PhD Thesis

Programmed-Death Receptor (PD-1) and its Role in T cell Biology-

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PD-1 Shapes Memory-phenotype CD8 T cell Subsets in a Cell-intrinsic Manner

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1. ABREVIATIONS

AICD- Activated induced cell death

APCs- Antigen Presenting Cells

BCL2- B cell lymphoma-2 family

BCR- B cell receptor

BIM- BCL2 interacting-mediator of cell death)

BLIMP1- B lymphocyte-induced maturation protein 1

CCR7- CC chemokine R-7

CFA- Complete freund's adjuvant

CFSE - Carboxyfluorescein Succinimidyl Ester

CHS- Contact hypersensitivity reaction

DC- Dendritic cells

EOMES- Eomesodermin

Fh- Follicular helper

GC- Germinal centres

GzmB- Granzyme B

HEVs- High endothelial venules

HIP- Homeostatic induced proliferation

HP- Homeostatic proliferation

i.p- intra peritoneal

Id2- DNA-binding protein inhibitor 2

Id2-Inhibitor of DNA binding 2

IFA- Incomplete freund's adjuvant

IFN- Interferon

IL- Interleukin

ITAM- Immunoreceptor tyrosine-based activation motif

ITSM- immunoreceptor tyrosine-based switch motif

JAK- Janus protein tyrosine kinase

JNK- c-Jun N-terminal kinase

KLF2- Krüppel-like factor 2

KLRG1- Killer cell lectin-like receptor subfamily G member 1

LAT- Linker for activation of T cells

Lck- Leukocyte-specific protein tyrosine kinase

LCMV-Lymphocytic choriomeningitis virus

LFA-1- Lymphocyte function associate antigen 1

LIP- lymphopenia-induced proliferating

MAPKs- Mitogen Activated Protein Kinases

MHC- Major histocompatibility complex

MP- Memory-phenotype

MPECs- Memory precursor effector cells

mTOR- Mammalian target of rapamycin

NF-κB- Nuclear factor κ-light-chain-enhancer of activated B cells

NK- Natural Killer

PD-1 -Programmed Death-1

PDK1- phosphoinositide-dependent kinase 1

PI3K- Phosphoinositide 3-kinase

PLCγ- phospholipase C γ

p-MHC- Peptide-MHC complex

S.c – subcutaneous

S1P- Sphinogosine-1- phosphate

SLE- Systemic lupus erythematosus

SLECs- Short-lived effector cells

SLO- Secondary lymphoid organs

SLP-76- SH2 domain containing leukocyte protein of 76 kDa

SNARF-1- carboxylic acid, acetate succinimidyl ester

SOCS-1 suppressor of cytokines signalling-1

STAT- Signal transducers and activator of transcription

T reg- regulatory T cells

T_{CM}- Central memory T cell

TCR- T cell receptor

T_{DIMs}- death intermediate memory T cells

T_{EM}- Effector memory T cell

Th- T helper cell

TLR- Toll-like receptor

TNF-α Tumor necrosis factor α

TRAIL- Tumor necrosis factor-related apoptosis inducing Ligand

TRAPS- Transmembrane adaptor proteins

ZAP-70- Zeta-chain-associated protein kinase 70

2. ABSTRACT

Memory-phenotype (MP) T cells, found in unimmunized mice, display phenotypic and functional traits of memory cells and provide essential protection against infections, playing a role in both innate and adaptive immune responses. Mechanisms governing homeostasis of these MP T cells remain ill defined. In this paper, we reveal a crucial role of the negative co-stimulator Programmed Death-1 (PD-1) in regulating developmental fates of memory-phenotype cells. Thus, in lymphoid organs and tissues of PD-1 KO mice a marked accumulation of functional effector memory-(T_{EM}) phenotype CD8 T cells was observed. T_{EM}-phenotype cells from PD-1 KO mice exhibit decreased proliferation but increased survival potential. These cells could produce effector molecules constitutively, in response to phorbol esters or through bystander activation by innate stimuli. Similarly, in lymphopenia-induced proliferating (LIP) CD8 T cells, whereby normally naïve T cells acquire a memory-phenotype, skewing towards a T_{EM} phenotype was prominent in the absence of PD-1. Acquisition of the T_{EM}-phenotype was a CD8 T cell-intrinsic phenomenon as demonstrated by mixed bone marrow transfer experiments. Importantly, adoptively transferred PD-1 KO CD8 central memory (T_{CM}) cells converted into the T_{EM}-phenotype indicating that PD-1 sets a major checkpoint in the $T_{CM} \rightarrow T_{EM}$-phenotype differentiation process. This was reflected by distinct patterns of gene expression of PD-1 KO T_{CM}-phenotype cells revealed by global transcriptional analysis. Additionally, adoptively transferred PD-1 KO T_{EM}-phenotype cells converted to a lesser degree to a T_{CM}-phenotype. Together, these data suggest that PD-1 shapes memory-phenotype CD8 T cell subsets.

2. ΠΕΡΙΛΗΨΗ

Τα T λεμφοκύτταρα που εμφανιζουν φαινότυπο κυττάρων μνήμης (Memory Phenotype-MP) και απαντώνται σε μη ανοσοποιημένους ποντικούς,έχουν λειτουργικά χαρακτηριστικά κλασικών κυττάρων μνήμης και παρέχουν προστασία έναντι μολύνσεων παίζοντας ρόλο τόσο στήν εγγενή όσο και στήν επίκτητη ανοσία. Οι μηχανισμοί που διέπουν την ομοιόσταση αυτών των κυττάρων παραμένουν ασαφείς.Στη διδακτορική αυτή διατριβή αποκαλύψαμε ένα κρίσιμο ρόλο που παίζει ο αρνητικός συνενεργοποιητής Programmed Death 1 (PD-1) στήν εξελικτική τύχη αυτών των MP κυττάρων. Στα λεμφικά όργανα και ιστούς ποντικών, στούς οποίους έχει απαλειφθεί γενετικά το γονίδιο PD-1 (PD-1KO ποντίκια), παρατηρείται μιά σημαντική συσσώρευση λειτουργικών MP CD8 T λεμφοκυττάρων και συγκεκριμένα δραστικών λεμφοκυττάρων μνήμης(T Effector Memory-TEM).Αυτά τα κύτταρα έχουν μειωμένη ικανότητα πολλαπλασιασμού αλλά αυξημένη δυνατότητα επιβίωσης σε σχέση με του αγρίου τύπου. Επίσης παράγουν δραστικά μόρια μετά απο έκθεση σε εστέρες φορβόλης ή ενδογενή ερεθίσματα. Παρομοίως κατά τον πολλαπλασιασμό τών παρθένων CD8 κυττάρων που έπεται μιάς λεμφοπενίας, μια κατάσταση που οδηγεί σε εμφάνιση T λεμφοκυττάρων τύπου μνήμης, παρατηρείται μετάπτωσή τους κυρίως σε T_{EM} στα PD-1 KO ποντίκια. Η υιοθέτηση αυτού του φαινότυπου αποτελεί ένα γεγονός συνυφασμένο με τον γονότυπο μόνο τών CD8 T λεμφοκυττάρων και αποδείχθηκε με πειράματα χιμαιρισμού. Επιπρόσθετα κύτταρα κεντρικής μνήμης (T_{CM}) από PD-1 KO ποντίκια που μεταφέρθηκαν σε νέους ιστολογικά συμβατούς αποδέκτες μετέπεσαν στη συντριπτική πλειοψηφία τους σε κύτταρα δραστικής μνήμης (T_{EM}) ενώ το αντίστροφο αφορούσε ένα πολύ μικρό ποσοστό των μεταφερθέντων κυττάρων. Από τα παραπάνω και από τα αποτελέσματα τής μεταγραφικής ανάλυσης ολόκληρου του μεταγραφώματος τών CD8 T_{CM} κυττάρων καταλήγουμε στο συμπέρασμα ότι το μόριο PD-1 παίζει καθοριστικό ρόλο στη διαμόρφωση τών υποτύπων τών κυττάρων μνήμης.

3. INTRODUCTION

3.1- Innate and adaptive immunity

Immunity consists of a complex system of defence mechanisms against invading pathogens. Many different cell types orchestrate the immune response. As a first line of defence, macrophages, natural-killer cells (NK) and dendritic cells (DC) act immediately by the release of various cytokines and effector molecules, conferring partial protection, known as innate immunity. Additionally, DC take up invading pathogens components, migrate to local lymph nodes and there they participate in the activation of antigen-specific T lymphocytes. These lymphocytes mediate a more robust antigen-specific response and following pathogen clearance, persist as memory cells for many years (adaptive immunity) [1] [2].

Naïve T cells entering the periphery from the thymus constantly interact with antigens that are presented as peptide fragments bound to the major histocompatibility complex (MHC). MHC molecules can be characterized as MHC class I and MHC class II, which interact with CD8 T cells and CD4 T cells respectively. More specifically, intracellular antigens are processed in antigen presenting cells (APCs) into peptides and are presented on MHC class I molecules, which are recognized by T-cell receptors (TCR) on cytotoxic CD8 T cells. Extracellular antigens, on the other hand, are processed by the endocytic pathway of the APC and typically bind to the MHC class II molecules which are recognized by T-helper CD4 T cells.

In a steady state, in the absence of infection, APCs continuously cross-present self-antigen bound to the MHC complexes to naive T cells. Naïve T cells that receive this low level of self peptide/MHC complex stimulation continue to circulate through the blood and secondary lymphoid tissues. This self-antigen recognition, called tonic TCR signals, along with interleukin -7(IL-7) is necessary for naïve T cell survival [3].

Prior to antigen interaction, naïve T cells continuously scan the body for pathogens by migrating through the blood and secondary lymphoid organs (SLO). T cells enter SLOs from the blood via specialized capillaries called high endothelial venules (HEVs) present within paracortical (T-cell rich) regions of lymph nodes and a variety of receptors are important for this process. Typically L-selectin (CD62L) on T cells, aids the initial tethering of cells to various adhesion molecules called addresins on HEVs, CCR7 (CC chemokine R-7), which binds to CCL21, results in the immobilization and activation of T cells, while LFA-1 (Lymphocyte function associate antigen 1) binds to ICAM1 (Intercellular adhesion molecule 1) and stimulates T cell transmigration into SLOs. After T cells have entered lymph nodes, chemotactic motility is dependent on CCR7 interactions with CCL21 and CCL19, which

retain T cells in SLOs, facilitating immune-surveillance. Egression of T cells back to the blood stream and lymph is mediated by S1P (Sphinogosine-1- phosphate) [4].

Upon pathogen recognition, APCs become primed, upregulate the expression of MHC and co-stimulatory molecules and thus become 'licensed' to display the specific antigen to activate T and B lymphocytes. DCs also up-regulate the synthesis, production and surface expression of inflammatory cytokines, including IL-12, and type I interferons (IFN) which further activate the immune response. Presentation of the specific antigen to the TCR, triggering by co-stimulatory molecules, together with signals from the cytokine environment in the SLO, leads to the clonal expansion of T cells and subsequent differentiation into antigen-specific effector T cells [5]. CD8 T cells mediate their effector functions through production of cytokines such as IFN-γ and TNF-α and/or cytolytic mechanisms. CD4 T cells, depending on the variety of signals and cytokines can differentiate into at least four lineages of effector cells: Th1, Th2, Th17 and follicular helper T (Tfh) cells. Th1 cells, via the production of IL-2, IFN-γ and TNF, are essential for controlling intracellular pathogens such as viruses and certain bacteria. They also provide cytokine-mediated "help" to CD8 cytotoxic T cells. Th2 cells are essential for humoral responses by B cells and protect against extracellular pathogens via the production of IL-4, IL-5, IL-10 and IL-13. Th17 cells protect surfaces (e.g., skin, lining of the intestine) against extracellular bacteria, and possibly fungi, via the production of IL-17 and IL-22 [6]. Follicular helper T cells also provide help to B cells in germinal centres (GC) enabling them to develop into antibody-secreting plasma cells, and their signature cytokine to aid this process is IL-21. In addition, there is another related subset that dampens rather than promotes immune responses called regulatory T cells (T reg) which produce TGF-β and IL-10 [7]. It was originally thought that different Th cells were 'committed' to their path but recently evidence suggests that under certain conditions, seemingly committed T cells indeed possess plasticity and may interconvert [8].

A final arm of adaptive immunity is attributed to B cells and their humoral responses. This protection stems from a combination of sustained antibody titers and long-lived memory B cells (MBCs), the former deriving from long-lived plasma cells (PCs). MBC's are generated in GC's in response to a T cell-dependent Ag. It is within a GC that Ag-specific B cells are selected and undergo somatic hypermutation (SHM) of Ig V genes, yielding cells with increased affinity for antigen (Ag). As a result, MBC's rapidly differentiate into high-affinity plasma cells following a re-encounter with the specific Ag. Neutralizing antibodies (Ab) present in serum are the fastest form of protection against a re-encounter from a pathogen [9-10]. Follicular helper CD4 (Fh) T cells are instrumental to the formation and maintenance of GC and MBC's [11].

During the course of an immune response, inflammatory cues at the site of infection or in draining lymph nodes, induce dramatic changes in cell's homing patterns and in lymph node architecture. This is primarily to increase the probability of rare naive T cells to encounter their cognate antigen and also for the recruitment of other effector cells to the site of infection. In order to produce a strong primary immune response, proliferation of antigen-specific T cells occurs rapidly resulting in clonal expansion of these cells. Upon pathogen clearance, most effector T cells die by the end of the immune response and a small percent (~5%) of the effector cells survive as memory T cells. The principal feature of memory T cells is their ability to rapidly respond and protect the host from secondary encounter from the same pathogen, described in full below.

3.2- Phenotypical, migrational and functional properties of memory T cell subsets

In 1999 Lanzavecchia et. al. identified 2 different subsets of memory ($CD44^{hi}$) T cells in humans [12] and since then fundamental similarities between memory T cell populations in mice and humans have been observed [13]. Memory CD8 and CD4 T cells can be subdivided into two main functional subtypes; effector memory T cells (T_{EM}) and central memory T cells (T_{CM}) according to combinations of surface markers, such as chemokine receptors, interleukin receptor components and functional properties [14]. T_{CM} cells constitutively express CCR7 and CD62L, two receptors that are required for homing to T cell rich areas of SLO's [12]. Following TCR re-triggering these cells produce mainly IL-2 and rapidly proliferate [15]. This propensity to rapidly proliferate gives them the 'stem cell' properties often attributed to these cells. T_{EM} cells are phenotypically characterized by $CD44^{hi}$ $CCR7^{lo}$ $CD62L^{lo}$ and have been shown to circulate the blood and spleen and migrate to tissues and organs such as lung, liver and kidneys. Effector memory T cells, like their name suggests, are poised with rapid effector function, such as lytic activity and production of IFNγ, but have reduced ability to proliferate compared to T_{CM} cells [16]. A potential new group of memory T cell have been identified as tissue-resident memory T cells (T_{RM}) [17]. These cells permanently reside in peripheral tissues and especially mucosal sites, and are possibly the first line of defence upon re-infections. They express similar activation markers to T_{EM} cells, therefore contributing to the delay of their identification, with the addition of CD69 and intergrin CD103 [4]. However it is still unclear if these cells are T_{EM}'s that have lost their circulating ability and therefore remain in tissues, or if they are a distinct subgroup of memory T cell.

3.3- Dissecting the signals required for CD8 T cell memory generation and differentiation.

The transition from a naive T cell to a memory cell is a complex process that requires accumulation of multiple signals and the complexity of the signals determining the relative memory cell potential is only really starting to be understood. An adaptive immune response to an acute infection that results in the generation of memory T cells can be roughly categorized into 3 phases: Activation, clonal expansion and contraction (figure 3.1).

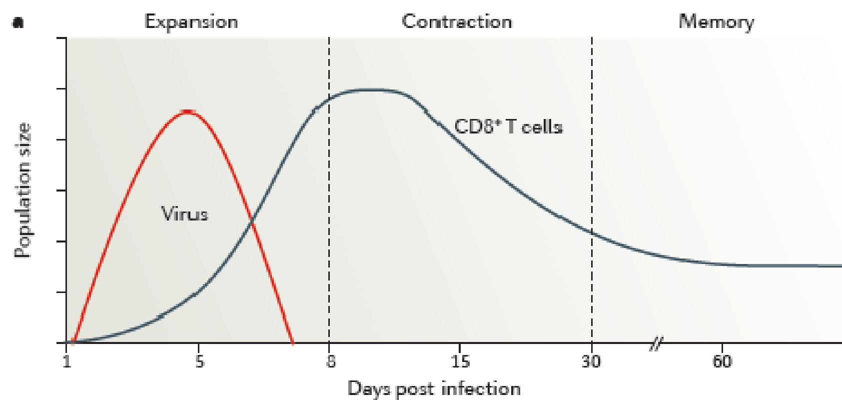


Figure 3.1 – Kinetics of a T cell response. During an acute viral infection, antigen-specific T cells rapidly proliferate (during the expansion phase) and differentiate into cytotoxic T lymphocytes (CTLs) that mediate viral clearance. Most of these cells die over the next several weeks during the contraction phase of the response. Only a small percentage of effector T cells (5–10%) survive and further develop into functional mature memory CD8^{+} T cells. Source Kaech et all Nat. Rev. Immunol. 2012

3.3.1- Activation phase

In the initial activation phase, naïve T cells receive 3 signals upon encounter with mature antigen presenting cells (APC). Signal 1 or TCR signalling results from recognition of cognate antigen bound to MHC complexes on the surface of 'licenced' APC. This signal provides the specificity to the immune response. Features of the TCR-pMHC interactions are antigen abundance, duration, affinity and efficiency. Studies assessing the role of antigen dose/strength of signal and duration of antigenic stimulation, suggest that the culmination of these signals via the TCR is required in moderation; too strong results in activated induced cell death (AIDC), too weak and cells die by neglect, while the 'just right' signals results in optimal effector and memory generation [18] [14]. One of the most initial events upon TCR activation is the upregulation of the early activation marker CD69 on effector T cell. CD69

inhibits the expression of S1PR1 on the surface of the cell [19], a receptor that interacts with S1P, which mediates T cell egression out of the lymph nodes. Therefore this crucial event augments the time the cell resides in the lymph node, and therefore its chances of receiving essential signals and instructions to promote productive T cell proliferation and differentiation.

Signal 2, or co-stimulation, has been shown to be a critical parameter in determining the developmental fate of memory T cells [20-26]. Members include CD28 (CD28, CTLA-4, PD-1, ICOS, BTLA), TNF/ TNFR (OX40, CD27, 4-1BB, CD30, GITR, and HVEM) and integrin (LFA-1, VLA-4) families, and they can be further classified into co-stimulatory and co-inhibitory molecules as depicted in **figure 3.2**. Co-stimulatory molecules synergise with TCR transduction pathways while co-inhibitory molecules inhibit downstream events. Programmed death-1 (PD-1) is one such immunoreceptor that negatively regulates TCR and B cell Receptor (BCR)-signaling upon engagement of one of its ligands (PD-L1 and PD-L2) [27-28]. Differences in the kinetics of expression of each co-stimulatory and co-inhibitory molecule suggest that each one may have distinct roles during the phases of the immune response and in instructing T cell fate. These signalling pathways synergize with TCR signal transduction and are therefore important for regulating activation, clonal expansion, effector functions, and survival of T cells (discussed in full below) **see figure 3.3**. Co-stimulatory and co-inhibitory molecules have been shown to regulate memory T cell development, with a consensus that co-stimulation promotes formation of antigen-specific memory cells, whereas co-inhibition impedes it [14]. Further elucidating the mechanisms of TCR and co-stimulatory signaling synergism, and when they take place in the immune response, will greatly benefit the therapeutic targeting of co-stimulatory molecules for vaccine improvement.

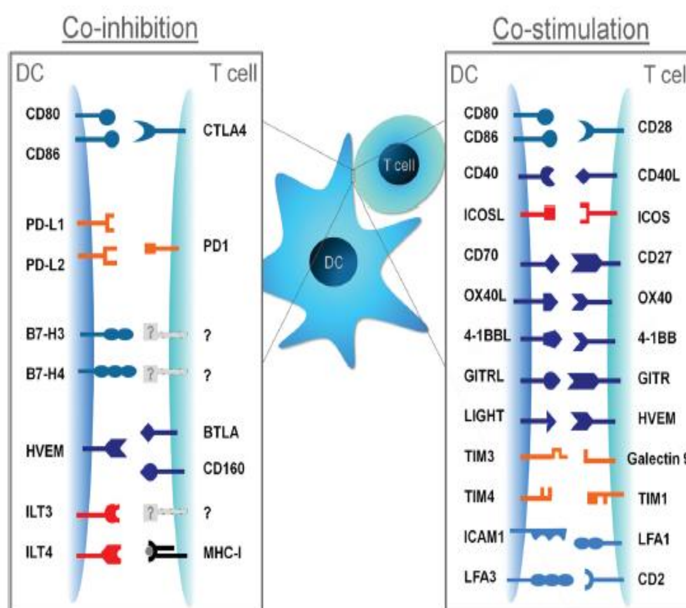


Figure 3.2- Co-stimulatory and co-inhibitory molecules and their cognate ligands. Source Bakdash et. al. Front. Immunol. 2013

Signal 3, or inflammatory signals, are from cytokines such as IL-12, Type I IFN and/or Toll-like receptor (TLR) ligands, as well as IL-2 [29]. It has become more and more appreciated that pro-inflammatory signals, during activation of naive T cells mediate crucial aspects of memory generation and differentiation. The nature of the pathogen determines the pro-inflammatory cytokine released and the expression of TLR on APC that will direct memory programming and development. Recent work suggests that the initial gene program is triggered by TCR and co-stimulatory signals and can only continue when inflammatory signalling is present, demonstrating the importance of this third signal [30]. Importantly, IL-12, type I interferons (IFNα and IFNβ) and IL-2 also induce PI3K, p38MAPK pathways, similar to TCR and CD28 signalling, suggesting another potential early interplay of the three signals at priming. IL-2/IL-2R signalling has also been linked to effector versus memory differentiation, by regulating expression of various transcription factors and genes associated with effector cells or memory cells fates [31] (as discussed below). There is an emerging theme that higher levels of inflammatory cytokines favour the formation of short-lived effector cells (SLEC) rather than long lived memory cells [32-33] [13]. Taken together, signals 1, 2 and 3 are closely linked *in vivo* during infection and the duration or the relative amount of these signals can affect the number, phenotype, function and long term fate of effector T cells and memory generation.

3.3.2- Expansion phase

Once primed, CD8 T cells follow a tightly orchestrated expansion phase where T cell clones rapidly proliferate and differentiate into effector T cells, which have potent effector functions such as lytic activity and cytokine release. IL-2 plays a crucial role in this initial activation and proliferation of T cells clones. Activated T cells rapidly upregulate IL-2Rα (CD25), start to produce IL-2 and this has both autocrine and paracrine effects that induce rapid proliferation of T cell clones [31, 34]. During the expansion phase, activated T cells also modify their ability to home and localize to different tissues based on changes of trafficking molecules on their cell surface. More specifically, L- selectin (CD62L) and CCR7, chemokine receptors that mediates homing to lymph nodes are down-regulated, while CD44 and LFA-1, are up-regulated, activation markers which mediate homing to peripheral tissues. There is compelling *in vivo* evidence that T cells upregulate different homing molecules required for migration to specific locations ie CCR9 for gut homing [35] or CCR4 for skin homing [36], and these seems to be influenced by the site of initial T cell priming [4]. Once in the correct vicinity, effector T cells locate their target cells and exert their effector functions. CD8 T cells

develop into cytotoxic T cells which target and kill pathogen-infected cells directly by the exocytosis of granules of cytolytic enzymes, including perforin and granzyme B (GzmB) [37], as well as the production of cytokines including IFN-γ and TNFα [38]. The importance of IL-12 and type I IFNs for optimal T cell expansion has also been demonstrated. These cytokines have been shown to regulate the balance of pro-apoptotic and anti-apoptotic BCL-2 family members in the proliferating T cells, and therefore alter survival potential of the expanding T cells [39].

3.3.3- Contraction phase

As the effector T cells eradicate the pathogen during the immune response, T cell interaction with the reduced inflow of antigen-bearing APC results in the decreased capacity of the APC to produce stimulatory cytokines leading to competition by the T cells for survival signals. In the third phase (contraction phase), when the threat has been overcome, 90-95% of effector cells die by apoptosis. The molecular mechanisms controlling contraction of antigen specific T cells are incompletely understood. At least two types of cell death can occur in activated T cells during the contraction phase: activation-induced cell death (AICD), also called Ag-driven apoptosis, and activated T cell autonomous cell death (ACAD), also called growth factor withdrawal-induced apoptosis [40]. IL-2 has been implicated in the AICD since sustained presence of IL-2 and IL-2Rα expression on T cells induces the expression of the death receptor FAS on the effector T cell, promoting apoptosis. Effector T cells compete for homeostatic cytokines (such as IL-7 and IL-15) availability and once these cytokines are withdrawn upon pathogen clearance, inevitably some cells die by neglect. IL-15 also promotes the expression of anti-apoptotic BCL-2 in T cells therefore having a protective role on effector CD8 T cells [41] and support the generation of memory cells [42]. Interestingly, IL-7Rα (CD127) is rapidly down regulated after activation on most effector cells, while a small fraction of these T cells regain expression of IL-7Rα after the infection has subsided and become long lived memory cells, implying that only IL-7Rα^{hi} cells are capable of surviving the contraction phase [43]. IL-2 signalling potently suppresses IL-7Rα expression and therefore IL-2Rα down-regulation is also shown to be a requirement for long lived memory T cells [44]. Inflammatory cytokines also have been shown to influence the contraction process, namely by up regulating the expression of pro-apoptotic molecule, specifically BIM a member of the BCL-2 family [40]. IL-2 and IL-12 are important for regulating the expression of a transcription factor Tbet in the responding T cells. Tbet is highly expressed on short lived effector cells while its expression is lost in long lived memory cells, and thus determines which cells survive contraction. Also, IFNγ signalling is thought to play a role in the reduction of CD8 memory T cells [45]. These data indicate that strong antigen driven signals and pro-inflammatory milieu drive T cells to become terminally

differentiated effector T cells that are destined for rapid death following clearance of the pathogen; the opposite, a reduced inflammatory environment, augments the formation of long lived memory T cells.

3.3.4- Recall and maintenance of memory CD8 T cells.

The cardinal features of memory T cells are their ability to rapidly respond to re-encountered pathogens and their capability to persist for many years [46]. Memory T cells exhibit altered homing patterns, increased TCR avidity, enhanced proliferation and cytokine production, all of which enable them to respond with increased vigour and potency to future encounter with the same pathogen. The different activation markers on memory cells, as discussed above, reflect their ability to home to different tissues. Circulating T_{EM} cells are recruited into inflamed tissues within hours to days of a pathogen re-infection, and they are followed by large numbers, days later, by secondary effector T cells possibly generated by recall T_{CM} proliferation, via IL-2, in secondary lymphoid tissues. T_{RM} cells have an advantage of being at site of entry of many pathogens and are therefore likely to be required for frontline defences against fast replicating pathogens [13]. However, little is known about the balance between tissue residence and circulating T_{EM} cells and the importance of each subgroup in protective immune responses. It is important to note that tissue microenvironments also provide developmental cues to 'fine tune' tissue-specific T cell memory phenotypes and therefore play a role in optimizing local protective immunity. For example, local production of TNF-β induces integrin expression on memory T cells after migration into the small intestines; this integrin being an important molecule for the local T cell maintenance [47]. Also T_{CM} cells can down-regulate their CD62L upon egression into tissues and obtain a T_{EM} phenotype.

However, despite the heterogeneity of memory subgroups, the hallmark of all memory T cell's fitness results in the expression of anti-apoptotic molecules and responsiveness to homeostatic cytokines [3]. The antigen-independent self-renewal of memory T cells, termed 'basal homeostatic proliferation' assures specific T cell survival and maintenance over many years [48] [49]. Basal homeostatic proliferation is defined as the *in vivo* turnover of memory cells observed under steady-state conditions. Basal homeostatic proliferation is ''non-productive'' i,e, cell numbers are not increased and should be distinguished from proliferation under conditions of lymphopenia ('acute homeostatic proliferation'), discussed below, or in response to antigenic stimuli ('antigen-driven proliferation') [48].

3.4- Signal transduction and transcriptional regulation of CD8 T cell differentiation

Antigen recognition leads to the redistribution of TCR-CD3 complexes, along with co-stimulatory and adhesion proteins, into a defined immunological synapse necessary for productive activation of T cells. The combination of these signals promotes a number of signaling cascades that ultimately determine cell fate through regulating cytokine production, cell survival, proliferation, and differentiation (**see figure 3.3**).

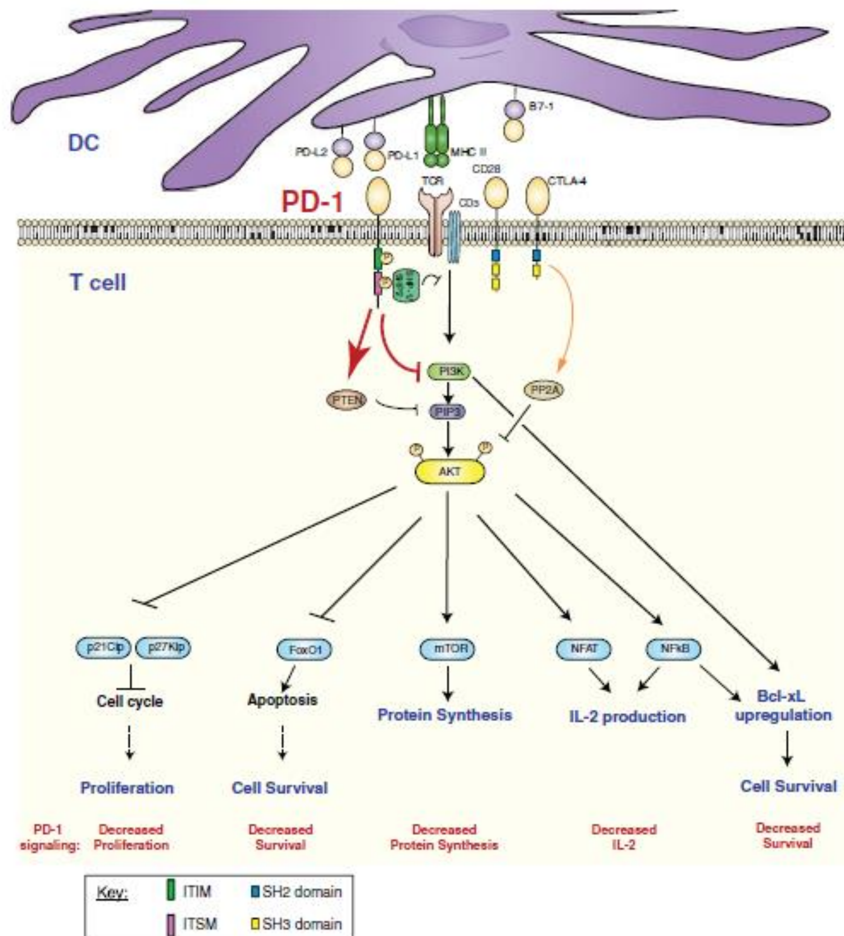


Figure 3.3- TCR signalling events and PD-1 Ligation of TCR and PD-1 leads to tyrosine phosphorylation (P) of the ITIM and ITSM of PD-1. Binding of the ITSM by SHP-1 or SHP-2 results in the dephosphorylation of proximal signalling molecules. This effectively attenuates the activation of the PI3K and Akt pathways. PD-1 signaling may result in decreased T-cell proliferation, survival, protein synthesis, and IL-2 production. (Red arrows and text indicate consequence of PD-1-mediated signalling). Source: Fransisco et al . Immunol Rev. 2010

More specifically, upon engagement of the TCR with pMHC molecules, the initial event is the phosphorylation of ITAMS (Immunoreceptor tyrosine-based activation motifs) in the cytoplasmic domains of CD3 zeta chain, by the Src-family kinases Lck and Fyn [50]. This leads to recruitment of the kinase Zap70 (ζ-chain–associated protein kinase 70 molecule).

Zap-70 activation induces recruitment and activation of scaffold molecules or TRAPS (Transmembrane Adaptor proteins) such as LAT (linker for activation of T cells) and SLP-76 (SH2-domain-containing leukocyte protein-76) [51]. These molecules provides a multitude of SH2- and SH3-binding sites for the transmission of downstream signalling events and play crucial roles in spatial and temporal regulation of the formation of immunological synapses at the T-cell–APC interface [52].

Importantly, LAT provides a platform for the integration of many positive and negative signals from a multitude of receptors (eg cytokine and chemokine receptors) that help cells sense the environment and 'decide' accordingly on their fate. Additionally, positive and negative co-stimulatory molecules regulate TCR signalling by modulating the phosphorylation state of kinases like Lck and Fyn. LAT and SLP-76 also help to localize a variety of molecules, such as PLCγ (phospholipase C γ), to the plasma membrane and aid their phosphorylation. These transducers then activate enzymatic signalling cascades and change conformations and binding capacities of secondary signalling molecules such as DAG (diacylglycerol) and IP3 (inositoltrisphosphate) . IP3 binds to its receptor on the surface of the endoplasmic reticulum and induces an increase in intracellular calcium triggering the activation of NFAT (Nuclear factor of activated T-cells) signalling pathway. NFATs, together with AP-1 transcription factors (Jun/Fos) bind to DNA response elements and induce the expression of genes related to T cell activation such as IL-2 and other effector molecules. Activation of Ras by DAG leads to the downstream activation of MAPKs (Mitogen Activated Protein Kinases) pathways which constitute a large kinase network that regulates a variety of physiological processes, such as cell growth, differentiation, and apoptotic cell death. Phosphorylation of PI3K (phosphatidyli- nositol 3-kinase) by Lck and Fyn, leads to the generation of several inositol phospholipids including PIP2 and PIP3 (phosphatidylinositol 3,4-bisphosphate). PIP3 recruits PDK1 (phosphoinositide-dependent kinase 1) to the plasma membrane and activates it. Activated PDK1 then phosphorylates serine/threonine kinase Akt and PKCθ. Phosphorylation of PKCθ leads to the eventual activation of NF-κB. Akt and NF-κB are powerful signalling molecules which translocate to the nucleus and mediate many diverse biological processes such as glucose transport, glycolysis, glycogen synthesis, cell proliferation and inhibition of apoptosis. [29, 53-54]. Akt also regulates diverse cellular process that impact CD8 T cells fates and so appears to be situated in a position to coordinate the convergence of the CD8 T cell-fate-determining pathways.

Recently, there have been great discoveries in the molecular aspects that regulate the effector to memory cell transition, and several transcriptional factors (TF) have been identified. These TF are regulated by downstream effector molecules of PI3K/Akt signalling such as mTOR (Mammalian target of rapamycin) and FOXO's. A recent review highlights

the importance of the reciprocal regulation of pairs of transcription factors in this development process [55]. According to the current paradigm, these TF pairs function like antagonistic genetic switches for cell fate decisions that allow for the simultaneously development of short-lived effector cells and long lived memory precursors, during the course of an immune response. The most well characterized TF pairs include, T-bet/Eomesodermin (EOMES), Inhibitor of DNA binding 2 (Id2)/Id3, B lymphocyte-induced maturation protein 1(BLIMP1)/BCL-6 and STAT3 /STAT4. In each pair stated above, the first mentioned TF has been shown to be highly expressed in and associated with effector T cells function and development, while the latter regulate the development of memory T cells. More specifically, as the expression or the activity of EOMES, BCl-6, Id3 and STAT4 increases in the cell, this triggers a cascade of events in the cell that help to maintain memory properties such as long term survival, proliferative potential and ability to self renew. Taken together, it is clear that multiple interrelated signalling pathways, influenced by factors such as signal strength/duration and exposure to cytokines, culminate in the graded expression of competing sets of TF and these play a pivotal role in the programming of T cells fates during memory generation.

3.5- Models of memory cell differentiation

How memory T cells develop and the relationship between effector and memory T cells has been actively debated in the literature for many years and several models have been proposed to explain the divergent developmental fates of the T cell progeny (**see figure 3.4**). When, where and how memory T cells and their subsets are formed is an area of intense study and great controversy. There are several factors that contribute to the problems in finding models that fit all scenarios. Firstly, the sheer complexity and heterogeneity in memory T cell generation. Secondly the differences in experimental systems implemented, such as *in vitro* versus *in vivo* models or different TCR-transgenic mice models together with differences in temporal (primary vs secondary infections) and topical (lymphoid or non lymphoid tissues) evaluations techniques. Thirdly, the undefined time frame in which memory cells arise in these different settings and fourthly, the lack of clear phenotypic markers that categorize effector and memory cells efficiently. Despite these difficulties several theoretical models have been proposed. There are numerous recent reviews that are dedicated to this topic [29, 56-58]. The models attempt to address several important basic questions. Firstly, do effector and memory T cells originate from a separate lineage, secondly are memory T cells pre-programmed/have a pre-existing potential or do they

acquire memory traits and if so at what stage of activation? Thirdly, what is the origin of memory T cell diversity and how is inter-conversion between memory subsets regulated?

Self renewing effector model. [57] This model assumes that the activation of naïve T cells results in the generation of self renewing 'pre-T_{CM}' independent of passing through an effector cell. These cells circulate the lymphoid tissues and when required can differentiate to T_{EM} and/or terminal differentiate into effector cells, acquiring effector functions and the ability to migrate to the periphery and eradicate the infection, without having self renewal potential. It is proposed that T_{CM} cells that develop directly from naive T cell and retain the rapid replicative capacity of naive cells are predicted to have a biological advantage and can potentially eradicate the pathogen faster. In contrast, a T_{CM} that slowly develops from a senescent effector cell or T_{EM} may have a distinct disadvantage of generating adequate numbers of effector cells to confer protection during a secondary infection. The conversion between $T_{EM} \rightarrow T_{CM}$ cells was not addressed in this model.

Decreasing-potential model. [58] This model suggests that every effector T cell has the potential to develop into a memory cell but exposure to inflammation and antigen for longer periods of time can further differentiate effector cells into terminal effector cells and decrease their potential to become memory cells. T cells acquire the memory phenotype in progressive stages of differentiation after initial priming events. The steps of differentiation depend on the variety of stimulation by antigen and/or signals 2 and 3. This model hypothesises that T cells that undergo maximum differentiation will become effector cells and will subsequently die after the infection is eliminated, while the ones that differentiate the least, will increase their survival potential and become long lived memory cells. With regards to plasticity among memory T cells subsets research from Ahmed's group have demonstrated after an acute LCMV infection [15] that long-term persistence of memory T cells is primarily in the form of T_{CM}.

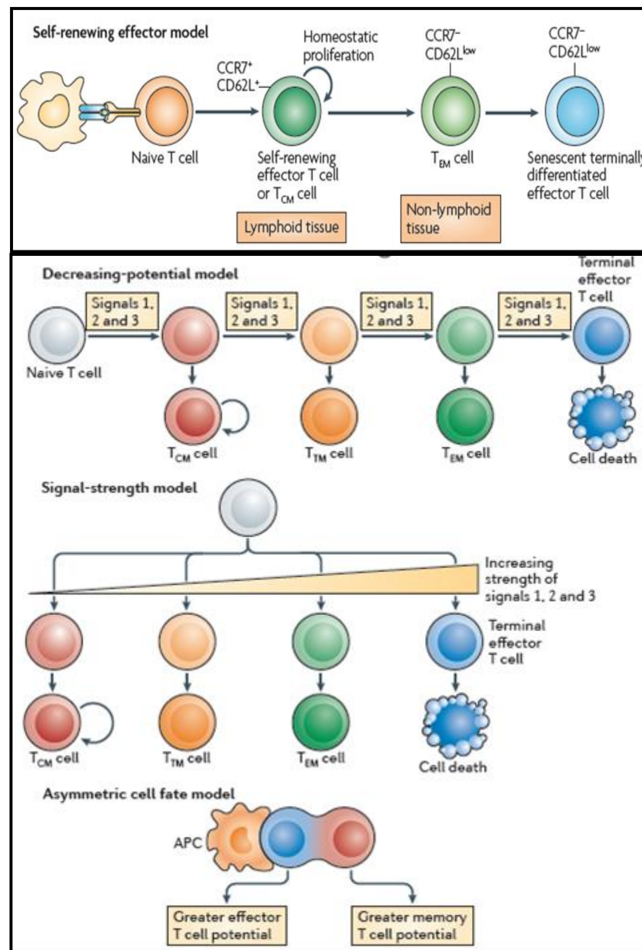


Figure 3.4 - Models for generating effector and memory T cell heterogeneity. Source: Kaech et al. Nat. Rev. Immunol. 2012 and Ahmed et al, 2009 Nat. Imm. rev

Moreover, T_{EM} cells converts to T_{CM} over time, in the absence of antigen, and gain the ability to undergo self renewal via homeostatic proliferation. They also showed that this $T_{EM} \rightarrow T_{CM}$ conversion was programmed during the period of initial *in vivo* T cell priming, and was dependent on the magnitude of the infection and the duration of antigenic stimulation; a lower amount of priming antigen resulted in more rapid differentiation of $T_{EM} \rightarrow T_{CM}$ [18]. Their results also demonstrate that, at least in the spleen, T_{CM} convert to effector cells and subsequently to T_{EM} only in the presence of antigen [15].

