

# T cell receptor signal strength in T<sub>reg</sub> and iNKT cell development demonstrated by a novel fluorescent reporter mouse

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The ability of antigen receptors to engage self-ligands with varying affinity is crucial for lymphocyte development. To further explore this concept, we generated transgenic mice expressing GFP from the immediate early gene *Nr4a1* (*Nur77*) locus. GFP was up-regulated in lymphocytes by antigen receptor stimulation but not by inflammatory stimuli. In T cells, GFP was induced during positive selection, required major histocompatibility complex for maintenance, and directly correlated with the strength of T cell receptor (TCR) stimulus. Thus, our results define a novel tool for studying antigen receptor activation in vivo. Using this model, we show that regulatory T cells (T<sub>reg</sub> cells) and invariant NKT cells (iNKT cells) perceived stronger TCR signals than conventional T cells during development. However, although T<sub>reg</sub> cells continued to perceive strong TCR signals in the periphery, iNKT cells did not. Finally, we show that T<sub>reg</sub> cell progenitors compete for recognition of rare stimulatory TCR self-ligands.

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Abbreviations used:  $\alpha$ -GalCer,  $\alpha$ -galactosylceramide; BAC, bacterial artificial chromosome; DP, double positive; IEL, intraepithelial lymphocyte; iNKT cell, invariant NKT cell; MFI, mean fluorescence intensity; mOVA, membrane OVA; OVAp, OVA peptide; pI:pC, polyinosinic:polycytidylic acid; pMHC, peptide MHC; RIP, rat insulin promoter; RTE, recent thymic emigrant; SP, single positive.

T cells encounter several checkpoints as they develop, and their fate often relies on the strength of signal perceived by the antigen receptor. For example, CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) thymocytes with low affinity for self-peptide MHC (pMHC) ligands undergo positive selection, whereas those with high affinity undergo negative selection (Starr et al., 2003). Multiple studies suggest that DP thymocytes are exquisitely sensitive and exhibit a broader range of recognition of pMHC than mature T cells (Davey et al., 1998; Lucas et al., 1999). Nonetheless, mature T cells continue to perceive low-affinity self-pMHC ligands in the periphery, and these interactions are essential for survival and effector function (Kirberg et al., 1997; Stefanová et al., 2002; Lo et al., 2009). Thus, the ability of the TCR to distinguish pMHC ligands of different affinity is a fundamental principal of immunological tolerance and homeostasis.

Although the affinity model explains the lineage commitment of a majority of T cell progenitors, some T cell subsets seem to have survived strong TCR signals during development as displayed by their activated phenotype (Baldwin et al., 2004; Kronenberg and Rudensky, 2005;

Kronenberg and Gapin, 2007). CD4<sup>+</sup>Foxp3<sup>+</sup> regulatory T cells (T<sub>reg</sub> cells), invariant NKT cells (iNKT cells), and CD8 $\alpha$ <sup>+</sup> intraepithelial lymphocytes (IELs) are hypothesized to be positively selected via strong TCR signals in the thymus (Leishman et al., 2002; Godfrey et al., 2004; Zhou et al., 2004). In the case of T<sub>reg</sub> cells, Jordan et al. (2001) first demonstrated that expression of a neo-self-antigen in the thymus of mice with TCRs specific for that antigen promoted the development of T<sub>reg</sub> cells. Yet use of a TCR with an intrinsically lower affinity for the same neo-self-antigen failed to select T<sub>reg</sub> cells, suggesting a role for strong TCR signals. Moreover, T cells transduced with TCRs cloned from T<sub>reg</sub> cells underwent homeostatic expansion in lymphopenic recipients to a greater extent than cells transduced with receptors cloned from conventional T cell (T<sub>conv</sub> cell) CD4 T cells, which is consistent with the idea that T<sub>reg</sub> cells recognize self-pMHC more avidly (Hsieh et al., 2006). Studies of the TCR repertoire from T<sub>reg</sub> and

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$T_{conv}$  CD4 T cells illustrated that they are equally diverse but different from each other (Hsieh and Rudensky, 2005). However, these TCR repertoires were not entirely unique; thus, others have suggested that  $T_{reg}$  cells are not shaped by agonistic interaction with self but rather by some stochastic event (Pacholczyk et al., 2006, 2007). In addition, when  $T_{reg}$  TCR transgenics were created, no overt thymic clonal deletion was observed (Bautista et al., 2009; Leung et al., 2009), nor was self-reactivity evident. Thus, it remains unclear precisely what type of TCR signals are involved in  $T_{reg}$  cell development in the thymus.

iNKT cells are CD1d-restricted  $\alpha\beta$  T cells that recognize lipid antigens. In the steady-state, they have a memory phenotype and have been proposed to develop after agonist or stimulatory interaction with a lipid self-ligand in the thymus, yet the precise ligand remains unidentified (Kronenberg and Gapin, 2007). Finally, CD8 $\alpha$  IELs have an activated phenotype and were increased in transgenic models in which the cognate stimulatory antigen was also present (Leishman et al., 2002). Thus, the term agonist selection has been applied to all three subsets, indicating encounter with a stimulatory (presumed high affinity) TCR ligand during development.

Short of cloning TCRs and identifying the selecting ligand in the thymus, it is difficult to know if a given T cell perceives a strong or weak TCR signal during development. Therefore, we sought to make a reporter mouse in which the level of a fluorescent protein reflects the strength of antigen receptor signal. We generated a transgenic mouse in which we inserted GFP into the *Nr4a1* (Nur77) locus of a bacterial artificial chromosome (BAC). Nur77 is an immediate early gene up-regulated by TCR stimulation in thymocytes and T cells (Osborne et al., 1994). It is an orphan nuclear receptor whose function in T cells is not completely understood, although data suggest it may play a role in thymocyte apoptosis (Cainan et al., 1995; Cho et al., 2003). In a microarray screen, we showed that thymocytes undergoing both positive and negative selection induced *Nr4a1* but to different expression levels (Baldwin and Hogquist, 2007). Thymocytes undergoing positive selection showed a twofold increase in *Nr4a1* expression, whereas those undergoing negative selection showed a 10-fold increase. Together, these observations suggested that a Nur77 reporter mouse might be a useful system for understanding the role of TCR signal strength during T cell development.

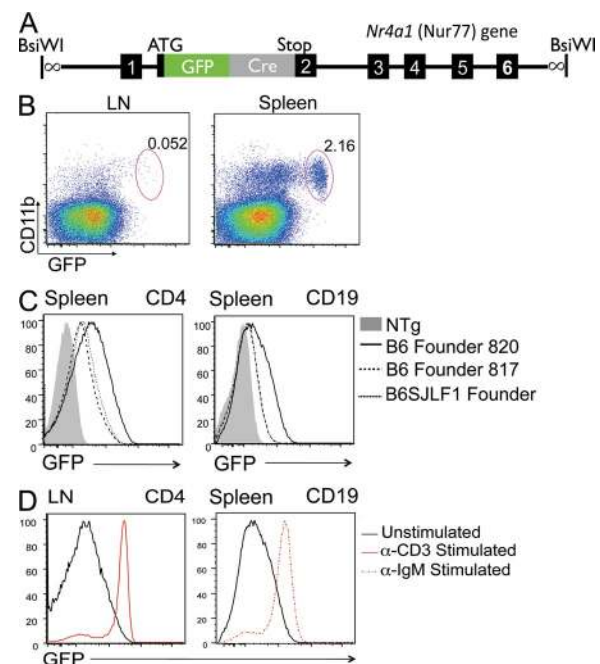
In this study, we report that GFP is up-regulated by antigen receptor stimulation in Nur77<sup>GFP</sup> mice, but unlike CD69, another common marker of T cell activation, it is not induced by inflammatory stimuli. Furthermore, the level of GFP expressed during acute activation reflects the strength of TCR stimulation, and the low basal level of GFP expressed in mature naive T cells is dependent on continued interaction with MHC. We applied this novel tool to study the TCR signal strength perceived by different T cell subsets during development.

