Regulation of T cell-dendritic cell interactions by IL7 governs T cell activation and homeostasis

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Abstract

Interleukin-7 (IL7) plays a central role in the homeostasis of the T cell compartment by regulating T cell survival and proliferation. Whether IL7 can influence TCR signalling in T cells remains controversial. Here, using IL7 deficient hosts and TCR transgenic T cells that conditionally express IL7R, we examined antigen specific T cell responses \textit{in vitro} and \textit{in vivo} to viral infection and lymphopenia to ask whether IL7 signalling influences TCR triggered cell division events. \textit{In vitro}, we could find no evidence that IL7 signalling could co-stimulate T cell activation over a broad range of conditions suggesting that IL7 does not directly tune TCR signalling. \textit{In vivo}, however, we found an acute requirement for IL7 signalling for efficiently triggering T cell responses to influenza A virus challenge. Furthermore, we found that IL7 was required for the enhanced homeostatic TCR signalling that drives lymphopenia induce proliferation by a mechanism involving efficient contacts of T cells with dendritic cells (DCs). Consistent with this, saturating antigen presenting capacity \textit{in vivo} overcame the triggering defect in response to cognate peptide. Thus, we demonstrate a novel role for IL7 in regulating T cell-DC interactions that is essential for both T cell homeostasis and activation \textit{in vivo}.
Introduction

The cytokine interleukin 7 (IL7) plays a vital role in regulating the homeostasis and function of the T cell compartment. Mice lacking either the cytokine\(^1\) or its specific receptor\(^2\), IL7R\(\alpha\) (CD127), have a profound block at the CD4\(^-\) CD8\(^-\) double negative stage of thymic development. Consequently, thymi are severely reduced in size and the mice are profoundly lymphopenic, having very few mature peripheral T cells. IL7 also plays a central role in regulating the homeostasis of the peripheral T cell compartment. It is essential for survival of naïve CD4 and CD8 T cells\(^3\)\(^-\)\(^5\) and is also an important factor in the long term survival of CD4\(^6\) and CD8\(^7\)\(^-\)\(^9\) memory cells. Additionally, IL7 has been implicated in the generation of memory cells from effectors\(^10\)\(^,\)\(^11\).

During immune responses, IL7R is down-regulated following activation\(^3\) and is not thought to participate in the effector response, rather handing over its responsibilities to other \(\gamma c\) cytokines such as IL2 and IL15. It is unclear, however, whether IL7 signals play any role in the initial priming and activation events, a point at which T cells are still expressing IL7R\(\alpha\) and receiving IL7 signals. Initial studies of polyclonal \(Il7ra^{-/-}\) mice found their T cells to be hyporesponsive to TCR mediated signalling\(^12\) suggesting that IL7 can influence TCR signalling. However, these mice are profoundly lymphopenic and their T cells have an abnormal activated phenotype often associated with lymphopenic mice. Later analysis of naïve \(Il7ra^{-/-}\) OT1 TCR transgenic T cells reported normal T cell activation\(^3\). However, studies of tumour immunity find that lymphopenia can enhance anti-tumour responses\(^13\)\(^-\)\(^15\), and in some studies increased bioavailability of IL7 has been directly implicated in the mechanism\(^14\). In the homeostasis of naïve T cells, there is evidence that IL7 can synergise with TCR signals to promote both survival\(^4\) and in the induction of TCR dependent homeostatic proliferation\(^3\)\(^,\)\(^16\). IL7 signalling can affect cell cycle by inhibiting p27\(^kip\), a negative regulator of cell cycle\(^17\), while evidence for convergence of TCR and IL7 signalling comes from studies of human memory cells which show that FoxO3a, a pro-apoptotic transcription factor, is a
downstream target of both TCR and IL7 signalling. A more recent study has suggested that IL7 can directly influence sensitivity of TCR signalling by tuning CD8 co-receptor expression. In humans, IL7 production by DCs was found to affect CMV specific CD8 T cell responses in vitro.

Previously, we have described a tetracycline inducible IL7R transgenic mouse model in which TCR transgenic T cells conditionally express IL7R. T cells from these mice generate effectors cells with comparable function to controls, but examining primary responses to live influenza virus in vivo, revealed that far fewer effectors were generated compared to WT control cells early in the response at d7. In the present study, we used the same system to investigate whether there was any evidence IL7R signalling could influence TCR dependent T cell activation and proliferation. In vitro, we could find no evidence that either induction or blockade IL7 signalling had any affect on TCR sensitivity or activation. In contrast, in vivo an absence of IL7R expression or IL7 cytokine resulted in a significant defect in triggering of T cell proliferative responses both to influenza A virus and to lymphopenia. Rather than influencing TCR signalling directly, we found evidence that for both antigen and lymphopenia induced proliferation, IL7 was in fact required for efficient interactions of T cells with DCs and that the failure to trigger responses was a failure to make sufficient contacts with antigen presenting DCs.
Materials and Methods

Mice.