Signal strength model [5] This model, also called the *Progressive differentiation model*, attempts to explain the heterogeneity of memory cell subsets. According to this model the strength of the initial priming signals (signals 1, 2, and 3) in a collective manner, affects the fate of the naïve cell. Thus different hierarchical thresholds of signals control the different proliferation and differentiation of memory T cells during the course of an immune response, leading to the generation of various intermediates and effector T cells. Accordingly, strong signals give rise to short lived effector cells, intermediate signals favour T_{EM}, and weaker

signals T_{CM}. As in most of the models the commitment to memory generation is established at the priming phase. Finally, in the absence of re-stimulation the majority of memory cells persist as T_{CM}, while a proportion of these cells differentiates into T_{EM} to replenish the effector memory pool in the presence of homeostatic cytokines IL-7 and IL-15 [59]. This model differs from the previous described model in that different cells fates can be specified early during the response, in a more divergent manner, according to the intensity of the signals received, rather than in a linear stepwise manner driven by successive rounds of stimulation.

Asymmetrical cell fate model proposed by Reiner et al [60]. This model (also called bifurcative model) again attempts to explain the cell fate heterogeneity of daughter cells. It theorises that progeny cells are committed very early on during the priming phase (at the first cell division) to become short lived effector or long lived memory cells. Hence, asymmetric segregation of proteins and mRNA, involved in cell signalling and fate specification, ensure asymmetrical division of the progeny T cell into two genetically distinct daughter cells [61]. The daughter T cell that is formed proximal to the APC was more likely to contribute to the effector T cell subset and the distal daughter T cell more likely to generate memory T cells. A study supporting this model demonstrated that transfer of a single naïve cell into a lymphosufficent host produced effectors, T_{CM} and T_{EM} memory populations after vaccination [62]. A more recent study generated naive T cells that carry unique DNA tags (barcodes) [63] for 'tracking' the progeny cells. Importantly, both studies demonstrated that effector and memory T cells are progeny of the same naive T cells. The interconverion between T_{CM} and T_{EM} cells was not addressed in either study.

It is important to note that the above models are not mutually exclusive and ultimately they achieve the same end result; a multitude of cells, each armed with specific effector functions and abilities, with some cells being able to persist for a long time as memory T cells.

3.6- Homeostasis of memory CD8 T cells

Homeostasis of memory cell numbers is important for the maintenance of stable memory pool over the course of the host's life time. It is now readily accepted in the field, that proliferation of memory T cells is not only driven by antigenic stimulation but also by homeostatic cytokines (called homeostatic proliferation) primarily via the γ-chain cytokines IL-15 and IL-7. In a steady state, memory cells divide every 2-3 weeks, without the need for antigenic stimulation and are primarily maintained through signals received via cytokine receptors [3].

The IL-15 receptor (IL-15R) is comprised of three chains: IL-15Rα, IL-15Rβ and IL-15Rγ. The α-chain is unique to IL-15R, the β-chain is shared by IL-2R and the γ-chain is common to a variety of cytokines including IL-2, IL-4, IL-7 IL-9 and IL-21. Memory cells 'sense' the levels of IL-15 in the environment by possessing the IL-15Rβ chain (CD122) and more than 50% of memory cells express this receptor subunit. While IL-15 signalling appeared to be dispensable for antigen-specific CD8 memory T cell generation and function during primary responses, in its absence, memory T cell pool slowly decline, and this was due to their inability to homeostatically proliferate [64]. IL-15 is produced by a variety of non- T cells, including DC's, and IFN's has been shown to induce IL-15 production and vice versa; for this reason IL-15 is often considered a bridge between innate and adaptive immune system. IL-15 binds to its receptor and activates JAKs/STATs pathway (specifically JAK3 and STAT-5) and the signalling is negatively regulated by SOCS-1 (suppressor of cytokines signalling-1) [65]. IL-15R signalling has also been attributed to the survival memory CD8 T cells by the up-regulation of anti-apoptotic molecules such as BCL-2, and down regulation of TRAIL (tumor necrosis factor-related apoptosis inducing Ligand) [66]. Thus, IL-15 has been shown to be a requirement for the survival and maintenance of memory cells by preventing apoptosis during the transition of effector to memory cells and maintaining their basal homeostatic proliferation.

IL-7 is another common γ-chain cytokine that has been shown to be important for maintenance of memory cells. Increasing the levels of IL-7 by over-expression can overcome the requirement of memory cells for IL-15 [67]. IL-7 also acts through the JAK/STAT pathway, activating anti-apoptotic factors, such as BCL-2, and is negatively regulated by SOCS1 [68]. IL-7 plays an important role on survival and homeostasis of CD8 memory T cells and the IL-7Rα chain (CD127) is upregulated on memory cells, while having no expression on effector cells. IL-7 signalling augments the transaction from effector to memory cells, and for this reason this receptor has recently been described as a marker for long lived memory CD8 T cells [43]. Collectively, γ-chain cytokines play a major role in the generation, maintenance and function of memory T cells. Interestingly IL-2, IL-7 IL-15 have been shown to induce expression of PD-1 and its ligands *in vitro* [69], therefore applying a natural break to proliferating T cells.

3.7- Differences in CD8 and CD4 T effector and memory generation and maintenance.

The dissection of CD4 memory differentiation and generation is less clear than for CD8 T cells, perhaps due to their limited cell frequencies *in vivo*, their extensive effector lineage heterogeneity and later development of MHC class II/peptide-multimer technology [70]. Similar to CD8 T cells, CD4 effector T cells undergo a differentiation process, depending on the nature of cytokines produced by the innate immune system. This involves the expression of lineage specific transcription factors that control the ability to produce certain effector cell types. For example, differentiation in the presence of IL-12 promotes expression of the transcription factor T-bet, which commits cells to the Th1 program of producing IFN-γ. Alternatively, differentiation in the presence of IL-4 promotes expression of the transcription factor GATA-3, which commits cells to the Th2 program of producing IL-4. A key question in the immune memory field is how the CD4 effector cells present at the peak of the primary response relate to the memory cells that survive the contraction phase. There is mounting evidence for plasticity in the CD4 Th lineages further complicating the situation [8]. A strong case can be made that some Th1 effector cells simply return to a quiescent state and become Th1 effector memory cells, with the 'classic' properties ascribed to T_{EM} cells [71]. There is also evidence that Th2 effector cells can become T_{EM} cells after the contraction phase of the immune response [72]; however, the evidence for this is not as extensive as that for Th1 cell memory. The case for the entry of Th17 effector cells and Tregs into the memory cell pool is less clear [73]. Interestingly some Th1 effector cells generated in response to certain infections in mice, seem to become $CCR7^{+}$ effector cells and, subsequently, memory cells that have low expression of T-bet and lack other lineage-defining transcription factors [74]. It has been proposed that these cells are possibly T_{CM} like cells, since upon re-stimulation they produce IL-2 rather than Th1 associated cytokines. It is also possible that these stem-like cells are not committed towards any Th lineage and following reactivation, can potentially generate secondary effector cells of several Th lineages [75]. This raises the question of how naive T cells 'decide' which path to follow. Similar to CD8 T cells generation, strong stimulation is needed for commitment to one of the CD4 T_{EM} cell lineages, whereas weaker stimulation favors the generation of less-committed T_{CM} cells [73] [76].

With regards to the maintenance of CD4 T cell memory, there is still some controversy whether antigen, MHC class II molecules [77] [78] and the common γ-chain cytokines are required for survival of CD4 memory T cells. Some studies providing evidence that IL-15 and IL-7 may not be required for the maintenance of CD4 memory cells [79]; while others provide evidence that IL-7 may indeed be important [80]. In a study that directly compared stability of antigen-specific CD4 and CD8 memory T cells [81], CD4 T cells were found to decay faster over time then their CD8 counter parts, and they attributed this to a reduced ability to be

rescued from apoptosis. With regard to costimulatory molecules, there is evidence that OX-40 and its ligand promote BCL-2 expression, which helps in sustaining CD4+ T cell survival [82]. Interestingly, in the absence of OX-40 [25] or ICOS [83] signalling in mice, a reduction in CD4 T_{EM} cells was observed. Other co-stimulation receptors CD27 [84] 4–1BB [85] and have also been shown to contribute to the generation and maintenance of memory CD4 T cells.

3.8- CD4 T cells help in CD8 T memory generation

Help mediated by CD4 T cells has been shown to be crucial for CD8 T cell memory cell generation in several models, however the exact underlying molecular and cellular mechanisms of this help are still elusive. What is readily regarded as the hallmark of 'helpless' CD8 T cells is that these cells are defective in the memory recall responses upon secondary stimulation. Studies with adoptive transfer of TCR-transgenic cells demonstrate that 'helpless' CD8 memory T cells were capable of robust primary expansion, but were not able to proliferate upon re-exposure nor to maintain their numbers via homeostatic proliferation. In one study, this defect was attributed to the increased expression of the death receptor TRAIL and the CD8 memory T cells underwent AICD upon re-stimulation [66]. Another study postulates that CD4 help is mediated through CD40 and CD40L pathways. In this model, activated CD4 T cells upregulate CD40L and engage CD40 on DC and this results in full maturation and 'licensing' of DC to then go on to fully activate CD8 T cells [86]. Importantly, Usherwood et al have demonstrated that, at least in a acute viral infection model, the recall responses by 'helpless' CD8 T cells are restricted by the up-regulation of PD-1 on these cells and blocking PD-1 pathways restores their functional defect [87].

3.9- Memory phenotype cells

3.9.1- Origins

Even in the absence of intentional immunization, small numbers of T cells, with phenotypical, functional and genetic characteristics of memory cells, are observed. Such 'spontaneously' generated cells are called memory phenotype (MP) cells and accumulate in animals and humans with age. MP cells are thought to be generated from a combination of environmental and self antigens [49]. There is a multitude of innocuous antigens found in the gut flora of the host or in the environment that can trigger the generation of MP cells. Unequivocal evidence for the involvement of self antigens in the development of MP cells comes from the findings

that these cells are present in humans before birth [88], and in mice kept in germ-free and even antigen-free housing [89]. Additionally, it has been shown that typical MP cells arise in large numbers when naïve T cells are adoptively transferred to lymphopenic hosts. In these setting the 'space' created, triggers naïve T cells to acutely proliferate, developing into protective $CD44^{hi}$ MP cells without passing through an effector phase [3, 29]. This has been shown with adoptive transfers of polyclonal naive and a variety of TCR transgenic naive cells and therefore it is proposed that LIP is directed at various self peptide-MHC complexes. LIP is boosted by the presence of elevated homeostatic cytokines such as IL-7 and IL-15 found in lymphopenic hosts [90]. LIP can arise in various situations of lymphopenia, such as, at birth in neonates [91], after some viral infections or radio- and chemo-therapy treatments [92]. Since a large majority of MP cells arise in the absence of antigen, homeostasis via cytokines plays a major role in their regulation and maintenance, as described above [93]. In addition to the involvement of γ-chain cytokines, the transition of naïve T cells into MP cells may involve other mechanisms, such as loss of negative signals. A transient loss of contact with the inhibitory action of regulatory T cells, or interruption of contact with negative co-inhibitory molecules on T cells, and their respective ligands on dendritic cells, might also result in MP cells formation [49], but this remains to be evaluated.

The mechanisms that govern the generation of CD4 MP cells are slightly different to CD8 T cells owning to their intrinsic lack of ability to proliferate in response to IL-15 and IL-7, this is partly due to the fact that CD4 T cells have a reduced expression of IL-2Rβ (CD122) receptor, and therefore reduced responsiveness to IL-15. A possible factor limiting the homeostatic proliferation of naive $CD4^{+}$ T cells is IL-7-mediated down-regulation of the expression of MHC class II [94]. Although CD4 MP do homeostatically proliferate, it happens to a lesser degree to CD8 MP cells [95]. The main mechanism thought to generate CD4 MP cells is cross-reactivity of TCR with different pMHC complexes, at least for humans, who are constantly exposed to a myriad of organisms. Intriguingly, recent work in humans found an abundance of MP cells in healthy adults for foreign antigens that the individuals had never encountered such as HIV, CMV and influenza [96]. The ability to induce immunological memory independent of infection or classical vaccination represents significant therapeutic potential. Further work is needed in order to fully understand the mechanisms for the generation of MP cells, while their protective capacities are undisputable, as discussed below.

3.9.2- Functional importance of memory phenotype cells

Similar to antigen-specific memory T cells, memory phenotype cells have been shown to rapidly proliferate, produce IFN-γ and other effector cytokines after TCR stimulation and therefore are important in adaptive immune responses [97] [98]. CD8 MP cells have also been show to effectively lyse infected cells upon simulation and provide comparable protection against bacterial infections in vivo as Ag-experienced memory CD8 T cells [99]. CD8 MP cells have similar dependence on CD4 T helper cells for functional protection, emphasizing again their similarities to true memory T cells.

Memory T cells have been recently correlated with a variety of autoimmune conditions and diseases. As mentioned above, memory T cells can develop and expand in an antigen independent manner, via homeostatic turnover and this process is amplified in conditions of lymphopenia. Autoreactive memory T cells can potentially arise by two means: by self cross-reactivity with pathogen associated antigens or by dysregulation during homeostatic mechanisms [100] [101] [102]. Armed with their ability to home to tissues and mediate rapid effector responses, these memory cells have been associated with the pathology of many autoimmune diseases such as type I diabetes, psoriasis, rheumatoid arthritis (RA), multiple sclerosis (MS), Crohn disease [103]. Particularly T_{EM} cells, due to their ability to home to tissues, have been found to be enriched in psoriatic plaques [104-105], in inflamed synovial fluids of RA patients [106-107], and in cerebrospinal fluid of MS patients [108]. For all these disorders, levels of circulating memory cells, especially T_{EM} cells, are correlated with worsening conditions, while a reduction is associated with a clinical improvement. Importantly, T_{EM} cells are now being exploited as target candidates for the treatment of some autoimmune diseases. In one such approach, inhibiting a K+ ion channel (Kv1.3) exclusively expressed in T_{EM} cells may hold therapeutic promise for MS patients [109]. Genetic silencing of Kv1.3 in human T cells leads to an expansion of T_{CM} cells and a depletion of T_{EM} cells, highlighting the functional importance of the Kv1.3 channel in the T_{EM} population [110].

Additionally, MP cells display important innate immune responses, providing early protection against pathogens during primary responses. Several groups have observed that administration of innate immune activators such as LPS or poly:IC (powerful inducers of IFN-I) causes strong non-antigen-specific stimulation of heterogeneous $CD44^{hi}$ $CD8^{+}$ T cells *in vivo* [111-112]. A proportion of MP cells produce IFN-γ in response to IL-12, IL-18 and IFN-α/β produced by activated macrophages and DC (bystander activation) **(see figure 3.5**) [112-113]. Since it was shown that none of these cytokines were able to directly stimulate MP cells in vitro it was proposed that a possibly common, effector cytokine was activating the MP cells indirectly. This cytokine was shown to be IL-15, which is produced and presented to T cells by APC upon stimulation with IFN-α/β and IFN-γ [114]. IL-15 preferentially stimulates MP CD8 T cells as consequence of MP CD8 T cells expressing very

high levels of IL-2Rβ (CD122) [93]. Expression of high affinity receptors for this cytokine allows memory, but not naive CD8 T cells to proliferate, in the absence of TCR stimulation [93].

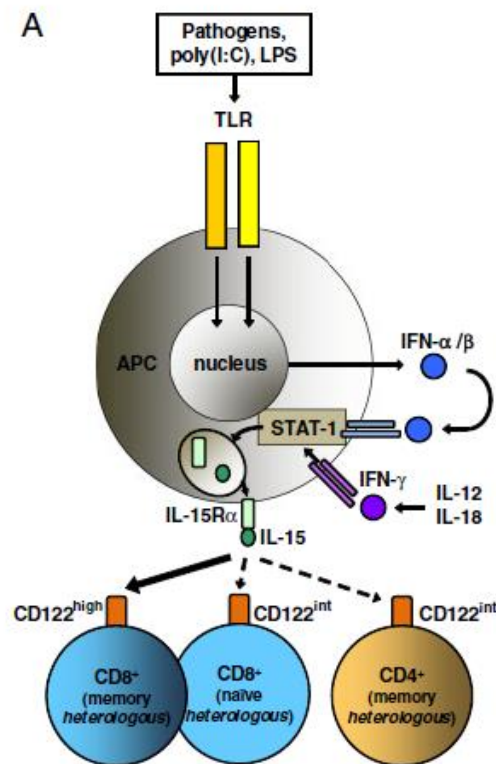


Figure 3.5- Pathways leading to bystander T-cell activation

Bystander activation of CD4 T cells is less efficient as compared with that of CD8 MP cells due to the lower degree of expression of cytokine receptor IL-2Rβ. However, unrelated $CD44^{hi}$ MP CD4 T cells have been reported to undergo a low degree of bystander proliferation upon virus infection and following administration of poly(I:C) or LPS [115] [116]. IFN-γ production by bystander-activated memory T cells may have profound, wide spread effects on immune responses, due to the fact that the effects of IFN-γ are independent of Ag specificity. Therefore, IFN-γ can be produced by polyclonal memory T cells of various specificities in response to early bacterial and viral stimuli. Furthermore, memory T cells due to their activation status are able to circulate to peripheral tissues, particularly under inflammatory conditions. Moreover the local IFN-γ released by memory CD8 T cells may be important in further stimulating APC and promoting Th1-type responses, pro-inflammatory conditions and immuno-pathology.

3.10- Role of PD-1 in T cell responses

Programmed Death-1 (PD-1) [117], as mentioned above, is a co-inhibitory receptor that belongs to the CD28/CTLA-4 family and negatively regulates TCR and BCR -signaling upon engagement of one of its ligands: PD-L1 and PD-L2 [27-28]. Apart from the established role of PD-1 in peripheral T cell tolerance (see below), its role in immunity and infection is also well described [118] [119] [120]. PD-1 is inducibly expressed on CD4, CD8 T cells, NK cells, B cell and monocytes. The common γ-chain cytokines can also induce PD-1 expression on T cells. The two PD-1 Ligands differ in their expression pattern with expression of PD-L2 being more restricted than PD-L1 [121]. PD-L2 also called B7-DC, is inducibly expressed on activated DC and macrophages, while PD-L1 (B7-H1) is constitutively expressed on T and B cells, DC's, macrophages, mesenchymal stem cells. Importantly PD-L1 is also constitutively expressed on non-hematopoietic cell types including vascular endothelial and epithelial cells, certain tissues cells, such as hepatocytes and pancreatic islet cells, and also sites of immune privilege [122] **See figure 3.6.** This expression pattern of PD-L1 places it in a central role in limiting the pathology associated with 'overaggressive' T cells during immune responses to persistent infections and also in regulation and protection against auto-reactive T and B cells, as will be discussed below. PD-L1 expression is further upregulated by type I and II IFN's and other pro-inflammatory cytokines.

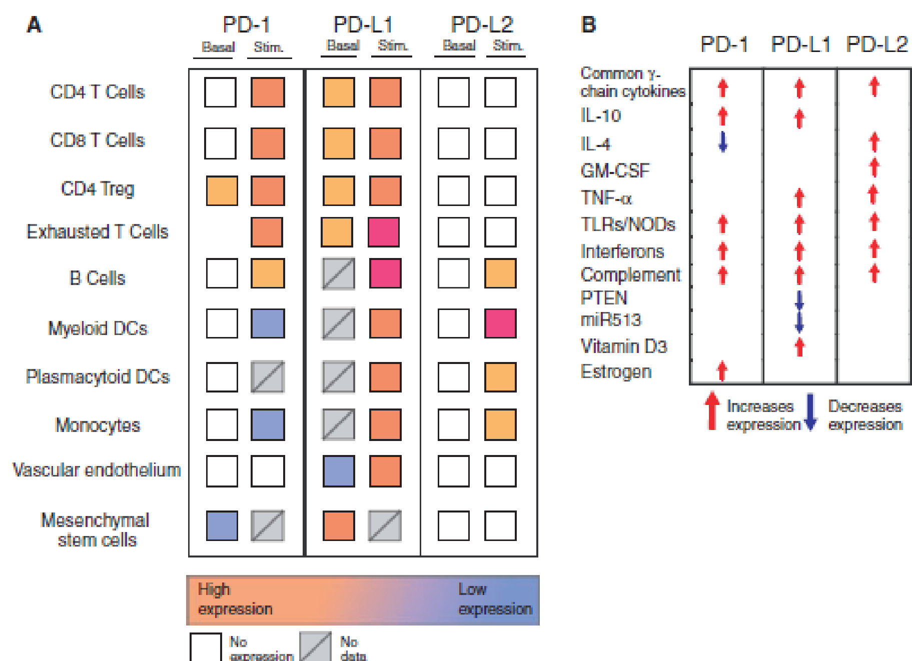


Figure 3.6- Relative expression of PD-1 and its ligands (A) Comparison of expression of PD-1, PD-L1 and PD-L2 on immune and non-immune cells in naive or activated states. (B) Factors that regulate expression of PD-1, PD-L1, and PD-L2. Regulation of expression on specific cell types is discussed in detail in the text. There are

some differences in expression of human and mouse PD-1, PD-L1, and PD-L2 expression. Murine expression is summarized in this figure. Expression of human PD-L1 differs from mouse PD-L1 in that human PD-L1 is primarily an inducible molecule. Source: Fransisco et al . Immunol Rev. 2010

The PD-1 receptor is a cell surface monomer consisting of a single immunoglobin variable-like domain and a cytoplasmic domain containing a immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM) [123]. Mutagenesis studies indicate that the tyrosine within the ITSM motif is essential for PD-1 function in T cells and B cells [28]. The protein tyrosine phosphatases SHP-2 (SRC homology), and to a lesser degree SHP-1, binds to the ITSM sequence in the PD-1 cytoplasmic tail. Recruitment of these phosphatases leads to the dephoshorylation of effector molecules activated by TCR-pMHC, such as Syk and PI3K. This in turn blocks activity of Akt kinase, thereby suppressing glucose metabolism, expression of BCL-2 family survival proteins and proliferation via IL-2. Therefore, the expression of PD-1 on T cells and the extent of PD-1-PD-L engagement, regulates the threshold of T cell activation and the quantity of the resulting cytokine production and possible fates of responding T cells [118]. Importantly exogenous IL-2, IL-7 and IL-15 can overcome the inhibitory effects of PD-1 [124] [125]. PD-1 and its ligands play a central role in interactions between host defences against pathogenic microbes, and this no better highlighted by the fact that some viruses exploit this pathway to evade host immune effector mechanisms [126].

A recent study by Usherwood et. al., showed the importance of PD-1 in an acute infection model. After infection with vaccinia virus, in the absence of PD-1 the virus-specific CD8 T cells expanded to a greater magnitude and had more robust recall responses with enhanced IL-2 production [127]; therefore, demonstrating that PD-1 compromise CD8 T cell responses during acute infections. Previous work from the same laboratory, showed that PD-1 blockage can rescue the defective recall responses of CD8 T cells generated in the absence of CD4 T cell ‘help’ [87] therefore suggesting that PD-1 signaling contributes to the functional impairment of “helpless” CD8 T cells. Several studies suggest that PD-1-PD-L pathway may regulate immune-mediated tissue damage during viral infections by blocking the damage caused by overaggressive T cells at sites of infection [128] [129].

The extent of literature on PD-1 and chronic infection underscores the importance of the molecule in T cell-mediated responses to persistent viral infections [130-132]. Mice model of chronic infections have shown that initial T cell responses are elicited, but as the T cells continue to respond to un-cleared infection, they become ‘exhausted’ and a variety of phenotypic and functional defects arise as the responding cells lose their ability to proliferate

and produce effector molecules. Normally when an infection has been resolved, PD-1 molecules, which have been transiently expressed on virus specific effector T cells, are down-regulated. However, PD-1 is highly and persistently expressed on virus specific CD8 T cells in chronic infections and correlates with the "exhausted" T cell phenotype, which is reversed upon PD-1 neutralization, resulting in decreased viral load [131-132].

PD-1 signalling has also been shown to play a role in Treg responses, and ligation of PD-1 augments Treg mediated-suppressive responses. Moreover, PD-1 signaling induces generation of iTregs from naive T cells by attenuation of Akt-mTOR pathway [133]. Additionally PD-1:PD-L pathway also may control the complicated dynamic interactions among Tregs, effector T cells, and APCs; constitutive expression of PD-L1 and PD-1 on Tregs may help to negatively regulate formation of stable and productive immunological contacts [120].

The role of PD-1 in the generation, maintenance, and function of MP CD8 T cells is less clear. MP CD8 T cells express PD-1, especially in aged mice, but to a lesser extent compared to MP CD4 T cells [134] and most PD-1-expressing MP CD8 T cells belong to the T_{EM} phenotype. Interestingly, PD-1 expression on MP $CD8^{+}CD122^{hi}$ T cells defines an IL-10-producing regulatory T cell population [135]. In settings of lymphopenia, a short-lived $PD\text{-}1^{+}$ fraction has been identified among homeostatically proliferating (LIP) CD8 T cells, characterized by poor functional responses [136]. Therefore, PD-1-PD-L interactions on the one hand are critical for protecting the host against pathology associated with overaggressive T cells responses to infections, while at the same time signalling restrict the ability to induce strong immune responses against infectious agents and result in exhausted cells and virus persistence.

The first indication of the importance of PD-1 in immune tolerance came from PD-1-deficient mice, which developed strain-specific autoimmunity. PD-1-/- mice in a C57BL/6 (B6) genetic background, spontaneously develop arthritis and glomerulonephritis due to Ab-antigen complex development and lymphocyte infiltration into tissues. The percentage of diseased mice and the severity increase with age [137]. PD1-/- Balb/c mice develop lethal dilated cardiomyopathy due to antibodies against cardiac troponin [138]. Neutralization of the PD-1 or gene ablation in NOD mice (model for autoimmune type I diabetes) results in acceleration of diabetes cases and severity, [139, 140 , 141].

Several studies also suggest a significant role of PD-1 and its ligands in human autoimmune diseases. Polymorphisms in PD-1 gene have been associated with systemic lupus erythematosus (SLE), type I diabetes, RA, Grave's disease and MS [120]. In patients with multiple sclerosis, treatment with IFN-β increases levels of PD-L1 mRNA, suggesting that

the anti-inflammatory activity of IFN-β is partly due to the increased expression of PD-L1 [118]. Auto antibodies against PD-L1 have been found in the serum of patients with rheumatoid arthritis and a soluble form of PD-1 have been associated with the active disease [28, 118].

There is accumulating evidences that tumors exploit PD-1-dependent immune suppression for immune evasion. The expression of PD-L1 and PD-L2 has been found on a wide variety of solid tumors and hematologic malignancies, and a strong correlation between PD-Ls expression on tumor cells and unfavorable prognosis has been demonstrated for various cancers [142] [122] [143]. There are currently four anti-PD-1 agents in the clinic: MDX-1106/BMS-936558/ONO-4538, CT-011, MK-3475, and AMP-224. The first three are reported to be PD-1 blocking mAbs, while the last is a PD-L2/IgG1 fusion protein [144]. To date, most clinical experience with PD-1 blockade has been gained with MDX-1106. Phase 1 studies are in progress to assess its safety and antitumor activity in patients with selected advanced solid tumors. An ongoing follow-up trial of biweekly MDX-1106 administration has already shown durable anti-tumor responses in one third of patients [145]. Clinical activity was also observed in patients with melanoma, renal cell carcinoma, colorectal cancer and non-small cell lung cancer (NSCLC). Importantly, tumor cell surface expression of PD-L1 in pretreatment biopsies emerged as a potential biomarker of response [144]. The results from those clinical trials are extremely promising and subsequent studies involving more patients are highly anticipated [146]. The recent cancer clinical trials with PD-1 pathway blockade should drive the use of this therapy into other clinical applications such as the control of HIV [147] and chronic viruses like hepatitis C [122].

4. RATIONALE AND OBJECTIVES:

Spontaneously generated memory-phenotype (MP) T cells, found in naïve mice, exhibit phenotypic and functional traits of memory cells and provide important protection to the host. MP cells can develop, expand and exert their effector functions in an antigen-independent manner, via homeostatic proliferation and bystander activation mechanisms [97, 99] [112]. The enhanced activity of MP cells, provide robust protection against infection [97, 148] and have been shown to improve anti-tumor responses [149]. The ability to induce immunological memory independent of antigen has significant therapeutic potential. However, tight regulation of MP cells is essential in order to obtain an equilibrium; fast and efficient protection of the host against pathogens, opposed to overaggressive immune responses leading to immunopathologies. Additionally, it has been well documented that

dysregulation during homeostatic proliferation can result in autoreactive memory T cells [100, 102]. Equipped with their ability to home to tissues and mediate rapid effector responses, T_{EM} cells have been associated with the pathology of many autoimmune diseases [103].

There are numerous studies indicating that the integration of signals 1,2 and 3 received by T cells during priming, largely determines the differentiation into memory T cell subsets with a consensus that T_{EM} cells require greater signal strength [14]. In agreement with the above, co-stimulatory molecules such as OX-40 and ICOS, promote differentiation towards the active T_{EM} phenotype [20, 24-26]. However, mechanisms governing homeostasis and differentiation of MP T cells, although similar to typical antigen-specific memory T cell generation, remain ill defined. Therefore, it is important to determine the factors that affect the formation of MP cells and their subsets. The role of the negative co-stimulator PD-1 in MP cell generation has not been evaluated. Therefore, we aimed to assess the role of PD-1 in regulating developmental fates of MP CD8 T cells.

Specific objectives:

- To investigate the contribution of PD-1 in memory phenotype subset homeostasis by assessing the affect of PD-1 ablation in the MP cell pool of non-immunized naive mice.
- To delineate the mechanism(s) involved in MP T cell differentiation and how the absence of PD-1 shapes these process(es) by assessing cell function, proliferation, survival and inter-conversion of MP cell subsets.
- To attempt to assess the role of PD-1 in antigen-specific memory T cell subset formation by utilizing a model of CHS and TCR (F5) transgenic mice model.

5. MATERIALS AND METHODS

5.1- Mice

PD-1 KO [150], GFP-transgenic mice [151], DsRed-transgenic mice [152], F5 TCR-transgenic [153], Rag-1 KO [154] have been previously described. All mice were backcrossed to the C57BL/10 background for 10 generations. C57BL/10 (referred to as wild type, WT) and C57BL/10.PD-1-deficient mice (PD-1 KO) were used in the present study. Mice were maintained in the Institute of Molecular Biology and Biotechnology (IMBB) colony. All experiments were approved by the General Directorate of Veterinary Services, Region Crete.

5.2- Reagents

The following fluorescent-conjugated monoclonal antibodies as well as Annexin V-FITC, propidium iodide and FACS™ Lysing Solution were purchased from BD Pharmigen ™: anti-CD3e (cl:145-2C11), anti-CD8a-APC (cl:53-6.7), anti-CD8b-APC, anti-CD4-FITC (cl: GK1.5) anti-CD4-PerCP, anti-CD69-PE (cl:H1.2F3), anti-CD62L-PE (cl:MEL-14), anti-CD62L-PE-Cy7, anti-CD62L-FITC, anti-CD44-PerCP-Cy5 (cl:IM7), anti-CD44-PE, anti-CD25-PE(cl:Pc61), anti-CD122-PE(cl:TM-β1), anti-IFN-γ-PE, anti-IL-2-PE, anti-Vβ11-PE (Cl:RR3-15), anti-Ly6C-PE (cl:AL-21), anti-PD-1 PE (CD279, cl:J43) and anti-CD90.2 (Thy-1.2, cl:30-H12). Anti-CD127-PE (cl:A7R34), anti-Ki-67-PE (cl:SolA15), anti-IgG2a, κ- PE and anti BrdU-APC(Cl:3D4) were from eBioscience, anti-Granzyme B-PE (clone GB12) were from Invitrogen. For CCR7 staining, a fusion of the CCL19 chemokine and the Fc fragment, plus PE-labeled anti-human IgG Fcγ fragment was used (eBioscience). Cell cultures were performed in RPMI, supplemented with 10% fetal bovine serum (FBS), penicillin (100U/ml) and streptomycin (100μg/ml), 2-mercaptoethanol (5x10-5 M) all from Gibco, Carlsbad, CA. E.coli LPS was from InvivoGen, PMA and Complete Freund's adjuvant (CFA), 2,4-dinitrofluorobenzene (DFNB) Percoll solution, and DNase I from Sigma-Aldrich. Complement and Lympholyte-M (CL5031) were from Cedarlane labs. 3[H] thymidine was obtained from Amersham Biosciences. CFSE (Carboxyfluorescein Succinimidyl Ester, Invitrogen), SNARF-1 (carboxylic acid, acetate succinimidyl ester) were from Molecular Probes, and MACS magnetic beads separation system was from Miltenyi Biotec. IL-12 and IL-15 were obtained from Peprotech while IL-18 was from R&D.

5.3- Lymphocyte suspensions, Cell staining and flow cytometry

Single-cell suspensions were prepared from tissues and cells were stained for extracellular markers for 30 min at 4°C in 1xPBS, 1% BSA, 0.02% NaN_3. For analysis of lymphocytes from peripheral blood, at least 100µl of blood was collected from the tail vain of the mouse into eppendorfs containing heparin (90u/ml).The blood was washed and then antibody staining was performed as above. The erythrocytes are lysed by incubating for 15mins with FACS™ Lysing Solution (Becton Dickinson) according to manufactures instructions then washed before analysis. Acquisition was carried out on a FACSCalibur and data were analyzed with WinMDI or FlowJo software. The significance of all data was evaluated by Student's t-test and where significant, p values are shown.

5.4- Isolation of lymphocytes from liver and lung

Mice were sacrificed and perfused via the left ventricle with 20 ml ice-cold PBS. Tissues were then teased over a filter. For lungs, Lympholyte-M (Cedarlane labs, CL5031) was used according to manufacturer's instructions. Briefly, equal volumes of lympholyte solution were placed underneath the cell suspension by using a pasteur pipette and centrifuge at 1000-1500g (2400rpm) for 20 mins at RT. After centrifuge there was a well defined layer of lymphocytes at the inter phase and these cells were removed and place in a new tube and washed by centrifuging at 800g (1950rpm) for 10 mins to pellet the lymphocytes. Lymphocytes were then counted and stained as described above. Cell suspensions from livers were spun at 550g. The cell pellet was resuspended in RPMI and overlaid onto 33% (v/v) Percoll solution (Sigma) followed by centrifugation at 800 g for 30 mins. Remaining cells after aspiration were washed twice with RPMI by centrifugation at 800 g for 5 mins at 4°C. Subsequent removal of red blood cells was performed by water lysis and lymphocytes were stained as above.

5.5- In vivo or in vitro stimulation and intracellular cytokine staining

For cytokine production, splenocytes were incubated for 4 h in the presence of GolgiPlug ™ (BD Biosciences) and 50 ng/ml of PMA and 500 ng/ml of ionomycin (both Sigma-Aldrich) or untreated. In some experiments 3 mo old WT and PD-1 KO mice were challenged with 50µg of LPS (E.coli 0111:B4) (Sigma-Aldrich) or PBS for 4 h and were then sacrificed and splenocyte suspensions were incubated at $1x10^6$/ml with GolgiPlug™. Cells were washed and stained for surface markers, as described above. Then, cells were fixed and rendered

permeable by using the Cytofix/Cytoperm^{TM} Kit (BD Biosciences) according to manufacturer's instructions, and subsequently stained for intracellular cytokines and analyzed by flow cytometry. For *in vitro* experiments with hIL-15 (Peprotech), $3x10^4$ $CD8^+CD44^{hi}CD62L^{lo}$ purified cells (purity >95%) pooled from 4 mo mice were incubated in duplicate wells 100 ng/ml cytokine and analyzed on day 7. In other experiments spleenoctyes from WT and PD-1KO mice were incubated with IL-15, IL-12 (Peprotech) and IL-18 (R&D) for various time periods.

5.6- Cell sorting and Adoptive Transfer

$CD8^+$ T cells were purified from spleen with the negative selection MACS magnetic beads separation system (Miltenyi Biotec) according to manufacturer's instructions. Purified $CD8^+$ GFP^+ T cells were stained with anti-CD44 PerCP- Cy5, anti-CD8-APC, and anti-CD62L-PE for the purification of T_{EM} ($CD8^+CD44^{hi}CD62L^{lo}$), T_{CM} ($CD8^+CD44^{hi}CD62L^{hi}$), or naïve cells ($CD8^+CD44^{lo}$) and sorted by Dako Cytomation MoFlo T High-Performance Cell Sorter**.** $1.5x10^5$ cells were then adoptively transferred into WT and PD-1 KO mice. Cell fate was analyzed after 42 d on the basis of CD62L and CD44 expression on donor-derived GFP^+CD8^+ cells. In the case of naïve cells, recipients were sub-lethally irradiated (450 rads).

5.7- CFSE and SNARF-1 staining

Cell proliferation was assayed by labeling with CFSE (Carboxyfluorescein Succinimidyl Ester, Invitrogen), (Molecular Probes) as previously described [155] or SNARF-1 (carboxylic acid, acetate succinimidyl ester). Briefly, 10-20 x 10^6/ml of purified cells were incubated with 10 μM CFSE in PBS, for 10 min at 37°C. Labeling was stopped with 5 vol of ice-cold HBSS 5% FCS, for 5min on ice, followed by three washing cycles in HBSS, 5% FCS. For SNARF-1 (carboxylic acid, acetate succinimidyl ester) labelling, purified cells were incubated with 25 μM SNARF-1 essentially in the same way as with CFSE, except from labelling time being 30 min.

5.8- BrdU incorporation and Ki-67 analysis

7 mo old PD-1 KO and WT mice were fed daily with 0.8 mg/ml of BrdU (Sigma) for one week. On day 7 the mice were sacrificed and splenocytes were stained as above. For BrdU analysis, cells were treated as previously described [156]. Briefly, cells were treated with

FACS Lysing Solution (BD Biosciences), followed by overnight fixation in 1% paraformaldehyde containing solution. Cellular DNA was then denatured with 50 Kunitz units of DNase I (Sigma) before being stained with anti-BrdU (BD Biosciences). For Ki-67 analysis 7 mo mice were sacrificed and splenocytes were stained as above. Cells were then treated for 15 min with FACS Lysing Solution, followed by fixation at 4°C in 1% paraformaldehyde and 0.05% Nonidet-P40 for 30 min. Cells were then blocked with mouse Fcγ receptor (CD16/CD32, BD Biosciences) for 15 min, and then immediately stained with Ki-67 for 30 min at 4°C. Cells were then analyzed by flow cytometry.

5.9- Microarray hybridizations and analysis

Spleen cells from 7 mo old WT and PD-1 KO mice were sorted for CD8 T cells and in separate experiments for CD8 T_{CM}, as described above. RNA was then extracted by standard procedures according to manufacturer's instructions (Quiagen). For genome-wide expression analysis of these cell populations, synthesis of double stranded cDNA and biotin labelled cRNA was performed according to the instructions of the manufacturer (Affymetrix, USA). Fragmented cRNA preparations were hybridized to full mouse genome oligonucleotide arrays (430 V2.0; Affymetrix, USA). Initial data extraction and normalization within each array was performed by means of the GCOS software (Affymetrix). Microarrays complied with the Minimum Information for Microarray Experiments (MIAME) and are available at ArrayExpress (E-MEXP-XXX and E-MEXP-XXX). Expression intensities from the PD-1 KO CD8 and T_{CM}-phenotype CD8 T cells and corresponding controls were log transformed and normalized within and between arrays with the quantile normalization method using the R open statistical package (http://www.r-project.org/). Two-tail, pair-wise analysis or a two-way analysis of variance was used to extract the statistically significant data from each group of mice by means of the Spotfire Decision Site software package 7.2 v10.0 (Spotfire Inc., MA, USA). The criteria for significance were set at $p \leq 0.05$ and a ≥ ±1.5-fold change in gene expression. The Affymetrix 430 V2.0 arrays include several internal controls to insure accurate and reproducible measurement of gene expression changes. For each probe set, signals were considered to be valid when they were marked as "Present" (for more information see www.affymetrix.com) and exhibited a signal higher than 40 in at least one microarray hybridization. All probe sets with a signal below 40 were set to be equal to 40. If there were discrepancies in the direction of expression between multiple probe sets, the gene was not included. Significant over representation of 5th level gene ontology terms describing "biological process" annotation (GOTERM_BP_5) was identified with the NIAID DAVID website (http://www.david.abcc.ncifcrf.gov)

5.10- Generation of mixed bone marrow chimeras

Bone marrow was obtained from femurs of GFP-transgenic and PD-1 KO mice; mature T cells were first depleted by the use of anti-CD90.2 (BD Biosciences) plus complement (Cedarlane Labs), according to manufacturer's instructions. Contamination of bone marrow cells with mature T cells was less than 0.1%. A mixture of 10^7 WT and PD-1 KO bone marrow cells at a 1:1 ratio was injected intravenously into DsRed mice lethally irradiated with 950 rads. Cells from these chimeras were analyzed after 8 weeks.

