1 **Title**

2 **Inflammatory signals are sufficient to elicit TOX expression in mouse and human CD8 T cells**

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4 **Authors**

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17

18 **Competing interests**

19 The authors declare no competing interests.

20

21 **Abstract**

22 T cell receptor (TCR) stimulation leads to expression of the transcription factor TOX. Prolonged TCR
 23 signaling, such as encountered during chronic infections or in tumors, leads to sustained TOX
 24 expression, which induces a state of exhaustion or dysfunction. While CD8 memory T cells (T_{mem}) in
 25 specific pathogen-free laboratory mice typically do not express TOX, functional human T_{mem} show
 26 heterogeneous TOX expression levels. Whether TCR-independent mechanisms can alter TOX
 27 expression in human and murine T_{mem} has not been defined. We report that human and mouse T_{mem}
 28 increase TOX expression following stimulation with inflammatory cytokines IL-12, IL-15, and IL-18. TOX
 29 and PD-1 expression patterns often appear to be directly correlated, however, we found that TOX is not
 30 necessary for cytokine-driven expression of PD-1. Together, these observations highlight that
 31 inflammation is sufficient to alter TOX and PD-1 expression and that the signals regulating TOX
 32 expression appear well conserved in human and murine T_{mem} .

33

34 Introduction

35 T cell exhaustion (i.e. dysfunction) is driven by chronic TCR stimulation with cognate antigen
 36 (Ag)^{1, 2, 3}. It describes a differentiation state in which T cells have diminished capacity to respond to
 37 stimulatory inputs and limited effector capacity^{2, 3, 4}. The purpose of T cell exhaustion during chronic
 38 infections may be to limit tissue pathologies when pathogen cannot be immunologically eliminated^{5, 6}.
 39 Though exhaustion could be considered an immunologic concession during chronic infection, it also
 40 occurs in tumors and causes an attenuated anti-tumor cytotoxic T cell response⁷. Thus, mechanistically
 41 understanding and therapeutically overcoming T cell exhaustion has been a major goal of tumor
 42 immunotherapy. Chronic TCR stimulation elicits a program that leads to constitutively high expression of
 43 programmed cell death 1 (PD-1)⁸. PD-1 is an inhibitory receptor which is expressed by activated and
 44 exhausted T cells and often used as a biomarker to infer T cell functionality⁹. When bound to its ligands,
 45 PD-1 negatively regulates T cell function². Therapeutic targeting of PD-1 with monoclonal antibodies,
 46 also referred to as immune checkpoint inhibitors, can reinvigorate a subset of these PD-1 expressing T
 47 cells^{2, 10, 11, 12}.

48 A set of recent studies demonstrated that the transcription factor, thymocyte selection-associated
 49 high mobility group box (TOX) protein, drives or stabilizes this TCR-mediated T cell dysfunction and PD-
 50 1 upregulation^{6, 13, 14, 15, 16}. When stably expressed, TOX drove Ag-specific T cell exhaustion in mouse
 51 models of chronic lymphocytic choriomeningitis virus (LCMV) infection, transplantable B16 melanoma,
 52 and inducible hepatocellular carcinoma^{6, 13, 14}. Further, putative tumor Ag-specific CD8 T cells isolated
 53 from primary human breast, ovarian, and skin cancer samples, as well as those specific for hepatitis C
 54 virus (HCV), mirrored this phenotype, suggesting TOX dictates exhaustion programs in humans, too^{6, 13,}
 55 ¹⁴. Of note, TOX expression by HCV-specific T cells was reduced following treatment and clearance of
 56 the infection but still detectable at higher levels than in T cells from HCV infections that spontaneously
 57 resolved and among T cells specific for influenza A virus (IAV)⁶. Mechanistic insight was provided by
 58 targeted deletion of TOX in Ag-specific cytotoxic T cells, which diminished PD-1 expression and restored
 59 functionality at the expense of cell survival^{6, 13}. Therefore, TOX concedes activation and effector function
 60 for exhaustion (i.e. PD-1 expression) and T cell survival during chronic TCR stimulation. In instances of

brief TCR engagement, TOX is transiently induced to a level lower than that of exhausted T cells, but with limited known functional consequence^{6, 13, 14}.

While the requirement for TOX has been well defined in the context of TCR-mediated dysfunction, there is nascent evidence that TOX expression by itself is not indicative of T cell exhaustion. Recent studies illustrated that TOX expression is detected in some functional CD8 memory T cells (T_{mem}), for instance in CD8 effector memory (T_{EM}) and effector memory CD45RA-expressing (T_{EMRA}) subsets¹⁷. CD8 T_{mem} specific for the latent viruses, cytomegalovirus (CMV) and Epstein-Barr virus (EBV), had elevated TOX expression, compared to those specific for acute infections, which further suggests that TCR signals are critical in regulating TOX expression¹⁷. In a second study, it was shown that a fraction of the human T_{mem} population expresses *TOX* transcripts amongst other signature genes typically associated with T cell exhaustion¹⁸. The observation that functional human memory T cells express TOX also led to questioning whether TOX is functionally conserved between mouse and human T cells¹⁹. Further complicating TOX and exhaustion, the murine tissue resident memory T cell (T_{RM}) transcriptome is characterized by concomitant expression of transcripts encoding *Tox*, exhaustion markers, TCR signaling components, and cytotoxic molecules, well after initial priming events^{20, 21}. While the role of TOX in these TOX-expressing populations with and without signs of T cell exhaustion is not fully understood, these data suggest that TOX expression by memory T cells cannot be reliably used to extrapolate T cell function.

