Ablation of Transplanted HTLV-I Tax-Transformed Tumors in Mice by Antisense Inhibition of NF-κB

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Mice transgenic for the human T cell leukemia virus (HTLV-I) Tax gene develop fibroblastic tumors that express NF-κB–inducible early genes. In vitro inhibition of NF-κB expression by antisense oligodeoxynucleotides (ODNs) inhibited growth of these culture-adapted Tax-transformed fibroblasts as well as an HTLV-I–transformed human lymphocyte line. In contrast, antisense inhibition of Tax itself had no apparent effect on cell growth. Mice treated with antisense to NF-κB ODNs showed rapid regression of transplanted fibrosarcomas. This suggests that NF-κB expression may be necessary for the maintenance of the malignant phenotype and provides a therapeutic approach for HTLV-I–associated disease.

Human T cell leukemia virus type I (HTLV-I) is the etiologic agent of adult T cell leukemia (*1*). The HTLV-I–encoded *tax* gene has been implicated in leukemogenesis. This gene encodes a 40-kD protein that causes transcriptional transactivation of viral gene expression and also activates expression of such cellular promoters as the interleukin-2 receptor (IL-2R) α chain, granulocyte-macrophage colony-stimulating factor (GM-CSF), *fos*, platelet-derived growth factor, IL-6, nerve growth factor, and transforming growth factor–β (*2*). There are two independent pathways for the action of Tax on transcription (*2*). The first affects the c-rel–related family of nuclear transcription factors that bind to NF-κB sites and are important for the normal activation of lymphocytes. NF-κB response sequences occur in a number of genes, including the HIV long terminal repeat (LTR) and the IL-6 promoter (*3–5*). The other effect of Tax, whereby Tax activates its own promoter through three Tax-responsive elements (TREs), is thought to occur independently of NF-κB. Similar sequence motifs have been identified in *fos* (*6*).

Transgenic mice that express Tax develop fibrosarcomas by 9 months of age (*7*). We have established cloned cell lines from fibroblastic mouse tumors derived from transgenic C57Bl/6 mice. Transfer of these cell lines to syngeneic mice resulted in rapid growth in situ of fibroblastic tumors (*8*). These tumors are well established (approximately 7 mm in diameter) by 10 days after injection, and the mice die from localized effects of the tumor by 2 months after injection. All of these cell lines expressed high levels of growth-related genes, similar to the expression in tumors from which they were derived. Analysis of a prototype malignant cell line (B line) is shown in Fig. 1A, lane 1. Expression of these genes was at least ten times greater than those found in unstimulated Balb/3T3 cells (lane 4). In contrast, these cells do not express the T cell–specific factors IL-2 receptor (*8*), IL-2, or gamma interferon (IFN-γ) (*9*).

To compare the importance of Tax and NF-κB proteins in the activation and maintenance of cell transformation, we used 3′ terminal phosphorothioate (PS)-modified antisense oligodeoxynucleotides (ODNs) (*10, 11*) to inactivate expression of these proteins in transformed cell lines. The ODNs were modified with PS and purified according to published procedures (*12*). The ODNs extended over the translational initiation sites of both Tax and NF-κB mRNAs. The Tax-specific sense ODNs had no effect on cell growth or gene expression (Fig. 1A, lane 2). Previous analyses of cells treated with Tax antisense ODNs demonstrated an approximately tenfold decrease in Tax protein production. This led to a corresponding tenfold decrease in transactivation of the HTLV-I LTR in transient assays (*8*). Figure 1A, lane 3, shows a slight decrease in the levels of Fos RNA, which is consistent with direct cyclic AMP response element activating transcription factor (CRE/ATF) activation of this promoter by Tax (*6*). However, no alteration in the levels of many other growth-related genes was seen, suggesting that they were not directly activated by Tax.