5.11- Proliferation of T cells from F5 TCR Tg mice

Single-cell suspensions of spleens of F5 and F5.PD1 mice were prepared in HBSS supplemented by 5% FCS 10 mM HEPES, 100U/ml penicillin-streptomycin, 2 mM L-glutamine. Erythrocytes were removed by osmotic lysis technique and spleenocytes (1 x10^6/ml) were stimulated with irradiated (2000 rad) C57BL/10 splenocytes (3x10^6/ml) preloaded with the appropriate concentration of influenza NP68 peptide (NP366–374) as previously described [155]. Thymidine incorporation was assayed essentially as previously described [155]. Normalization on equal numbers of CD8 T cells was performed keeping a constant ratio of C57BL/10 splenocytes:CD8 T cells. After 40 h of culture, cells were pulsed with 1µCi 3[H] thymidine (Amersham Biosciences) for 6 h. Cells were harvested with the INSEL CELL HARVESTER MODEL CH3 H/W, on special filters. Incorporated radioactivity was measured using a Beckman beta counter.

5.12- Contact hypersensitivity responses. Hapten-specific T cells were generated as previously described [157]. Briefly, 25 µl of 0.5% DNFB (Sigma) in acetone:olive oil (4:1) were applied to 2 cm^2 area of shaved dorsal skin of WT and PD-1 KO mice. After 5 d, animals were challenged with 10 µl of a 0.15% of DNFB solution on both sides of the right ear, and the solvent alone on the left ear, and repeated on day 38. After 48 h ear swelling measurement was performed with a digital calliper. Hapten-specific memory cells were then extracted by removing challenged ears and incubating them with 400 u/ml of collagenase (Sigma) for 50 min at 37ºC. Cells were then filtered and stained appropriately.

5.13- PCR and primers

PD-1 WT FOR: 5´- G CCAGCTAAGAGGCCACAGCTA -3´

PD-1 WT REV: 5´- CAGAGTGTCGTCCTTGCTTCCA -3´

PD-1 KO FOR: 5´- TTGTGTAGCGCCAAGTGCCCAGCG -3´

PD-1 KO REV: 5´- CGGTGCTCTCTGTGGAGGGTCTG -3´

dsRED FOR: 5´- AAGGTGTACGTGAAGCACCC -3´

dsRED REV: 5´- TCCACGTAGTAGTAGCCCGG -3´

The reactions were in a final volume of 100μl and contained 1X buffer PCR (Minotech), 2.5 mM MgCl2, 0,2 mM of a mixture of the four dNTPs (Promega), 0.5mM of each primer, 0.8μl of each cDNA and 2.5 IU Taq polymerase 5u/μl (Minotech). The PCR reaction carried out for the expression of PD-1 was as follows: 30 sec denaturation at 96°C, 30 sec hybridization at 63°C, 30 sec elongation at 72°C. The number of cycles was 35. The PCR reaction carried out for the expression of Dsred was as follows: 30 sec denaturation at 94°C, 30 sec hybridization at 55°C, and 30 sec stage replication at 72°C. The number of cycles was 35. In both cases, each reaction was followed by an additional elongation for 3 min at 72°C.

6. RESULTS

6.1- Increased numbers of CD8 and CD4 T_{EM}-phenotype cells in lymphoid organs and tissues of PD-1 KO mice.

We phenotypically examined the periphery of young (2-4 mo) and middle aged (7-14 mo) C57/B10 (WT) and C57/B10.PD1 KO (PD-1 KO) mice. There was a significant increase in the number of spleenocytes in the PD-1 KO mice compared to WT (152 $x10^6$ vs 128 $x10^6$, **figure 1A**) at middle age, and this increase appeared to start at an early age. Three coloured FACS analysis with anti-CD3,-CD4,-CD8 revealed significant increases in total numbers of CD4 T cells in the PD-1KO mice compared to WT at both in young mice (31 $x10^6$ vs 22 $x10^6$, p=0.02, **figure 1B, right**) and middle aged mice groups in spleen (29 $x10^6$ vs 19 $x10^6$, p=0.0003, **figure 1B, right**). Although a similar trend was found for $CD8^+$ T cells, there was no significant difference in the numbers between WT and PD1-KO mice in either age group (**figure 1B, left**).

Next in order to characterize the populations of expanded cells found in the PD-1 KO mice a variety of markers where used to identify CD8 and CD4 T cells sub-categories. CD44 is a marker for antigen experienced cells and therefore can distinguish from naïve T cells ($CD44^{lo}$). Consistent with the literature that shows that memory phenotype ($CD44^{hi}$) cells increase with advancing age [49], analysis of WT splenoctyes revealed a 2-fold increase in $CD8^+CD44^{hi}$ T cells, and similar fold increase in $CD4^+CD44^{hi}$ (**figure 1C)** when comparing young and middle aged mice. Interestingly, in the absence of PD-1, higher numbers of $CD8^+CD44^{hi}$ (**figure 1C left)** and $CD4^+CD44^{hi}$ (**figure 1C, right)** T cells were found compared to age matched WT counterparts, and this difference started at an early age (2-4mo).

Recently, there have been advances in the understanding of memory phenotype (MP) cells and it is clear that these cells are a highly heterogeneous population. $CD44^{hi}$ MP T cells can be subdivided into two main functional subtypes; central memory (T_{CM}) and effector memory T cells (T_{EM}) according to combinations of expression of surface markers, such as chemokine receptors and interleukin receptor components (CD62L, CD44, CCR7, Ly6c) [14]. In accordance with the literature [25],[23] a combination of CD62L (L-selectin) and CD44 were used to identify these two populations, in this study. CD8 and CD4 T cells were identified as naïve by $CD62L^{hi}$ $CD44^{lo}$, T_{CM} by $CD62L^{hi}$ $CD44^{hi}$, and T_{EM} with $CD62L^{lo}$ $CD44^{hi}$ as can be seen in **figure 2A**.

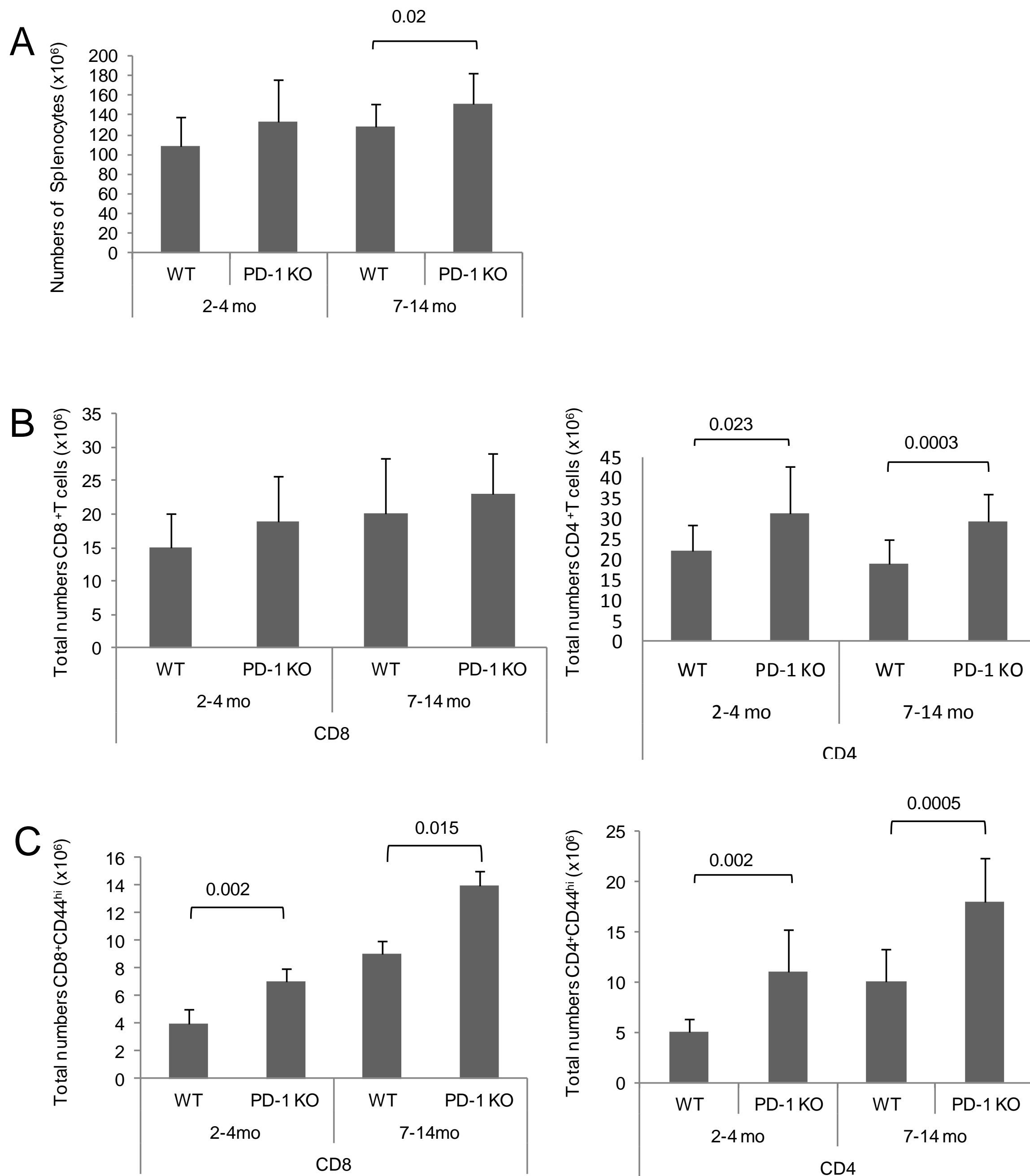


Figure 1– Increase in spleenocytes, CD4 and CD8 T cells and MP (CD44^{hi}) cells in PD-1 KO mice. A, Young (2-4 mo) or middle-aged (7-14 mo) PD-1 KO and WT control mice were sacrificed and spleen cell suspensions were analysed. Graphs show mean values of splenocytes with error bars indicating SD. **B,** Spleen cell suspensions were analysed by flow cytometry with anti-CD3, anti-CD4 and anti-CD8. Graphs show mean values of CD8 (left) and CD4 (right) T cells in spleens of WT and PD-1KO mice at two age groups (2-4 and 7-14 mo old) Error bars indicate SD. **C,** Graphs depict mean values of CD8^{+}CD44^{hi} (left) and CD4^{+}CD44^{hi} (right) cell of WT and PD-1 KO mice with error bars indicating SD, n=13 per group. Data are representative of 3 independent experiments

It was apparent, from this analysis, that the population expanding in the PD-1 KO belongs to the T_{EM} MP population, with a 7-fold increase in CD8 T_{EM} ($7x10^6$±1.78 vs $1x10^6$±0.35, p=0.0001, **figure 2B, left**) and 2-fold increase in CD4 T_{EM} ($14x10^6$ ± 3.82 vs $6x10^6$ ±1.58, p=0.0001, **figure 2B, right**) from middle aged PD-1 mice compared to age-matched controls. No significant differences were found in other subsets (naïve or T_{CM}) of CD8 and CD4 populations between the PD-1 KO and WT at either age group (**figure 2B**).

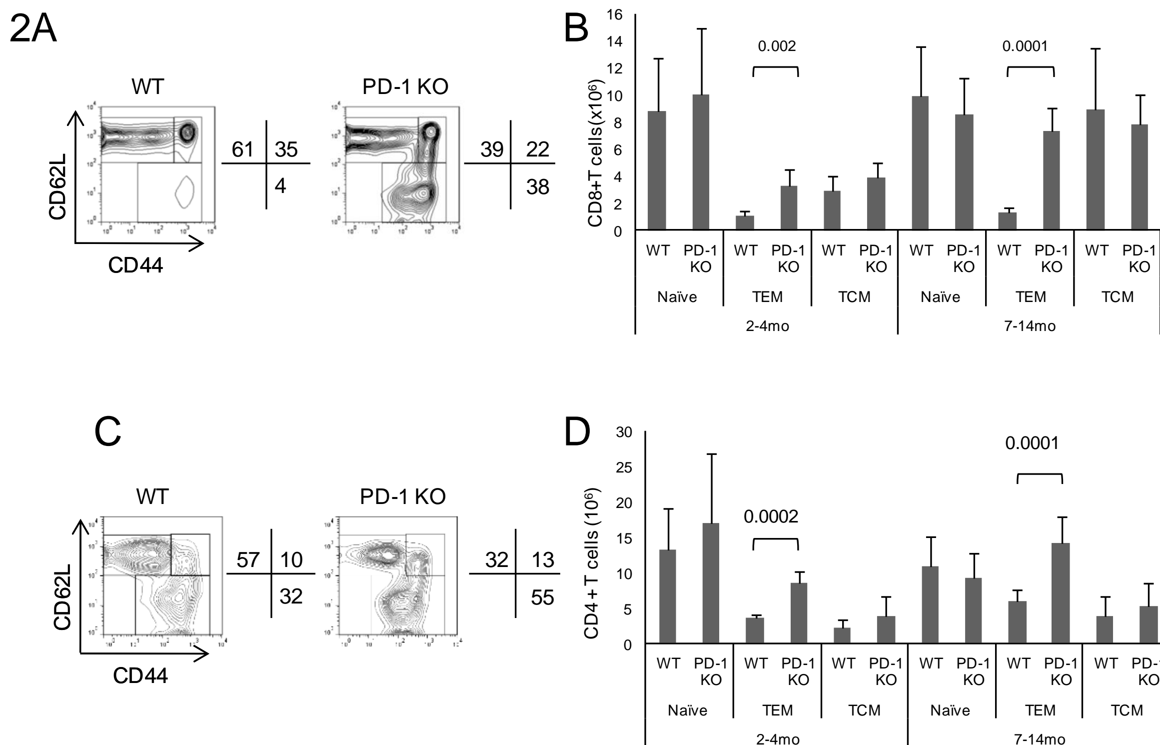
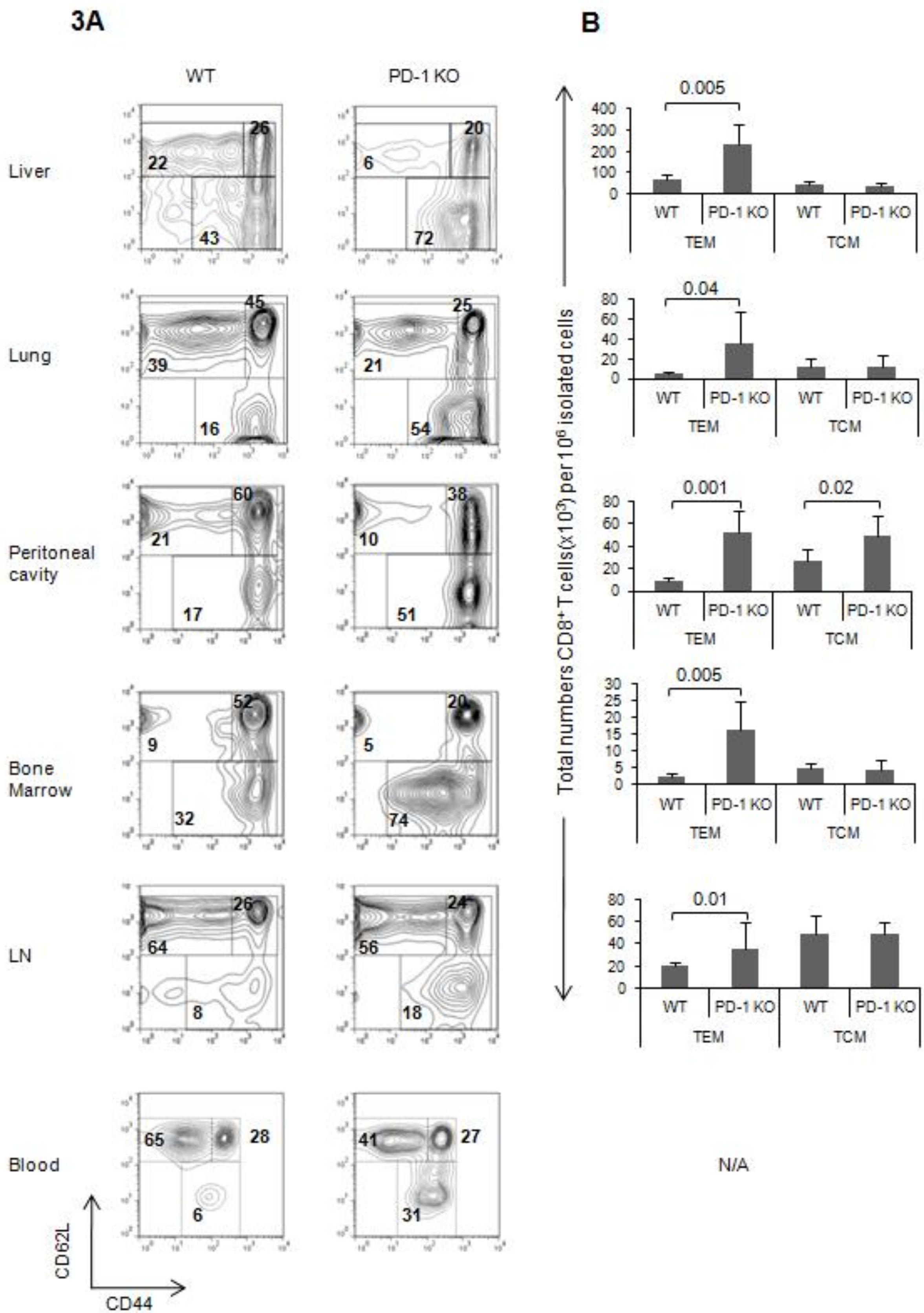


Figure 2– Increased numbers of CD8$^+$ and CD4$^+$ T_{EM} cells in spleen of PD1-KO compared to WT controls. Splenocytes were categorized phenotypically by flow cytometry into naïve (CD44^{lo}CD62L^{hi}), T_{CM}-(CD44^{hi}CD62L^{hi}), and T_{EM}-phenotype (CD44^{hi} CD62L^{lo}) cells in young and middle-aged PD-1 KO and WT mice. **A**, Representative dot plots from middle-aged mice are shown gated on CD8$^+$ T cells with percentages of cell subset in each region. **B,** Total numbers of naïve, T_{EM} and T_{CM}-phenotype CD8$^+$ cells, with error bars indicating the SD. **C,** Representative dot plots from middle-aged mice are shown gated on CD4$^+$ T cells with percentages of cell subset in each region. **D,** Total numbers of naïve, T_{EM} and T_{CM}-phenotype CD4$^+$ cells, with error bars indicating the SD. n=13 per group

Since T_{EM} cells have been shown to reside preferentially in non lymphoid organs we isolated lymphocytes from various tissues including liver, lung, peritoneal cavity and bone marrow from middle aged PD-1 KO and WT mice. As expected, in all tissues of the WT mice there was greater percentage of T_{EM} MP cells than in the mesenteric LN or spleen (**figure 3**). When comparing WT and PD-1KO mice, in all tissues examined, there was a marked

increase in the percentage of CD8 T_{EM}–phenotype cells in the absence of PD-1(**figure 3A**).



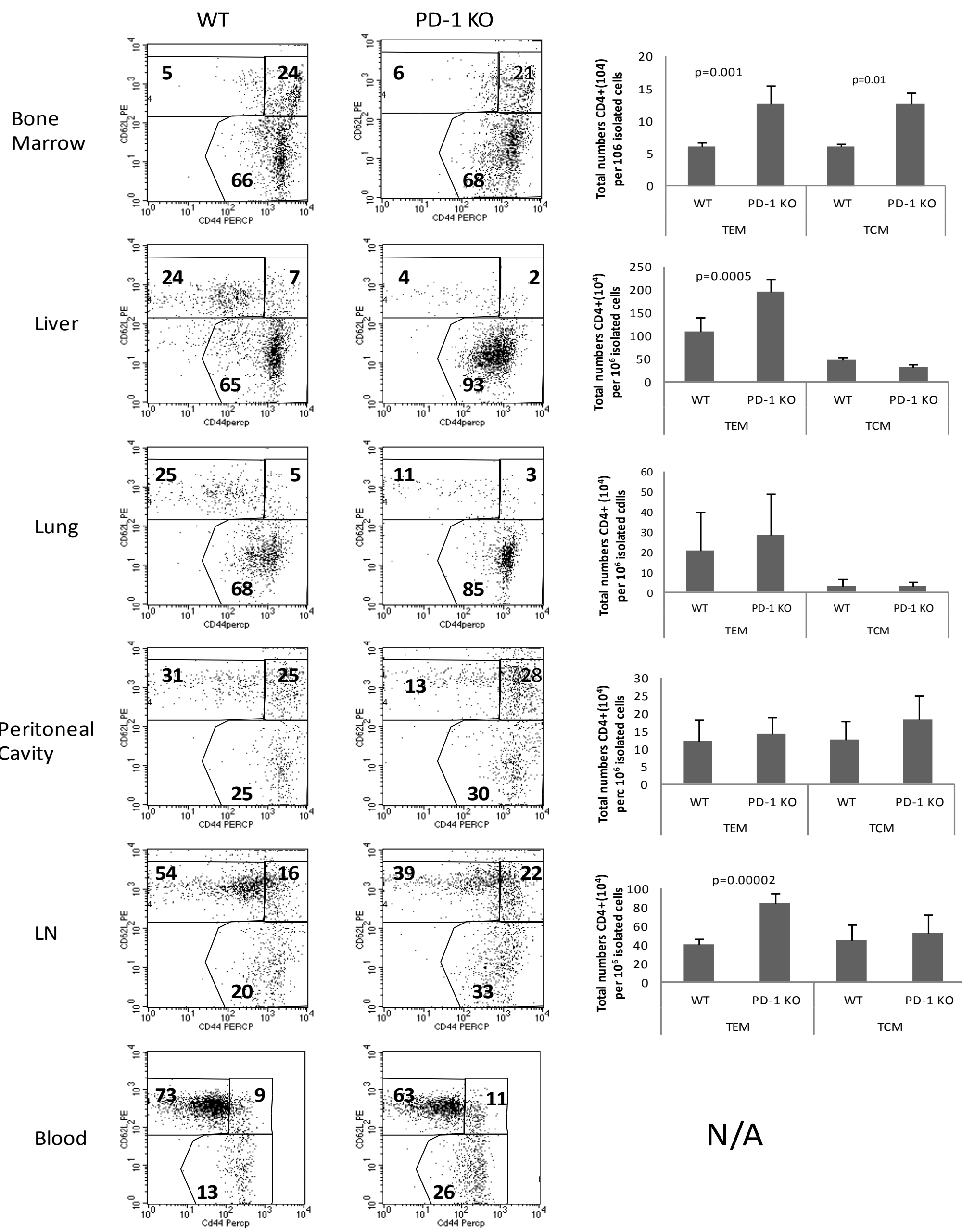


Figure 3– Increase in CD8$^+$ and CD4$^+$ T_{EM}-phenotype cells in various lymphoid and non-lymphoid tissues of PD-1 KO mice. WT and PD-1KO mice were sacrificed at 9 months of age and cell suspensions from various lymphoid and non-lymphoid tissues were characterized phenotypically by flow cytometry into naïve ($CD44^{lo}CD62L^{hi}$), T_{CM} ($CD44^{hi}CD62L^{hi}$), and T_{EM} ($CD44^{hi}CD62L^{lo}$). **A**, Representative dot plots gated on CD8 T cells are shown with percentages of cells per region. **B**, Total numbers of CD8+ T_{EM} and T_{CM}-phenotype cells per 10^6 isolated cells are shown, with error bars indicating SD. **C**, Representative dot plots gated on CD4 T cells are shown, as in A. **D**, Total numbers of CD4+T_{EM} and T_{CM}-phenotype as in B. The results are representative of 3 individual experiments with at least 2 mice per group.

Considering that the recovered CD8 T cells were more in all PD-1 KO tissues examined, T_{EM}-phenotype cells were from ~3-fold (in liver, $235x10^3$ ± 89 vs $71x10^3$ ± 24, p=0.005) to 8 fold (in bone marrow, $16x10^3$ ± 8.72 vs $2x10^3$ ±1.27, p=0.005) more abundant compared to tissues from WT animals (**figure 3B**). When analysing CD4 T_{EM}-phenotype cells in tissues from PD-1KO mice, a similar trend was observed, however to a lesser degree (**figure 3C and D).** The above observed differences could not be attributed to aberrant migration patterns of T_{EM} MP cells to the tissues since the same trend was also observed in LN of PD-1 KO mice (**figure 3B and C**).

We decided to focus the rest of the study on CD8 T cells for several reasons. Firstly, the defined lineages and functions ascribed to memory CD8 T cells contrast the more inherent plasticity in populations of memory CD4 T cells at all stages of their development allowing easier analysis and dissection of the role of PD-1 in memory generation of the former. Secondly, we had previously utilized and characterized TCR Tg F5 naive mice, in which most CD8 T cells recognise a defined antigen, making it an appropriate tool to study the kinetics of CD8 memory generation and at which point PD-1 puts its break on memory generation.

6.2- Phenotypic and functional analysis of CD8 T cells MP subsets.

As mentioned above, there is a variety of markers that are used to distinguish T_{EM} from T_{CM} cells. The chemokine receptor CCR7 has been shown to be co-regulated with CD62L. Thus, in order to confirm that the accumulating MP population indeed have a T_{EM} phenotype, and not just a dysregulation of the CD62L receptor, CD8 T cells from spleens of middle aged mice were co-stained with CD62L and CCR7. **Figure 4** shows that CCR7 and CD62L are indeed co-regulated.

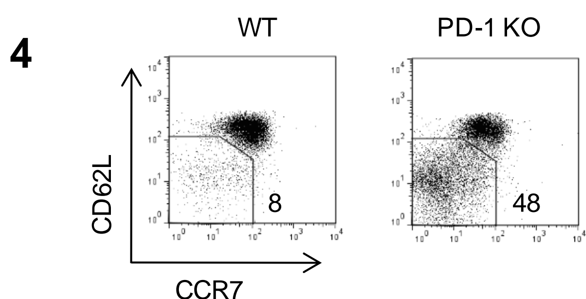


Figure 4- CCR7 is co-regulated with CD62L. Splenocytes from 9 mo old mice were analyzed for co-expression of CCR7 and CD62L gated on $CD8^+$ T cells. Representative dot plots are shown with percentages of cells per region.

Ly6c is an antigen that is important for migration lymphocytes to lymph nodes [158]. It is used to distinguish between T_{EM} and T_{CM}-phenotype cells, being highly expressed on the latter [159]. FACS analysis was performed on spleenocytes from middle-age WT and PD-1KO mice. As expected, when gating on CD8 T_{CM-} (**figure 5A**) and T_{EM}-phenotype cells (**Figure 5B**), the majority of T_{CM} in the WT were $Ly6C^{hi}$ (~80%, **figure 5A)**, while the T_{EM}-phenotype cells were mostly $Ly6C^{lo}$ (**Figure 5B**). When comparing WT and PD-1KO mice, there was a higher percentage of $Ly6C^{hi}$ T_{EM} MP cells in the WT mice (33% vs 13%, **Figure 5B**). However, $Ly6C^{hi}$ cells numbers were increased in spleens of the PD-1KO mice compared to the WT mice, when considering absolute numbers, ($797x10^3$±115 vs $365x10^3$ ±73, p=0.0008, **figure 5C**) due to the 7-fold increase in the numbers of T_{EM}-phenotype cells found in the absence of PD-1.

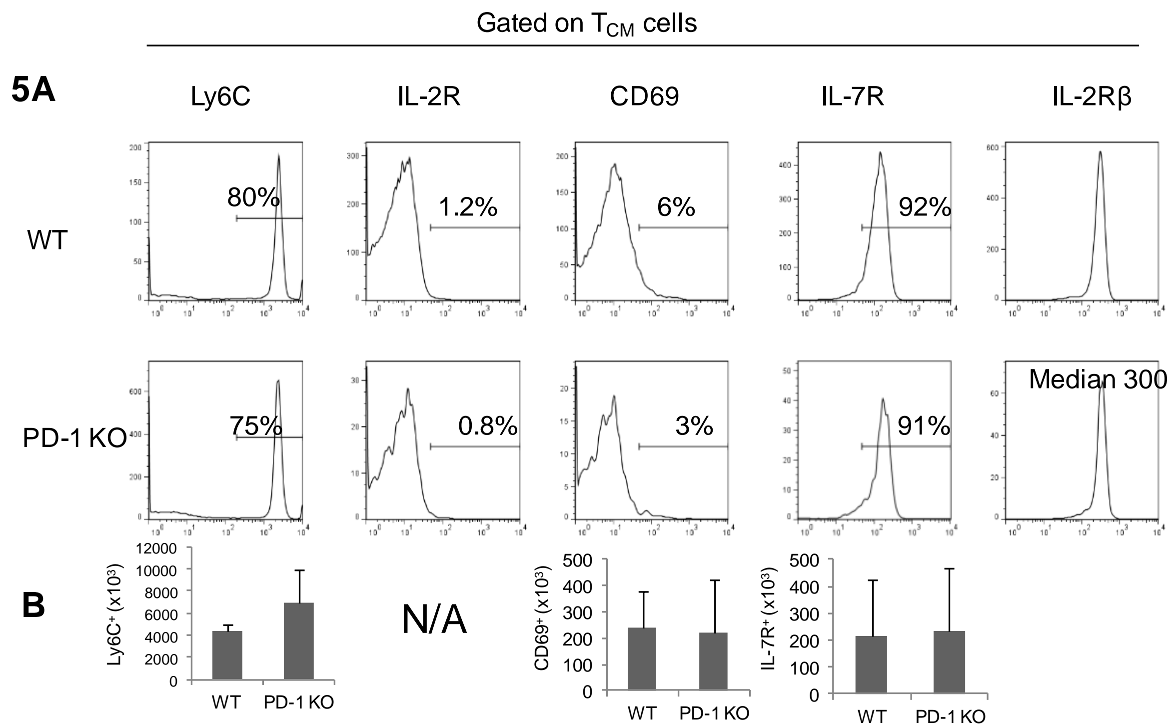
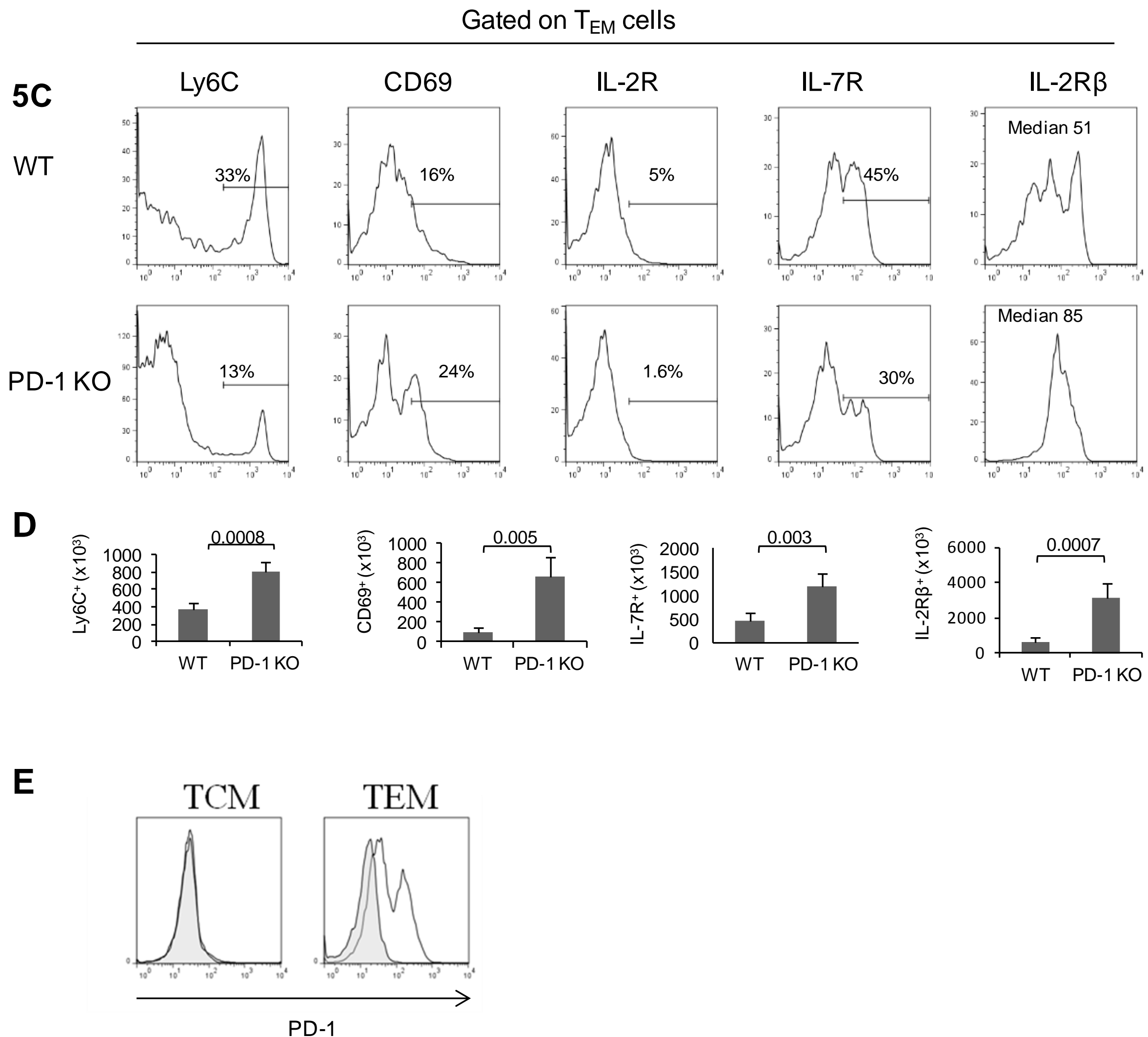


Figure 5- Phenotypic characterization of accumulated CD8 $^+$ T_{CM} and T_{EM}-phenotype cells in PD-1 KO mice. Spleens from 9 mo old PD-1 KO and WT mice were analyzed by flow cytometry. **A**, Representative dot plots show expression of various surface markers gated on CD8 T_{CM}- ($CD44^{hi}CD62L^{hi}$) phenotype cells **B**, Absolute numbers of various surface markers as indicated, gated on T_{CM}-phenotype cells, with error bars indicating SD. **C**, Representative dot plots show expression of various surface markers gated on CD8 T_{EM}-phenotype ($CD44^{hi}$ $CD62L^{lo}$) cells**. D,** Absolute numbers of various surface markers as indicated , with error bars indicating SD. Data represent 2-3 individual experiment with 3 mice per group. **E,** Representative histogram with PD-1 expression on T_{CM} and T_{EM} WT cells compared to isotype control (shaded)



CD44^{hi} cells represent a mixed population of recently activated and resting MP cells. In order to assess the contribution of these subpopulations, further phenotypic characterisation was undertaken. The expression of CD69, an early activation marker and IL-2Rα (CD25) a marker for IL-2 responsiveness, both highly expressed on recently activated effector T cells were assessed on T_{EM} cells from WT and PD-1 KO mice (**figure 5B**). Despite the increase in CD69$^+$ cells among T_{EM}-phenotype cells of PD-1 KO mice compared to WT (24% vs 16%, **figure 5B**), no IL-2Rα expression was found on these cells, indicating that these cells could not be typical effector T cells (**figure 5B**).

IL-7 plays an important role on survival and homeostasis of CD8 MP T cells and the IL-7R (CD127) is up-regulated on memory cells, while having no expression on effector cells. IL-7R was equally expressed on T_{CM} from both WT and PD-1 KO (~90%, **figure 5A**). Despite the slight decrease in IL-7Rα ^{hi} T_{EM} found in the PD-1KO compared to WT (30% vs 45%,

figure 5B), there was a 3 fold increase of IL-7Rα^{hi} T_{EM}-phenotype cells when considering total numbers ($1179x10^3$±287 vs $459x10^3$ ±169, p=0.0036,**figure 5C**).

IL-2Rβ (CD122) is vital for IL-15 responsiveness and survival of CD8 naïve and memory T cell. IL-2Rβ is also commonly used as a memory marker [160]. Mean expression of IL-2Rβ on T_{CM} was similarly high between WT and PD-1 KO cells (287 MFI vs 300 MFI, **figure 5A**). When examining T_{EM} phenotype-cells, PD-1 KO mice had an increase in MFI compared to WT counterparts (85 MFI vs 5 MFI, **figure 5B**) indicating a possible increase in responsiveness of these cells to IL-15 in the absence of PD-1. Interestingly, PD-1 KO T_{EM}-phenotype cells expressed an intermediate level of IL-2Rβ compared to WT T_{EM}-phenotype cells, where distinct IL-2Rβ^{lo} and IL-2Rβ^{hi} populations were found (**figure 5A**). Importantly, a proportion of MP cells, described by Boymen et. al, were shown to have a similar phenotype to cells involved in chronic viral infections with semi-activated phenotype expressing CD69, low IL-7Rα and low CD62L [161]. Interestingly, the accumulated T_{EM}-phenotype CD8 T cells in the PD-1 KO mice were found to have some characteristics of these semi-activated chronically stimulated cells (figure 5C and D). When examining the expression of PD-1 on WT middle aged mice, PD-1 was found to be highly expressed on T_{EM}-phenotype WT cells, in accordance to the literature [134], while having no expression on T_{CM} cells compared to isotype control (**figure 5D**). After extensive phenotypical analysis of spleenocytes from middle aged mice it was clear that the T_{EM}-phenotype cells accumulating in the PD-1KO are consistent with an effector memory T cell phenotype.

Next we wanted to examine whether the accumulating $CD8^+$ T_{EM}-phenotype cells in the PD-1 KO mice were armed with increased effector memory function. Granzyme B (GzmB) has been reported as a key molecule important for the lytic activity associated with CD8+ T_{EM} cells. GzmB expression was assessed ex vivo on CD8 T cells subsets, and was found to be specifically high on the T_{EM}-phenotype cells, while having uniformly low expression on T_{CM} and naïve cells, regardless of genotype (**figure 6A**). Importantly, there was a significantly higher population of GzmB^{hi} cells in the T_{EM} MP cells from PD-1 KO compared to WT controls (23% versus 5%, **figure 6A**).

One principal feature of memory cells is the ability to evoke fast recall responses to previously encountered antigens. Since the antigens in these mice are not defined, a previously described short stimulation protocol with phorbol esters was adopted, in order to re-stimulate CD44^{hi} MP cells, while preventing the priming of naïve cells. Since it was not possible to assess IFN-γ production by T_{EM} subsets due to rapid shedding of CD62L after TCR-stimulation [162], we performed this assay on isolated CD8+ T_{EM}-phenotype cells. As shown in **figure 6B** a higher proportion of T_{EM}-phenotype CD8 T cells from PD-1KO mice

produced IFN-γ compared to WT counterparts (61%±8.9 vs 48%±4.8, p=0.01, **figure 6A and B**).

