Regulatory CD56\textsuperscript{bright} natural killer cells mediate immunomodulatory effects of IL-2R\textalpha-targeted therapy (daclizumab) in multiple sclerosis

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Administration of daclizumab, a humanized mAb directed against the IL-2R\textalpha chain, strongly reduces brain inflammation in multiple sclerosis patients. Here we show that daclizumab treatment leads to only a mild functional blockade of CD4\textsuperscript{+} T cells, the major candidate in multiple sclerosis pathogenesis. Instead, daclizumab therapy was associated with a gradual decline in circulating CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells and significant expansion of CD56\textsuperscript{bright} natural killer (NK) cells \textit{in vivo}, and this effect correlated highly with the treatment response. \textit{In vitro} studies showed that NK cells inhibited T cell survival in activated peripheral blood mononuclear cell cultures by a contact-dependent mechanism. Positive correlations between expansion of CD56\textsuperscript{bright} NK cells and contraction of CD4\textsuperscript{+} and CD8\textsuperscript{+} T cell numbers in individual patients \textit{in vivo} provides supporting evidence for NK cell-mediated negative immunoregulation of activated T cells during daclizumab therapy. Our data support the existence of an immunoregulatory pathway wherein activated CD56\textsuperscript{bright} NK cells inhibit T cell survival. This immunoregulation has potential importance for the treatment of autoimmune diseases and transplant rejection and toward modification of tumor immunity.

CD25 | IL-2 | immunoregulatory natural killer cells

M ultiple sclerosis (MS) is an inflammatory/demyelinating disease of the CNS that is one of the leading causes of neurological disability in young adults (1). It is believed that MS is a T cell-mediated autoimmune disease, and therefore the search for new therapies focuses on agents that affect lymphocyte function. Daclizumab (Zenapax), a humanized mAb that blocks the IL-2 binding site on the IL-2R\textalpha chain, CD25 (i.e., Tac epitope), is among these novel agents (2). The IL-2R complex is comprised of three subunits: IL-2R\beta (CD122), and IL-2R\gamma (CD132). CD122 and CD132 have intracellular signaling motifs and together form the intermediate-affinity (K_{\text{diss}} = 0.1–1 nM) IL-2R. CD25 binds IL-2 with low (K_{\text{diss}} = 10 nM) affinity, but when it associates with CD122/CD132 it stabilizes the complex to form the high-affinity (K_{\text{diss}} = 10 pm) receptor (3). CD25 is present at low levels in resting human T cells (with the exception of T regulatory cells) but is significantly up-regulated on activated T cells, enabling them to receive a high-affinity IL-2 signal (4). Therefore, it is believed that the blockade of CD25 will result in selective functional inhibition of activated T cells (5). Although it has been demonstrated that daclizumab (or the original murine anti-Tac mAb) inhibits early IL-2R signal transduction events (6, 7) and blocks T cell activation and expansion \textit{in vitro} (8), a comprehensive characterization of its \textit{in vivo} effects is still lacking.

We recently concluded a phase II, open-label, baseline-versus-treatment crossover trial of daclizumab in 10 MS patients with incomplete therapeutic response to IFN-\gamma. Daclizumab showed a profound inhibitory effect on brain inflammatory activity (78% reduction) and subsequent stabilization of disability progression (9). Both the inhibition of brain inflammation by daclizumab and reappearance of inflammation after cessation of the therapy developed gradually over a period of 2–3 months, consistent with the hypothesis that daclizumab induced gradual and prolonged immunomodulatory changes \textit{in vivo}. Based on these results, we initiated a second trial to test whether the inhibition of brain inflammation is maintained during long-term daclizumab monotherapy. Here we present \textit{in vivo} observations complemented by \textit{in vitro} experiments from a total of 22 MS patients from both phase II trials of daclizumab in MS that suggest a mechanism of action of daclizumab via a regulatory circuit between innate and adaptive immune responses that involves the action of immunoregulatory CD56\textsuperscript{bright} natural killer (NK) cells on T cells.