F5 7r\(^{-}\) TreIL7R rTA\(^{hucD2}\) tetracycline inducible IL7R transgenic mice (TetIL7R) have been described previously\(^{10}\). Breeders and weaned pups were fed doxycycline (dox) in food (3mg/gm) in order to induce IL7R expression. (F5 Rd1\(^{-}\)xC57Bl/6JCD45.1)F1 mice were used as controls throughout. These strains and recombinase activating gene-1 deficient (Rd1\(^{-}\)) mice, F5 Rd1\(^{-}\), \(\beta 2m\)^{-} Rd1\(^{-}\), Rd1\(^{-}\) Rd1\(^{-}\), Rd1\(^{-}\) Rd1\(^{-}\) mice were bred in a conventional colony free of pathogens at the National Institute for Medical Research, London. All lines used were of H-2\(^{b}\) haplotype. Animal experiments were done according to institutional guidelines, with ethical approval from the Home Office.

In vitro activation of F5 T cells

Lymphocytes were teased from lymph nodes and spleen of donor mice and single cell suspensions prepared. Cells were labelled with 2\(\mu\)M carboxyfluorescein diacetate succininidyl ester (CFSE, Molecular Probes) in Dulbecco's PBS (GibcoBRL) for 10 minutes at 37\(^\circ\)C and washed twice and unfractionated cells cultured (10\(^{6}\)/T/ml) in complete RPMI 1640 media (Sigma) supplemented with 10% FCS(Sigma), glutamine(Sigma), 2mercaptoethanol(Sigma) and antibiotics(Sigma). Where used, IL7 and IL15 was supplemented at 10ng/ml. T cells were activated with either NP68\(^{21}\) or NP34\(^{22}\) peptide in PBS for short term stimulation (<4h) or complete media proliferation assays.

Flow cytometry

Flow cytometry was carried out using 2-5 x 10\(^{6}\) lymph node or spleen cells. Cell concentrations were determined using a Scharf Instruments Casy Counter. Cells were incubated with saturating concentrations of antibodies in 100\(\mu\)l PBS-BSA (0.1%)-Azide (1 mM) for 1 h at 4
°C followed by three washes in PBS-BSA-Azide. mAb used in this study were as follows: APC-TCR (H57-597) (eBioscience), FITC-CD11c (eBioscience), PE-CD69 (eBioscience), PE-CD86 (eBioscience), PE-IL7R (eBioscience), bio-I-A^b (eBioscience), APC- and bio-CD45.1 (eBioscience), APC-CD5, APC-CD44 (Leinco Technologies), bio-CD44 (eBioscience), APC-, PerCP- and PE-CD8 (eBioscience). Biotinylated mAb staining was detected using PerCP steptavidin (Pharmingen) and PE-TR steptavidin (Caltag Laboratories). bio-I-A^b staining was detected using PE-steptavidin (Pharmingen) for T cells and PerCP steptavidin for mature DCs. PE-pZap70 (BD Biosciences), PE-Bcl-2 (BD Pharmingen), active PE-Caspase 3 (BD Pharmingen) and APC-pSTAT5 (BD Biosciences) staining of paraformaldehyde fixed samples was carried out according to manufacturers instructions. Four and five colour cytometric staining was analysed on a FACSCalibur and LSR Instruments (Becton Dickinson), respectively, and data analysis performed using FlowJo V8.5 software (TreeStar).

**Labeling and adoptive transfer of T cells**

Lymphocytes were teased from lymph nodes and spleen of donor mice and single cell suspensions prepared. Cells were labelled with 2µM CFSE and transferred into recipient mice via tail vein injections. Mice further challenged with influenza A virus (A/NT/60-68) were injected i.v. with 1-100 haemagglutinating (HA) units of virus or two i.p. injections of NP68 peptide at the dose indicated at 0h and 18h after cell transfer. After 72h, spleen of recipient mice were taken from host mice and splenocytes analysed by FACs for expression of CD8, TCR, CD45.1 and CFSE. Triggering and burst size of proliferative responses were calculated using FlowJo analysis software and refer to the precursor population.
Results

IL7 does not affect TCR sensitivity in vitro

In order to determine whether IL7 could affect T cell activation, we first assessed the affects of IL7 signalling on proximal TCR signalling, triggering and proliferation of TCR transgenic T cells in vitro. T cells of F5 mice express a transgenic TCR specific for a peptide of nucleoprotein (NP) of influenza A (Flu) virus\textsuperscript{21}. T cells from these mice were stimulated with 10nM agonist peptide (NP68)\textsuperscript{21} in the presence or absence of high dose of IL7 (10ng/ml). Neither phosphorylation of Zap70 kinase, which peaked at 30 minutes, or subsequent induction of CD69 at 90 minutes post-stimulation, was affected by the presence of IL7 (Fig. 1A). IL7 signalling was intact in these activated T cells since both IL7R expression (Fig. S1A) and induction of phosphoSTAT5 (pSTAT5) was identical to non-NP68 stimulated cells during these early stages of activation (Fig 1A). IL2 also induces STAT5 phosphorylation\textsuperscript{23} and pSTAT5 could be detected in IL7 free cultures by 4hrs (Fig. S1B). However, this pSTAT5 level was also unaffected by the presence of IL7 either at 4h (Fig. 1A) or 24h (Fig. S1C). Consistent with this, Bcl2 expression levels were not modulated by IL7 in peptide stimulated cultures.

