Direct and immune-mediated cytotoxicity of interleukin-21 contributes to anti-tumor effects in mantle cell lymphoma

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Key points

- IL-21 activates IL-21R dependent singling to mediate direct cytotoxicity of mantle cell lymphoma cells.
- Indirect effects of IL-21 on immune effector cells also contribute to anti-tumor effects against mantle cell lymphoma.
Abstract

Mantle cell lymphoma (MCL) is a distinct subtype of non-Hodgkin lymphoma characterized by overexpression of cyclin D1 in 95% of patients. MCL patients experience frequent relapses resulting in median survival of 3-5 years, requiring more efficient therapeutic regimens. Interleukin 21 (IL-21), a member of the IL-2 cytokine family, possesses potent anti-tumor activity against a variety of cancers not expressing the IL-21 receptor (IL-21R) through immune activation. Previously, we established that IL-21 exerts direct cytotoxicity on IL-21R-expressing diffuse large B cell lymphoma cells. Herein we demonstrate that IL-21 possesses potent cytotoxicity against MCL cell lines and primary tumors. We identify that IL-21-induced direct cytotoxicity is mediated through STAT3-dependent cMyc up-regulation, resulting in activation of Bax and inhibition of Bcl-2 and Bcl-X₁. IL-21-mediated cMyc up-regulation is only observed in IL-21-sensitive cells. Further, we demonstrate that IL-21 leads to NK-cell dependent lysis of MCL cell lines that were resistant to direct cytotoxicity. In-vivo treatment with IL-21 results in complete FC-muMCL1 tumor regression in syngeneic mice via NK and T-cell dependent mechanisms. Together, these data indicate that IL-21 has potent anti-tumor activity against MCL cells via direct cytotoxic and indirect, immune-mediated effects.
Introduction

Mantle cell lymphoma (MCL) is a morphologically distinct subtype of lymphoma that accounts for 6-8% of non-Hodgkin lymphomas (NHL). MCL is characterized by the chromosomal translocation (11;14)(q13;q32) that juxtaposes $CCND1$ to the immunoglobulin heavy chain ($IGH$) gene enhancer region\(^1\). This translocation leads to constitutive overexpression of cyclin D1, resulting in early expansion of neoplastic B cells in the lymphoid follicle mantle zone, contributing to increased cell proliferation.

MCL is a highly aggressive disease with a median overall survival (OS) of 3 to 5 years\(^2-4\). Although treatment with conventional chemotherapy results in an overall response of 60-80%, the majority of patients relapses and succumb to MCL\(^5\). The addition of the anti-CD20 antibody rituximab to first line therapy led to improved complete remission rates, but did not prolong progression free and OS\(^6\). Consolidation with autologous stem cell transplantation improved response rates and duration, but did not result in lasting remissions\(^7\). Studies incorporating cytarabine (ARA-C) as part of the initial regimens led to marked increases in complete response rates and prolonged survival, yet failed to lead to cure of patients\(^8\). Consequently, there is an urgent need to develop newer therapeutic approaches for MCL.

Interleukin-21 (IL-21), a member of the IL-2 cytokine family, is mainly secreted by CD4\(^+\) T, natural killer (NK) and Th17 cells\(^9\). Upon binding of IL-21 to IL-21 receptor (IL-21R), which contains the common cytokine receptor γ chain (γc), Jak-1 and Jak-3 are activated, leading to STAT1, STAT3 and STAT5 phosphorylation. Dimerization of phosphorylated STAT proteins results in nuclear translocation and transcription of target genes. IL-21 exerts diverse regulatory effects on NK, NKT and B cells\(^10\). IL-21 induces B-cell proliferation, differentiation or apoptosis.
depending on the cellular context and type of stimulus\textsuperscript{10-14}. Surprisingly, unlike other $\gamma_c$ family members, IL-21 exhibits pro-apoptotic effects on activated and naïve B-cells\textsuperscript{15}.

IL-21 anti-tumor activity was demonstrated in multiple preclinical studies as single agent or in combination with chemotherapy\textsuperscript{16-24} and was evaluated in clinical trials for renal cell carcinoma and metastatic melanoma\textsuperscript{24-26}. In solid tumors not expressing IL-21R, the anti-tumor effects of IL-21 are mediated via NK or/and CD8$^+$ T-cell activation\textsuperscript{21,27,28}.

We have previously demonstrated that IL-21 exerts direct cytotoxicity on IL-21R expressing diffuse large B cell lymphoma (DLBCL) cell lines and primary tumors \textit{in-vitro} and \textit{in-vivo}\textsuperscript{18}. In our study IL-21-induced cell death was mediated via STAT3 dependent cMyc up-regulation, resulting in activation of the intrinsic apoptosis pathway. \textit{In-vitro} studies also demonstrated that IL-21 exerts direct cytotoxicity in chronic lymphocytic leukemias (CLL) and MCL cell lines, but via different mechanisms: caspase-8 activation leading to Bid cleavage followed by caspase-3 activation\textsuperscript{17,19} and STAT1 activation, respectively\textsuperscript{22}. The distinct cellular mechanisms of IL-21-mediated cytotoxicity in different B cell tumors were unexpected and surprising. However, in the case of MCL, the report was based on \textit{in-vitro} studies in only 2 cell lines. To reconcile these findings and to more carefully examine the potential role of IL-21 for MCL therapy, we analyzed the direct IL-21-mediated effects on survival, proliferation and apoptosis in a large set of MCL cell lines and primary tumors. Further, we also examined the indirect immunostimulatory effects of IL-21 signaling on NK and T cells and their contribution to its anti-tumor activity in MCL.
Materials and Methods

Reagents, antibodies, cell lines, primary tumors and in-vitro studies.