## RESULTS

### A Nur77<sup>GFP</sup> transgenic mouse reports antigen receptor activation in lymphocytes

To create a fluorescent reporter that would be activated by antigen receptor signaling in lymphocytes, we inserted a GFP-Cre fusion protein at the start codon of the *Nr4a1* (Nur77) gene in a BAC (Fig. 1 A). The Cre recombinase gene was included for fate mapping experiments that are not reported in this study. One B6  $\times$  SJL F1 and two C57BL/6J founders were generated. Each founder expressed a slightly different overall level of GFP, but the pattern of expression was identical, and endogenous Nur77 expression was consistent with GFP expression (Fig. S1 A). All three showed normal lymphoid and myeloid development (unpublished data). A subset of myeloid lineage cells in the spleen expressed high levels of GFP in the steady-state (Fig. 1 B), whereas mature T and B lymphocytes expressed low levels of GFP (Fig. 1 C).

To determine whether TCR stimulation induced GFP expression, we injected Nur77<sup>GFP</sup> mice with 50  $\mu$ g anti-CD3 i.v. and 12 h later harvested lymphocytes. We observed robust induction of GFP (Fig. 1 D, left) and CD69 on T cells (not depicted) after  $\alpha$ -CD3 stimulation. We also stimulated bulk splenocytes with 10  $\mu$ g  $\alpha$ -IgM in vitro for 3 h. Again, robust



**Figure 1. A Nur77<sup>GFP</sup> BAC transgenic mouse expresses GFP upon TCR activation.** (A) A GFP-Cre fusion protein was inserted at the start site of the *Nr4a1* (Nur77) gene of a BAC construct and used to generate B6 or B6.SJLF1 transgenic lines. (B) GFP was highly expressed in a subset of myeloid cells of the spleen but not lymph node. (C) T and B lymphocytes expressed a low level of GFP. Three founder lines showed similar cell-specific patterns of GFP expression, but higher levels were observed in the B6-820 line ( $n = 5$  mice). (D) GFP was up-regulated in T cells 12 h after anti-CD3 injection in vivo or in B cells after 3 h of anti-IgM treatment in vitro ( $n = 4$  mice and three experiments).

GFP expression was observed in B cells (Fig. 1 D, right) but not T cells. Thus, we conclude that GFP expression can be induced after lymphocyte antigen receptor activation both in vitro and in vivo.

Initial microarray experiments showed differential expression of *Nr4a1* in thymocytes undergoing positive versus negative selection. In light of this, we asked whether the level of GFP induced in T cells would correlate with the strength of TCR signal perceived. We used K<sup>b</sup>/OVA-specific OT-I TCR transgenic mice, for which many variant peptide ligands have been characterized (Hogquist et al., 1994; Daniels et al., 2006). DP thymocytes from OT-I/Nur77<sup>GFP</sup> mice lacking the transporter associated with antigen process 2 gene (*Tap*<sup>o</sup>) were stimulated with APCs pulsed with OVA peptide (OVAp) variants in vitro. In Fig. 2 A, these are listed according to stimulatory strength, with the cognate OVAp on the left and the weakest variant (E1) on the right. The level of GFP induced by each directly correlated with its stimulatory activity (Fig. 2 A). Interestingly, even the low-affinity variant E1 and the self-peptide  $\beta$ -CAT induced GFP above the background level (control peptide p815; Fig. 2 A, inset). Neither of these weak peptides stimulates OT-I T cells to proliferate, but they support positive selection of OT-I in organ cultures

(Hogquist et al., 1994; Santori et al., 2002) and in vivo (Stefanski et al., 2001).

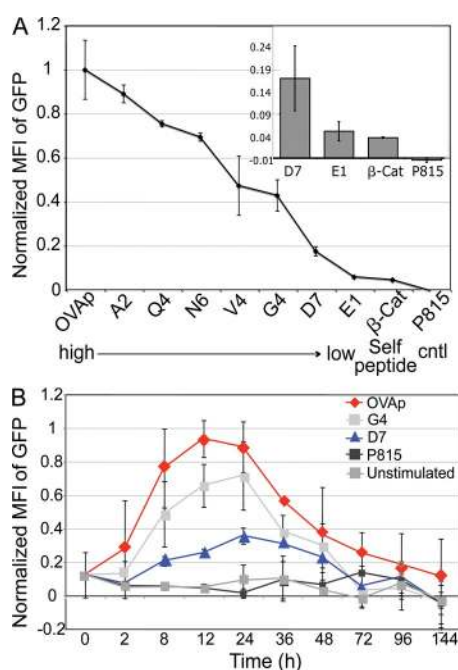
GFP up-regulation was transient after TCR stimulation with maximum expression observed between 12 and 24 h (Fig. 2 B). The up-regulation of endogenous Nur77 protein was also determined in parallel, and endogenous levels also correlated with strength of stimulus (Fig. S1 B), although peak induction of endogenous Nur77 occurred earlier than GFP, presumably reflecting the time required for maturation of a fully fluorescent GFP and the greater stability of GFP (Fig. S1 C).

Analogous experiments were performed in vivo using OT-I/Nur77<sup>GFP</sup> cells transferred into mice and infected with strains of *Listeria monocytogenes* expressing variants of the OVAp. As seen with peptides in vitro, the level of GFP in OT-I T cells in vivo reflected the stimulatory strength of the variant peptide ligand, even in the context of an infection (Fig. S2). Thus, the Nur77<sup>GFP</sup> mouse has the potential to be a sensitive reporter of TCR signal strength both in vitro and in vivo.

### GFP expression is induced by positive selection and maintained by tonic MHC signals

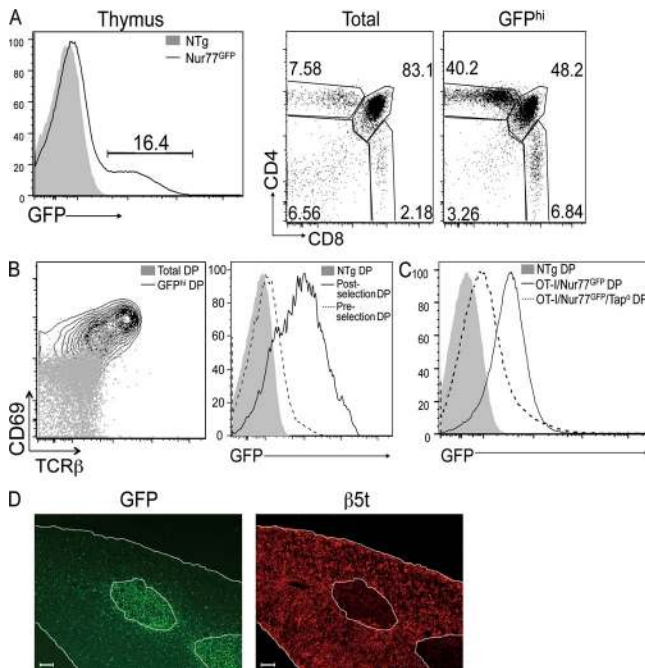
Because *Nr4a1* message was up-regulated during positive selection (Baldwin and Hogquist, 2007) and GFP could be induced by low-affinity TCR ligands (Fig. 2 A), we sought to determine whether GFP was up-regulated by positive selection in vivo. In the thymus of Nur77<sup>GFP</sup> mice, only a fraction of cells expressed GFP (Fig. 3 A). Further analysis revealed that the GFP<sup>+</sup> population was enriched for DP dull, CD4, and CD8 single-positive (SP) cells (Fig. 3 A, right). Among DP thymocytes, the GFP<sup>+</sup> cells were high for CD69 and the TCR- $\beta$  chain (Fig. 3 B, dot plot), suggesting that induction of GFP occurred at the time of positive selection (Fig. 3 B, histogram). Furthermore, we observed very low expression of GFP in DP thymocytes from OT-I *Tap*<sup>o</sup> (nonselecting) mice compared with OT-I (selecting) control mice (Fig. 3 C), demonstrating that positive selection induced GFP in vivo. Consistent with the induction of GFP expression during positive selection, the majority of GFP bright cells were located in the medulla with only a few GFP-positive thymocytes found in the cortex (Fig. 3 D).

