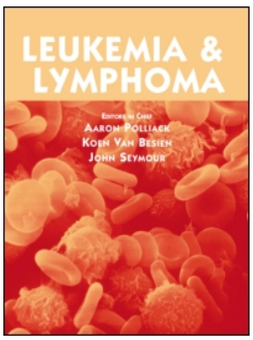

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REVIEW

Interleukin 21 – its potential role in the therapy of B-cell lymphomas

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ABSTRACT

Interleukin-21 (IL-21), a member of IL-2 cytokine family, has pleotropic biological effects on lymphoid and myeloid cells. During the past 15 years, since the discovery of IL-21, great advances have been made regarding its biological activity and the mechanisms controlling IL-21-mediated cellular responses, especially in hematological malignancies. Preclinical studies have shown that IL-21R is expressed on healthy and neoplastic B-cells and exogenous IL-21 can induce direct apoptosis of IL-21R expressing B-cell non-Hodgkin lymphomas (NHL), making it a potentially attractive anti-lymphoma therapy. However, in some hematological malignancies such as multiple myeloma, Hodgkin lymphoma and Burkitt lymphoma, IL-21 can induce proliferation of neoplastic B-cells. In NHL, the underlying mechanism of cell death was found to be different between the various subtypes, including activation of different JAK/STAT signal transduction pathways or other factors. Immunomodulatory effects of IL-21 have also been reported to contribute to its anti-tumor effects as described by earlier studies in solid tumors and B-cell associated malignancies. These effects are predominantly mediated by IL-21's ability to activate cytolytic activities by NK-cells and $CD4^+$/$CD8^+$ T-cells. In this review, we provide an overview of IL-21's effects in NHL, results from clinical trials utilizing IL-21, and propose how IL-21 can be therapeutically exploited for treating these lymphomas.

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Introduction

IL-21 induces diverse regulatory effects on healthy and tumor cells, including proliferation, differentiation and apoptosis. The ultimate outcome of IL-21 treatment is determined by the type of cell, stage of differentiation and type of stimulus. Despite the inherent complexity in understanding the pleotropic effects of IL-21, we have now begun to understand the therapeutic actions of this cytokine. IL-21 has already been investigated as a potential therapeutic agent in several cancers, including solid tumor as well as hematological malignancies and demonstrated promising activity as anti-cancer therapy in several studies. The challenge now is to optimize its anti-tumor effects for specific tumors as a single agent or as component of combination regimen with other anti-cancer agents to maximize therapeutic effects. In this review, we first summarize the basic biology of IL-21/IL-21R and effect of IL-21 on the various components of the immune system. Second, we discuss the anti-tumor effects of IL-21 that are mediated by immune system and finally, we describe the potential of IL-21 therapy in various lymphomas by outlining results from several preclinical and clinical studies.

Interleukin-21 (IL-21)-Interleukin-21 receptor (IL-21R) distribution

Interleukin-21 (IL-21) is a member of the γ-common chain (γ_c) receptor cytokine family and IL-21 cDNA shows extensive open reading frame homology with IL-2, IL-4, and IL-15.[1,2] IL-21 is a four helix bundle cytokine predominantly secreted by natural killer T (NKT) cells, T follicular helper (T_{FH}) cells and T_H17 cells, with lower levels of production by other lymphocyte subpopulations.[3–6] IL-21 can amplify both innate and adaptive immune responses. It acts as a modulator of the immune system by regulating the differentiation of B-cells to plasma cells, $CD4^+$ T cells to NK T cells and by increasing macrophage and dendritic cell proliferation.[1,7,8] A detailed review on the biology of IL-21 can be found in several other references.[9,10]

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Functional IL-21R is preferentially expressed by T-cells, B-cells, NK cells, some myeloid cells and keratinocytes, and is absent in other tissues or solid tumors.[1,2] The IL-21R was discovered in 2000 by genomic and cDNA sequencing of an open reading frame that was presumed to encode a type I cytokine receptor.[1] IL-21 signaling requires the common γ_c for signaling.[11] Similar to other members of the type I cytokine family, upon binding to its cognate receptor, IL-21 activates the Janus tyrosine kinase-signal transducer and activator of transcription (Jak-STAT) signaling pathway and leads to expression of IL-21 responsive genes. Jak1 and Jak3 are activated upon IL-21 binding to the γ_c in a similar fashion as other members of the γ_c family of cytokines activate their cognate receptors.[2,11,12] Activation of these two Jak kinases leads to phosphorylation of STAT1, STAT3 and to a lesser extent STAT5a and STAT5b.[13,14] STAT3 seems to be a critical transcription factor in IL-21 signaling as T-cells lacking STAT3 have defective IL-21 signaling.[14] IL-21 signaling can also activate PI3-kinase and RAS/MAPK pathways to modulate cell proliferation, protein translation, and survival.[14,15]