To measure triggering and proliferation, T cells from these mice were labelled with CFSE and stimulated with a wide range of NP68 concentrations in the presence or absence of high dose of IL7 (10ng/ml). Analysis of T cell proliferation at 72h revealed that addition of IL7 had no effect on either the proportion of cells triggered into division (Fig. 1B) or the size of their proliferative burst at any antigen dose (Fig. 1C). It was possible that IL7 signalling might only modulate suboptimal TCR stimuli but not stimuli induced by high avidity ligands such as NP68. Therefore, we also tested the affects of IL7 stimulation on T cell responses to NP34, a NP peptide with weak agonist/antagonist properties \textsuperscript{22}. As expected, triggering (Fig. 1B) and proliferation (Fig. 1C) of F5 T cells to this peptide was much reduced compared to NP68 induced responses. However, IL7
signalling had no influence on proliferative responses to the weaker NP34 peptide ligand.

Furthermore, titrating IL7 (between 1-50ng/ml) also failed to reveal any effects on F5 T cell responses (data not shown).

We could find no evidence that induction of IL7 signalling could affect T cell activation, so we next asked whether a complete loss of IL7 signalling had any effect. Recent studies have implicated a role for IL7R signalling in tuning T cell TCR responsiveness by modulating CD8 expression\(^{19}\). To examine this possibility further, we took advantage of a mouse model in which F5 mice conditionally express IL7R using the tetracycline regulatory system (F5 TreIL7R rtTA\(^{hUCD2}\) II7r\(^{+/−}\), F5 TetIL7R hereon, see Materials and Methods)\(^{10}\). Induction of IL7R expression by feeding mice doxycycline (dox) throughout life overcomes the block in thymic development that normally occurs in II7r\(^{−/−}\) F5 mice and allows the generation of a normal peripheral compartment of F5 T cells\(^{10}\). Peripheral T cells from F5 TetIL7R mice taken off dox food for 7d (IL7R- F5 T cells hereon) cease to express IL7R (Fig. 1D and S2). Analysis of IL7R- F5 T cells did reveal a subtle reduction in CD8 expression (Fig. 1D), although not to the extent described elsewhere\(^{19}\). We have previously assessed the proliferative capacity of IL7R- F5 T cells in experiments similar to those described here (Fig. 1B-C), and could find no defect in their ability to proliferate to antigen in vitro\(^{10}\). However, to be certain that the slight reduction in CD8 expression by IL7R- F5 T cells was not having an influence earlier in the response, we also examined CD69 up-regulation at 24h.

Induction of CD69 expression was entirely normal over a wide range of antigen doses, regardless of IL7R expression (Fig. 1E). In conclusion, we could find no evidence that either blockade or stimulation of IL7 signalling in F5 T cells had any direct affect on TCR responsiveness or activation in vitro.

**Impaired triggering of IL7R- F5 T cells in response to Flu challenge**

We next tested whether IL7 signalling could affect T cell responses to viral challenge in vivo. F5 T cells from CD45.1\(^{+}\) control F5 mice (IL7R+ F5 T cells) and CD45.2\(^{+}\) IL7R- F5 T cells were
labelled with CFSE and co-transferred into Rag1−/− recipients. Host mice were then immediately challenged with A/NT/60-68 influenza A virus. To assess triggering and proliferation of F5 T cells, recipients were culled at d3 and CFSE profile of donor populations assessed by FACs. Mice challenged with flu virus underwent a clear burst of cell divisions at day 3 not observed in unchallenged hosts (Fig. 2A). When CFSE profiles of control and IL7R- F5 T cells in the same host were compared, a highly significant (p<0.0008) and reproducible difference in the frequency of cells triggered to proliferate was apparent. The proportion of F5 T cells triggered into division in the absence of IL7R expression was reduced more than two-fold compared with control F5 T cells (Fig. 2B). While there was a clear reduction in the frequency of triggered cells, the profile of dividing IL7R- F5 T cells appeared normal. The average burst size by control F5 T cells was 2.4±0.4 divisions at day 3 compared with 2.4±0.3 for IL7R- F5 T cells from F5 TetIL7ROFF mice. This selective defect in triggering was also reflected in the physical size of the cells responding. Dividing cells from both populations exhibited identical increases in cell size following their activation, while undivided IL7R- F5 T cells were noticeably smaller than undivided control F5 T cells, most likely due to the requirement for IL7 signalling for the maintenance of naïve T cell size24. Significantly, we could find no evidence that death of undivided or dividing IL7R- F5 T cells could account for the observed triggering defect (Fig. S3 and Fig. S4).

To test how robust the defect in triggering of IL7R− F5 T cells was, we extended the experiment to challenge groups of mice with a range of different flu doses. Immunising recipient mice with flu doses between 1U and 100U resulted in a range of T cell triggering responses broadly proportional to the dose of flu administered (Fig. 3A). Triggering of IL7R− F5 T cells was significantly reduced over all the doses of flu tested, compared with control F5 T cells co-transferred in the same host (Fig. 3B and 3C). Interestingly, of cells successfully triggered to divide, we found no difference in proliferation between IL7R+ and IL7R− F5 T cells (Fig. 3D) at any
of the flu doses, suggesting that the defect observed in the absence of IL7R expression was restricted to initial triggering and not subsequent proliferation.

