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Denileukin diftitox (DD), a fusion protein comprising IL-2 and diphtheria toxin, was initially expected to enhance antitumor immunity by selectively eliminating regulatory T cells (Tregs) displaying the high-affinity IL-2R (α-β-γ trimers). Although DD was shown to deplete some Tregs in primates, its effects on NK cells (CD16+CD56−CD20−CD4+CD45RA+Foxp3+) and to enhance antitumor immunity, are still unknown. To address this question, cynomolgus monkeys were injected i.v. with two doses of DD (8 or 18 µg/kg). This treatment resulted in a rapid, short-term, reduction in detectable peripheral blood resting Tregs (CD4+CD45RA+Foxp3+) and a transient increase in the number of activated Tregs (CD4+CD45RA−Foxp3high), followed by their partial depletion (50–60%). In contrast, all NK cells were deleted immediately and durably after DD administration. This difference was not due to a higher binding or internalization of DD by NK cells compared with Tregs. Coadministration of DD with IL-15, which binds to IL-2R-βγ, abrogated DD-induced NK cell deletion in vitro and in vivo, whereas it did not affect Treg elimination. Taken together, these results show that DD exerts a potent cytotoxic effect on NK cells, a phenomenon that might impair its antitumoral properties. However, coadministration of IL-15 with DD could alleviate this problem by selectively protecting potentially oncolytic NK cells, while allowing the depletion of immunosuppressive Tregs in cancer patients. The Journal of Immunology, 2012, 188: 000–000.

Materials and Methods

Animals and treatments

Five- to seven-kilogram male cynomolgus macaques (Charles River Primates, Wilmington, MA) were used. The studies were performed under protocols approved by the National Institutes of Health guidelines for the care and use of primates and the Massachusetts General Hospital Subcommittee on Animal Research. DD (Ontak; Eisai, Woodcliff Lake, NJ) was given i.v. at high doses: 18 µg/kg given twice on two consecutive days or 8 µg/kg given four times weekly. In DD–IL-15 coadministration experiments, two doses (10 or 50 µg/kg) of IL-15 (Insight Genomics, Falls Church, VA) were given to each animal i.v. with each DD infusion.

Flow cytometry analyses

PBMCs were analyzed via cell surface staining using mAbs directed against the following Ags: CD3 (SP34), CD4 (L200), CD8 (RPA-T8), CD16 (3G8), CD20 (2H7), CD45RA (5H9), CD56 (B159), CD62L (SK11), CD95 (DX-2), NKG2A (Z199); isotype-matched control mAbs were also used. To
assess intracellular protein expression of Foxp3 (PCH101), Ki67 (B56), and CD125 (BN3), cells were permeabilized using Fixation/Permeabilization solution (eBioscience, San Diego, CA), following the manufacturer’s instructions. Cells were analyzed on a FACSCalibur or Accuri Flow Cytometer using FlowJo software.

Cytotoxic and competition assays

PBMCs from nonhuman primates were cultured for 12–72 h in complete media (RPMI 1640, 10% FCS, 12 ml HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, and 2 mM glutamine) with DD (0–5 nM), followed by staining for specific populations.

NK cells were sorted into CD16+CD8−NK2A+CD3− (20), and CD4+ Tregs were sorted into resting Tregs (R-Tregs; CD45RA−CD25++) and activated Tregs (A-Tregs; CD45RA+CD25++) using a FACSaria cell sorter. Each population was cultured in complete media, with or without DD, in the presence of different concentrations of IL-2 (Invitrogen, Carlsbad, CA) or IL-15 (R&D Systems, Minneapolis, MN). Cytotoxicity was determined by 7-aminoactinomycin D (7-AAD; BD, San Diego, CA). The viability was analyzed as a percentage relative to the level of pre-incubation.