While the role of TCR signals in initiating and maintaining PD-1 and TOX expression has been well established, relatively little remains known about non-TCR signals that could regulate their expression in T cells²². We considered that cytokine-mediated stimuli could also affect TOX expression levels without promoting the induction of T cell exhaustion. First, pro-inflammatory cytokines, like IL-15, can induce PD-1 without agonist TCR signals. Second, T_{RM} that are likely not detecting cognate Ag still upregulate PD-1 and other markers associated with exhaustion^{20, 21, 23, 24}, yet rely on IL-15 signaling for maintenance in some tissues^{25, 26}. Thus, inflammatory signals could provide an explanation for some of the seemingly disparate results of TOX expression and T cell function. Here, we show that pro-inflammatory cytokines were sufficient to induce TOX expression in the absence of agonist TCR signals

88 in both mouse and human CD8 T_{mem}, while concurrently inducing expression of cytotoxic molecules.
 89 Together, these data demonstrate that TOX expression per se does not indicate TCR-mediated
 90 dysfunction or even a recent TCR signals. We also demonstrate that PD-1 expression is still upregulated
 91 in TOX-deficient T cells indicating that TOX is not necessary for PD-1 expression. Overall, our data
 92 reveal new TCR-independent mechanisms that shape TOX and PD-1 expression heterogeneity in T_{mem}
 93 and indicate that these mechanisms are conserved in both mouse and human T cells. Though these
 94 findings ultimately complicate the use of TOX exclusively as an exhaustion biomarker, they implicate
 95 TOX in inflammation-driven programs of memory T cell activation.

96

97 Results

98 Cytokine stimulation induces TOX expression in murine CD8 T_{mem}

99 The pro-inflammatory cytokines IL-12, IL-15, and IL-18 elicit interferon- γ (IFN γ) and granzyme B
100 (GzmB) expression in mouse and human CD8 T_{mem} in the absence of agonist TCR signals^{27, 28, 29}. We
101 first sought to determine if these cytokines could also induce TOX expression in a TCR-independent
102 manner. To generate a well-defined population of CD8 T_{mem}, we transferred congenically-marked OT-I
103 CD8 T cells, which express a TCR specific for the SIINFEKL peptide of OVA, into wildtype C57BL/6J
104 animals followed by infection with OVA-expressing vesicular stomatitis virus (VSV-OVA) (**Fig. 1a**). We
105 waited ≥ 60 days before using these mice for subsequent experiments (referred to as VSV-OVA OT-I
106 memory mice) (**Fig. 1a**). We isolated T cells from the spleens and LNs from VSV-OVA OT-I memory
107 mice using negative-selection magnet-activated cell sorting (MACS) prior to ex vivo stimulation
108 experiments (**Fig. 1a**). This was done to ensure that cytokines act directly on T cells³⁰. As a negative
109 control, we cultured bulk T cells in media alone (mock) and as a positive control, we stimulated T cells
110 with anti-CD3/CD28 microbeads (**Fig. 1a**). We used a combination of rIL-12, rIL-15, and rIL-18 (IL-
111 12/15/18) to induce IFN γ and GzmB expression in a TCR-independent manner (**Fig. 1a**). We found that
112 IL-12/15/18 stimulation induced PD-1 expression in OT-I T_{mem}, but the increase in expression was
113 markedly higher after TCR ligation (**Fig. 1b**). PD-1 frequency and median fluorescence intensity (MedFI)
114 in OT-I T_{mem} increased throughout the duration of IL-12/15/18 stimulation (**Fig. 1b**). Similarly, TCR and
115 IL-12/15/18 stimulation induced TOX upregulation in OT-I T_{mem} (**Fig. 1c**). Next, we measured TCF1
116 expression, a transcription factor needed for memory T cell self renewal that is lost in terminally
117 exhausted T_{mem}^{31, 32, 33, 34}. Alongside increasing PD-1 and TOX levels, both TCR- and IL-12/15/18-
118 mediated stimulation led to significant loss of TCF1 expression in OT-I T_{mem} (**Fig. 1d**). In sum, these data
119 indicate that phenotypes often associated with exhaustion can be induced by TCR-independent,
120 cytokine-mediated T_{mem} activation. Finally, we sought to determine whether stimulation similarly affected
121 endogenous CD8 T_{mem} and CD8 T_{naïve}. IL-12/15/18 stimulation significantly increased TOX expression in
122 endogenous CD8 T_{mem} but was not observed to the same degree in CD8 T_{naïve} (**Supplemental Fig. 1a,**
123 **b**). This CD8 T_{mem}-specific response is, too, reflected in IL-12/15/18-mediated upregulation of PD-1

(**Supplemental Fig. 1c, d**). This is likely, in some degree, to the different propensities of T cell subsets (both major and memory) to become efficiently activated by cytokines³⁵ and differences in cytokine receptor expression (particularly naïve T cells, which require TCR-mediated activation to induce IL-12R and strongly increase IL-18R expression^{36, 37}). Much akin to OT-I T_{mem}, TCR stimulation dramatically increased both TOX MedFI and PD-1 expression across endogenous subsets (**Supplemental fig. 1a-d**), though the fold change in TOX staining intensity was most pronounced in CD8 T_{mem} (**Supplemental fig. 1a**). Though IL-12/15/18 stimulation increases TOX MedFI in transgenic and endogenous CD8 T_{mem}, it is initially to a lower degree than that of TCR-stimulated cells (**Fig. 1c, Supplemental fig. 1a**). Since short-term TCR- and IL-12/15/18-stimulation could dramatically augment TOX and PD-1 expression in CD8 T_{mem} from VSV-OVA OT-I memory mice, we next sought to test if TOX and PD-1 upregulation compromises functionality.

135

136 **Functional CD8 T_{mem} express TOX and PD-1 and effector proteins**

137 We isolated T cells from VSV-OVA OT-I memory mice as outlined for Fig. 1. We stimulated T
138 cells in the presence of Golgi inhibitors and found that OT-I T_{mem} produced substantial amounts of IFN γ
139 after IL-12/15/18 or TCR stimulation(**Fig. 2a**); yet IFN γ -expressing OT-I T_{mem} demonstrated higher TOX
140 and PD-1 expression than those that failed to make IFN γ (**Fig. 2b, c**). Similarly, OT-I T_{mem} that produced
141 GzmB post-stimulation also demonstrated increased TOX and PD-1 expression (**Supplemental fig. 2a,**
142 **b**). Together, these data indicate that TOX and PD-1 expression are elevated in activated, functional
143 CD8 T_{mem} and suggest that TOX expression is also part of a cytokine-driven T cell activation program.