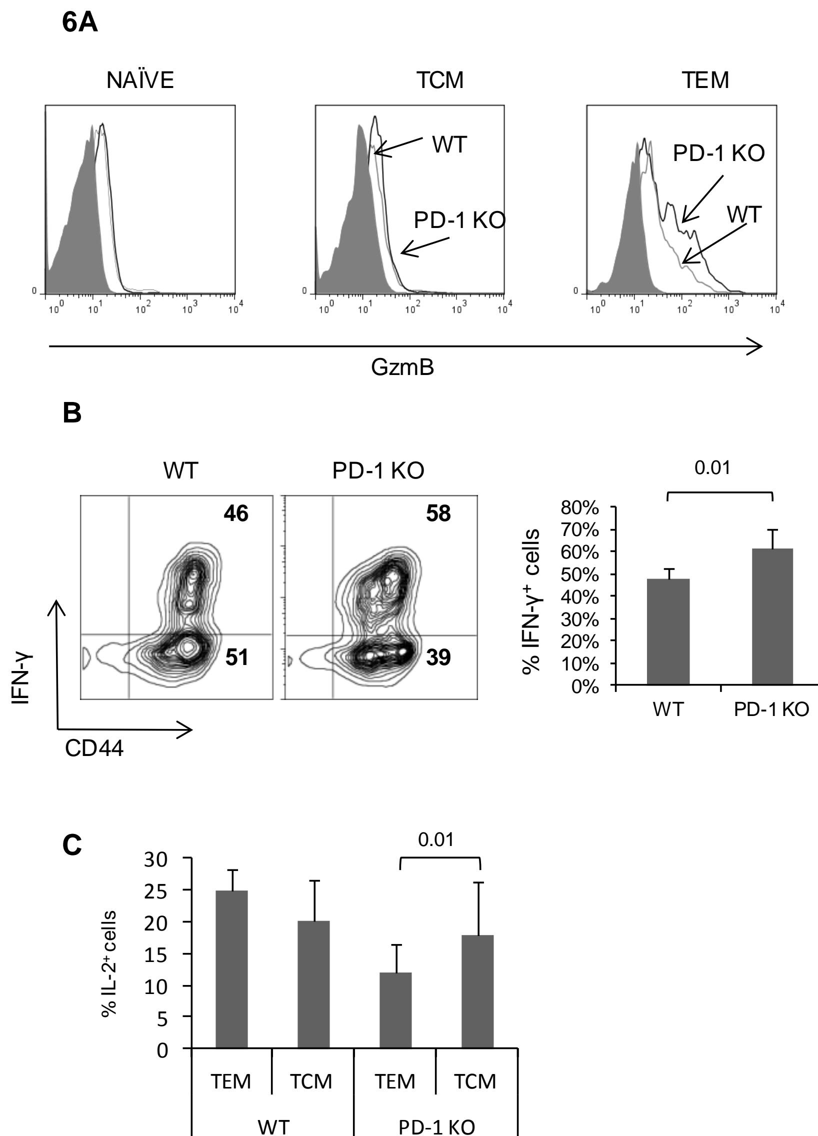
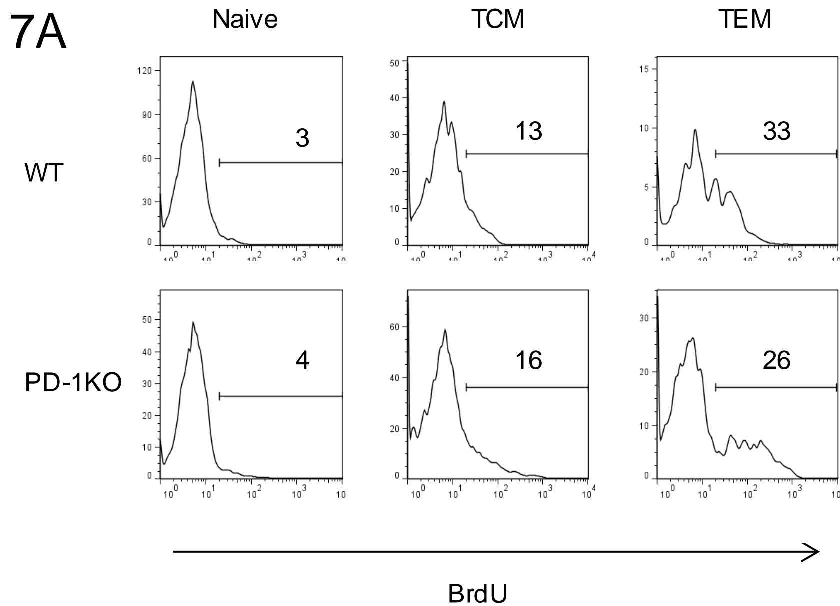
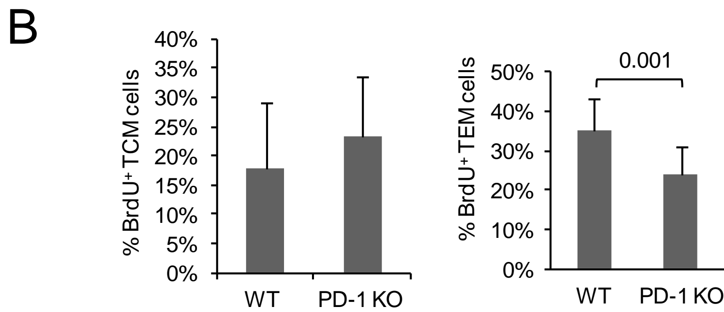


Figure 6- CD8 T_{EM} phenotype cells from PD-1 KO mice display significantly enhanced characteristics of effector memory T cells. Spleens from 9 mo old PD-1 KO and WT mice were analyzed by flow cytometry. **A,** Intracellular Granzyme B (GzmB) staining on $CD8^+$ subsets. Shaded histogram denotes staining with isotype control. Data are representative of 3 individual experiments with 2 mice per group **B**, IFN-γ production by purified $CD8^+$ $_{TEM}$ phenotype cells, after brief *ex vivo* stimulation. Representative dot plots of IFN-γ production by purified T_{EM}-phenotype $CD8^+$ cells.(left) and mean percentages with error bars indicating SD (right). Data represent 3 individual experiments with 8 pooled spleens per group. **C,** IL-2 production by purified $CD8^+$ T_{EM} and T_{CM}-phenotype cells from WT and PD-1 KO mice after brief *ex vivo* stimulation. Graph depicts mean percentages with error bars indicating SD. Data represent 3 individual experiments with 8 pooled spleens per group.

T_{CM}-phenotype cells are characterized by fast proliferation and IL-2 production [16]. Assayed in the same way as above, the production of IL-2 by purified T_{CM} and T_{EM} phenotype cells of PD-1 KO mice were compared. A larger percentage of T_{CM} cells produced IL-2 compared to T_{EM} cells (18%±8.1 vs 12%±4.4, p=0.01, **figure 6C**). There was no significant difference in the production of IL-2 between T_{CM} and T_{EM}-phenotype cells in the WT mice **(figure 6C)**. Interestingly, there was much less IL-2 produce by PD-1 KO T_{EM} cells compared to WT counterparts. Conclusively, T_{EM}-phenotype CD8 T cells are substantially expanded in lymphoid organs and tissues of PD-1 KO mice and they display significantly enhanced characteristics of effector memory T cells.

The rate of MP cell turnover is about one division every 2 weeks [111]. The increased number of T_{EM}-phenotype cells, found in the PD-1 KO mice, could result from an increased ability of these cells to proliferate. To address this question, 7 mo old WT and PD-1 KO mice were fed for 7 days with BrdU. Mice were sacrificed and BrdU analysis on CD8 T cell subsets was undertaken. As expected, naïve cells from both WT and PD-1 KO mice were almost all uniformly $BrdU^-$ (**figure 7A**). There was no significant difference in the turnover of T_{CM} cells between genotypes. Interestingly the percentage of $BrdU^+$ CD8 T_{EM}-phenotype cells was significantly less in the PD-1 KO mice, suggesting that they cycle slower than their WT counterparts (24%±7 vs 36%±8, p=0.001, **figure 7A and B**). Turnover of CD8 subsets was also assessed by Ki-67 staining, another marker of proliferation, and similar results were observed (**figure 7C**)





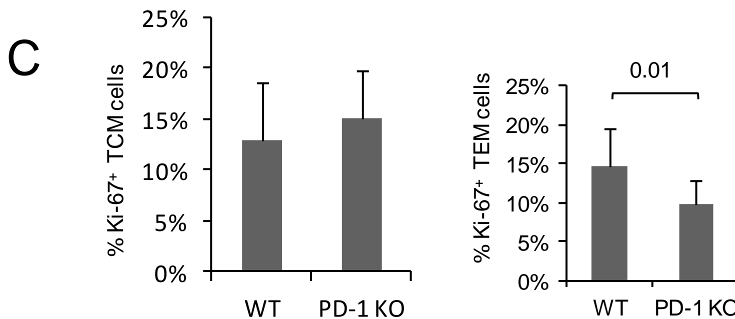


Figure 7- No differences in basal proliferation of CD8$^+$ MP subsets in PD-1 KO compared to WT. 7 mo old PD-1 KO and WT mice were fed BrdU for 7 days and spleenocytes were analyzed by flow cytometry. **A**, Representative histograms show expression of BrdU+ cells gated on CD8$^+$CD44^{lo}CD62L^{hi} (naïve) CD44^{hi}CD62L^{hi} (T_{CM}) and CD44^{hi}CD62L^{lo} (T_{EM}) memory-phenotype cells. Numbers indicate percentages. **B**, Mean percentages of BruU$^+$ T_{CM} and T_{EM}-phenotype cells, with error bars indicating SD. Data represent 3 individual experiments with 4 mice per group. **C**, Mean percentages of Ki67$^+$ T_{CM} and T_{EM}-phenotype cells, with error bars indicating SD. Data represent 2 individual experiments with 4 mice per group.

Since differences in proliferation in the MP T_{EM} subset could not account for the increase in numbers of T_{EM} cells found in the PD-1KO mice, next the survival of these cells was

assessed by *ex vivo* Annexin V-binding assay. CD8 T_{EM} phenotype cells from WT mice bound consistently more Annexin V compared to T_{CM} cells (**figure 8A).** Interestingly, the proportion of Annexin V^+ T_{CM}-phenotype cells from the PD-1KO mice were ~2 fold higher compared to WT T_{CM} (7.38%±1.69 vs 3.43%±0.45, p=0.001, **figure 8A**). Additionally, there was a significant decrease in the binding of Annexin V when comparing T_{EM}-phenotype cells from WT and PD-1 KO mice (44.77%±4.83 vs 29.35%±5.79 p=0.0001 **Figure 8A**). This data indicate that, in the absence of PD-1, T_{CM}–phenotype cells are more prone to apoptosis while T_{EM} phenotype cells survive better. Interestingly when gating on $CD62L^{hi}$, $CD62L^{int}$ and $CD62L^{lo}$ MP PD-1KO CD8 T cells we observed a correlation of CD62L downregulation with increased Annexin V-binding (**Figure 8B**). This may suggest that the increased Annexin V-binding of PD-1 KO T_{CM}-phenotype cells reflects their predisposition to become ($CD62L^{lo}$) T_{EM} cells. Collectively, this data indicate that an enhance propensity to survive rather than their faster proliferation rate augment the accumulation of T_{EM} cells in the PD-1KO mice.

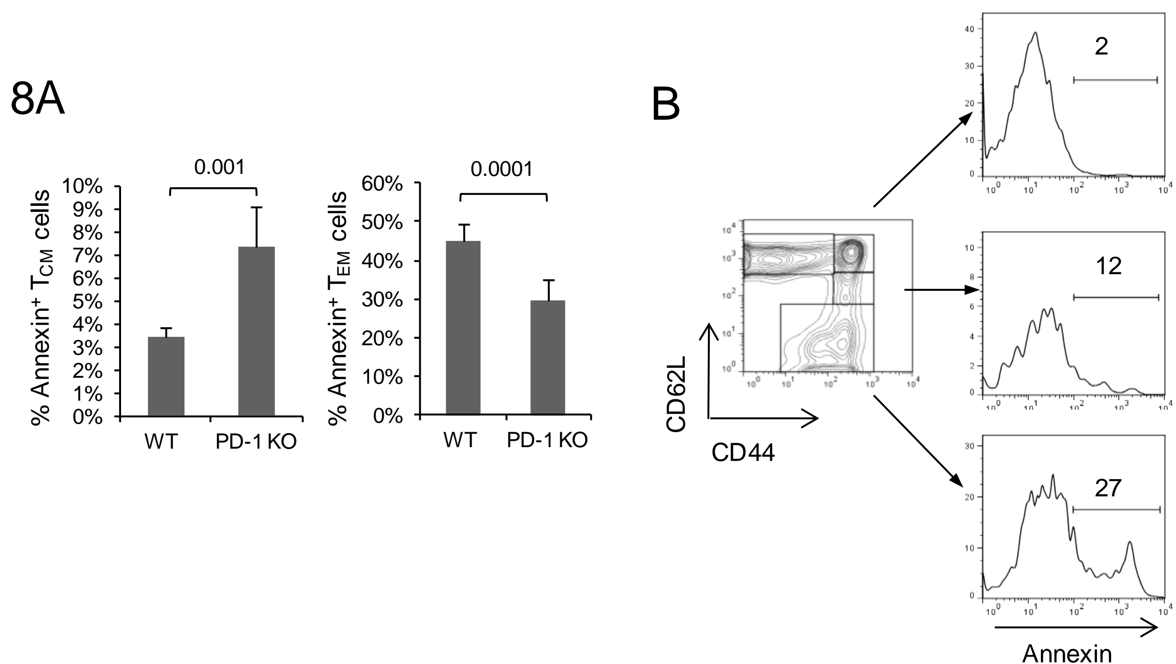


Figure 8- Contribution of cell death in CD8 T cell memory phenotype subsets. Spleens from 7mo old PD-1 KO and WT mice were analyzed by flow cytometry. **A**, Mean percentages of Annexin V^+ cells among $CD44^{hi}CD62L^{hi}$ (T_{CM}) and $CD44^{hi}CD62L^{lo}$ (T_{EM}) -phenotype cells, gated on live cells as confirmed by propidium iodide staining. **B**, Annexin V-binding on $CD8^+$ $CD44^+$ $CD62L^{hi}$, $CD62L^{int}$ and $CD62L^{lo}$ subpopulations from spleens of 7 mo old PD-1 KO mice. Numbers indicate percentage of Annexin V^+ cells. Data represent 3 individual experiments with 3 mice per group

6.3- Molecular characterization of PD-1 KO MP CD8 T cells

To gain further insights into the functional role of PD-1 in the generation and maintenance of memory-phenotype CD8 T cells, with the collaboration of Dr G. Garinis, we scanned the complete mouse transcriptome of CD8 T cells derived from 7-month old PD-1 KO and WT spleens. First, all significantly differentially expressed genes from the PD-1 KO and WT spleens were classified as having increased or decreased expression. Two-tail, pairwise analysis of variance of Affymetrix complete mouse genome arrays revealed 537 probe sets, representing 483 annotated genes with significantly changed expression patterns between WT and PD-1 KO CD8 T cells ($p \leq 0.05$, 1.2 fold change up- or down regulated, (**Supplementary Table S1**), a number that significantly exceeds the number of genes that are expected to occur by chance under these selection criteria. Using this dataset, we then sought to identify those biological functions with a significantly disproportionate number of responsive genes relative to those printed on microarrays (**Figure 9A**). Subsequent analysis of these processes led us to identify an upregulation of genes related to cytotoxic T cell function, including GzmB, GzmK, IFN-γ, and perforin (Prf1) but also IL-10 (**Figure 9B**) further supporting our previous flow cytometry assessment on IFN-γ and Granzyme B (**Figure 6A and B**). Further analysis revealed an upregulation of both co-stimulatory (4-1BB, CD9) and co-inhibitory molecules (CTLA-4, Tim-3, Lag-3). With respect to T cell death, Caspases 1 and 4 and Serpin6Ba were upregulated whereas DR6 (Tnfrsf21) was found to be downregulated in PD-1 KO CD8 T cells. Of particular notice is the high upregulation (11,7-fold) of IEX-1 (Ier3*)* which has been demonstrated to promote accumulation of effector/memory CD8 T cells through inhibition of apoptosis, resulting in a lupus-like disease [163]. Furthermore, transcription factors Id2, Eomes and Blimp-1 previously known to be related to effector/memory fate decisions were upregulated [164]. Thus, PD-1 KO CD8 T cell demonstrate distinct transcriptional responses bearing both enhanced cytotoxic function but also suppression/exhaustion; the end-effect of this transcriptional program is nevertheless the production of a set of critical effector and cytolytic molecules, which reflects the skewing of CD8 T cell fraction towards a T_{EM}-phenotype in the PD1-KO mice, presented in this study. These results are, at least in part, consistent with effector memory-associated genes as demonstrated by gene expression profiling of memory CD8 T cell subsets in humans, where T_{EM} cells were found to strongly express genes with known importance in CD8 T cell effector function [165]. However, since we are dealing with a mixed CD8 population we can not discriminate between upregulation of gene on a per cell basis and upregulation due to overrepresentation of T_{EM} cells among PD-1 KO CD8 T cells.

9A

Function Annotation	p-Value	# Molecules
Accumulation of T lymphocytes	1,94E-06	10
Activation of cytotoxic T cells	2,02E-05	8
Activation-induced cell death of T lymphocytes	2,97E-04	6
Anergy of T lymphocytes	1,65E-05	5
Apoptosis of T lymphocytes	3,52E-08	23
Cell cycle progression	1,17E-06	51
Cell death of T lymphocytes	3,53E-08	25
Cytotoxicity of lymphocytes	1,83E-04	12
Differentiation of T lymphocytes	4,16E-06	24
Lymphocyte homeostasis	6,41E-14	53
Proliferation of T lymphocytes	4,23E-07	33
T cell development	6,29E-14	52
T cell migration	7,08E-08	21
Transcription	5,98E-06	80

B

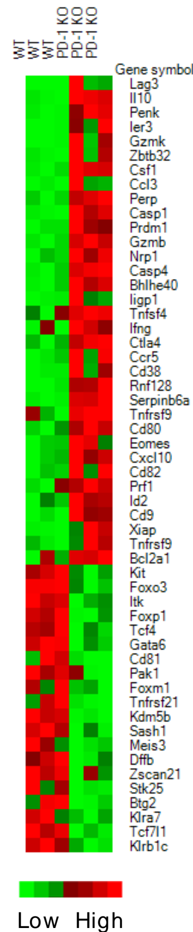


Figure 9. Microarray data analysis of sorted CD8+ T cells from PD-1 KO and WT mice. Splenocytes from PD-1 KO and WT mice were sorted for CD8+cells. Transcriptional profiles from sorted cells were then compared, n=3. **A**, Table showing the functions, p-values and number of molecules per category as assessed by DAVID microarrays software. **B,** Heatmap depicting the relative normalized expression of selected genes that are significantly different in expression between WT and PD-1 KO T_{CM}-phenotype CD8 cells.

6.4- PD-1 pathway prevents differentiation of LIP-memory CD8 T cells to T_{EM}-phenotype

Lymphopenia induced proliferation (LIP) of naïve T cells contributes to the maintenance of the T cell pool and the progressive accumulation of MP cells. Naïve T cells undergoing lymphopenia-induced homeostatic proliferation acquire a memory-phenotype similar to central memory cells without passing through an effector phase [3, 29], and become capable of mediating protective immunity against pathogens [99]. In order to examine the role of PD-1 in LIP we purified naïve ($CD44^{lo}$) CD8+ T cells from GFP.WT and GFP.PD-1 KO mice and transferred them to sub-lethally irradiated WT hosts.

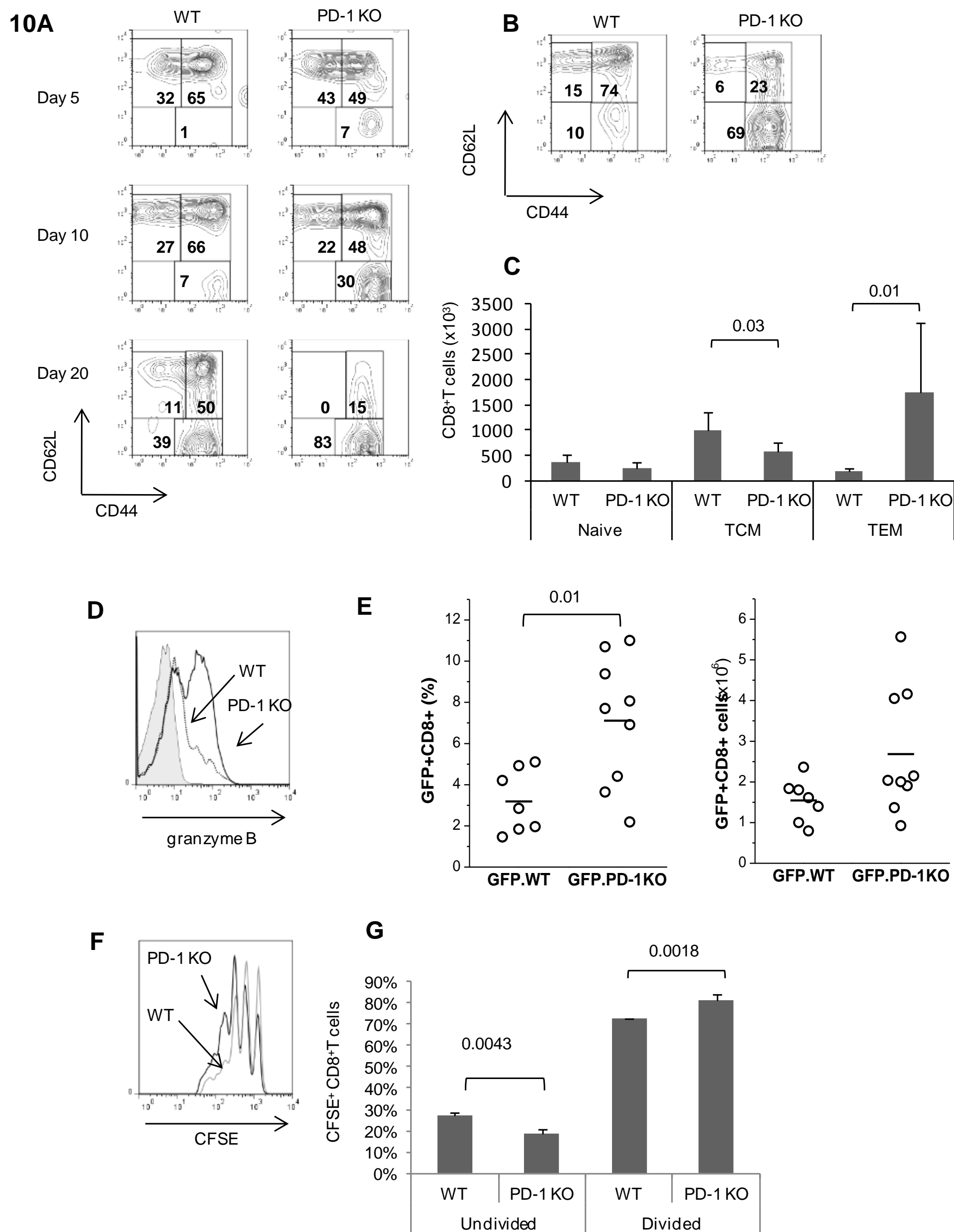


Figure 10- Fate of naïve WT and PD-1 KO CD8$^+$ cells transferred to sublethally irradiated WT hosts. GFP$^+$CD8$^+$CD44^{lo} T cells from spleens of 2-4 mo old PD-1 KO and WT mice were isolated by FACS sorting. Purified cells were then adoptively transferred into sublethally irradiated WT host mice. **A**, Donor-derived GFP$^+$ CD8$^+$ cells in hosts' blood were examined for CD8, CD44 and CD62L expression, on days 5,10 and 20. Numbers indicate percentages in each region. Data are representative of one experiment with 3 mice per group. **B**, Spleenocytes were analyzed as in A, on day 20. Numbers indicate percentages in each region. Plots are representative of 3 individual experiments. (WT, n=7; PD-1 KO, n=9). **C**, Total numbers of GFP$^+$ MP CD8 T cell subsets found in spleen on day 20. Error bars indicate SD. **D**, Ex vivo GzmB expression on day 20, gated on T_{EM}-phenotype CD8 cells (shaded region, isotype control; dashed line, GFP.WT; solid line GFP.PD-1 KO). **E**, Percentages and total numbers of donor-derived GFP$^+$ CD8$^+$ cells in spleens of irradiated WT hosts. Data are representative of 3 individual experiments with at least 2 mice per group. **F**, CFSE profiles of donor-derived CD8$^+$ cells in host spleens on day 5 (thick line, PD-1 KO; thin line, WT). Data are representative of two experiments with 3-4 mice per group. **G**. Mean percentages of undivided and divided CFSE+ CD8+ donor derived T cells in spleen, as in F. Error bars indicate SD.

At an early time point (day 5) after transfer, host's blood was analyzed for the presence of donor-derived MP subtypes by staining with anti- CD8, -CD62L and -CD44. Analysis of donor-derived cells in blood, revealed that initially both WT and PD-1 KO naïve donor cells gave rise mostly to T_{CM}-phenotype cells (**figure 10A**, upper panel, day 5) while at later time points (day 10 and 20), T_{EM}-phenotype cells progressively emerged, when PD-1 KO cells were transferred. Even at early time points there was clearly a difference in MP subsets between the donor derived WT and PD-1-deficient cells (**figure 10A**). This difference was even more pronounced at day 20 in spleens, as can be seen by the significant increase in numbers of T_{EM}-phenotype cells ($193x10^3$±57 vs $1749x10^3$±1382, p=0.010, **figure 10C**). There was also a parallel reduction in numbers of T_{CM} ($994x10^3$±372 vs $564x10^3$±207, p=0.028, **figure 10B and C**) between donor-derived GFP.WT and GFP.PD-1 deficient cells. These results suggest that in the absence of PD-1, T_{EM} cells accumulate at the expense of other CD8 subsets. GzmB was analyzed *ex vivo* on T_{EM}-phenotype cells, as a marker of functionality; importantly, a much larger fraction of PD-1 KO-derived T_{EM} cells were $GzmB^{hi}$ (**figure 10D**). This correlated increased effector memory function with the augmented numbers of T_{EM}–phenotype cells found in the absence of PD-1.

Interestingly, recovery of PD-1 KO- derived CD8 T cells was superior to WT-derived; however this reached statistical significance only when percentages **(Figure 10E, left)**, but not absolute numbers (**Figure 10E, right**), were compared, due to variation between experiments. To address directly the possibility that the lack of PD-1 from transferred CD8 T cells results in more robust lymphopenia-induced proliferation, we purified $CD8^+$ $CD44^{lo}$ naïve T cells from WT and PD-1 KO mice and labeled them with CFSE before adoptive transfer to irradiated WT hosts. On day 5, we examined CFSE profiles of donor-derived CD8 T cells; PD-1 KO cells exhibit a modestly advanced proliferation rate, as shown by a representative overlay of CFSE profiles from WT and PD-1 KO derived cells gated on $CD8^+$cells (**figure 10F**). Mean percentages of $CD8^+CFSE^+$ undivided and divided cells show that PD-1 KO cells had undergone more divisions on average compared to WT counterparts (**figure 10G**) indicating a role of PD-1 signals in delaying CD8 T cell proliferation in a lymphopenic environment. In conclusion, our results show that PD-1 signaling in CD8 T cells can modulate the homeostasis of the memory-phenotype pool by impeding proliferation and regulates T_{CM} to T_{EM} subset differentiation in lymphopenic conditions. The fact that we transferred purified naïve WT or PD-1 KO $CD8^+$ T cells and hosts were always WT, is suggestive of a CD8 T cell-intrinsic mechanism.

6.5- Accumulation of T_{EM}-phenotype CD8 T cells depends on cell-intrinsic mechanisms

In order to determine whether the accumulating $CD8^{+}$ T_{EM} MP cells in the PD-1 KO mice is a result of a T cell-intrinsic property rather than a secondary effect from other cells or altered cytokine milieu in PD-1KO mice, we performed mixed bone-marrow chimera experiments. Consequently, we transferred mixtures consisting of equal numbers of PD-1 KO and GFP.WT bone marrow cells to lethally irradiated DsRed.WT hosts. In these settings, PD-1 KO and WT CD8 T cells mature and respond to the same environmental cues and any observed differences should be attributed to intrinsic factors. Eight weeks after transfer we analyzed thymi, spleens, and lymph nodes from hosts and the ratios of donor-derived WT and PD-1 KO T cells were evaluated. Analysis of thymi showed equal contribution of WT- and PD-1 KO-derived cells in thymocytes and similar percentages of CD8 single positive (SP) cells (**Figure 11A**). The mean PD-1 KO to WT ratio for these chimeric mice was 1.0 for CD8+ SP thymocytes, (**Figure 11A**, right), suggesting that PD-1 KO bone marrow cells had no general thymic developmental advantage over WT counterparts. In contrast, the majority of donor-derived CD8 T cells in spleens were of PD-1 KO origin ($3.31x10^{6}±0.92x10^{6}$ vs $1.61x10^{6}±0.47x10^{6}$, p=0.002, **Figure 11B**) suggesting that post thymic events are the cause of increased PD-1 KO-derived peripheral CD8 T cells.

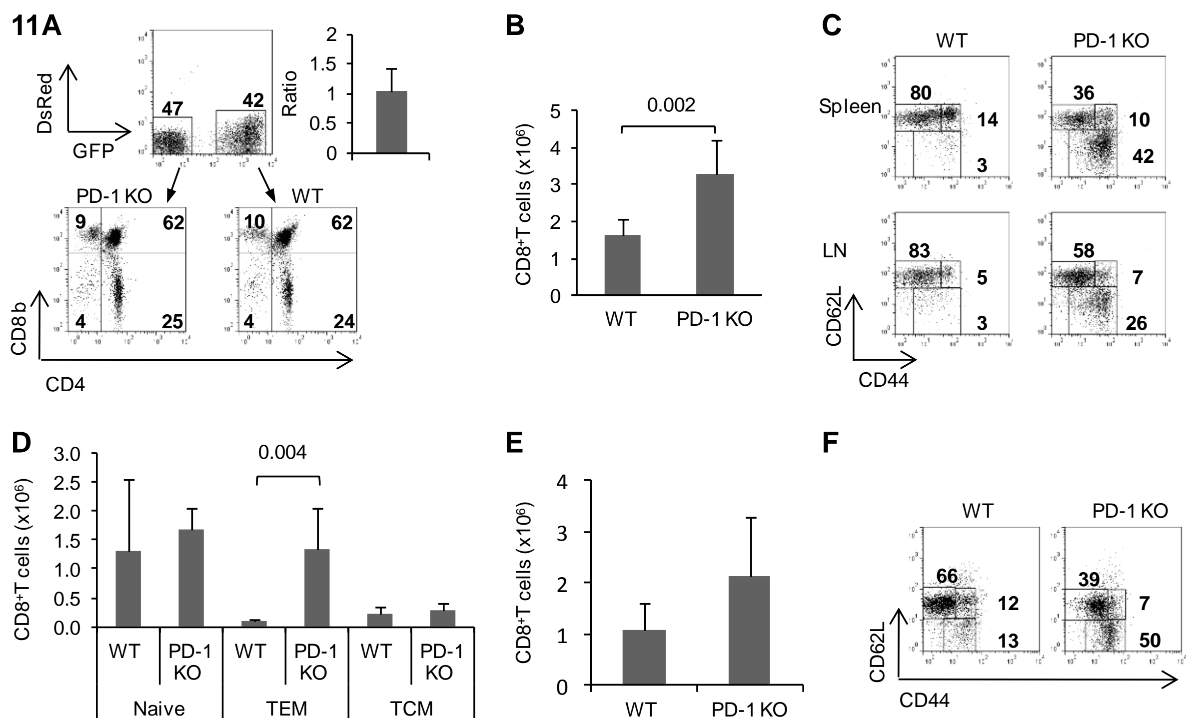


Figure 11- T cell-intrinsic increase in PD-1 KO CD8$^+$ T_{EM}-phenotype cells. Donor-derived WT (GFP$^+$DsRed$^-$) and PD-1 KO (GFP$^-$DsRed$^-$) CD8$^+$ T cells from thymi, spleens and lymph nodes were analyzed by flow cytometry 8 weeks after bone marrow reconstitution in irradiated DsRed hosts. **A**, Representative dot plots with donor-derived WT (GFP+DsRed-) and PD-1 KO (GFP-DsRed-) thymocytes. On gated populations the expression of CD4 and CD8 was analysed. Numbers indicate percentages in each region (upper left: CD8 SP; lower right: CD4 SP; upper right: DP; lower left: DN). Column represents the average value of PD-1 KO/WT CD8 SP thymocyte ratios with error bar indicating SD. Data are representative of 2 individual experiments, n=6. **B**, Total numbers of CD8$^+$ WT and PD-1 KO cells in spleens with error bars indicating SD. **C**, Donor-derived WT (GFP$^+$DsRed$^-$) and PD-1 KO (GFP$^-$DsRed$^-$) CD8$^+$ T cells from spleens and mesenteric lymph nodes were further analyzed for expression of CD44 and CD62L. Numbers indicate percentages in each region. Data are representative of 3 individual experiments, with 3-4 mice per group. **D**, Total numbers of WT and PD-1 KO CD8$^+$ T cell subsets in spleens with error bars indicating SD. **E**, Similar analysis of donor-derived WT (GFP$^-$ DsRed$^-$) and PD-1 KO (GFP$^+$DsRed$^-$) CD8$^+$ T cells from spleens after bone marrow reconstitution in irradiated DsRed host as in C. **F,** Total numbers of CD8$^+$ WT and PD-1 KO cells in spleens with error bars indicating SD. Data are representative of 1 individual experiments, with 3 mice per group

Further subtype analysis in spleens and mesenteric lymph nodes showed that there was a significantly higher proportion of T_{EM}-phenotype cells in CD8 T cell populations of PD-1 KO origin (**Figure 11C and D**). Similar results in spleen were obtained when we transferred mixtures of GFP.PD-1 KO and WT bone marrow cells to DsRed.WT hosts (**Figure 11E and F**), indicating that the GFP transgene in donor-derived cells had no effect in the observed phenotype. These results demonstrate that the absence of PD-1 results in accumulation of CD8 T_{EM}-phenotype cells in a cell-intrinsic manner.

6.6- PD-1 negatively regulates interconversion of T_{CM} and T_{EM}-phenotype CD8 T cells.

In order to investigate whether aberrant conversion between MP subsets contributes to accumulation of T_{EM}-phenotype CD8 T cells in PD-1 KO mice, we purified both T_{EM}- and T_{CM}-phenotype CD8 T cells from GFP.WT or GFP.PD-1 KO spleens and transferred them separately to WT or PD-1 KO mice respectively. **Figure 12A** upper panel, shows the purity of T_{CM}-phenotype CD8 T cells. When analyzing host mice that received T_{CM}-phenotype cells, little conversion of T_{CM}→T_{EM} cells was found in WT mice after 42 days (**Figure 12A**, lower panel, left). In PD-1 KO mice however, a striking conversion of T_{CM}→T_{EM}-phenotype was observed (**Figure 12A**, lower panel, right, ~80% of donor-derived cells from PD-1 KO hosts that received T_{CM} CD8 T cells were of a T_{EM}-phenotype). This was accompanied by a substantially higher recovery of PD-1 KO T_{EM}-phenotype donor-derived cells compared to

WT counterparts ($102x10^3$±$109x10^3$ vs $0.99x10^3$±$0.98x10^3$, p=0.008 **Figure 12B**). This was also true, but to a lesser degree, for PD-1 KO T_{CM}-phenotype donor-derived cells ($30.5x10^3$±26.2 vs $10x10^3$±$2.4x10^3$, p=0.020 **Figure 12B**). Similar degree of abnormal conversion and high recoveries were also obtained when PD-1 KO T_{CM} cells were transferred to WT hosts but not when WT T_{CM} cells were transferred to PD-1 KO mice (**Figure 11C and D**), indicating that the above described phenomenon was a result of the lack of PD-1 in donor T_{CM} cells.

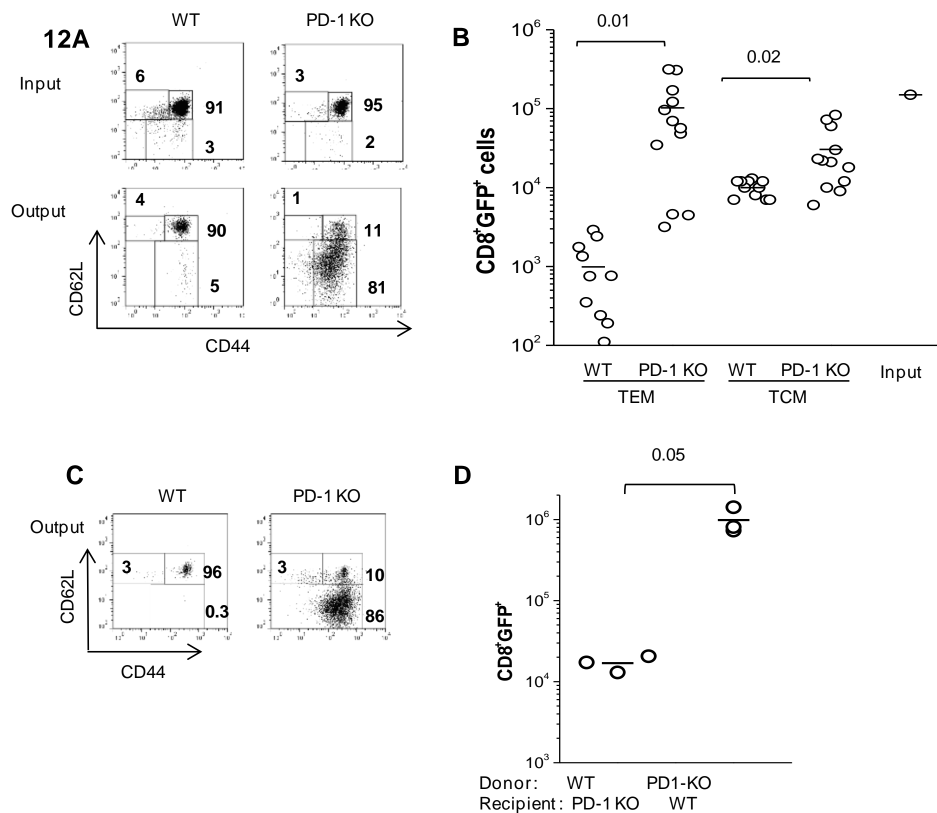


Figure 12- Fates of CD8 T_{CM} memory-phenotype cells in adoptive transfer experiments. **A** Purified GFP^+CD8^+ T_{CM}-phenotype cells from 5-7 mo old GFP.WT and PD-1 KO were adoptively transferred into WT and PD-1 KO mice., Representative dot plots with CD62L and CD44 expression on purified T_{CM}-phenotype cells before adoptive transfer (upper panel) and on day 42 on donor-derived GFP^+CD8^+ cells (lower panel). Numbers indicate percentages in each region. Data are representative of 4 individual experiments (WT, n=10; PD-1 KO, n=12). **B**, Total numbers of recovered GFP^+ $CD8^+$ T_{EM}- and T_{CM}-phenotype cells from WT and PD-1 KO host spleens as in A. For comparison, the numbers of transferred cells per host (input) are indicated. **C .** GFP.WT and GFP.PD-1 KO T_{CM}-phenotype CD8+ cells were adoptively transferred into PD-1 KO and WT mice respectively and analyzed on day 42 as in A. **D**, Total numbers of recovered GFP^+ $CD8^+$ cells from WT and PD-1 KO host spleens. Data represent an individual experiment with 3 mice per group.

It was possible that accumulating PD-1 KO T_{EM}-phenotype cells may arise from overt proliferation of residual T_{EM} cells in the purified T_{CM} “preparation”. To exclude this, we analyzed Ki-67 expression in GFP$^+$ PD-1 KO T_{CM}- and T_{EM}-phenotype cells on days 21 and 42 after transfer of GFP$^+$ T_{CM}-phenotype cells. Ki-67 expression was lower in the T_{EM}-phenotype subset compared to T_{CM}-phenotype when analyzed in the same host (**Figure 13A**), thus showing that GFP$^+$ T_{CM}-phenotype cells in PD-1 KO hosts were not outnumbered by vast proliferation of contaminant T_{EM}-phenotype cells. For the same purpose we transferred purified SNARF-1 labeled GPF.PD-1 KO T_{CM}-phenotype cells to PD-1 KO hosts, and compared dye intensity dilution in GFP$^+$ T_{CM}- and T_{EM}-phenotype cells. No consistent difference was observed when profiles for these subsets were overlayed (**Figure 13B**). These data indicate that accumulated T_{EM}-phenotype cells, after PD-1 KO T_{CM} cell-transfers, do not originate from overt expansion of residual co-transferred T_{EM} cells.

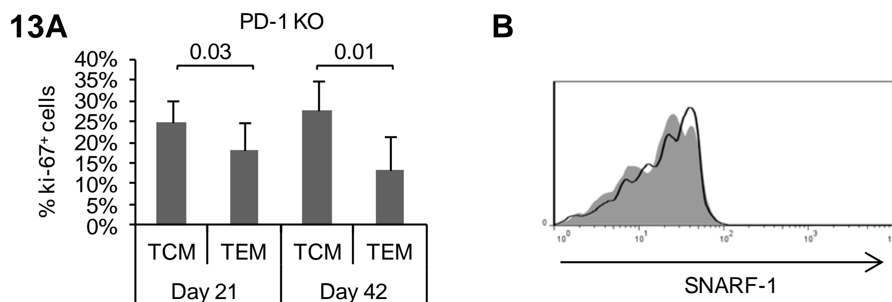


Figure 13- Comparison of proliferation of CD8 T cell subsets among donor derived GFP.PD-1 KO cells after adoptive transfer of T_{CM} cells. A, Mean percentages of Ki-67$^+$ cells among donor-derived GFP.PD-1 KO CD8$^+$ subsets on day 21 and 42 after transfer, with error bars indicating SD. Data are representative of 2 individual experiments, with 3 mice per group. **B**, SNARF-1 profiles of donor-derived CD8+ PD-1 KO T_{CM}- and T_{EM}-phenotype cells in host spleens on day 13 (thick line, PD-1 KO T_{CM}; Shaded, PD-1 KO T_{EM}). Data are representative of one experiment with 4 mice per group.