Biology of IL-21

IL-21 has pleiotropic effects on various components of immune systems. This section describes the effect of IL-21 on normal lymphocytes (Figure 1(A)).

Effects on T-cells

IL-21's effects on T-cells are varied, and include enhancement of proliferation and cytotoxic function. IL-21 can increase proliferation of $CD4^+$ T cells and stimulate their differentiation into T_H17 cells together with IL-6.[6,16–18] The activation of $CD8^+$ T cells by $CD4^+$ T cells is important for the production of cytotoxic T cells (CTLs), which play an important role in mediating T-cell dependent anti-tumor effects. IL-21 stimulates proliferation of both human and mouse $CD8^+$ T cells and induces production of perforin and granzymes, thus increasing their cytolytic functional capacity.[16,19–22] These effects on T cells require co-stimulation of the T-cell receptor (TCR) or co-activation by additional cytokines, such as IL-2, IL-15 and IL-7. Results obtained from an independent study demonstrated that IL-21 leads to expansion of CTLs by

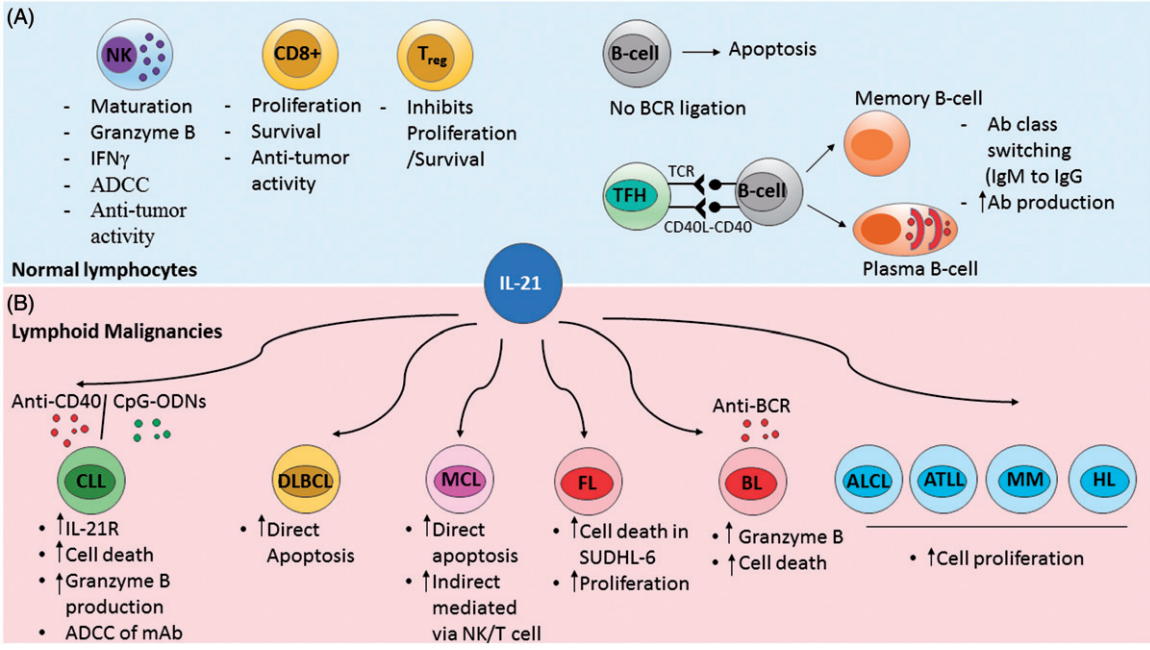

Figure 1. Differential effects of IL-21 on normal and tumor lymphocytes. IL-21 has diverse effects on various lymphocyte population. (A) B-cell compartment: In the absence of stimulation, B-cells follow apoptosis upon IL-21 encounter. In the presence of B-cell receptor signaling (BCR) and/or interaction with T-cell, IL-21 induces class switch recombination and antibody production, together with differentiation and maturation of B-cells into antibody producing plasma cells or memory cells. IL-21 also affects proliferation, differentiation and maturation of NK-cells and T-cells. (B) IL-21 induces distinct effects in different types of B-cell malignancies as indicated in text below every subtype. Anti-tumor effects of IL-21 are mediated via IL-21R to induce direct apoptosis and/or immune cell activation dependent indirect apoptosis mechanisms. NK: natural killer cells; CLL: chronic lymphocytic leukemia; DLBCL: diffuse large B-cell lymphoma; MCL: mantle cell lymphoma; FL: follicular lymphoma; MM: multiple myeloma; BL: Burkitt lymphoma; ALCL: anaplastic large cell lymphoma; ATLL: adult T-cell leukemia/lymphoma; HL: Hodgkin lymphoma.

suppressing the expansion of Foxp3 expressing T_{reg} *in vitro*. Importantly, IL-21 has an advantage over IL-2 because unlike the latter, it does not promote T_{reg} expansion and thereby has lower systemic toxicity.

Effects on NK cells