In contrast, inhibition of either the p50 or p65 subunits of NF-κB had marked effects on endogenous cytokine gene expression (Fig. 1B). Interleukin-6 expression was inhibited more than GM-CSF expression, whereas there was no apparent effect on c-Fos, Tax, or actin expression. Inhibition of p65 was slightly more effective than p50 in three independent experiments. This result demonstrated specificity of the ODNs for the NF-κB–responsive genes. To quantitate functional effects of this inhibition, we performed transient chloramphenicol acetyltransferase (CAT) expression assays on an NF-κB–dependent promoter. The plasmid HIV-CAT was used because this retroviral LTR contains two copies of an NF-κB target sequence and has shown to be highly NF-κB–responsive (*3*). Figure 1C shows greater than 20-fold inhibition of CAT expression in the presence of NF-κB p65 antisense ODNs. In contrast, no effect on HIV-CAT expression was seen with Tax antisense ODN treatment (*9*). This was further confirmed by means of an electrophoretic mobility shift analysis (EMSA) of nuclear extracts (Fig. 1D). Analysis of unmanipulated cells (lanes 1 and 6) revealed three bands of NF-κB complexes as previously described (*13*). Unlabeled NF-κB consensus oligonucleotide competitively inhibited all complexes (lanes 2 and 3). In contrast, an oligonucleotide with a single G

Table 1. Time course analysis of NF-κB p65 sense- and antisense-treated mice. Tumor growth rates were determined by weight at the indicated times. Tumors were excised, and adventitial tissue was carefully removed. Tumor capsules were not removed. At each time point, the average and standard deviations of tumor weights from identically treated mice were determined. Statistical analyses of variance (ANOVA) (*24*) revealed a significant difference between the sense and antisense treatment groups. $F(1,8) = 133.8$; $P < 0.001$. ND, not done. Groups at 8 and 15 days were compared by Student's t test.

Time (days)	Tumor growth rate (mg)		
	No treatment	Sense	Antisense
0	440, 256	ND	ND
4	774, 824	604, 892	300, 220
8	1224, 1256, 1187 (1319 ± 182)	1112, 1414, 1094 (1207 ± 358)*	250, 203, 94 (182 ± 80*)
15	3365, 6624, 4164 (4718 ± 1698)	2708, 2766, 4021, 5727, 3884 (3821 ± 1228)†	225, 154, 99, 60, 85, 30 (109 ± 70.4)†
60	ND	8033, 6457	40, 20

*$P < 0.02$. †$P < 0.01$.

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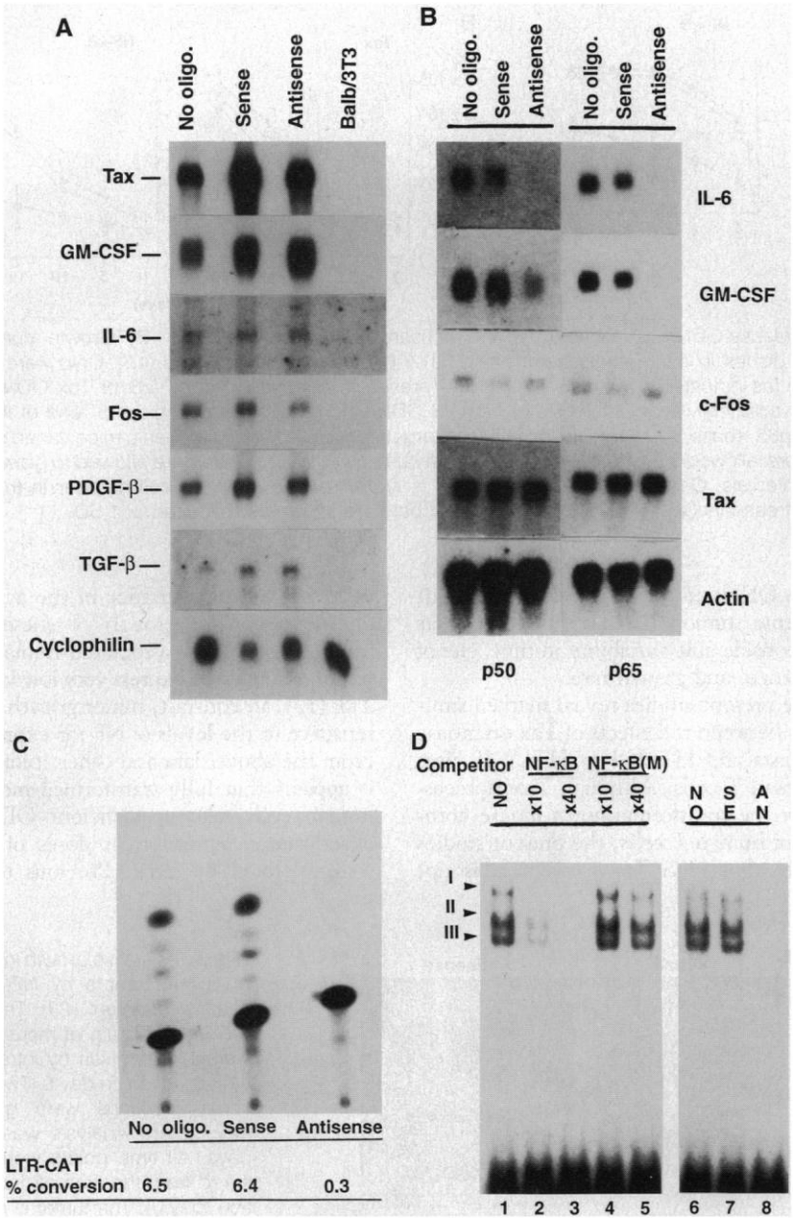