In addition, we purified T_{EM}-phenotype CD8 T cells from GFP.WT or GFP.PD-1 KO spleens and transferred them separately to WT or PD-1 KO mice respectively. **Figure 14A**, upper panel, shows the purity of transferred cells. When analyzing mice that received T_{EM}-phenotype cells, $T_{EM} \rightarrow T_{CM}$ conversion was moderate for WT donor cells, whereas a smaller proportion of recovered PD-1 KO donor cells bore the T_{CM} phenotype, consistent with less $T_{EM} \rightarrow T_{CM}$ conversion (**Figure 14A**, lower panel). A significantly higher recovery of T_{EM}-phenotype PD-1 KO donor-derived cells was observed ($68.4 \times 10^3 \pm 84$ vs $10.9 \times 10^3 \pm 9.7$, $p=0.05$, **Figure 14B**) which may also be partly attributed to their enhanced survival (see **figure 8A**). Conclusively, these results provide strong evidence that PD-1 regulates

differentiation of T_{CM}- to T_{EM}-phenotype CD8 cells in non-immunized, naïve mice both by inhibiting T_{CM}→T_{EM} and by promoting T_{EM}→T_{CM} conversion.

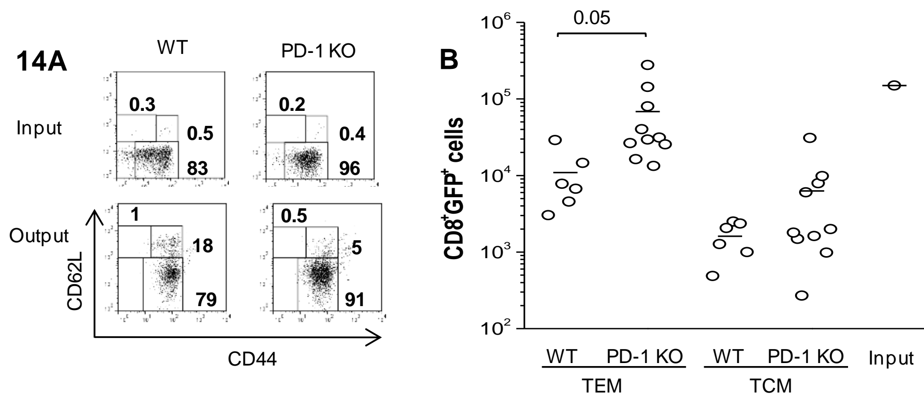


Figure 14- Fates of CD8 T_{EM} memory-phenotype cells in adoptive transfer experiments Purified GFP^+CD8^+ T_{EM}-phenotype cells from 5-7 mo old GFP.WT and GFP.PD-1 KO were adoptively transferred into WT and PD-1 KO mice. **A**, Representative dot plots with CD62L and CD44 expression on purified T_{EM}-phenotype cells before adoptive transfer (input, upper panel) and on day 42 on donor-derived GFP^+CD8^+ cells; (output, lower panel). **B**, Total numbers of recovered GFP^+ $CD8^+$ T cell subsets from WT and PD-1 KO host as in A,(WT, n=6; PD-1 KO, n=10). For comparison, the numbers of transferred cells per host (input) are indicated.

IL-15 signaling plays a major role in homeostasis of memory CD8 T cells [93, 160]. Differential expression of IL-2Rβ on PD-1 KO T_{EM}-phenotype CD8 cells (see **Figure 5D**) prompted us to investigate their response to IL-15 in vitro. **Figure 15A** shows that culture of purified WT T_{EM}-phenotype CD8 T cells with IL-15 for 7 days resulted in conversion of a substantial fraction of these cells to the T_{CM}-phenotype and the effect appeared to be dose dependent (**Figure 15A upper and lower panels)**. On the contrary, most of purified PD-1 KO cells retained their T_{EM}- phenotype. As a result, recovered PD-1 KO T_{EM}-phenotype CD8 T cells were double their WT counterparts ($5.6x10^4$±0.10 vs $2.6x10^4$±0.18, p=0.0071, **Figure 15B**), while opposite results for recovery of T_{CM} were found ($0.93x10^4$±0.19 vs $4.89x10^4$±0.22, p=0.0032, **Figure 15B**). These experiments suggest that differential response of WT and PD-1 KO T_{EM}-phenotype CD8 cells to the IL-15 cytokine could partly account for their accumulation in PD-1 KO mice.

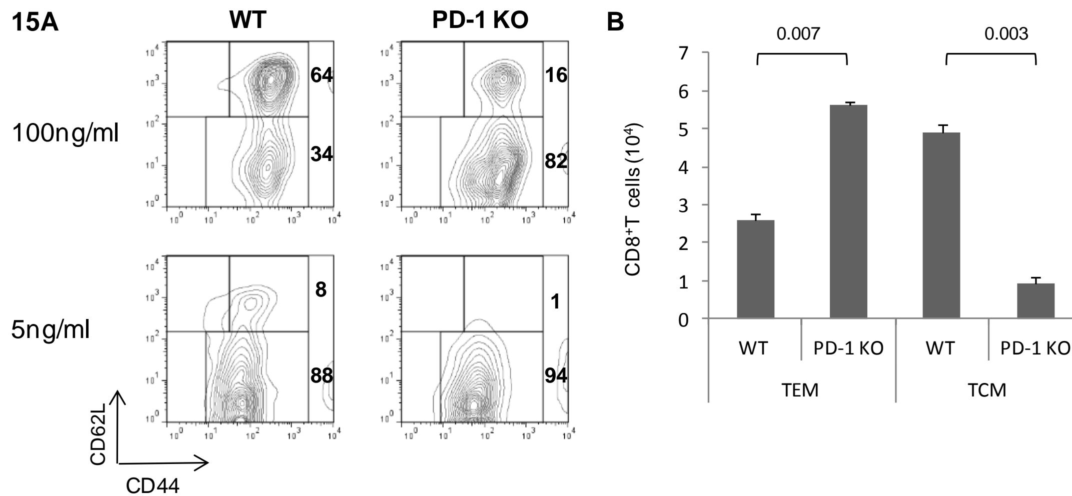


Figure 15-Responsiveness to IL-15 *in vitro* by CD8 T_{EM} cells from PD-1 KO and WT mice. Spleenocytes from WT and PD-1 KO mice were sorted for $CD8^{+}CD44^{hi}$ $CD62L^{lo}$ (T_{EM}) cells as described in materials and methods. **A,** $3x10^{4}$ T_{EM} cells were cultured for 7d in medium containing 100ng/ml (upper panel) and 5ng/ml (lower panel) of IL-15. Cells were analysed by flow cytometry after staining with CD8, CD44 and CD62L. **B**, Graphs indicate mean values of recovered live CD8 T cell subsets after culture with 100ng/ml of IL-15 . Eror bars representing S.D. Data are representative of 3 separate experiments with 8 pooled mice per group.

6.7- Absence of PD-1 exerts genome-wide gene expression changes in T_{CM}-phenotype CD8 cells

We have shown that transferred T_{CM}-phenotype CD8 cells from PD-1 KO mice, but not WT, can give rise predominantly to a T_{EM}-phenotype population (**Figure 12A**). Analysis of T_{CM}-phenotype CD8 cells for CD69, Ly6C, IL-2R, IL-7R, IL-2Rβ surface expression revealed indistinguishable patterns between PD-1 KO and WT cells (**Figure 5A**). To examine whether T_{CM} -phenotype CD8 cells from PD-1 KO mice had already adopted a different transcriptional profile at the time of transfer, with the collaboration of Dr G. Garinis we performed transcriptome analysis, on T_{CM}-phenotype CD8 cell subpopulations derived from PD-1 KO and WT spleens. First, all significantly differentially expressed genes between the PD-1 KO and WT T_{CM}-phenotype CD8 cells were classified as having increased or decreased expression. Two-tail, pairwise analysis of variance of Affymetrix complete mouse genome arrays revealed 237 annotated genes with significantly changed expression patterns between WT and PD-1 KO T_{CM} CD8 cells ($p \leq 0.05$, 1.5-fold change up- or down-regulated) (**Supplemental Table 2**) a number that significantly exceeds the number of genes that are expected to occur by chance under these selection criteria. Using this dataset, we then

identified those biological processes with a significantly disproportionate number of

16A

Function Annotations	p-Value	# Molecules
Activation-induced cell death of T lymphocytes	1,43E-05	10
Apoptosis of T lymphocytes	2,96E-12	42
Cell death of T lymphocytes	9,75E-15	50
Cell division process of lymphocytes	6,78E-07	18
Expansion of T lymphocytes	1,39E-07	21
Growth of T lymphocytes	2,88E-07	22
Immune response of T lymphocytes	2,75E-06	16
Lymphocyte homeostasis	2,35E-26	107
Lymphocyte migration	1,07E-05	35
Proliferation of T lymphocytes	1,28E-09	60
Stimulation of T lymphocytes	1,58E-05	13
Survival of lymphocytes	2,44E-08	26
T cell development	3,15E-25	103
Transcription	2,50E-15	181

B

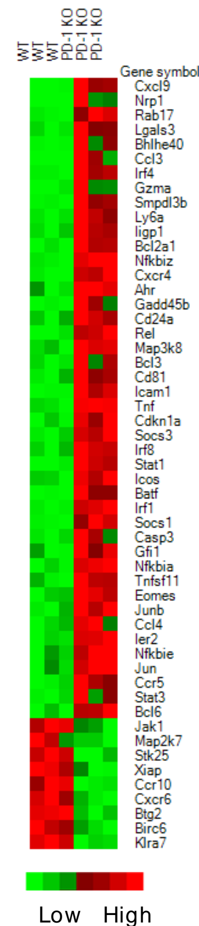


Figure 16- Microarray data analysis of sorted CD8^{+} T$_{CM}$ -phenotype cells from PD-1 KO and WT mice. Splenocytes from PD-1 KO and WT mice were sorted for CD8^{+}CD44^{hi}CD62L^{hi} cells. Transcriptional profiles from sorted cells were then compared, n=3. **A**, Table showing the functions, p-values and number of molecules per category as assessed by DAVID microarrays software. **B**, Heatmap depicting the relative normalized expression of selected genes that are significantly different in expression between WT and PD-1 KO T$_{CM}$-phenotype CD8 cells.

responsive genes in the T$_{CM}$-phenotype CD8 cell subset relative to those contained in the Affymetrix arrays as shown in **Figure 16A**. Selected genes and the magnitude of over- or under-expression are graphically depicted in **Figure 16B**. Among these, there are genes involved in T cell co-stimulation (CD24, Icos, ICAM1, Tnfsfr1b (TNFR2)), apoptosis/survival (Bcl2a1, Bcl3, TNF, Xiap), signal transduction (Jak1, Map3k8 (Tpl-2), Gadd45b, Socs3) as well as T cell migration/adhesion/inflammation (Ccl3, Cxcl9, Nrp1, (Neuropilin-1), Lgals3 (Galectin-3)). Differentially expressed transcription factors included Rel, STAT1, Irf4, Icrf8, and the less characterized Atf3, Ahr and Bhlhe40 (Dec1). Ahr is able to modulate CD62L

expression in primary responses [166] and under certain conditions diminishes memory CD8 pool but not CD8 cell responses [167]. Bhlhe40 transcription factor, which has recently been shown to be important in generation of Tregs cells [168] is one of the most up-regulated genes in PD-1 KO T_{CM} CD8 cells (3.8-fold). Interestingly, up-regulation of IL-12Rb1 was accompanied by increased expression of genes previously characterized as positively regulated by IL-12 and/or IFN-α/β such as Gadd45b, Bcl3, TNF, Lgals3, Ccl3, Bhlhe40, Cdkn1a, and Atf3 and IL12Rb1 itself [39, 169]. Importantly when these cytokines are used as signal 3 on CD8 T cells they down-regulate CD62L and CCR7 more efficiently than signal 1 and 2 alone [169].

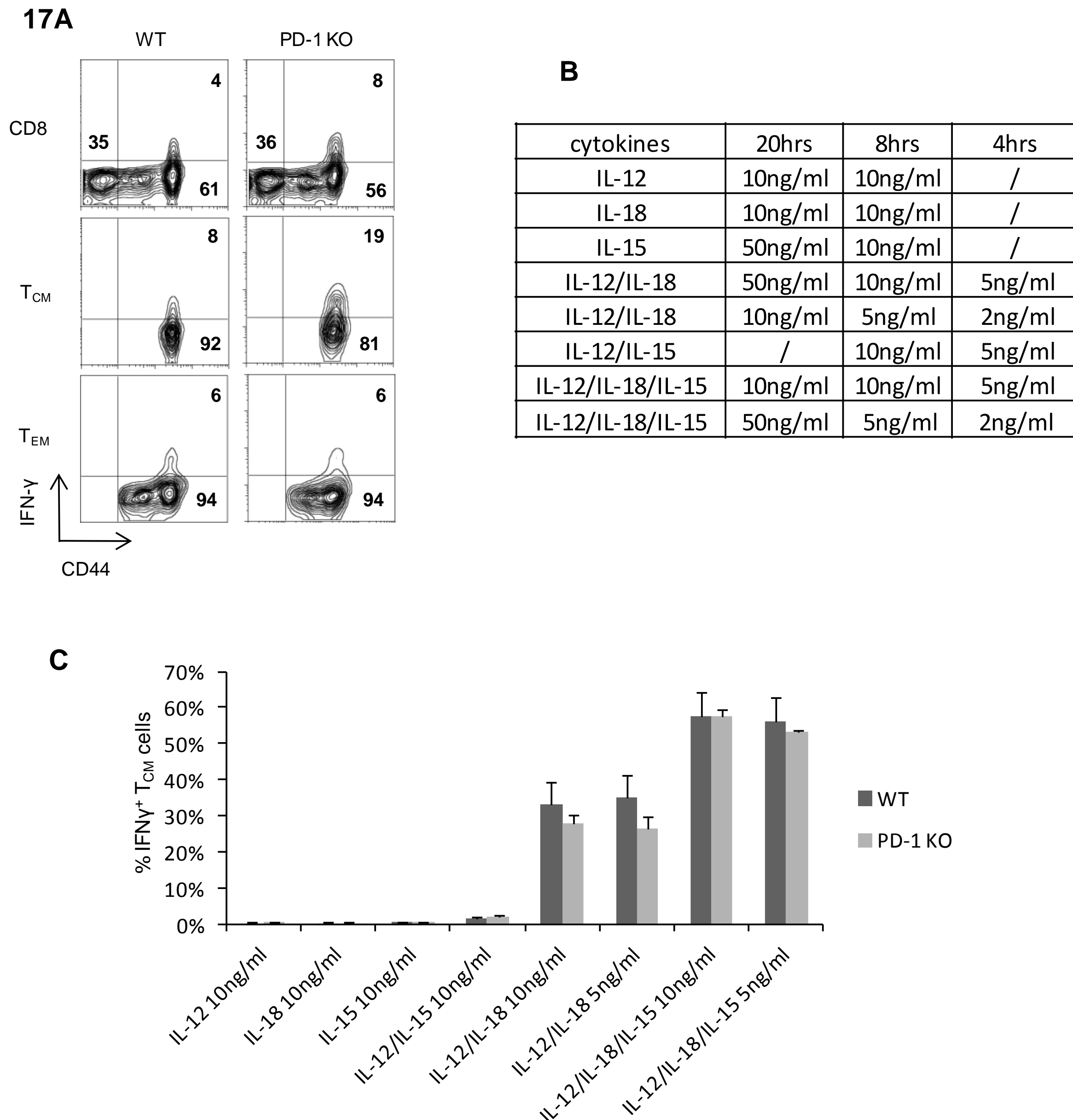
Overall our results show that PD-1 KO T_{CM}-phenotype CD8 cells bear a distinct gene expression profile and ablation of PD-1 pathway had exerted an impact before the acquisition of the T_{EM}-phenotype. This may indicate that in transfer experiments PD-1 KO T_{CM}-phenotype cells are already pre-programmed, at least at the transcriptional level, to differentiate to T_{EM}-phenotype cells. Additionally, their profile indicates that T_{CM}-phenotype CD8 T cells may respond differently to IL-12 and IFN-α/β cytokines.

6.8- Superior bystander production of IFN-γ by T_{CM}-phenotype PD-1 KO CD8 cells after innate stimulus.

MP CD8 T cells have been shown to produce IFN-γ driven by IL-12, and IFN-α/β produced by macrophage/dendritic cells, in response to infection or a defined innate stimulus [112-113]. Given our microarray results that imply an increased response of PD-1 KO T_{CM}-phenotype CD8 cells to these cytokines (**figure 15**), we injected WT and PD-1 KO mice with LPS and analyzed CD8 T cells for IFN-γ production 4 hours after injection. A higher fraction of PD-1 KO CD8 T cells was IFN-γ producers (**Figure 17A,** upper panel). When we analyzed T_{EM} and T_{CM} subsets we found that a larger percentage of PD-1 KO T_{CM}-phenotype cells produced IFN-γ *ex vivo* (**Figure 17A**, middle panel). No difference in IFN-γ production was observed between T_{EM}-phenotype cells from WT and PD-1 KO mice (**Figure 17A**, lower panel). These results show increased indirect response of PD-1 KO T_{CM}-phenotype CD8 cells to LPS, probably through IL-12 and/or IFN-α/β, and imply a greater bystander innate response of PD-1 KO MP CD8 T cells to various pathogens.

In order to assess more directly the responsiveness of MP cells to these cytokines we incubated splenocytes for different time periods (4h, 8h, 20h) with various concentrations of recombinant IL-12, IL-15 combined with recombinant IL-18 in vitro (**see figure 17B**). Splenocytes were also incubated with IFNa/b (1000u/ml) and IL-18 (10-100 ng/ml) for 6hrs. Under no conditions did T_{CM} of PD-1 KO CD8 T cells produced IFN-γ to a higher extent compared to WT (**figure 17B)**. Nevertheless, this does not exclude a role of these cytokines

in the increased production of IFN-γ by PD-1 KO T_{CM} CD8 cells *in vivo* in the context of inflammatory milieu induced by LPS; the incubation with these cytokines seems not to fully simulate *in vivo* inflammatory conditions produced by the LPS-injection. Therefore it remains open the possibility that in certain inflammatory conditions PD-1 KO T_{CM}-phenotype cells exhibit differential responses to IL-12/IFN-a/b.



cytokines	20hrs	8hrs	4hrs
IL-12	10ng/ml	10ng/ml	/
IL-18	10ng/ml	10ng/ml	/
IL-15	50ng/ml	10ng/ml	/
IL-12/IL-18	50ng/ml	10ng/ml	5ng/ml
IL-12/IL-18	10ng/ml	5ng/ml	2ng/ml
IL-12/IL-15	/	10ng/ml	5ng/ml
IL-12/IL-18/IL-15	10ng/ml	10ng/ml	5ng/ml
IL-12/IL-18/IL-15	50ng/ml	5ng/ml	2ng/ml

Figure 17- IFN-γ production *ex vivo* and *in vitro* by memory-phenotype $CD8^+$ T cell subsets. A Spleens from 3 mo old PD-1 KO and WT mice were analyzed after LPS injection, by flow cytometry. Representative dot plots of IFN-γ production by total $CD8^+$ and MP subsets. Data are representative of 2 individual experiments with 4 mice per group. Numbers show the percentages of cells in each quadrant. B, Table indicating the combinations of cytokine, concentrations and time points used in various experiments. C. Spleenocytes from WT and PD-1KO mice were incubated *in vitro* with indicated concentration of cytokines for 8hrs before being analysed for CD44

and CD62L expression by flow cytometry. Gated on T_{CM}-phenotype cells graphs show average IFN-γ production by WT and PD-1 KO cells. Data are representative of 3 experiments with 3 mice per group.

6.9- PD-1 affects the generation of hapten-specific CD8 memory T cells in contact hypersensitivity reaction.

The above data clearly demonstrate the importance of PD-1 in shaping memory-phenotype subsets. We next wanted to investigate whether PD-1 is also involved in responses of memory CD8 T cells against defined antigenic challenges in tissues. Thus, we used a described modification [157] of the classical short-term contact hypersensitivity (CHS) protocol, to generate memory CD8 T cells against the hapten 2,4-dinitrofluorobenzene (DNFB) (**Figure 18A**). CHS to haptens is an inflammatory response of the epidermis to epicutaneous sensitization and subsequent challenge with a hapten, in which the immune response is mediated by hapten-specific CD8 T cells [170]. The generation of hapten-specific memory was manifested by the induction of a substantial ear swelling measured 2 days after the second challenge. As shown in **Figure 18B**, ears of PD-1 KO mice exhibited a significantly more pronounced swelling, as a result of the elicited CD8 T cell inflammatory response, compared to WT(19.83 $x10^{-2}$ mm ±3.54 $x10^{-2}$ mm vs 11.5$x10^{-2}$mm±3.01 $x10^{-2}$ mm, p=0.00015, **Figure 18B**). The same day we isolated lymphocytes from ear tissue and phenotypically characterized them. We found elevated numbers of CD8 T lymphocytes in challenged ears of PD-1 KO mice compared to WT challenged ears (2737±1170 vs 601±342, p=0.00054, **Figure 18C and 18D, upper panel**) and the majority had acquired the $CD44^{hi}CD62L^{lo}$ phenotype (**Figure 18D, lower panel**). Therefore, almost 4 times more $CD44^{hi}CD62L^{lo}$ T_{EM} CD8 cells were extracted from PD-1 KO than from WT ears (2459±1185 vs 500±297, p=0.019 **Figure 18E**). Both in WT and PD-1 KO mice, unchallenged ears bore almost no T cells (**figure 18E**). These data show that the lack of PD-1 results in more efficient generation of memory responses in contact hypersensitivity, accompanied by enhanced recruitment of memory cells into the inflamed tissue.

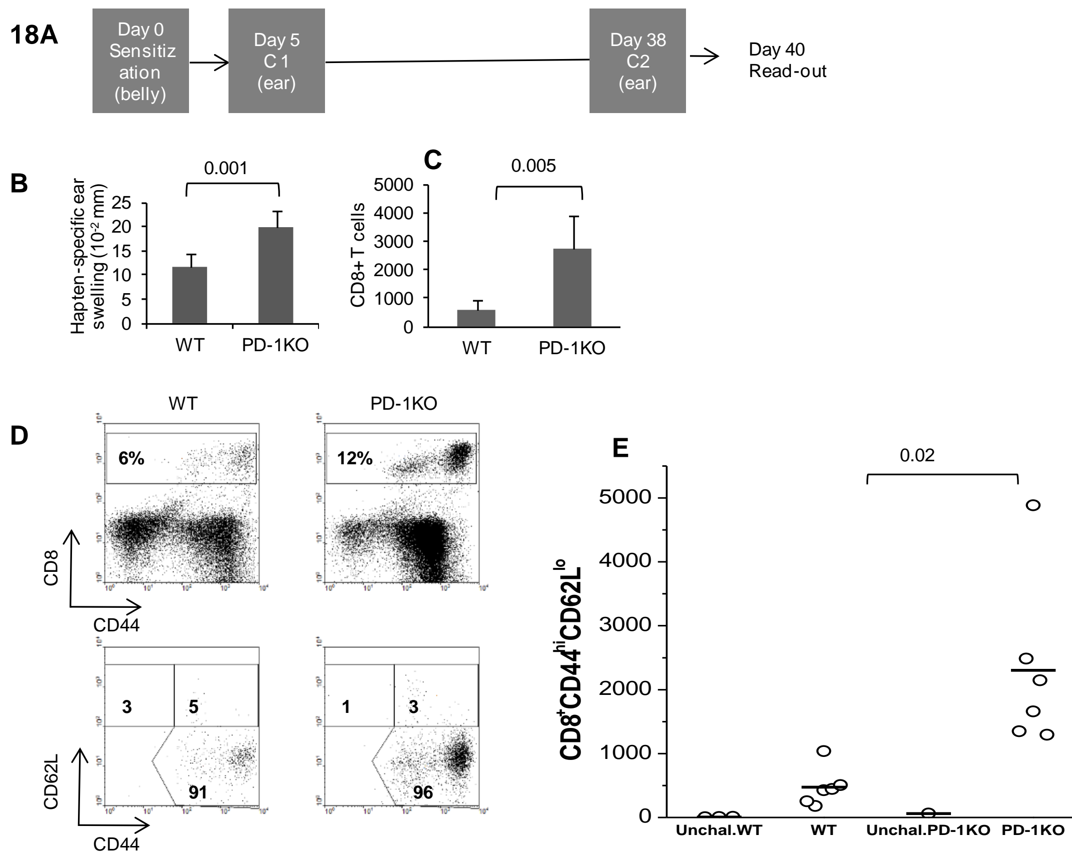
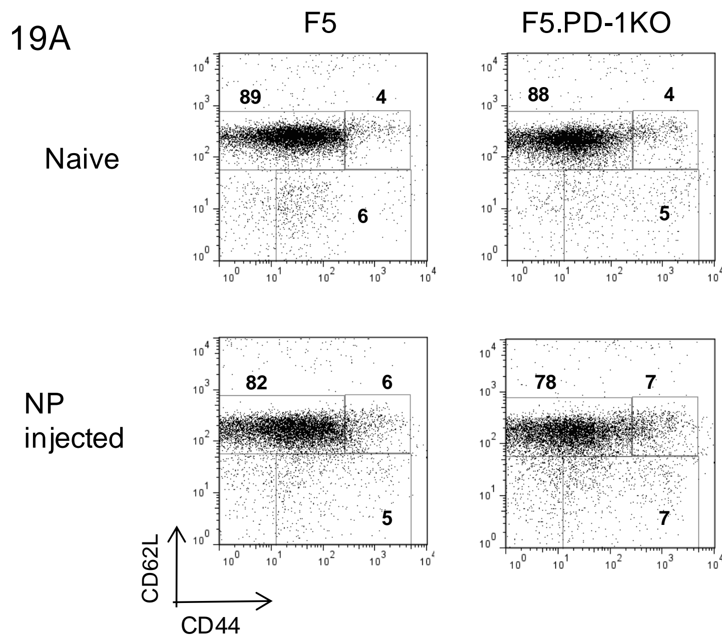


Figure 18- Generation of hapten-specific memory cells in WT and PD-1 KO mice. **A,** CHS to DNFB was performed by sensitization on the ventral skin of WT and PD-1 KO mice on day 0. Mice received 2 challenges on the ear on day 5 and day 38. **B**, Ear thickness was measured 48 h after the second challenge. The results are expressed as mean hapten-specific ear swelling by measuring differences in thickness between challenged and unchallenged ears. **C,** Lymphocytes from ears were extracted and stained with anti- CD8, anti-CD44 and anti-CD62L. Total numbers of isolated CD8 T cells from ears of PD-1 KO and WT mice on day 40. **D,** Representative dot plots of CD8+ T cells isolated from ears (upper panel) and CD44 and CD62L expression gated on CD8+ T cells (lower panel). Numbers indicating percentages in each region **E**, Graph indicates recovered total numbers of $CD44^{hi}CD62^{lo}$ CD8+ T cells per 1 cm^2 of ear tissue from challenged and unchallenged ears. Data are representative of 3 individual experiments with at least 2 mice per group.

6.10- Attempt to induce antigen-specific CD8 T cell memory in influenza nucleoprotein (NP)-specific T-cell receptor transgenic mice (F5).

In order to investigate the role of PD-1 in antigen specific-memory induction, previously described influenza nucleoprotein (NP)-specific T-cell receptor transgenic mice (F5) were used (Mamalaki, Elliott et al. 1993). The majority of CD8+ T cells from these animals express the transgenic TCR and recognize antigen in association with the D^b MHC class I molecule. F5 TCR transgenic mice backcrossed to PD-1 KO mice, was considered to be an

suitable model in which to determine the role of PD-1 in memory generation, after the administration of the specific peptide NP68.



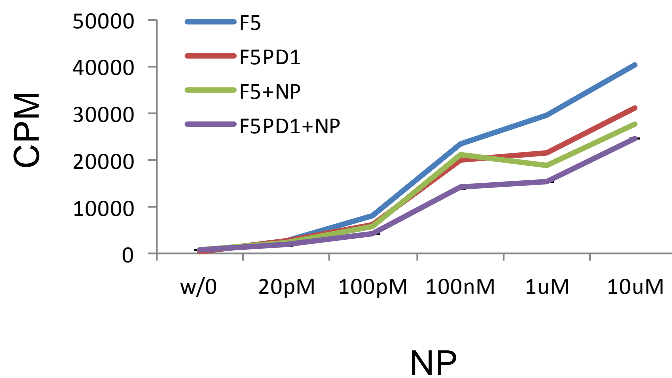


Figure 19- Attempt to induce antigen-specific memory in TCR-transgenic F5 mice.3 mo old F5 and F5 PD1 mice were injected s.c. with 75nmol of peptide together with CFA followed by a second injection of peptide with IFA on day 21. Mice were sacrificed on day 40, and F5 cells were identified as CD8+Vβ11+ and characterized phenotypically into naïve (CD44^{lo}CD62L^{hi}), T_{CM} (CD44^{hi}CD62L^{hi}), and T_{EM} (CD44^{hi}CD62L^{lo}). **B** Spleenocytes from F5 and F5.PD-1KO mice were cultured with indicated concentrations of NP for 48 hrs, thymide was added for the last 6 hours. Graph shows average thymidine incoportation (CPM, Counts per minute), Naive F5 and F5.PD-1 mice are shown for comparison. Figures are representative of 1 experiment with 2 mice per group.

Therefore, F5 and F5.PD-1KO mice were injected with peptide NP68 and complete Freund's adjuvant (CFA) subcutaneously, then 21 days later they were re-injected with NP68 and incomplete Freund's adjuvant (IFA). Mice were sacrificed on day 46, spleenocytes were stained and assessed by FACS. F5 clones were identified by anti-CD8α and anti-Vβ11 (recognizing the F5 transgenic TCR β-chain) and characterized phenotypically into naïve ($CD44^{lo}CD62L^{hi}$), T_{CM} ($CD44^{hi}CD62L^{hi}$), and T_{EM} ($CD44^{hi}CD62L^{lo}$) subsets (**figure 19**). In the absence of NP immunization ~90% of peripheral $CD8^{+}Vb11β^{+}$ T cell clones had a naive phenotype ($CD62L^{hi}CD44^{lo}$) (**figure 19A, upper panel**) consistent with the literature [171]. When comparing naive mice to NP68-injected mice, it was apparent from this analysis that very few $CD8^{+}Vb11^{+}$ T cells had differentiated into $CD44^{hi}$ cells and more specifically, there was almost no generation of T_{EM} cells, compared to non-injected control mice (**Figure 19A, lower panel**). In order to assess recall responses to NP by F5 clones, spleenocytes from immunizied mice were cultured with various concentrations of NP *ex vivo* for 48 hrs. There was no enhanced thymidine incorporation, as it would be expected by memory T cells in recall responses, compared to spleenocytes from naive non-injected control mice, regardless of genotype (**Figure 19B**). These preliminary data suggest that this injection regime of NP68 does not effectively result in adequate generation of memory T cells. The result could not be explained fully by the dilution of memory cell numbers by the constant thymic output of naive cells over a 46 day period, since similar results were found by Marvell's group in both euththymic and thymectomized F5 TCR transgenic mice [171]. Further experiments are needed to identify the reasons for the above.

A second protocol was implemented where we transferred spleenocytes from F5 and F5PD-1, containing $16x10^{6}$ $CD8^{+}Vβ11^{+}$ cells and adoptively transferred them to Rag-1 KO mice. The lymphopenic environment in the host of Rag-1 KO mice, similar to irradiated WT mice, induces LIP and activation of naive T cells. Some mice (group B) were injected subcutaneously with NP68 together with CFA on day 5, followed by a second injection of NP68 with IFA on day 26. A control group of mice (group A) were left non-immunized, in order to compare the effects of LIP to the injected group. Spleenocytes of both groups were stained and analysed on day 46. In the non-injected control group A, analysis of donor-derived CD8+Vb11β+ cells from WT and PD-1KO cells revealed that about 50% of the F5 clones had converted to activated $CD44^{hi}$ memory phenotype cells, regardless of cell origin (**figure 20A**). When comparing memory subsets between donor-derived WT and PD1-KO cells, there was an obvious skewing towards a T_{EM} phenotype in the absence of PD-1 (**figure 20A, upper panel**), a phenomenon observed in LIP of naïve ($CD44^{lo}$) polyclonal

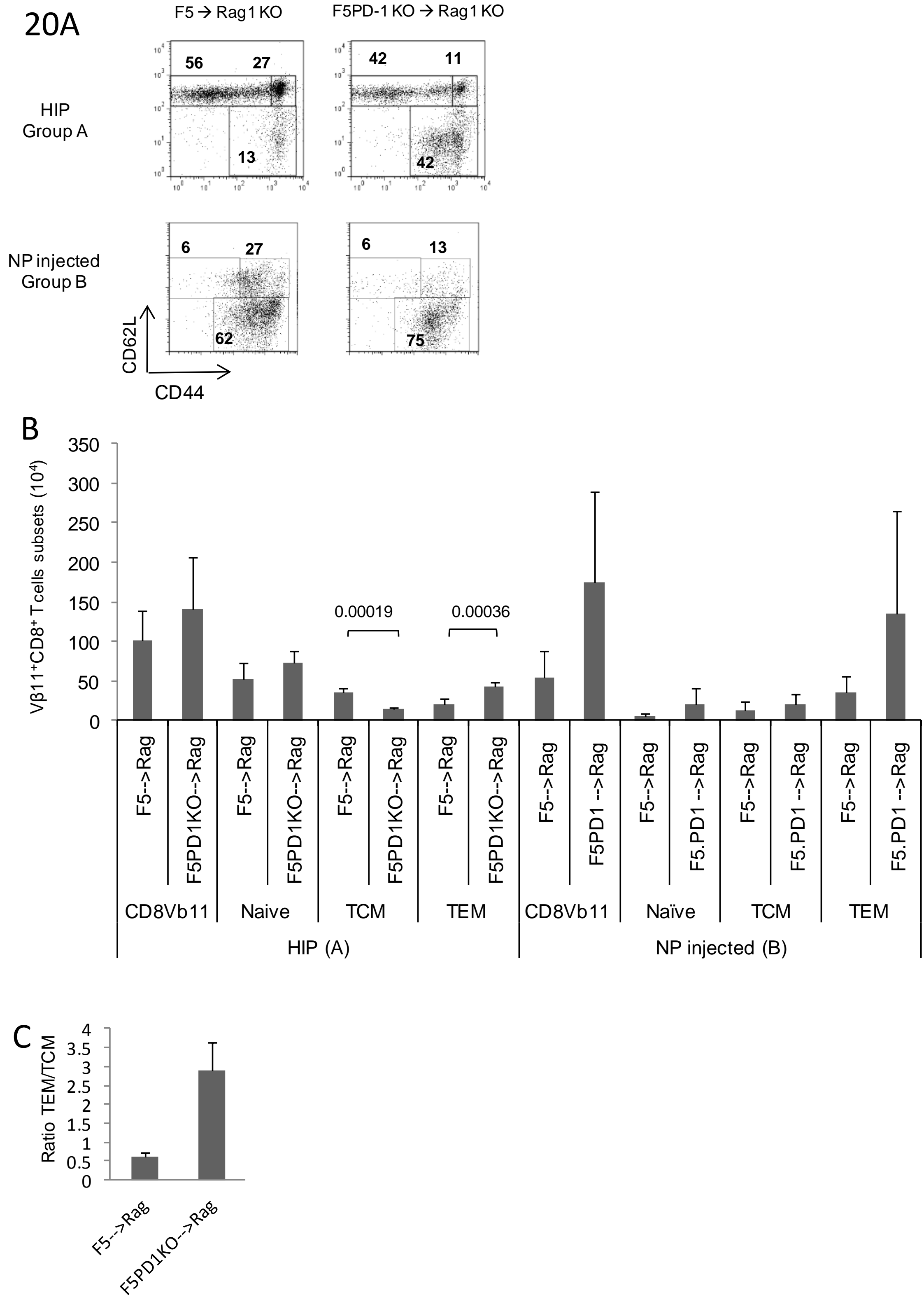
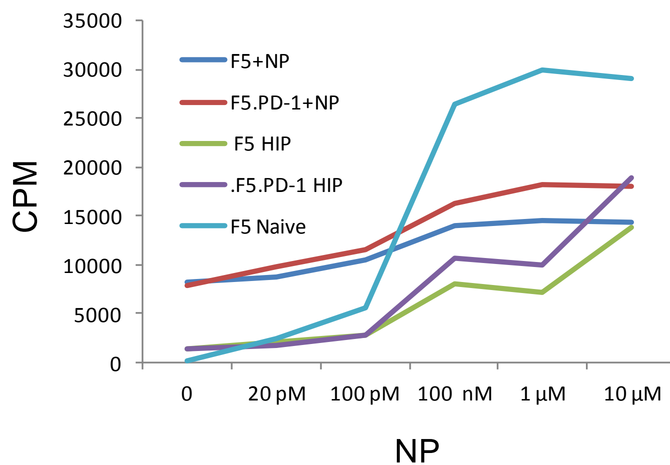


Figure 20- PD-1 signalling impedes HIP and differentiation of adoptively transferred TCR-transgenic cells into Rag-1KO mice. Splenocytes containing 16x10^{6} CD8+Vβ11+ cells from TCR-transgenic F5 and F5.PD-1 KO mice were adoptively transferred into Rag-1 KO mice. On day 5, some mice were injected with 75nmol of peptide together with CFA ,followed by a second injection of peptide with IFA on day 26(Group B). Mice were sacrificed on day 46, and donor-derived cells were identified as CD8+Vβ11β+ and characterized phenotypically into naïve (CD44^{lo}CD62L^{hi}), T_{CM} (CD44^{hi}CD62L^{hi}), and T_{EM} (CD44^{hi}CD62L^{lo}). Some mice were not injected (Group A) **A**,

Representative figure of memory subsets gated on donor derived $CD8^{+}Vb11\beta^{+}$ F5 cells from WT and PD-1KO of groups A and B. **B**, Recovery of CD8 T cells subsets from group A (left) and Group B (right). Figures are representative of 2 experiments with at least 5 mice per group. **C**, Ratios of T_{EM}/T_{CM} gated on $CD8^{+}Vb11\beta^{+}$ F5 cells from non injected mice. **D**, F5 and F5.PD-1KO donor -derived cells were cultured with varying concentrations of NP for 48 hrs ex vivo, thymide was added for the last 6 hours. Graph shows average thymidine incoportation (CPM,Counts per minute), Naive F5 is shown for comparison. **E**, Percentage of IFNγ production by donor -derived WT and PD-1KO cells from group B, incubated ex vivo with and without NP for 5 hrs. Data represent values of individual mice from one experiment, n=5.

CD8 T cells (**figure 10B and C**). When examining total recovered donor-derived cells, despite being no significant difference in numbers of $CD8^{+}V\beta11^{+}$ cell numbers (**figure 20B**), there was a 2 fold increase in T_{EM} donor-derived cells from PD-1 KO compared to WT mice ($42X10^{4}\pm7.2$ vs $21X10^{4}\pm6.6$, p=0.0003, **figure 20B**) and a corresponding decrease in number of T_{CM} phenotype cells ($15X10^{4}\pm2.4$ vs $35X10^{4}\pm6.1$, p=0.0002, **figure 20B**). Therefore the ratio of T_{EM}/T_{CM} compared to F5 controls had clearly been altered from 0.6, for the WT cells compared to 2.9, in the absence of PD-1 (**figure 20C).** When comparing mice from Group B, that had received NP68 injections, there was no significant increase in numbers of T_{EM} cells, compared to T_{EM} cells from non-immunized F5 mice in group A ($35.9X10^{4}\pm20.2$ vs $19.2X10^{4}\pm7.7$, p=0.078, **figure 20B).** When comparing WT and PD-1 KO cells in host spleens there was a skewing towards a T_{EM} phenotype in the PD-1 KO mice (**figure 20B).** However these differences were not significant, due to the extent of variation between mice within each group. This could be possibly attributed to the varying success of the emulsification process of CFA with NP68 between experiments. In order to assess memory recall responses in these mice, spleenocytes from injected and non-injected mice were placed in culture with various concentrations of NP68 for 48 hrs. When thymidine incorporation by these cells was assessed, it was clear that cells were cycling, even in the absence of added NP68 in culture of cells from group B mice. (**figure 20D**). This was perhaps due to residual NP found in mice after injections, even at this late time point, since spleenocytes from mice in group A (which had not received the injection of NP) were not cycling.

D



E

	W/O NP	With NP	Fold Change
F5 donor cells	0.79%	4.76%	6.03
	2.36%	9.49%	4.02
	8.89%	10.63%	1.20
	3.20%	6.00%	1.88
	5.89%	6.25%	1.06
F5.PD-1 donor cells	0.69%	2.33%	3.38
	2.66%	3.77%	1.42
	2.53%	6.23%	2.46
	0.83%	2.55%	3.07
	0.97%	1.15%	1.19

Similarly, when analysing percentage of IFN-γ by donor-derived cells *ex vivo*, F5 clones were able to produce IFN-γ even when NP68 was not added *in vitro*. (**Figure 20E**). Therefore recall responses to NP in these cells were difficult to assessed or interpret accurately. Despite this, there were no differences in fold change between F5 and F5PD-1 KO donor derived cells (**Figure 20E**). Moreover, since the mice in group A had already significant differences in numbers of T_{CM} and T_{EM} cells between PD-1KO and WT donor-derived cells, we were unable to distinguish the effect of LIP from the additional affect of NP, on memory generation.

