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# Engagement of lymphoma T cell receptors causes accelerated growth and the secretion of an NK cell-inhibitory factor

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## Abstract

The development of T cell lymphomas in mice that constitutively express a single T cell receptor is surveilled by the action of NK cells. We investigated the effects of engaging the lymphoma TCR in this mouse model. We stimulated lymphoma cells expressing an ovalbumin-specific TCR in vivo using listeria monocytogenes as a vehicle. Infections with listeria expressing ovalbumin but not with control bacteria caused a stable change in lymphoma cells that allowed its growth in mice with normal NK cells. TCR engagement furthermore enhanced lymphoma growth in NK-cell-depleted mice suggesting a lymphoma-intrinsic change that lead to accelerated growth. The ability to grow in mice without prior NK cell depletion did not appear to be accompanied by changes in the recognition of lymphoma by NK cells. Rather, lymphoma immunization was associated with a decrease in NK cell numbers: Leukemic phases were observed for all mice starting three to eight weeks after immunizations, and leukemias were succeeded by the disappearance of NK cells from blood. We also observed strong decreases of NK cell numbers in spleens at the time of death. Co-culture experiments showed decreases in the ability of NK cells to proliferate in response to IL-15 when post-immunization lymphoma cells were present in a mechanism that did not require direct cell contact. Together these data suggest that TCR engagement caused intrinsic changes in T cell lymphoma cells resulting in both accelerated in vivo growth and in the secretion of a factor that caused NK cell disappearance.

## 1. Introduction

Tumor immunosurveillance has been described to be mediated by multiple arms of the immune system. Tumor development requires the escape from these tumor-limiting mechanisms [1]. A three-step process named “immune-editing” has been postulated to explain such changes. Alterations that allow escape involve both modifications to tumor

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Author contributions
Conceived and designed the experiments: SD, TAW, JRM. Performed the experiments: SD, JRM. Analyzed the data: SD, JRM. Wrote the paper: SD, TAW, JRM.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data
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proteins themselves resulting in invisibility from the eyes of surveilling immune cells. And immune processes can be corrupted by actions of tumors to misdirect immune action or even convert normally immune-enhancing into -suppressing activities.

We have recently described a murine model of T cell lymphoma development [2]. We have shown that lymphomagenesis in mice that constitutively express a single TCR is limited by the action of NK cells. The inability to generate lymphomas or even regrow established lymphomas under conditions of NK cell presence caused us to hypothesize that T cell lymphomas must possess the ability to induce an additional and necessary step to escape this immunosurveillance and complete T cell lymphoma development.

A role for TCR engagement in T cell lymphoma development has been proposed in 1982 [3] though its role remains poorly understood [4]. The expressions of the TCR, its helper molecules and downstream signaling molecules are maintained in the majority of human T cell lymphomas despite frequent losses of other T cell-specific surface proteins. Further support comes from the detection of mutations that mimic parts of the TCR-induced signaling cascade in human T cell lymphomas. Such mutations have been reported for ALK, DUSP22, ITK-Zap70, among others [4]. T cell receptor signaling is initiated by dendritic cells presenting peptides via MHC, and the lymphoma microenvironment is rich in such APCs often exerting immune-inhibiting activities [5,6]. Dendritic cells are believed to support cutaneous T cell lymphomas in a mechanism that stimulates lymphoma TCRs via DC-mediated presentation of lymphoma protein [7]. A number of mouse models also support a role for TCR in lymphomagenesis. Using a mouse model of peripheral T cell lymphoma, Wang et al. [8] showed the requirement of intact TCR signaling for T cell lymphoma development.

Here we take advantage of known TCR specificities in our T cell lymphoma model. We investigate downstream effects of TCR engagement on lymphoma cells. We show that initiating the TCR signaling cascade resulted both in lymphoma-induced NK cell disappearance and in NK cell-independent lymphoma growth acceleration.

## 2. Materials and methods

### 2.1. Mice

C57BL/6 and CD90.1$^{+}$ mice were bred in our own animal colony. Animal care and all animal procedures were done in accordance with National Institutes of Health (NIH) guidelines and was approved by the Animal Care and Use Committee of the NCI. In vivo antibody treatments were done i.p. as follows: anti-CD122, 25 μg twice weekly, Bio X Cell; anti-NK1.1, 25 μg twice weekly, Bio X Cell. The lymphoma cell line SJ3 was routinely passaged into NK cell-depleted mice by i.p. injections of approximately $10^5$ lymphoma cells following anti-NK1.1 treatments.

To determine the levels of short-term proliferation, splenocytes from mice carrying either SJ3S or SJ3R (splenocytes contained both CD90.1$^{-}$ normal splenocytes and CD90.1$^{+}$ SJ3 cells) were labelled with CFSE and injected into CD90.1$^{-}$ mice ($10^7$ cells each, i.v.) that had been depleted of NK cells (anti-NK1.1, 25 μg 4 and 1 days prior). Levels of CFSE were

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determined 48 h later both in the transferred normal splenocytes ($CFSE^+$, $CD90.1^-$) and in SJ3 cells ($CFSE^+$, $CD90.1^+$).