Reagents, acquisition of primary tumors and statistical methods are described in the Supplemental Materials and Methods.

MCL cell lines: Mino, HBL-2, SP-53, Jeko-1, IRM-2, G-519, L-128, Z-138 and DLBCL cell line RC-K8 were cultured in RPMI 1640 medium (Gibco BRL) supplemented with 10% heat inactivated fetal bovine serum (FBS; Mediatech), 2mM glutamine (Gibco BRL), and 100U/L penicillin/streptomycin (Gibco BRL). HeLa and 293T cells were cultured in Dulbecco modified Eagle medium (Mediatech) supplemented with 10% FBS, 2mM glutamine, and penicillin/streptomycin. The FC-muMCL1 mouse lymphoma cell line, generated from the splenocytes of a 1 year old male Eμ-cyclin D1 transgenic C57BL/6 mouse injected with pristane, was a kind gift of Dr. M.R. Smith (Cleveland Clinic, Ohio)29. These cells were maintained in RPMI 1640 medium supplemented with 15% FBS, 1mM non-essential amino acid, 1mM Na-pyruvate, 2mM glutamine, and penicillin/streptomycin.

Mice studies

To assess the in-vivo efficacy of IL-21 in a murine MCL model, four week old female C57BL/6 mice (Charles River) were subcutaneously (s.c.) inoculated with FC-muMCL1 cells (10×10^6 cells/100μl RPMI) into the right flank. All mice were housed according to institutional animal care and use committee (IACUC) guidelines in accordance with an approved protocol and monitored on a daily basis for tumor growth. Once tumor volume reached 100mm^3, mice were divided into two experimental groups (n=5/group) and treated intratumorally with PBS (50μl) or IL-21 (10μg) twice a day in the first week and once a day for the following week. Tumor-bearing mice were assessed every two days for weight loss and tumor volume by
standard calipers. Mice were sacrificed once the tumor volume exceeded 1500mm³ or if weight loss of more than 10% total body weight was observed. For toxicity studies, tumor bearing C57BL/6 mice were treated *i.v.* with IL-21 or PBS. Starting at 4 days after first injection, liver, kidney, lungs, heart, brain, and spleen were extracted at different time intervals, stained with Hematoxylin and Eosin (H&E) and examined by histology. Complete blood cell count and serum chemistry analyses were performed during follow up.

**In-vivo lymphocyte subsets depletion**

For *in-vivo* CD4, CD8 and NK cells depletion, C57BL/6 mice were treated *i.v.* with anti-mouse CD4 (GK1.5), CD8 (2.43) antibodies (BioXcell) and anti-asialo GM1 (Wako Chemicals) on days -7, -5 and 0 (where day 0 is the day of tumor inoculation). Animals treated with control Ig with irrelevant specificity were used as non-depleted controls. The antibody injections were repeated *i.v.* every 6 days until the end of study. Depletion of lymphocyte subsets were confirmed by flow cytometry. Control or NK, CD4 and CD8 depleted mice were implanted with FC-muMCL1 cells and treated as described above.

**Results**

**MCL cell lines and primary tumors express IL-21R on cell surface**

Analysis of cell-surface IL-21R expression in a panel of 8 MCL cell lines demonstrated heterogeneous expression levels, with highest levels on Mino cells (Figure 1A). All cell lines were IL-21R positive. MCL cells isolated from primary tumors also expressed IL-21R at different levels and proportions of MCL cells (4 MCL tumors had more than 90% cells expressing IL-21R and 3 tumors had IL-21R expression in 18 to 56% of cells, Supplemental Figure S1A-B).
IL-21 triggers cell death of MCL cell lines and primary tumors

We next assessed the effects of IL-21 on apoptosis and cell death. It has been previously reported that IL-21 directly induces growth arrest and apoptosis in the MCL cell lines Mino and SP-53. To expand these studies, we initially analyzed IL-21 dose response by treating Mino cells with increasing cytokine concentrations (Supplemental Figure S2). IL-21-induced cell killing plateaued at the 100ng/mL, similar to our previous study in DLBCL. The 100ng/ml concentration of IL-21 was previously commonly used in multiple studies and can be achieved in patients’ serum using IL-21-doses evaluated in clinical trials. Consequently, we selected 100ng/ml IL-21 dose and analyzed its cytotoxic effects in additional MCL cell lines by flow-cytometry using YO-PRO and PI staining assay. Cell lines and primary tumors were defined as “sensitive/responsive” to IL-21 if IL-21 treatment induced greater than 15% increase in cell death compared to control cells (Figure 1B). IL-21 led to a marked increase in cell death of Mino, HBL-2 and SP-53 cells compared to untreated counterparts. In contrast, Jeko-1, IRM-2, L-128, Z-138 and G-519 cells were resistant to IL-21 treatment (percentage increase in cell death < 15% upon IL-21 treatment, Figure 1B). Time-course analysis over 5 days revealed that IL-21–induced apoptosis was time-dependent with progressive transition from early apoptotic cells (YO-PRO+/PI) to late apoptotic cells (YO-PRO+/PI+) (Supplemental Figure S3). Noticeably, IL-21-induced killing was specific to MCL cell lines as non-B cells (293T) were not affected (Figure 1B).