Like mature SP thymocytes, naive T cells in the periphery expressed GFP, although at slightly lower levels (Fig. 4 A, right), suggesting a decay of GFP with maturation. In fact, there was a modest stepwise decrease in GFP expression during development with semimature SP thymocytes expressing the highest level of GFP, followed by mature SP, then recent thymic emigrants (RTEs; HSA<sup>hi</sup>Qa2<sup>lo</sup>), and finally naive non-RTE T cells (Fig. S3). Nonetheless, naive non-RTE T cells expressed a level of GFP in the steady-state that was significantly above background and did not vary with age. Interestingly, this basal level of GFP was higher in CD4 than CD8 T cells, which is consistent with some models that have proposed that the CD4 coreceptor delivers a stronger signal than the CD8 coreceptor (Veillette et al., 1988; Legname et al., 2000). Memory phenotype CD4 and CD8 T cells did not express significantly different levels of GFP when compared with their



**Figure 2. The level of GFP expression reflects TCR signal strength and is transient.** (A) OT-I/*Tap*<sup>o</sup>/Nur77<sup>GFP</sup> thymocytes were co-cultured for 3 h with B6 splenocytes pulsed with the peptide SIINFEKL (OVAp) or the indicated altered peptide ligands, listed in order of decreasing potency. The MFI of GFP was normalized to the level observed with OVAp stimulation ( $n = 5$ ). cntl, control. (B) Splenocytes from OT-I/Nur77<sup>GFP</sup> mice were cultured with the indicated peptides for various lengths of time. The data represent the mean normalized GFP levels from six different experiments of at least six mice. Error bars indicate standard deviation.

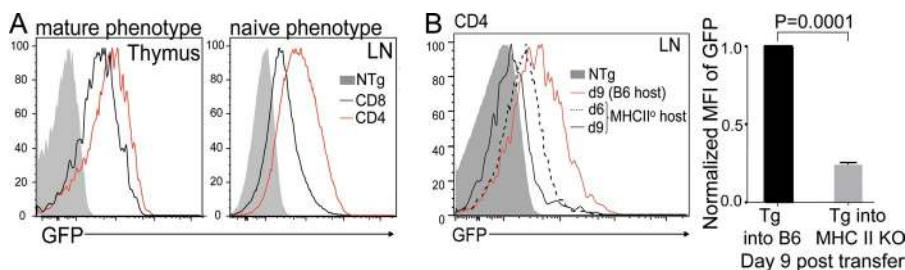




**Figure 3. GFP expression is induced during positive selection.** (A) Flow cytometric analysis of GFP in total thymocytes (left). Dot plots (right) show CD4 and CD8 expression on total or GFP-positive thymocytes from Nur77<sup>GFP</sup> mice. (B) The GFP<sup>+</sup> DP population was enriched for CD69<sup>+</sup>TCR-β<sup>+</sup> cells (dot plot). CD69<sup>+</sup>TCR-β<sup>hi</sup> (postselection) DP thymocytes expressed higher levels of GFP compared with CD69<sup>+</sup>TCR-β<sup>lo</sup> (preselection) DP thymocytes (histogram overlay). (C) GFP expression of DP thymocytes from WT or Tap-deficient mice. NTg, nontransgenic. (D) Immunofluorescence analysis of GFP in the Nur77<sup>GFP</sup> thymus, with the cortical region defined by staining for the β5t proteasome subunit. Data are representative of >10 mice from at least three independent experiments. Bars, 100 μm.

naive counterparts (unpublished data). Finally, in the absence of the co-stimulatory molecule CD28, T cells expressed identical levels of GFP compared with CD28-sufficient T cells (Fig. S4).

To determine whether the GFP levels in naive T cells reflect tonic TCR stimulation by self-pMHC, we adoptively transferred Nur77<sup>GFP</sup> CD4 T cells into congenic WT or MHC II-deficient hosts and analyzed them after 6 or 9 d. The GFP level in naive phenotype, non-RTE CD4 T cells was maintained after adoptive transfer into WT recipients (Fig. 4 B, red line). In contrast, GFP was lost from CD4 T cells in MHC II-deficient recipients. These data suggest that TCR signals maintain GFP expression in peripheral T cells.



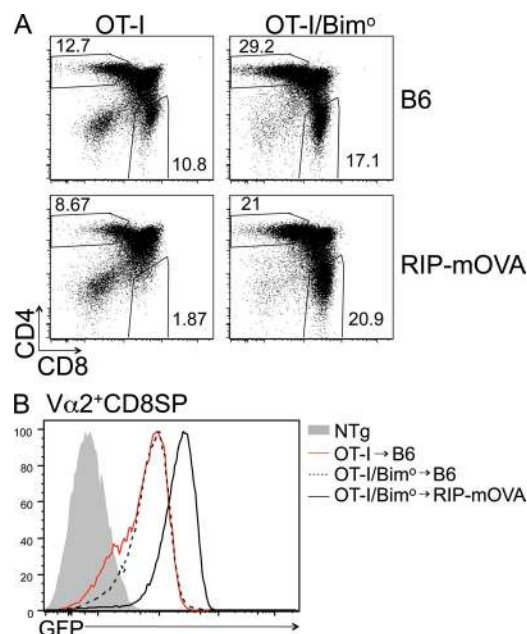
**Figure 4. GFP expression is maintained in the steady-state by tonic MHC signals.** (A) Analysis of GFP levels in mature CD4 and CD8 SP thymocytes (left; defined as HSA-10Qa2<sup>hi</sup>) or naive phenotype CD4 and CD8 lymph node T cells (right; defined as CD44<sup>lo</sup>CD69<sup>-</sup>CD25<sup>-</sup>). Data are representative of >10 mice. (B) 1–2 × 10<sup>6</sup> polyclonal Nur77<sup>GFP</sup> CD4 T cells were transferred into B6 or I-A<sup>b</sup>-deficient (MHC II<sup>o</sup>) recipients and analyzed 6 or 9 d later. Bar graph shows the mean GFP level on cells adoptively transferred into I-A<sup>b</sup>-deficient recipients normalized to the level on CD4 T cells in B6 recipients. Data are representative of 11 mice from four independent experiments. Error bars indicate standard deviation. NTg, nontransgenic; Tg, transgenic.