Fig. 1. Antisense inhibition of Tax or NF-κB protein in vitro. (**A**) Northern (RNA) analysis of early genes in Tax or (**B**) NF-κB antisense–treated cells. Line B cells were treated with 40 μg of sense or antisense ODNs per milliliter for 48 hours. Polyadenylated RNA was extracted from tissues, digested with proteinase K, and purified by the FastTrack method (*19*) (Invitrogen, San Diego, California). Samples were denatured and run on formaldehyde–1.4% agarose gels, and this was followed by blotting onto nylon membrane and ultraviolet fixation. Cloned cDNAs were used for generation of probes. Probes were labeled by the random primer method with [^{32}P]dATP plus deoxycytidine 5′-triphosphate (dCTP). Specific activities were 1×10^9 cpm/μg. (**C**) NF-κB transactivation assays. Line B cells were pretreated with p65 NF-κB oligonucleotides for 8 hours. The cells were then transfected with 5 μg of an HIV LTR-CAT construct. CAT activity was analyzed after an additional 48 hours. As an internal control, 5 μg of CMV–β galactosidase (Clontech, San Francisco) was cotransfected and analyzed by 4-methylumbelliferyl β-D galactoside assay (*20*). Transfections used Lipofectin (Bethesda Research Laboratories, Bethesda, Maryland) as per Felgner *et al.* (*21*). The level of CAT activity was determined by thin-layer chromatography with [^{14}C]chloramphenicol. Results were quantitated by scintillation counting of the extracted radiographic spots and are shown below. (**D**) Electrophoretic mobility shift assay. Three micrograms of nuclear extract from untreated (NO), sense-treated (SE), or antisense-treated (AN) cells (cell line B) were incubated with a double-stranded ODN encoding the NF-κB consensus target sequence (*14*). Labeled ODN (1×10^4 cpm; approximately 0.86 ng) was used. Procedures were as published (*13*). Three NF-κB–specific bands (I, II, and III) (*13*) are readily identified. Competition assays are shown in the left panel. The ratios of molar excess of unlabeled consensus ODN (NF-κB) or mutant NF-κB(M) are shown. Duplicate parallel plates were used for Northern blot, CAT, and EMSA analyses, and these experiments were repeated three times.

to C substitution in the binding site (*14*) was unable to block the formation of complexes (lanes 4 and 5). These data confirm the specificity of this EMSA assay for detection of NF-κB complexes. Treatment of these cells with sense ODNs to p65 had no effect on complex formation (lane 7). In contrast, use of p65 antisense ODNs specifically ablated all complex formation (lane 8). This occurred in a dose-dependent manner.