We next evaluated the effects of IL-21 on 7 de novo, untreated primary human MCL specimens. IL-21 induced cell death in 3 of 7 primary tumors, with all susceptible tumors expressing IL-21R in more than 90% of cells (Figure 1C and Supplemental Figure S1A-B). The remaining 4 primary tumors were resistant to IL-21 treatment irrespective of the percentage of
cells expressing IL-21R (Supplemental Figure S1A-B). These findings implied that IL-21R expression is not sufficient for IL-21-induced cell death, since not every cell line and primary tumor with surface IL-21R expression exhibited cell death upon IL-21 treatment (Supplemental Figure S1C). Noticeably, none of the normal IL-21R expressing B-cells obtained from blood or lymph nodes of healthy volunteers were killed by IL-21, suggesting specificity of cytotoxic effects to the tumor cells (Figure 1D). Overall, our data demonstrate a non-uniform response of MCL cell lines and primary tumors to IL-21’s direct cytotoxicity.

**IL-21 induces signaling via tyrosine phosphorylation of STATs in MCL cell lines and primary tumors**

Previous reports have indicated that IL-21 can activate STAT family members STAT1 and STAT3\(^{18,19,22}\) and to a lesser extent STAT5\(^{18}\). To this end, we stimulated MCL cell lines with IL-21 (100ng/mL) and analyzed tyrosine phosphorylation of STAT proteins by immunoblotting. IL-21 stimulation resulted in phosphorylation of STAT1 (pSTAT1) and STAT3 (pSTAT3) in IL-21 responsive and resistant MCL cell lines. Noticeably, pSTAT1 and pSTAT3 levels were not significantly different between the resistant and responsive cell lines (Figure 2A). Similar to cell lines, IL-21 induced STAT1 and STAT3 phosphorylation in both responsive and resistant primary MCL specimens (Figure 2B). IL-21 also induced phosphorylation of STAT5 in some of the analyzed cell lines and primary tumors irrespective of IL-21-induced apoptosis (Supplemental Figure S5).

**IL-21 induced apoptosis of MCL cells is cMyc-dependent**

Since STATs activation was observed in both IL-21 responsive and resistant cell lines, we analyzed pathways downstream of these proteins to elucidate key players mediating IL-21-induced apoptosis. In DLBCL we have previously demonstrated that IL-21 induces cell death by
STAT3-mediated induction of cMyc. To examine for a similar intracellular signaling mechanism in MCL, we analyzed cMyc protein levels following IL-21 treatment in responsive and resistant MCL cell lines and primary tumors. Sensitive MCL cell lines (Mino, HBL-2 and SP-53) and a primary tumor (Specimen-1) exhibited significant increases in cMyc protein levels at 24 hours post IL-21 treatment (Figure 3A). In contrast, IL-21 did not induce an increase in cMyc protein levels in resistant cell lines (Jeko-1, L-128 and G-519) and primary cells (Specimen-4), suggesting that cMyc may play a critical role in IL-21-induced cell death in MCL (Figure 3A).

To determine whether cMyc up-regulation is required for IL-21’s apoptotic effects, we knocked down cMyc using specific siRNAs. cMyc knockdown prevented cMyc up-regulation following IL-21 treatment and blocked IL-21–induced cell death as opposed to nontargeting control siRNA (p<0.005; Figure 3B). To further confirm the role of cMyc in mediating the cytotoxic effects of IL-21, we transfected IL-21 resistant MCL cell lines with a cMyc expressing vector. Transient cMyc overexpression mildly (not statistically significantly) increased the rate of spontaneous cell death in the G-519 but not L-128 MCL cell lines. Importantly, cMyc overexpression markedly increased IL-21-induced cytotoxicity in both of these previously IL-21-resistant MCL cell lines (p<0.05; Figure 3C). Overall, these data suggest that cMyc up-regulation is imperative for direct IL-21 cytotoxicity in MCL, similarly to DLBCL.