Binding and internalization of DD

DD was labeled using the Alexa Fluor 647 Protein Labeling Kit (Invitrogen), following the manufacturer’s instructions. Cells were analyzed on a FACSCalibur or Accuri Flow Cytometer using FlowJo software. DD-internalization analyses were performed by imaging flow cytometry using the ImageStreamX Imaging Flow Cytometer (Amnis, Seattle, WA). For these analyses, nuclei were counterstained with DAPI, and the data were analyzed with IDEAS software version 4.0 (Amnis). Only live cells were gated. For quantification of internalization, 2 × 10^6 PBMCs were cultured with Alexa Fluor 647-labeled DD (5 nM), and cells were analyzed after 3 or 6 h. We used multiple surface markers to identify Tregs (CD45RA−FITC, CD25-PE, CD4−allophycocyanin–Cy7) and NK cells (CD16- FITC, CD8-PE) and identified the internalization of Alexa Fluor 647-labeled DD using a quantitative morphology-based feature (IDEAS software version 4.0).

Statistical analyses

Statistical significance was examined with a two-tailed Student t test, with p values < 0.05 considered significant. Statistical analysis was performed using SPSS software (SPSS, Chicago, IL).

Results

In vitro effects of DD administration on peripheral blood leukocyte subsets

Following i.v. administration of high-dose (18 µg/kg × 2) DD to cynomolgus monkeys, the total number of PBLs decreased rapidly from 3500/mm³ to 800/mm³ (day 2). This effect was transient, because the lymphocyte counts started to increase by day 5 and returned to pretreatment levels by day 10 post-DD administration (Fig. 1A). Because this population contains only 2–4% Tregs, we can conclude that this effect largely reflected the elimination of Teffs. Next, we tested a lower multiple-dose administration protocol (8 µg/kg × 4, weekly). Again, a transient reduction in the total lymphocyte count was observed, but to a much lesser degree (Fig. 1B). This prompted us to use this regimen in this study aimed at more selective Treg depletion, as previously used in clinical trials (1).

In fact, early selective depletion of Tregs was not observed, with the actual frequency initially increasing from days 2 to 10 after DD injection (Fig. 1C). Subsequently, similarly to observations reported in humans (1), both the absolute count and the percentage of Foxp3+CD4+ T cells among total CD4+ T cells gradually decreased and remained low (<50%) for several weeks (Fig. 1C). To more precisely evaluate the in vivo effects of DD on specific cell subpopulations, we applied the phenotypic definition of human Treg subpopulations proposed by Miyara et al. (21) to cynomolgus monkeys. Our preliminary studies showed that monkey Tregs can similarly be separated into A-Tregs (CD4+CD45RA−Foxp3++) and R-Tregs (CD4+CD45RA+Foxp3+) (Fig. 1D). The suppressive function of each Treg subpopulation was also similar to human Tregs: A-Tregs possess significantly greater suppressive function than do R-Tregs (data not shown). The current study revealed that the initially increased numbers of peripheral blood Tregs corresponded to A-Tregs (Fig. 1E), exhibiting a phenotype identical to that expressed by human A-Tregs: CD62L−, CD31−, CD152 (CTLA-4)+, Ki-67−, and CD95+ (Supplemental Fig. 1). In contrast, DD treatment resulted in a marked and immediate decrease in the frequency of R-Tregs (60–80%), which remained low for 2 mo (Fig. 1F).

The most striking effect observed immediately after initial DD injection was the complete depletion of NK cells (CD16+CD8− NK2A+CD3−). The frequency of NK cells remained low for 20 d, and it gradually returned to pretreatment levels by days 30–40 (Fig. 1G). NKT cells (CD16+CD8−NK2A+CD3+) were also depleted (data not shown). Altogether, these results show that DD administration to monkeys resulted in a significant, but very transient (2–d), depletion of Teffs following each i.v. infusion. At the same time, DD triggered a profound and sustained depletion of R-Tregs and NK cells and a short-term expansion of A-Tregs. Therefore, unlike initially anticipated, DD exerts multiple and variable effects on various peripheral blood leukocytes in primates.

