IL-2–dependent tuning of NK cell sensitivity for target cells is controlled by regulatory T cells

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The emergence of the adaptive immune system took a toll in the form of pathologies mediated by self-reactive cells. Regulatory T cells (T reg cells) exert a critical brake on responses of T and B lymphocytes to self- and foreign antigens. Here, we asked whether T reg cells are required to restrain NK cells, the third lymphocyte lineage, whose features combine innate and adaptive immune cell properties. Although depletion of T reg cells led to systemic fatal autoimmunity, NK cell tolerance and reactivity to strong activating self- and non-self–ligands remained largely intact. In contrast, missing-self responses were increased in the absence of T reg cells as the result of heightened IL-2 availability. We found that IL-2 rapidly boosted the capacity of NK cells to productively engage target cells and enabled NK cell responses to weak stimulation. Our results suggest that IL-2–dependent adaptive-innate lymphocyte cross talk tunes NK cell reactivity and that T reg cells restrain NK cell cytotoxicity by limiting the availability of IL-2.

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Abbreviations used: β2M, β2-microglobulin; DT, diphtheria toxin; DTX, DT treatment.

The interaction between diverse cell types of the immune system facilitates or restrains specific immune functions. This provision of help or suppression optimizes immune responses triggered by the recognition of non-self– or altered-self–ligands while preventing pathologies caused by self-reactivity and excessive immune-mediated inflammation. Regulatory T cells (T reg cells) expressing the transcription factor Foxp3 exert a critical brake on responses of T and B lymphocytes. A third lymphoid cell lineage represented by NK cells is capable of detecting MHC class I molecules (self), stress-induced ligands (altered-self), or foreign (e.g., viral) proteins (non-self). Traditionally regarded as innate immune cells, NK cells share important characteristics with cells of the adaptive immune system, in particular $CD8^+$ T cells (Sun and Lanier, 2011; Vivier et al., 2011). The capacity of NK cells for antigen-specific differentiation and memory formation (O'Leary et al., 2006; Sun et al., 2009) and their ability to recognize a much broader repertoire of foreign and self-antigens than previously appreciated (Paust et al., 2010) imply that there is a need for stringent regulation of their reactivity.

The current view of NK cell tolerance is that inhibitory receptors serve as a cell-intrinsic mechanism to restrain spurious NK cell activation and that NK cell functional maturation is linked to the acquisition of inhibitory receptor expression (Joncker and Raulet, 2008; Elliott and Yokoyama, 2011). Although some of these inhibitory receptors are also expressed by T cells and might tune their function, T cell tolerance to self critically depends on the negative regulation by T reg cells (Kim et al., 2007). These considerations raised the question as to whether NK cell tolerance to self as well as responses to non-self or missing-self require T reg cell–mediated suppression to keep them in check. Alternatively, the dual expression of inhibitory and activating receptors by NK cells might circumvent the need for this cell-extrinsic control.

T reg cell control of NK cells could be mediated via suppression of dendritic cell function or the secretion of inhibitory soluble mediators. It is also possible that T reg cells exhibit distinct context-dependent means to suppress innate lymphocytes. Such a mechanism would place T reg

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cells at the intersection of innate and adaptive immunity. Finally, T reg cells could restrain the actions of effector T cells and restrict adaptive help for innate lymphocytes. Although numerous studies have suggested that NK cells can affect adaptive immune responses (Sun and Lanier, 2011), there is little evidence of adaptive immunity directly affecting NK cell responses in vivo. In this regard, IL-2, which is predominantly produced by T cells, has been used for the activation and expansion of both mouse and human NK cells (Henney et al., 1981; Caligiuri et al., 1993). Recent studies implicated T cell–derived IL-2 in mouse NK cell responses to infection (Bihl et al., 2010; Lee et al., 2012) and the activation of human NK cells in vitro (Horowitz et al., 2012). IL-2–deficient mice have impaired NK cell responses (Kündig et al., 1993), and early work also suggested that NK cells can be limited through the competition for IL-2 (Su et al., 1994). Because IL-2 is one of the major targets of regulation by T reg cells (Gasteiger and Kastenmuller, 2012; Josefowicz et al., 2012), we hypothesized that T reg cells could limit T cell–derived IL-2, which helps the activation of NK cells.

To address these issues, we explored the effect of acute T reg cell ablation on NK cell reactivity. Although depletion of T reg cells led to systemic fatal T cell–mediated autoimmunity, it did not affect NK cell tolerance to strong activating self-ligands. However, NK cell reactivity toward missing-self targets was enhanced in the absence of T reg cells and depended on the availability of IL-2 and activated T cells. In addition to its known functions, IL-2 acted to rapidly boost NK cell engagement of target cells and enabled NK cell–mediated killing in response to weak, suboptimal stimuli that in the absence of IL-2 were unable to efficiently elicit NK cell cytotoxic activity. Our experiments therefore have revealed the adaptive control of NK cell functional affinity and suggest that T reg cells provide an important second check for NK cell responsiveness by restricting this previously unappreciated form of IL-2–dependent T cell help.

RESULTS

NK cell reactivity to activating ligands is independent of T reg cells

A previous study revealed an increase in numbers of NK cells upon diphtheria toxin (DT)–mediated ablation of T reg cells in *Foxp3*^{DTR} mice, in which T reg cells express the human DT receptor (DTR) under the control of the *Foxp3* locus (Kim et al., 2007). To test whether NK cell reactivity was restrained by T reg cells, we isolated NK cells at different times after T reg cell depletion and stimulated with IL-12 and IL-18, PMA and ionomycin, or plate-bound agonistic antibody specific for the activating NK cell receptor Ly49D. We were unable to detect significant differences in the reactivity of NK cells to stimulation via Ly49D. In four out of six experiments, IFN-γ production and degranulation were comparable in DT-treated versus control mice at days 2 and 6 after DT administration (Fig. 1 A). In two experiments, we observed a moderately increased NK cell response on day 2 of DT treatment (DTX). These occasionally observed increased responses disappeared when CD4$^+$ T cells were depleted in addition to T reg cells; similar results were obtained when NK cells were stimulated with NKp46 antibody (not depicted). Responsiveness of NK cells to IL-12 and IL-18 or PMA and ionomycin measured by IFN-γ production was only slightly increased on day 2 of DTX, whereas by day 6, NK cell reactivity was comparable with controls and even reduced at later time points (day 10; Fig. 1 A). This was in contrast to the progressively increasing activation of CD4$^+$ and CD8$^+$ T cells upon T reg cell depletion (not depicted).

Next, we examined the cytotoxic activity of NK cells in vitro in a ^{51}Cr-release assay using YAC-1 target cells, which are missing-self targets whose killing is dependent on NKG2D signaling (Jamieson et al., 2002). We found that the cytotoxicity of NK cells isolated on days 2 and 6 after initial DT administration was comparable with that of NK cells from mock-treated mice (Fig. 1 B). To test whether the ability of NK cells to kill target cells in vivo was limited by T reg cells, we transferred splenocytes from mice expressing the transgene-encoded MCMV glycoprotein m157 (m157tg), an activating ligand for Ly49H, and found that NK cell–mediated target cell rejection was comparable in T reg cell–depleted and control mice (Fig. 1 C). We then analyzed the responsiveness of inhibitory receptor-negative NK cells, which have been proposed to achieve tolerance by dampening stimulatory signaling (Fernandez et al., 2005). These Ly49C/I$^-$ NKG2A$^-$ cells exhibited comparable IFN-γ production and even slightly reduced degranulation when stimulated with NKp46 and Ly49D antibodies on day 6 of T reg cell depletion (not depicted).