Results

Daclizumab Therapy Has only Marginal Effects on Functional T Cell Responses \textit{in Vivo}. To assess T cell functions in daclizumab-treated patients, the average proliferation from two baseline samples (months –2 and 0) was compared with the average of three treatment samples (months 1.5, 3.5, and 5.5) for each patient. We saw no inhibition of T cell proliferation to polyclonal (plate-bound anti-CD3/CD28, IL-2, and IL-15) stimuli when daclizumab was not present in culture media (data not shown). Only modest (∼20%) but significant inhibition of CD4\textsuperscript{+} (but not CD8\textsuperscript{+}) T cell proliferation was observed when daclizumab was added to culture medium at 10 μg/ml [the peak concentration achieved \textit{in vivo} at 1 mg/kg every 4 weeks i.v. dosing (10)] (Fig. 4, which is published as supporting information on the PNAS web site). The antiproliferative effect of daclizumab was further analyzed by titrating the TCR stimulus and IL-2 (Fig. 4B and data not shown). In these experiments the inhibition of CD4\textsuperscript{+} T cell proliferation by daclizumab was most pronounced at lower levels of TCR stimulation, and the inhibitory effect could be overcome by high amounts of IL-2. No significant inhibition of cytokine production (IL-2, IL-4, IL-6, IL-8, and IFN-\gamma; ELISA and intracellular cytokine staining) from these cultures was observed during daclizumab therapy (data not shown).

The lack of a direct functional inhibition of adaptive immune responses by daclizumab was supported by \textit{in vivo} observations that...
Expression of IL-2R chains, 

Lymphocytes subpopulations (n = 22)

<table>
<thead>
<tr>
<th>Markers examined by flow cytometry</th>
<th>Baseline mean</th>
<th>IFN-β therapy + dacliz., mean</th>
<th>% change</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>All lymphocytes</td>
<td>24.39</td>
<td>23.81</td>
<td>−2.39</td>
<td>NS</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>1,748</td>
<td>1,633</td>
<td>−6.56</td>
<td>NS</td>
</tr>
<tr>
<td>%</td>
<td>51.6</td>
<td>48.68</td>
<td>−5.67</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Absolute no.</td>
<td>917</td>
<td>815</td>
<td>−11.20</td>
<td>0.009</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>18.68</td>
<td>17.19</td>
<td>−7.97</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>%</td>
<td>333</td>
<td>295</td>
<td>−11.53</td>
<td>0.015</td>
</tr>
<tr>
<td>CD56dim lymphocytes</td>
<td>7.49</td>
<td>10.52</td>
<td>+40.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Absolute no.</td>
<td>124</td>
<td>168</td>
<td>+35.62</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Expression of IL-2R chains, 

CD56dim NK cells, % 24.07 24.32
CD56dim NK cells % 3.30 8.78 +166.10 <0.001
CD56dim T cells, % 2.61 2.80 +7.30 NS
γ/δ-T cells, % 2.96 3.09 +6.65 NS

Absolute numbers are per microliter of whole blood. NS, not significant; Dacliz., daclizumab.

Delayed-type hypersensitivity responses (i.e., skin test results) against the recall antigens tetanus, candida, and mumps at the end of the daclizumab dosing were comparable to those observed at baseline.

Thus, contrary to published in vitro data (6, 7), daclizumab therapy had only marginal effects on adaptive immune responses in vivo.

Daclizumab Therapy Leads to Modest Declines in Circulating CD4+ and CD8+ T Cells and to a Robust Expansion of CD3−/CD56bright NK Cells.

To consider possible cell-depleting properties of daclizumab, changes in lymphocyte subpopulations were monitored by flow cytometry during the daclizumab trial. By comparing the average of two baseline samples with the average of two therapy samples for each patient (Table 1), small but highly statistically significant decreases were observed in CD4+ and CD8+ T cell counts (6–12%) as well as a significant expansion of CD4−/CD8εdim lymphocytes (~35%).