In vivo effects of DD treatment on NK cells and Tregs

Next, we investigated the in vitro effects of DD on different monkey leukocyte subsets. A-Tregs (CD4+CD45RA−CD25++), R-Tregs (CD4+CD45RA+CD25−), and Teffs (CD4+CD45RA+CD25−) were sorted by FACS and cultured in the presence or absence of DD (5 nM) for 72 h. The frequencies of viable cells were determined via 7-AAD staining at different time points from 12 to 72 h. As shown in Fig. 2A, the percentages of viable Teffs remained constant throughout the entire period. We could not evaluate the in vivo effect of DD on the survival of A-Tregs, because these cells die rapidly, even in the absence of DD. In the absence of DD, the frequency of live R-Tregs remained constant for 72 h, whereas DD treatment resulted in a 70% lethality among R-Tregs (Fig. 2A). In addition, exposure of R-Tregs to DD upregulated their expression of Foxp3 and CD95 for the first 12 h, indicating that these cells had been activated (data not shown). Altogether, these results show that in vitro DD treatment did not affect the survival of Teffs, whereas it activated all Tregs and caused the death of R-Tregs.

The percentages of live NK cells among PBMCs treated with DD (0.5–5 nM) decreased over time, in a dose-dependent manner, while no effect was observed in controls incubated with medium alone (p < 0.001) (Fig. 2B). To determine whether DD directly affected NK cells, NK cells were sorted by FACS and incubated with 5 nM DD for up to 72 h. NK cell viability decreased significantly as early as 12 h after exposure to DD (Fig. 2C). Addition of IL-2 to the culture inhibited the effect of DD on NK cell death in a dose-dependent manner at 24 h (Fig. 2D), thereby confirming that DD mediated its effect via IL-2R. A similar cytotoxic pattern was also observed in human NK cells (data not shown).

These in vitro results recapitulate the effects observed following in vivo DD administration, including some, but minimal, depletion of Teffs, a rapid and massive cell death among NK cells and R-Tregs, and upregulation of the expression of various activation markers on Tregs. Finally, it is noteworthy that the rapid and spontaneous death of A-Tregs observed in vitro, but not in vivo, suggests that homeostatic survival of this Treg subset relies on some factors absent in our cell cultures.

Binding and internalization of DD by NK cells and Tregs

In another set of experiments, we studied whether the differential effects of DD on NK and Tregs is associated with differences in its...
binding and/or uptake by these cells. To address this question, NK cells, Teffs, and each of the Treg subsets were isolated and incubated with DD (5 nM) labeled with the fluorescent dye, Alexa Fluor 647, for different periods of time ranging from 0 to 24 h. The percentages of viable cells bound to DD were evaluated by FACS. As shown in Fig. 3A, 20% of Teffs and NK cells were

\[ \text{(FIGURE 1)} \]

In vivo effects of DD on various PBLs in monkeys. Reduction in total peripheral lymphocyte counts with high-dose DD (18 μg/kg × 2) (A) and with multiple low doses (8 μg/kg × 4, weekly) (B). (C) Absolute count of CD4⁺CD25⁺Foxp3⁺ Tregs slowly decreased to <50% of baseline levels over 2 mo following DD treatment. (D) Tregs were divided into CD4⁺RA⁺Foxp3⁺ (R-Tregs) and CD4⁺RA⁻Foxp3⁺⁺ (A-Tregs) populations. A marked transient expansion of A-Tregs was seen in all three recipients (E), whereas R-Tregs consistently decreased (F). (G) Sequential low-dose DD treatments induced immediate and long-term depletion of NK cells from peripheral circulation. Arrows indicate the administration of DD.
bound to DD after 2 h; in contrast, 40 and 65% of R-Tregs and A-Tregs, respectively, were labeled with DD. Subsequently, the number of Teffs bound to DD remained unchanged, whereas the numbers of NK cells and R-Tregs bound to DD increased over time and reached 53 and 60%, respectively, at 6 h, 74.8 and 76.2% at 12 h, and 90% at 24 h. Finally, the number of DD-labeled A-Tregs reached 90% at 12 h and remained constant until the end of the assay. Likewise, the mean fluorescent intensity, representing the level of DD binding/individual cell, increased faster and was consistently higher in Tregs than in NK cells. Representative histograms are shown in Supplemental Fig. 2A and 2B. These results show that both Treg subsets bind DD in an accelerated fashion compared with NK cells. Therefore, the greater cytotoxic effect of DD on NK cells compared with Tregs cannot be attributed to increased binding to IL-2R by the NK cell population.