While IL-15 is the most critical to the early proliferation of NK cells, IL-21 induces the maturation of NK cells and also enhances their cytotoxic function.[8,16,23–26] IL-21 decreases proliferation of mature NK cells if they were previously stimulated with IL-15. Of note, IL-21R$^{-/-}$ mice have fully functional NK cells, suggesting that it is not required for NK cell development. In immature NK cells, low dose of IL-21 results in increased cell proliferation while at high doses and in combination with IL-2 and IL-15, IL-21 decreased NK cell proliferation. IL-21-stimulated NK cells also express increased levels of CD16 (FcγRIIIa),[1] which mediates antibody-dependent cell mediated cytotoxicity (ADCC), a major mechanism of cell killing by monoclonal antibodies (mAbs). Finally, co-stimulation with IL-15 and IL-21 synergistically increases IFN-γ production by NK cells.[27] IL-21 also exerts modulatory effect on NKT cells, a unique population of T cells that participate in autoimmunity, allergy, infection and tumor rejection.[26,28] IL-21 results in proliferation of NKT cells upon CD3 stimulation but only in combination with IL-2 or IL-15. Of note, NKT cells can produce their own IL-21 after stimulation.

Effects on B-cells

IL-21 induces variable effects on B-cells, ranging from proliferation, differentiation or apoptosis, determined by the context of stimulation and cell type.[29,30] IL-21 can induce pro-apoptotic effects on activated and naïve B-cells but this effect is modulated by the presence of other stimulatory molecules.[31,32] For instance, IL-21 increases growth and differentiation of murine B lymphocytes that received both B-cell receptor (BCR) and T-cell help mediating signals. Conversely, it induces apoptosis of murine B lymphocytes post toll like receptor (TLR) stimulation, which can be prevented by CD40 stimulation.[33] IL-21-induced apoptosis of mouse B-cells is mediated by upregulation of pro-apoptotic proteins (especially Bim) and down-regulation of anti-apoptotic proteins, including Bcl-X_L.[33,34] The effects of IL-21 on human non-neoplastic B-cells have been confined to regulation of B-cell activation and differentiation. Specifically, co-stimulation by IL-21 and anti-CD40 Ab results in human B-cell proliferation, while IL-4 and BCR co-stimulation decreases B-cell proliferation.[16,34]

In vivo studies have demonstrated that overexpression of IL-21 in murine B-cells lead to increased numbers of isotype-switched memory cells, immature transitional cells, and plasma cells, resulting in increased levels of IgG and IgM in serum. Increased IgE serum levels are observed in IL-21R$^{-/-}$ mice, both at baseline and in response to immunization. IL-21 also plays a central role in the differentiation of human primary B-cells into plasma cells by inducing expression of AID, an enzyme which catalyzes class switch recombination (CSR).[7,35] Studies have shown that IL-21 stimulates CSR and secretion of IgG and IgA in post-switch CD40-stimulated IgM^{+} memory B-cells.[36]