The Tac epitope of CD25, the molecular target of daclizumab, was blocked throughout the duration of daclizumab therapy (>95% inhibition at trough levels; Table 1). However, the CD25 epitope detected by a mAb (7G7) that binds outside of the Tac epitope (11, 12) persisted on cell surfaces and was selectively down-modulated (~32%) on CD4+ T cells by daclizumab. Additionally, daclizumab therapy led to a significant expansion (~45%) of CD122bright/CD4− lymphocytes.

Because the CD4−/CD8εdim cells and CD4−/CD122bright cells expanded during daclizumab treatment could represent the same cellular population, i.e., NK cells, the immunology protocol was modified during the second phase II trial (n = 12) to prospectively examine NK cell markers. Almost all CD4−/CD8εdim lymphocytes were CD122bright CD3−/CD56+ NK cells. Furthermore, daclizumab therapy led to a selective expansion of CD56bright NK cells (~200%). CD56dim NK cells, CD56+ T cells, and γ/δ-T cells were not expanded (Table 1).

Daclizumab Therapy Leads to Activation and Expansion of CD56bright NK Cells Through an IL-2-Dependent Mechanism.

The next logical question was how does daclizumab therapy lead to highly selective expansion of CD56bright NK cells?

Comparing the expression of effector molecules on NK cells by flow cytometry from samples before and during daclizumab therapy, we observed significant modulation of expression patterns on CD56bright but not CD56dim NK cells or T cells (Fig. 5A, which is published as supporting information on the PNAS web site). CD56bright cells consistently expressed the highest levels of IL-2Rβ chain (CD122) among all lymphocytes, and this expression was further enhanced during daclizumab therapy (Δ = +30.3%, P = 0.004). We observed surface expression of CD25 only in a subgroup of CD56bright NK cells, and the expression levels did not change significantly by daclizumab therapy (Fig. 5A and B). Compared with CD56dim cells, CD56bright NK cells expressed the IL-7Rα chain and had consistently higher expression of all adhesion molecules and chemokine receptors studied (CD44, CXCR3, and CCR7). Both IL-7Rα chain (Δ = +22.9%, P = 0.027) and CD44 (Δ = +53.8%, P = 0.001) were further up-regulated by daclizumab treatment. Neither population expressed IL-4Rα and IL-15Rα chains (data not shown). Most NK cell effector molecules examined (CD2, CD94/NKG2A, NKG2D, Nkp46, KIR2DL4, and TRAIL) were expressed in higher levels on CD56bright as compared with CD56dim NK cells, and NKG2A (Δ = +82.3%, P = 0.001), NKG2D (Δ = +28.7%, P = 0.009), Nkp46 (Δ = +44.7%, P = 0.027), and KIR2DL4 (Δ = +30.5%, P = 0.002) were further up-regulated on these cells during daclizumab therapy (Fig. 5A). CD16 and perforin (intracellularly) had higher expression on CD56dim as compared with CD56bright NK cells. CD16 was down-modulated in daclizumab therapy samples on both CD56dim (Δ = −31.8%, P = 0.032) and CD56bright (Δ = −51.4%, P = 0.02) NK cells, whereas no significant changes in perforin expression were observed.

Because the detected changes in expression patterns on CD56bright NK cells during daclizumab therapy affected molecules that are known to be regulated by IL-2 (i.e., IL-2Rβ, IL-7Rα, and KIR2DL4) (13) one plausible explanation for the expansion of these cells during daclizumab treatment was their heightened ability to receive IL-2/IL-15 signals by means of the intermediate-affinity IL-2R. Although it was previously reported that CD56bright NK cells proliferate to picomolar doses of IL-2 (14) and IL-15 (15), it was the role of CD25, not CD122, that was traditionally emphasized in this phenomenon (16). We were able to address the role of CD25 versus CD122 in the proliferation of NK cells during the daclizumab trial: the prospectively collected 5- (and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assays were reanalyzed, and gating on CD4−/CD8εdim lymphocytes (i.e., NK cells) revealed that the proliferation of these cells during daclizumab treatment was their heightened ability to receive IL-2/IL-15 signals by means of the intermediate-affinity IL-2R. Although it was previously reported that CD56bright NK cells proliferate to picomolar doses of IL-2 (14) and IL-15 (15), it was the role of CD25, not CD122, that was traditionally emphasized in this phenomenon (16).