**Acute requirement for IL7 for efficient T cell triggering**

Cessation of IL7R expression in F5 TetIL7R mice was achieved by withdrawal of dox from diet of mice for seven days. We therefore wished to determine whether the requirement for IL7 signalling was acute, acting at the time of challenge, or whether chronic stimulation from IL7 was required to condition T cells for optimal T cell responsiveness. To determine the temporal requirement for IL7 signalling for efficient T cell triggering, we compared responses to flu challenge of mixtures of IL7R+ and IL7R− F5 T cells in either *Rag1*−/− or *Il7*−/− *Rag1*−/− hosts. As expected, in *Rag1*−/− hosts IL7R− F5 T cells exhibited a clear defect in triggering (Fig. 4A) as already described. Strikingly, in *Il7*−/− *Rag1*−/− hosts the frequency of control IL7R+ F5 and IL7R− F5 T cells triggered following flu challenge was virtually identical (Fig. 4A). The ratio of IL7R− F5 to control IL7R+ F5 T cells successfully triggered into division was 0.87 in IL7 deficient hosts compared with 0.45 in control hosts (Fig. 4B), suggesting an acute IL7 requirement for efficient triggering of T cells at the time of antigen encounter.

**IL7 signalling facilitates efficient T cell - DC interaction**

To further investigate the mechanism by which IL7 could affect T cell triggering, we examined F5 T cell proliferative responses to lymphopenia. Lymphopenia induced proliferation (LIP) is driven by TCR signals from self-peptide MHC complexes (spMHC) but also has a profound requirement for IL7 signals3,25. In the absence of IL7, F5 T cells also fail to undergo LIP16. Therefore, we first asked whether the homeostatic TCR signals that drive LIP were affected by the absence of IL7, or whether IL7 facilitated LIP by a mechanism independent of TCR signalling. To assess homeostatic TCR signalling, we examined CD5 expression levels. CD5 is a negative regulator of TCR signalling and its expression levels are tuned by homeostatic signals
through the TCR\textsuperscript{4,26}. F5 T cells transferred to \textit{Rag1}\textsuperscript{+/-} hosts up-regulated CD5 consistent with an increase in homeostatic TCR signals from spMHC driving LIP (Fig. S5A). CD5 up-regulation was Class I MHC dependent as levels were down-regulated on the same cells transferred to Class I MHC deficient β2m\textsuperscript{+/-} \textit{Rag1}\textsuperscript{+/-} lymphopenic hosts (Fig. S5A). While F5 T cells transferred to \textit{Rag1}\textsuperscript{+/-} hosts underwent a rapid and sustained up-regulation of CD5 (Fig. 5A), the same cells transferred to \textit{Il7}\textsuperscript{-/-} \textit{Rag1}\textsuperscript{-/-} hosts remained unchanged for the first week and only started to gradually increase expression thereafter (Fig. 5A), suggesting that homeostatic TCR signalling to donor F5 T cells was not increased in lymphopenic hosts in the absence of IL7, despite being completely devoid of host T cells. CD8 expression by F5 T cells was similar following transfer to \textit{Rag1}\textsuperscript{-/-} or \textit{Il7}\textsuperscript{-/-} \textit{Rag1}\textsuperscript{-/-} hosts but up-regulated in Class I deficient hosts (Fig. 5A).

If IL7 signalling could directly tune or amplify TCR signalling induced by spMHC, this would explain the failure of F5 T cells to receive enhanced homeostatic TCR signalling in \textit{Il7}\textsuperscript{-/-} \textit{Rag1}\textsuperscript{+/-} hosts. An alternative possibility was that in the absence of IL7, there was a failure of spMHC ligand to induce the enhanced TCR signalling required for LIP. To ask whether IL7 was affecting TCR engagement with spMHC ligand, we measured TCR down-modulation on donor F5 T cell populations. TCR is down modulated following stimulation by pMHC complexes\textsuperscript{27,28} and we found the same was also true of spMHC mediated stimulation of TCR during LIP. Following transfer of F5 T cells to \textit{Rag1}\textsuperscript{+/-} hosts, TCR levels were down modulated due to increased engagement by spMHC in the absence of host T cells since the same cells transferred to Class I deficient \textit{Rag1}\textsuperscript{+/-} hosts up regulated TCR expression in the absence of spMHC ligands (Fig. S5A). Maximal down modulation of TCR in \textit{Rag1}\textsuperscript{+/-} hosts took between 14 and 21 days, but at early time points was greatest on those cells that had divided the most, possibly because that had received more stimulation from spMHC to divide (Fig. 5B). However, all T cells down-modulated TCR to a similar level by day 21, regardless of division history suggesting that the whole donor population was subject to enhanced engagement of TCR by spMHC in these lymphopenic hosts. In contrast, in
IL7 deficient hosts there was only limited down-modulation of TCR (Fig. 5B), suggesting that, in the absence of IL7, TCR engagement by spMHC was hardly increased. Even undivided F5 T cells in Rag1−/− underwent greater down-modulation of TCR than undivided cells in I17−/−Rag1−/− hosts.