**IL-21 induces MCL apoptosis via STAT3 and Bax**

cMyc is known to induce apoptosis via up-regulation of proapoptotic Bax and down-regulation of anti-apoptotic Bcl-2 and Bcl-X<sub>L</sub>. Thus, we analyzed the expression of these proteins in MCL cell lines and primary tumors following IL-21 treatment. IL-21 treatment resulted in Bax up-regulation and downregulation of Bcl-2 and Bcl-X<sub>L</sub> in the IL-21 sensitive cell
line Mino but not in the IL-21 resistant line Jeko-1 (Figure 4A). Moreover, increased Bax expression was observed in sensitive but not resistant primary tumors (Supplemental Figure S4). These findings suggest that similarly to DLBCL, IL-21-induced cMyc up-regulation modulates expression of Bcl-2 family proteins to trigger cell death.\(^{18}\)

Bax is reported to be essential for cMyc–induced apoptosis.\(^{33,34,36}\) To demonstrate that observed changes in Bax expression might contribute to IL-21-induced apoptosis in MCL, siRNAs were used to knock-down Bax. Bax knockdown markedly decreased apoptosis after IL-21 treatment (Figure 4B). These findings suggest that Bax is a downstream effector of cMyc-induced apoptosis, as we reported in DLBCL.\(^{18}\)

We next interrogated the upstream signaling cascade mediating the IL-21-induced increase in cMyc expression. cMyc is a known target of STAT3,\(^{37-39}\) which was shown to be activated in MCL cell lines and primary tumors upon IL-21 stimulation (Figure 2A). We knocked down STAT3 expression using specific siRNAs to analyze the role of STAT3 in regulating cMyc expression following IL-21 stimulation. STAT3 knockdown completely blocked apoptosis after IL-21 stimulation (p<0.001; Figure 4C), similarly to our results in DLBCL.\(^{18}\) Taken together, these findings confirm the role of STAT3 in mediating IL-21-induced apoptosis in MCL.

However, previously published data suggested that IL-21-induced apoptosis of MCL cells is mediated via STAT1 dependent signaling.\(^{22}\) To interrogate the potential contribution of STAT1 to IL-21-induced apoptosis in MCL, we knocked down STAT1 using specific siRNAs. In contrast to the previous report, complete STAT1 knockdown did not affect IL-21-induced apoptosis in the Mino cell line (Figure 4D). siRNA-induced STAT3 and STAT1 knockdown was specific (Supplemental Figure S5A), excluding the possibility of concomitant knockdown of
STAT1 by the STAT3 siRNAs. Gelebart et al previously used IL-21 at doses of 20 and 50ng/mL, while we used IL-21 at a dose of 100ng/mL. Repetition of experiments using IL-21 at doses of 20 and 50ng/mL also demonstrated that IL-21-induced MCL killing was not mediated by STAT1 (Supplemental Figure S5B). We further validated that IL-21 induces MCL killing by activation of STAT3 and not STAT1 by overexpressing dominant negative (DN) STAT1 and STAT3 proteins in Mino cells before treatment with IL-21. In absolute concordance with our STAT3 and STAT1 siRNA-mediated knock-down studies, DN STAT3 but not DN STAT1 protein ameliorated IL-21-induced apoptosis (p=0.007, Supplemental Figure S6). Taken together, our findings demonstrate that IL-21-induced STAT3 activation causes a prohibitively high induction in cMyc expression, leading to Bax up-regulation and Bcl-2 and Bcl-XL downregulation, resulting in apoptosis in IL-21 sensitive cells. Furthermore, based on our findings, it is potentially possible to predict MCL sensitivity to direct IL-21 cytotoxicity by evaluating the change in cMyc expression following in-vitro stimulation with IL-21.

Baseline mitochondrial priming does not correlate with IL-21 sensitivity in MCL cell lines

To further exclude the possibility of nonspecific IL-21-induced killing, we examined baseline mitochondrial priming in IL-21 sensitive and resistant cell lines. It was previously shown that baseline cell mitochondrial priming to apoptosis may determine sensitivity of cancer cells to different cytotoxic agents\textsuperscript{40,41}. Mitochondrial priming is controlled by the BCL-2 family of pro-apoptotic and anti-apoptotic proteins\textsuperscript{42,43}. We adopted the BH3 profiling technique to determine the magnitude of response of mitochondria to peptides derived from the BH3 domains of pro-apoptotic proteins via measuring cytochrome C release. The greater the loss of cytochrome C caused by the BH3-only peptides, the cells are more primed for death. We BH3 profiled MCL cell lines to determine their priming status using different concentrations of BIM.
BH3 peptide (that promiscuously inhibits all anti-apoptotic members)\textsuperscript{43} and PUMA BH3 peptide (that inhibits response to sensitizers such as BAD, NOXA and HRK)\textsuperscript{43}. The peptides gain access by diffusion through a plasma membrane that has been permeabilized with low concentrations of digitonin. We found that Mino cells showed highest level of priming, as evident by lowest IC50 for both BIM and PUMA peptides compared to Jeko-1, Z138 or L-128 cell lines (Supplemental Figure S7). Indeed, this data correlates with cytotoxicity data of IL-21 where Mino cell line showed the highest cell death in response to IL-21. However, IL-21 responsive cell line HBL-2 showed lower priming compared to resistant Jeko-1 cells. These findings suggest that mitochondrial priming alone may not serve as a predictive biomarker to determine IL-21’s response in MCL cells. Further, these finding suggest that IL-21 is inducing apoptosis not only in MCL cells that are primed to die.