### Thymocytes undergoing negative selection express high levels of GFP

If GFP levels reflect the strength of the TCR signal perceived, one would predict higher GFP expression in thymocytes undergoing negative selection compared with positive selection. To test this, we used the OT-I/rat insulin promoter (RIP)–membrane OVA (mOVA) system, in which negative selection occurs via clonal deletion in CD8 SP thymocytes (Kurts et al., 1997). However, cells undergoing clonal deletion are rapidly cleared by thymic macrophages (Surh and Sprent, 1994). Thus, we created OT-I/Bim<sup>o</sup>/Nur77<sup>GFP</sup> transgenic mice, in which deficiency of the proapoptotic molecule Bim prevented apoptosis. Accordingly, bone marrow chimeras were created using OT-I/Nur77<sup>GFP</sup> or OT-I/Bim<sup>o</sup>/Nur77<sup>GFP</sup> mice as donors and B6 or RIP-mOVA mice as recipients (Fig. 5). As expected, we observed efficient positive selection of OT-I in B6 recipients and efficient deletion in RIP-mOVA recipients (Fig. 5 A, left). Moreover, OT-I/Bim<sup>o</sup> cells underwent efficient positive selection in B6 recipients, but Bim deficiency completely rescued OT-I cells from deletion in RIP-mOVA recipients (Fig. 5 A, right; and Fig. S5). Interestingly, GFP expression was substantially higher in OT-I/Bim<sup>o</sup> thymocytes rescued from deletion in RIP-mOVA recipients compared with cells undergoing positive selection in B6 recipients (Fig. 5 B). Therefore, we conclude that GFP levels were induced to higher levels by negative selection signals as compared with positive selection stimuli.

### Induction of GFP is TCR specific

Many immunological studies use the expression of CD69 as a read-out for TCR activation. One caveat to this is that CD69 expression can be induced by inflammatory stimuli such as type I interferons (Sun et al., 1998; Shioh et al., 2006), thereby limiting its use as a marker of TCR stimulation in infection or inflammatory settings. We sought to determine whether inflammatory stimuli would also induce GFP expression in Nur77<sup>GFP</sup> mice. Neither polyinosinic:polycytidylic acid (pI:pC; Fig. 6 A) nor LPS (Fig. 6 B) induced GFP expression, although both induced CD69 up-regulation. To confirm this observation in the context of an infection, we transferred OT-I/Nur77<sup>GFP</sup> T cells into

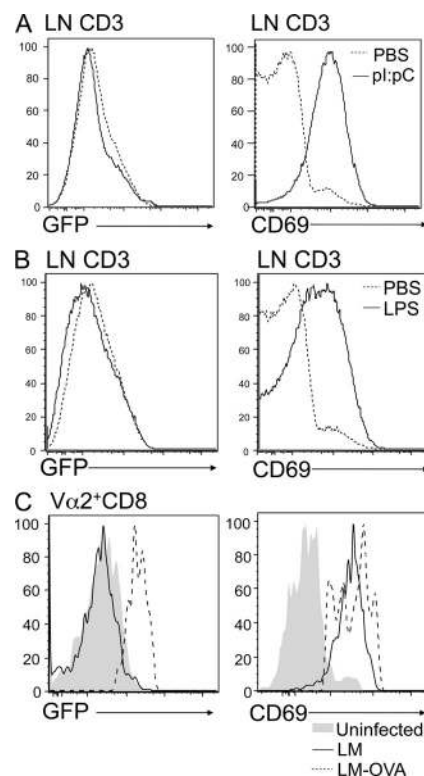


**Figure 5. Thymocytes undergoing negative selection express higher levels of GFP compared with those undergoing positive selection.** OT-I/Nur77<sup>GFP</sup> mice or OT-I/Bim<sup>o</sup>/Nur77<sup>GFP</sup> mice were generated and used as bone marrow donors.  $5\text{--}10 \times 10^6$  bone marrow cells were injected into lethally irradiated B6 or RIP-mOVA recipients. (A) Expression of CD4 and CD8 on thymocytes from the indicated chimeric mice. (B) GFP expression on Vα2<sup>+</sup> CD8SP from the indicated chimeric mice. Representative data are from five experiments with more than five mice. NTg, nontransgenic.

B6 recipient mice and infected them with *L. monocytogenes* that did or did not express OVA. Only when the pathogen expressed the OVA antigen was GFP up-regulation observed in OT-I T cells (Fig. 6 C). Together, these results suggest that GFP expression is driven by antigen receptor signaling in T cells in Nur77<sup>GFP</sup> mice and not by other homeostatic or inflammatory signals.

#### T<sub>reg</sub> cells express higher levels of GFP than T<sub>conv</sub> cells

Because GFP levels reflected TCR signal strength in Nur77<sup>GFP</sup> mice, we sought to use these mice to test whether T<sub>reg</sub> cells perceive stronger TCR signals compared with T<sub>conv</sub> cells during development. Thymic Foxp3<sup>+</sup> T<sub>reg</sub> cells expressed approximately twofold higher mean fluorescence intensity (MFI) for GFP than conventional CD4SP (Fig. 7). A higher level of GFP in T<sub>reg</sub> cells might arise if they were developmentally younger than T<sub>conv</sub> cells because we observed a slight decrease in GFP as cells matured in the thymus (Fig. S3). This is unlikely because a previous study showed that thymic T<sub>reg</sub> cells are, on average, developmentally more mature than conventional CD4SP (McCaughy et al., 2007). Furthermore, thymic T<sub>reg</sub> cell progenitors (defined as CD4SP, CD25<sup>+</sup>, CD122<sup>hi</sup>, Foxp3<sup>+</sup>; Burchill et al., 2008) had an even higher GFP expression level (Fig. 7). Finally, a twofold higher GFP level was also observed in peripheral T<sub>reg</sub> cells compared with T<sub>conv</sub>

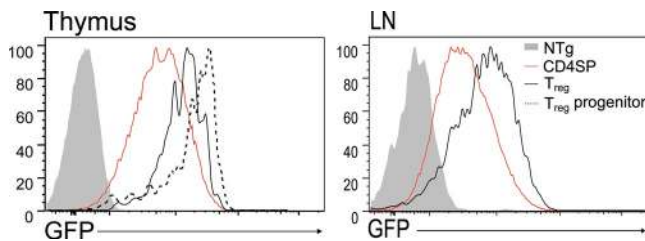


**Figure 6. Induction of GFP is TCR specific.** (A and B) Nur77<sup>GFP</sup> mice were injected i.v. with pl:pC (A) or LPS (B). After 6 h, cells were analyzed for GFP (left) and CD69 (right) expression. (C)  $5 \times 10^6$  OT-I/Nur77<sup>GFP</sup> lymph node cells were adoptively transferred into B6 recipients and infected with *L. monocytogenes* expressing the OVAp (LM-OVA) or not (LM). GFP expression on Vα2<sup>+</sup>CD8<sup>+</sup> transferred cells was evaluated after 12 h. Histograms show representative data from three independent experiments with at least three mice.

CD4<sup>+</sup> T cells (Fig. 7 and Fig. S6). These data together suggest that T<sub>reg</sub> cells perceive stronger TCR signals than T<sub>conv</sub> cells during development and that this perception continues in the periphery. Interestingly, the GFP histograms for T<sub>reg</sub> and T<sub>conv</sub> cells are not completely distinct, but overlap. This is consistent with TCR repertoire studies (Hsieh et al., 2006; Pacholczyk et al., 2006), which showed that some clones are unique to T<sub>reg</sub> cells, some are unique to T<sub>conv</sub> cells, and some are shared.