Next, we compared NK cells and Tregs for their ability to internalize DD. Monkey PBMCs (2 × 10^6) were cultured with 5 nM of DD for up to 72 h. The viability of NK cells after exposure to DD decreased significantly compared with medium alone. ***p < 0.001. IL-2 competitively inhibited the effects of DD on NK cells in a dose-dependent fashion at 24 h. Each experiment was performed at least three times using two animals.

**FIGURE 2.** In vitro effects of DD treatment on NK cells and Tregs. (A) R-Tregs and Teffs were sorted by FACS and incubated with 5 nM of DD for up to 72 h. Although Teffs were resistant, even after 72 h of exposure to DD, the viability of R-Tregs decreased to 27% after 72 h. **p < 0.005, compared with medium alone. Cell death was measured by 7-AAD. (B) Monkey PBMCs were incubated with various concentrations of DD, and the percentage of NK cells among PBMCs decreased over time in a dose-dependent manner. **p < 0.005, ***p < 0.001. (C) NK cells were sorted by FACS and incubated with 5 nM DD for up to 72 h. The viability of NK cells after exposure to DD decreased significantly compared with medium alone. ***p < 0.001. (D) IL-2 competitively inhibited the effects of DD on NK cells in a dose-dependent fashion at 24 h. Each experiment was performed at least three times using two animals.

**FIGURE 3.** Binding and internalization of DD by NK cells and Tregs. (A) Isolated A-Tregs, R-Tregs, and NK cells were incubated with Alexa Fluor 647-labeled DD and analyzed by FACS for DD binding. During the initial 6 h, DD bound to both A-Tregs and R-Tregs faster than to NK cells. (B) For quantification of internalization, 2 × 10^6 PBMCs were incubated with Alexa Fluor 647-labeled DD and analyzed after 3 or 6 h.
of DD for 3 or 6 h. The presence of intracellular Alexa Fluor 647-labeled DD in Tregs and NK cells was determined by imaging with an ImageStreamX Imaging Flow Cytometer using a quantitative morphology-based feature (representative figures are shown in Supplemental Fig. 3A–C). The percentages of R-Tregs/A-Tregs bound to DD were 62.9/97.4% at 3 h and 72.5/99.1% at 6 h. Among these cells, DD-internalized R-Tregs/A-Tregs accounted for 89/83% and 94/85% of the cells at 3 and 6 h, respectively (Fig. 3B). Therefore, although NK cells were more susceptible than Tregs to DD-mediated cytotoxicity, they exhibited a lower ability to bind and internalize DD.

Influence of IL-15 on DD-mediated depletion of NK cells and Tregs

IL-15 is essential to the differentiation and homeostasis of NK cells and has been used to expand NK cells and enhance their functions (22–24). At the same time, IL-15 is known to compete with IL-2 through IL-2/15Rβ-subunits with IL-2R. Alternatively, IL-15 does not bind to IL-2Rα (25–27). Based upon these considerations, we hypothesized that IL-15 could competitively suppress the lethal effect of DD on NK cells but not on Tregs.