We next asked whether the failure of TCR to engage spMHC in the absence of IL7 was in fact secondary to a failure of F5 T cells to make sufficient contacts with spMHC expressing DCs. We quantified T cell-DC contact on the basis of passive acquisition of Class II MHC molecules by T cells that occurs as a consequence of T cells-DC interactions29. Mouse T cells cannot synthesise Class II MHC themselves29, but do acquire surface expression as a consequence of cell-cell interactions with Class II MHC+ cells, mostly DCs in Rag1−/− lymph nodes and specific staining was not observed on CD8 T cells from I-Ab−/− mice (Fig. S5B). Higher levels of Class II MHC were found on F5 T cells in Rag1−/− hosts than in replete F5 Rag1−/− hosts, and interestingly, enhanced expression appeared dependent on host Class I MHC expression (Fig. S5C), also implying a role for spMHC recognition for enhancing T cell-DC interactions. Following transfer to T cell deficient Rag1−/− hosts, F5 T cells had higher levels of I-Ab molecules on their surface, suggesting increased contact with DCs. It is likely that this increased T-DC interaction in the absence of competing host T cells resulted in the increase in TCR-spMHC engagement that delivers enhanced homeostatic TCR signals and ultimately drives LIP. Significantly, little increase in I-Ab staining of F5 T cells was observed on cells transferred to IL7 deficient hosts in the first week (Fig. 6A), suggesting that IL7 was also required for the enhanced T cell-DC contacts that result in the induction of LIP. The failure to enhance Class II acquisition in the absence of IL7 could not be attributed to changes in host DCs in I17−/−Rag1−/− hosts, as DCs in these mice were present in normal numbers and of normal phenotype compared to those in Rag1−/− hosts (Fig. S6). However, as for CD5 expression, Class II expression did gradually increase thereafter and it is notable that a small subset of F5 T cells did undergo divisions in IL7 deficient hosts, most likely induced by IL15 (Fig. S3A). To confirm that acquisition of Class II molecules was dependent on IL7R signalling to F5 T cells, we also compared
I-A\textsuperscript{b} staining on IL7R+ and IL7R- F5 cells following co-transfer to \textit{Rag1}\textsuperscript{−/−} hosts. Interestingly, analysis pre-transfer revealed that IL7R- F5 T cells had slightly higher basal I-A\textsuperscript{b} staining than IL7R+ F5 controls, probably due to reduced competition for DCs in F5 TetIL7R\textsuperscript{ind} mice that have 3-4 fold fewer F5 T cells than controls\textsuperscript{10}. Significantly though, only IL7R+ F5 T cells increased Class II expression following transfer (Fig. 6B) and, consistent with our experiments employing IL7 deficient hosts (Fig.6A), levels were significantly greater than on IL7R- F5 T cells in the same host.

**Antigen dose overcomes the triggering defect in IL7R deficient F5 T cells**

The evidence from our studies of LIP by F5 T cells suggested a novel role for IL7 influencing efficient T cell-DC interactions. Normal activation of IL7R- F5 T cells \textit{in vitro} suggested that T cell-DC conjugate formation was essentially normal in the absence of IL7R signalling. The reduced DC contact we detected \textit{in vivo} may therefore reflect a reduction in the number T cell-DC contacts rather than a qualitative reduction in individual interactions. Only a proportion of control F5 T cells were successfully triggered during flu challenge (Fig. 3), suggesting that antigen is limiting in this challenge and therefore successfully activation would depend on T cells finding DCs presenting antigen. The triggering defect in IL7R- F5 T cells to flu challenge may therefore reflect impairment in efficient location of DCs presenting flu antigen as compared with IL7R+ control F5 T cells. If true, then the triggering defect should be overcome by saturating the antigen presenting capacity \textit{in vivo}. To test this hypothesis, we examined antigen responses of IL7R- F5 cells in a setting in which antigen presenting capacity would not be limiting \textit{in vivo}. This was achieved by challenging mice with a range of doses of soluble NP68 peptide, such that at high doses, antigen presenting capacity would become saturated \textit{in vivo}. As before, CFSE labelled IL7R- F5 T cells and control IL7R+ F5 cells were co-transfered to \textit{Rag1}\textsuperscript{−/−} hosts. Mice were challenged with a broad range of NP68 peptide doses and at d3, the frequency of cells triggered into divisions determined. As was the case for responses to flu, the size of the responding population was proportional to antigen dose (Fig. 7A).
When triggering responses of control IL7R+ and IL7R- F5 T cells were compared over a range of NP68 doses, a similar defect in triggering was evident amongst the IL7R- F5 T cells as observed in response to flu challenge. Significantly, however, as antigen dose increased, the defect was overcome such that triggering of control IL7R+ F5 and IL7R- F5 T cells became indistinguishable (Fig. 7B and 7C). Crucially, this also confirmed that IL7R- F5 T cells in these mice were normally responsive and demonstrated that the observed triggering defect was not a consequence of non-responsiveness in a subset of the IL7R- F5 T cells.
Discussion

Whether cytokines such as IL7 can influence the sensitivity of T cells to TCR stimulation remains controversial. There are conflicting reports from a variety of different systems, and little consensus on mechanism. In the present study, we examined both antigen and lymphopenia induced proliferation of F5 T cells to ask whether IL7 could affect the TCR signalling that is required to induce these responses. We found no evidence that IL7 signalling could directly tune TCR responsiveness \textit{in vitro}, but found evidence \textit{in vivo} of a novel mechanism by which IL7 could augment CD8 T cell stimulation by affecting the ability of CD8 T cells to interact with antigen presenting DCs.