The Expansion of CD56bright NK Cells on Daclizumab Therapy Correlates with Decreased Brain Inflammation.

The relationship between the daclizumab-induced expansion of CD56bright NK cells and its therapeutic effect was examined by correlating the inhibition of contrast-enhancing lesions on brain MRI (an established measure of brain inflammation) with the percentage of CD56bright NK cells. Using either nonparametric Spearman or parametric Pearson correlations (after logarithmic transformation of the data to achieve normality and linearity assumption of parametric correla-
Investigators counting the live cells in culture was assessed by two independent gated cells. The rate of T cell proliferation by CFSE (0.000927) and highly significant (P < 0.0001; P < 0.026) when only long-term data points were evaluated. There was a trend that did not reach significance (P = 0.079) when only short-term data points were considered.

Fig. 1. Correlation between expansion of CD56<sup>high</sup> NK cells and inhibition of brain inflammatory activity during the daclizumab trial. Percentages of CD56<sup>high</sup> NK cells were averaged from two combination therapy samples (months 0–5.5; IFN-β plus daclizumab, open circles) and two monotherapy samples (months 6.5–15.5; daclizumab, target circles) for each patient (n = 12) and correlated with the average number of contrast-enhancing lesions on brain MRI during 5.5 combination therapy months and 12 monotherapy months.

Fig. 2. Inhibitory effect of daclizumab on T cell survival in <i>in vitro</i> cultures appears to be mediated by NK cells and requires NK–T cell contact. (A) PBMC or NK-depleted PBMC (by CD56 microbeads) from the same samples were stained with CFSE, polyclonally activated (plate-bound CD3/CD28) for 72 h in the presence/absence of daclizumab, washed and reseeded in T cell media enriched for IL-7 and IL-15 (with or without daclizumab), and followed for long-term survival. Corresponding flow cytometry profiles of equivalent proportions of cultures (CFSE proliferation together with intracellular cytokine staining for IL-2) at day 8 after stimulation are depicted from a representative patient from samples before and during daclizumab therapy. (B and C) Similar experimental design to A except that the NK cells that were depleted from PBMC were added into TW cultures at a 1:10 NK:T cell ratio so that T cells and NK cells were not in contact. (B Left) Rate of T cell proliferation by CFSE dilution at day 6 (no. of mitoses per 100 gated cells). (B Right) Thymidine incorporation from the same cultures at same time point (cpm). (C) T cell survival at day 18 of culture was assessed by two independent investigators counting the live cells in culture by light microscopy using trypan blue exclusion and by calculating the final number of T cells based on the proportions of CD3<sup>+</sup> T cell in the cultures analyzed by flow cytometry. For each of the three panels, T cells in the PBMC are compared with T cells in NK-depleted PBMC, and depleted NK cells were placed in TW. Each plot represents representative experiment from two to five patients.
TW and separated from the T cells by a semipermeable membrane (Fig. 2 B and C). Early after stimulation (day 6) the rate of T cell proliferation (CFSE dilution: no. of mitoses per 100 surviving T cells) was only minimally inhibited by daclizumab (Fig. 2B Left). However, the thymidine incorporation (cpm), which reflects the total number of proliferating cells in the PBMC culture, was already decreased at day 6 by >50% by daclizumab and >70% by IL-2-blocking Ab (Fig. 2B Right). Because this discrepancy between CFSE dilution (which focuses on the proportion of surviving cells) and thymidine incorporation (which reflects the absolute number of cells that were in place in the culture and are still proliferating) indicated lack of survival of T cells in PBMC versus NK-depleted PBMC, cultures were followed in long-term survival assays (Fig. 2C). When NK cells were not in contact with T cells, daclizumab had absolutely no effect on long-term survival of activated T cells, whereas very few T cells survived in PBMC cultures where T cells were in contact with NK cells. In contrast to IL-2 blockade, daclizumab affected T cell survival only when NK cells were in contact with T cells.