First, we compared the ability of IL-15 to compete with DD for binding to NK cells and Tregs. Purified NK cells or R-Tregs were cultured with Alexa Fluor 647-labeled DD for 24 h in the presence of either IL-2 or IL-15. As expected, DD binding to both NK cells and Tregs was inhibited in the presence of its “natural competitor,” IL-2. Alternatively, IL-15 efficiently blocked the binding of DD to NK cells but not Tregs (Fig. 4A, 4B). It is noteworthy that IL-15 was more efficient in blocking DD binding than was IL-2 itself, a result suggesting that IL-15 displays a higher affinity for IL-2Rβγ-heterodimer than its natural ligand, IL-2. These results prompted us to investigate whether IL-15 could differentially alter the cytotoxic effects of DD on NK cells and Tregs. Purified monkey NK cells or Tregs were exposed to DD for 24 h in the presence of either IL-15 or IL-2. As shown in Fig. 4C and 4D, IL-15 effectively prevented DD-mediated NK cell death in a dose-dependent fashion. In fact, IL-15 inhibited DD-induced lethality more efficiently than did IL-2 itself (p < 0.005) (Fig. 4C). In striking contrast, IL-15 had no effect on DD-mediated death of Tregs (Fig. 4D). Finally, we evaluated whether IL-15 could selectively block DD-mediated killing of NK cells, but not Tregs, in a mixed cell population. PBMCs (2 × 10^5 cells) were cultured with labeled DD for 24 h in the presence of different doses of IL-15 or IL-2 cytokines. NK cells, R-Tregs, and A-Tregs were evaluated for their viability and binding to DD. Similarly to that observed with isolated cell subsets, IL-15 bound preferentially to

![Image](http://www.jimmunol.org/)

**FIGURE 4.** Influence of IL-15 on DD-mediated depletion of NK cells and Tregs. (A and B) Isolated monkey NK cells and R-Tregs were exposed to 5 nM DD labeled with Alexa Fluor 647 in the presence of various concentrations of either IL-15 or IL-2. IL-15 was more effective than was IL-2 in preventing DD from binding to NK (A), whereas IL-2 more efficiently blocked binding of DD to R-Tregs (B). As a comparison, the binding of DD to Teffs in both conditions are also shown. These analyses were performed at 24 h. (C) IL-15 was more effective than was IL-2 in preventing isolated NK cell death induced by DD at 24 h. Prevention of cell death occurred in a dose-dependent fashion. (D) Although the addition of 5 nM IL-2 prevented the cell death of purified R-Tregs exposed to DD, 5 nM IL-15 did not prevent the cell death of R-Tregs. Each experiment was performed at least three times using two animals. *p < 0.05, **p < 0.005, ***p < 0.001.
NK cells and averted their death, whereas it had no effect on Tregs (Supplemental Fig. 4A–D).

**IL-15 prevents in vivo DD-mediated depletion of NK cells but not Tregs**

To investigate the in vivo effects of IL-15, two monkeys were injected i.v. with DD (8 µg/kg) along with IL-15 (10 or 50 µg/kg). Two control animals were treated with IL-15 alone. As previously reported, IL-15 induced a massive leukocyte extravasation, resulting in peripheral blood lymphopenia during the first few days postinjection (28). Remarkably, IL-15 treatment resulted in a rapid and complete recovery of NK cells after DD treatment (Fig. 5A). In contrast, IL-15 did not affect in vivo depletion of Tregs induced by DD (Fig. 5B). These observations suggest that IL-15 might be a useful therapeutic agent to selectively protect NK cells from elimination in subjects being treated with DD.

**Discussion**

Individual IL-2R subunits are expressed on various lymphoid cell populations, whereas coexpression of CD25 (IL-2Rα), CD122 (IL-2Rβ), and CD132 (IL-2Rγ) subunits that form the high-affinity IL-2R, is essentially confined to CD4+Foxp3+ Tregs and a few activated “conventional” Teffs. Based upon this principle, the IL-2/DT fusion protein was expected to preferentially bind to and delete Tregs while sparing other leukocytes. Actually, our study shows that DD has multiple and distinctive effects on various leukocyte subsets in monkeys. This study confirms that DD treatment does mediate a partial (50–60%) and prolonged depletion of primate Tregs in vivo. However, our observations also show that this phenomenon is more complex than initially anticipated: DD elicited an early expansion of Tregs displaying an activated phenotype (A-Tregs), and DD-mediated Treg depletion is restricted to R-Tregs. The observation that many DD-exposed Tregs initially become activated before dying after 12–36 h is reminiscent of another study in which T cells cultured with IL-2–DT first displayed elevated levels of cytoplasmic mRNA coding for IL-2R, c-myc, and IFN-γ, followed by a reduction in these mRNA levels by 20 h (29). Following activation/expansion, it is likely that these A-Tregs ultimately succumb to the effects of the DT or via apoptosis caused by the absence of continuous exposure to exogenous IL-2 (30). Conversely, although our in vitro assays clearly show that some R-Tregs are killed via DD exposure, R-Treg reduction due to conversion to A-Tregs cannot be ruled out in this study.