IL7 has a number of pleiotropic effects on T cells that could, in principle, contribute to the enhancement of TCR stimulation we observed. While IL7 is an essential survival factor for naïve T cells\textsuperscript{24}, we found no evidence that this played any role in the regulating T cell triggering. Although IL7R- F5 T cells died gradually in the long term, we observed no detectable loss of IL7R- F5 T cells over the 3 day duration of the flu challenge. Following successful triggering \textit{in vivo}, proliferation and blast transformation was normal in the IL7R- F5 T cells, as is effector generation from these cells\textsuperscript{10}. Apoptosis of proliferating IL7R- F5 T cells could not account for the triggering defect since both Bcl2 expression and Caspase-3 activation were identical between IL7R+ and IL7R- F5 cells during flu responses. As well as promoting survival, IL7 signalling pathways have been described to influence cell division through the inhibition of the cell cycle inhibitor p27\textsuperscript{kip}, and some rescue of proliferation in IL7 deficient hosts is observed in p27\textsuperscript{kip} deficient T cells\textsuperscript{17}. However, we found that IL7R- F5 T cells express normal levels of p27\textsuperscript{kip} (data not shown). Other factors, such as FoxO3a\textsuperscript{18}, have also been identified as targets downstream of both TCR and IL7 signalling pathways. We do not therefore exclude the possibility that pathways activated downstream of IL7R signalling may, under certain circumstances, converge with TCR induced
pathways and be a mechanism to enhance T cell proliferation. Indeed, we have suggested such a model in the past\textsuperscript{4,16}. However, in the present study we could find no obvious role for IL7 as a co-stimulator of T cell activation with either high or low avidity ligands, over a broad range of antigen and cytokine doses. Other studies have suggested that IL7 can tune T cell reactivity by modulating co-receptor expression\textsuperscript{19}. While we did observe a small but reproducible reduction in CD8 expression by IL7R- F5 T cells (~15%), neither this nor the complete absence of IL7R expression had any detectable affect on CD69 up-regulation or T cell proliferation \textit{in vitro} \textsuperscript{10}, neither was CD8 expression by IL7R+ F5 T cells influenced by IL7 deficiency \textit{in vivo}. Therefore, we could find no evidence to suggest that activating IL7 signalling can directly influence T cell responses to TCR ligands.

Arguably the strongest evidence for cross talk between TCR and IL7 signalling comes from studies of lymphopenia induced proliferation. LIP is triggered by TCR signals induced following engagement of receptor with spMHC in lymphopenia. IL7 is also essential for induction of LIP\textsuperscript{3,16,25} but its precise role is not understood. While p27\textsuperscript{kip} is clearly a target of IL7 signalling\textsuperscript{17}, it’s ablation in T cells could only slightly rescue proliferation in the absence of IL7 and so does not completely account for the role IL7 in facilitating LIP. We found that induction of LIP was associated with increased homeostatic TCR signalling resulting from spMHC recognition\textsuperscript{30} that occurred as a result of enhanced interactions of T cells with DCs in secondary lymphoid organs. It is not surprising that such increased contact should occur in the absence of host T cell competition and we previously found LIP to be inversely proportional to the number of F5 T cells transferred and hence level of T cell competition\textsuperscript{31}. What was surprising was that in the absence of IL7, the increase in homeostatic TCR signalling was not observed and neither was there any evidence of enhanced contact with DCs, in spite of the lack of competing host T cells. It therefore appears that a key role for IL7 exists at the level of initial delivery of TCR signalling by influencing the ability of T cells to interact and receive signals from pMHC on DCs, rather than tuning or modulating ongoing TCR signalling.
The mechanism by which IL7 is influencing T cell-DC interactions to enhance priming remains to be fully elucidated. Chemokines CCL19 and CCL21 regulate both migration and motility of naïve T cells\textsuperscript{32} and have been recently implicated in regulating delivery of IL7 signalling in lymph nodes by specific attraction of T cells to IL7 secreting fibroblastic reticular cells\textsuperscript{33} while inactivation of G\(\alpha_i\)-coupled receptors, such as CCR7, with pertussis toxin efficiently inhibits LIP but not T cell survival\textsuperscript{34}. In addition, Foxo1 and IL7 signalling can regulate CD62L and Klf2 expression, implying a role for IL7 signalling in LN homing and trafficking\textsuperscript{35}. However, we found neither IL7 nor IL7R deficiency had any affect on CCL21 induced transmigration of F5 T cells \textit{in vitro} (unpublished data). Another study found evidence that IL7 specifically regulates CD4 T cell homeostasis by directly modulating Class II expression on plasmacytoid DCs\textsuperscript{36}, raising the intriguing possibility that homeostasis of CD4 and CD8 T cells by DCs and IL7 could be achieved by distinct mechanisms relying on different DC subpopulations.

Another function of IL7 is in the control of T cell size and metabolism independently of survival, through the activation of PI3Kinase dependent pathways\textsuperscript{24} and regulation of glucose metabolism\textsuperscript{37}. Consistent with this, IL7R- F5 T cells were smaller than control IL7R+ F5 T cells and likely also had reduced metabolism. Indeed, we found that CFSE labelling of IL7R- F5 T cells, a process dependent on active uptake of dye, was consistently lower compared with control F5 T cells. The reduced size did not affect the ability of cells to activate or blast transform but reduced cellular metabolism may have had an impact on the ability of the naïve T cells to traffic around lymphoid tissues and thereby the rate with which they could interact with DCs. The ability of T cells to actively migrate around lymph nodes scanning DCs for antigen is vital for efficient initiation of immune responses. In lymphopenia, enhanced IL7 signalling to F5 T cells results in significant growth\textsuperscript{31} and the increase in metabolism may allow a more rapid scanning rate of DCs that would account for the increased level of DC contact we detected and, consequently, the delivery of more intense signalling from spMHC. This would also explain how spMHC ligands can
trigger T cells into division in lymphopenia but not replete conditions. A failure to efficiently scan DCs would also explain why IL7R- F5 T cells were less successful at finding DCs presenting peptide under conditions of limiting antigen such as following flu challenge. However, for those cells that did successfully find a flu antigen presenting DC, priming and activation would proceed normally, as we observed. Consistent with this view, when we saturated the antigen presenting compartment of the mice with soluble peptide, thereby removing any advantage conferred to more motile cells that could scan more DCs, triggering rates amongst IL7R- and IL7R+ F5 T cells became identical. In conclusion, our data reveal a novel role for IL7 in regulating the ability of T cells to interact with DCs and thereby influence both T cell priming and homeostasis.