Immune-system mediated role of IL-21 in cancer treatment

IL-21 has obvious potential as an anti-tumor agent, because of its ability to enhance the cytotoxic activity of both CD8^{+} T cells and NK cells. Mounting evidence has demonstrated an anti-tumor role of IL-21 in solid tumors as well as in other types of cancers using studies in mouse models, cell lines and clinical trials in patients.[37] As detailed below, IL-21 has been validated as an effective immunotherapeutic agent for cancer by various therapeutic approaches: IL-21 administered alone as a systemic therapy, in combination with mAbs, co-administration with tumor cell vaccines or in conjunction with adoptively-transferred T cells.

Anti-tumor effects of IL-21 in non-hematopoietic tumors

IL-21 demonstrated anti-tumor activity in solid tumors devoid of IL-21R, both in preclinical murine models and clinical trials. These effects are largely indirect and mediated by IL-21-induced terminal differentiation of NK cells and eliciting protective T cell responses via CD8^{+} T cells and CTLs.[8] Early mouse studies carried out using systemic administration of plasmid DNA encoding murine IL-21 resulted in tumor regression of B16 melanoma and MCA205 fibrosarcoma.[38] Reduction of tumor growth was also observed in additional syngeneic murine tumor models irrespective of the method of IL-21 delivery (implantation of IL-21 transfected tumor cells, treatment with recombinant IL-21 (rIL-21) or IL-21 encoding plasmid delivery). It has been reported that constitutive expression of IL-21 prevents initiation of pancreatic or colon cancers via NK cell-mediated cell lysis.[39,40] Furthermore, genetically modified IL-21 secreting murine mammary

adenocarcinoma cells prevented tumor development in syngeneic mouse models.[41] A study by Daga et al. demonstrated that in a glioblastoma model, IL-21 treatment was able to reduce tumor size in wild type but not in B-cell deficient mice, suggesting B-cell dependency for its antitumor activity.[42]

The anti-tumor efficacy of IL-21 was also compared with other cytokines, as a single agent or in combination. Zeng et al. reported that tumor regression achieved by intraperitoneally (i.p.) delivered IL-21 outperformed the effects of other cytokines (IL-2 or IL-15).[19] Interestingly, IL-15 enhanced the potency of IL-21 when used in combination, resulting in long-term survival of B16 melanoma bearing mice.[19] These cooperative anti-tumor effects were attributed to increased proliferation of both memory and naïve $CD8^+$ T cells and interferon-gamma (IFNγ) production. Of note, synergy between IL-21 and IL-7 was relatively weak and completely absent when used in combination with IL-2.[22]

Taken together, IL-21 affects components of both the innate and adaptive immune systems and the contribution of individual cell subset to *in vivo* anti-tumor effects of IL-21 are tumor model and host microenvironment specific.

Immune-mediated anti-tumor effects of IL-21 in tumors of hematopoietic origin

Activation of the tumor host microenvironment via immunotherapy is becoming an increasingly attractive approach to treat cancer. However, this concept is somewhat underappreciated in the treatment of non-Hodgkin lymphomas (NHLs). A recent study investigated whether indirect effects of IL-21 on various immune cells contribute to its anti-tumor activity. *Ex vivo* treatment with IL-21 resulted in lysis of tumor cells when incubated with NK cells. Using a syngeneic mouse model of mantle cell lymphoma (FcmuMCL-1), we demonstrated that IL-21 treatment resulted in complete tumor regression and prolonged animal survival.[43] To evaluate the direct contribution of distinct effectors to IL-21 therapeutic efficacy, immune cell subsets were depleted, showing that IL-21-mediated anti-tumor effects are dominated by enhanced activity of $CD4^+$ T and NK cells. This study provided the first evidence for IL-21-induced anti-tumor activity that is mediated by immune effector cells in a lymphoma tumor model.[43]

Combination immunotherapy approaches to activate NK cells and enhance ADCC activity demonstrated improved therapeutic efficacy in B-cell lymphoma tumor models. Strategies combining cytokines with mAb have been attempted to boost NK-cell mediated ADCC, a major mechanism of cell-killing by mAbs.[44–46] Several groups have demonstrated that when combined with a therapeutic antibody, IL-21 enhances ADCC against mAb coated tumor cells. This was demonstrated using anti-CD20 antibody (rituximab) against NHLs including CLL, MCL and DLBCL as well as with anti-HER2 antibody (trastuzumab) in breast cancer.[24,37,47,48] A phase I clinical trial of IL-21 in combination with rituximab for B-cell lymphoma also showed clinical activity, as described in a later section.[47]