IL-2-Activated NK Cells Can Kill Activated Autologous T Cells. Because of contact dependence, we considered cytotoxicity a possible explanation for the NK-mediated inhibition of T cell survival. Chromium-release cytotoxicity assays were performed with negatively selected NK cells as effectors and autologous resting or polyclonally activated T cells as targets (Fig. 3). Anti-CD3/CD28 activation of T cells was used initially but was replaced by PMA/ionomycin in subsequent experiments to exclude the possibility of Ab-dependent cellular cytotoxicity.

Freshly isolated NK cells from healthy donors did not exert significant cytotoxicity against resting T cells (Fig. 3A Left). In contrast, after overnight culture with IL-2 (10 units/ml) NK cells readily killed activated autologous T cells, irrespective of the presence or absence of daclizumab (Fig. 3A Right).

Freshly isolated NK cells from MS patients during daclizumab therapy showed cytotoxicity toward activated autologous T cells (PMA/ionomycin) without need for an IL-2 activation step. When comparing NK cells isolated from the same patient before (baseline) and during daclizumab therapy, greater NK cytotoxicity was found in the therapy samples (Fig. 3B). We observed a mild degree of cytotoxicity toward autologous activated T cells by resting NK cells even when isolated from some healthy donors and MS patients before daclizumab therapy (i.e., at baseline) (Fig. 3B and data not shown).

Because only CD56 bright NK cells were expanded during daclizumab therapy and low cytotoxicity potential has been attributed to these cells previously (14), CD56 bright and CD56 dim NK cells were sorted (>99% purity) from patients under daclizumab therapy by using flow cytometry and were tested for their cytotoxicity against autologous T cells (Fig. 3C). Both populations were comparable cytotoxic toward activated T cells. Addition of daclizumab did not enhance this cytotoxicity further. Whereas CD56 bright NK cells had virtually no cytotoxicity toward resting T cells, CD56 dim NK cells killed resting T cells equally as well as activated T cells. This cytotoxicity of CD56 dim NK cells was completely abrogated by anti-CD16 Ab, whereas the same Ab had only a mild inhibitory effect on the cytotoxicity of CD56 bright NK cells (Fig. 3C, red).

To assess whether NK-mediated killing of activated T cells may occur in vivo, the relationship between the expansion of CD56 bright NK cells and decline in CD4+ and CD8+ T cells was analyzed for individual patients. A significant correlation was found between the expansions of CD56 bright NK cells and contractions of CD4+ (R Spearman = -0.458; P = 0.0277) and CD8+ (R Spearman = -0.623; P = 0.00154) T cells in daclizumab-treated MS patients (Fig. 6, which is published as supporting information on the PNAS web site).

**Discussion**

We initiated a clinical trial of daclizumab in MS based on the hypothesis that in MS patients lymphocytes are chronically activated, rendering them functionally dependent on high-affinity...
IL-2R signaling. However, despite extensive collected data, we observed virtually no signs of inhibition of adaptive immune responses in daclizumab-treated patients. This apparent discrepancy from in vitro studies (6, 7) can be explained by the redundancy in cytokine systems in vivo. In contrast to predictions from in vitro experiments, immune responses in IL-2-deficient mice are not suppressed. These animals show normal lymphocyte development and mount normal cytotoxic T and B cell responses against viruses but have reduced NK cell activity (18). By 5 weeks they develop uncontrolled proliferation of T cells resulting in autoimmunity (19). Therefore, although IL-2 is not required for the lymphocyte activation in vivo, it controls excessive lymphoproliferation. However, IL-2 deficiency is not equivalent to daclizumab therapy, because daclizumab blocks only high-affinity IL-2 signaling while permitting signaling through the intermediate-affinity receptor (4). Indeed, daclizumab and IL-2-blocking Ab had divergent effects on T cell survival in NK-depleted PBMC cultures (Fig. 2 B and C), and daclizumab-treated patients had increased, not decreased, NK cell numbers (Table 1) and functional activities (Fig. 3B).