7. DISCUSSION

In this study, we describe a previously unrecognized role of PD-1 in memory-phenotype T cell formation and particular in shaping MP subset development. More specifically, we found an increase in CD8 and CD4 $CD44^{hi}$ T cell numbers in the absence of PD-1 (Figure 1B) and in particular we identified a substantial increase in $CD44^{hi}CD62L^{lo}CCR7^{lo}$ T cells, categorized as T_{EM}-phenotype cells [14] in spleen and tissues and even lymph nodes of PD-1 KO mice (Figure 1, Figure 2). This phenomenon was more prominent with advancing age (Figure 1C) and could not be attributed to advanced basal proliferative capacity of T_{EM} cell's (Figure 7). The number of IL-7Rα ^{hi} (CD127) and IL-2$Rα^{hi}$ (CD122) T_{EM}-phenotype CD8 T cells was considerably higher in PD-1 KO spleens (Figure 5D), consistent with a memory-phenotype [15, 43, 86]. CD69 is considered an early activation marker and it could be argued that this could better characterize an effector, rather than a T_{EM} phenotype. However, while a proportion of PD-1 KO T_{EM}-phenotype CD8 T cells express CD69 (Figure 5C) the majority should not be recently activated cells, because no IL-$2R^{hi}$ (CD25) subpopulation was identified (Figure 5C). Moreover, recently activated, typical effector cells would decay fast in a 42-day period, something not observed in our experiments (Figure 14B). These accumulated T_{EM} cells, in the absence of PD-1, also seem to have enhanced effector memory characteristics as shown by higher expression of GzmB directly *ex vivo* (Figure 6A) and IFN-γ after short activation with phorbol esters (Figure 6B).

It still remains largely unclear why a proportion of naive T cells differentiate into memory-phenotype cells in naive unimmunized mice. Some potential mechanisms involved in the conversion of naïve T cells to MP cells have been speculated [49]. It is generally thought that MP cells are generated in response to self- rather than foreign antigens and also via homeostatic proliferation mechanisms. Sprent and colleagues have defined two distinct subsets of CD8 MP cells; those that are dependent on MHC class I interactions for their formation and survival and those that are driven more by homeostatic mechanisms and dependent on IL-15 [161]. They suggested that MP cells that are maintained via TCR interactions are generated as a result of chronic TCR triggering in response to self-antigen recognition. It has become more appreciated how important tonic TCR self-antigen MHC signals are for the maintenance of naive T cell viability. As naive T cells are resting cells, the intensity of TCR signalling is presumed to be below the threshold needed to induce activation. Therefore subtle alterations in the intensity of tonic TCR signalling may play a role in their activation. Upon the removal of the break by PD-1 on TCR signal transduction it is possible that these weak TCR signals become stronger and capable of T cell activation, proliferation and acquisition of MP phenotype.

Co-stimulatory and co-inhibitory molecules have been shown to regulate memory T cell development, with a consensus that co-stimulation promotes formation of antigen-specific or MP cells whereas co-inhibition impedes it. In line with the above, the loss of another co-inhibitory molecule BTLA-4 resulted in the increased number of MP cells [23]. However, variable data exist on correlation between TCR-signal strength modulated by positive and negative co-stimulators and developmental fate towards T_{EM} and T_{CM} subsets. For example, while enhancement of TCR signals by OX-40 [25] and ICOS [20] promote accumulation of effector memory T cells, stronger TCR signals in the absence of BTLA lead to accumulation of central memory T cells [23]. Our results, which show that ablation of the PD-1 pathway drives MP CD8 T cells preferentially to T_{EM}-phenotype, are in agreement with the notion that increased signal strength [14] and/or duration [18] favors skewing towards T_{EM} subset (see figure 3.4 -Models for generating effector and memory T cell heterogeneity).

A recent study showed that vaccinia virus-specific PD-1 KO CD8 T cells are skewed towards T_{CM} after acute infection [127]. This does not conflict with our data since it has been shown that the type of pathogen affects memory differentiation pathways, with vaccinia-virus (but not LCMV) typically leading to fast emergence of T_{CM} CD8 T cells [172]. Moreover, in most acute infections CD8 T cells rapidly stop encountering antigen (for vaccinia virus, infection is fully resolved within 2 weeks) [173] and without any circumstantial or deliberate re-stimulation, typically the majority of antigen-specific memory cells belong to the T_{CM} subset. On the contrary, repetitive/continuous stimulation, either by infection or vaccination [174-176] promotes generation of cells belonging to the T_{EM} subset; repetitive antigenic stimulation has been shown to induce progressive decrease of CD62L surface expression [176]. Masopust et al demonstrated, using three different prime-boost vaccinations regimes, that repeated immunization led to the preferential accumulation of T_{EM} cells [177]. This suggests that memory CD8 T cell differentiation is influenced by the cumulative history of Ag experience. Importantly, in the settings of acute infection, PD-1 is shown to be expressed only transiently on CD8 T cells, whereas on chronically stimulated cells, sustained expression is observed [131, 178]. Freeman et al have demonstrated that PD-1 inhibition is greatest at lower amounts of antigen which suggests that PD-1 might be more effective at attenuating weak TCR signals rather than strong ones [179]. A study by Goldberg et al. compared PD-1 expression in different contexts (in response to self verses infectious antigens) and showed that PD-1 expression upon self-antigen is rapidly up-regulated, while minimal upon encounter with microbial antigens. They suggested that pro-inflammatory signals associated with microbial infection suppress PD-1 expression on CD8 T cells encountering antigen. The absence of these signals in the context of self-antigen encounter allowed for rapid PD-1 up-regulation [180]. In line with this, PD-1 expression was found to be highest on WT CD8 T_{EM}

phenotype cells (figure 5E), the MP subtype most effect by the removal of the PD-1 break. Therefore, with a different mode of PD-1 signaling (i.e. transient vs. sustained) transition to different memory developmental pathways may take place. So, it is probable that settings of acute infection [127], on one hand, and response to a plethora of antigens –many of them self and repetitively encountered- on the other, could have a different impact on memory fate of PD-1 KO CD8 T cells.

A further possible explanation for the acquisition of T_{EM} MP cells in the absence of PD-1 is that there are alterations in transcription factors that regulated the generation of MP subsets. KLF2 (Krüppel-like factor 2) has been proposed as an important TF that regulates migratory activity of T cells by regulating the expression of CD62L [181-182]. Strong signals promotes efficient PI3K activation and blocks KLF2 activity which results in shedding of CD62L and an acquisition of a T_{EM} phenotype, while weaker ones permits KLF2 re-expression and therefore CD62L, resulting in T_{CM} phenotype [183-184]. Additionally, FoxO (Forkhead box O) transcription factors may also contribute to this regulation; FoxO1 promotes expression of KLF2 in mature T cells. Our microarray results on purified T_{CM} PD-1KO CD8 T cells show a decreased expression of FoxO3 compared to WT cells; importantly Fox01 and FoxO3 TF have been shown to have some overlapping functions [185]. Additionally, FoxO3 has been shown to intrinsically regulate the development of CD8 memory T cell; a higher number of memory cells were detectable in FoxO3a KO mice compared with WT mice [186] all thought analysis of subsets was not assessed in this study. Evaluation of phosphorylated-Akt, -FOXO-1/3a or KLF2 mRNA, after incubation of WT and PD-1 KO cells with various stimuli (TCR triggering and/or cytokines) and how these correlate with CD62L expression, would help to assess the role of these TF in the acquisition of T_{EM} phenotype found in the PD-1 KO mice.

As mentioned above, a proportion of memory phenotype cells have been found to be preferentially dependent on homeostatic cytokine IL-15 for their proliferation, survival and bystander activity. These MP cells seem to be MHC-independent, since they survive in MHC-class I deficient mice for long periods of time and so are thought to be maintained and regulated primarily via cytokines [161]. They are highly sensitive to local changes in concentration of γ-chain cytokine, and high levels can trigger their proliferation. Importantly, our results from mixed bone marrow transplantation experiments (Figure 11), adoptive transfer of T_{CM} (Figure 12) CD8 T cells and transfers of naïve cells to lymphopenic hosts (Figure 11) strongly indicate that the accumulation of PD-1 KO T_{EM} phenotype cells, is at least partly, a CD8 T cell-intrinsic effect. These findings argue against the changes in cytokine concentrations in the host as the underlying cause of increased memory-phenotype T_{EM} cells found in the PD-1 deficient mice. However, it does not rule out the altered

expression of γ-chain cytokine receptors on MP cells, and therefore their altered responsiveness to these cytokines as a possible mechanism for increased T_{EM} cells found in the absence of PD-1 (discussed further below).

MP cells closely resemble memory T cells generated under lymphopenic conditions (LIP-memory cells). Homeostatic proliferation (HP) of naïve T cells is a further mechanism that results in the acquisition of typical MP cells and this occurs both in lymphopenic, and in lymphosufficient hosts. Given the abundance of MHC–self peptide ligands that can trigger both positive selection of T cells in the thymus and homeostatic proliferation in the periphery, it is of great importance to understand how homeostatic proliferation is controlled and appears self-limiting. We show that the PD-1 pathway plays a vital role in regulating this process, since CFSE profiles of adoptively transferred naïve PD-1 KO CD8 T cells into lymphopenic mice showed that these cells divided more compared to WT cells (figure 10F). Although LIP and HP in lymphsufficient hosts differ with regards to the concentrations of γ-chain cytokines, present in higher levels in the former, they are both triggered by similar mechanisms; an anti-self response directed to various self-pMHC complexes and this triggering results in augmented responsiveness to γ-chain cytokines [49, 90, 97]. Therefore this data reveal a vital inhibitory checkpoint via PD-1 signalling that controls the pace of LIP which could account partly for the increase in MP cells found in the PD-1 KO naive mice. Furthermore, it has been described that the majority of naïve T cells undergoing lymphopenia-induced proliferation acquire a central memory-phenotype [3, 29] and this can be also seen from our WT data (10A-C) and by others [23]. Importantly, LIP of adoptively transferred naïve PD-1 KO CD8 T cells gave rise to large numbers of T_{EM}-phenotype cells compared to WT (Figure 10B-C) in the spleen at day 20. This illustrates that PD-1 again shapes memory-phenotype CD8 T cell subsets under conditions of lymphopenia.

Numerous models have been proposed to explain the lineage relationship of T_{EM} and T_{CM} antigen-specific memory T subsets (see figure 3.4 Models for generating effector and memory T cell heterogeneity). Even less is known about MP subset ontology, however, depending on the settings, both T_{CM}→T_{EM} [187] and T_{EM}→T_{CM} [23] have been documented. Interestingly we found when we transferred naïve CD8 T cells into irradiated hosts, T_{CM} cells appear first (day 5, Figure 10A), followed by substantial accumulation of T_{EM} cells in blood of PD-1 KO (day 20, Figure 10A) and in spleen (Day 20, Figure 10B) which does not take place in WT donor cells to the same extent. Due to the fact that T_{EM} cells accumulated at the expense of T_{CM} cells in the PD-1 KO during LIP (there was a drop in T_{CM} numbers with a paralleled increase in T_{EM} numbers, figure 10C) strongly suggests that there is lineage relationship between T_{CM} and T_{EM} cells in this setting.

Taken together, these data imply that increased duration of signal, in the absence of PD-1, favors T_{EM} differentiation, and this is most probably through a T_{CM} intermediate cell. These observations correlate well with the massive $T_{CM} \rightarrow T_{EM}$ conversion (Figure 12A and 12B) of transferred purified CD8 PD-1 KO T_{CM} cells in lymphosufficient mice where we provided "extra time" and therefore chances for the transferred cells to receive repeated stimuli, inside the host, to differentiate to T_{EM} cells. Our microarray results on purified CD8 PD-1 KO T_{CM}-phenotype cells, that exhibit a discrete expression profile compared to WT counterparts (Figure 16), further reinforcing the concept of T_{CM} cells being aberrant in the absence of PD-1 (discussed further below).

In adoptive transfer experiments of purified CD8 T_{EM} MP cells into lymph sufficient hosts, we found reduced $T_{EM} \rightarrow T_{CM}$ conversion in the absence of PD-1 (figure 14) indicating that PD-1 signaling regulates the inter-conversion between these MP subsets by inhibiting $T_{CM} \rightarrow T_{EM}$ conversion while promoting the reverse. Additionally, we found that culturing purified CD8 T_{EM}-phenotype WT cells with IL-15 resulted in moderate conversion to T_{CM} cells, while reduced conversion was seen again in PD-1 KO cells (figure 15). Differences in IL-2Rβ expression between the PD-1 KO and WT T_{EM} cells and therefore responsiveness to IL-15, could account, at least in part, for these differences in MP subset conversion found in the current study. In support of the above findings, it was shown that activated CD8 cells cultured in a high dose of IL-2 differentiated into T_{EM}, and those cultured in IL-15 (a less strong signal) became T_{CM} cells [188].

Naïve T cells undergoing LIP have been shown to acquire a memory-phenotype and become capable of mediating protective immunity against pathogens [99]. Importantly, we show that a much larger fraction of PD-1 KO-derived T_{EM} cells were $GzmB^{hi}$ when assayed directly *ex vivo* (Figure 10D). It could be argued that some PD-1 KO-derived T cell clones recognize self-antigens with high affinity, and being autospecific, acquire their abnormally "activated" T_{EM} phenotype during LIP. However, it does not seem to be the case since abnormal T_{EM}/T_{CM} ratios were also observed when TCR-transgenic F5.PD-1 KO CD8 T cells (which recognize an influenza nucleoprotein epitope) were transferred to Rag-1 KO lymphopenic recipients (Figure 20A) where it is highly improbable that F5 cells could recognize their cognate antigen in the host mice.

We found an increase in IL-7$R\alpha^{hi}$ T_{EM}-phenotype CD8 T cells in the absence of PD- 1 when considering total numbers (figure 5). Memory precursor effector cells (MPEC), generated during acute infections, are identified by the expression of IL-7Rα [43] and lack KLRG1 (killer cell lectin-like receptor sub-family group 1), a marker for short lived effector cells (SLEC). SLEC undergo apoptosis during the contraction phase while MPEC are long lived and have

stem-like renewal properties. The generation of effector CD8 T cell subsets varies greatly among different infection settings due to the fact that they are highly affected by the amount of inflammatory mediators and therefore affect the memory T cell pool [33]. However, it is still not clear if MPEC's are generated in the same way durring MP cell formation, and it has even been proposed that MP cells are formed without passing throught an effector phase [3, 29]. Thus, it is unlikely that these data represent altered numbers of memory precursor cells in the PD-1 KO mice compared to WT mice. Studies to assess if PD-1 effects the memory precursor cells formation early on during an immune response could only be achieved in an antigen-specific model of memory T cell formation, where analysis with KLRG1 could be undertaken to clarify more precisely the contributions of SLEC and MPEC populations.

At which points during the generation of memory T cells is the inhibitory role of PD-1 important? It is readily regarded that memory heterogeneity and phenotypic differences are directly related to the degree of antigenic stimulation, co-stimulatory signals, division history, cytokine exposure and other priming-associated events. Only 24 hrs of stimulation appears adequate to elicit an instructional program of clonal expansion, expression of effector functions and differentiation into memory cells [189]. It is important to note that T-cell programming refers to the process by which events during priming imprint heritable patterns of gene expression on T cells. PD-1 most likely asserts its breaks at initial priming events, since PD-1 is expressed on activated T cells 24 hrs after TCR stimulation [190] and more importantly PD-1 is reported to be induced on T cells before their first division following antigen encounter *in vivo* [180]. This data would argue that PD-1 is certainly in the 'right place and the right time' to assert its break on T cell priming. Importantly PD-1 may help to negatively regulate formation of stable and productive immunological contacts [120]. Therefore, in its absence T cells could form more stable interactions resulting in stronger signals and more activation towards T_{EM} phenotype. Interestingly, a study by Goldberg et al. demonstrated the role of PD-1 in early fate decisions of CD8 T cells; PD-1 blockade being critical during the priming phase of a CD8 T cell response rather than the effector phase [180]. While other studies have demonstrated the importance of PD-1 inhibition at the effector phase [131] our data from adoptive transfer of naive polyclonal (Figure 10) and naive F5 transgenic T cells (figure 20A, group A) demonstrate a clear inhibitory role of PD-1 during LIP of naïve T cells. Due to the inability to induce antigen-specific memory CD8 T cell subsets in the F5 mice model, with the current immunization regime, we were unable to identify more precisely the role of PD-1 on memory formation, as discussed further below. It is generally accepted, and incorporated into several models of memory cell generation (see figure 3.4- Models for generating effector and memory T cell heterogeneity), that all phases of an immune response (activation, expansion, differentiation, contraction and memory

formation) are programmed shortly following antigenic stimulation in a cell autonomous manner. Therefore, it is difficult to identify the exact time point during which PD-1 asserts its break on memory formation. However, these models do not exclude the potential influence that other factors might have on memory T cell development after the initial priming events.

Factors during the weeks/months following priming, including accessibility to continued antigen and various co-stimulatory molecules, cytokine availability, chemokines, and anatomical location also have qualitative and quantitative influences on the developing memory T cells [191] [189]. Our results from transfers of purified populations of CD8 T_{CM} (figure 12) and T_{EM} (figure 14) MP cells suggest that PD-1 signalling is also important in regulating the interconversion of these MP subtypes. Further experiments would address the issue of whether the fate of PD-1 KO-donor CD8 MP subsets was already predetermined at the time of transfer or post-transfer intervention on WT MP subsets would be sufficient to promote differentiation. Anti-PD-1 blocking antibodies administered at time of WT T_{CM} transfer would help to clarify this issue. However, our microarray results, showing a discrete expression profile on PD-1 KO CD8 T_{CM} cells (Figure 16) argues in favor of the first scenario.

Although memory T cells are relatively stable, they nonetheless undergo changes in phenotype, function and location over time [192]. As discussed previously, repeated re-exposure of antigen by memory T cells results in a gradual loss of CD62L and the adoption of a T_{EM} phenotype. Anatomical location and the tissue microenvironment itself might contribute to the adoption and maintenance of different memory phenotypes [192] [189]. For example, different tissues affect MP subsets differentially with respect to available cytokines, co-stimulatory ligand expression, immune accessory cells and antigen persistence. In addition, when circulating T_{CM} memory cells enter skin, liver, lung, and intestinal mucosa sites, they shed CD62L, upregulate 'tissue-specific' markers and acquire effector functions and protective abilities upon encountering distinct environmental cues [193] [194]. This is biologically significant, since it is important for the host to be able to adapt to the changing conditions during an immune invasion and regulate T cell migratory properties and therefore protection.

CD62L plays a pivotal role in the T cell homing and initial tethering of leukocytes to the endothelium and to other leukocytes. It could be argued that shedding of CD62L in PD-1 KO cells results in their altered location and therefore altered secondary responses; the current paradigm proposes that strict regulation of effector CD8 T cell access to lymph nodes is essential for functional immune responses. It is thought that T cells that home frequently to lymph nodes during their transition from effector to memory stages preferentially adopt T_{CM} phenotype and functional attributes. However, another study has shown that this is CD62L

independent [195]; memory CD8 T cell development and memory lineage commitment was found to be unaltered by manipulations that enhanced or prevented CD62L-mediated lymph node homing. However, it is unclear if loss of CD62L expression and subsequent reduction of T cell homing to lymph nodes is a consequence rather than the cause of more T_{EM} differentiation found in the PD-1 KO mice. In the present study PD-1 KO T_{EM} cells were found to be accumulated in all tissues examined (Figure 3). However, the fact that PD-1 KO T_{EM} cells were accumulated also in blood, spleen and mesenteric lymph nodes, argues in favour of the former. This suggests that accumulation could not be attributed just to a global alteration in T cell trafficking, neither solely to the dysregulation of CD62L, since CCR7 was also downregulated on the accumulated T_{EM} cells (figure 4). As discussed above, CD69 was found to be expressed higher on T_{EM} cells from PD-1 KO mice in spleens. Notably CD69 has also been used as a marker for resident memory T cells (T_{RM}) [47]. It would be interesting to assess CD69 expression in conjunction with CD44 and CD62L from all tissues to assess the contribution of T_{RM} in the accumulated T_{EM} fractions, found in the absence of PD-1.

Apart from their role in driving HP, γ-chain cytokines have also been shown to play a major role in the maintenance of memory-phenotype CD8 T cells, by supporting their survival. Importantly, T_{EM}-phenotype CD8 T cells from middle aged PD-1 KO mice survived better compared to WT counter parts, while the opposite was found for T_{CM}-phenotype cells (Figure 8). Homeostatic cytokines are important for providing survival signals to MP cells by regulating the balance of pro- and anti-apoptotic molecules. It would be interesting to assess the levels of the apoptotic molecules in PD-1KO T_{EM} cells. Models to explain how memory CD8 T-cell numbers are maintained have not been experimentally addressed. A recent study identified a unique population of CD8 T_{CM} cells called T_{DIMs} (death intermediate memory T cells) within a population of TCR-Tg memory and endogenous memory cells, following memory generation to LCMV infection [196]. They showed that these non-functional T_{DIM} cells originate during homeostatic turnover of T_{CM} cells, have a reduced proliferative capacity, a $CD62L^{lo}$ $CD27^{lo}$ phenotype, and bind highly to annexin V, compared to $CD62L^{hi}$ T_{CM} counterparts. They propose a model, to explain stable memory cell numbers, in which a dividing T_{CM} generates a daughter cell with 'self renewing' properties and a T_{DIM} cell that ultimately dies. Given that CD62L is also a marker used to discern T_{CM} from T_{EM} cells, the authors distinguish T_{DIM} cells from T_{EM} cells, since T_{DIM} were unable to produce effector molecules, such as IFNγ, a prerequisite for T_{EM} phenotype cells. In our study, the percentage of T_{EM} cells in the WT that produce IFN-γ upon TCR stimulation is less than 50% (see figure 6B), therefore it could be possible that some $CD62L^{lo}$ cells in fact represent non-functional T_{DIM} cells. Interestingly, when examining cell death potential in CD8 T_{CM} and T_{EM} subsets from PD-1 KO mice, we found an inverse correlation between CD62L expression

and annexin V binding (figure 8B); as T_{CM} cells lost CD62L expression they increased their ability to bind with annexin V. Ultimately, the T_{EM} cells of PD-1 KO mice were less annexin V^+ compared to WT cells. Thus in the absence of PD-1, T_{EM}-phenotype CD8 T cells were rescued from cell death. Perhaps some of the cells we identify as T_{EM} cells could be in fact T_{DIM} derived from T_{CM} turnover, and in the absence of PD-1 negative signals survive better compared to WT counterparts. This would correlate with the documented capacity of PD-1 signaling to induce T cell apoptosis [197]. However, the fact that upon transfer of $1.5x10^5$ purified T_{CM}- or purified T_{EM}-phenotype PD-1 KO cells we recovered similar numbers ($\sim 1x10^5$) of PD-1 KO T_{EM}-phenotype cells (Figure 12B, 2nd column vs. Figure 14B, 2nd column) strongly implicates increased rates of $T_{CM} \rightarrow T_{EM}$ conversion as the major determinant of PD-1 KO T_{EM}-phenotype cell accumulation, rather than enhanced survival alone. This suggests that the enhanced survival is not the major mechanism for the accumulating T_{EM} population found in the PD-1 KO mice, but does not rule it out as having an effect.

Overall, the emerging picture is that naïve WT or PD-1 KO CD8 T cells encounter antigens (commensal, environmental or self-antigens) in the periphery of an unimmunized mouse and undergo priming and/or homeostatic proliferation; many of these initially acquire a T_{CM} phenotype, which in PD-1 KO cells is aberrantly transient and a large proportion of them develops stable characteristics of T_{EM} cells. In addition to that, the resulting PD-1 KO T_{EM}-phenotype cells have a moderate survival advantage over the WT ones (Figure 8A) thus further intensifying the effect of enhanced conversion.

Importantly memory phenotype T cells can develop and expand in an antigen-independent manner via bystander activation and homeostatic driven proliferation mechanisms. Armed with their ability to home to tissues and mediate rapid effector responses, it is of vital importance to balance the protective role of memory T cells and their potential over-aggressive responses that can result in pathology. Importantly, we showed that T_{CM}-phenotype PD-1 KO CD8 cells produced more IFN-γ per cell after an innate stimulus (Figure 17A) therefore suggesting they are more sensitive to bystander activation. IFN-γ production by memory T cells have profound, wide spread effects on innate and adaptive immune responses such as enhanced antigen presentation and promoting pro-inflammatory Th1 immune responses. Considering this data and the expression profile of PD-1 ligands (Figure 3.6-Relative expression of PD-1 and its ligands), it would suggest that PD-1 signaling plays a pivotal role in regulating memory T cells responses. Furthermore, dysregulation during homeostatic proliferation can potentially result in autoreactive memory T cells [100] [101] [102]. The more tissue-specific autoimmune phenotypes of PD-1 KO mice at a late age [139] [137, 198] contrast markedly with the multi-organ autoimmunity observed within the first few

weeks of birth for CTLA-4 KO mice [199]. These findings support the idea that PD-1 is part of a system that fine-tunes immune responses, in contrast to the "on-off switch" mediated by the B7-1/B7-2-CD28/CTLA-4 system. It is of notice that compared to respective WT T_{EM} phenotype CD8 T cells a much larger fraction of PD-1 KO LIP T_{EM}-phenotype cells produces high levels of GzmB *ex vivo* (Figure 6A). Given the fact that most of these cells recognize self-ligands, although with low affinity [92], it is reasonable to think that they could have an autoreactive potential. In line with this hypothesis, Thangavelu *et al.*, although not examining GzmB expression on T cells, have shown in a recent report that PD-1 KO recent thymic emigrants cause a lethal autoimmune-like disease in chronically lymphopenic hosts [200]. In the light of the above, it would be interesting to extending the time course of our bone marrow transplantation studies to assess this lethality.

Due to the fact that the antigen, time of activation and differentiation into MP cells are all unknowns in the polyclonal mice, we used two different mouse models to attempt to delineate the role of PD-1 in the generation of CD8 T memory cells to a specific antigen. The first model we used was the contact hypersensitivity reaction (CHS) to the hapten 2,4-dinitrofluorobenzene (DNFB). An earlier study demonstrated that the blockade of PD-1 with anti-PD-1 mAb, at sensitization enhanced and prolonged the ear swelling induced by the hapten challenge, suggested a regulatory role for PD-1 in CHS at the effector phase [201]. We show that PD-1 is also important in CHS responses at the memory phase by extending the course of the study and also include a challenge time point after the effector phase that corresponds to the memory phase in other experimental systems [157]. The superior generation of an Ag-specific memory T cells in the PD-1 KO mice was manifested by the more substantial ear swelling following recall exposure to DNFB (figure 18B). CD8 T_{EM} are most likely responsible for this secondary immune response and in the absence of PD-1 these responses are heightened, with increase numbers of infiltrated T cells (figure 18 B and C). More studies would be needed to clarify firstly, if the T_{EM} cells were already present after the first challenge (on day 5) or if they were recruited during the recall phase of the immune response, after the second challenge (day 38).

Unfortunately, we were unsuccessful in our attempts to dissect the exact time frame where PD-1 signaling on CD8 T cells is sufficient to impose a break towards a T_{EM}-phenotype, with the current immunization regime in TCR Tg F5 mice (figure 18). Our preliminary results from F5 mice, resulted in the generation of very few $CD44^{hi}$ cells after injection with NP in either F5 nor in F5.PD-1 KO mice (figure 19). Marvel et al have also shown similar results; a small proportion of $CD44^{hi}$ T cells are present in naive F5 mice and this percentage does not increase after one NP injection. They showed that a second injection, within a 24hr time period, doubled these numbers [171] however, the majority of the $CD44^{hi}$ cells that resulted

after immunizations gave rise to a predominantly T_{CM} phenotype. Additionally, the 3-5% of cells found in F5 naive mice, which the authors describe as $CD44^{hi}$ $CCR7^{lo}$ T_{EM} cells were heterogeneous in terms of antigen specificity and were NP-independent. Moreover, these numbers did not increase after single or double injections with NP. These naturally occurring MP cells have been shown to be present in other TCR Tg mice and even in Rag-1 KO backgrounds [97]. These data suggest that NP-specific memory CD8 T cells with a T_{EM} phenotype are not generated in these priming conditions and further studies with alternative priming conditions are required. There are several possible explanations for these findings. Firstly, TCR affinity plays a major role in the generation of memory T cell subsets and perhaps the F5 T cells affinity for the cognate antigen is too low for adequate priming and the acquisition of a T_{EM} phenotype. Secondly, it has been shown that in situations of high antigen competition, for example as a result of low DC:T cells ratio, the majority of the resulting memory T cells are a T_{CM} phenotype [202]. Due to the fact that most of the T cells in the F5 mice are of one specificity, this could result in a high competition for antigen and could result in the differentiation to T_{CM} phenotype cells. Additionally, memory CD8 T cells developed in CD4 deficient animals contain an increased proportion of T_{CM} cells [203]. Although F5 TCR Tg mice contain CD4 cells they are in much lower frequency compared to polyclonal mice. This was presumably due to the lack of maturation signals to DC, which has also been shown to result in the generation of a memory population that is skewed towards a T_{CM} phenotype. Thus, despite the use of CFA in our priming experiments, it appears insufficient in generating mature DC. This could also explain the proliferation results, since 'helpless' CD8 T cells have also been shown to have defect recall responses (figure 19B). Therefore, although the use of TCR Tg systems have clear advantages, a variety of possible factors influenced the development of T_{EM} cell in the F5 Tg mice and therefore limits the approaches effectiveness in studying the role of PD-1 on memory T cell formation.

When examining CD4 T cells in polyclonal naïve mice, MP cells were also found to be increased in the PD-1 KO mice compared to WT counterparts and the increase in numbers were primarily within the T_{EM} subset (Figure 1 and 2). It has been demonstrated that CD4 and CD8 T cells have distinct co-stimulatory requirements for memory generation [204]. These data, albeit preliminary, suggests that PD-1 signalling can also affect CD4 T cell memory subset development. A full and precise analysis of phenotype, gene expression and functional differences of CD4 T cell memory subsets in the absence of PD-1 would be highly informative, and is presently underway. Additionally, a sub-goal would be to investigate whether the normal balance between Th1, Th2 and Th17 lineages has been perturbed in memory CD4 T cells from PD-1 KO mice, an imbalance which often triggers autoimmune reactions. A CD4 T cell adoptive transfer model of colitis [205-206] could be adopted since it

is a widely used model to dissect the initiation, induction, and regulation of immunopathology mediated by CD4 T cells. This model provides an easy and effective way to identify the reactive potential of T_{EM} cells and we hypothesis that the onset and severity of colitis would be more severe upon transfer of T_{EM} cells that lack PD-1.

In conclusion, our results show that PD-1 signaling in CD8 T cells can modulate the homeostasis of memory-phenotype pool by inhibiting differentiation towards a functional T_{EM}-phenotype, most probably through a T_{CM}-phenotype intermediate. PD-1 signaling in CD8 T cells also promotes $T_{EM} \rightarrow T_{CM}$ conversion (Figure 14). In the absence of PD-1 CD8 T_{EM}-phenotype cells survive better than WT counterparts (Figure 8A) thus further increasing the effect of enhanced conversion found in the PD-1 KO mice. Accumulated T_{EM}-phenotype cells harbor potent functional properties (Figure 6A and 6B) and this could result in altered host responses against pathogens, environmental or self-antigens in the absence of an intact PD-1 pathway. Additionally, PD-1 KO MP CD8 cells may elicit superior bystander protective responses against pathogens as suggested by LPS-driven IFN-γ production, especially by T_{CM}-phenotype cells (Figure 17A). These findings can be clinically important especially in the settings of currently developing treatments with antagonistic anti-PD-1 or anti-PD-L1 antibodies in cases of certain malignancies or chronic infections [132, 207]. Equally important, manipulation of PD-1 pathway could enhance efficacy of certain vaccination regimens where production of T_{EM} cells is critical [174, 208]. Further studies may include a more precise analysis of accumulated antigen specificities as well as the exact time frame where PD-1 signaling on T cells is sufficient to impose a break towards T_{EM}-phenotype differentiation in naïve or immunized mice.

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Programmed Death-1 Shapes Memory Phenotype CD8 T Cell Subsets in a Cell-Intrinsic Manner

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Memory phenotype T cells, found in unimmunized mice, display phenotypic and functional traits of memory cells and provide essential protection against infections, playing a role in both innate and adaptive immune responses. Mechanisms governing homeostasis of these memory phenotype T cells remain ill-defined. In this study, we reveal a crucial role of the negative costimulator programmed death-1 (PD-1) in regulating developmental fates of memory phenotype cells. Thus, in lymphoid organs and tissues of PD-1 knockout (KO) mice a marked accumulation of functional effector memory (T_{EM}) phenotype CD8 T cells was observed. T_{EM} phenotype cells from PD-1 KO mice exhibit decreased proliferation but increased survival potential. These cells could produce effector molecules constitutively, in response to phorbol esters or through bystander activation by innate stimuli. Similarly, in lymphopenia-induced proliferating CD8 T cells, whereby normally naive T cells acquire a memory phenotype, skewing toward a T_{EM} phenotype was prominent in the absence of PD-1. Acquisition of the T_{EM} phenotype was a CD8 T cell–intrinsic phenomenon as demonstrated by mixed bone marrow transfer experiments. Importantly, adoptively transferred PD-1 KO CD8 central memory T (T_{CM}) cells converted into the T_{EM} phenotype, indicating that PD-1 sets a major checkpoint in the T_{CM} to T_{EM} phenotype differentiation process. This was reflected by distinct patterns of gene expression of PD-1 KO T_{CM} phenotype cells revealed by global transcriptional analysis. Additionally, adoptively transferred PD-1 KO T_{EM} phenotype cells converted to a lesser degree to a T_{CM} phenotype. Collectively, these data suggest that PD-1 shapes memory phenotype CD8 T cell subsets. *The Journal of Immunology*, 2013, 190: 6104–6114.

Memory phenotype (MP) T cells are found in normal, unimmunized mice and display phenotypic and functional traits of memory cells; they account for 10–20% of T cells in young mice, and their number increases with age. It is thought that they are generated as a result of lifetime exposure to various environmental Ags, self-Ags (1), or even simply by homeostatic expansion mechanisms. Signaling by IL-7 and/or other common γ-chain cytokines, such as IL-15, can induce naive T cells to undergo homeostatic proliferation and convert into cells with a memory phenotype (2). Apart from their role in secondary adaptive immune responses, MP cells seem to display important innate immune responses that provide early protection against pathogens during a primary response mostly by producing IFN-γ (bystander activation) in response to IL-12, IL-18, and IFN-α/β produced by macrophages and dendritic cells (3, 4). MP and Ag-specific memory CD8 T cells can be broadly divided into central memory (T_{CM}) and effector memory (T_{EM}) cells based on differential expression of CCR7 and CD62L and different properties regarding effector functions, migration to lymphoid organs or tissues, as well as proliferation in response to Ag or cytokines (5). T_{CM} cells are $CD44^{hi}CD62L^{hi}CCR7^{hi}$ and migrate preferentially to lymph nodes, whereas T_{EM} cells are $CD44^{hi}CD62L^{lo}CCR7^{lo}$ and are mostly located in spleen, peripheral tissues, and bone marrow. T_{EM} cells provide immediate effector functions at the site of pathogen entry through production of lytic molecules such as perforin and granzymes as well as IFN-γ (5–8).

Several models have been proposed to explain the lineage relationship of T_{EM} and T_{CM} Ag-specific memory subsets. The question of memory subset interconversion has been addressed in different experimental systems, and both conversion of T_{EM} to T_{CM} cells (9, 10) and T_{CM} to T_{EM} cells have been reported (11–13). Importantly, recent studies have shown that a single naive precursor cell is able to give rise to all different memory subsets (14, 15). Although mechanisms governing subset differentiation of memory T cells is the subject of intense investigation, homeostasis of MP T cell subsets is less well studied.

Costimulation has been shown to be a critical parameter in determining the developmental fate of memory T cells (16–22). Programmed death-1 (PD-1) is an immunoreceptor that belongs to the CD28/CTLA-4 family and is expressed (among others) on activated CD4 and CD8 T cells. PD-1 negatively regulates TCR signaling upon engagement of one of its ligands PD-ligand 1 and PD-ligand 2 (23, 24). Apart from the established role of PD-1 in peripheral T cell tolerance, its role in immunity and infection is also well described. PD-1 is highly expressed on virus-specific

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Abbreviations used in this article: GzmB, granzyme B; KO, knockout; LIP, lymphopenia-induced proliferation; MP, memory phenotype; PD-1, programmed death-1; SNARF-1, seminaphtorhodafluor-1-carboxylic acid acetate succinimidyl ester; SP, single-positive; T_{CM}, central memory T; T_{EM}, effector memory T; WT, wild-type.

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CD8 T cells in chronic infections and is correlated with an "exhausted" T cell phenotype that is reversed upon PD-1 neutralization (25, 26). The PD-1 pathway can compromise CD8 T cell responses during some acute infections and contributes to the functional impairment of "helpless" CD8 T cells (27). The role of PD-1 in generation, maintenance, and function of MP CD8 T cells is less clear. MP CD8 T cells express PD-1, especially in aged mice, but to a lesser extent compared with MP CD4 T cells (28), and most PD-1–expressing MP CD8 T cells belong to the T_{EM} phenotype. Interestingly, PD-1 expression on MP $CD8^+$ $CD122^+$ T cells defines an IL-10–producing regulatory T cell population (29). In settings of lymphopenia, a short-lived PD-1^+ fraction has been identified among homeostatically proliferating (lymphopenia-induced proliferating, LIP) CD8 T cells, characterized by poor functional responses (30).

In this study we demonstrate a crucial role of PD-1 in differentiation of MP CD8 T cells. Our data reveal that PD-1 impedes accumulation of T_{EM} phenotype CD8 T cells through promoting their apoptotic death and by inhibiting conversion of T_{CM} to T_{EM} phenotype.

Materials and Methods

Mice

PD-1 knockout (KO) (31), GFP-transgenic (32), and DsRed-transgenic mice (33) have been previously described. All mice were backcrossed to the C57BL/10 background for 10 generations. C57BL/10 (referred to as wild-type, WT) and C57BL/10.PD-1–deficient mice (PD-1 KO) were used in the current study. Mice were maintained in the Institute of Molecular Biology and Biotechnology colony. All experiments were approved by the General Directorate of Veterinary Services, Region Crete.

Flow cytometry

Cells from spleen, thymus, lymph nodes, and blood were prepared for flow cytometry as previously described (34). The following Abs, as well as annexin V-FITC and propidium iodide, were purchased from BD Pharmigen: anti–CD8a-allophycocyanin, anti–CD8b-allophycocyanin, anti–CD69-PE, anti–CD62L-PE, anti–CD62L-PE-Cy7, anti–CD62L-FITC, anti–CD44-PerCP-Cy5, anti–CD44-PE, anti–CD25-PE, anti–CD122-PE, anti–CD4-PerCP, anti–IFN-γ-PE, and anti–IL-2-PE. Anti–CD127-PE, anti–Ki-67-PE, and anti–BrdU-allophycocyanin were from eBioscience; anti–granzyme B (GzmB)-PE (clone GB12) was from Invitrogen. For CCR7 staining, a fusion of the CCL19 chemokine and the Fc fragment, plus PE-labeled anti-human IgG Fcγ fragment, was used (eBioscience). Acquisition was carried out on a FACSCalibur and data were analyzed with WinMDI or FlowJo software. The significance of all data was evaluated by a Student *t* test and, where significant, *p* values are shown.

BrdU incorporation and Ki-67 analysis

Seven-month-old PD-1 KO and WT mice were fed daily with 0.8 mg/ml BrdU (Sigma-Aldrich) for 1 wk. On day 7 the mice were sacrificed and splenocytes were stained as described above. For BrdU analysis, cells were treated as previously described (35). Briefly, cells were treated with BD FACS lysing solution (BD Biosciences), followed by overnight fixation in 1% paraformaldehyde containing solution. Cellular DNA was then denatured with 50 Kunitz units of DNase I (Sigma-Aldrich) before being stained with anti-BrdU (BD Biosciences). For Ki-67 analysis, 7-mo-old mice were sacrificed and splenocytes were stained as above. Cells were then treated for 15 min with BD FACS lysing solution, followed by fixation at 4˚C in 1% paraformaldehyde and 0.05% Nonidet P-40 for 30 min. Cells were then blocked with mouse FcγR (CD16/CD32; BD Biosciences) for 15 min and then immediately stained with Ki-67 for 30 min at 4˚C. Cells were then analyzed by flow cytometry.

Isolation of lymphocytes from liver and lung

Mice were sacrificed and perfused via the left ventricle with 20 ml ice-cold PBS. Tissues were then teased over a filter. For lungs, Lympholyte-M (Cedarlane Laboratories, catalog no. CL5031) was used according to the manufacturer's instructions. Cell suspensions from livers were spun at $550 \times g$. The cell pellet was resuspended in RPMI 1640 and overlaid onto 33% (v/v) Percoll solution (Sigma-Aldrich) followed by centrifugation at $800 \times g$ for 30 min. Remaining cells after aspiration were washed twice with RPMI 1640 by centrifugation at $800 \times g$ for 5 min at 4˚C. Subsequent removal of RBCs was performed by water lysis.