144

145 **Induction of TOX and PD-1 is heterogeneous in CD8 T_{mem}**

146 To ensure that our data were not solely reliant on OT-I T cells, we also generated gBT-I memory
147 mice using gBT-I TCR transgenic cells (specific for an epitope of the HSV2 gB protein) and a
148 recombinant, gB epitope-expressing LM strain (LM-gB) (**Supplemental Fig. 3b**). After stable contraction
149 of TCR transgenic T_{mem} (≥ 60 d), we conducted stimulation assays as previously outlined (**Fig. 1a**). IL-
150 12/15/18- or TCR-mediated stimulation led to comparable TOX upregulation in OT-I and gBT-I T_{mem} (**Fig**

151 **3a, b**). Similarly, PD-1 expression was comparable in OT-I and gBT-I T_{mem} after stimulation
 152 (**Supplemental Fig 3c, d**), with a concurrent loss of TCF1 expression (**Supplemental Fig. 3e, f**). We
 153 next asked if altering the nature of the priming infection could affect the ability to express TOX in
 154 response to cytokine-mediated activation at the memory stage. We adoptively transferred P14 transgenic
 155 T cells, a TCR transgenic specific for lymphocytic choriomeningitis virus (LCMV) gp33, followed by
 156 infection with LCMV Armstrong or Docile (**Supplemental Fig. 3g, h**). These LCMV strains elicit acute
 157 and chronic infections, respectively (the latter causing T cell dysfunction). We then stimulated (same
 158 culture set-up as outlined in **Fig. 1a**) T cells from these P14 memory mice. P14 T_{mem} from LCMV
 159 Armstrong infected mice readily upregulated PD-1 after TCR- or IL-12/15/18 stimulation (**Supplemental**
 160 **Fig. 3i**). The exhausted P14 T_{mem} from LCMV Docile-infected mice already uniformly expressed PD-1
 161 prior to stimulation; but IL-12/15/18 or TCR stimulation further increased surface PD-1 expression (via
 162 increased MedFI) (**Supplemental Fig. 3j**). P14 T_{mem} from LCMV Armstrong infected mice increased TOX
 163 expression after TCR or IL-12/15/18 stimulation (**Fig. 3c**). However, exhausted P14 T_{mem} from LCMV
 164 Docile-infected mice only significantly increased TOX expression after TCR stimulation (**Fig. 3d**) and
 165 showed significantly lower fold changes in TOX MedFI compared to P14 T_{mem} from LCMV Armstrong-
 166 infected mice. While differences between CD8 T_{mem} from acute and chronic infected are expected, the
 167 differences between gBT-I and OT-I (~3 to 4-fold increase in TOX expression) compared to P14 (up to
 168 ~2-fold) need to be interpreted with caution since the gBT-I, OT-I and P14 experiments used different
 169 TOX antibody clones (REA473 and TXRX10, respectively). Overall, our data indicate that T_{mem} that were
 170 generated by different acute infections increase TOX expression in response to pro-inflammatory
 171 cytokines suggesting that this a broadly applicable mechanism of TOX induction in the memory T cell
 172 compartment. We next sought to determine if PD-1 and TOX upregulation in response to stimulation was
 173 similarly recapitulated in human CD8 T cells.

174 175 **Cytokine stimulation induces TOX and PD-1 in human T_{mem}**

176 Using cryopreserved PBMCs from healthy, HIV-seronegative donors, we interrogated TOX and
 177 PD-1 expression by flow cytometry. We specifically gated CD8 T cells by a memory and naïve binary,

delineating CD8 T_{naïve} as CD45RO-negative CCR7-positive, with remaining cells as CD8 T_{mem}³⁸ (**Fig. 4a**), and interrogated basal TOX and PD-1 expression between these two subsets (**Fig. 4a**). Since PD-1 expression is heterogeneous in humans^{39, 40}, we measured TOX MedFI across PD-1 low-, medium-, and high-expressing events. We found that CD8 T_{mem} with the highest PD-1 expression also demonstrated significantly elevated TOX MedFI (**Fig 4b**), mirroring correlations of TOX and PD-1 expression in our mouse model as well as human HCV infections⁶. We next tested whether IL-12/15/18 stimulation increases PD-1 and TOX expression in T cell subsets and included mock and TCR stimulation conditions as negative and positive controls, respectively. We also included stimulations using rIL-6, rIL-15, or rIL-12 and rIL-18. We chose these additional conditions as IL-6 activates CD8 T_{naïve} (as evidenced by CD69 upregulation) and to discern individual activating contributions of each cytokine (**Supplemental Fig. 4a**). Across these conditions, IL-12/15/18- and TCR-mediated stimulations led to the most prominent increase of TOX staining intensity and PD-1^{hi} frequency in CD8 T_{mem} (**Fig. 4d**). We measured TCF1 expression after mock, IL-12/15/18, and TCR stimulation. A decrease in TCF1 expression accompanied an increase in TOX and PD-1 expression after IL-12/15/18 or TCR stimulation (**Supplemental fig. 4b**), akin to our mouse stimulation data. We further tested the degree of similarity between human and mouse T cells by measuring PD-1, TCF1, and TOX expression profiles in stimulated human CD8 T_{naïve}. Like mouse CD8 T_{naïve}, only TCR stimulation could lead to appreciable changes in TOX and PD-1 within human CD8 T_{naïve} (**Fig. 4c, Supplemental fig. 4c**). Since IL-6 can activate CD8 T_{naïve}, we used this condition to determine if PD-1 and TOX expression could occur in naïve T cells in the absence of a TCR signal. Despite inducing CD69 expression, we found that IL-6-mediated stimulation failed to increase TOX or PD-1 expression in CD8 T_{naïve} (**Supplemental fig. 4d**). Together, these data show that CD8 T_{mem} differentially expressed TOX, PD-1, and TCF1 at homeostasis and after both IL-12/15/18 and TCR stimulation. We next wanted to better define these changes across different memory T cell subsets.