### 2.2. Listeria monocytogenes

L. monocytogenes was handled as described [9]. Briefly, L. monocytogenes was grown in brain–heart infusion broth. For infections of mice, log phase cultures of L. monocytogenes expressing full-length ovalbumin or LCMV-gp protein were used. We induced TCR responses with $10^5$ cfu of L. monocytogenes i.p. that was followed by the presence of approximately 20,000 cfu/ml in the drinking water for two weeks. As previously described, ampicillin (1 mg i.v. each) was given 24 h after the end of each immunization [10].

### 2.3. Cytometry

For cytometric analyses, cells were blocked with anti-CD16/32 (15 min at RT) that was followed by a 30-min incubation on ice with the specific antibodies. All antibodies used are listed in the Supplementary Table S1. For biotinylated antibodies, an additional 15-min incubation on ice was done with streptavidin-PE-CY5.5 (Ebioscience). BrDU stains were done 12 h after the injection of 1 mg BrDU using the BrDU Flow Kit (BD Biosciences). We determined NK cell degranulation rates by co-incubating lymphoma cells and splenocytes at equal numbers ($10^6$ each in 24-well plates, 500 μl) in the presence of anti-CD107a (5 h, 6.25 μg/ml) and of Golgistop (BD Pharmingen, present during the last 4 h). Erythrocytes were lysed in PBMC samples after antibody staining using CAL-LYSE (Invitrogen). Data analysis was performed using FlowJo software.

### 2.4. In vitro NK cell growth assay

Normal splenocytes ($0.75 * 10^6$ per well of 24-well plates, CFSE-labeled, 500 μl total volume) were co-incubated with various numbers of ex vivo lymphoma cells in the presence of 500 pM murine IL-15 for five days. Half of the medium was exchanged at day 3. For transwell experiments (24-well plates, 0.4 μm pore size, Costar), $0.75 * 10^6$ CFSE-labeled splenocytes in the lower chamber were co-incubated with various numbers of lymphoma cells in the upper chamber in 500 μl total volume.

To generate supernatants from cultured SJ3R cells, we plated splenocytes from SJ3R-bearing mice at $10^6$ cells per ml in RPMI containing 10% FBS overnight that was continued by cultures in RPMI containing 5% FBS with three weekly additions of 20% to the existing volume. We harvested the resulting supernatants 4 weeks later and studied their effects on IL-15-induced NK cell proliferation. We cultured normal splenocytes in IL-15 for five days as described above in the presence of 20% control medium or supernatants.

### 2.5. SJ3 lysis assay

We performed an SJ3 lysis assay as previously described [2]. Briefly, we cultured splenocytes from mice that express the diphtheria toxin receptor from the NK1.1 promoter (48 h, 50% IL-15 complex derived from transfected 293 cells) in the absence or presence of diphtheria toxin (40 ng/L), and we verified NK cell numbers by FACS. We CFSE-labelled splenocytes from mice carrying either SJ3S or SJ3R as well as EL4 cells that are not recognized by NK cells, mixed them at a SJ3 to EL4 ratio of 10:1, and added them to the

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cultured splenocytes at NK cell to SJ3 ratios of 1:1 and 2:1 based on the NK cell numbers in cultures without diphtheria toxin. The number of SJ3 cells added to cultures with or without diphtheria toxin was equal based on their total numbers of splenocytes. We determined the numbers of surviving SJ3 and EL4 cells 20 h later by FACS based on their expressions of CD90.1 and CD90.2. We determined the percentages of SJ3 lysis based on the ratios of alive SJ3 to EL-4 cells in the presence or absence of NK cells using the formula 100–100*(SJ3/EL4-NK$^{+}$)/(SJ3/EL4-NK$^{-}$).

### 2.6. Statistical analyses

Analyses were done using Prism 7. Cell numbers, data from NK cell growth and lysis assays as well as from FACS-generated MFIs were compared using unpaired *t*-test, and two-tailed p-values are shown. P-values for mouse survival were calculated by Log-rank (Mantel-Cox) test.

## 3. Results

### 3.1. Lymphoma progressions caused by TCR engagement

We have previously shown that T cell lymphomas develop in mice that express a transgenic TCR and lack NK cells [2]. The resulting T cell lymphoma lines regrew in recipient mice only in the absence of NK cells [2]. We had done most experiments in mice that recognize a TCR with specificity for an MHC class I-restricted peptide of ovalbumin (OT1). We decided to utilize the known TCR specificity of the lymphoma line SJ3 to investigate potential effects of its TCR engagement with its cognate peptide. We speculated that engaging the TCR signaling pathways could induce changes in the SJ3 lymphoma line that would allow its survival in NK cell-harboring environments. An effective way of eliciting specific CD8 T cell responses in vivo is by delivering immunizing proteins with recombinant Listeria monocytogenes [11]. We infected mice by i.p. injections with L. monocytogenes that expressed either ovalbumin or the irrelevant LCMV-gp which was followed by i.p. transfers of $10^6$ cells of the lymphoma line SJ3 24 h later and by two weeks of the respective bacteria in the drinking water. To study a potential gain of resistance to NK cell-containing environments, at no time were mice depleted of NK cells. Fig. 1A shows that all mice succumbed to lymphoma disease following immunizations with the cognate ovalbumin, but no mice died following immunizations with the control protein. These data suggest that TCR engagement did indeed permit the survival and expansion of the lymphoma line SJ3 in mice with NK cell presence.