Systemic administration of cytokines has often failed to improve therapeutic efficacy due to the inability to achieve optimal concentration in the tumor bed and increased systemic toxicity.[49] To overcome these issues, fusion of cytokines to mAbs, referred to as fusokines or immunocytokines, were explored.[50] Williams et al. reported that a granulocyte marophage colony stimulating factor (GM-CSF) and IL-21 fusion protein (GIFT-21) activates an immune response and leads to anti-tumor responses in the B16 xenograft model.[51] A similar approach using fusion of anti-CD20 antibody with IL-21 (αCD20-IL-21 fusokine) is currently actively investigated in our laboratory.[52] We found that αCD20-IL-21 fusokine has superior anti-lymphoma activities than its individual components against NHL primary tumors and rituximab resistant tumor model *in vivo*.

Direct cytotoxic anti-tumor effects of IL-21 in NHLs

Apart from indirect immune mediating effects, IL-21 also induces direct effects on IL-21R-expressing malignancies originating from B lymphocytes (Figure 1(B)). An increasing number of laboratory studies have demonstrated anti-tumor effects of IL-21 in a variety of NHLs. Although in most cases, IL-21 treatment resulted in apoptosis of malignant B-cells (Figure 2), the underlying mechanism of cell death was found to be different among various types of lymphomas, as detailed below.

Results from preclinical studies of IL-21 in NHL

Chronic lymphocytic leukemia (CLL)

The first study examining the effects of IL-21 on CLL B-cells was carried out by De Totero and colleagues.[53] They found that IL-21R was expressed at variable levels in CLL B-cells, with 11 of 33 patients demonstrating little or no IL-21R expression, yet