The effects of Tax or NF-κB antisense ODN inhibition on in vitro growth of mouse cell lines are shown in Fig. 2A. A constant amount of ODN (40 μg/ml) was used, and growth rates were measured daily. The data in Fig. 2 show the percent confluence at day 6 as a function of serum concentration. Serum concentration has marked effects on the growth rate of cells; previous studies have demonstrated induction of nuclear translocation of NF-κB by serum (*4*). Therefore, the effects of ODNs are displayed over a wide range of growth conditions. Treatment with Tax-specific ODNs [which caused a 90% inhibition of Tax expression (*8*)] had no apparent effect on the growth rates of cells. In contrast, p65 antisense ODNs had marked effects on the growth of line B, with no apparent effect on Balb/3T3 cell growth at all serum concentrations. Similar results were obtained when the concentration of NF-κB ODN was varied and serum concentration was fixed or with other Tax-expressing fibrosarcoma lines (*9*). The p50 ODNs were slightly less effective at the same concentration but gave a similar profile. Mixtures of both p50 and p65 ODNs had an additive inhibitory effect (*9*). NF-κB antisense treatment of the HTLV-I–transformed human cell line MT2 (*15*) led to a 14-fold inhibition of growth when assayed at 20 days (Fig. 2B). In contrast, only a twofold difference was seen in the growth of the control human T cell line Jurkat (*9*).

It is difficult to predict the behavior of tumor growth in animals on the basis of transient in vitro assays. We therefore examined the effects of ODN-mediated suppression on unmanipulated B line cells that were transferred to syngeneic C57Bl/6 mice. Once tumors were established (7 days after injection in the hind limb), mice were treated with three intraperitoneal injections of 40 μg of sense and antisense ODNs per gram of mouse body weight at 3-day intervals, as described (*16*). Western blot analysis of tumors exposed to Tax antisense ODN treatment showed virtually complete suppression of Tax expression at the 40 μg/g dose (*8*). No perturbation in the growth rate of tumors was seen in the animals treated with Tax antisense ODNs (*9*). However, whereas treatment with antisense to p65 caused regression of tumors,

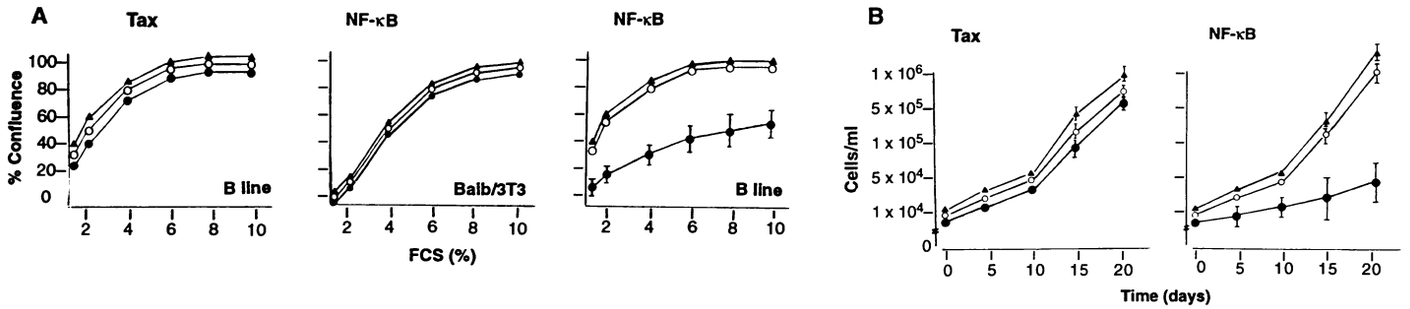

Fig. 2. Growth curves of cells treated with Tax or NF-κB antisense ODNs. (**A**) Balb/3T3 cells or cell line B were cultured in 10-cm dishes in the presence of varying concentrations of fetal calf serum (FCS) (as indicated on the abscissa). The amount of cells plated (1×10^4 per milliliter) was calculated to give approximately 30% confluence. The p65 sense or antisense ODNs (20 μg/ml) were added, and cultures were allowed to grow for 6 days. The ODNs were replenished at 3-day intervals. Cells were counted and expressed as percent confluence. Mock treatment (▲), sense (○), and antisense (●) are indicated. (**B**) Growth curves of antisense-treated HTLV-I–transformed MT-2 cells (*15*). Cells were grown in FCS (4%) in the presence or absence of NF-κB or Tax ODNs. The synthesis of new PS ODN was required because the 5′ end of the Tax translation initiation target sequence in the transgenic mice varied slightly from that in the native human virus (*22*). Cells were allowed to grow for up to 20 days. Growth is expressed as absolute cell number in triplicate six-well dishes. Error bars are shown as the mean ± 1 SD.