In vivo or in vitro stimulation and intracellular cytokine staining

For cytokine production, splenocytes were incubated for 4 h in the presence of GolgiPlug (BD Biosciences) and 50 ng/ml PMA and 500 ng/ml ionomycin (both from Sigma-Aldrich) or untreated. For all experiments culture medium was RPMI 1640 (Biosera) supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin-streptomycin, 2 mM L-glutamine, and 50 μM 2-ME. In some experiments 3-mo-old WT and PD-1 KO mice were challenged with 50 μg LPS (*Escherichia coli* 0111:B4) (Sigma-Aldrich) or PBS for 4 h and were then sacrificed and splenocyte suspensions were incubated with GolgiPlug. Cells were washed and stained for surface markers, as previously described. Cells were then fixed and rendered permeable by using a Cytofix/Cytoperm kit (BD Biosciences), according to the manufacturer's instructions, and subsequently stained for intracellular cytokines and analyzed by flow cytometry.

Transfer of sorted $CD8^+$ T cell subsets

$CD8^+$ T cells were purified from spleen with the negative selection MACS magnetic beads separation system (Miltenyi Biotec) according to the manufacturer's instructions. Purified $CD8^+GFP^+$ T cells were stained with anti–CD44-PerCP-Cy5, anti–CD8-allophycocyanin, and anti–CD62L-PE for the purification of T_{EM} ($CD8^+CD44^{hi}CD62L^{lo}$), T_{CM} ($CD8^+CD44^{hi}CD62L^{hi}$), or naive cells ($CD8^+CD44^{lo}$) and sorted with a Dako MoFlo T high-performance cell sorter. Cells (1.5×10^5) were then adoptively transferred into WT and PD-1 KO mice. Cell fate was analyzed after 42 d on the basis of CD62L and CD44 expression on donor-derived GFP^+CD8^+ cells. In the case of naive cells, recipients were sublethally irradiated (450 rads).

For SNARF-1 (seminaphtorhodafluor-1-carboxylic acid acetate succinimidyl ester; Molecular Probes) labeling, purified cells (10–20 × 10^6/ml) were labeled with 25 μM SNARF-1 in PBS, for 30 min at 37˚C, as described (34).

Microarray hybridizations and analysis

Spleen cells from 7-mo-old WT and PD-1 KO mice were sorted for CD8 T_{CM} cells as described above. RNA was then extracted by standard procedures according to manufacturer's instructions (Qiagen). For genome-wide expression analysis of these cell populations, synthesis of double-stranded cDNA and biotin-labeled cRNA was performed according to the instructions of the manufacturer (Affymetrix). Fragmented cRNA preparations were hybridized to full mouse genome oligonucleotide arrays (GeneChip mouse genome 430 2.0 array; Affymetrix). Initial data extraction and normalization within each array were performed by means of GeneChip operating software (Affymetrix). Microarrays complied with the Minimum Information About a Microarray Experiment and are available at ArrayExpress (http://www.ebi.ac.uk/arrayexpress, accession number E-MTAB-1569). Expression intensities from the PD-1 KO T_{CM} phenotype CD8 T cells and corresponding controls were log transformed and normalized within and between arrays with the quantile normalization method using the R open statistical package (http://www.r-project.org/). Two-tailed, pairwise analysis or a two-way ANOVA was used to extract the statistically significant data from each group of mice by means of the Spotfire Decision Site software package 7.2 version 10.0 (TIBCO Spotfire, Somerville, MA). The criteria for significance were set at $p \leq 0.05$ and a ± 1.5-fold or more change in gene expression. The Affymetrix 430 2.0 arrays include several internal controls to ensure accurate and reproducible measurement of gene expression changes. For each probe set, signals were considered to be valid when they were marked as "present" (for more information, see http://www.affymetrix.com) and exhibited a signal >40 in at least one microarray hybridization. All probe sets with a signal <40 were set to be equal to 40. When there were discrepancies in the direction of expression between multiple probe sets, the gene was not included. Significant overrepresentation of fifth-level gene ontology terms describing "biological process" annotation (GOTERM_BP_5) was identified with the National Institute of Allergy and Infectious Diseases Database for Annotation, Visualization and Integrated Discovery Web site (http://www.david.abcc.ncifcrf.gov)

Generation of mixed bone marrow chimeras

Bone marrow was obtained from femurs of GFP-transgenic and PD-1 KO mice. Mature T cells were first depleted by the use of anti-CD90.2 (BD Biosciences) plus complement (Cedarlane Laboratories), according to manufacturers' instructions. Contamination of bone marrow cells with mature T cells was <0.1%. A mixture of 10^7 WT and PD-1 KO bone marrow cells

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at a 1:1 ratio was injected i.v. into DsRed mice lethally irradiated with 950 rads. Cells from these chimeras were analyzed after 8 wk.

Results

Increased numbers of T_{EM} phenotype CD8 T cells in lymphoid organs and tissues of PD-1 KO mice

We analyzed splenocytes from young (2- to 4-mo-old) and middle-aged (7- to 14-mo-old) C57BL/10 (WT) and C57BL/10.PD-1 KO (PD-1 KO) mice for the presence of $CD8^{+}CD44^{hi}$ (MP CD8) cells. As expected (1), middle-aged WT mice had accumulated more $CD8^{+}CD44^{hi}$ T cells than did young WT ones (9.6 versus 3.8 × 10^{6}; Fig. 1A). Splenocytes from either young or middle-aged PD-1 KO mice contained slightly, but significantly, higher numbers of MP CD8 T cells compared with WT mice of respective age (Fig. 1A). When we further categorized these cells to T_{CM} phenotype ($CD44^{hi}CD62L^{hi}$) or T_{EM} phenotype ($CD44^{hi}CD62L^{lo}$) we found that young and middle-aged PD-1 KO mice contained ~3- and ~5.5-fold, respectively, more T_{EM} phenotype CD8 cells in spleen than did their WT counterparts (Fig. 1B, 1C). As expected, T_{EM} phenotype cells expressed low levels of CCR7, as shown by co-regulation of CD62L and CCR7 expression on WT and PD-1 KO CD8 T cells (Fig. 1D). Naive and T_{CM} phenotype CD8 T cell numbers were not significantly different between WT and PD-1 KO mice in any age group (Fig. 1C).

Because T_{EM} cells migrate preferentially to tissues, we analyzed $CD8^{+}$ T cells isolated from liver, lung, peritoneal cavity, and bone marrow. In all tissues the percentage of T_{EM} phenotype cells among $CD8^{+}$ T cells was significantly higher in PD-1 KO mice. Similar results were obtained in blood (Fig. 2A). When we consider that recovered $CD8^{+}$ T cells were more numerous in all PD-1 KO tissues examined, T_{EM} phenotype cells were from ~5-fold (in bone marrow) to ~9-fold (in lung) more abundant when compared with tissues from WT animals (Fig. 2B). It is possible that the observed differences were due to increased preference of PD-1 KO T_{EM} phenotype CD8 cells to migrate from lymph nodes to tissues. However, when lymph nodes from WT and PD-1 KO mice were examined, the same trend was observed; that is, T_{EM} phenotype CD8 T cells were significantly more numerous in lymph nodes from PD-1 KO mice (Fig. 2).

Phenotypic and functional analysis of PD-1 KO T_{EM} phenotype CD8 T cells

$CD44^{hi}CD62L^{lo}CCR7^{lo}$ T_{EM} phenotype cells have been reported to express CD127 (IL-7Rα) and CD122 (IL-2Rβ-chain), whereas they lack CD25 (IL-2Rα). We investigated expression of several activation/memory markers on the surface of accumulated PD-1 KO T_{EM} phenotype CD8 T cells (Fig. 3A); we found that T_{EM} phenotype cells from both PD-1 KO and WT mice were $CD25^{-}$, consistent with a memory and not a recently activated effector phenotype. CD122 was found to be expressed on a larger fraction of PD-1 KO T_{EM} phenotype cells compared with WT (93 versus 65%), suggesting a possible role of IL-15 in the homeostasis of the accumulated cells (1). Although CD127 was expressed on a slightly lower percentage of T_{EM} cells from PD-1 KO mice, the absolute number of CD127 T_{EM} phenotype $CD8^{+}$ cells was found to be 3-fold higher compared with WT spleens as a consequence of increased numbers of T_{EM} cells in spleen of PD-1 KO mice (Fig. 3B). Interestingly, there was a percentage of PD-1 KO and WT T_{EM} phenotype $CD8^{+}$ cells that expressed the early activation marker CD69, and this was increased in the PD-1 KO cells (Fig. 3A). However, these cells could not be typical effectors because they were uniformly $CD25^{-}$.

GzmB is one of the most important effector molecules produced by armed cytotoxic CD8 T cells. GzmB expression was assayed ex vivo in WT and PD-1 KO CD8 T cells from middle-aged mice. In T_{EM} phenotype CD8 T cells there was a discrete $GzmB^{hi}$ population that was significantly larger when cells came from PD-1 KO mice (5 versus 23%) (Fig. 3C).

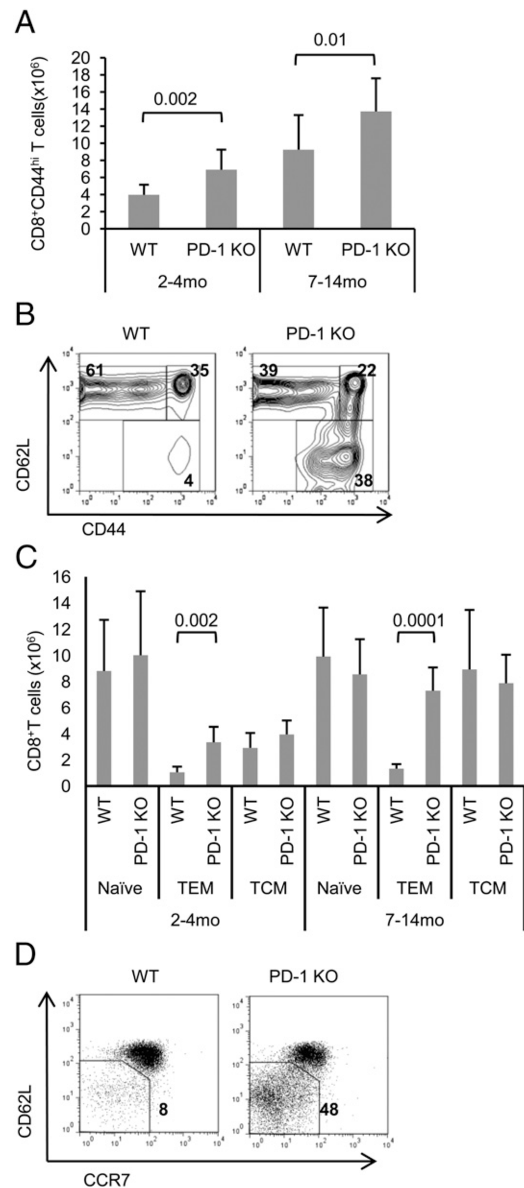

FIGURE 1. Increased numbers of T_{EM} phenotype $CD8^{+}$ cells in spleen of PD-1 KO mice. Young (2- to 4-mo-old) or middle-aged (7- to 14-mo-old) mice were sacrificed and spleen cell suspensions were analyzed by flow cytometry. (**A**) Total $CD8^{+}CD44^{hi}$ spleen cell numbers of WT and PD-1 KO mice. Bars indicate mean values with error bars showing SD (n = 13/group). (**B**) $CD8^{+}$ splenocytes cells were further categorized phenotypically into naive ($CD44^{lo}CD62L^{hi}$), T_{CM} ($CD44^{hi}CD62L^{hi}$), and T_{EM} phenotype cells ($CD44^{hi}$ $CD62L^{lo}$) in spleen of middle-aged mice. Representative dot plots from middle-aged mice are shown with percentages of cell subsets in each region. (**C**) Total numbers of naive, T_{EM}, and T_{CM} phenotype $CD8^{+}$ cells with error bars indicating the SD. Results are representative of three individual experiments with three mice per group. (**D**) Splenocytes from 9-mo-old mice were analyzed for coexpression of CCR7 and CD62L gated on $CD8^{+}$ T cells. Representative dot plots are shown with percentages of cells per region.

One of the cardinal features of memory CD8 T cells is the fast recall responses, for example, production of effector molecules after brief stimulation with phorbol esters. In such an assay, IFN-γ is accumulated only in $CD44^{hi}$ MP cells and not in naive CD8

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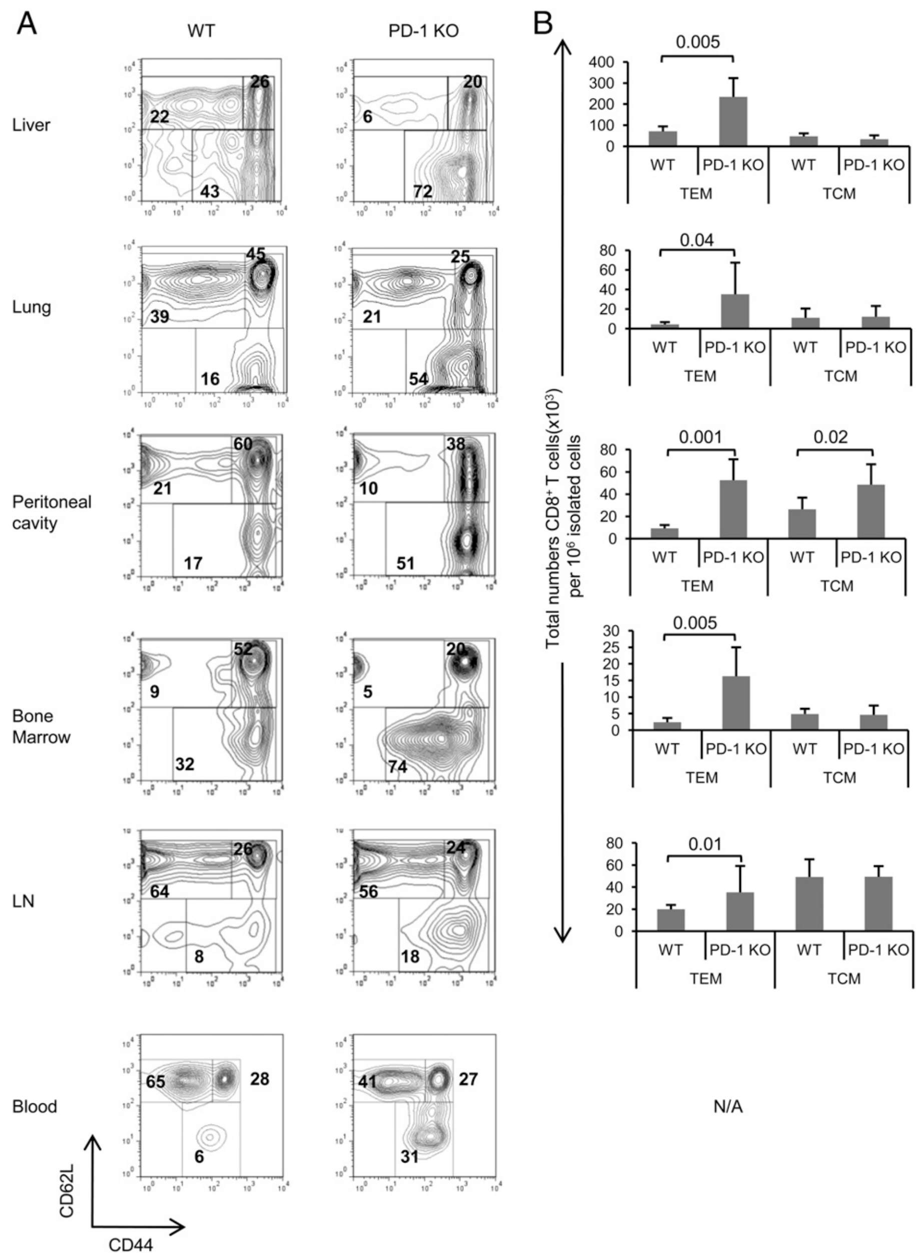

FIGURE 2. Increased numbers of T_{EM} phenotype $CD8^+$ cells in lymphoid and nonlymphoid tissues of middle-aged PD-1 KO mice. Nine-month-old WT and PD-1 KO mice were sacrificed and cell suspensions from various lymphoid and nonlymphoid tissues were categorized phenotypically by flow cytometry into naive ($CD44^{lo}CD62L^{hi}$), T_{CM} ($CD44^{hi}CD62L^{hi}$), and T_{EM} ($CD44^{hi}$ $CD62L^{lo}$) $CD8^+$ cells. (**A**) Representative dot plots are shown with percentages of cells per region. (**B**) Total numbers of T_{CM} and T_{EM} phenotype cells per 10^6 isolated cells are shown with error bars indicating SD. The results are representative of three individual experiments with at least two mice per group.

T cells. Because it was not possible to assess IFN-γ production by T_{EM} and T_{CM} subsets owing to rapid shedding of CD62L after TCR stimulation (36), we performed this assay on isolated $CD8^+$ $CD44^{hi}CD62L^{lo}$ T_{EM} phenotype cells. As shown in Fig. 3D and 3E, a higher proportion of T_{EM} phenotype CD8 T cells from PD-1 KO spleens produced IFN-γ. Additionally, a smaller percentage of PD-1 KO T_{EM} cells produced IL-2 compared with PD-1 KO T_{CM} cells (Fig. 3F), in agreement with previously described subset phenotypes (8, 10).

To investigate whether accumulation of PD-1 KO T_{EM} phenotype $CD8^+$ cells is due to increased proliferation, we analyzed cell cycle by Ki-67 expression and BrdU incorporation assays. Both of these experiments showed that PD-1 KO T_{EM} phenotype cells cycle slower than do their WT counterparts, thus strongly suggesting that their accumulation is not due to enhanced rate of proliferation (Fig. 3G, 3H). Next, we wanted to examine whether cell survival is involved in accumulation of PD-1 KO T_{EM} phenotype cells. Ex vivo annexin V binding assays showed that a higher percentage of WT T_{EM} phenotype cells was annexin V^+ (Fig. 3I), indicating a contribution of survival in the accumulation of PD-1 KO T_{EM} phenotype cells.

In conclusion, T_{EM} phenotype CD8 T cells are substantially accumulated in lymphoid organs and tissues of PD-1 KO mice where they display significantly enhanced characteristics of T_{EM} cells, and decreased potential to apoptosis may contribute to their accumulation.

PD-1 pathway prevents differentiation of LIP memory CD8 T cells to T_{EM} phenotype

Naive T cells undergoing lymphopenia-induced homeostatic proliferation acquire a MP similar to central memory cells without passing through an effector phase (37, 38), and they become capable of mediating protective immunity against pathogens (39). To examine whether PD-1 mutation perturbs normal development of LIP memory T cells we transferred purified naive ($CD44^{lo}$) GFP. WT or GFP.PD-1 KO $CD8^+$ T cells to sublethally irradiated WT hosts. CD8 T cell subset analysis showed that by day 20, a significant population of T_{EM} phenotype PD-1 KO cells arose and

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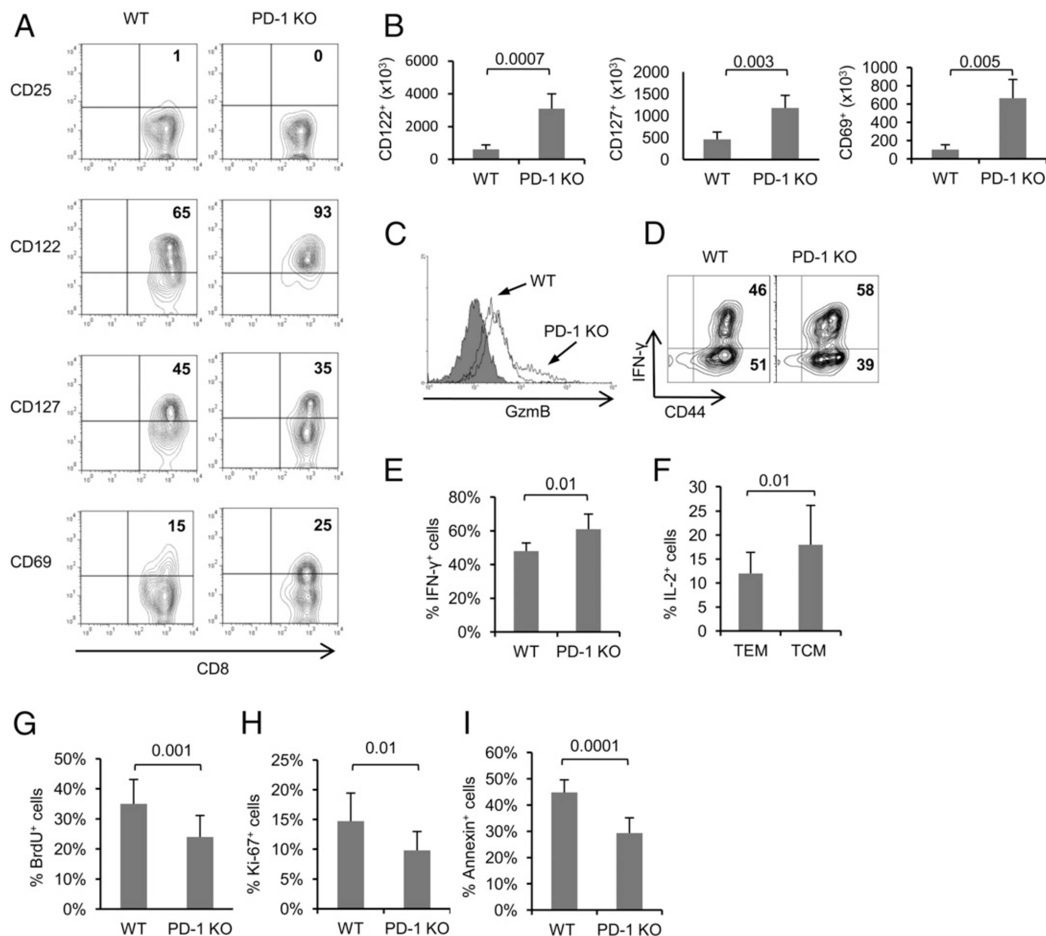

FIGURE 3. Phenotypic and functional characterization of accumulated T_{EM} phenotype cells in PD-1 KO mice. Spleenocytes from 9-mo-old PD-1 KO and WT mice were analyzed by flow cytometry. (**A**) Representative dot plots show expression of various surface markers gated on T_{EM} ($CD8^{+}CD44^{hi}$ $CD62L^{lo}$) phenotype cells. Numbers indicate percentages in each region. (**B**) Absolute numbers as indicated with error bars depicting SD. Data represent two to three individual experiments with three mice per group. (**C**) Intracellular GzmB staining on freshly isolated T_{EM} phenotype $CD8^{+}$ cells. Shaded histogram denotes staining with isotype control. Data are representative of three individual experiments with two mice per group. (**D**) Representative dot plots of IFN-γ production by purified T_{EM} phenotype $CD8^{+}$ cells after brief ex vivo stimulation. (**E**) Mean percentages as in (D) with error bars indicating SD. Data represent three individual experiments with eight pooled spleens per group. (**F**) IL-2 production by purified PD-1 KO T_{CM} and T_{EM} phenotype $CD8^{+}$ cells as in (E). (**G**) PD-1 KO and WT mice were fed with BrdU and then mice were sacrificed on day 7 and splenocytes were analyzed by flow cytometry. Bars show mean percentages of $BrdU^{+}$ cells among T_{EM} phenotype CD8 cells with error bars indicating SD. Data represent three individual experiments with four mice per group. (**H**) Mean percentages of Ki-67^{+} cells among T_{EM} phenotype CD8 cells. Data represent two individual experiments with four mice per group. (**I**) Mean percentages of annexin V^{+} cells among T_{EM} phenotype CD8 cells gated on live cells as confirmed by propidium iodide staining. Data represent three individual experiments with three mice per group.

became by far the predominant one in host spleens (Fig. 4A). Higher numbers of PD-1 KO T_{EM} phenotype cells were recovered when compared with WT, with a parallel decrease in the number of PD-1 KO T_{CM} phenotype cells (Fig. 4B). These results might suggest that in the absence of PD-1, T_{EM} cells accumulate at the expense of the other CD8 subsets. Importantly, a much larger fraction of PD-1 KO-derived T_{EM} cells were $GzmB^{hi}$ when assayed directly ex vivo (Fig. 4C). Additionally, analysis of transferred cells at earlier time points in the host's blood (day 5) revealed that initially both WT and PD-1 KO naive donor cells gave rise mostly to T_{CM} phenotype cells (Fig. 4D); at later time points T_{EM} phenotype cells progressively emerged and formed the largest subpopulation by day 20, when PD-1 KO cells were transferred. This suggests that PD-1 regulates T_{CM} to T_{EM} subset differentiation in lymphopenic conditions. The fact that we transferred purified naive WT or PD-1 KO $CD8^{+}$ T cells and hosts were always WT is suggestive of a CD8 cell–intrinsic mechanism.

In conclusion, our results show that PD-1 signaling in CD8 T cells can modulate the homeostasis of the MP pool by impeding differentiation toward a functional T_{EM} phenotype, most probably from a T_{CM} phenotype intermediate.

Accumulation of T_{EM} phenotype CD8 T cells depends on cell-intrinsic mechanisms

To examine further whether the effect of PD-1 was indeed intrinsic to the CD8 T cells, we performed mixed bone marrow chimera experiments transferring mixtures consisting of equal numbers of PD-1 KO and GFP.WT bone marrow cells to lethally irradiated DsRed.WT hosts. In these settings, PD-1 KO and WT CD8 T cells mature and respond to the same environmental cues, and any observed differences should be attributed to intrinsic factors. Eight weeks after transfer we analyzed thymi, spleens, and lymph nodes from hosts and the ratios of donor-derived WT and PD-1 KO T cells were evaluated. Analysis of thymi showed equal contribution of WT- and PD-1 KO-derived cells in thymocytes and similar percentages of CD8 single-positive (SP) cells (Fig. 5A). The mean PD-1 KO/WT ratio for these chimeric mice was 1.0 for $CD8^{+}$ SP thymocytes (Fig. 5A, *right*), suggesting that PD-1 KO bone

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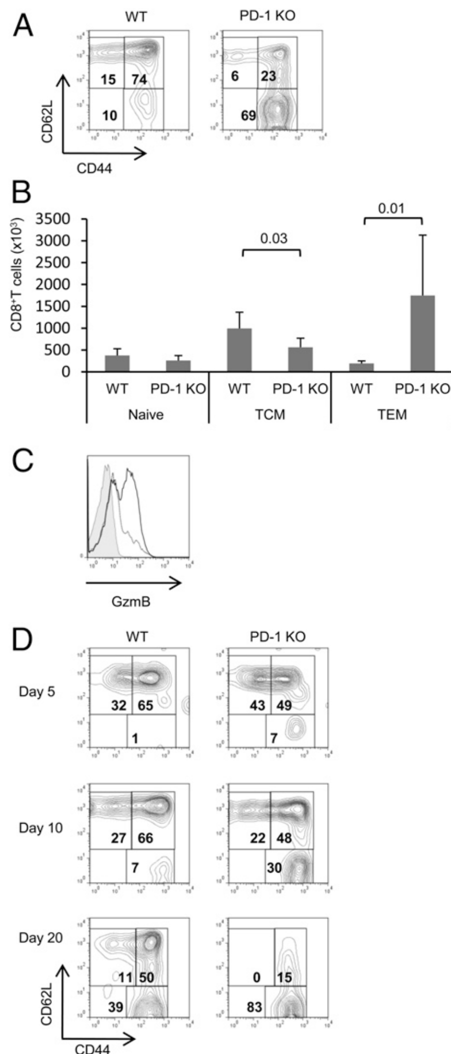

FIGURE 4. Fate of naive WT and PD-1 KO CD8$^+$ cells transferred to sublethally irradiated WT hosts. GFP$^+$CD8$^+$CD44^{lo} cells from spleens of 2- to 4-mo-old PD-1 KO and WT mice were isolated by FACS sorting. Purified cells were then adoptively transferred into sublethally irradiated WT mice. On day 20, mice were sacrificed and spleens were analyzed. (**A**) Spleenocytes were analyzed for CD8, CD44, and CD62L expression. Numbers indicate percentages in each region. Plots are representative of three individual experiments (WT, n = 7; PD-1 KO, n = 9). (**B**) Total numbers of GFP$^+$ MP CD8 T cell subsets found in spleen. Error bars indicate SD. (**C**) Ex vivo GzmB expression on day 20, gated on T_{EM} phenotype CD8 cells (shaded region, isotype control; thin line, GFP.WT; thick line, GFP.PD-1 KO). (**D**) GFP$^+$CD8$^+$ cells in hosts' blood were examined, as in (A), on days 5, 10, and 20. Numbers indicate percentages in each region. Data are representative of one experiment with three mice per group.

marrow cells had no general thymic developmental advantage over WT counterparts. In contrast, the majority of donor-derived CD8$^+$ cells in spleens were of PD-1 KO origin (Fig. 5B), suggesting that postthymic events are the cause of increased PD-1 KO-derived peripheral CD8 T cells. Further subtype analysis in spleens and mesenteric lymph nodes showed that there was a significantly higher proportion of T_{EM} phenotype cells in CD8 T cell populations of PD-1 KO origin (Fig. 5C, 5D). The same results in spleen were obtained when we transferred mixtures of GFP.PD-1 KO and WT bone marrow cells to DsRed.WT hosts (Fig. 5E, 5F), indicating that the GFP transgene in donor-derived cells had no effect in the observed phenotype. These results demonstrate that the absence of PD-1 results in accumulation of CD8 T_{EM} phenotype cells in a cell-intrinsic manner.

PD-1 regulates interconversion of T_{CM} and T_{EM} phenotype CD8 T cells

To investigate whether aberrant conversion between MP subsets contributes to accumulation of T_{EM} phenotype CD8 T cells in PD-1 KO mice, we purified both T_{EM} and T_{CM} phenotype CD8 T cells from GFP.WT or GFP.PD-1 KO spleens and transferred them separately to WT or PD-1 KO mice, respectively. Fig. 6A (*upper panel*) shows the purity of T_{CM} phenotype CD8 T cells. When analyzing host mice that received T_{CM} phenotype cells, little conversion of T_{CM} to T_{EM} cells was found in WT mice after 42 d (Fig. 6A, *lower panel*, *left*). In PD-1 KO mice, however, a striking conversion of T_{CM} to T_{EM} phenotype was observed (Fig. 6A, *lower panel*, *right*) (~80% of donor-derived cells from PD-1 KO hosts that received T_{CM} CD8 T cells were of a T_{EM} phenotype). This was accompanied by a substantially higher recovery of PD-1 KO T_{EM} phenotype donor-derived cells (Fig. 6B). This was also true but to a lesser degree for PD-1 KO T_{CM} phenotype donor-derived cells. Similar degrees of abnormal conversion and high recoveries were also obtained when PD-1 KO T_{CM} cells were transferred to WT hosts but not when WT T_{CM} cells were transferred to PD-1 KO mice (Supplemental Fig. 1), indicating that the above-described phenomenon was a result of the lack of PD-1 in donor T_{CM} cells.

It was possible that accumulating PD-1 KO T_{EM} phenotype cells might have arisen from overt proliferation of residual T_{EM} cells in the purified T_{CM} cell "preparation." To exclude this, we analyzed Ki-67 expression in GFP$^+$ PD-1 KO T_{CM} and T_{EM} phenotype cells on days 21 and 42 after transfer of GFP$^+$ T_{CM} phenotype cells. Ki-67 expression was lower in the T_{EM} phenotype subset compared with T_{CM} phenotype when analyzed in the same host (Fig. 6C), thus showing that GFP$^+$ T_{CM} phenotype cells in PD-1 KO hosts were not outnumbered by vast proliferation of contaminant T_{EM} phenotype cells. For the same purpose we transferred purified SNARF-1–labeled GPF.PD-1 KO T_{CM} phenotype cells to PD-1 KO hosts and compared dye intensity dilution in GFP$^+$ T_{CM} and T_{EM} phenotype cells. No consistent difference was observed when profiles for these subsets were overlaid (Fig. 6D). These data indicate that accumulated T_{EM} phenotype cells, after PD-1 KO T_{CM} cell transfers, do not originate from overt expansion of residual cotransferred T_{EM} cells.

Additionally, we purified T_{EM} phenotype CD8 T cells from GFP. WT or GFP.PD-1 KO spleens and transferred them separately to WT or PD-1 KO mice, respectively. Fig. 6E (*upper panel*) shows the purity of transferred cells. When analyzing mice that received T_{EM} phenotype cells, T_{EM} to T_{CM} conversion was moderate for WT donor cells, whereas a smaller proportion of recovered PD-1 KO donor cells bore the T_{CM} phenotype, consistent with less T_{EM} to T_{CM} conversion (Fig. 6E, *lower panel*). A significantly higher recovery of T_{EM} phenotype PD-1 KO donor-derived cells was observed (Fig. 6F), which may be partly attributed to their enhanced survival.

In conclusion, these results provide strong evidence that PD-1 regulates differentiation of T_{CM} to T_{EM} phenotype CD8 cells in nonimmunized, naive mice both by inhibiting T_{CM} to T_{EM} conversion and by promoting T_{EM} to T_{CM} conversion.

Absence of PD-1 exerts genome-wide gene expression changes in T_{CM} phenotype CD8 cells

We have shown that transferred T_{CM} phenotype CD8 cells from PD-1 KO mice, but not WT, can give rise predominantly to a T_{EM} phenotype population (Fig. 6A, 6B). Analysis of T_{CM} phenotype CD8 cells for CD69, Ly6C, CD25, CD127, and CD122 surface expression revealed indistinguishable patterns between PD-1 KO

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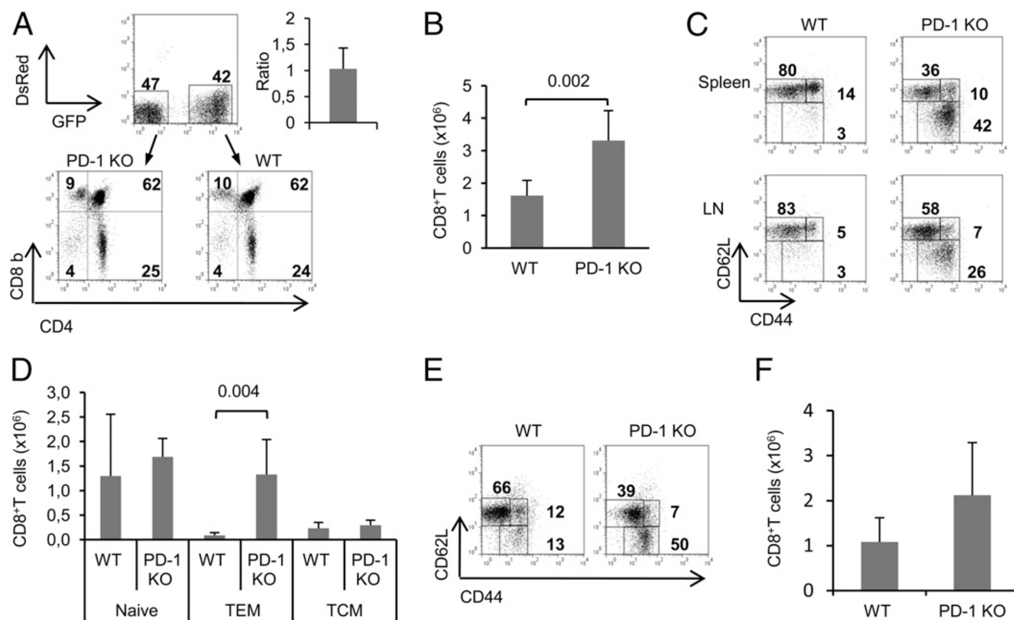

FIGURE 5. T cell–intrinsic increase in PD-1 KO CD8$^+$ T_{EM} phenotype cells. Donor-derived WT (GFP$^+$DsRed$^-$) and PD-1 KO (GFP$^-$DsRed$^-$) CD8$^+$ T cells from thymi, spleens, and lymph nodes were analyzed by flow cytometry 8 wk after bone marrow reconstitution in irradiated DsRed hosts. (**A**) Representative dot plots with donor-derived WT (GFP$^+$DsRed$^-$) and PD-1 KO (GFP$^-$DsRed$^-$) thymocytes. The expression of CD4 and CD8 was analyzed in gated populations. Numbers indicate percentages in each region (*upper left*, CD8 SP; *lower right*, CD4 SP; *upper right*, double-positive; *lower left*, double-negative). Column represents the average value of PD-1 KO/WT CD8 SP thymocyte ratios with error bar indicating SD. Data are representative of two individual experiments ($n = 6$). (**B**) Total numbers of CD8$^+$ WT and PD-1 KO cells in spleens with error bars indicating SD. (**C**) Donor-derived WT (GFP$^+$DsRed$^-$) and PD-1 KO (GFP$^-$DsRed$^-$) CD8$^+$ T cells from spleens and mesenteric lymph nodes (LN) were further analyzed for expression of CD44 and CD62L. Numbers indicate percentages in each region. Data are representative of three individual experiments with three to four mice per group. (**D**) Total numbers of WT and PD-1 KO CD8$^+$ cell subsets in spleens with error bars indicating SD. (**E**) Similar analysis of donor-derived WT (GFP$^-$DsRed$^-$) and PD-1 KO (GFP$^+$DsRed$^-$) CD8$^+$ T cells from spleens after bone marrow reconstitution in irradiated DsRed host as in (C). Data are representative of one individual experiment with three mice per group. (**F**) Total numbers of CD8$^+$ WT and PD-1 KO cells in spleens with error bars indicating SD, as in (E).

and WT cells (Supplemental Fig. 2). To examine whether T_{CM} phenotype CD8 cells from PD-1 KO mice had already adopted a different transcriptional profile at the time of transfer, we performed transcriptome analysis on T_{CM} phenotype CD8 cell subpopulations derived from PD-1 KO and WT spleens. First, all significantly differentially expressed genes from the PD-1 KO and WT T_{CM} phenotype CD8 cells were classified as having increased or decreased expression. Two-tailed, pairwise ANOVA of Affymetrix complete mouse genome arrays revealed 237 annotated genes with significantly changed expression patterns between WT and PD-1 KO T_{CM} CD8 cells ($p \leq 0.05$, 1.5-fold change up- or downregulated) (Supplemental Table I), a number that significantly exceeds the number of genes that are expected to occur by chance under these selection criteria. Using this dataset, we then identified those biological processes with a significantly disproportionate number of responsive genes in the T_{CM} phenotype CD8 cell subset relative to those contained in the Affymetrix arrays as shown in Fig. 7A. Selected genes and the magnitude of over- or underexpression are graphically depicted in Fig. 7B. Among these, there are genes involved in T cell costimulation (*CD24*, *Icos*, *ICAM1*, *Tnfsfr1b* [TNFR2]), apoptosis/survival (*Bcl2a1*, *Bcl3*, *TNF*, *Xiap*), signal transduction (*Jak1*, *Map3k8* [Tpl-2], *Gadd45b*, *Socs3*), as well as T cell migration/adhesion/inflammation (*Ccl3*, *Cxcl9*, *Nrp1*, [neuropilin-1], *Lgals3* [galectin-3]). Differentially expressed transcription factors included Rel, STAT1, Irf4, Icrf8, and the less characterized Atf3, Ahr, and Bhlhe40 (Dec1). Ahr is able to modulate CD62L expression in primary responses (40) and under certain conditions it diminishes memory CD8 pool but not CD8 cell responses (41). Bhlhe40 transcription factor, which has recently been shown to be important in generation of regulatory T cells (42), is one of the most upregulated genes in PD-1 KO T_{CM} CD8 cells (3.8-fold). Interestingly, upregulation of *IL12Rb1* was accompanied by increased expression of genes previously characterized as positively regulated by IL-12 and/or IFN-α/β, such as *Gadd45b*, *Bcl3, TNF*, *Lgals3*, *Ccl3*, *Bhlhe40*, *Cdkn1a*, and *Atf3* and *IL12Rb1* itself (43–45). Importantly, when these cytokines are used as signal 3 on CD8 T cells they downregulate CD62L and CCR7 more efficiently than signal 1 and 2 alone (43).

Because many of the annotated genes functions were related to cell death, we compared ex vivo annexin V binding between WT and PD-1 KO T_{CM} phenotype CD8 T cells. A higher percentage of PD-1 KO T_{CM} phenotype cells were annexin V$^+$, indicating an increased propensity to apoptosis (Fig. 7C). When gating on CD62L^{hi}, CD62L^{int}, and CD62L^{lo} MP PD-1 KO CD8 T cells, we observed a correlation of CD62L downregulation with increased annexin V binding (Fig. 7D). This may suggest that the increased annexin V binding of PD-1 KO T_{CM} phenotype cells reflects their predisposition to become (CD62L^{lo}) T_{EM} cells.