201

202 **Inflammation-induced PD-1 and TOX expression occur in most but not all CD8 T_{mem} subsets**

203 To test if inflammation-induced PD-1 and TOX expression differs across human CD8 T_{mem} subsets, we used CD45RO and CCR7 staining to further delineate central memory (T_{CM}) (CD45RO⁺

204

CCR7⁺), T_{EM} (CD45RO⁺ CCR7⁻), and T_{EMRA} (CD45RO⁻ CCR7⁻) subsets^{38, 41} (**Fig. 5a**). When we measured TOX, PD-1, and TCF1 expression across these subsets, we noted that a substantial fraction of CD8 T_{EM} events were PD-1^{hi}, and both CD8 T_{EM} and T_{EMRA} expressed elevated and lower levels of TOX and TCF1, respectively, at homeostasis (**Fig. 5b**). While this observation is in line with the initial report demonstrating TOX heterogeneity in human CD8 T_{mem} subsets¹⁷, it remained unknown if these CD8 T_{mem} subsets are equally capable of further TOX upregulation after stimulation. We observed that TOX, PD-1, and TCF1 expression kinetics in CD8 T_{CM} and T_{EM} largely resembled one another, with both IL-12/15/18 and TCR stimulation increasing the frequency of PD-1^{hi} events and TOX MedFI, but decreasing TCF1 MedFI (**Fig. 5c**). It is worth noting, that while TCF1 MedFI in CD8 T_{CM} drops profoundly after IL-12/15/18 or TCR stimulation, the loss in frequency of TCF1-expressing cells (as defined by subjective gating) is not as pronounced as what we observed in CD8 T_{EM} (**Supplemental fig. 5a**). While IL-12/15/18- and TCR-mediated stimulation were both able to significantly increase the frequency of PD-1^{hi} events and lower TCF1 MedFI in CD8 T_{EMRA}, the degree of these changes was less pronounced than in CD8 T_{CM} or T_{EM} (**Fig. 5c**). Moreover, CD8 T_{EMRA} did not significantly upregulate TOX expression after TCR stimulation. This, however, was not due to inability to be stimulated, as CD8 T_{EMRA} readily expressed the activation marker CD69 after cytokine- or TCR-mediated stimulation (**Supplemental Fig. 5a**). Finally, it is worth noting that when stimulated with IL-15 alone, CD8 T_{CM}, unlike CD8 T_{EM} and T_{EMRA}, fail to significantly express PD-1 (**Supplemental fig. 5b**).

We next interrogated T cells with defined TCR specificity, specifically influenza A virus (IAV)-specific CD8 T cells using HLA-A*02 tetramers loaded with the GILGFVFTL peptide (**Fig. 6a**). We examined this CD8 T_{mem} population because these cells were reported to not express appreciable levels of TOX at homeostasis, likely owing to their T_{CM} phenotype¹⁷. Within our sample set, IAV-specific CD8 T cells were predominantly T_{CM} in half of the HLA-A*02 PBMC donors (**Fig. 6a**). Nevertheless, all IAV-specific CD8 T cells were able to substantially upregulate TOX and PD-1 expression after IL-12/15/18 stimulation (**Fig. 6b**), indicating that CD8 T_{mem} low for TOX and PD-1 at homeostasis, can also contribute to TOX and PD-1 heterogeneity after recent activation. Alongside testing IAV-specific CD8 T cells, we also interrogated the effects of stimulation in mucosal associated invariant T (MAIT) cells. We selected

232 this population because 1) MAIT cells are non-conventional T cells, recognizing bacterial metabolites as
 233 Ags presented on MHC-related 1 (MR1)⁴², 2) inflammation is necessary for sustained MAIT cell effector
 234 function^{43, 44} and 3) MAIT cells are near-uniformly T_{EM} when defined by CD45RO and CCR7⁴⁵. We
 235 identified MAIT cells using MR1 tetramers loaded with the 5-OP-RU metabolite⁴⁶, which largely fell into
 236 our T_{EM} gate (**Fig. 6c**). Like IAV-specific CD8 T cells, IL-12/15/18 stimulation led to substantial TOX and
 237 PD-1 upregulation in MAIT cells (**Fig. 6d**). Since inflammation is necessary for sustained MAIT cell
 238 effector function, we asked if MAIT cells are differentially capable of responding to other cytokine
 239 combinations. Alongside IL-12/15/18, IL-15 alone, or IL-12 and IL-18 in unison could significantly
 240 increase both the PD-1^{hi} frequency and TOX MedFI of MAIT cells, but not IAV-specific T cells
 241 (**Supplemental fig. 6a, b**). Together, these data indicate that this cytokine-driven activation program is
 242 conserved across conventional and innate-like T cells.

243

244 Cytokine stimulation-induced PD-1 expression is independent of TOX

245 Finally, since PD-1 and TOX upregulation appeared tightly associated following cytokine-driven
 246 activation, we next asked if this association is mechanistic in nature. If TOX is necessary for PD-1
 247 expression, it would allow to use the surface-expressed PD-1 as a surrogate for the intracellularly
 248 expressed TOX. TOX expression appears to drive PD-1 expression in a number of contexts, as
 249 exhausted T_{mem} dramatically downregulated PD-1 after TOX deletion or knockdown^{6, 13, 15, 47}. Conversely,
 250 T cell transduction with TOX-encoding constructs leads to PD-1 upregulation^{13, 14, 15, 47}. While TOX
 251 controls PD-1 expression during exhaustion, the role of TOX is less clear in activation. To dissect the
 252 function of TOX in stimulation-mediated PD-1 upregulation, we used wildtype (WT) and *Tox*^{-/-} P14 T_{mem}.
 253 To generate these P14 T_{mem}, we adoptively transferred wildtype or knockout P14 T cells into C57BL/6J
 254 hosts, which we subsequently infected with LCMV Armstrong to form a T_{mem} population (**Supplemental**
 255 **fig. 7a**). To determine if TOX deficiency alters stimulation-induced PD-1 upregulation, we cultured
 256 MACS-isolated T cells from WT and *Tox*^{-/-} P14 memory mice (28 days post LCMV Armstrong infection)
 257 in the presence of mock, IL-12/15/18, or TCR stimulation (**Supplemental fig. 7a**). Both WT and *Tox*^{-/-}
 258 P14 T_{mem} increased PD-1 expression after IL-12/15/18 or TCR stimulation (**Fig. 7a, b, c**). Together these