**IL-21 induces indirect cytotoxicity through NK-cell mediated lysis**

Apart from IL-21R-dependent direct cytotoxicity, IL-21-mediated immune cell activation is also known to contribute to its anti-tumor effects against a variety of cancers\textsuperscript{21,28,44,45}. To clarify the contribution of IL-21-induced immunomodulatory effects to its anti-MCL activity, we performed cell cytotoxicity assays using NK cells isolated from peripheral blood. As opposed to the direct cytotoxicity, immune-mediated effects of IL-21 are commonly induced by lower IL-21 concentrations (e.g. 10ng/mL)\textsuperscript{45}. Consequently, human NK cells were stimulated overnight with IL-21 (10 ng/mL) or PBS. IL-21 significantly enhanced NK cell cytolytic activity against Jeko-1 (Figure 5A) and MCL primary tumors (Figure 5B), while B-cells isolated from healthy volunteers were unaffected (Figure 5B). Consequently, primary MCLs and MCL cell lines that are resistant to direct IL-21-mediated cytotoxicity may be sensitive to IL-21-activated NK-cell mediated cell lysis.
Preclinical studies have demonstrated that IL-21 augments the ADCC activity of antibody coated target cells by enhanced NK cell activation in CLL primary tumors and animal B-cell lymphoma and breast cancer models\textsuperscript{17,45}. However, this phenomenon was not explored in MCL tumors. To extend these findings, we tested the ability of IL-21 to enhance ADCC against MCL primary tumors coated with an anti-CD20 mAb (rituximab). IL-21-activated NK cells exhibited significantly increased rituximab-specific ADCC activity against tested primary tumors (p<0.005 and p<0.01; Figure 5C).

**In-vivo IL-21 treatment induces tumor regression in a syngeneic murine model of MCL**

IL-21’s proapoptotic activity in MCL cell lines and primary tumors suggests that IL21 may be useful to treat MCL patients. Consequently, we examined the *in-vivo* efficacy of IL-21 against syngeneic, immunocompetent MCL mouse model, FC-muMCL1 derived from splenocytes of E\(_{\mu}\)-cyclin D1 transgenic C57BL/6 mouse\textsuperscript{29}. Impressively, IL-21 treatment resulted in complete MCL regression in all animals (Figure 6A), resulting in significantly longer survival compared to control mice (p<0.0001, Figure 6B). IL-21 treated mice remained disease free for more than 6 months and no tumors were detected upon extensive postmortem analyses of the IL-21 treated mice. Similar to previous reports in animals and humans\textsuperscript{20,24,30}, the IL-21 treatment was well tolerated and the mice continued to gain weight during treatment and showed no signs of toxicity. We did not detect any changes in blood cell counts, electrolytes, kidney and liver functions (not shown). Further, extensive pathological studies of normal organs did not reveal any pathological findings (Supplemental Figure S8A-L).

To determine whether the observed IL-21 activity on the FC-muMCL1 tumors and immune microenvironment was direct or indirect, we examined the effects of *in-vitro* IL-21 treatment on FC-muMCL1 cell death. Despite expression of IL-21R and pSTAT-3 activation
upon IL-21 treatment, we did not observe cMyc upregulation and hence FC-muMCL1 cells remained unaffected by *in-vitro* IL-21 treatment (Figure 6C and D). Overall, these results indicate that IL-21 does not exhibit direct cytotoxic effects on FC-muMCL1 cells, suggesting that the observed *in-vivo* IL-21 anti-tumor effects in the syngeneic mouse model were immune-mediated, as reported in other solid tumors types\textsuperscript{21}.

**In-vivo anti-tumor effects of IL-21 are dependent on NK and T cells**

Earlier studies demonstrated that IL-21 may induce anti-tumor immunity through NK-cells and/or T-cell (CD4\textsuperscript{+} or CD8\textsuperscript{+}) activation, depending on the tumor model\textsuperscript{21,28}. To elucidate the direct contribution of distinct effectors to IL-21 therapeutic efficacy, we carried out cell subset depletion experiments using specific monoclonal antibodies. Mice were first depleted of lymphocytes (NK, CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells) followed by subcutaneous tumor implantation with FC-muMCL1 cells. Each depletion was cell-subset specific and did not affect other cellular subsets (Supplemental Figure S9A-C). Depletion of NK cells, CD4\textsuperscript{+} or CD8\textsuperscript{+} cells did not significantly alter the rate of tumor engraftment and animal survival (Supplemental Figure S9D). IL-21 treated NK cell depleted mice showed significantly shorter survival compared to non-depleted controls (p<0.01), with 80% of animals dying from the lymphoma, suggesting that the IL-21 anti-tumor effect is NK cell-dependent (Figure 7A). CD4\textsuperscript{+} depletion also abrogated the IL-21 anti-tumor effects, resulting in shorter animal survival compared to non-depleted IL-21 treated animals (p<0.05, Figure 7B). Furthermore, NK and CD4\textsuperscript{+} cell depleted animals treated with IL-21 exhibited significantly shorter survival compared to IL-21 treated NK depleted mice (p=0.002). The survival of these animals was similar to the survival of animals not receiving IL-21 treatment. CD8\textsuperscript{+} T-cell depletion only minimally decreased survival in IL-21 treated animals (Figure 7C). There was no statistically significant difference in the survival between animals
depleted of NK cells only versus animals concomitantly depleted of both NK and CD8⁺ T-cells (Figure 7C). Overall, these depletion studies indicate that IL-21-mediated anti-MCL effects on the FC-muMCL1 tumors require both NK and CD4⁺ T-cells with minimal contribution from CD8⁺ T cells.