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Authorship

Manoj Saini – Performed experiments, interpreted data.

Claire Pearson - Performed experiments.

Benedict Seddon – Performed experiments, interpreted data and wrote manuscript.

The authors have no conflicts of interest.
References

Figure legends

Figure 1 – IL7 does not affect T cell activation in vitro

(A) Total LN cells from F5 Rag1−/− mice were cultured (10⁶/ml) with 10nM NP68 in the presence or absence of IL7 (10ng/ml). Histograms are of pZap70 and CD69 levels on CD8 gated T cells in unstimulated (grey fill) or NP68 stimulated cultures with (solid line) or without (broken line) IL7 for the times indicated. pSTAT5 staining is shown for IL7 stimulated cells cultured with (solid line) or without (broken line) NP68 peptide compared with unstimulated cultures lacking IL7 and NP68 (grey fill). (B-C) Total F5 LN cells were labeled with CFSE and cultured (10⁶/ml) with a range of peptide doses. At day 3, CFSE profile of viable CD8⁺ T cells was analysed by FACS. Graphs show the percent cells triggered into division (B) and mean division of triggered cells (C) in response to different doses of agonist NP68 peptide (circles) or the weak agonist NP34 (diamonds) in the absence (empty symbols) or presence (filled symbols) of IL7 (10ng/ml). (D) Histograms show expression of IL7R, TCR and CD8 by IL7R+ F5 T cells from control (solid line) and IL7R- F5 T cells from F5 TetIL7R mice off dox for 7 days (grey fill). Histogram of IL7R by CD4⁺CD8⁺ DP F5 thymocytes (broken line) is shown as negative control. (E) The graph shows CD69 expression at 18h by IL7R⁺ F5 splenocytes of control mice (filled circle) and CD8⁺ IL7R⁺ F5 splenocytes from F5 TetIL7R mice off dox for 7 days (empty circle), stimulated with different doses of NP68 peptide. Data are representative of three or more experiments.

Figure 2 -Defective triggering of F5 T cells in the absence of IL7R expression

F5 T cells from CD45.1⁺ control F5 and CD45.1⁻ F5 TetIL7R mice off dox for 7 days were CFSE labeled, mixed 1:1 ratio and transferred (3 x 10⁶ total T cells/mouse) to groups of Rag1⁻/⁻ hosts. Groups of recipient mice (n=3) were further challenged with flu virus i.v. (24U/mouse). At day 3 post transfer, mice were culled and donor populations among host splenocytes analysed by FACS. (A) Dot plots are of CFSE vs CD45.1 expression by CD8⁺TCRhi cells from naïve or flu challenged
recipients. (B) Histograms are of CFSE profiles for CD45.1+ control F5 T cells (IL7R+ F5) and CD45.1− F5 T cells from F5 TetIL7R donors (IL7R− F5). Numbers indicate the average % of F5 T cells triggered into division ± s.d. in each case. *p<0.008, n=6. (C) Histograms show cell size as determined by FSc signal for divided and undivided control (sold line) and IL7R- (grey fill) F5 T cells. Data are representative of six independent experiments.

**Figure 3 - T cell triggering is defective over a wide range of antigen doses**

(A) Control F5 T cells were CFSE labeled and transferred to Rag1−/− hosts (3 x 10⁶/mouse) and groups of mice were challenged with a range of flu doses. At day 3, mice were culled and the responding T cell population in the spleen analysed by FACS. The scatter plot shows the percent of F5 T cells triggered into division in individual mice challenged with different doses of flu virus. (B-D) F5 T cells from CD45.1+ control F5 and CD45.1− F5 TetIL7R mice off dox 7 days were CFSE labeled, mixed at a 1:1 ratio and transferred (3 x 10⁶ total T cells/mouse) to groups of Rag1−/− hosts challenged with either 1.6, 6, 24 or 100U flu virus. At day three, mice were culled and CFSE profile of CD8+ TCRhi CD45.1+ control (IL7R+ F5) and CD45.1− F5 TetIL7R F5 T cells (IL7R− F5) analysed by FACs. Plot shows ratio of IL7R− F5 : IL7R+ F5 cells triggered into division as a function of Flu dose (B). Scatter plot shows of the percent of F5 T cells triggered into division for IL7R+ F5 (x axis) vs IL7R− F5 (y axis) cells in the same recipient challenged with the flu dose indicated (C). The scatter plot shows the mean division of triggered IL7R+ F5 (x axis) vs IL7R− F5 T cells in individual hosts (D). Data are pooled from three independent experiments.