259 data indicate that TOX alone is not necessary for PD-1 upregulation in cytokine-stimulated CD8 T_{mem} and
260 suggest other transcription factors are sufficient to drive PD-1 expression in the absence of TOX.
261
262

263 Discussion

264 TOX has been foremost studied in TCR-mediated exhaustion of mouse CD8 T cells in context of
 265 tumor or chronic infection^{6, 13, 14}. A recent study reported TOX expression in functional circulating human
 266 CD8 T_{mem}, suggesting TOX expression does not necessarily dictate dysfunction^{17, 18}, which led to the
 267 speculation that TOX may have distinct roles across species, specifically mice and humans¹⁹.
 268 Alternatively, TOX expression heterogeneity in humans may simply reflect the more complex
 269 environment that human T cells are exposed to in every day life, that may not be readily appreciable in
 270 specific pathogen-free mice, such as routine inflammatory events in barrier tissues. Thus, we asked if
 271 pro-inflammatory cues could be sufficient to increase TOX expression and contribute to TOX
 272 heterogeneity. While inflammation has been previously shown to enhance TCR-mediated TOX
 273 upregulation (in a VEGF-A-dependent manner that necessitates initial TCR signaling)⁴⁸, our findings are,
 274 to the best of our knowledge, the first to demonstrate TOX expression in the absence of agonist TCR
 275 signals. Transient IL-12/15/18 and TCR stimulation increased PD-1 and TOX expression in most CD8
 276 T_{mem}. In mouse, dysfunctional P14 T_{mem} from LCMV Docile infected mice still increased surface PD-1
 277 expression after TCR stimulation, while IL-12/15/18 had little to no effect on TOX expression. Similarly,
 278 human T_{EMRA} showed limited to no increase in TOX expression following exposure to IL-12/15/18. The
 279 underlying mechanisms will require further investigation, but one could speculate that the cytokine
 280 stimulation is simply not potent enough to further enhance the already ongoing effector or activation
 281 program in these two memory T cell subsets. The notion that TOX, but also PD-1 expression can
 282 indicate an ongoing effector or activation program in CD8 T cells is important, since PD-1 and (now also)
 283 TOX are used as biomarkers of T cell exhaustion^{49, 50, 51}. Of note, certain features of general activation
 284 programs of CD8 T_{mem} appear to be well conserved and have also been reported as transcriptomic
 285 overlap of tissue-resident, recently-activated, and exhausted CD8 T cells⁵². While infection parameters
 286 and inflammatory events are well defined in mouse model studies, most human studies remain agnostic
 287 in regard to the infection and activation history of Ag-specific T cells. This in turn makes it difficult to
 288 correctly interpret the underlying reason for expression of PD-1 and TOX by human T cells.

289

Our data emphasize the need for conservative interpretation of TOX in regard to activation and exhaustion and also caution against interpreting TOX expression purely through the lens of recent TCR-mediated activation. TOX expression has been predictive of T cell exhaustion and unfavorable outcome in hepatocellular carcinoma animal models and clinical samples⁵³, in line with the paradigm of TOX-mediated TCR-dependent T cell dysfunction. However, other studies have yielded contradictory data. Meta analyses of TOX expression in breast cancers reported TOX levels paradoxically correlating with increased immune cell function and favorable prognosis⁵⁴. This is perplexing, as in tumors, TOX expression is associated with T cell dysfunction^{6, 13, 14}. This discrepancy could in part be explained by TOX upregulation during activation, akin to what we observed during T cell activation in TCR-dependent and -independent stimulations. Thus, our data stress that all possible activation pathways of TOX and PD-1 induction must be considered before interpreting TOX as a biomarker of T cell dysfunction. A well done human study that interrogated TOX heterogeneity found elevated TOX in CMV- and EBV-specific CD8 T_{mem} and hypothesized recent viral reactivation provided cognate Ag to facilitate TCR-mediated upregulation of TOX. This is certainly a plausible explanation, but our data highlight the need to also consider recent exposure to inflammation as a critical parameter affecting TOX expression. Conventional CD8 T_{EM} and T_{EMRA} (the predominant phenotype of CMV- and EBV-specific CD8 T cells) express elevated levels of TOX basally, T_{CM} (including IAV-specific CD8 T cells) and innate-like MAIT cells can, too, upregulate TOX expression following inflammation-mediated activation. Importantly, our data highlight that this mechanism of TOX expression is conserved across species, conventional CD8 T_{mem} subsets, and innate-like MAIT cells.

Since pro-inflammatory cytokines can concurrently induce TOX and PD-1 expression, these signals may drive TOX heterogeneity in other contexts. P14 tissue-resident memory T cells showed increased *Tox* expression at homeostasis, which has been observed 90 days post priming with LCMV Armstrong²⁰. Since the acute infection is cleared well before this timepoint, it is unlikely that continued TCR signaling by cognate Ag drives this phenotype, despite elevated transcripts encoding mediators of TCR signaling²⁰. IL-15, however, is likely present within the tissue microenvironment. IL-15 has been implicated in T_{RM} maintenance^{25, 26}, and transcriptional profiles indicative of IL-15/STAT5 signaling can be

317 detected in human T_{RM} ^{23, 24}. Thus, IL-15 in tissue microenvironments may also contribute to TOX
318 heterogeneity. Future work will be necessary to dissect the role of these inflammatory cues versus other
319 signals that can shape T_{RM} phenotype, such as co-stimulation and tonic TCR signaling²¹.