Daclizumab inhibits CD4+ T cell proliferation in vitro, particularly under conditions of low-potency stimuli and at low IL-2 concentrations (Fig. 4) that preferentially occur during stimulation by autoantigens. However, this inhibition is dose-dependent and is fully reversible when daclizumab fails to saturate CD25-binding sites, which occurs in patients 6–8 weeks after cessation of daclizumab dosing (data not shown). Therefore, the sole inhibition of T cell function by daclizumab does not appear to explain the gradually developing and lasting therapeutic effect observed during a daclizumab trial in MS (9). In contrast, daclizumab therapy resulted in a gradual expansion of CD56bright NK cells that correlated strongly with the decrease in brain inflammatory activity (Fig. 1). Expanded CD56bright NK cells limited survival of activated T cells in vitro in a contact-dependent manner, and NK cells isolated from treated patients were directly cytotoxic to autologous activated T cells. Finally, we observed a gradual decline in CD4+ and CD8+ T cells in daclizumab-treated patients (Table 1), and the correlation between expansion of CD56bright NK cells and decline in T cell numbers (Fig. 6) indirectly supports the hypothesis of NK cell-mediated immunoregulation of activated T cells in vivo.

CD56bright NK cells have been labeled “immunoregulatory” based on their ability to secrete cytokines (20) and home to lymph nodes (21) and tissues (22) and their expansion in humans during states characterized by increased immune tolerance (23, 24) such as pregnancy. Animal studies demonstrated a regulatory role of NK cells in autoimmunity in general (25–28), and in particular in experimental autoimmune encephalomyelitis (EAE) (25, 27), the animal model of MS. In an informative study Zhang et al. (25) depleted NK cells before immunization of susceptible mice with encephalitogenic myelin oligodendrocyte glycoprotein35–55 with the decrease in brain inflammatory activity (Fig. 1). Expanded CD56bright NK cells limited survival of activated T cells in vitro in a contact-dependent manner, and NK cells isolated from treated patients were directly cytotoxic to autologous activated T cells. Finally, we observed a gradual decline in CD4+ and CD8+ T cells in daclizumab-treated patients (Table 1), and the correlation between expansion of CD56bright NK cells and decline in T cell numbers (Fig. 6) indirectly supports the hypothesis of NK cell-mediated immunoregulation of activated T cells in vivo.

Immunological Assays. Blood sample collection. Blood samples were collected between 0830 hours and 1130 hours and were processed within 2 h. Lymphocytapheresis were collected during baseline, at month 5.5 of IFN-β therapy versus treatment (5.5 months; IFN-β plus daclizumab i.v. infusions at 1 mg/kg twice every 2 weeks and then five times every 4 weeks) crossover trial in 10 MS patients with complete response to IFN-β therapy. The clinical/MRI results of this trial are reported elsewhere (9). Additionally, we included immunological data on 12 MS patients from an ongoing second phase of the daclizumab trial. This new trial has an identical trial design for the first 5.5 months (IFN-β plus daclizumab combination therapy) but has an added extension during which IFN-β is withdrawn and patients continue on daclizumab monotherapy (1 mg/kg every 4 weeks) for an additional 12 months (months 6.5–15.5). Although the monotherapy extension phase is ongoing, all patients completed the IFN-β/daclizumab combination therapy to give us total of 22 patients with an entirely identical trial design. Both trials were approved by the National Institute of Neurological Disorders and Stroke Institutional Review Board.