Together, these results suggest that under physiological conditions, the NK cell reactivity to strong activating ligands was independent of T reg cells. NK cells can have both proinflammatory and immunoregulatory functions, but the ablation of NK cells did neither alleviate nor facilitate the systemic myelo- and lymphoproliferative syndrome resulting from T reg cell elimination in *Foxp3*^{DTR} mice (Fig. 1, D–F). Although these experiments do not formally exclude potential T reg cell–dependent modulation of NK cell self-reactivity under physiological conditions, they are consistent with the observation that acute T reg cell ablation did not lead to the excessive generalized activation of NK cells (Fig. 1, A–C).

Self-reactive NK cells remain tolerant in the absence of T reg cells

Although we did not observe a marked increase in the ability of NK cells to produce IFN-γ or to kill target cells upon T reg cell ablation, the assessment of in vivo NK cell reactivity at a population level could conceal T reg cell–mediated control of a small number of self-reactive NK cells. Therefore, we tested a role of T reg cells in the maintenance of tolerance of NK cells with a known specificity for self-ligands. Analogous to the T cell anergy that results from continuous exposure to a cognate self-antigen, NK cells that encounter activating ligands during differentiation similarly exhibit peripheral tolerance (Oppenheim et al., 2005; Sun and Lanier, 2008; Tripathy et al., 2008). In the case of T cells, T reg cells are thought to be

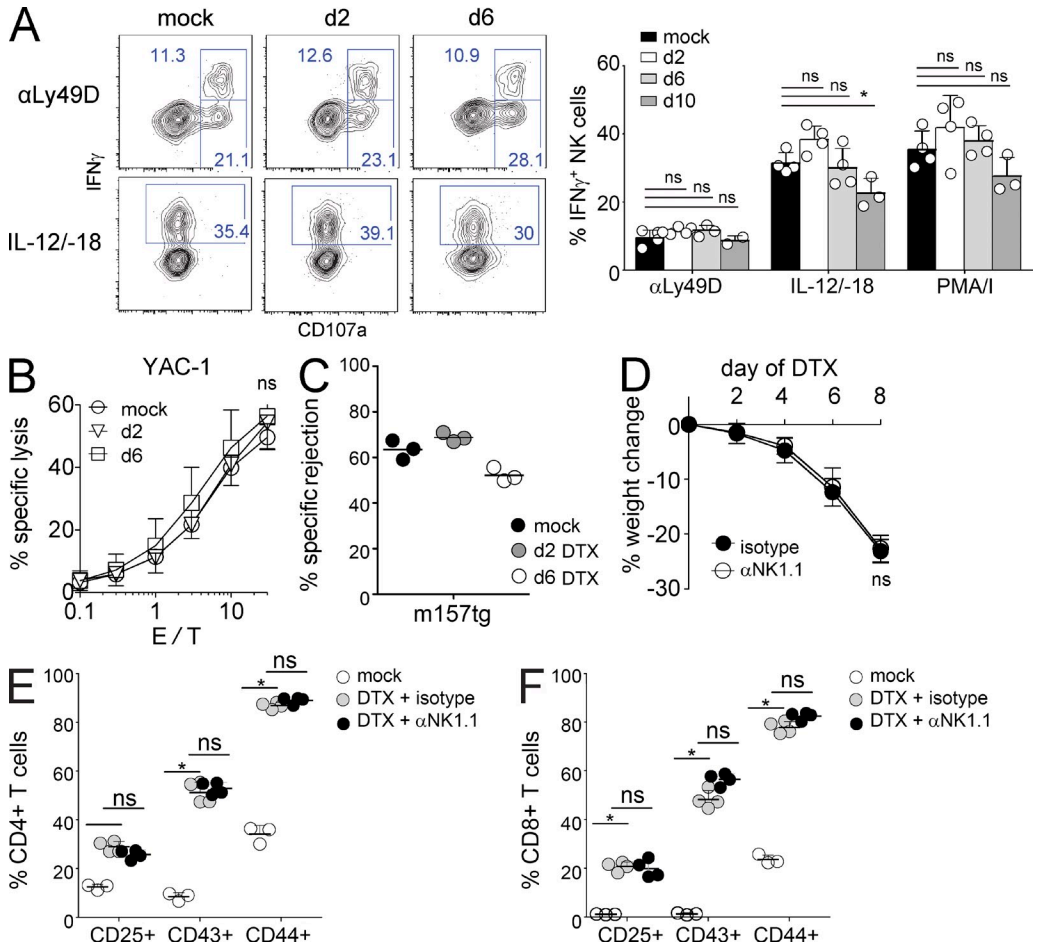

Figure 1. NK cell reactivity to activating ligands is independent of T reg cells. *Foxp3*^{DTR} mice were subjected to DTX to deplete T reg cells or mock treated, and splenic NK cells were analyzed at the indicated days after the first DTX. (A) IFN-γ production and degranulation (measured by CD107a cell surface expression) of NK cells stimulated at the indicated days of DTX for 5 h with Ly49D antibody, IL-12 and IL-18, or PMA/ionomycin (PMA/I). (B) NK cells were isolated on days 2 and 6 of DTX, and their cytotoxicity was tested using YAC-1 target cells in a 5-h ^{51}Cr release assay in vitro. (C) Specific in vivo rejection 14 h after the injection of fluorescently labeled m157tg target and CD45.1 wild-type control cells on day 2 or 6 of DTX. (D) Weight loss in DT-treated mice that were additionally depleted of NK cells or treated with an isotype control. (E and F) Analysis (day 8 of DTX) of cell surface activation markers on CD4$^+$ (E) or CD8$^+$ (F) T cells treated as in D. The data are shown as mean ± SD and are representative of two to four independent experiments (n = 3–4 per group). *, P < 0.05; ns, not significant.

essential to maintain this state of tolerance keeping peripheral autoreactive T cells at bay (Kim et al., 2007). To assess a role for T reg cells in the restraint of NK cells that exhibit self-reactive activating receptors, we generated *Foxp3*^{DTR} mice that transgenically express m157 (m157tg; Tripathy et al., 2008). NK cells continuously exposed to m157 as self-ligand in m157tg mice exhibit decreased amounts of Ly49H and a Ly49H receptor–specific unresponsiveness but can be activated through other activating receptors (Tripathy et al., 2008). As expected, NK cells in m157tg *Foxp3*^{DTR} mice similarly down-regulated Ly49H and were hyporesponsive to its stimulation. Depletion of T reg cells did not trigger expansion of Ly49H$^+$ NK cells nor did it rescue Ly49H expression or IFN-γ production in response to Ly49H engagement (Fig. 2, A and B). NK cell responses to Ly49D stimulation were comparable in *Foxp3*^{DTR} mice expressing or lacking m157tg in the presence of T reg cells and upon their depletion. NK cells isolated from T reg cell–depleted mice expressing or lacking m157tg killed YAC-1 target cells as efficiently as NK cells from mock-treated animals. However, NK cells from m157tg mice were unable to lyse m157-expressing Ba/F3 (Ba/F3-m157) cells, and this state of unresponsiveness was not reversed upon ablation of T reg cells (Fig. 2 C). NK cells from m157tg mice maintained tolerance in the absence of T reg cells not only upon short-term (2 d) but also upon long-term (6–10 d) ablation of T reg cells when systemic T cell autoimmunity developed (Fig. 2 A and not depicted). Thus, T reg cells are dispensable for the maintenance of NK cell tolerance to activating self.