treatment with sense caused no diminution in growth (Fig. 3A). We analyzed a total of 13 antisense-treated and 22 controls over time and determined growth rates by weighing excised tumors. Clear growth inhibition was seen as early as 8 days after the first treatment, and marked differences occurred by 15 days (Table 1). Histologic analysis of these tumors was also performed, and typical results are shown in Fig. 3, B to E. Samples obtained from mice treated with sense NF-κB ODN for 8 days revealed a morphology characteristic of growing tumors (Fig. 3B). Treatment with antisense ODN showed focal tumor necrosis with inflammatory infiltrate by 4 days (Fig. 3C), followed by widespread segmental necrosis by 8 days (Fig. 3D). By 15 days (Fig. 3E), fibrotic tissue and tumor capsule with occasional inflammatory cells were all that remained. Beyond 15 days, it was difficult to identify the tumor site. Untreated mice, or those treated with sense ODNs, died between 8 and 12 weeks, whereas antisense ODN–treated mice have been followed for up to 5 months without evidence of recurrence of tumors. None of these mice were given ODN injections beyond the first 9 days of treatment. These studies were repeated with a different Tax-transformed cell line (PX-1) derived from Tax transgenic mice with a mixed haplotype background (*7*). Tumors were therefore grown in nude mice. These tumors also showed significant differences in growth between the antisense (695 ± 196 mg) and control groups (1525 ± 201 mg) when assayed at 15 days (four mice per treatment group). A similar pattern of growth retardation without complete ablation was also seen when B line tumor experiments were performed in nude mice. This suggests an important role for the immune system in antisense-mediated tumor ablation. So far, we have been unable to perform large-scale controlled experiments with antisense ODNs in the original (uncultured) transgenic tumors because these tumors show considerable variability in time, site of occurrence, and growth rate.

The present studies reveal marked similarities between the effects of Tax on mouse fibroblasts and HTLV-I or HTLV-II virus on human T cells. Although Tax is necessary for the transformation of mouse fibroblasts or human T cells, the present studies indicate that high Tax expression is not necessary for maintenance of the activated phenotype or for growth of these cells. Similarly, HTLV-I–associated human lymphomas frequently express very low levels of Tax (*17*). In contrast, tumor growth is very sensitive to the levels of NF-κB expression. From the above data and other studies (*8*), it appears that fully transformed mouse or human cells take up sufficient ODNs to effect tumor regression at doses of ODN well tolerated by mice. Previous toxicity