Next we studied whether the changes that had been induced in T cell lymphoma cells via in vivo TCR engagement were stable in nature. We re-designated SJ3 cells that remained sensitive to NK cell presence as SJ3S, and cells derived from mice after ovalbumin immunization as SJ3R. We isolated SJ3S and SJ3R cells from NK cell-depleted or ovalbumin-immunized lymphoma-bearing mice, respectively, and transferred them into immuno-competent but non-immunized mice. Fig. 1B shows that SJ3R lymphoma cells that had been TCR-stimulated in their previous host environment were still able to expand and kill its subsequent recipient while SJ3S cells were unable to do so. Thus, engaging the TCR

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on lymphoma cells caused stable changes that allowed its growth under conditions of NK cell presence.

### 3.2. Accelerated growth of lymphoma cells in response to TCR signaling

We tested whether in vivo TCR engagement had altered lymphoma cells unrelated to NK cell activities. We investigated whether rates of proliferation were changed by inducing TCR signaling cascades. We observed that in ex vivo cultures, a larger portion of SJ3R than SJ3S cells had divided as measured by CFSE dilution within 24 h (Fig. 2A). We also determined rates of early in vivo proliferation 48 h after transfers into NK cell-depleted recipients. Fig. 2B shows that the levels of CFSE remained equally high in non-lymphoma splenocytes that had been transferred from both SJ3S- and SJ3R-bearing mice (gray areas in right FACS plots). CFSE levels for both SJ3S and SJ3R were lower indicating that nearly all SJ3 cells had divided, but the levels of CFSE dilution was significantly higher for SJ3R when compared with SJ3S cells indicating increased numbers of proliferation cycles. We also determined proliferation rates for both SJ3S and SJ3R at a later stage of lymphoma disease after the appearance of leukemic cells. Analyses of proliferation markers ex vivo showed the expression of Ki67 in nearly all lymphoma cells regardless of prior TCR engagement (Fig. 2C). However, determining the actual number of cells that had undergone S phase 12 h after BrDU injections still showed small but consistent increases in lymphoma cells after in vivo TCR engagement (Fig. 2C, SJ3R). Lastly, we studied whether transfers of SJ3S versus SJ3R would lead to different survival rates in NK cell-depleted mice. We choose depletions with an antibody against CD122 that in our hands causes the most complete NK cell depletions in vivo [10,12]. We determined that the expression levels of CD122 on both SJ3S and SJ3R cells are similarly low (Fig. 2D, left), and substantially lower than the CD122 expression on “memory phenotype” CD8 T cells that are the only CD8 cells deleted by anti-CD122 injections. Fig. 2D (right) shows that survival rates were significantly lower in NK cell-depleted mice after transfers of SJ3R when compared with SJ3S lymphoma cells. Together these data show that the engagement of the TCR on SJ3 T cell lymphomas caused its accelerated growth in vitro and in vivo.

### 3.3. Surface phenotype changes in lymphomas after TCR engagement

T cell lymphomas that developed in TCR-transgenic mice in the absence of NK cells displayed a distinct phenotype [2]. We investigated potential changes of surface molecules to explain behavioral changes of lymphoma cells after TCR engagement. One explanation for the ability of post-immunization lymphoma cells to grow in mice with normal NK cells is that NK cell recognition could be impaired. The majority of surface expression remained unchanged (see Table S1 for a complete list of antibodies used). Fig. 3A depicts all surface expressions that were significantly different between SJ3 lymphoma cells grown in the absence of NK cells (SJ3S) versus lymphoma cells that emerged in response to in vivo TCR engagement in mice without NK cell deletions (SJ3R). When compared to iso type controls (gray) and levels in normal CD8 cells (solid line) in top row, the activation markers CD25 and ICOS were higher on SJ3R than SJ3S cells as was the marker for central memory T cells CD62L and a marker for thymic development CD30 (bottom row of FACS panels, graphs below show MFIs and p-values). Importantly, TCR signaling had caused increases in MHC class I though the level on SJ3R remained lower than on normal CD8 T cells