Overall, our results show that PD-1 KO T_{CM} phenotype CD8 cells bear a distinct gene expression profile, and ablation of the PD-1 pathway had exerted an impact before the acquisition of the T_{EM} phenotype. This may indicate that in transfer experiments PD-1 KO T_{CM} phenotype cells are already preprogrammed, at least at the transcriptional level, to differentiate to T_{EM} phenotype cells where further reprogramming takes place. Moreover, their profile indicates that T_{CM} phenotype CD8 T cells may respond differently to IL-12 and IFN-α/β cytokines.

Superior bystander production of IFN-γ by T_{CM} phenotype PD-1 KO CD8 cells after innate stimulus

MP CD8 T cells have been shown to produce IFN-γ driven by IL-12, as well as IFN-α/β produced by macrophage/dendritic cells, in

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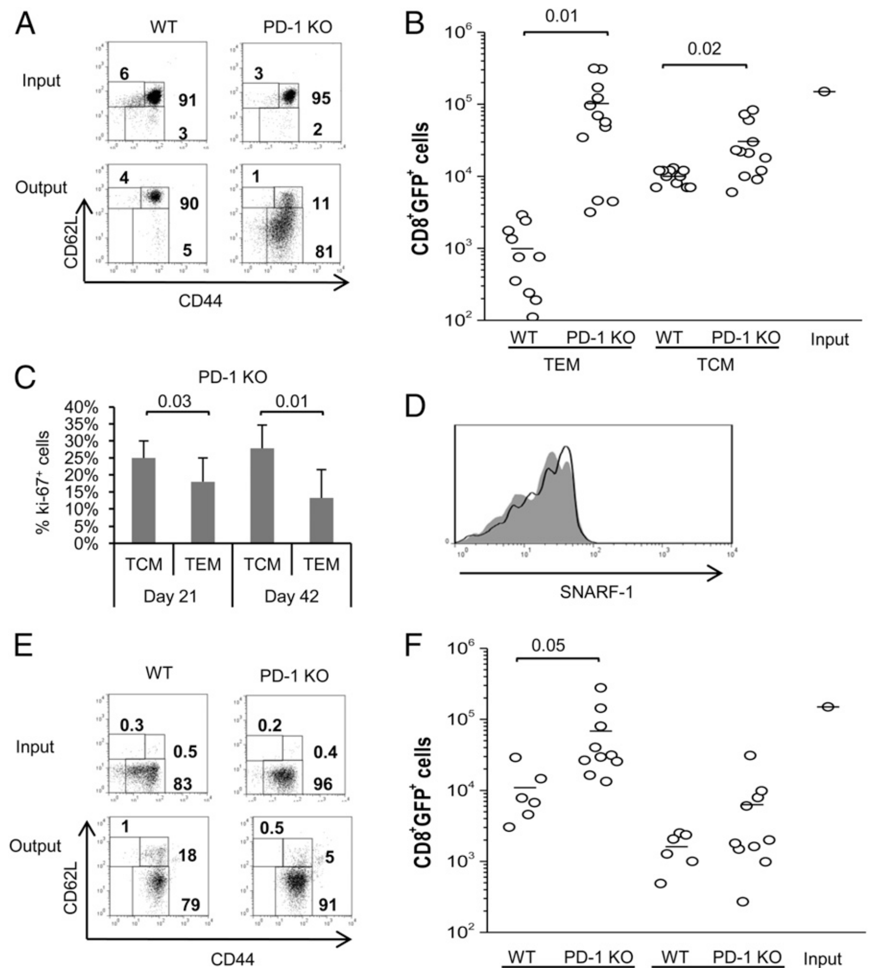

FIGURE 6. Fates of memory CD8 T cell subsets in adoptive transfer experiments. Purified GFP^+CD8^+ T_{CM} phenotype cells from 5- to 7-mo-old GFP.WT and PD-1 KO were adoptively transferred into WT and PD-1 KO mice. (**A**) Representative dot plots with CD62L and CD44 expression on purified T_{CM} phenotype cells before adoptive transfer (*upper panel*) and on day 42 on donor-derived GFP^+CD8^+ cells (*lower panel*). Numbers indicate percentages in each region. Data are representative of four individual experiments (WT, $n = 10$; PD-1 KO, $n = 12$). (**B**) Total numbers of recovered GFP^+ $CD8^+$ T_{EM} and T_{CM} phenotype cells from WT and PD-1 KO host spleens as in (A). For comparison, the numbers of transferred cells per host (input) are indicated. (**C**) Mean percentages of Ki-67^+ cells among donor-derived GFP.PD-1 KO $CD8^+$ subsets on days 21 and 42 after transfer with error bars indicating SD. Data are representative of two individual experiments with three mice per group. (**D**) SNARF-1 profiles of donor-derived CD8- PD-1 KO T_{CM} and T_{EM} phenotype cells in host spleens on day 13 (thick line, PD-1 KO T_{CM}; shaded area, PD-1 KO T_{EM}). Data are representative of one experiment with four mice per group. (**E**) Purified GFP^+CD8^+ T_{EM} phenotype cells from 5- to 7-mo-old GFP.WT and GFP.PD-1 KO mice were adoptively transferred into WT and PD-1 KO mice. Input and output of T_{EM}-transferred cells, as in (A). (**F**) Total numbers of recovered GFP^+ $CD8^+$ cell subsets from WT and PD-1 KO host as in (B) (WT, $n = 6$; PD-1 KO, $n = 10$).

response to infection or a defined innate stimulus (3, 4). Given our microarray results that imply an increased response of PD-1 KO T_{CM} phenotype CD8 cells to these cytokines, we injected WT and PD-1 KO mice with LPS and analyzed CD8 T cells for IFN-γ production shortly after injection. A higher fraction of PD-1 KO CD8 T cells was IFN-γ producers (Fig. 8, *upper panel*). When we analyzed T_{EM} and T_{CM} subsets we found that a larger percentage of PD-1 KO T_{CM} phenotype cells produced IFN-γ ex vivo (Fig. 8, *middle panel*). No difference in IFN-γ production was observed between T_{EM} phenotype cells from WT and PD-1 KO mice (Fig. 8, *lower panel*). These results show increased indirect response of PD-1 KO T_{CM} phenotype CD8 cells to LPS, probably through IL-12 and/or IFN-α/β, and they imply a greater bystander innate response of PD-1 KO MP CD8 T cells to various pathogens. However, incubation of PD-1 KO splenocytes with various concentrations of IL-12 or type I IFN together with IL-18 did not result in superior production of IFN-γ by PD-1 KO MP CD8 subsets in vitro (not shown). Nevertheless, this does not exclude a role of these cytokines in the increased production of IFN-γ by PD-1 KO T_{CM} CD8 cells in vivo in the context of an inflammatory milieu induced by LPS.

Discussion

In this study we describe a previously unrecognized role of PD-1 in MP CD8 T cell formation and particularly in shaping MP subset development. We have identified a substantial increase in $CD44^{hi}$ $CD62L^{lo}CCR7^{lo}$ CD8 T cells, categorized as T_{EM} phenotype (5), in spleen and tissues and even lymph nodes of PD-1 KO mice (Figs. 1, 2). This phenomenon was more prominent with advancing age (Fig. 1C). Despite some quantitative differences in expression of memory markers on T_{EM} cells from PD-1 KO and WT mice, the number of $CD127^{hi}$ and $CD122^{hi}$ T_{EM} phenotype CD8 T cells is considerably higher in PD-1 KO spleens (Fig. 3B), consistent with an MP (46, 47). Although a proportion of PD-1 KO T_{EM} phenotype cells express CD69 (Fig. 3A), most should not be recently activated cells because no $CD25^{hi}$ subpopulation was identified (Fig. 3A). Moreover, recently activated, typical effector cells would decay fast in a 42-d period, something not observed in our experiments (Fig. 6F). These accumulated T_{EM} cells, in the absence of PD-1, seem to have enhanced effector memory characteristics, as shown by higher expression of GzmB directly ex vivo (Fig. 3C) and IFN-γ after short activation with phorbol esters (Fig. 3D, 3E). The differences in expression of cytokine receptors found (Fig. 3A) could reflect an altered responsiveness to homeostatic cytokines in the PD-1 KO mice. Further studies will determine the contribution of these cytokines in the altered homeostasis of MP cells found in the PD-1 KO mice.

Costimulatory and coinhibitory molecules have been shown to regulate memory CD8 T cell development, with a consensus that costimulation promotes formation of Ag-specific or MP cells whereas coinhibition impedes it. However, to date, variable data exist on correlation between TCR signal strength modulated by positive and negative costimulators and developmental fate toward T_{EM} and T_{CM} subsets. For example, whereas enhancement of TCR signals by OX-40 (21) and ICOS (16) promote accumulation of T_{EM} cells, stronger TCR signals in the absence of BTLA lead to

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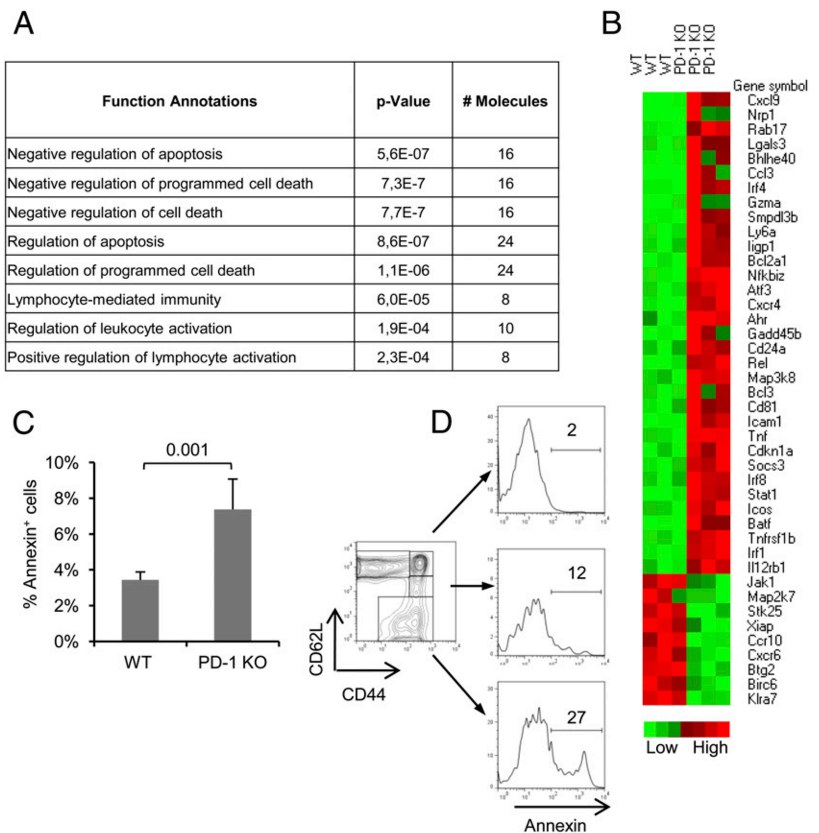

Function Annotations	p-Value	# Molecules
Negative regulation of apoptosis	5,6E-07	16
Negative regulation of programmed cell death	7,3E-7	16
Negative regulation of cell death	7,7E-7	16
Regulation of apoptosis	8,6E-07	24
Regulation of programmed cell death	1,1E-06	24
Lymphocyte-mediated immunity	6,0E-05	8
Regulation of leukocyte activation	1,9E-04	10
Positive regulation of lymphocyte activation	2,3E-04	8

FIGURE 7. Microarray data analysis of sorted CD8$^+$ T_{CM} phenotype cells from PD-1 KO and WT mice. Splenocytes from PD-1 KO and WT mice were sorted for CD8$^+$CD44^{hi}CD62L^{hi} cells. Transcriptional profiles from sorted cells were then compared (n = 3). (**A**) Table showing the functions, p values, and number of molecules per category as assessed by Database for Annotation, Visualization and Integrated Discovery microarray software. (**B**) Heat map depicting the relative normalized expression of selected genes that are significantly different in expression between WT and PD-1 KO T_{CM} phenotype CD8 cells. (**C**) Mean percentages of annexin V$^+$ T_{CM} phenotype CD8 cells gated on live cells as confirmed by propidium iodide staining. Data represent three individual experiments with three mice per group. (**D**) Annexin V binding on CD8$^+$CD44$^+$ CD62L^{hi}, CD62L^{int}, and CD62L^{lo} subpopulations from spleens of 7-mo-old PD-1 KO mice. Numbers indicate percentage of annexin V$^+$ cells. Data are representative of three experiments with three animals per group.

accumulation of T_{CM} cells (19). Our results, which show that ablation of the PD-1 pathway drives MP CD8 T cells preferentially to a T_{EM} phenotype, are in agreement with the notion that increased signal strength (5) and duration (9) favor skewing toward a T_{EM} cell subset. Homeostatic proliferation of adoptively transferred naive PD-1 KO CD8 T cells gave rise to large numbers

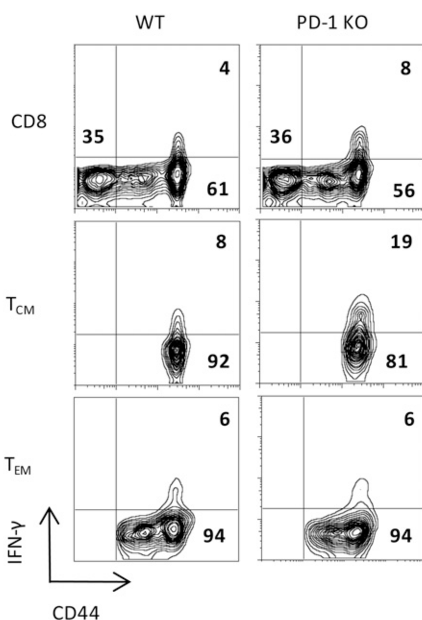

FIGURE 8. IFN-γ production ex vivo by MP CD8$^+$ subsets. Spleens from 3-mo-old PD-1 KO and WT mice were analyzed after LPS injection by flow cytometry. Representative dot plots of IFN-γ production by total CD8$^+$ and MP subsets. Data are representative of two individual experiments with four mice per group. Numbers show the percentages of cells in each quadrant.

of T_{EM} phenotype cells (Fig. 4A, 4B, 4D), as MP CD8 T cells closely resemble memory cells generated under lymphopenic conditions (LIP memory cells) (1). Interestingly, T_{CM} cells appear first (day 5, Fig. 4D), followed by substantial accumulation of T_{EM} cells in blood of PD-1 KO (day 20, Fig. 4D) and in spleen (day 20, Fig. 4A), which does not take place in WT donor cells to the same extent. This implies that increased duration of signal, in the absence of PD-1, favors T_{EM} differentiation through a T_{CM} intermediate. This observation correlates well with the massive T_{CM} to T_{EM} conversion of transferred purified PD-1 KO T_{CM} phenotype CD8 T cells in lymphosufficient mice (Fig. 6A, 6B) where we provided "extra time" to the transferred cells, inside the host, to differentiate to T_{EM} cells.

Enhanced survival of PD-1 KO T_{EM} phenotype cells compared with WT may play an additional role in their accumulation (Fig. 3I). However, the fact that upon transfer of 1.5×10^5 purified T_{CM} or purified T_{EM} phenotype PD-1 KO cells we recovered similar numbers ($\sim 1 \times 10^5$) of PD-1 KO T_{EM} phenotype cells (Fig. 6B, *second column* versus Fig. 6E, *second column*) strongly implicates increased rates of T_{CM} to T_{EM} conversion as the major determinant of PD-1 KO T_{EM} phenotype cell accumulation, rather than enhanced survival alone.

Our results from mixed bone marrow transplantation experiments (Fig. 5), adoptive transfer of T_{CM} CD8 T cells (Fig. 6A, 6B, Supplemental Fig.1), and transfers of naive cells to lymphopenic hosts (Fig. 4) strongly indicate that the accumulation of PD-1 KO T_{EM} phenotype cells is, at least partly, a CD8 T cell–intrinsic effect. Further experiments would address the issue of whether the fate of PD-1 KO donor T_{CM} cells was already predetermined at the time of transfer or whether posttransfer intervention on WT T_{CM} cells would be sufficient to promote T_{CM} to T_{EM} differentiation. However, our microarray results, showing a discrete expression profile on PD-1 KO T_{CM} cells (Fig. 7A, 7B), argue in favor of the first scenario.

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A recent study showed that vaccinia virus–specific PD-1 KO CD8 T cells are skewed toward T_{CM} after acute infection (48). This does not conflict with our data because it has been shown that the type of pathogen affects memory differentiation pathways, with vaccinia virus (but not lymphocytic choriomeningitis virus) typically leading to fast emergence of T_{CM} CD8 T cells (49). Moreover, in most acute infections, CD8 T cells rapidly stop encountering Ag (for vaccinia virus, infection is fully resolved within 2 wk) (50), and without any circumstantial or deliberate restimulation, typically most Ag-specific memory cells belong to the T_{CM} subset. On the contrary, repetitive/continuous stimulation, either by infection or vaccination (51–53), promotes generation of cells belonging to the effector memory subset; repetitive antigenic stimulation has been shown to induce progressive decrease of CD62L surface expression (53). Importantly, in the settings of acute infection, PD-1 is shown to be expressed only transiently on $CD8^+$ T cells, whereas on chronically stimulated cells, sustained expression is observed (25, 54, 55). Therefore, with a different mode of PD-1 signaling (i.e., transient versus sustained) transition to different memory developmental pathways may take place. Thus, it is probable that settings of acute infection (48), on the one hand, and response to a plethora of Ags, with many of them repetitively encountered, on the other hand, could have a different impact on memory fate of PD-1 KO CD8 T cells. Further experiments are needed to determine whether PD-1 has the same effect on differentiation of MP phenotype cells and Ag-specific memory cells following multiple re-exposure to Ag.

Note that compared with respective MP CD8 T cells a much larger fraction of PD-1 KO LIP T_{EM} phenotype cells produces high levels of GzmB ex vivo (Fig. 4C). Given that most of these cells recognize self-ligands, although with low affinity (56), it is reasonable to think that they could have an autoreactive potential. In line with this hypothesis, Thangavelu et al. (57), although not examining GzmB expression on T cells, have shown in a recent report that PD-1 KO recent thymic emigrants cause a lethal autoimmune-like disease in chronically lymphopenic hosts.

Overall, the emerging view is that naive WT or PD-1 KO CD8 T cells encounter Ags (commensal, environmental, or self-Ags) (2, 58) in the periphery of an unimmunized mouse and undergo conventional priming or homeostatic proliferation; many of these initially acquire a T_{CM} phenotype, which in PD-1 KO cells is aberrantly transient and a large proportion of them develops stable characteristics of T_{EM} cells. Additionally, resulting PD-1 KO T_{EM} phenotype cells have a moderate survival advantage over the WT ones (Fig. 3I), thus further intensifying the effect of enhanced conversion.

In conclusion, our results show that PD-1 signaling in CD8 T cells can modulate the homeostasis of the MP pool through inhibiting differentiation toward a functional T_{EM} phenotype, most probably through a T_{CM} phenotype intermediate. These accumulated T_{EM} phenotype cells harbor potent functional properties (Fig. 3C–E) and this could result in altered host responses against pathogens, environmental Ags, or self-Ags in the absence of an intact PD-1 pathway. Additionally, PD-1 KO MP CD8 cells may elicit superior bystander protective responses against pathogens as suggested by LPS-driven IFN-γ production, especially by T_{CM} phenotype cells (Fig. 8). These findings can be clinically important, especially in the settings of currently developing treatments with antagonistic anti–PD-1 or anti–PD-ligand 1 Abs in cases of certain malignancies or chronic infections (26, 59). Equally important, manipulation of PD-1 pathway could enhance efficacy of certain vaccination regimens where production of T_{EM} cells is critical (51, 60). Further studies may include a more precise analysis of accumulated Ag specificities as well as the exact time frame where PD-1 signaling on CD8 T cells is sufficient to impose a break toward T_{EM} phenotype differentiation in naive or immunized mice.

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Disclosures

The authors have no financial conflicts of interest.

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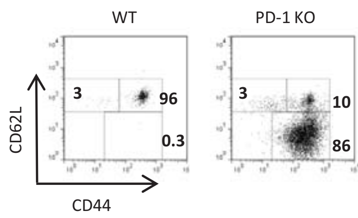
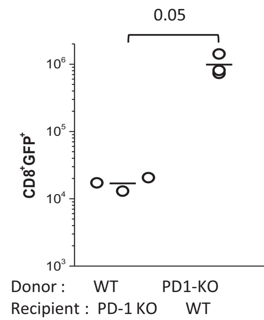
S1A



B



S1. T_{CM}→T_{EM} conversion is host independent. GFP.WT and GFP.PD-1 KO T_{CM}-phenotype CD8+ cells were adoptively transferred into PD-1 KO and WT mice respectively and analyzed on day 42 as in 6A and 6B. Data represent an individual experiment with 3 mice per group.

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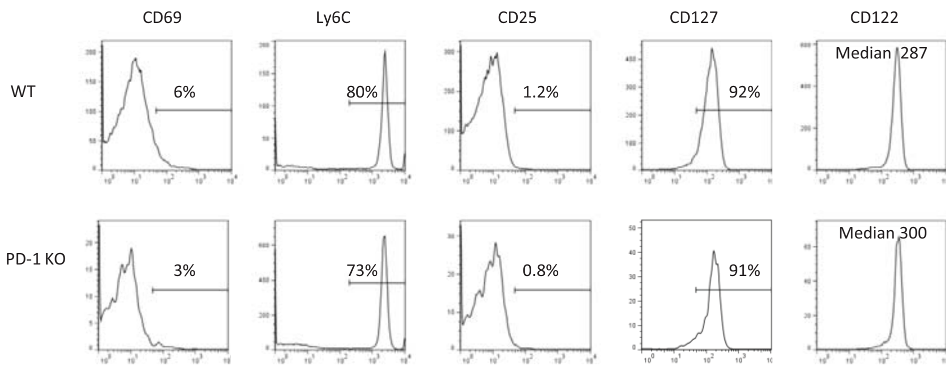
S2



S2. Expression of surface markers on T_{CM} phenotype CD8 cells. Spleens from 9 mo old PD-1 KO and WT mice were analyzed by flow cytometry. Representative histograms show expression of various surface markers gated on $CD8^{+}CD44^{hi}CD62L^{hi}$ (T_{CM}) memory-phenotype cells**.**

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Table S1. Genes differentially expressed between PD-1 KO and WT CD8 T_{CM}-phenotype cells

Gene Title	Gene Symbol	Representative Public ID	Fold Difference (PD-1 KO / WT)
Immunoglobulin heavy chain 6 (heavy chain of IgM)	Igh-6	BC018365	5.48
chemokine (C-X-C motif) ligand 9	Cxcl9	NM_008599	4.93
neuropilin 1	Nrp1	AK011144	4.69
RAB17, member RAS oncogene family	Rab17	NM_008998	4.52
similar to anti-glycoprotein-B of human Cytomegalovirus immunoglobulin Vl c	LOC100047222 /	M35669	4.16
lectin, galactose binding, soluble 3	Lgals3	X16834	4.09
basic helix-loop-helix family, member e40	Bhlhe40	NM_011498	3.87
chemokine (C-C motif) ligand 3	Ccl3	NM_011337	3.69
interferon regulatory factor 4	Irf4	U34307	3.62
Immunoglobulin kappa chain variable 14-111	Igkv14-111	U62386	3.43
sclerostin domain containing 1	Sostdc1	BC021458	3.35
receptor (calcitonin) activity modifying protein 1	Ramp1	NM_016894	3.34
Immunoglobulin heavy chain (gamma polypeptide)	Ighg	BC025447	3.17
transmembrane protein 2	Tmem2	BC019745	3.00
granzyme A	Gzma	NM_010370	2.98
Eph receptor A3	Epha3	M68513	2.95
sphingomyelin phosphodiesterase, acid-like 3B	Smpdl3b	NM_133888	2.81
immunoglobulin heavy chain 3 (serum IgG2b) /// Immunoglobulin heavy chai	Igh-3 /// Ighg	S69212	2.79
carbonic anhydrase 2	Car2	NM_009801	2.72
similar to immunoglobulin kappa-chain /// similar to Ig kappa chain V-V regior	LOC100047162 /	U55641	2.63
cell adhesion molecule 1	Cadm1	NM_018770	2.62
UDP glucuronosyltransferase 1 family, polypeptides	Ugt1a1 /// Ugt1a1	D87867	2.61
lymphocyte antigen 6 complex, locus A	Ly6a	BC002070	2.53
histocompatibility 2, class II antigen A, beta 1	H2-Ab1	M15848	2.51
growth arrest and DNA-damage-inducible 45 beta	Gadd45b	AI323528	2.50
interferon inducible GTPase 1	Iigp1	BM239828	2.48
macrophage expressed gene 1	Mpeg1	L20315	2.46
deoxyribonuclease 1-like 3	Dnase1l3	BC012671	2.44
histocompatibility 2, class II antigen A, alpha	H2-Aa	BE688749	2.41
similar to RIKEN cDNA 1100001H23 gene /// phospholipase B domain conta	LOC100045163 /	NM_025806	2.39
histocompatibility 2, class II antigen E beta	H2-Eb1	NM_010382	2.36
family with sequence similarity 84, member A	Fam84a	BC002154	2.34
B-cell leukemia/lymphoma 2 related protein A1a, A1b, A1d related protein A1	Bcl2a1a /// Bcl2a	L16462	2.26
myeloid leukemia factor 1	Mlf1	AF100171	2.23
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, z	Nfkbiz	AB026551	2.21
CD74 antigen (invariant polypeptide of major histocompatibility complex, clas	Cd74	BC003476	2.20
activating transcription factor 3	Atf3	BC019946	2.17
chemokine (C-X-C motif) receptor 4	Cxcr4	D87747	2.16
aryl-hydrocarbon receptor	Ahr	BE989096	2.16
cytochrome P450, family 17, subfamily a, polypeptide 1	Cyp17a1	NM_007809	2.15
tetraspanin 3	Tspan3	NM_019793	2.13
predicted gene 10883	Gm10883	BG966217	2.12
myristoylated alanine rich protein kinase C substrate	Marcks	AW546141	2.11
CD83 antigen	Cd83	NM_009856	2.08
RIKEN cDNA 5430435G22 gene	5430435G22Rik	AV293314	2.08
LPS-induced TN factor	Litaf	AV360881	2.04
desmoglein 2	Dsg2	BG092030	2.03
high mobility group nucleosomal binding domain 3	Hmgn3	AV018952	2.01
protein kinase inhibitor beta, cAMP dependent, testis specific	Pkib	AV047342	1.99
a disintegrin and metallopeptidase domain 8	Adam8	NM_007403	1.98
CD24a antigen	Cd24a	NM_009846	1.97
reticuloendotheliosis oncogene	Rel	NM_009044	1.97

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mitogen-activated protein kinase kinase kinase 8	Map3k8	NM_007746	1.95
immunoglobulin kappa chain variable 32 (V32)	Igk-V32	U25103	1.93
N-acylethanolamine acid amidase	Naaa	BI106821	1.93
nuclear receptor subfamily 4, group A, member 2	Nr4a2	NM_013613	1.93
fascin homolog 1, actin bundling protein (Strongylocentrotus purpuratus)	Fscn1	NM_007984	1.93
nuclear receptor subfamily 4, group A, member 1	Nr4a1	NM_010444	1.91
B-cell leukemia/lymphoma 3	Bcl3	NM_033601	1.90
proteoglycan 2, bone marrow	Prg2	NM_008920	1.89
NEDD4 binding protein 1	N4bp1	NM_030563	1.89
predicted gene 7202	Gm7202 /// Igk-C	BF301241	1.88
early growth response 1	Egr1	NM_007913	1.87
chloride intracellular channel 4 (mitochondrial)	Clic4	BB814844	1.86
proviral integration site 1	Pim1	NM_008842	1.85
immunoglobulin joining chain	Igj	BC006026	1.85
plasma glutamate carboxypeptidase	Pgcp	BB468025	1.83
nuclear receptor subfamily 4, group A, member 3	Nr4a3	BE980583	1.83
arrestin domain containing 4	Arrdc4	BC017528	1.82
RIKEN cDNA 2010002N04 gene	2010002N04Rik	BI963682	1.82
FERM domain containing 4B	Frmd4b	BG067753	1.80
phosphodiesterase 4B, cAMP specific	Pde4b	BM246564	1.80
CD81 antigen	Cd81	NM_133655	1.79
SKI-like	Skil	U36203	1.79
intercellular adhesion molecule 1	Icam1	BC008626	1.79
secretory leukocyte peptidase inhibitor	Slpi	NM_011414	1.79
ornithine decarboxylase, structural 1	Odc1	S64539	1.78
Kv channel interacting protein 3, calsenilin	Kcnip3	AF300870	1.77
tumor necrosis factor	Tnf	NM_013693	1.77
cyclin-dependent kinase inhibitor 1A (P21)	Cdkn1a	NM_007669	1.76
protease, serine, 2	Prss2	BI348548	1.76
hemochromatosis	Hfe	AJ306425	1.75
Kruppel-like factor 10	Klf10	NM_013692	1.74
septin 4	Sept4	AW208509	1.74
carbohydrate sulfotransferase 2	Chst2	NM_018763	1.74
Notch-regulated ankyrin repeat protein	Nrarp	BI696369	1.73
suppressor of cytokine signaling 3	Socs3	NM_007707	1.73
TGFB-induced factor homeobox 1	Tgif1	NM_009372	1.73
---	---	BC027249	1.73
CD38 antigen	Cd38	BB256012	1.72
ring finger protein 19B	Rnf19b	AK015966	1.70
similar to sprouty 1 /// sprouty homolog 1 (Drosophila)	LOC100046643 /	NM_011896	1.70
predicted gene 1409	Gm1409	U48716	1.70
RAR-related orphan receptor alpha	Rora	BI660199	1.69
secreted phosphoprotein 1	Spp1	NM_009263	1.69
allograft inflammatory factor 1	Aif1	NM_019467	1.69
serine (or cysteine) peptidase inhibitor, clade A, member 3G	Serpina3g	BC002065	1.69
interferon regulatory factor 8	Irf8	BG069095	1.68
oocyte secreted protein 1	Oosp1	NM_133353	1.67
SAM domain, SH3 domain and nuclear localization signals, 1	Samsn1	NM_023380	1.66
hypothetical protein LOC100047091 /// transmembrane protein 163	LOC100047091 /	AK011522	1.65
ets variant gene 6 (TEL oncogene)	Etv6	BB068442	1.65
signal transducer and activator of transcription 1	Stat1	AW214029	1.64
carbohydrate (keratan sulfate Gal-6) sulfotransferase 1	Chst1	NM_023850	1.64
dual specificity phosphatase 16	Dusp16	NM_130447	1.64
inducible T-cell co-stimulator	Icos	AB023132	1.63
TSC22 domain family, member 1	Tsc22d1	BB357514	1.63
histocompatibility 2, class II, locus Mb1 /// histocompatibility 2, class II, locus	H2-DMb1 /// H2-D	NM_010387	1.63
family with sequence similarity 110, member A	Fam110a	AK005776	1.63
arachidonate 5-lipoxygenase activating protein	Alox5ap	BC026209	1.61
syndecan 1	Sdc1	BI788645	1.60

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basic leucine zipper transcription factor, ATF-like	Batf	NM_016767	1.59
cystatin C	Cst3	AF483486	1.59
---	---	AFFX-18SRNAMu	1.58
ectonucleotide pyrophosphatase/phosphodiesterase 2	Enpp2	BC003264	1.58
v-myc myelocytomatosis viral oncogene homolog 1, lung carcinoma derived	Mycl1	BG064871	1.58
lactate dehydrogenase B	Ldhb	AV219418	1.58
SLIT-ROBO Rho GTPase activating protein 2	Srgap2	AK005172	1.58
asparagine synthetase	Asns	BC005552	1.58
GTP cyclohydrolase 1	Gch1	NM_008102	1.57
acyl-CoA synthetase long-chain family member 4	Acsl4	AB033886	1.57
component of Sp100-rs /// predicted gene 7582	Csprs /// Gm7582	BB148221	1.57
tumor necrosis factor receptor superfamily, member 1b	Tnfrsf1b	M60469	1.57
purinergic receptor P2Y, G-protein coupled, 14	P2ry14	AF177211	1.56
histone cluster 1, H3a , b ,c , d, e, f, g, h, i/// H3b /// histone cluster 2, H3c1 //	Hist1h3a /// Hist1	NM_019469	1.56
chemokine (C-C motif) receptor-like 2` v	Ccrl2	AJ318863	1.56
dual specificity phosphatase 8	Dusp8	NM_008748	1.56
glycoprotein 49 A /// leukocyte immunoglobulin-like receptor, subfamily B, me	Gp49a /// Lilrb4	U05264	1.55
metallothionein 2	Mt2	AA796766	1.55
interferon regulatory factor 1	Irf1	NM_008390	1.55
homeodomain interacting protein kinase 2	Hipk2	AK016742	1.55
MARCKS-like 1	Marcksl1	NM_010807	1.55
myomesin 2	Myom2	BB474208	1.54
prion protein	Prnp	BE630020	1.53
CD22 antigen	Cd22	AF102134	1.53
prostate transmembrane protein, androgen induced 1	Pmepa1	AV370981	1.53
family with sequence similarity 129, member A	Fam129a	NM_022018	1.53
interleukin 12 receptor, beta 1	Il12rb1	NM_008353	1.53
guanylate binding protein 3	Gbp3	NM_018734	1.53
cell division cycle associated 5	Cdca5	NM_026410	1.52
TBC1 domain family, member 8	Tbc1d8	BC005421	1.52
neutrophil cytosolic factor 4	Ncf4	NM_008677	1.52
cysteinyl leukotriene receptor 2	Cysltr2	NM_133720	1.52
RASD family, member 2	Rasd2	BC026377	1.52
creatine kinase, brain	Ckb	BG967663	1.52
CDK5 regulatory subunit associated protein 1	Cdk5rap1	NM_025876	1.51
chymotrypsin-like elastase family, member 1	Cela1	BC011218	1.51
EH-domain containing 1	Ehd1	NM_010119	1.51
LPS-induced TN factor	Litaf	AV360881	1.51
CDC28 protein kinase 1b	Cks1b	NM_016904	1.51
proviral integration site 2	Pim2	NM_138606	1.51
dual specificity phosphatase 2	Dusp2	L11330	1.50
carbohydrate sulfotransferase 11	Chst11	NM_021439	1.50
predicted gene 11275	Gm11275	NM_013550	1.50
tissue inhibitor of metalloproteinase 2	Timp2	M93954	1.50
sperm associated antigen 5	Spag5	BM208112	1.50
WW domain-containing oxidoreductase	Wwox	NM_019573	1.50
lysosomal trafficking regulator	Lyst	NM_010748	-1.50
hypothetical LOC100270747	LOC100270747	BB200448	-1.50
BRCA1/BRCA2-containing complex, subunit 3	Brcc3	AI462244	-1.51
zinc finger protein 790	Zfp790	BG068796	-1.51
Janus kinase 1	Jak1	BQ032637	-1.51
annexin A6	Anxa6	AK013026	-1.51
mitogen-activated protein kinase kinase 7	Map2k7	AW541674	-1.52
serine/threonine kinase 25 (yeast)	Stk25	NM_021537	-1.52
acylphosphatase 1, erythrocyte (common) type	Acyp1	NM_025421	-1.53
O-linked N-acetylglucosamine (GlcNAc) transferase	Ogt	BF681886	-1.53
casein kinase 2, alpha 1 polypeptide	Csnk2a1	AK011501	-1.53
ATP-binding cassette, sub-family C (CFTR/MRP), member 5	Abcc5	AV150520	-1.54
PRP39 pre-mRNA processing factor 39 homolog (yeast)	Prpf39	BB460975	-1.54

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zinc finger protein 82	Zfp82	BM230481	-1.54
translin-associated factor X	Tsnax	BM119928	-1.54
intraflagellar transport 80 homolog (Chlamydomonas)	Ift80	BC013814	-1.55
zinc finger protein 260	Zfp260	L36316	-1.55
CD93 antigen	Cd93	BB039247	-1.55
high mobility group 20A	Hmg20a	AI987819	-1.56
DNA segment, Chr 4, Wayne State University 53, expressed	D4Wsu53e	BE652553	-1.56
DEAD (Asp-Glu-Ala-Asp) box polypeptide 10	Ddx10	AK019495	-1.56
zinc finger protein 709	Zfp709	BC021921	-1.56
poliovirus receptor	Pvr	BB049138	-1.57
carbohydrate (N-acetylgalactosamine 4-sulfate 6-O) sulfotransferase 15	Chst15	AK019474	-1.57
minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cer	Mcm6	BB099487	-1.57
ring finger protein, transmembrane 1	Rnft1	AK002624	-1.57
T-cell receptor gamma, variable 4	Tcrg-V4	NM_011558	-1.57
myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila);	Mllt6	AY050217	-1.57
pigeon homolog (Drosophila)	Pion	BB637972	-1.57
S-adenosylhomocysteine hydrolase-like 1	Ahcyl1	BB831090	-1.58
RNA binding motif protein 6	Rbm6	BB706030	-1.60
zinc finger protein 623	Zfp623	BB333454	-1.60
protein-L-isoaspartate (D-aspartate) O-methyltransferase domain containing	Pcmtd2	BM117243	-1.60
X-linked inhibitor of apoptosis	Xiap	BF134200	-1.61
SH3-domain kinase binding protein 1	Sh3kbp1	AK018032	-1.61
guanylate cyclase activator 1B	Guca1b	BC018480	-1.61
killer cell lectin-like receptor subfamily B member 1C	Klrb1c	NM_008527	-1.61
ATPase, Na+/K+ transporting, beta 1 polypeptide	Atp1b1	NM_009721	-1.62
RIKEN cDNA 2610030H06 gene	2610030H06Rik	BB410125	-1.63
similar to splicing factor, arginine/serine-rich 1/// splicing factor, arginine/serir	LOC100048559 /	X66091	-1.63
predicted gene 14430 /// predicted gene 14434 /// predicted gene, OTTMUS(	Gm14430 /// Gm1	C77009	-1.63
putative homeodomain transcription factor 2	Phtf2	BM228625	-1.64
praja1, RING-H2 motif containing	Pja1	BM199789	-1.64
neuronal PAS domain protein 2	Npas2	BG070037	-1.65
---	Fas	BG976607	-1.65
dopa decarboxylase	Ddc	AF071068	-1.67
SET domain containing 4	Setd4	BF020504	-1.67
zinc finger protein 329	Zfp329	AK014562	-1.68
glutamine repeat protein 1	Glrp1	NM_008132	-1.68
bromodomain PHD finger transcription factor	Bptf	BB380312	-1.68
chemokine (C-C motif) receptor 10	Ccr10	AF215982	-1.68
ring finger protein 157	Rnf157	BB246182	-1.69
tetraspanin 32	Tspan32	AF175771	-1.70
eukaryotic translation initiation factor 4, gamma 1	Eif4g1	BF227830	-1.71
RIKEN cDNA 1110057K04 gene	1110057K04Rik	BB534387	-1.72
centrosomal protein 350	Cep350	BC019716	-1.73
B-cell translocation gene 2, anti-proliferative	Btg2	NM_007570	-1.73
ArfGAP with FG repeats 2	Agfg2	BC003330	-1.73
far upstream element (FUSE) binding protein 1	Fubp1	BB488001	-1.73
bromodomain adjacent to zinc finger domain, 1B	Baz1b	BB253608	-1.74
chemokine (C-X-C motif) receptor 6	Cxcr6	NM_030712	-1.74
expressed sequence C78339	C78339	BG075168	-1.75
RIKEN cDNA 4933411K20 gene	4933411K20Rik	NM_025747	-1.76
splicing factor, arginine/serine-rich 7	Sfrs7	BC014857	-1.77
pleckstrin homology domain containing, family A member 5	Plekha5	BG067450	-1.77
tetratricopeptide repeat domain 14	Ttc14	BC021448	-1.78
cyclic nucleotide gated channel alpha 1	Cnga1	U19717	-1.79
neural precursor cell expressed, developmentally down-regulated 4	Nedd4	NM_010890	-1.79
liver glycogen phosphorylase	Pygl	NM_133198	-1.82
alpha thalassemia/mental retardation syndrome X-linked homolog (human)	Atrx	BB825830	-1.83
myeloblastosis oncogene	Myb	BC011513	-1.85
tubulin tyrosine ligase-like family, member 3	Ttll3	NM_133923	-1.85

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CXXC finger 5	Cxxc5	AK015150	-1.86
RUN and FYVE domain-containing 2	Rufy2	AI852705	-1.91
GRIP and coiled-coil domain containing 2	Gcc2	BC027339	-1.96
baculoviral IAP repeat-containing 6	Birc6	BC026990	-2.00
---	---	BC021831	-2.04
utrophin	Utrn	AI788797	-2.09
pinin	Pnn	AV135835	-2.44
killer cell lectin-like receptor, subfamily A, member 7	Klra7	U10095	-3.10

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