#### STAT5 signaling does not increase GFP levels in T<sub>reg</sub> or T<sub>conv</sub> cells

Although it is proposed that avid interactions with self-ligands are required for T<sub>reg</sub> cell development, γc cytokines (IL-2 and to a lesser extent IL-15 and IL-7) are also known to be crucial (Burchill et al., 2007; Vang et al., 2008). To address the potential contribution of cytokine signaling to GFP expression, we isolated thymocytes and lymphocytes from Nur77<sup>GFP</sup> mice and cultured them for 3–12 h with 25 ng/ml IL-2 and observed no increase in GFP expression (unpublished data). A previous study showed that constitutive expression of STAT5 (Stat5b-CA) increased the frequency and number of Foxp3<sup>+</sup> T<sub>reg</sub> cells (Burchill et al., 2003). Therefore, we generated Nur77<sup>GFP</sup>/Stat5b-CA mice.



**Figure 7.  $T_{reg}$  cells express higher levels of GFP compared with  $T_{conv}$  cells.** Lymphoid organs from Nur77<sup>GFP</sup> mice were analyzed by flow cytometry.  $T_{reg}$  cells were defined as CD4SP<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup>.  $T_{reg}$  cell progenitors were CD4SP<sup>+</sup>CD25<sup>+</sup>CD122<sup>hi</sup>Foxp3<sup>+</sup>. Data are representative of more than eight mice from eight experiments. NTg, nontransgenic.

Thymocytes and lymphocytes were harvested and analyzed for Foxp3 expression and total GFP. As previously described (Burchill et al., 2003), we observed an increase in  $T_{reg}$  cells in Nur77<sup>GFP</sup>/Stat5b-CA mice in both the thymus and the periphery when compared with WT littermate controls (Fig. 8 A). However, the total MFI of GFP in  $T_{reg}$  cells from Nur77<sup>GFP</sup>/Stat5b-CA thymocytes did not increase but rather decreased in both the thymus ( $42 \pm 2\%$  decrease) and the periphery ( $32 \pm 8\%$  decrease; Fig. 8 B), whereas GFP expression in conventional CD4 thymocytes and lymphocytes did not change (Fig. 8 C). These data suggest that  $\gamma$ c cytokine signaling via Stat5 does not account for the increased expression of GFP observed in the CD4<sup>+</sup>Foxp3<sup>+</sup> population. Rather, the decrease in the total GFP MFI in the  $T_{reg}$  cell population of Stat5b-CA mice likely reflects the recruitment of low-affinity TCR clones normally found in the naive repertoire into the  $T_{reg}$  cell population as previously suggested (Burchill et al., 2008).

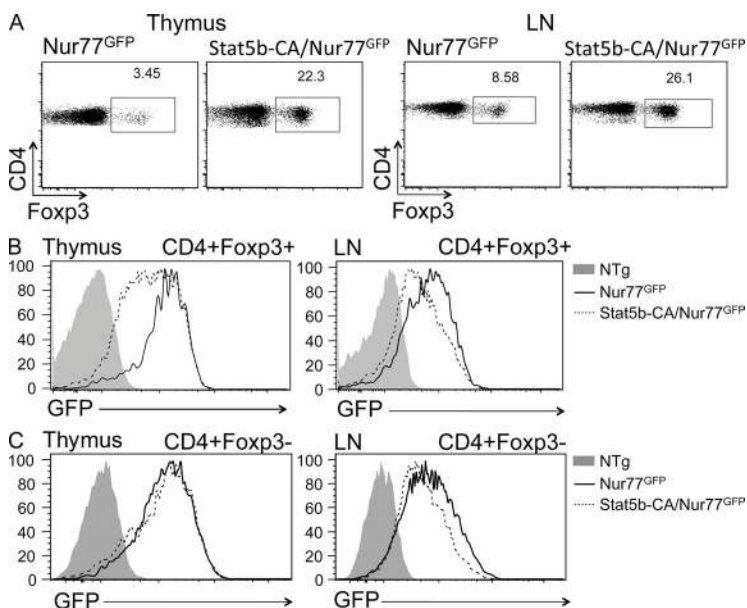
#### $T_{reg}$ cells compete for strong TCR ligands in the thymus

Previous studies with  $T_{reg}$  TCR transgenic mice showed that development of the Foxp3<sup>+</sup>  $T_{reg}$  cell lineage is impaired at high

precursory frequency, suggesting that  $T_{reg}$  cell progenitors compete for a limited factor during development (Bautista et al., 2009; Leung et al., 2009). This factor might be a cytokine, given the profound requirement for  $\gamma$ c cytokine signaling during  $T_{reg}$  cell development (Burchill et al., 2007). Alternatively,  $T_{reg}$  cell progenitors might compete for recognition of rare high-affinity self-ligands during development. In the Nur77<sup>GFP</sup> mouse, the level of GFP reflects TCR signal strength; thus, we postulated that if  $T_{reg}$  cell development were limited by competition for high-affinity ligands, GFP would be increased at low precursor frequency of a TCR specific for high-affinity ligands. Alternatively, if  $T_{reg}$  cell development were limited by non-TCR factors, GFP would not increase with less competition for the selecting ligand. To test this, using a mixed bone marrow chimera strategy, we generated animals with varying frequencies of Nur77<sup>GFP</sup>/G113 TCR transgenic precursors. G113 was cloned from a naturally occurring  $T_{reg}$  cell, although its TCR specificity is unknown (Hsieh et al., 2006). Similar to what was previously reported (Bautista et al., 2009), we observed that monoclonal G113 mice have an almost undetectable frequency of CD25<sup>+</sup>  $T_{reg}$  cells in the thymus (Fig. 9 A) or periphery (not depicted). Interestingly, the level of GFP was not higher on intact G113 CD4SP compared with the polyclonal CD4SP population (Fig. 9 A, top row). This suggests that  $T_{reg}$  cell-encoded TCRs do not have an intrinsically higher affinity for ubiquitous self-antigens and that both  $T_{reg}$  and conventional CD4 precursors are positively selected through similar (low) affinity interactions in the cortex. In contrast to monoclonal G113 mice, when chimerism was  $<1\%$ , the frequency of CD4<sup>+</sup>CD25<sup>+</sup> cells dramatically increased, as previously reported (Bautista et al., 2009). Surprisingly, the level of GFP increased on all G113 precursors (Fig. 9 A) when they were at low precursor frequency, with an inverse relationship between percent chimerism and the GFP MFI (Fig. 9 B). This was true for both CD25<sup>+</sup> and CD25<sup>+</sup> G113 CD4SP thymocytes. GFP did not increase on non- $T_{reg}$  cell (OT-II) precursors in analogous control chimeras (Fig. 9 B). These data demonstrate that thymic  $T_{reg}$  cell precursors compete for interactions that lead to strong TCR stimulations and imply that the high-affinity self-antigens that support  $T_{reg}$  cell development are rare.