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(Fij 3A). TCR signaling also decreased the expression of the NKG2D ligand Mult1 slightly but significantly (Fig. 3A). Both changes could alter the recognition of lymphoma cells by NK cells. We therefore studied whether SJ3S and SJ3R were different in their ability to induce NK cell degranulation and NK cell-mediated lysis (Fig. 3B). We observed that the ability to activate NK cells ex vivo was similar for both lymphomas regardless of prior TCR engagement (Fig. 3B, left). The results that we obtained from lysis assays remained inconclusive (Fig. 3B, right). As previously described, NK cell-mediated lysis of SJ3 cells is slow [2], and no lysis was seen after six hours. We observed significantly higher percentages of cell lysis for SJ3S than SJ3R after 20 h (Fig. 3C). However, lysis levels may have been affected by the NK cell-inhibitory factor secreted by SJ3R (see below). Together these data suggest that since NK cell degranulation as measured by CD107 stains is necessarily preceded by a lymphoma recognition step, changes of lymphoma surface molecules induced by in vivo TCR engagement are insufficient to account for the ability of post-activation lymphoma cells to grow in mice with NK cell presence.

### 3.4. Lymphoma immunization is associated with NK cell disappearance

An alternative way for lymphoma cells to escape NK cell surveillance could result from effects on NK cell numbers and functions. We studied potential effects of the presence of TCR-activated SJ3R T cell lymphomas on NK cells in vivo. We first analyzed PBMCs at weekly intervals following immunizations and lymphoma transfers. Fig. 4A, top depicts an example of FACS analyses from a single mouse while graphs below show percentages of SJ3R and NK cells among PBMC for each individual mouse: While initial leukemic appearances of lymphoma cells (CD90.1$^{+}$) varied between three and eight weeks post immunization, these leukemic phases were consistently succeeded by the disappearance of NK cells (NK1.1$^{+}$) from blood. We then enumerated NK cells in spleens at the time when mice bearing lymphoma cells after their in vivo TCR engagement had succumbed to the lymphomas; it revealed an average decrease of nearly 90% when compared with control spleens for NK cells identified as CD3$^{-}$/CD19$^{-}$/NK1.1$^{+}$, and a decrease to near zero for NK cells identified as CD3$^{-}$/CD19$^{-}$/NKp46$^{+}$, (Fig. 4B). These data suggest that immunizations confer onto T cell lymphoma cells the ability to delete NK cells thus allowing their survival in hosts without prior NK cell deletions.

### 3.5. A lymphoma-generated soluble factor inhibits NK cell proliferation in vitro

We tested whether we could recreate elements of the lymphoma-mediated NK cell suppression under tissue culture conditions. We used splenic NK cells from untreated mice grown in IL-15 as targets and co-cultured these with pre- and post-immunization lymphoma cells at various target-to-effector ratios. Fig. 5A shows that while both pro- (top) and post-immunization (bottom) lymphoma cells inhibited IL-15-induced NK cell proliferation as measured by the CFSE dilution, the effect was observed with fewer post-immunization lymphoma cells present (4:1 splenocytes-to-lymphoma ratio versus 4:2 for pre-immunization cells). The presence of post-immunization lymphoma cells was associated with decreased numbers of live NK cells after 5 days of co-culture (Fig. 5B, left) an effect not caused by pre-immunization cells. We further investigated whether a direct cell contact with lymphoma cells was necessary for the NK cell inhibition. Similar decreases in NK cell numbers were achieved if both cell types had been separated by membranes with 0.4 μm

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pore size (Fig. 5B, right). To gain further proof for the secretion of an NK cell-inhibitory factor, we cultured SJ3R cells for four weeks and used the resulting supernatants to study their effects on the IL-15-induced proliferation of normal splenic NK cells. Comparisons to SJ3S supernatants could not be made since SJ3S proved refractory to our efforts of culturing. Fig. 5C shows that a presence of 20% supernatants from SJ3R cultures resulted in substantial and significant decreases of the percentages of NK cells after five days of cultures. These effects of relatively low percentages of supernatants within the cultures also suggests that the effect is caused by a factor within the supernatants rather than by nutrient depletion from the culture medium. Together these data suggest that a portion of the mechanism of lymphoma-induced NK cell inhibition can be re-capitulated under in vitro conditions. The results of these co-culture experiments suggest that the TCR stimulation increased the generation of a soluble factor by lymphoma cells that affected a decrease of the proliferation and number of NK cells.

## 4. Discussion

The necessity of TCR signaling in the generation of T cell lymphomas has been a long-standing concept [3]. This concept is supported by the relative intactness of TCR signaling pathways in most human T cell lymphomas, in contrast to other pathways such as those initiated by cytokine binding [4]. Further support comes from mutations that frequently activate portions of the TCR signaling cascade in the same tumors. In addition, mouse models appear to conclusively demonstrate a need for intact TCRs in some models [8,13].