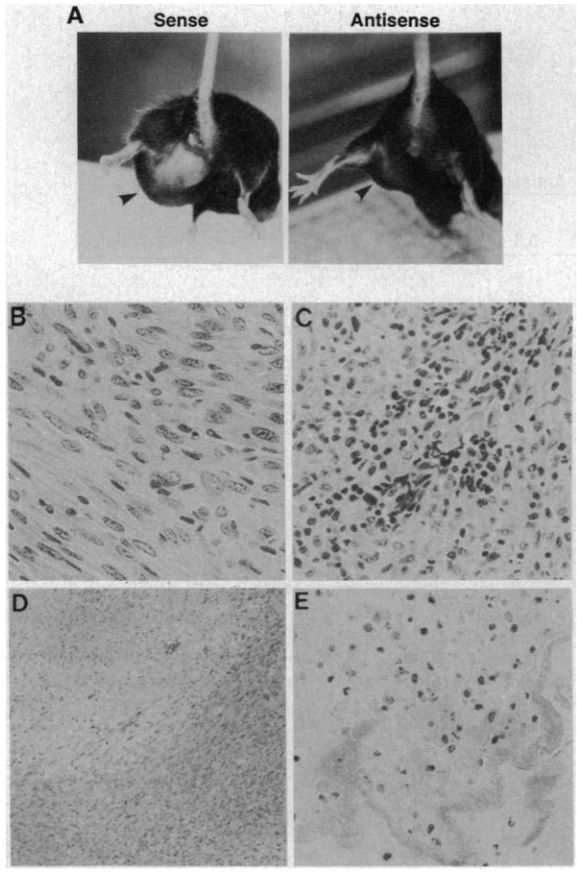

Fig. 3. (**A**) In vivo growth inhibition of B line tumors by NF-κB antisense treatment (*23*). The ODN (40 μg per gram of mouse body weight) was given by intraperitoneal injection on day 0. Two additional injections were given 3 days apart. Analysis was at 15 days. All time points were measured from the time of first injection (day 0). The tumor is indicated by an arrow. (**B** to **E**) Histologic analysis of tumors from similarly treated mice. (**B**) Tumors taken 8 days after treatment with sense NF-κB p65 ODN and stained with Hematoxylin and Eosin (H&E) (magnification, ×400). (**C**) Tumors taken 4 days after treatment with antisense ODN (H&E, magnification ×400); (**D**) Tumors taken 8 days after treatment with antisense ODN (H&E, magnification ×100). (**E**) Tumors taken 15 days after treatment with antisense ODN (H&E, magnification ×400).

studies suggest that mice readily tolerate at least 2.5 times this amount (*16*). The reason for this difference in susceptibility of Tax-transformed and normal cells to NF-κB ODNs is unclear. It may represent a difference in the requirement of intracellular NF-κB for survival or a difference in the ability to internalize the ODNs. Previous studies have correlated the activation or growth state of cells with their ability to take up oligonucleotides by receptor-mediated endocytosis (*18*). The Tax- or virus-transformed tumor cells appear highly activated, which may cause them to take up more ODNs (*8*).

Histologic analysis showed marked and widespread tumor necrosis, and even this short window of treatment was apparently sufficient to prevent tumor recurrences. This suggests that these ODNs may provide a valuable approach to therapy of HTLV-I–associated adult T cell leukemia, which has proved largely refractory to other modalities.

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Alterations in Signal Transduction Molecules in T Lymphocytes from Tumor-Bearing Mice

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Impaired immune responses occur frequently in cancer patients or in tumor-bearing mice, but the mechanisms of the tumor-induced immune defects remain poorly understood. In an in vivo murine colon carcinoma model (MCA-38), animals bearing a tumor longer than 26 days develop CD8$^+$ T cells with impaired cytotoxic function, decreased expression of the tumor necrosis factor–α and granzyme B genes, and decreased ability to mediate an antitumor response in vivo. T lymphocytes from tumor-bearing mice expressed T cell antigen receptors that contained low amounts of CD3γ and completely lacked CD3ζ, which was replaced by the Fc$_\epsilon$ γ-chain. Expression of the tyrosine kinases p56^{lck} and p59^{fyn} was also reduced. These changes could be the basis of immune defects in tumor-bearing hosts.

Cancer patients and tumor-bearing mice have impaired immune functions manifested by decreased delayed-type hypersensitivity, decreased lymphocyte lytic function, and a decreased lymphocyte proliferative response (*1*). A variety of mechanisms, including the action of suppressor cells, the production of suppressor factors by the tumor cells, deletion of tumor-specific clones, and diminished production of lymphokines, have been proposed to explain the altered immune response in cancer patients (*2*). The precise nature and prevalence of these defects has not been elucidated. In an in vivo tumor model, the progressive growth (>26 days) of a subcutaneous implant of MCA-38 resulted in decreased lytic function by the CD8$^+$ T lymphocytes that was associated with decreased expression of mRNA for tumor necrosis factor–α and granzyme B and the complete loss of the ability of adoptively transferred cells to mediate an antitumor effect in vivo (*3*). However, proliferation, lymphokine production, and lymphokine receptor up-regulation in CD4$^+$ T cells were comparable in normal and tumor-bearing mice. Cells with suppressor function were not detected, nor was the production of transforming growth factor–β detected in the lymphocytes from tumor-bearing mice or in the MCA-38 tumor cells.

In an effort to understand the molecular basis of these functional defects, we assessed the structure and function of the T cell antigen receptor (TCR) (*4*). No differences were detected between normal, purified splenic T cells and those from tumor-bearing mice in fluorescence intensity (an indicator of receptor number) or in the percentage of cells expressing the TCRαβ heterodimer (Fig. 1A) or the CD3 complex (Fig. 1B). Flow cytometry demonstrated that splenic T cells from tumor-bearing mice express Thy-1.2 (98%) and TCRαβ (98%) and have a normal CD4:CD8 ratio. They did not express natural killer (NK) cell markers: NK1.1 was <1%, and CD16 (Fc receptors) was <1%. No skewing of the T cell

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