320 Previous studies have demonstrated that TOX ablation or knockdown leads to PD-1
321 downregulation in models of exhaustion^{6, 13, 15, 47}, and conversely, introduction of TOX-expressing
322 constructs enhances PD-1 expression^{15, 47}. Similarly, our data showed a close correlation in regards to
323 TOX and PD-1 expression levels, but we found that PD-1 expression could be induced in stimulated $Tox^{-/-}$
324 P14 T_{mem} . Of note, these $Tox^{-/-}$ P14 T_{mem} lack exon 5, which abrogates the ability to function as a
325 transcription factor, but the truncated protein is still expressed and detected by the TOX antibody. Alfei et
326 al. previously showed that the early wave of effector cells formed from $Tox^{-/-}$ $T_{naïve}$ expressed significant
327 levels of PD-1 independently of functional TOX. However, TOX was required for the expression of high
328 levels of PD-1 at later stages, once the initial population of exhausted effector T cells had been replaced
329 by a proliferation competent TCF1 progenitor population³¹. Together, these data suggest that long-term
330 expression of PD-1 requires TOX, but activation-induced expression of PD-1 is TOX-independent. In the
331 absence of TOX, PD-1 expression could be driven TOX2, which can induce PD-1 expression in CD8 T
332 cells^{15, 47}; however, it remains unclear if TOX2 is also upregulated by transient TCR- or cytokine-
333 mediated stimulation. Similarly, how different activating signals integrate to regulate TOX expression also
334 requires further studies: while inflammatory cues increase TOX expression in memory T cells, increased
335 IL-12 signaling during the priming of $T_{naïve}$ has been shown to limit subsequent TOX expression at steady
336 state^{55, 56}.

337 Overall, our data suggest that the mechanisms that regulate TOX expression, both at
338 homeostasis and after transient TCR or cytokine stimulation, are remarkably similar and quite possibly
339 highly conserved between humans and mice. Our data further highlight the need to consider TOX and
340 PD-1 expression as prominent indicators of ongoing activation and effector programs in T_{mem} instead of
341 exclusive biomarkers of exhaustion.

342

343 **Materials and methods**

344

345 **Mice**

346 Mouse protocols and experimentation conducted at the Fred Hutchinson Cancer Research
347 Center were approved by and in compliance with the ethical regulations of the Fred Hutchinson Cancer
348 Research Center's Institutional Animal Care and Use Committee. Experiments performed at the
349 Technical University of Munich were in compliance with institutional and governmental regulations in
350 Germany and approved by the veterinarian authorities of the Regierung von Oberbayern in Germany. All
351 animals were maintained in specific pathogen-free facilities and infected in modified pathogen-free
352 facilities. Experimental groups were non-blinded, animals were randomly assigned to experimental
353 groups, and no specific method was used to calculate sample sizes.

354

355 We purchased 6-week-old female C67BL/6J mice from the Jackson Laboratory; *Tox*^{-/-} P14 mice
356 (P14 *Tox*^{tm1c(KOMP)Wtsi}; *Mx*^{Cre}; *Rosa26-STOP-eYFP*) were generated as previously described⁶. Both WT
357 and *Tox*^{-/-} P14 mice, OT-I mice, and gBT-I mice were maintained on CD45.1 congenic backgrounds. We
358 euthanized mice in accordance with institutional protocols and subsequently collected spleens and lymph
359 nodes (LNs) for experimentation.

360

361 **Development of memory mice**

362 We prepared a single-cell suspension of LN cells that were harvested from female OT-I, P14, or
363 gBT-I mice by mechanically passing LN tissue through a 70-100µm strainer. To enrich transgenic T cells,
364 we used MACS with a CD8 negative selection kit (Miltenyi Biotec).

365

366 For OT-I memory mice, we adoptively transferred 1×10^4 OT-I T cells in sterile 1x PBS i.v. per
367 C57BL/6J recipient, and subsequently infected recipients i.v. with $1-2 \times 10^7$ PFU OVA-expressing
368 vesicular stomatitis virus (VSV-OVA) or 4×10^3 CFU OVA-expressing *Listeria monocytogenes* (LM-
369 OVA). For gBT-I memory mice, we adoptively transferred 5×10^4 gBT-I T cells i.v. and subsequently

infected recipient mice i.v. with or 4×10^3 CFU herpes simplex virus 2 (HSV2) glycoprotein B (gB)-expressing *L. monocytogenes* (LM-gB). We allowed ≥ 60 days to pass after initial VSV or LM infections before assaying tissues.

373

For P14 memory mice, we adoptively transferred 2×10^3 WT P14 T cells i.v. and subsequently infected recipient mice i.v. with 2×10^5 PFU LCMV Armstrong clone (LCMV Arm.) or 2×10^6 PFU LCMV Docile clone (LCMV Doc.). For *Tox*^{-/-} P14 memory mice, we adoptively transferred 2×10^3 *Tox*^{-/-} P14 memory mice and subsequently infected with 2×10^5 PFU LCMV Arm.; we allowed 28 days to pass after initial LCMV infection before assaying tissues.

379

380 **Human PBMC and study approval**

Twenty-three healthy, HIV-uninfected adults were recruited by the Seattle HIV Vaccine Trials Unit (Seattle, Washington, USA) as part of the study “Establishing Immunologic Assays for Determining HIV-1 Prevention and Control.” These samples are also known as the Seattle Area Control (SAC) Cohort. All participants were provided and signed informed consent, and the Fred Hutchinson Cancer Research Center Institutional Review Board approved the study protocol.