While most emphasis has been given to finding evidence for a general TCR requirement in T cell lymphomagenesis [8,13], less effort has been made to delineate the downstream effects of TCR signaling that confers growth advantage to T cell lymphomas. It appears generally assumed that TCR engagement endows lymphoma cells with a proliferative advantage similar to its effect on non-transformed T lymphocytes [4]. We have indeed observed such an increase of proliferation following TCR engagement in vivo causing reduced mouse survival under NK cell-depleted conditions. However, TCR engagements of non-transformed T lymphocytes also affects mechanism that are not or not exclusively connected to their own expansion. These include the generation of cytokines that orchestrate response types such as Th1, Th2, Th9, etc. to respond to demands for particular immune responses. Investigating the type of downstream effects of engaging the TCR of lymphoma cells in our model allowed us to observe a response that may be part of such a complex orchestration: The ability to reduce the NK cell response to IL-15 may reflect a physiological response in which signaling by a specialized T cell would cause the attenuation of an NK response thus allowing subsequent alternative immune responses to take over.

It appears that the evolution of T cell lymphoma development has allowed to highjack this mechanism of NK cell response attenuation not for the purpose of orchestrating efficient responses but to affect its own survival. In this, T cell lymphomas in our model are not alone. The outcome of the TCR engagement in our lymphoma model, corrupting the NK cell arm of the immune system, is shared by a number of human tumors. Patients suffering from both AML and CML often exhibit defects of NK cell numbers, functions and phenotypes [14,15]. It is intriguing to speculate that similar mechanisms mediate the NK

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cell suppressing effects in AML/CML patients as they do in our T cell lymphoma model. However, this would require addressing the diverse origins of the NK cell suppressor from either monocytic cells in AML or T lymphocytic tumors in our model. One possible answer would be that the effect of NK cell suppression may not be direct in either or in both diseases. Answering this question would require the identification of the NK cell-inhibiting factor that we have been unable to achieve so far.

Our data may also have clinical implications. A disruption of the interactions between antigen-presenting cells and T cell lymphomas may decrease lymphoma growth rates and may attenuate lymphoma-induced NK cell inhibitions. Also, drugs that interfere with cascades downstream of the TCR such as ITK inhibitors may produce similar outcomes [16]. Lastly, boosting NK cell numbers and/or functions in vivo [17] or transferring ex vivo-expanded NK cells as applied to patients with AML [18] may also be of use in the treatment of T cell lymphomas.

In summary, our data suggest that the TCR engagement of T cell lymphoma cells constitute a tumor-promoting step that results in accelerated growth and the perturbation of surveilling NK cells.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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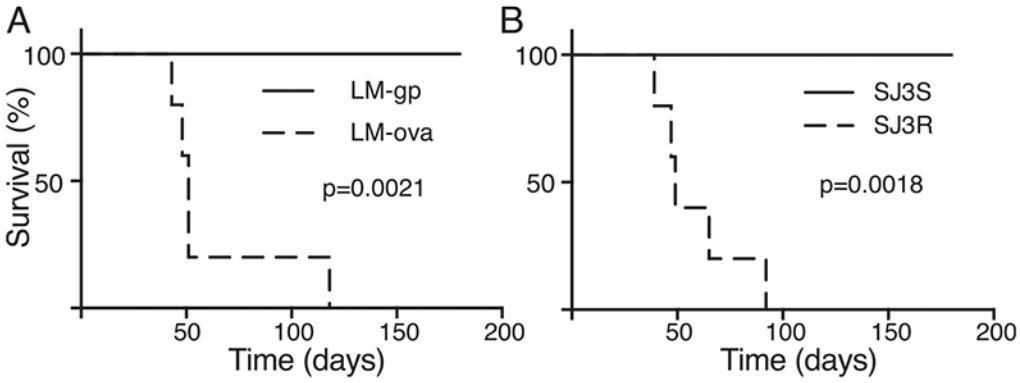


**Fig. 1.**

TCR engagement overcomes NK cell surveillance of T cell lymphomas. (**A**) SJ3 lymphoma cells were transferred into fully immune-competent mice (5 mice each without prior NK cell depletion, $10^6$ cells i.p. for all transfers in this figure) 24 h after immunizations with listeria monocytogenes that either expressed the cognate antigen ovalbumin or the unrelated protein LCMVgp. The same bacteria were added into the drinking water for an additional two weeks. Lymphoma growth in NK cell presence occurred after ovalbumin immunizations resulting in the death of recipient mice. (**B**) The resulting lymphomas (SJ3R) but not its pre-immunization precursors (SJ3S) regrew after transfer into immune-competent mice (5 mice each without prior NK cell depletion and without further immunizations) indicating a stable change to lymphoma cells.