T reg cells limit NK cell reactivity against missing-self targets

On the basis of the observation that NK cell reactivity and tolerance to activating ligands were regulated independently of T reg cells, we speculated that NK cell–intrinsic inhibitory

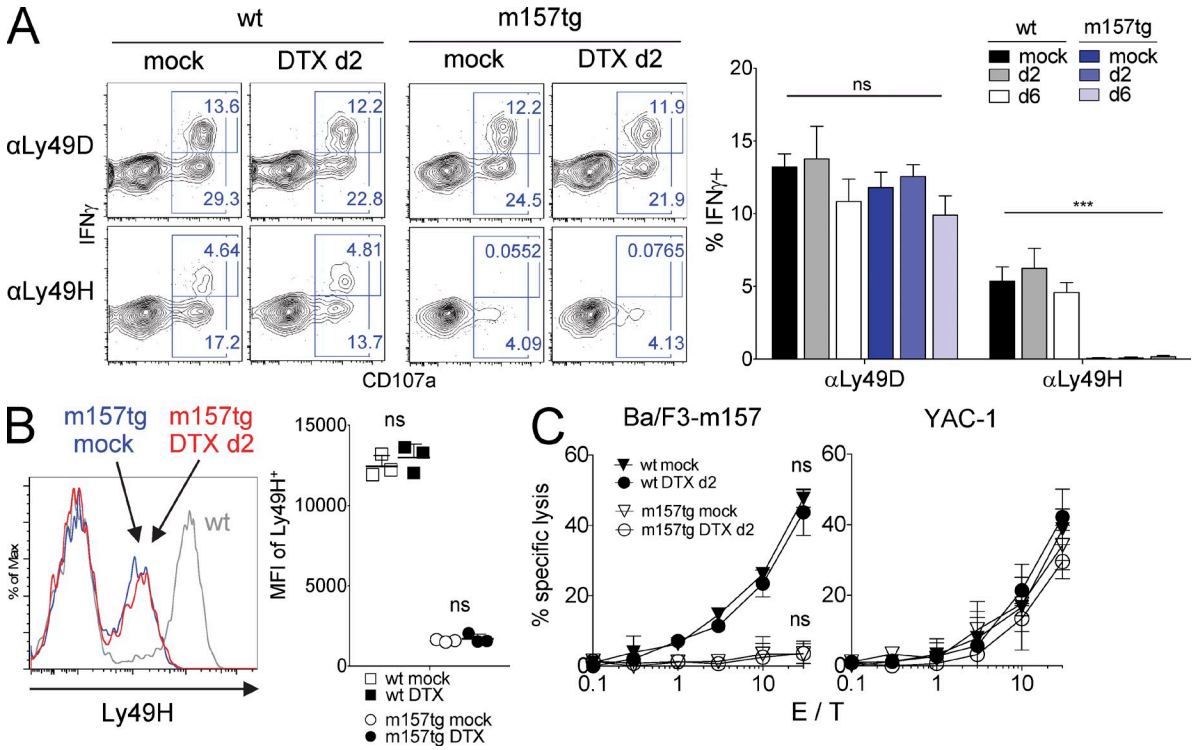

Figure 2. Self-reactive NK cells remain tolerant in the absence of T reg cells. *Foxp3*^{DTR} mice (wt) or m157tg *Foxp3*^{DTR} (m157tg) mice were analyzed 2 and 6 d after T reg cell depletion or mock treatment. (A) IFN-γ production and degranulation (cell surface expression of CD107a) of splenic NK cells stimulated for 5 h with Ly49D or Ly49H antibodies. (B) Surface expression of Ly49H on NK cells isolated from mock- or DT-treated m157tg *Foxp3*^{DTR} or *Foxp3*^{DTR} mice (MFI, mean fluorescence intensity). (C) In vitro lysis of Ba/F3-m157 or YAC-1 target cells by NK cells isolated from mock- or DT-treated m157tg *Foxp3*^{DTR} or *Foxp3*^{DTR} mice. The data are shown as mean ± SD and are representative of three independent experiments ($n = 3$ per group). ***, $P < 0.001$; ns, not significant.

mechanisms obviated the requirement for T reg cell–mediated suppression. Therefore, we sought to explore whether T reg cells influenced NK cell function in the absence of cell-intrinsic inhibitory signaling. Interactions of NK cell inhibitory receptors with self–MHC class I molecules prevent licensed functionally mature NK cells from attacking cells that provide suboptimal stimulation too weak to overcome the activation thresholds imposed by inhibitory receptors (Elliott and Yokoyama, 2011). In the absence of this continuous inhibition, lack of self-recognition enables the killing of cells that down-regulate MHC class I molecules as the result of oncogenic transformation or viral infection. To determine whether T reg cells safeguard NK cell cytotoxicity against missing-self in vivo, we cotransferred splenocytes from β2-microglobulin (β2M)–deficient and m157tg mice as targets for missing-self (loss of inhibitory signals because of MHC class I deficiency)– and non-self–recognition (engagement of an activating viral ligand in the presence of MHC class I), respectively (Fig. 3 A). To exclude a potential effect of different composition of experimental and control target cell populations (e.g., lack of CD8$^+$ T cells in β2M-deficient mice), the specific rejection was assessed for CD19$^+$ B cells. We observed a modest increase in β2M-deficient target cell rejection on day 2 after T reg cell depletion followed by a significant increase on day 6, whereas the rejection of m157tg targets was comparable in mock-treated or T reg cell–depleted mice (Fig. 3, B and C). The target cell rejection in T reg cell–depleted mice was largely mediated by NK cells because NK1.1 antibody–mediated depletion of NK cells in DT-treated control mice reduced the observed rejection of β2M-deficient and m157tg target cells to background levels (7.7 ± 5.3% and 6.4 ± 5.0%, respectively; not depicted). Increased NK cell cytotoxicity was unlikely the consequence of NK cell expansion because absolute NK cell numbers were only slightly increased, with differences reaching statistical significance when the data from several experiments were pooled (Fig. 3 D). Importantly, enlarged spleens in T reg cell–depleted mice also harbored proportionally more target cells, leading to comparable ratios of NK effector to control target cells in mock- and DT-treated mice (Fig. 3, E and G). Moreover, the enhanced rejection was specific to β2M-deficient target cells because NK cell activity against m157tg targets coinjected into the same animals was unaffected. NK cell–dependent rejection of target cells lacking classical MHC class I molecules H-2K^b and -D^b was similarly increased in the absence of T reg cells (Fig. 3 F). These data suggested that T reg cells control the activation of NK cells in the absence of inhibitory receptor engagement.