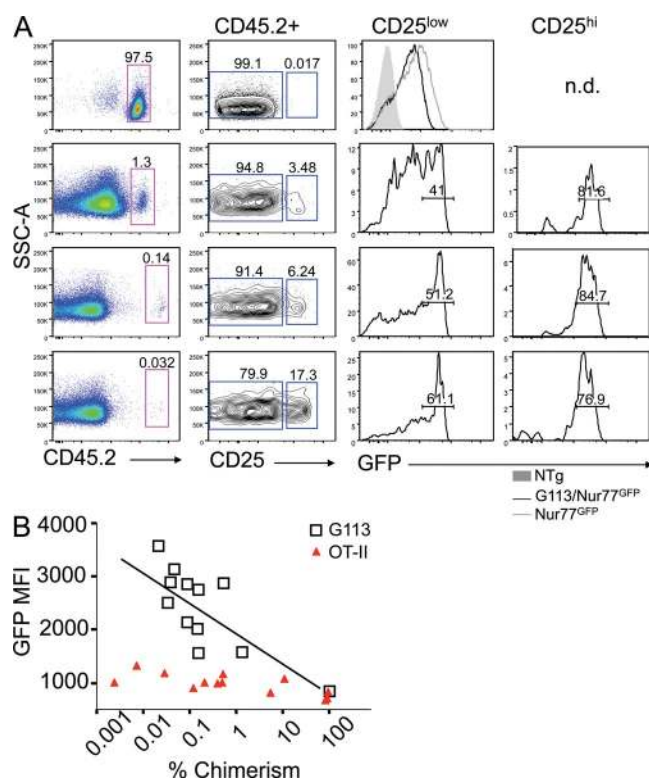
#### NKT cells express high levels of GFP during thymic selection

iNKT cells have been described as autoreactive by design with a preponderance of indirect data suggesting



**Figure 8. Stat5 signaling does not increase GFP in  $T_{reg}$  or  $T_{conv}$  cells.** Stat5b-CA/Nur77<sup>GFP</sup> and Nur77<sup>GFP</sup> mice were generated. (A) Increased frequency of CD4<sup>+</sup>Foxp3<sup>+</sup>  $T_{reg}$  cells in Stat5b-CA/Nur77<sup>GFP</sup> mice in both the thymus and periphery. (B) Histogram overlays of CD4<sup>+</sup>Foxp3<sup>+</sup> cells of Stat5b-CA/Nur77<sup>GFP</sup> and Nur77<sup>GFP</sup> control thymocytes (left) and lymphocytes (right). (C) Histogram overlays of CD4<sup>+</sup>Foxp3<sup>+</sup> CD4 T cells from Stat5b-CA/Nur77<sup>GFP</sup> and Nur77<sup>GFP</sup> mice. Data are representative of three experiments with four mice per group. NTg, nontransgenic.



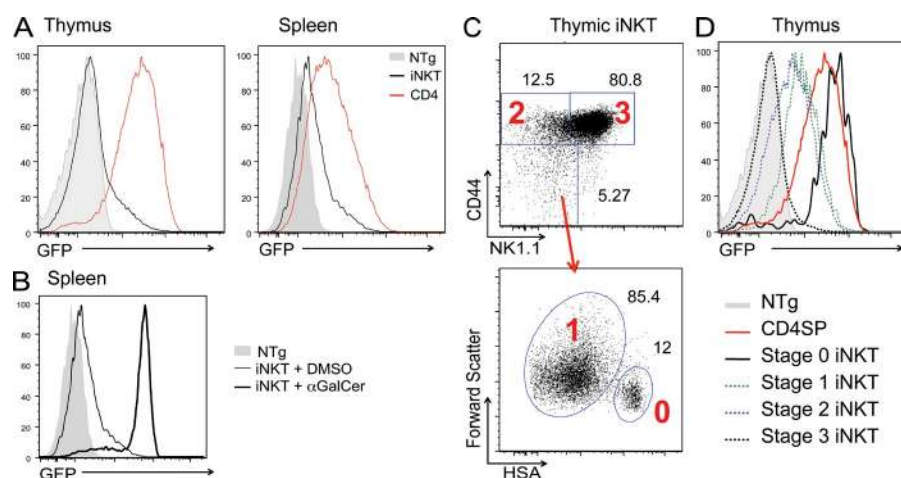


**Figure 9. T<sub>reg</sub> cells compete for strong TCR ligands during development.** G113/Rag1<sup>0</sup>/Nur77<sup>GFP</sup> (CD45.2<sup>+</sup>) and WT (CD45.1<sup>+</sup>) bone marrow were mixed at various ratios and used to reconstitute lethally irradiated recipients (CD45.1<sup>+</sup>). Chimerism was determined by analyzing the G113<sup>+</sup> T cell fraction from lymphoid tissue. 8 wk after transplant, lymphoid organs were harvested and analyzed by flow cytometry. (A) Dot plots on the far left show percent chimerism from select animals ranging from 97 to 0.032% G113 donor-derived T cells. Histograms on the right show GFP after gating on CD45.2<sup>+</sup> $\alpha$ 2 $\beta$ 6 $\alpha$ CD4SP that were either CD25 negative or positive. (B) Similar mixed bone marrow chimeras were set up with OT-II/Nur77<sup>GFP</sup> bone marrow. Graph shows the GFP MFI on  $\alpha$ 2 $\beta$ 6 $\alpha$ CD4SP thymocytes (for G113) or  $\alpha$ 2 $\beta$ 6 $\alpha$ CD4SP (for OT-II). Data are representative of three experiments with more than five mice. n.d., not determined. NTg, nontransgenic.

that precursors interact with a stimulatory self-lipid ligand, which remains incompletely identified (Bendelac et al., 2001; Gapin, 2010). Surprisingly then, both thymic and splenic iNKT cells expressed very low levels of GFP in Nur77<sup>GFP</sup> mice (Fig. 10 A). To confirm that iNKT cells could in fact up-regulate GFP after TCR stimulation, we injected mice i.p. with 5  $\mu$ g  $\alpha$ -galactosylceramide ( $\alpha$ -GalCer). There was robust induction of GFP in splenic CD1d tetramer-positive cells (Fig. 10 B). The lack of GFP in thymic iNKT cells was seemingly contradictory to data implicating agonistic TCR stimulation in thymic selection of iNKT cells. However, it is well known that iNKT cells undergo cell division after selection (Benlagha et al., 2002) and that mature iNKT cells can be retained in the thymus for extremely long periods of time (Berzins et al., 2006). Thus, to inquire more specifically about the intensity of TCR stimulation during iNKT cell selection, we sought to evaluate the earliest iNKT cell precursors, previously named stage 0 precursors, which can be identified as binding CD1d/ $\alpha$ -GalCer tetramer, and are HSA<sup>hi</sup>, CD44<sup>lo</sup>, NK1.1<sup>+</sup> (Benlagha et al., 2005). Because such cells are rare in the thymus, we performed CD1d/ $\alpha$ -GalCer tetramer-based magnetic enrichment. As expected, the majority of thymic iNKT cells were stage 3 mature cells (CD44<sup>hi</sup>NK1.1<sup>+</sup>), with a smaller subset of stage 2 and 1 cells (NK1.1<sup>+</sup>; Fig. 10 C). CD44<sup>+</sup> iNKT cells were further defined as stage 1 (HSA<sup>lo</sup>) or stage 0 (HSA<sup>hi</sup>). Interestingly, stage 0 iNKT cell progenitors expressed a higher level of GFP than age-matched conventional CD4SP, suggesting that they are indeed selected on stronger TCR ligands than T<sub>conv</sub> CD4 T cells. However, unlike T<sub>conv</sub> and T<sub>reg</sub> CD4 T cells, most iNKT cells lost GFP expression after maturation in the thymus and persisted like this in the periphery (Fig. 10 A).