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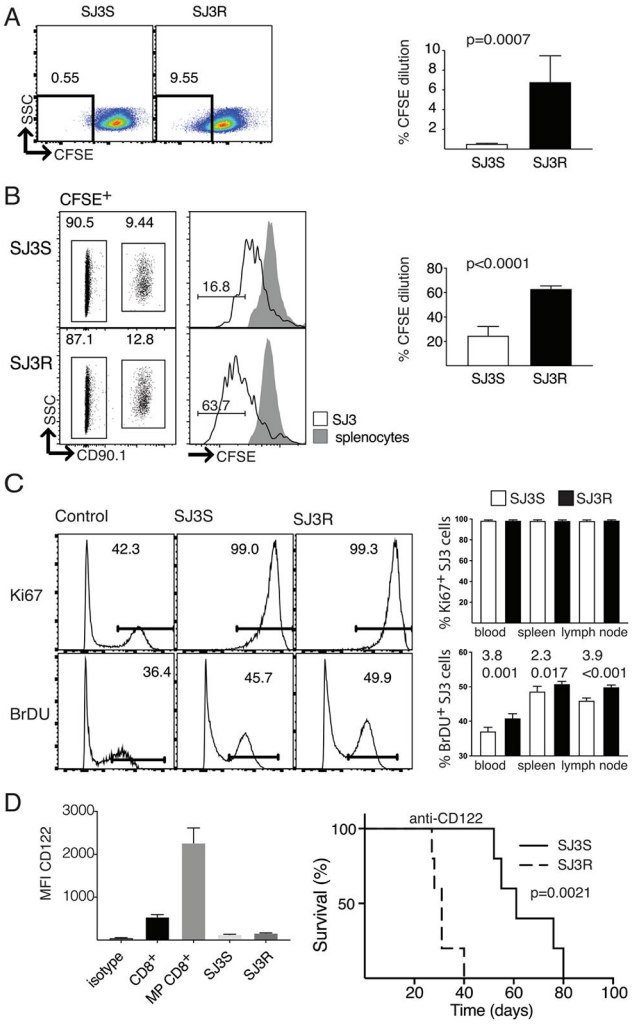


**Fig. 2.**

In vivo TCR signaling caused accelerated lymphoma growth. (**A**) Ex vivo proliferation rates differed between post-immunization (SJ3R) when compared with pre-immunization (SJ3S) lymphomas. Lymphoma cells from SJ3R-bearing mice retained higher ex vivo proliferation rates as shown by increased CFSE dilution rates of 24-hour cultures, n = 5. (**B**) The proliferation rates of CFSE-labelled SJ3S and SJ3R differed within 48 h after transfers. We transferred donor splenocytes that harbored SJ3S or SJ3R into NK cell-deleted recipient mice. FACS analyses targeted transferred cells by gating on $CFSE^+$ cells. We compared CFSE dilution rates of transferred normal donor splenocytes ($CD90.1^-$) versus transferred SJ3S and SJ3R ($CD90.1^+$). CFSE dilutons did not differ between transferred normal donor splenocytes while dilution rates were higher for SJ3R when compared to SJ3S, 5 mice each. (**C**) Proliferation differences were less pronounced at later stages of lymphoma development. We performed ex vivo analyses of proliferation markers in mice after the appearance of leukemic cells. It showed that nearly all lymphoma cells expressed Ki67 for both lymphomas, FACS panels on left depict an example of control T cells and tumor cells in spleens, graphs show averages of 5 mice on right. Analyzing BrDU incorporation rates 12 h after BrDU injections revealed small but significant differences. Numbers above bars

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represent mean differences and p-values, 5 mice each. (**D**) Survival rates differed for mice carrying either SJ3S or SJ3R in the absence of NK cells. The expression rates of CD122 are shown on the left for total and 'memory phenotype" (MP) CD8 T cells as well as for SJ3S and SJ3R. The graph on the right shows that survival rates were significantly shorter for SJ3R-bearing mice even when NK cells had been depleted for the duration of the experiment by anti-CD122 injections.