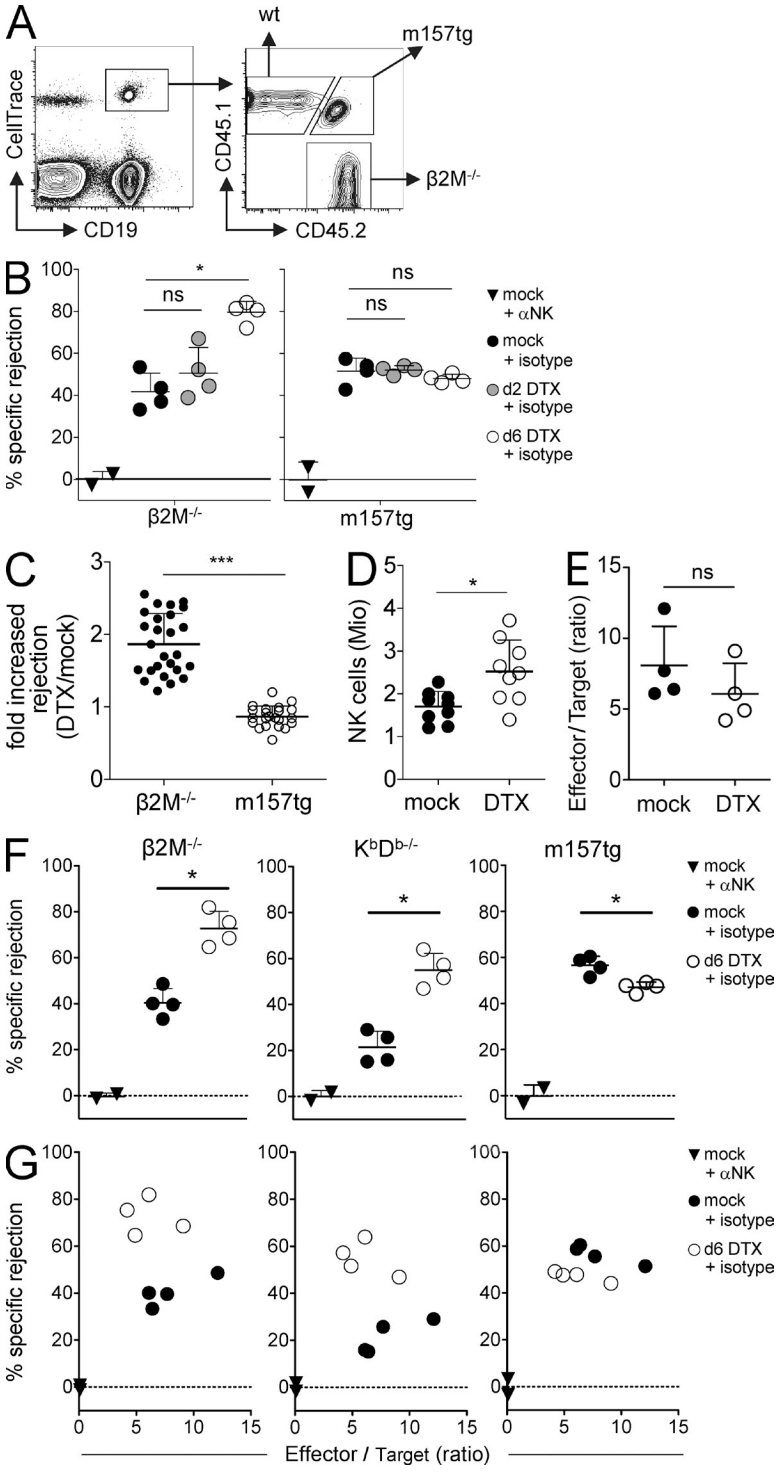

Figure 3. T reg cells limit NK cell missing-self–recognition. *Foxp3*^{DTR} mice were subjected to DTX to deplete T reg cells or mock treated. On day 2 or 6 of DTX, congenically marked, fluorescently labeled target cells were injected, and specific rejection was calculated from the ratios of targets and wild-type cells recovered after 14 h from the spleens of experimental mice as compared with mice that received NK cell depleting antibody 2 d before transfer of target cells. (A) Representative analysis of recovered target cells. (B) Specific rejection of β2M-deficient and m157tg target cells. (C) Rejection (fold increase) of target cells in individual DT-treated versus mean rejection in control mice. The results represent combined analysis of mice (n = 26) from seven independent experiments. (D) Absolute numbers of splenic NK cells in control or DT-treated mice. The data shown are pooled from two individual experiments. (E) Ratio of CellTrace$^-$ CD3$^-$ CD19$^-$ NK1.1$^+$ endogenous effector cells to transferred CellTrace$^+$ CD19$^+$ CD45.1$^+$/CD45.2$^-$ wild-type control target cells. (F and G) In vivo rejection of indicated target cell types in day 6 DTX versus mock-treated mice. Specific rejection (F) and its relationship to effector to target cell ratios in vivo (G) are shown. The data are shown as mean ± SD and are representative of three or more independent experiments. *, $P < 0.05$; ***, $P < 0.001$; ns, not significant.

T reg cells restrict IL-2–dependent T cell help for missing-self–recognition

Interestingly, the increased cytotoxicity against β2M-deficient targets was abolished upon concomitant depletion of CD4$^+$ and CD8$^+$ T cells (not depicted), suggesting that the presence of activated T cells rather than the lack of T reg cells was responsible for increased NK cell cytotoxicity in DT-treated mice. Based on these findings, it seemed likely that T cell–dependent factors helped the NK cells to gain function. Multiple studies suggested that lymphokines, particularly IL-2, can activate NK cells in vitro, or when therapeutically administered in vivo (Henney et al., 1981; Caligiuri et al., 1993). IL-2–dependent help has been proposed to play a role for IFN-γ production by NK cells during infection (Bihl et al., 2010;

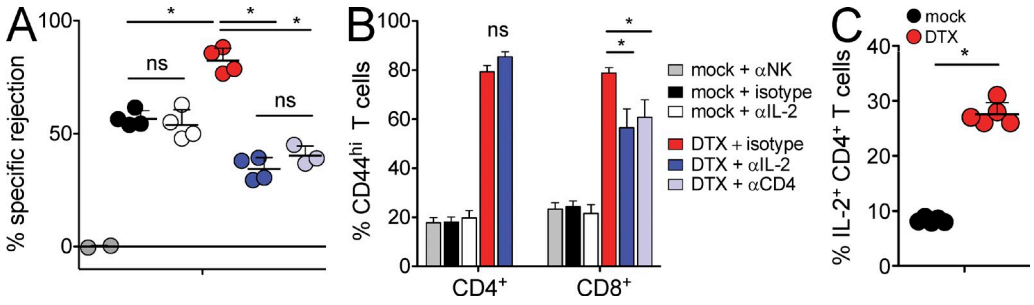

Figure 4. T reg cells restrict IL-2–dependent T cell help for missing-self–recognition. *Foxp3*^{DTR} mice were subjected to DTX to deplete T reg cells or mock treated. In addition, mice received antibody GK1.5 to deplete CD4+ T cells, IL-2 blocking antibody JES6-1A12, or isotype control IgG, as indicated. On day 6 of DTX, labeled target cells were injected and spleens were analyzed 14 h later. (A) Specific rejection of β2M-deficient target cells. (B) Frequency of activated CD44^{hi} splenic T cells in mice treated as indicated. (C) Frequency of CD4+ T cells producing IL-2 upon 4-h stimulation with PMA and ionomycin. The data are shown as mean ± SD and are representative of three independent experiments (n = 3–4 per experimental group). *, P < 0.05; ns, not significant.