**IL-21 promotes NK cell and CD4⁺ T cell activation and cytotoxic function**

Because NK and CD4⁺ T cells accounted for tumor inhibition by IL-21, we next investigated the effects of IL-21 on cytotoxic function of these cell subsets. IL-21 markedly activated NK cells, as measured by activation marker CD69 on C57BL/6 mice-derived NK cells treated with IL-21 both in-vitro and in-vivo (Supplemental Figure S10A-C). Furthermore, NK cells enriched from spleens of IL-21-treated C57BL/6 mice demonstrated significantly increased cytolytic activity against FC-muMCL1 cells at various T:E ratios compared to PBS-treated mice (p<0.001). Similarly, CD4⁺ T cells stimulated with IL-21 demonstrated enhanced in vitro lysis of FC-muMCL1 cells compared to unstimulated controls. This enhanced lysis was observed at T:E ratio of 1:10 (p<0.001, Figure 7E) with no further increase at increasing T:E ratio (data not shown). Moreover, IL-21 mediated effector activities of NK cells and CD4⁺ T cells were found to be restricted to the lymphoma cells since both cell types did not affect the lysis of normal B-cells isolated from peripheral blood of healthy volunteers (Figure 5B and 7F). Overall, these results suggest that the observed anti-tumor activity of IL-21 in the FC-muMCL1 syngeneic mouse model was mediated by stimulation of immune effectors and not by direct cytotoxicity. In contrast to animal studies, IL-21 enhanced NK but not CD4⁺ T cell cytolytic activity in human MCL cells line Jeko-1 (Figure 5A and not shown).
Discussion

MCL is characterized by a relatively short survival. Development of novel targeted therapeutic modalities is urgently needed to improve MCL patient survival. Herein we demonstrate that IL-21 can induce cell death of MCL cell lines and primary tumors and extend the survival of mice harboring syngeneic MCL. The anti-MCL activities of IL-21 were mediated via direct (IL-21R-STAT3-cMyc-dependent) and indirect (immune-mediated-NK-T-cell-dependent) cytotoxic effects. Further, our studies demonstrate that IL-21R was necessary, but not sufficient, for direct cytotoxic effects of IL-21 on MCL cells, since a subset of IL-21R expressing MCL cell lines and primary tumors were unresponsive to IL-21 therapy. Nevertheless, MCL cells resistant to the direct cytotoxic effects of IL-21 may still be killed in-vivo via indirect immune-mediated mechanisms. Contrary to our earlier study in DLBCL, where most cell lines and primary tumors were responsive to IL-21, MCL cells showed a heterogeneous response. Such varied response to IL-21 was also observed in CLL cells.

We demonstrate that IL-21 induced apoptosis of MCL cells is mediated through activation of the STAT3-cMyc pathway, leading to up-regulation of pro-apoptotic (Bax) and down-regulation of anti-apoptotic (Bcl-2 and Bcl-XL) proteins. In agreement with our study in DLBCL, IL-21-induced direct cytotoxic effects in MCL were STAT3 but not STAT1-dependent, since STAT1 knockdown by several siRNAs and use of DN STAT1 failed to rescue IL-21-induced cell death. Our report is consistent with a previous report showing that MCL cell lines are sensitive to IL-21 treatment, except that we found the cytotoxic effects to be dependent on STAT-3 instead of STAT1. Although the reasons for this difference is unclear, it is possible that the siRNAs used by Gelebart et al may non-specifically targeted STAT3, since in contrast to
our study, Gelebart et al\textsuperscript{22} did not evaluate the potential role of STAT3 in mediating IL-21’s direct cytotoxicity.

Apart from our study in DLBCL, STAT3 has been implicated in the induction of apoptosis in additional studies. STAT3 promoted cell death upon triggering terminal differentiation and apoptosis in myeloid leukemia M1 cells or during involution of mammary glands\textsuperscript{46}. Molecular factors determining whether STAT3 activation would result in cell survival or cell death still remain poorly understood. Sutherland et al showed that loss of SOCS3 is associated with the conversion of STAT3 into a proapoptotic transcription factor\textsuperscript{47}. However, in our previous study in DLBCL we demonstrated that SOCS3 expression was induced upon IL-21 treatment in both resistant and sensitive DLBCL cell lines\textsuperscript{18} and thus could not explain the mechanism behind proapoptotic effects of STAT3. It is likely that the strength and duration of STAT3 phosphorylation may contribute to the final phenotypic outcome, and studies exploring this possibility are needed.