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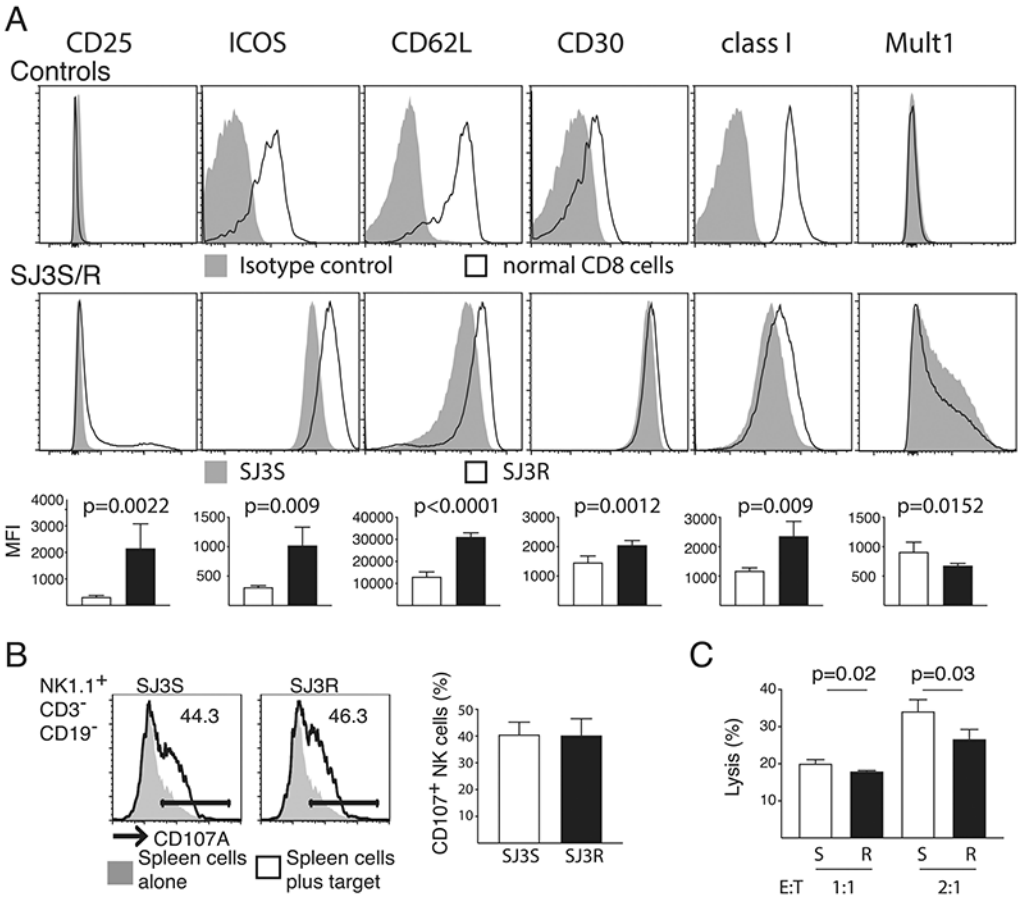


**Fig. 3.**

Phenotypical changes in lymphoma cells after in vivo TCR engagement. (**A**) We observed significant expression changes for CD25, ICOS, CD62L, and for CD30. Top row panels show iso type controls and the specific stains on normal CD8 T cells, bottom row panels compare SJ3S with SJ3R cells. Bar graphs show differences of MFI averages of 5 independently grown lymphomas. Amounts of surface MHC class I and Multi were also altered that we have previously shown to be involved in NK cell recognition [2]. No changes were observed in the remaining previously analyzed surface markers [2]. (**B**) Lymphoma cells prior to (SJ3S) and after in vivo immunization (SJ3R) were used as target cells and revealed a similar ability to induce the degranulation of NK cells as measured by CD107 stains. A representative experiment on the left and averages on right are shown, n = 3. (**C**) Lysis assays using splenic NK cells as effectors revealed differences between SJ3S and SJ3R as target cells within 20 h, n = 3.

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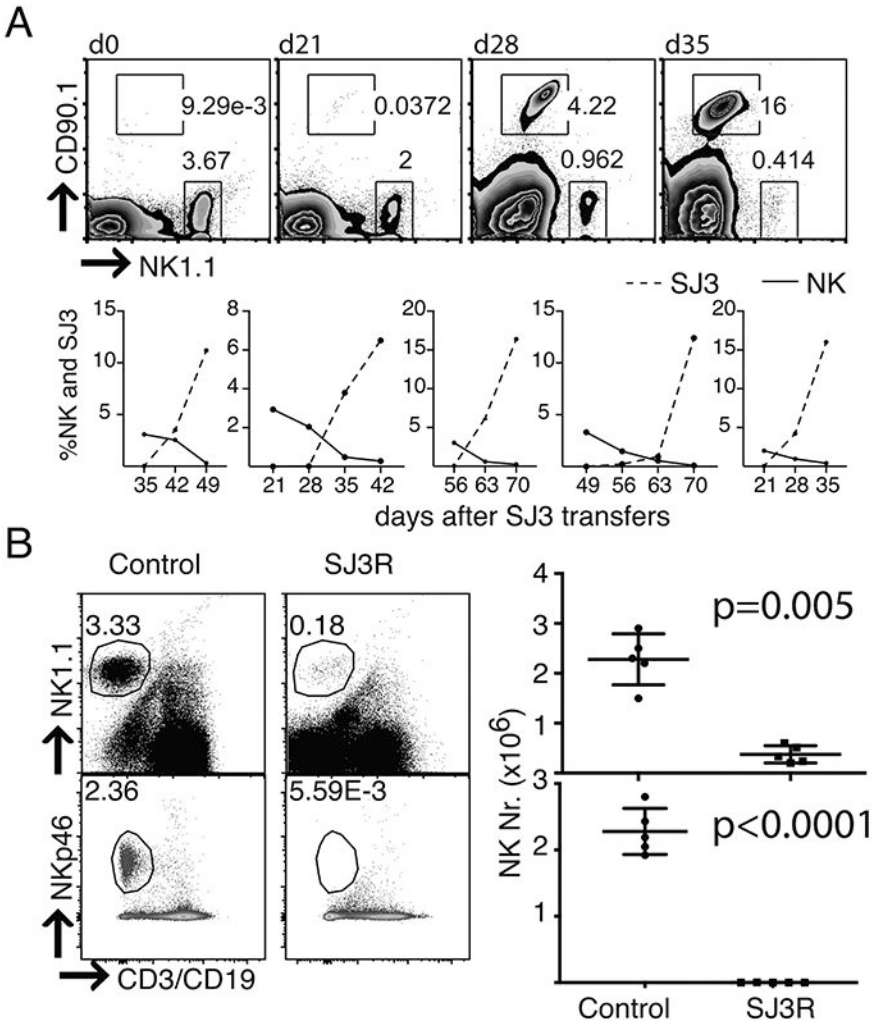