Horowitz et al., 2010; Lee et al., 2012). It was not known, however, whether in the absence of infection T cells help NK cells through provision of IL-2 and whether T reg cells have the capacity to interfere with this help. To test whether increased amounts of IL-2 could account for the T cell–dependent increase of NK cell cytotoxicity in T reg cell–depleted mice, we treated these and control mice with IL-2 blocking antibody JES6-1A12 or isotype control IgG. IL-2 neutralization prevented an increase in NK cell cytotoxicity against missing-self targets in the absence of T reg cells (Fig. 4 A). Importantly, rejection of m157tg MHC class I–sufficient targets was not affected by IL-2 blockade (not depicted). Therefore, increased levels of IL-2 selectively boosted cytotoxicity against missing-self. Neutralization of IL-2 did not prevent the activation of T cells in T reg cell–depleted mice as we found similar frequencies of CD44^{hi} and IFN-γ–producing CD4+ T cells and only a modest reduction of activated CD8+ T cells in JES6-1A12– or isotype control–treated mice (Fig. 4 B). Interestingly, depletion of CD4+ T cells, which readily produce IL-2 in T reg cell–depleted mice (Fig. 4 C), fully recapitulated the effect of IL-2 blocking antibodies (Fig. 4 A). Together, these data suggested that CD4+ T cell–derived IL-2 activated NK cells in the absence of T reg cells. The neutralization of IL-2 in mock-treated mice harboring T reg cells did not alter NK cell cytotoxicity. Therefore, T reg cells appeared to limit amounts of IL-2 under steady-state conditions and, thereby, dampen NK cell activation. Thus, T reg cells restrained the IL-2–dependent CD4+ T cell help for NK cell cytotoxicity in the absence of inhibitory receptor signaling. In contrast, killing of MHC-sufficient m157tg targets, which can engage both activating and inhibitory receptors, was not affected by T reg cell depletion with or without the concomitant depletion of effector T cells or the neutralization of IL-2.

IL-2 tunes NK cell–target cell conjugate formation and cytotoxicity

These observations raised the question of how IL-2 could affect the NK-dependent rejection of missing-self targets while not affecting non-self targets. Previously, effects of IL-2 on the NK cell expansion and on granzyme B and perforin mRNA translation have been suggested to increase cytotoxicity against a broad range of targets (Fehniger et al., 2007). We hypothesized that the observed selective impact of the relief from T reg cell–mediated control on the in vivo responsiveness of NK cells against MHC class I–deficient targets was a consequence of a short-term effect of IL-2 on proximal events in target cell recognition or NK cell activation rather than a long-term generalized potentiation of NK cell cytotoxicity. Interestingly, a recent study suggested that IL-2 rescues the function of human WASP-deficient NK cells through actin reorganization (Orange et al., 2011). This observation in conjunction with our data raised the possibility that IL-2 can act early on target cell recognition by wild-type NK cells. To test this hypothesis, we performed live cell imaging of the interactions of FACS-purified NK cells with RMA-S target cells. In contrast to their unstimulated, spherically symmetrical counterparts, IL-2–pretreated NK cells appeared elongated and readily adhered to target cells (Fig. 5 A). To visualize productive encounters with their targets, NK cells were preloaded with the calcium-sensitive dye Fura-2 to monitor activation-induced Ca^{2+} flux. We found that IL-2–treated NK cells had a significantly increased contact efficiency as determined by the number of target cell contacts that cells underwent before initiating Ca^{2+} flux for the first time (Fig. 5 B). Furthermore, whereas only ∼20% of mock-stimulated NK cells eventually engaged with target cells in a manner leading to the initiation of Ca^{2+} flux during the 45-min observation period, Ca^{2+} flux was observed in ∼70% of NK cells that had been preincubated with 250 U/ml IL-2 (for 60 min; Fig. 5 C). Consistent with these results, a brief exposure to IL-2 amplified up to approximately fourfold the NK cell conjugate formation with MHC class I–deficient RMA-S target cells in a dose-dependent manner but had a much reduced effect (∼1.2 increase) on the conjugate formation with Ba/F3-m157 cells (Fig. 5, D and E). In addition, punctual IL-2 stimulation was sufficient to increase cytotoxicity of NK cells (Fig. 5 F). It is noteworthy that IL-2 differentially affected NK cell activity against the two types of targets, boosting RMA-S lysis by >5-fold, whereas lysis of Ba/F-m157 was increased only marginally (∼1.4-fold). Therefore, the ability of IL-2 to modulate NK cell cytotoxicity