Although STAT3 knockdown prevented IL-21-induced cell death, pSTAT3 levels were not significantly different between resistant and responsive MCL cell lines/primary tumors. This observation implies that STAT3 activation alone is not sufficient to mediate direct cytotoxic effects of IL-21 and hence cannot be used to predict anti-tumor responses to IL-21 in MCL cells. This prompted us to elucidate the underlying mechanisms of IL-21-induced direct cytotoxicity in MCL cell lines. In concordance with our findings in DLBCL\textsuperscript{18}, IL-21-induced cMyc up-regulation was observed only in cell lines and primary tumors responsive to IL-21, while resistant cell lines did not up-regulate cMyc. cMyc is a known target of STAT3 and has been implicated in regulating cell growth, differentiation and apoptosis\textsuperscript{38}. cMyc expression levels determine cell fate; low cMyc-levels promote cell proliferation while high cMyc levels induce
cell death\textsuperscript{48,49}. Knockdown of cMyc prevented IL-21-induced increases in cMyc expression, thus reducing MCL cell apoptosis. Correspondingly, cMyc overexpression in resistant MCL cell lines facilitated IL-21-induced cytotoxicity. Overall, these results are in agreement with our previous study that cMyc up-regulation is indispensable for IL-21-induced direct cytotoxicity. Moreover our data corresponds to studies from other groups that showed a link between STAT3 and cMyc activation\textsuperscript{50}. Based on these findings we propose that IL-21-mediated cMyc up-regulation may be utilized as a predictor of IL-21-induced direct cytotoxicity in MCL cell lines and primary tumors and should be evaluated in clinical settings.

In this study we have used a syngeneic MCL mouse model to determine IL-21 activity in-vivo. This allowed us to effectively examine the contribution of immune effector cells towards IL-21-induced anti-tumor activity. Using the FC-muMCL1 lymphoma model we demonstrate that IL-21 completely inhibits tumor growth, and the observed in-vivo anti-tumor effect was indirect and likely attributed to both NK-cell and CD4\textsuperscript{+} T-cell subsets, since depletion of these cells ameliorated IL-21 anti-tumor effects. Moreover, IL-21 also enhanced NK and CD4\textsuperscript{+} T cells cytolytic activity as demonstrated by increased lysis of \textsuperscript{51}Cr-labeled target tumor cells. NK cells were implicated in IL-21-mediated anti-tumor activity in solid tumor models of melanoma. Wang et al. reported that NK cell depletion completely abrogated IL-21’s anti-tumor activity\textsuperscript{21}. CD8\textsuperscript{+} but not CD4\textsuperscript{+} T cells also contributed to the IL-21 anti-tumor effects in this model\textsuperscript{21}. In contrast, CD8\textsuperscript{+} T cells were the major mediators of the IL-21 anti-tumor effects in a syngeneic mouse model of breast adenocarcinoma. Independent studies in syngeneic thymoma, B16 melanoma and renal carcinoma models showed increased CD8\textsuperscript{+} T-cell infiltration upon IL-21 treatment\textsuperscript{28}. In these studies, depletion of CD8\textsuperscript{+} but not CD4\textsuperscript{+} T cells or NK and NKT cells ameliorated IL-21 mediated anti-tumor responses\textsuperscript{21,27,28}. Collectively, as emphasized by the
earlier studies in solid tumors, the contribution of individual cell subset to *in-vivo* anti-tumor effects of IL-21 are tumor model and host microenvironment specific. Our study provides the first comprehensive evidence for IL-21-induced immune effectors-mediated anti-lymphoma activity.

In summary, we demonstrate that IL-21 exerts direct and indirect immune mediated cytotoxicity against MCL. A recent clinical trial of IL-21 in combination with rituximab for relapsed or refractory B-cell lymphomas showed clinical responses in 8 out of 19 patients\(^5\). However, patients with MCL were not included in this study. Since our data demonstrated that IL-21 enhances the ADCC activity of rituximab in MCL primary tumors and cell lines by stimulatory effects on NK cells, clinical studies evaluating the activity and immune mechanisms of IL-21 in MCL patients are warranted.
Acknowledgement

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Conflict-of-interest disclosure: All the authors reviewed the manuscripts and agree with its content and do not have conflicts of interest relevant to this manuscript
Authors Contributions:

Shruti Bhatt performed most of the laboratory work, analyzed the data, and wrote the paper; Julie Matthews, Salma Parvin, Kristopher A. Sarosiek, Dekuang Zhao, Elif Isik and Xiaoyu Jiang performed laboratory experiments;

Anthony Letai supervised experiments;

Izidore S. Lossos conceptualized the idea of the study, supervised the experiments, analyzed the data, provided funding and wrote the paper.
References


Figure Legends

Figure 1. Flow cytometric analysis of IL-21R expression in MCL cell lines. (A) The indicated MCL cell lines were stained for IL-21R and analyzed by flow cytometry. Grey histograms represent IL-21R-stained population while white histograms represent background fluorescence observed with matched isotype control. HeLa cells were utilized as a negative control. Data is representative of 3 independent experiments. (B) MCL cell lines (Mino, HBL-2, SP-53, IRM-2, Z-138, L-128, G-519 and Jeko-1), DLBCL cell line (RCK8; positive control), 293T cell line (negative control), (C) neoplastic B-cells isolated from de novo primary MCL tumors and (D) normal B-cells isolated from peripheral blood (PBL) or lymph node (tonsil) were treated with IL-21 (100ng/mL) for 72 hours. The cell death was measured by YO-PRO and PI staining followed by flow cytometry analysis. IL-21 responsive and non-responsive cell lines and primary tumors are indicated as sensitive (S) and resistant (R), respectively. In panels A-B, data is representative of 3 independent experiments and error bars correspond to standard deviation between triplicate samples from single experiment.