## DISCUSSION

In this study, we introduced a unique BAC transgenic mouse useful for studying T cell activation in vivo. We showed that antigen receptor signaling was a major inducer of GFP in lymphocytes in Nur77<sup>GFP</sup> mice. GFP was not induced by



**Figure 10. NKT cells express higher levels of GFP immediately after selection but do not maintain it in the periphery.**

(A) Histograms show the level of GFP on conventional CD4SP or CD1d/ $\alpha$ -GalCer tetramer-binding iNKT cells in the thymus or spleen of Nur77<sup>GFP</sup> mice. Data are representative of more than five mice in at least three independent experiments. (B) GFP levels on splenic iNKT cells 6 h after injection with  $\alpha$ -GalCer or solvent control. (C) CD1d/ $\alpha$ -GalCer tetramer-binding iNKT cells were enriched from adult thymus using magnetic beads. Dot plots show the gating strategy used to identify subsets of thymic iNKT cells. (D) GFP levels on various staged iNKT cell populations compared with conventional CD4SP thymocytes. Data are representative of four experiments and more than nine mice. NTg, nontransgenic.

TLR (toll-like receptor) ligands or other inflammatory stimuli, did not require co-stimulation, and was dependent on MHC for induction and maintenance in T cells. This finding is surprising in light of evidence that mechanical force, hormones, growth factors, and cytokines could induce Nur77 expression at the transcriptional level in nonlymphoid tissues (Pei et al., 2005; Pols et al., 2007). The only other stimulus reported to induce Nur77 in T cells is the thymotoxic plastics stabilizer DBTC (di-*n*-butylin dichloride; Gennari et al., 2002), whose biochemical effect is not understood.

We observed that lymphocytes in the steady-state expressed a low level of GFP that was nonetheless consistently above the nontransgenic background. Polyclonal T and B lymphocytes expressed similar levels of GFP, and both responded to antigen receptor activation with rapid induction of GFP. Moreover, the thymic and peripheral expression pattern of GFP was consistent with antigen receptor regulation of signaling and TCR “tuning” and suggests this reporter mouse may be useful for imaging selection events in the thymus. Consistent with the idea that Nur77 expression is tightly regulated by the TCR, we found that adoptive transfer of CD4 T cells into an MHC II-deficient environment resulted in a loss of GFP expression that was otherwise maintained in the presence of MHC. This suggests that the tonic TCR signals perceived by T cells sustain the elevated GFP expression in the steady-state. Whether GFP expression in B cells requires tonic BCR signals is unknown. Naive CD4 T cells expressed higher basal levels of GFP compared with naive CD8 T cells. These data may suggest that CD4 T cells as a population express TCRs that perceive stronger self-pMHC signals through the TCR/coreceptor than CD8 T cells, but more experiments are required to address this hypothesis.

Using the Nur77<sup>GFP</sup> mouse as a reporter of TCR signal strength, we tested the idea that CD4<sup>+</sup>Foxp3<sup>+</sup>T<sub>reg</sub> cells perceive a stronger signal during thymic development than T<sub>conv</sub> CD4 T cells. We showed that polyclonal T<sub>reg</sub> cells expressed a higher level of GFP, implying they perceived stronger TCR stimulation upon selection and continued to do so in the periphery. These data are consistent with previous work with TCR transgenic mice in which coexpression of a neo-self-antigen and specific TCRs skewed the T cell repertoire to a higher frequency of CD4<sup>+</sup>Foxp3<sup>+</sup>T cells (Jordan et al., 2001; Kawahata et al., 2002). It is also consistent with the fact that mutations in LAT (linker of activated T cells) led to T<sub>conv</sub> cell development but not CD4<sup>+</sup>Foxp3<sup>+</sup> cell development (Koonpaew et al., 2006). Interestingly, we noted that there was distinct overlap in the GFP levels between T<sub>reg</sub> and non-T<sub>reg</sub> cells. This pattern is conceptually reminiscent of the repertoire analysis of T<sub>reg</sub> and non-T<sub>reg</sub> cells, which showed distinct receptor specificities that were found predominantly in one population or the other and some receptor specificities that were shared (Hsieh et al., 2006; Pacholczyk et al., 2006).

Stat5 signaling is known to be required for CD4<sup>+</sup>Foxp3<sup>+</sup>T<sub>reg</sub> cell development (Burchill et al., 2007). In light of this, it was possible that increased cytokine sensitivity and signaling in T<sub>reg</sub> cells accounts for the increased GFP expression. However,

the failure of IL-2 to increase GFP expression in vitro or Stat5b-CA to increase GFP in vivo suggested otherwise. In fact, T<sub>reg</sub> cells from Stat5b-CA mice showed an overall decrease in GFP MFI. This is consistent with the TCR repertoire analysis performed by Burchill et al. (2008) in the Stat5b-CA mice, in which they observed that overexpression of Stat5 diverted TCR clones from the naive population into the T<sub>reg</sub> cell repertoire. Because naive T cells expressed lower GFP when compared with T<sub>reg</sub> cells, this resulted in a decrease in the total GFP MFI of the T<sub>reg</sub> cell population in Stat5b-CA mice.

Using a T<sub>reg</sub> TCR transgenic model (G113) at low precursor frequency, we were able to provide evidence that T<sub>reg</sub> cells compete for strong TCR ligands during development. We observed that when there was high competition, as seen in the 100% G113 chimeras, there was no increase in the overall level of GFP on T<sub>conv</sub> cells, suggesting that the G113 TCR does not have an intrinsically higher affinity for ubiquitous (presumably positive selecting) self-antigens. However, the level of GFP in G113 cells was higher when the progenitor was present at low precursor frequencies. This finding implies that G113 precursors compete for rare higher affinity ligands, either because the proteins they are derived from are low abundance or because the APCs that process and present such ligands are not numerous. The Nur77<sup>GFP</sup> mouse may provide a useful tool to distinguish between these possibilities in the future. Interestingly, even at very low precursor frequencies, where all G113<sup>+</sup> thymocytes were GFP<sup>hi</sup>, not all were converted to the T<sub>reg</sub> cell lineage. This may suggest that there are other factors that also limit T<sub>reg</sub> cell development. Alternatively, it may reflect the delay between time of TCR stimulation and CD25 up-regulation and Foxp3 induction or clonal deletion of some of this population. A delay is consistent with work suggesting that Foxp3 is not required for the initial lineage decision in the thymus, but is downstream of a TCR signal and thus a delay in lineage differentiation (Gavin et al., 2007; Lin et al., 2007).

Finally, we show that iNKT cells also perceive a stronger TCR stimulus than T<sub>conv</sub> cells upon selection in the thymus. However, unlike T<sub>reg</sub> cells, iNKT cells do not continue to perceive this stimulus as they mature and emigrate to the periphery. Interestingly, the level of GFP on iNKT cells in the spleen and liver was so low that it suggests they receive very weak if any TCR stimulation in the steady-state. Given this, it is unclear why iNKT cells express intermediate levels of the T cell activation marker CD69, although it is well established that other stimuli can induce CD69 (Shiow et al., 2006). However, our findings are consistent with a published report that iNKT cells can persist long term in the absence of CD1d (McNab et al., 2005).

Many cell types in the body express CD1d (Bendelac et al., 1997). The glycosphingolipid iGb3 was identified as a potential self-lipid ligand for NKT cells, although it is not clear that it is the sole endogenous antigen that stimulates iNKT cells (Zhou et al., 2004; Gapin, 2010). There is emerging evidence that stimulatory lipids are continually catabolized



in lysosomes, and it was recently shown that when the catabolic enzyme  $\alpha$ -galactosidase is absent, CD1d<sup>+</sup> cells are able to activate iNKT cells (Bendelac et al., 1995; Zhou et al., 2004; Darmoise et al., 2010). Importantly, TLR signaling seems to inhibit  $\alpha$ -galactosidase activity, thereby allowing for iNKT cell activation in the context of infection (Darmoise et al., 2010). The Nur77<sup>GFP</sup> mice may therefore be useful in determining what types of infections and stimuli activate APCs to display self-lipids that then stimulate iNKT cells.