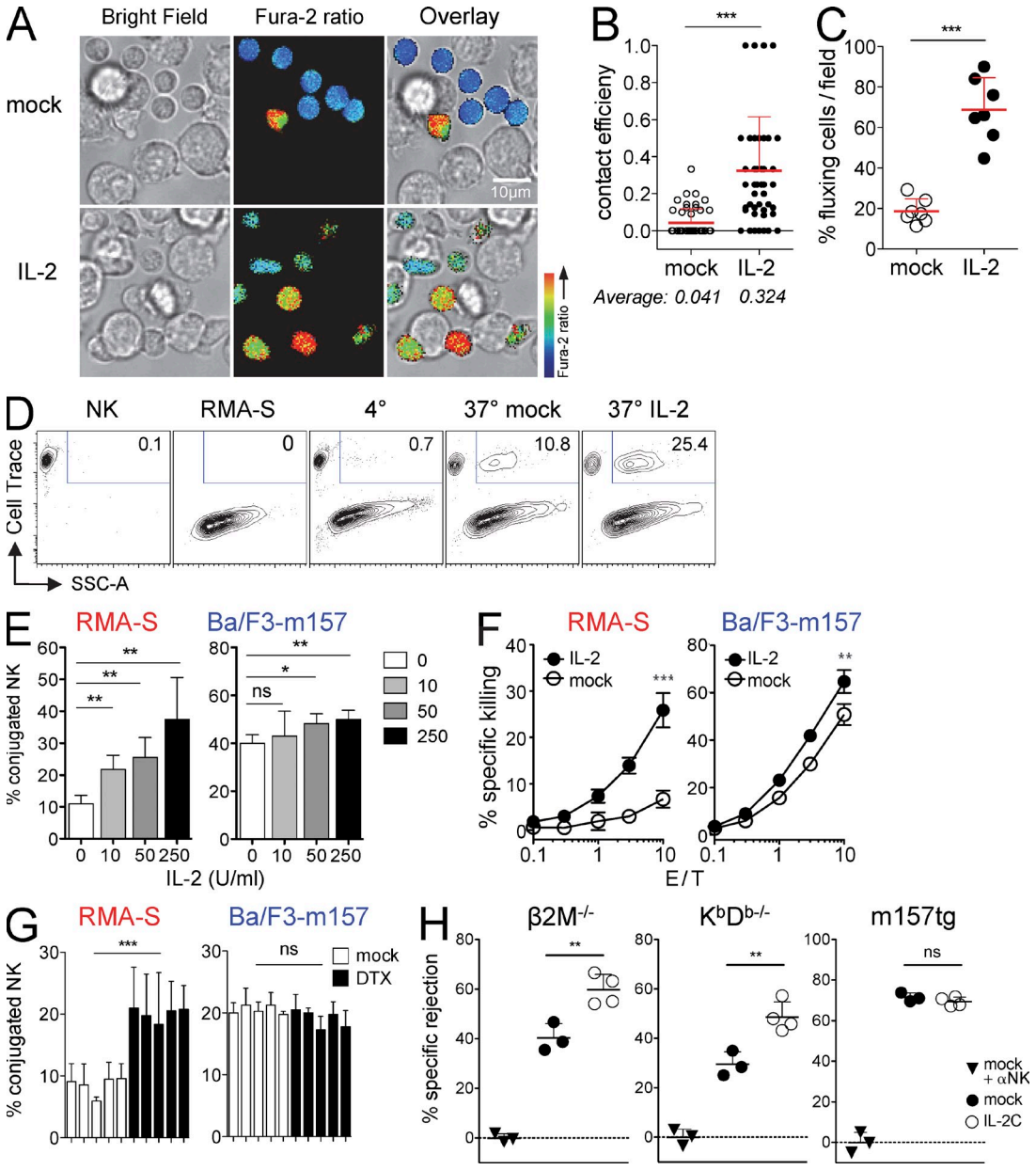

Figure 5. IL-2 tunes NK target cell conjugate formation, Ca^{2+} flux, and cytotoxicity. (A–C) FACS-purified NK cells were incubated for 40 min in IL-2 or medium and then loaded for an additional 20 min with Fura-2AM. Live-cell imaging was then performed on RMA-S–coated coverslips. (A) Representative images from the time-lapse analysis. (B) NK–target cell contact efficiency (1/n; with "n" being the number of target cell contacts until the first contact leading to Ca^{2+} flux). (C) Frequency of NK cells undergoing at least one Ca^{2+} flux per imaged field during the 45-min observation time. (D) Representative FACS plots of the conjugate formation. Numbers show the frequency of target cell conjugates as a percentage of $NKp46^+$ $CD3^-$ cells. (E) NK cells were pretreated with the indicated doses of IL-2 and incubated with RMA-S or Ba/F3-m157 target cells for 20 min. The percentage of NK cells conjugated to target cells is shown. (F) In vitro killing of the indicated targets by NK cells that were prestimulated with IL-2 for 1 h and then coincubated with Cr^{51}-labeled target cells for 5 h. (G) Conjugate formation with the indicated targets by NK cells FACS purified from T reg cell–depleted (day 6 DTX) or control mice. Each bar represents mean ± SD of five replicates per mouse. (H) In vivo rejection of targets by NK cells after brief (2 h) in vivo stimulation with CD122-targeted IL-2–antibody complexes. All data are representative of at least two independent experiments. Error bars indicate SD. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

directly correlated with its ability to increase target–cell conjugation. This observation raised the possibility that the preferential IL-2–dependent rejection of MHC class I–deficient versus m157tg target cells in mice subjected to T reg cell depletion (Fig. 3 B) could be explained by its differential impact on the ability of NK cells to engage target cells. To test this possibility, we FACS-purified NK cells from T reg cell–depleted and control mice and tested their ability to form conjugates with target cells. In agreement with the aforementioned in vivo and in vitro experiments, we found that in the absence of T reg

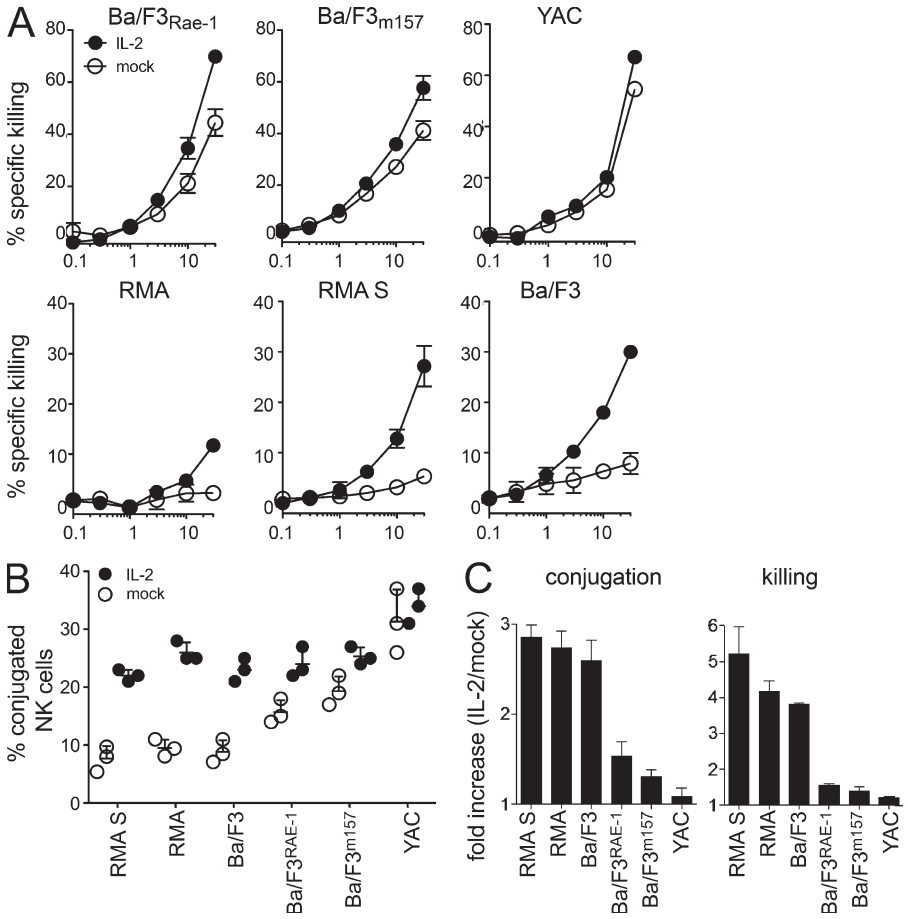

Figure 6. IL-2 rapidly increases NK cell adhesion to and killing of weak targets. (A and B) In vitro killing of (A) and conjugation with (B) the indicated target cells by freshly isolated NK cells that were cultured with 250 U IL-2/ml or medium alone for 1 h and subsequently coincubated with the target cells. (C) Aggregate plot of potentiation (fold increase) of conjugate formation and cytotoxicity (E/T ratio 30:1) in experiments shown in A and B, respectively. The data are representative of at least two independent experiments. Error bars indicate SD.

cells, NK cells had an increased ability to engage RMA-S cells, whereas NK cell conjugate formation with Ba/F3-m157 cells was unaffected (Fig. 5 G). Furthermore, targeting of IL-2 bound to S4B6 antibody to NK cells was sufficient to rapidly augment the rejection of MHC class I–deficient but not m157tg target cells (Fig. 5 H).

IL-2 rapidly increases NK cell adhesion to and killing of weak targets

The observation that IL-2 rapidly increased the adhesiveness of NK cells to RMA-S cells lacking MHC class I raised the question of whether this effect was specific for missing-self targets or whether it could also be relevant for a broad range of target cells. We found that a brief preincubation with IL-2 dramatically boosted cytotoxicity against RMA, RMA-S, and Ba/F3 cells, which in the absence of IL-2 were inefficiently killed. In contrast, IL-2 had little effect on the killing of target cells that were readily lysed by NK cells without IL-2 stimulation (Fig. 6 A). Consistently, IL-2 also boosted NK cell conjugation to these weak targets but had little impact on NK cell adhesiveness to strong targets (Fig. 6 B). Importantly, the ability of IL-2 to increase adhesiveness to weak suboptimal targets correlated with its effect on cytotoxicity (Fig. 6 C), suggesting that this adaptive cytokine is important for the regulation of NK cell functional affinity for weak target cells.