Analysis of the functional immune status. Functional analyses included skin testing to tetanus, candida, and mumps, all performed at baseline and at month 5.5 of daclizumab therapy, and prospective analysis of T cell proliferation by CFSE-based flow cytometry proliferation assay as described (36) bimonthly during baseline and treatment. Briefly, PBMC were isolated by density gradients, diluted to 1 × 10⁶ PBMC per ml in PBS, and stained with CFSE (Molecular Probes; 1 μM). After washing, PBMC were resuspended in serum-free X-vivo 15 medium (BioWhittaker, Walkersville, MD) and seeded in 24-well plates (2 × 10⁶ PBMC per well) with the following stimuli: plate-bound anti-CD3 (20 ng/ml; preincubated at 37°C for 2 h), plate-bound CD3/CD28 (CD28 at 10 μg/ml), IL-2 (50 units/ml), IL-15 (20 ng/ml) (all PeproTech), and unstimulated sample. After 72 h of incubation (37°C and 5% CO₂), 1 ml of supernatant from each culture was collected and stored (−20°C) for ELISA (Cyto-Sets from BioSource International). Ninety-six hours after stimulation cells were stained with anti-CD3, anti-CD4, anti-CD8, or anti-CD56 Ab and analyzed by flow cytometry. We calculated the total number of mitoses per 100 gated cells using the
of the cultures were stimulated for 5 h by PMA (20 ng/ml) and anti-CD28 (CD28/CD3) to study T cell survival (17). Days 8–10 after stimulation one-half (10 ng/ml) of each culture was washed and stimulated for an additional 60 h. Cells were then stained with CFSE and seeded in triplicate or equimolar concentration of control anti-CD25 Ab M-A251 (BD Pharmingen), which does not block the IL-2 binding site. After 72 h of incubation one-half of each culture was washed and stained for intracellular cytokine staining (IL-2, IL-4, IL-6, IL-10, IFN-γ, and perforin; all from BD Pharmingen). Remaining cells were washed for long-term survival until days 14–48 after stimulation by counting viable cells. The same experimental design was performed simultaneously on NK-depleted PBMC: before CFSE staining, NK cells were depleted by CD56 MACS microbeads (Miltenyi Biotec, Auburn, CA). Where indicated, separate wells were set up in parallel to test the effects of higher concentrations of IL-2 or daclizumab. Cells from these wells were harvested, and 5 × 10⁶ cells were plated overnight with 200 μCi (1 Ci = 37 GBq) of Na₂¹⁵Cr₂O₇ (MP Biomedicals). Freshly purified NK cells (negative selection via NK cell isolation kit II, MACS, Miltenyi Biotec) from frozen or freshly acquired apheresis samples were mixed with labeled targets (7 × 10⁴ per well) at the indicated effector-to-target ratio in 96-well plates in triplicate. Alternatively, NK cells were cultured overnight with IL-2 (10 units/ml) and IL-15 (10 ng/ml) or without IL-2. Supernatants were harvested and counted in a gamma counter. Lysis was calculated from the supernatant ⁵¹Cr with the spontaneous release subtracted. To test for Ab-dependent cellular cytotoxicity, CD16 blockade was performed by using mAb anti-CD16 (1 μg/ml; BD Pharmingen).

Statistical Analysis. To evaluate immunological changes from baseline to treatment, two baseline samples and two to three treatment samples were averaged for each individual and compared by the signed-rank test or, if permitted, by a paired t test. Correlations between MRI measures and immunological indices were assessed by nonparametric Spearman correlation or by regression analysis. When Pearson correlation was used, values were logtransformed (zero values were replaced by half the smallest non-zero value in the data set because ln 0 does not exist) to achieve normality and linearity of distribution required by parametric test. All statistics were performed using SIGMSTAT 2.03 (Jandel Scientific) with a preset limit of statistical significance of P < 0.05.

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