Historically, CD69 has been used to study T cell activation. However, CD69 expression is up-regulated by inflammatory stimuli (Shiow et al., 2006), whereas GFP in Nur77<sup>GFP</sup> mice was not. Therefore, this difference may make the Nur77<sup>GFP</sup> tool useful for determining whether certain populations of T cells, such as CD8 $\alpha\alpha$  IELs that express high levels of CD69, are being activated through their antigen receptor or whether the environmental stimuli cause the activated phenotype.

In light of the tight regulation of GFP expression by TCR ligation and the differential expression of GFP based on TCR signal strength, we propose that the Nur77<sup>GFP</sup> mouse may be a novel model for studying TCR signal strength in vivo. In addition, because inflammatory stimuli that induce CD69 expression fail to up-regulate GFP expression, we expect that these mice will be a useful tool for tracking activated T cells in several different experimental contexts such as acute and chronic infection, cancer, and transplantation.

## MATERIALS AND METHODS

**Mice.** A Nur77<sup>GFP</sup> targeting construct was created by insertion of a GFP-Cre fusion protein cDNA into the start site of the *Nr4a1* gene on a 167-kb BAC vector. An  $\sim$ 135-kb fragment from this vector was purified via BsiWI restriction sites and microinjected into C57BL/6J (B6) embryos at the Mouse Genetics Laboratory at the University of Minnesota. Alternatively, a 167-kb linearized DNA fragment was injected into B6  $\times$  SJL F1 embryos at the Transgenic and Chimeric Mouse Core Facility at the University of Pennsylvania.

B6 and B6.SJL (CD45.1 congenic B6) mice were obtained from the National Cancer Institute. MHC I-A<sup>b</sup>-deficient mice were obtained from Taconic. CD28-deficient mice were obtained from The Jackson Laboratory. G113 TCR transgenic mice were provided by C.-S. Hsieh (Washington University in St. Louis, St. Louis, MO), and Stat5b-CA mice were provided by M. Farrar (University of Minnesota, Minneapolis, MN). All animal experimentation was approved by and performed according to guidelines from the Institutional Animal Care and Use Committee at the University of Minnesota.

**Flow cytometry.** Cell surface staining was performed with antibodies from eBioscience, BD, or BioLegend. For intracellular Foxp3, cells were stained with the Foxp3 Staining Buffer set (eBioscience). For endogenous Nur77 detection, cells were fixed with fresh 4% PFA, vortexed well, and permeabilized with 0.1% Triton X-100. Antibody was used at 1:50 for staining. Biotinylated CD1d/ $\alpha$ -GalCer monomers were obtained from the tetramer facility at the National Institutes of Health. Isolation of CD1d/ $\alpha$ -GalCer binding cells via tetramer enrichment was performed as previously described (Matsuda et al., 2000). Samples were analyzed on an LSR II (BD). Data were processed with FlowJo software (Tree Star).

**Bone marrow chimeras.** Bone marrow was depleted of T cells with anti-Thy1.2 antibody and complement. Bone marrow was injected into lethally irradiated (1,000 rad) recipient mice. Chimeras were euthanized and analyzed at 8–12 wk after transplant.

**Immunofluorescence.** Tissue was harvested and immediately placed in 4% PFA in PBS overnight. Tissue was washed three times with PBS before being placed in 15% sucrose in PBS overnight. Tissue was then embedded in OCT compound and frozen with 2-methylbutane with dry ice and stored at  $-80^{\circ}\text{C}$  for long-term use. After cutting tissue sections, slides were dried for 30 min and then submerged in 0.1% Triton X-100 in PBS at room temperature for 5 min. Blocking was performed with 3% BSA in PBS before general antibody staining or endogenous GFP detection.

**Tetramer-based enrichment of thymic iNKT cells.** Enrichment of CD1d<sup>+</sup> cells was performed using an adult thymus as previously described (Matsuda et al., 2000). After tetramer enrichment, cell surface stains were performed, and a dump strategy (including B220, CD11c, Gr1, and CD25) was used to eliminate nonspecific events. The smallest gate (stage 0) included a mean of 115 events.

**In vivo and in vitro stimulation.** For stimulation with  $\alpha$ -CD3, 50  $\mu\text{g}$   $\alpha$ -CD3 was injected i.v., mice were euthanized, and tissues were harvested and analyzed 12 h later. Stimulation of NKT cells was performed by i.v. injection of 5  $\mu\text{g}$   $\alpha$ -GalCer 4–6 h before harvesting the spleen and liver. 50  $\mu\text{g}$  LPS and 100  $\mu\text{g}$  pI:pC were administered i.v., and tissues were harvested 6 h or 12 h later, respectively. For stimulation with  $\alpha$ -IgM, 10<sup>6</sup> bulk splenocytes were cultured with 10  $\mu\text{g}$  soluble  $\alpha$ -IgM for 3 h at 37 $^{\circ}\text{C}$  and then stained for FACS analysis. Plate-bound stimulation was performed by pre-coating 48-well plates with 10  $\mu\text{g}$   $\alpha$ -CD3 or 10  $\mu\text{g}$   $\alpha$ -CD2/CD3 and 50  $\mu\text{g}$   $\alpha$ -CD28 O/N at 4 $^{\circ}\text{C}$  and then culturing thymocytes and lymphocytes at 10<sup>6</sup> cells/well for 3 h at 37 $^{\circ}\text{C}$ . OT-I Tap<sup>o</sup> stimulation was performed by peptide pulsing APCs with saturating concentrations of peptides and then adding thymocytes at a 1:4 ratio. Cells were incubated for 3 h at 37 $^{\circ}\text{C}$  before FACS analysis.

***L. monocytogenes* infection.** 5  $\times$  10<sup>6</sup> OT-I (CD45.2<sup>+</sup>) lymphocytes were adoptively transferred in B6.SJL (CD45.1<sup>+</sup>) hosts. 24 h after transfer, mice were infected i.v. with 5  $\times$  10<sup>3</sup> CFU of *L. monocytogenes* or variants expressing either the OVAp or one of the OVA altered peptide ligands (Zehn et al., 2009) provided by M. Bevan (University of Washington, Seattle, WA). Mice were euthanized, and spleens were harvested 24 h after infection. Tissue was incubated with 5% collagenase D in serum-free HBSS for 30 min with mild agitation before performing cell surface staining.

**Statistical analysis.** Prism software (GraphPad Software) was used for statistical analysis. Paired and unpaired, two-tailed Student's *t* tests were used for data analysis and generation of *p*-values.

**Online supplemental material.** Fig. S1 shows that GFP and endogenous Nur77 reflect strength of antigen receptor signal but that GFP decays more slowly. Fig. S2 shows that GFP expression correlates with TCR signal strength during infection in vivo. Fig. S3 shows that GFP expression changes with developmental age. Fig. S4 shows that GFP expression is independent of CD28. Fig. S5 shows that peripheral OT-I T cells that escaped deletion in RIP-mOVA recipients expressed a high level of GFP. Fig. S6 shows the normalized MFI of GFP in various lymphocyte subsets. Online supplemental material is available at <http://www.jem.org/cgi/content/full/jem.20110308/DC1>.

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