**Fig. 4.**

In vivo immunizations of T cell lymphoma cells decrease NK cell numbers in vivo. (**A**) Weekly cytometric analyses of tail blood PBMCs from mice that had been immunized with ovalbumin-expressing L. monocytogenes and transferred with T cell lymphoma cells ($CD90.1^+$, recipients are $CD90.1^-$) revealed appearances of lymphoma cells in blood starting between three and eight weeks that was followed by a gradual decrease in the number of NK cells from the same blood samples. Representative of five mice, percentages of NK and SJ3 cells among PBMCs for each individual mouse are depicted in line graphs. (**B**) The growth of post-immunization T cell lymphomas was associated with a reduced number of splenic NK cells. We enumerated NK cell numbers in spleens from mice that had succumbed to the lymphomas. Representative stains at the left show reduced percentages of $NK1.1^+/CD3^-/CD19^-$ or of $NKp46^+/CD3^-/CD19^-$ NK cells among splenocytes. Splenic lymphoma cells had been excluded from the analysis based on size. Averages of five mice are shown on right.

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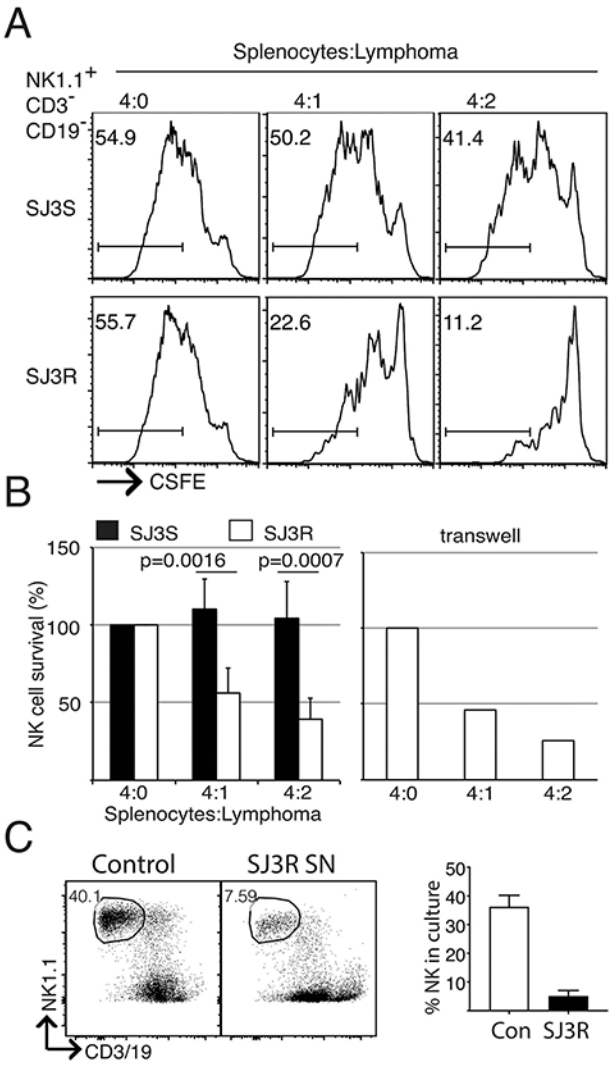


**Fig. 5.**

Post-immunization T cell lymphoma cells generate a soluble factor that suppresses NK cell proliferations in vitro. SJ3 lymphoma cells prior to (S) or after in vivo immunization (R) were co-cultured with normal CFSE-labeled splenocytes at the indicated ratios, and proliferation rates were estimated based on CFSE dilution. (**A**) A decrease caused by the presence of SJ3S was observed at its highest number only while SJ3R caused stronger NK cell inhibitions at lower lymphoma cell numbers. Representative of three experiments. (**B**) The presence of post- but not pre-immunization SJ3 lymphoma cells reduced the number of surviving NK cells after 5 days of culture, shown on left. Three experiments per data point. The physical separation of lymphoma and NK cells by trans-well membranes resulted in a similar NK cell inhibition as in B suggesting the generation of a soluble factor by post-immunization lymphoma cells, right. (**C**) IL-15-driven expansions of NK cell numbers were reduced by the presence of supernatants (20%) from cultured SJ3R cells in five-day spleen cultures. Left shows representative FACS panels, bar graph depicts means of 5 cultures.