotoxic CD8+ T cells in cancer and cancer immunotherapy. British journal of cancer, 124(2), pp.359-367.
10. Hoekstra, M.E., Vijver, S.V. and Schumacher, T.N., 2021. Modulation of the tumor micro-environment by CD8+ T cell-derived cytokines. Current opinion in immunology, 69, pp.65-71.
11. Wissinger, E. (n.d.). BiteSized Immunology: Cells T CD8+. British Society for Immunology. Retrieved August 30, 2024, from https://www.immunology.org/public-information/bitesized-immunology/cells/cells-t-cd8
12. Rowshanravan, B., Halliday, N. and Sansom, D.M., 2018. CTLA-4: a moving target in immunotherapy. Blood, The Journal of the American Society of Hematology, 131(1), pp.58-67.
13. Vanneman, M. and Dranoff, G., 2012. Combining immunotherapy and targeted therapies in cancer treatment. Nature reviews cancer, 12(4), pp.237-251.
14. National Cancer Institute, n.d. *CTLA-4*. [online] Available at: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/ctla-4 [Accessed 1 September 2024].