**Figure 4 – Acute requirement for IL7 for optimal triggering of T cells**

F5 T cells from CD45.1+ control F5 and CD45.1− F5 TetIL7R mice off dox 7 days were CFSE labeled, mixed at a 1:1 ratio and transferred (3 x 10⁶ total T cells/mouse) to groups of Rag1−/− or Il7−/− Rag1−/− hosts. Recipient mice were further challenged with flu virus (24U/mouse). At day 3, mice were culled and F5 T cells populations in spleen analysed by FACs. (A) Histograms show CFSE
profiles of CD8^+ TCR$^{hi}$ F5 T cells from the donor mice and in the hosts indicated. (B) Box plot shows the ratio of control F5 to F5 TetIL7R donor T cells triggered into division in the indicated hosts. Statistics: * comparing ratios in $Rag1^{-/-}$ and $Il7^{-/-}Rag1^{-/-}$ - p<0.016; comparing frequency of triggered control and F5 TetIL7R T cells in $Rag1^{-/-}$ hosts**, p<0.0001 and in $Il7^{-/-}Rag1^{-/-}$ hosts *** p=0.66.

Figure 5 – IL7 enhances T cell stimulation by DCs to induce LIP
(A) T cells from F5 $Rag1^{-/-}$ donors were transferred (2 x 10^6/mouse) to $Rag1^{-/-}$, $\beta2m^{-/-}Rag1^{-/-}$ or $Il7^{-/-}Rag1^{-/-}$ hosts (n=5/group). At various days, spleens were taken from recipient mice and stained for CD8, TCR and CD5. Histograms of CD5 expression 7 days after transfer of F5 T cells to $Rag1^{-/-}$ hosts (solid line), $Il7^{-/-}Rag1^{-/-}$ (broken line) hosts compared with expression by F5 T cells from control F5 $Rag1^{-/-}$ mice (grey fill). Histograms of CD8 expression are of F5 T cells 7 days after transfer to $Rag1^{-/-}$ hosts (solid line), $\beta2m^{-/-}Rag1^{-/-}$ hosts (grey fill) and $Il7^{-/-}Rag1^{-/-}$ hosts (broken line). Graph shows CD5 expression following transfer to $Rag1^{-/-}$ (solid) or $Il7^{-/-}Rag1^{-/-}$ hosts (empty) as percent of expression by F5 T cells from control mice. (B) F5 T cells were CFSE labelled and transferred (2 x 10^6/mouse) to $Rag1^{-/-}$ and $Il7^{-/-}Rag1^{-/-}$ hosts. At days 7, 14 and 21, lymphocytes were recovered and stained for expression of CD8 and TCR. Histograms are of representative CFSE profiles at day 14 and indicate gates used to examine cells that had undergone different number of division. Line graphs show TCR expression by F5 T cells that have undergone different numbers of divisions at d7 (empty square), d14 (half square) and d21 (filled square) after transfer into the hosts indicated normalised to expression in control F5 $Rag1^{-/-}$ mice.

Figure 6 - IL7 enhances T cell-DC interactions
(A) Histogram shows Class II MHC expression by F5 T cells recovered from spleen 7 days after transfer to $Il7^{-/-}Rag1^{-/-}$ (broken line), $Rag1^{-/-}$ (solid line) hosts (n=5 in each group) compared with
F5 T cells from control F5 mice (grey fill). Graph shows I-A\(^b\) staining following transfer to \(RagI^{+/}\) (solid) or \(IL7R^{+}\) \(RagI^{+/}\) hosts (empty) as percent of expression by F5 T cells from control mice. Data are representative of three or more experiments. (B) CD45.1+ IL7R+ and CD45.1- IL7R- F5 T cells were co-transferred to \(RagI^{+/}\) hosts (n=4) and cells analysed for Class II MHC expression 1 day later. Histograms show I-A\(^b\) staining by IL7R+ and IL7R- F5 T cells before (broken line) and after (solid line) transfer to \(RagI^{+/}\) hosts. Bar chart is MFI of I-A\(^b\) staining by the indicated F5 T cell donor following transfer to \(RagI^{+/}\) hosts (* \(p < 0.02\)).

**Figure 7 – Saturating antigen presenting capacity overcomes the triggering defect in IL7R- F5 T cells**

(A) Control F5 T cells were CFSE labeled and transferred to \(RagI^{+/}\) hosts (3 x 10\(^6\)/mouse) and groups of mice were challenged with a range of NP68 peptide doses at 0h and 18h after transfer. At day 3, mice were culled and the responding T cell population analysed by FACS. The scatter plot shows the percent of control F5 T cells triggered into division in individual mice challenged with different doses of peptide. (B) F5 T cells from CD45.1+ control F5 (IL7R+ F5) and CD45.1- F5 TetIL7R mice off dox 7 days (IL7R- F5) were CFSE labeled, mixed at a 1:1 ratio and transferred (3 x 10\(^6\) total T cells/mouse) to groups of \(RagI^{+/}\) hosts challenged with a range of NP68 peptide doses. At day three, mice were culled and CFSE profile of IL7R+ F5 and IL7R- F5 T cells analysed by FACs. Scatter plot shows the percent of F5 T cells triggered into division for IL7R+ F5 (x axis) vs IL7R- F5 (y axis) T cells in the same recipient (B) and ratio of IL7R- F5 : IL7R+ F5 as a function of peptide dose (C). Data are representative two independent experiments.
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Regulation of T cell-dendritic cell interactions by IL7 governs T cell activation and homeostasis

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