Supplementary Methods

Tolerogenic effect of MDSC

To determine the effect of N1IC in the susceptibility of T cells to the tolerogenic effect of tumor-associated MDSC *in vivo*, we used a model described previously (1-3). Briefly, 5 X 10^6 $CD8^+$ T cells from $CD45.2^+$ N1IC or $N1IC^{f/f}$ mice were adoptively transferred via tail vein into $CD45.1^+$ mice. Two days later, MDSC were sorted from mice bearing 3LL tumors for 17 days, pulsed with 2 μg/mL siinfekl for 1 hour, and 5 X 10^6 MDSC were adoptively transferred into mice previously injected with N1IC or $N1IC^{f/f}$ $CD8^+$ T cells. The same day, 4 X 10^6 DCs, previously generated from bone marrows cultured in medium containing GM-CSF (20 ng/mL) and IL-4 (10 ng/mL) for 6 days and exposed for the last 24 hours of culture to 2 μg/mL siinfekl and 1μg/mL LPS, were injected s.c.. An additional injection of siinfekl-pulsed MDSC was done 5 days later. Ten days after the initial immunization, draining lymph nodes were recovered and monitored for the accumulation of transferred $CD8^+$ T cells and for the production of IFNγ after *ex vivo* challenge with siinfekl by ELISpot (R and D systems).

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed using SimpleChip kits (Cell Signaling Technologies). Chromatin was prepared from $CD8^+$ T cells from N1IC and $N1IC^{f/f}$ mice activated with siinfekl for 48 hours, according to manufacturer's protocol. Chromatin was immunoprecipitated with antibodies against Notch 1, RBP-J, NF-κB p65, and Histone H3 (Cell signaling Technologies), or rabbit IgG (Sigma-Aldrich). Eluted and purified DNA was analyzed by qPCR with pre-validated primers against the Granzyme B promoter purchased from Qiagen.

Western Blot and Immunoprecipitation

Cellular protein lysates collected from activated N1IC and N1IC$^{f/f}$ CD8^{+} T cells or T cells sorted after 48 hours of co-cultures with tumor-infiltrating MDSC were electrophoresed in TrisGlycine gels, transferred to PVDF membranes, and immunoblotted with antibodies against Notch-1, Notch-2, granzyme B, perforin A, FasL, Runx3, T-bet, Eomes, and β-actin. Differentiation between cleaved Notch-1 and transgenic N1IC was based on molecular size (cleaved Notch-1 was 110 kDa, while transgenic N1IC was ~65 kDa). Membrane-bound immune complexes were detected using ECL western blotting detection reagent (GE Healthcare). Immunoprecipitations were achieved using 200 μg of protein extracts from activated N1IC and N1IC$^{f/f}$ CD8^{+} T cells and 2 μg anti-Notch-1 or control IgG antibodies. After overnight incubation, protein G plus-captured complexes were analyzed for Notch-1 (cleaved and transgenic), RBP-J, and NF-κB p65 by western blot. As input controls, we used 10 μg of extracts from each experimental group before immunoprecipitation.

Cytotoxicity

In vitro cytotoxicity assays were conducted as previously described (4). Percent of specific lysis was calculated as follows: [(experimental ^{51}Cr release − spontaneous ^{51}Cr release)/(maximum ^{51}Cr release − spontaneous ^{51}Cr release)] × 100, where spontaneous release is the radioactivity of target cells in the absence of effectors, and maximum release is the radioactivity released by the targets incubated in 0.1% Triton X-100 (Sigma). All determinations were done in triplicates. To determine the effect of N1IC on cytotoxicity *in vivo*, N1IC and N1IC$^{f/f}$ CD8^{+} T cells activated for 72 hours were adoptively transferred i.v. into normal mice. Next, mice received 5 X 10^{6} of a 1:1

ratio of siinfekl-loaded splenocytes labeled with a high concentration of CFSE (1 μM) and control peptide-loaded splenocytes labeled with low CFSE (0.1μM) (2, 5). Spleens were collected 1 day later, and the ratio of bright:dull CFSE-labeled targets was calculated by flow cytometry in the $CD8^{NEG}$ $CFSE^{+}$ population.

Quantitative PCR

Total RNA was isolated from activated T cells or tumor-derived MDSC using TRIzol (Life Technologies). RNA was converted into cDNA using Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Quantitative PCR was performed on an Applied Biosystems thermocycler (7900 HT) using Bio-Rad SYBR green supermix with primers against mouse Notch-2 forward (TATCGCCCAGACATTCTCGC) reverse (ACACACTGACGGGGATCAAC) from IDT or validated Quantitect primer assays for mouse Notch-1 (QT00156982), Jagged-1 (QT00115703), Jagged-2 (QT01043819), DLL1 (QT00113239), DLL3 (QT00113477), and DLL4 (QT01053598) from Qiagen. Relative expression was calculated using the delta-delta *Ct* method and normalized to reference gene Actin; forward (TGTGATGGTGGGAATGGGTCAGAA) reverse (TGTGGTGCCAGATCTTCTCCATGT).

Statistical Analysis

Statistical analyses were performed in SAS 9.3 (SAS Institute, Cary, NC). Tests were conducted at 5% significance level. Continuous data were checked for unequal variances with the Brown-Forsythe and Levene tests. Percentage data were arcsine transformed and further checked for unequal variances. Experimental groups differences of endpoints were assessed by ANOVA with the Satterthwaite correction for unequal variances using the MIXED procedure. Mean

comparisons were carried out with the Tukey procedure for all comparisons or with the Dunnet procedure for comparisons with the control group.

Reference List

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2 Nagaraj S, Gupta K, Pisarev V, Kinarsky L, Sherman S, Kang L, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Med 2007;13:828-835.

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4 Davila E and Celis E. Repeated administration of cytosine-phosphorothiolated guanine-containing oligonucleotides together with peptide/protein immunization results in enhanced CTL responses with anti-tumor activity. J Immunol 2000;165:539-547.

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