Figure 2. IL-21-induces activation of STAT1 and STAT3 in MCL cell lines and primary tumors. For panels A and B, MCL cell lines (Mino, HBL-2, SP-53, Jeko-1) and primary tumors (Specimens 1 and 4) were treated with IL-21 (100 ng/mL) for the indicated time periods. Whole cell lysates were prepared and subjected to immunoblotting to measure the levels of phosphorylated STAT1 and STAT3. Immunoblotting for nonphosphorylated STATs and GAPDH served as loading controls. Results in panels A and B are representative of 3 independent experiments.
Figure 3. Up-regulation of cMyc correlates with IL-21-induced direct cell death in MCL cells. (A) Immunoblotting for cMyc in IL-21 sensitive (Mino, HBL-2, SP-53, Specimen-1) and resistant (Jeko-1, G-519, L-128, Specimen-4) MCL cell lines and primary tumors treated with 100ng/mL IL-21 for 24h. cMyc protein levels quantified by densitometric analysis are displayed below each panel. (B) Mino cells transfected with cMyc siRNA and (C) L-128 and G-519 cells transfected with pcDNA3-cMyc plasmid were treated at 24h post transfections with IL-21 (100ng/mL). Percentage of cell death was quantified using flow-cytometry at 48h post treatment. Immunoblotting for cMyc at 24h post transfection were performed to confirm knock down or overexpression of cMyc. Results in panels A-C are representative of 3 independent experiments. GAPDH and actin served as loading controls (*p < 0.05 and ***p < 0.005).

Figure 4. IL-21 induces cell death via STAT3 and modulation of expression of Bcl-2 family genes but is independent of STAT1. (A) Immunoblotting for Bcl-2 family genes in IL-21 responsive (Mino) and resistant (Jeko-1) MCL cell lines following treatment with IL-21 at indicated time points. (B-D) Mino cells were first transfected with siRNA targeting STAT3 (B), Bax (C), and STAT1 (D) or control siRNA followed by treatment with IL-21 (100ng/mL) at 24h after transfection. Panels (A-D) indicate cell death analyzed by YO-PRO/PI staining at 48h post treatment. Immunoblotting for STAT-3, Bax and STAT1 was carried out at 24h post transfection to confirm proteins knockdown (B-D). Actin and GAPDH served as a loading control. Data is representative of 3 independent experiments. Error bars represent standard deviation between 3 experiments (*p < 0.05 and ***p < 0.005).

Figure 5. IL-21 increases NK-cell mediated lysis of MCL cells. Enriched human NK cells from peripheral blood were incubated with 51Cr-labeled (A) Jeko-1 and (B) MCL primary tumor cells coated with IL-21 at various effector/target (E/T) ratios as described in the Material and
Methods. In C, $^{51}$Cr-labeled MCL primary tumor cells were coated with rituximab alone or in the presence of IL-21, followed by incubation with NK cells at variable E/T ratios. Percentage of specific lysis was derived from $^{51}$Cr released by target cells. Data is representative of 3 independent experiments. Error bars represent standard deviation between triplicate wells (*p < 0.05, **p < 0.01, ***p < 0.0005 and ****p<0.0001).

**Figure 6. In-vivo IL-21 inhibits tumor growth and prolongs survival of MCL bearing mice.**

(A-B) C57BL/6 mice (n=5/group) were injected s.c. with FC-muMCL1 cells and treated with IL-21 or PBS as described in the Material and Methods. (A) Depicts tumor volume of lymphoma bearing mice and (B) illustrates Kaplan-Meier survival curves. (C) FC-muMCL1 cells were treated with 100ng/mL IL-21 for 72h and cell death was measured by YO-PRO and PI staining followed by flow-cytometry. (D) FC-muMCL1 cells were stimulated with 100ng/mL IL-21 in-vitro for 15 minutes or 24 hours followed by immunoblotting for phosphorylated STAT3 and cMyc, respectively. For panels A-D, data are representative of two independent experiments. Error bars represent standard error of the mean (SEM).

**Figure 7. In-vivo antitumor effect of IL-21 is NK and T cells dependent.** In A-B, C57BL/6 mice (n=5/group) depleted of (A) NK or treated with irrelevant Ig, (B) CD4$^+$ T or/and NK, and (C) CD8$^+$ T or/and NK cells were s.c. inoculated with FC-muMCL1 cells. Depicted here are Kaplan-Meier survival curves of mice treated with IL-21 or PBS (control) as described in the Material and Method. (D-E) FC-muMCL1 cells or (F) normal human B-cells from peripheral blood were labeled with $^{51}$Cr and incubated with purified mice NK cells (D) or mice CD4$^+$ T cells (E) or human CD4$^+$ T cells (F) at the indicated T/E ratios. Percentage of specific lysis was measured based on the $^{51}$Cr release. All samples were run in triplicates (n=3). Data in A-E are
representative of 2 independent experiments. (**p < 0.01 and ***p < 0.0005). Error bars represent standard error of the mean (SEM).
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Direct and immune-mediated cytotoxicity of interleukin-21 contributes to anti-tumor effects in mantle cell lymphoma

Shruti Bhatt, Julie Matthews, Salma Parvin, Kristopher A. Sarosiek, Dekuang Zhao, Xiaoyu Jiang, Elif Isik, Anthony Letai and Izidore S. Lossos