In addition to its effects on Tregs, DD treatment triggered some, although partial and short-lasting, depletion of effecttor T lymphocytes. Most importantly, exposure of monkey PBMCs both in vitro and in vivo caused a profound and durable elimination of NK cells. The phenotypic definition of macaque NK cells has been established (20, 31, 32), and we defined cynomolgus monkey NK cells as CD16+CD8+NKG2A+CD3 in this study. Although a recent study identified the presence of a minor CD8α− NK cell subpopulation among CD8−CD16−CD3−CD20−CD14− cells in rhesus monkeys (33), they are presumed to account for only <5–10% of total NK cells. Because the CD16+CD3−CD20−CD14− CD8− population also contains a substantial number of myeloid dendritic cells, we did not include CD8− NK cells in our analyses. Nevertheless, this observation might seem surprising, because only 10% of NK cells express the IL-2Rα subunit, whereas the vast majority of these cells displays IL-2Rβ-γ heterodimers (34). However, it is noteworthy that studies by Re et al. (18) showed that cells expressing the intermediate-affinity IL-2Rβ-γ could bind...
DD quite efficiently. Despite this, no NK cell depletion was observed in studies using the previous-generation IL-2/DT, DAB486–IL-2, at doses ranging from 1 to 10 nM (35). Subsequent re-engineering of this agent led to the development of DAB389–IL-2 (DD), which is used in the clinical market currently), which has a higher $K_d$ and longer half-life than does the original compound (36). Nevertheless, to our knowledge, no effect of DD on human or nonhuman primate NK cells has been reported (3, 4, 9). Our study shows unambiguously that DD, delivered at doses ranging from 0.5 to 5 nM, completely and durably eliminates NK cells in monkeys, an observation that has important implications for the design of antitumor therapies using this and future IL-2R–based immunotoxins. It remains unclear why DD treatment results in a complete depletion of NK cells, whereas a significant number of Tregs is apparently not eliminated. Our results showed that this phenomenon is not the result of differences in DD binding or internalization among these cell subsets. Therefore, it is likely that DD mediates different intracellular signals to NK and Tregs or that these two cell types display different sensitivity toward DT. Additionally, the rapid homeostatic expansion of DD known to occur after leukopenia may account for the recovery of some Tregs. It is also conceivable that DD converts Tregs to other subpopulations that are more resistant to the cytotoxic effects of DD. Further studies are required to test these hypotheses.

Of potential importance to future DD clinical trials, we observed that IL-15 bound preferentially to IL-2R on NK cells and protected them from elimination by DD, whereas it did not impact the depletion of Tregs in monkeys. This protective effect by IL-15 is presumably the result of competition between DD and IL-15. However, it is also possible that activation of NK cells via IL-2R, at doses ranging from 1 to 10 nM (35). Subsequent re-enforcement and Tregs depletion, whereas it spared and presumably potentiated NK cells. Together, with our results, this shows that in vivo IL-15 administration selectively activates NK cells through their IL-2R, without affecting Tregs.

In summary, our study shows that, although DD administration depletes a significant proportion of Tregs, it rapidly and durably eliminates all NK cells in nonhuman primates. Such depletion of potentially tumoricidal NK cells may explain why DD treatment refractory peripheral T-cell lymphoma with denileukin difftitox (ONTAK).

**References**


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**Disclosures**

The authors have no financial conflicts of interest.

**References**