In conclusion, our results suggest that IL-2 can rapidly enhance NK cell cytotoxicity by boosting their ability to engage weak target cells. T reg cells control this $CD4^+$ T cell–dependent tuning of NK cell responsiveness by limiting the amounts of available IL-2 and therefore regulate NK cell function by interfering with this previously unappreciated adaptive-innate lymphocyte cross talk.

DISCUSSION

An essential role for T reg cells in the immune homeostasis is highlighted by the finding that their depletion leads to the activation and expansion of $CD4^+$ and $CD8^+$ T cells and B cells (Kim et al., 2007). Here, we asked whether T reg cells restrain NK cells, a third lymphocyte lineage with the capacity to recognize self- and foreign antigens. Given the parallels between T and NK cell biology, we anticipated a prominent role for T reg cell–mediated suppression of NK cells. We found that T reg cells were dispensable for NK cell tolerance to an activating self-ligand and seemed to only modestly influence the responsiveness of NK cells to a non-self–ligand. However, we observed increased cytotoxicity of NK cells against missing-self targets in the absence of T reg cells. Because any gain of NK cell function in T reg cell–depleted mice was dependent on the presence of activated $CD4^+$ T cells and IL-2, these observations suggested that T reg cells restricted IL-2–dependent T cell help

for NK cell function rather than directly suppressing NK cells. These results provide experimental support for the idea that the therapeutic manipulation of T reg cells could influence NK cells mainly by indirect means (Barao et al., 2006; Terme et al., 2008; Galani et al., 2010). Our findings are consistent with the idea that $CD4^+$ T cells capable of activating other immune cell types are the intended targets of T reg cell–mediated regulation (Kim et al., 2007).

The notion that T reg cells restrain NK cells by limiting T cell help does not exclude the possibility that a particular local inflammatory environment might enable direct T reg cell–mediated control of NK cell responsiveness. For example, T reg cells may directly suppress NK cell responses in settings where they serve as major producers or exhibitors of TGF-β, an important negative regulator of both NK and effector T cell activation (Laouar et al., 2005; Li et al., 2006). Indeed, T reg cells isolated from tumors suppressed NKG2D expression and tumor cell lysis by NK cells in a TGF-β–dependent manner (Ghiringhelli et al., 2005; Smyth et al., 2006). However, inhibition of NK cells by these tumor-derived T reg cells was neutralized in the presence of IL-2 (Ghiringhelli et al., 2005).

Our study provides experimental evidence of IL-2–mediated T cell help for NK cells. Early studies indicated that NK cell function can be boosted upon provision of IL-2 in experimental and therapeutic settings (Henney et al., 1981; Caligiuri et al., 1993). IL-2–mediated T cell help has been suggested to occur in vivo in the context of infection (Bihl et al., 2010; Lee et al., 2012), but its role under physiological conditions or in autoimmunity remained unclear. Our observation that T reg cells restrict access of NK cells to IL-2 might explain why T cell help for NK cells has been difficult to observe in the presence of competent T reg cells. Neutralization of IL-2 had no effect on NK cell cytotoxicity in control mice, suggesting that basal IL-2 amounts produced by T cells are not accessible to NK cells in the presence of T reg cells. This might be explained by the lack of CD25 expression on resting mature NK cells in mice. Therefore, adaptive help for NK cells in the presence of T reg cells might only occur in situations of markedly increased IL-2 production or CD25 expression on NK cells. Interestingly, during systemic viral infection the induction of IL-12 and IL-18 can drive high-level CD25 expression on NK cells (Lee et al., 2012), which might allow NK cells to compete with T reg cells for IL-2. Thus, in addition to direct activation of NK cells, CD25-inducing therapeutic (e.g., intratumoral) delivery of IL-12 and IL-18 might enable NK cells to receive T cell help even in the presence of T reg cells (Ni et al., 2012).

In contrast to previously described effects of IL-2 (Henney et al., 1981; Caligiuri et al., 1993; Fehniger et al., 2007; Lee et al., 2012), our experiments revealed a context-dependent, immediate role of IL-2 for NK cells in vivo. The presence of IL-2 did not result in the generalized activation of NK cells because killing of m157 targets was not affected by the presence or absence of T reg cells. However, IL-2 increased the in vivo rejection of MHC class I–deficient target cells. Thus, IL-2–induced potentiation of NK cell effector function seemed to occur in the absence of inhibitory receptor signaling. However, IL-2 did not further boost NK cell responses to a strong activating signal in the presence of inhibitory ligands. Therefore, it seems likely that the balance between activating and inhibitory signals defines the impact of IL-2–dependent help on NK cell function and its control by T reg cells. IL-2 rapidly lowered the activation threshold of NK cells by tuning their ability to adhere to and engage with their targets, suggesting potential interactions of IL-2R with integrin or activating-receptor signaling pathways. Integration of activating and inhibitory signals occurs at the interface with the target cell. A study in T cells suggested that the IL-2R might be recruited to the immunological synapse (Maldonado et al., 2004). Furthermore, a cross talk between cytokine and activating receptor signaling in NK cells has been described for IL-15 and NKG2D (French et al., 2006; Horng et al., 2007). We found no evidence for the IL-2–dependent enhancement of Ly49H- and NKG2D-dependent interactions, but these results do not exclude IL-2–mediated potentiation of NK cell cytotoxicity through the priming of other activating receptor pathways (e.g., downstream of SLAM receptors). MHC class I–reactive inhibitory receptors could potentially block such a receptor cross talk and therefore explain the IL-2–dependent increase of NK cell responses against missing-self targets. Of particular interest in this regard are SHP tyrosine phosphatases, which associate with inhibitory receptors and can be recruited to the IL-2R complex and inhibit IL-2 signaling (Migone et al., 1998; Xiao et al., 2009). Interestingly, we found that IL-2 rapidly boosted the ability of NK cells to engage with their target cells. However, the IL-2–dependent modulation of conjugate formation and cytotoxicity was also observed when using MHC class I–sufficient RMA or Ba/F3 target cells. These results suggested that IL-2 drastically amplifies NK cell responsiveness against weak targets, but it had little effect on the conjugate formation with and the killing of strong targets such as YAC, Ba/F3-Rae1, and Ba/F3-m157 expressing high amounts of activating ligands. MHC class I–deficient and m157tg cells used in our in vivo experiments likely represent weak and strong targets, respectively. MHC class I–deficient cells express endogenous activating ligands that are normally insufficient to overcome the threshold imposed by inhibitory receptors, whereas m157tg cells express high-affinity viral ligands that activate NK cells even in the presence of inhibitory receptor signaling. Thus, IL-2–mediated help might be dispensable for NK cell responses to strong activating signals but is likely a critical determinant of NK cell–mediated cytotoxicity against weak targets.

Our results suggest that an IL-2–dependent adaptive-innate lymphocyte cross talk regulates NK cell function during early stages of target cell recognition. By restricting the availability of IL-2, T reg cells provide an additional checkpoint for NK cell activation. The ability of T reg cells to deprive NK cells from access to IL-2 might be of special relevance in autoimmune lesions, where autoreactive T cells produce IL-2 and could thereby unleash NK cell activity against weak tissue targets. Consistent with this idea, T reg cell depletion

in prediabetic BDC2.5/NOD mice rapidly activated NK cells (Feuerer et al., 2009), which appears to depend on T cell–derived IL-2 (in this issue, see Sitrin et al.). The different kinetics of NK cell activation in the BDC2.5/NOD and *Foxp3*^{DTR} mice used in our study might therefore be explained by the different kinetics of T cell activation.