386

387 **T cell isolation and in vitro stimulation**

We harvested spleen and LN from memory mice and mechanically prepared single-cell suspensions. We thawed $\sim 4 \times 10^7$ cryopreserved PBMC in human RP10 media (RPMI1640 supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin-streptomycin). To enrich bulk T cells from single cell suspensions, we respectively used mouse- and human-specific T cell negative isolation MACS (STEMCELL Technologies, Canada). We plated $0.5\text{--}1 \times 10^6$ T cells per well in 96-well V-bottom tissue culture plates. We cultured cells in human RP10 or mouse RP10 media (RPMI 1640 supplemented with 10% FBS, 2mM L-glutamine, 100 U/mL penicillin-streptomycin, 1mM sodium pyruvate, 0.05mM β -mercaptoethanol, and 1mM HEPES). To stimulate cells, we cultured mouse T cells in mouse RP10 with rIL-12, rIL-15, and rIL-18 (BioLegend) (each at 100ng/mL), with Dynabeads mouse

397 T-Activator (Thermo Fisher) anti-CD3/CD28 beads (at a 1:1 bead:cell ratio), or with media alone. For
 398 human T cell stimulations, we used human RP10 media with combinations of rIL-6 (BioLegend), rIL-12,
 399 rIL-15, and/or rIL-18 (Peprotech) (each at 100ng/mL), with Dynabeads human T-Activator (Thermo
 400 Fisher) anti-CD3/CD28 beads (at a 1:1 bead:cell ratio), or with RP10 alone. We cultured cells at 37°C,
 401 5% CO₂, sampling cells at 0, 24, and 48 hours for flow staining. For intracellular cytokine staining (ICS),
 402 we added GolgiPlug (BD Biosciences) at a 1:1,000 dilution 8 hours prior to cell harvest.

403

404 **Flow cytometric analysis**

405 We conducted all flow staining for mouse and human T cells on ice and at room temperature,
 406 respectively. All mouse and human flow panel reagent information, stain conditions, and gating are
 407 included in **(Supplemental Fig. 8-11, Supplemental tables 1-6)**. We conducted LIVE/DEAD fixable
 408 aqua or blue viability dye (AViD or BViD, respectively) or Zombie Near-IR viability dye (NIRViD) staining in
 409 1× PBS. For surface staining, we utilized FACSWash (1× PBS supplemented with 2% FBS and 0.2%
 410 sodium azide) as the stain diluent. For all TOX staining panels, we fixed cells with the FOXP3
 411 fixation/permeabilization buffer kit (Thermo Fisher) and conducted intranuclear stains using the FOXP3
 412 permeabilization buffer (Thermo Fisher) as diluent. To minimize day-to-day variation for TOX staining, we
 413 conducted all intracellular stains within a batch (0, 24, and 48-hour samples) at the same time. We
 414 resuspended cells in FACSWash and acquired events on a FACSSymphony, which we analyzed using
 415 FlowJo v10 (BD Biosciences). We conducted statistical testing using Prism v8 (GraphPad).

416

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611 **Figure Legends**

612

613 **Figure 1. Cytokine stimulation induces TOX expression in murine CD8 T_{mem}**

614 **a** Schematic of OT-I memory mouse generation (top) and subsequent stimulation assays (bottom). OT-I
615 T_{naïve} were transferred and expanded with VSV-OVA, then aged to stable memory contraction; after, T
616 cells were enriched from VSV-OVA expanded OT-I memory animals and stimulated with media alone
617 (mock), IL-12, -15, and -18 in combination (IL-12/15/18) (each at 100ng/mL), or anti-CD3/CD28
618 microbeads (TCR) at a ~1:1 bead:cell ratio. **b-c** expression of **b** PD-1, **c** TOX, and **d** TCF1 within
619 stimulated OT-I T_{mem} throughout experiment time course. TOX MedFI fold change in **c** was calculated
620 against average TOX MedFI from mock stimulations in a subset-specific, batch-specific, and timepoint-
621 specific manner. In **b** and **c**, bar chart symbols represent one animal at a unique timepoint/condition and
622 are connected by animal identity, with bar indicating mean; the indicated statistical significances were
623 calculated using Wilcoxon matched-pairs signed rank tests. In **b-d**, symbols in line plots comparing
624 stimulation conditions represent the mean across all animals for a specific timepoint/condition ± SD; the
625 indicated statistical significances were calculated using Mann-Whitney tests. Figures in **b** and **c** depict
626 results from *n* = 14 mice across 7 experiments. Figures in **d** depict results from *n* = 9 mice across 2
627 experiments. All representative flow plots are sourced from the same animal.

628

629 **Figure 2. TOX and PD-1 expression occur in functional CD8 T cells**

630 **a-c** Intracellular cytokine staining (ICS) in tandem with TOX interrogation. **a** Experiment schematic, in
631 which bulk T cells from VSV-OVA OT-I memory mice were stimulated (mock, black; IL-12/15/18, blue;
632 TCR, red). Cells were treated with GolgiPlug 18h into stimulation and harvested for flow staining and
633 analysis at 24h. **b, c** Expression of **b** TOX and **c** PD-1 in IFN γ ⁺ and IFN γ ⁻ OT-I T_{mem}. Representative
634 plots depict cells from the same animal across different stimulation conditions. Symbols in **b** and **c**
635 represent a T cell population within a unique animal with symbols connected by animal identity (*n* = 6
636 across 2 experiments). Bars represent mean and indicated statistical significances were calculated by
637 Wilcoxon matched-pairs signed rank test.

638

639 **Figure 3 Cytokine-mediated TOX induction is limited in exhausted T cells**

640 **a-b** Changes in TOX expression within LM-expanded TCR transgenic T_{mem} : OT-I, specific for OVA Ag
 641 and gBT-I, specific for gB Ag. MACS-enriched T cells from LM-expanded OT-I or gBT-I memory mice
 642 were stimulated with media alone (mock), recombinant IL-12, -15, and -18 in combination (IL-12/15/18)
 643 (each at 100ng/mL), or anti-CD3/CD28 microbeads (TCR) at a ~1:1 cell:bead ratio. **a, b** Representative
 644 TOX expression and TOX MedFI fold change during stimulation in LM-primed **a** OT-I and **b** gBT-I T_{mem} .
 645 **c-d** Changes in TOX expression within LCMV-specific TCR transgenic P14 T cells expanded by acute
 646 (Armstrong, Arm.) or chronic (Docile, Doc.) LCMV infection. **c, d** Representative TOX expression and
 647 TOX MedFI fold change during stimulation in P14 T cells primed by **c** LCMV Armstrong and **d** LCMV
 648 Docile. TOX MedFI fold change in **a-d** was calculated against average TOX MedFI within mock
 649 stimulation in a batch-specific, timepoint-specific manner. We calculated indicated statistical
 650 significances in **a-d** using Wilcoxon matched-pairs signed rank tests. Each symbol in **a-d** represents a
 651 sample at a unique timepoint/condition, with bars delineating mean, which are connected by donor in **a-d**
 652 ($n = 4$ LM-OVA expanded OT-I memory mice across 2 experiments; $n = 10$ LM-gB expanded gBT-I
 653 memory mice across 2 experiments; $n = 17$ LCMV Armstrong-expanded P14 memory mice across 4
 654 experiments; $n = 8$ LCMV Docile-expanded P14 memory mice across 2 experiments). Mouse identities
 655 are consistent between representative flow plots within the same generation/adoptive transfer condition.

656

657 **Figure 4. Inflammatory cytokines are potent inducers of TOX and PD-1 in human T_{mem}**

658 **a** Basal expression of TOX and PD-1 in CD8 T_{mem} and T_{naive} . **b** TOX MedFI across PD-1 low, medium,
 659 and high expressing CD8 T_{mem} . **c** Schematic detailing T cell isolation from cryopreserved PBMCs and
 660 subsequent stimulation with recombinant IL-6, IL-15, IL-12 and -18, IL-12 and -15 and -18 (all at
 661 100ng/mL, each), or anti-CD3/CD28 microbeads (TCR, 1:1 bead to cell ratio) and subsequent flow
 662 interrogation. **d** TOX expression (MedFI) and PD-1^{hi} frequency in CD8 T_{mem} throughout stimulation time
 663 course. **e, f** Comparison of TOX MedFI and PD-1^{hi} frequency in mock-, IL-12/15/18-, and TCR-stimulated
 664 **e** CD8 T_{mem} and **f** CD8 T_{naive} . In **a, b, d, e, f** we calculated indicated statistical significances by **a, d**

Wilcoxon matched-pairs signed rank tests, **b** Friedman test with Dunn's multiple comparisons tests, or **e**, **f** Mann-Whitney tests. In **a**, **d** each symbol represents a unique timepoint/treatment connected by donor with bars indicating mean **a** ($n = 23$ across four experiments) **d** ($n = 11$ across two experiments). In **e**, **f** each symbol represents the mean $\pm$ SD of the stimulation condition from $n = 23$ donors across four experiments. Representative plots from **a**, **d**, **f** are sourced from the same donor.

Figure 5. TOX and PD-1 upregulation are largely independent of T_{mem} subset

a-c Basal and stimulation-induced TOX and PD-1 expression in CD8 memory subsets. **a** Representative gating of CD8 T cells into naïve ($T_{\text{naïve}}$, grey), central memory (T_{CM} , orange), effector memory (T_{EM} , purple), and effector memory CD45RA-expressing (T_{EMRA} , green) subsets. **b** basal expression levels (MedFI) of TOX and TCF1 and frequency of PD-1^{hi} cells across CD8 T cell memory subsets. **c** TOX MedFI, PD-1^{hi} frequency, and TCF1 MedFI after mock (black), IL-12/15/18 (each at 100ng/mL, blue), or TCR (1:1 bead to cell ratio, red) stimulation in CD8 T_{CM} (left column), CD8 T_{EM} (center column), and CD8 T_{EMRA} (right column). Symbols in **b** and **c** represent unique samples (by timepoint/condition/subset) and are connected by donor identity, with bars representing mean. We determined statistical significances in **b** and **c** respectively using Friedman tests and Wilcoxon matched-pairs signed rank tests. **b** and **c** depict $n = 23$ donors across four experiments, except for TCF1 plots, which depict $n = 12$ donors across two experiments.

Figure 6. Stimulation induces TOX and PD-1 expression in conventional and innate-like T cells

a-b TOX and PD-1 induction in influenza A virus (IAV)-specific CD8 T cells. **a** Gating and memory phenotyping of IAV-specific CD8 T cells. **b** Induction of TOX and PD-1 in IAV-specific CD8 T cells by mock (black) or IL-12/15/18 (each at 100ng/mL, blue) stimulation. **c-d** TOX and PD-1 induction in mucosal associated invariant T (MAIT) cells. **c** Gating and memory phenotyping of MAIT cells. **d** Induction of TOX and PD-1 in MAIT cells by mock (black) or IL-12/15/18 (each at 100ng/mL, blue) stimulation. Representative plots in **a-d** are sourced from the same donor. Symbols in **a-d** represent unique samples (by timepoint/condition/subset) and are connected by donor identity, with bars

representing mean. We determined statistical significances in **b** and **d** using Wilcoxon matched-pairs signed rank tests. **a** and **b** depict $n = 8$ donors across two experiments; **c** and **d** depict $n = 23$ donors across four experiments.

Figure 7. TOX deficiency does not abrogate stimulation-induced PD-1 expression

a-c Stimulation-induced PD-1 expression in WT and $Tox^{-/-}$ P14 T_{mem} T cells were stimulated with media alone (mock), recombinant IL-12, -15, and -18 in combination (IL-12/15/18 or ILs) (each at 100ng/mL), or with anti-CD3/CD28 microbeads at an ~1:1 cell:bead ratio (TCR). **a**, **b** PD-1 MedFl and expression frequencies in **a** WT or **b** $Tox^{-/-}$ P14 T_{mem} over stimulation time course. **c** Comparison of PD-1 MedFl and expression frequencies between IL-12/15/18 (left) or TCR (right) stimulated WT and $Tox^{-/-}$ P14 T_{mem} . All indicated statistical significances were calculated using Mann-Whitney tests. Symbols in **a** and **b** represent the mean $\pm$ SD from all animals at a specific time/condition; and symbols in **c** represent stimulated P14 T_{mem} populations within a single animal ($n = 9$ WT P14 recipients and $n = 10$ $Tox^{-/-}$ P14 recipient across 2 experiments).

a

b

c

d