An important clinical implication of our findings is that IL-2 doses that override the IL-2 buffering capacity of T reg cells will likely boost NK cell cytotoxicity despite the presence of T reg cells. Indeed, treatment of type I diabetes patients with a combination of rapamycin and IL-2 increased NK cell numbers and β cell dysfunction despite markedly increased T reg cell numbers (Long et al., 2012). Our data also suggest that therapies that can increase IL-2 availability might have immediate effects on the activity of NK cells.

The indirect regulation of NK cell activity by T reg cells is likely relevant in the context of tumor growth. A recent report suggests increased control of MHC class I–deficient lymphomas by NK cells that expand in response to IL-2 from homeostatically activated T cells (Ni et al., 2012). Intriguingly, several tumors down-regulate MHC class I expression. Recently, frequent mutations in the β2M gene and the resulting loss of MHC class I expression have been reported in human B lymphoma patients (Challa-Malladi et al., 2011). One possible explanation of why these tumors are not sufficiently cleared by NK cells was provided by the observation that a large fraction of those tumors that lose MHC class I expression show concomitant mutations in the cell adhesion molecule CD58. These results together with our findings imply that NK target cell adhesion is a critical factor of tumor cell rejection likely controlled by T reg cells. Our data suggest that the provision of IL-2 either directly by NK cell–targeted IL-2–antibody complexes or indirectly might prove therapeutically beneficial in these patients. In summary, our study revealed that T reg cell control of IL-2 availability serves as an important checkpoint in the dynamic modulation of NK cell activation.

MATERIALS AND METHODS

Animals. *Foxp3*^{DTR} mice (Kim et al., 2007) used in this study have been backcrossed to C57BL/6 for >16 generations. m157tg mice (Tripathy et al., 2008) were provided by S.K. Tripathy (Washington University in St. Louis, St. Louis, MO). β2M$^{-/-}$ and K^{b}D$^{b-/-}$ mice were purchased from Taconic. All of the mice were bred and housed in the specific pathogen–free animal facility at the Memorial Sloan-Kettering Cancer Center and used in accordance with the guidelines approved by the Institutional Animal Care and Use Committee.

Cell depletions, IL-2 blockade, and treatment with IL-2–anti–IL-2 complexes. To deplete T reg cells, *Foxp3*^{DTR} mice were injected i.p. with 50 µg/kg DT (Sigma-Aldrich) every other day (q2d). NK cells and CD4^{+} and CD8^{+} T cells were depleted by i.p. injections of 300 µg PK136 or 400 µg GK1.5 or 2.43 antibody, respectively (BioXcell). To allow for complex formation, 1 µg of recombinant mouse IL-2 (PeproTech) was incubated for 10 min at room temperature with 5 µg anti–IL-2 antibody (S4B6-1; Bio-Xcell) before i.p. injection. To block IL-2 in vivo, mice received 200 µg of the indicated antibodies q2d i.p.

Analysis of phenotype, cytokine production, and degranulation. Cell suspensions were stained with fluorophore-conjugated antibodies purchased from eBioscience, BioLegend, or BD. Intracellular staining of Ki-67, Foxp3, and Eomes was performed using the Foxp3 mouse T reg cell staining kit (eBioscience). To analyze cytokine production and degranulation, 10^6 splenocytes/well were incubated on MaxiSorp 96-well plates (Thermo Fisher Scientific) coated with 10 µg/ml of agonist antibodies against Ly49D or Ly49H (provided by L. Lanier, University of California, San Francisco, San Francisco, CA) for 5 h in the presence of 1 µg/ml Brefeldin A (Sigma-Aldrich) and 2.5 µg/ml CD107a antibody. Alternatively, cells were stimulated as indicated with 20 ng/ml IL-12 and 10 ng/ml IL-18 (R&D Systems) or 50 ng/ml PMA and 500 ng/ml ionomycin. Intracellular cytokine staining was performed using the Perm/Wash kit (BD). For live/dead discrimination, propidium iodide or LIVE/DEAD fixable yellow dye (Molecular Probes) was used. Cells were analyzed on a LSRII cytometer (BD) and analyzed with FlowJo software (Tree Star).

Ca^{2+} imaging of NK cells. FACS-sorted NK cells were incubated in the presence or absence of 250 U/ml human IL2 (PeproTech) for 40 min and then loaded with 5 µg/ml Fura-2AM for 20 min and transferred into colorless RPMI (no phenol red) containing 5% FCS ± IL-2. 10^5 NK cells were mixed 1:1 with RMA-S cells and imaged in 8-well chamber slides (Thermo Fisher Scientific) using an inverted fluorescence video microscope (IX-81; Olympus) fitted with a 20×, 0.75 NA objective lens (Olympus). A Xe lamp (DG-4; Sutter Instrument) was used for fluorophore excitation, and data were collected on an electron-multiplying charge-coupled device camera (ImagEM; Hamamatsu Photonics). Time-lapse recordings were made using SlideBook software (Intelligent Imaging Innovations). In general, one brightfield image and one Fura-2 image were taken every 10 s for 45 min. Contact efficiency (Fig. 5 B) was defined as the reciprocal of the number of target cell contacts made by each NK cell before its initial Ca^{2+} flux. For NK cells that did not flux Ca^{2+} despite 12 contacts with target cells, the contact efficiency was set to 0.

In vitro conjugation assay. NK cells were stained with anti-NKp46 and FACS-purified using an Aria2 sorter (BD). NK cells were then incubated for 20–60 min in the presence or absence of 10–250 U/ml IL-2 in RPMI supplemental 10% or used directly ex vivo. 10^4 NK cells were pelleted with 3×10^4 target cells in 96-well V-bottom plates and incubated for 20 min. The plate was immediately transferred on ice, and samples were carefully resuspended in ice-cold PBS and analyzed on an LSRII flow cytometer.

In vitro killing assay. Splenic NK cells were negatively enriched and incubated in triplicates with ^{51}Cr-labeled target cells. Supernatants were assayed for ^{51}Cr release after 5 h of incubation at 37°C, and percentage of specific lysis was calculated.

In vivo killing assays. Splenocytes from CD45.1 BL6, CD45.1/CD45.2 m157tg, or CD45.2 β2M^{KO} mice were labeled with CellTraceViolet (Invitrogen), and $3–5 \times 10^6$ cells/each were transferred by i.v. injection. 12–14 h after transfer, splenocytes were isolated and analyzed by FACS. Specific lysis of CD19^{+} cells was calculated based on the relative numbers recovered from each donor population compared with the mean ratios of cells recovered from two to three NK-depleted mice and in the input mix.

Statistical analysis. All statistical analyses were performed using Prism5 software (GraphPad Software). Results are expressed as means ± SD. Differences between individual groups were analyzed for statistical significance using the nonparametrical Mann-Whitney test. Only when comparing groups of $n = 3$ (triplicate cultures in in vitro killing and conjugate formation assays in Figs. 1 B, 2 C, and 5, F and G) was the unpaired Student's t test used: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant.

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