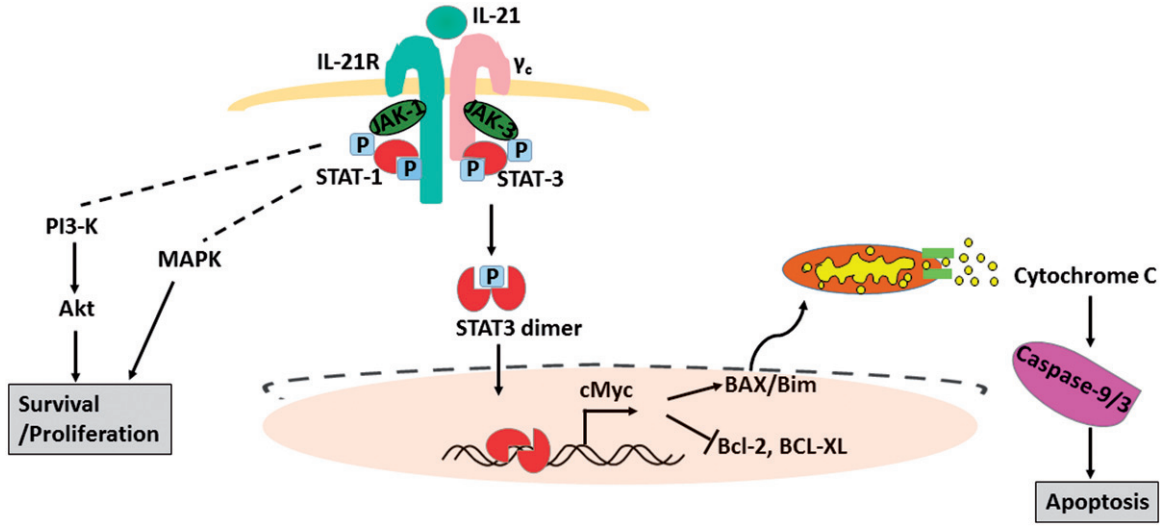

Figure 2. IL-21 signaling pathway. Interleukin-21 (IL-21) binding stabilizes the complex between the IL-21 receptor (IL-21R) and the common cytokine-γ chain, γc, leading to the activation of Janus kinase 1 (JAK1) and JAK3, which then activates signal transducer and activator of transcription (STAT) proteins by phosphorylation (predominantly STAT3 and STAT1, but also STAT5 in some instances). These STAT proteins dimerize, enter the nucleus to induce transcription. In DLBCL and MCL, activation of STAT-3 upregulates cMyc which then results in activation of pro-apoptotic proteins and inhibition of anti-apoptotic proteins to induce mitochondria dependent intrinsic pathway of apoptosis. IL-21 binding to IL-21R can also indirectly/directly activate the mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways. Bax: B-cell 2 associated X-protein; Bcl-XL: B-cell lymphoma-extra-large; Bcl2: B-cell lymphoma 2; Bim: BCL-2 interacting mediator of cell death.

co-stimulation with CD40 ligand activated resting CLL B-cells and increased IL-21R expression. IL-21R dependent signaling was found to be similar to that in normal B-cells, resulting in tyrosine phosphorylation of STAT-1, STAT-3 and STAT-5 in CD40-preactivated CLL B-cells, while resting CLL B-cells showed signaling only through STAT-3. As opposed to the proliferative effects of IL-15 on CLL cells, IL-21 led to apoptosis of CLL B-cells. Although apoptotic response to IL-21 was mild in resting CLL B-cells (observed in only six of 20 cases), anti-CD40 activated cells had a clearly more potent proapoptotic response. The authors found that IL-21-induced apoptosis of CLL B-cells led to activation of caspase 8 and caspase 3, suggesting a role for the extrinsic apoptotic pathway. In addition, IL-21 stimulated cleavage of Bid to its active form t-Bid as well as cleavage of p27 and PARP in these cells. Another study led by this group demonstrated that opposing effects of IL-15 and IL-21 on CLL B-cells may be manifested via activation of different signaling events: induction of Shc, ERK1/2, and STAT5 phosphorylation by IL-15 and activation of JAK1, STAT1, and STAT3 pathways by IL-21.[54]

A study led by George Weiner's group showed that apart from anti-CD40, co-stimulation with Toll-like receptor 9 (TLR-9) ligand CpG oligonucleotide (ODN) also upregulated IL-21R expression on CLL cells.[55] Simultaneous treatment with CpG ODN and IL-21 greatly increased apoptosis of CLL cells compared to IL-21 alone. Furthermore, they showed that the mechanism underlying the observed synergy between IL-21 and CpG ODN was due to increased secretion of granzyme B by CLL cells. When IL-21 was added to CpG ODN treated CLL B-cells, it induced apoptosis of untreated bystander CLL cells, which was blocked by treatment with anti-granzyme B antibody.

A subsequent study led by Gowda et al. also demonstrated that CLL patients' cells express variable levels of IL-21R, ranging from 10% to 80%.[56] Similar to the two studies described above, IL-21 induced direct apoptosis of CLL B-cells, which was further increased by pretreatment with anti-CD40 mAb or CpG oligonucleotide. Notably, levels of apoptosis were directly correlated with IL-21R expression (cells expressing >20% IL-21R showed >20% increase in apoptosis in all nine analyzed patients). The mechanism of IL-21R mediated apoptosis was found to be similar to that observed in murine B-cells, involving upregulation of BH3 family member Bim, occurring in all patients responsive to IL-21. The authors found that knock down of Bim abrogated IL-21 induced apoptosis, suggesting a direct contribution of Bim to the pro-apoptotic effects of IL-21. Extending these findings, they showed that IL-21 can be effectively combined with other drugs such as fludarabine and rituximab. Addition of IL-21 to fludarabine therapy enhanced the

cytotoxicity of CLL cells without affecting other immune cells expressing IL-21R. IL-21 enhanced direct cytotoxicity as well as ADCC of CLL cells when combined with rituximab.

Diffuse large B-cell lymphoma (DLBCL)

DLBCL is the most prevalent subtype of non-Hodgkin lymphoma with a 5-year survival of 50–60%. A comprehensive study performed in our laboratory examined the therapeutic role of IL-21 in DLBCL and demonstrated that DLBCL cell lines expressed variable levels of surface IL-21R.[57] In contrast to the relatively mild apoptotic effects on CLL tumors that were not pretreated with anti-CD40 or CpG ODN, IL-21-induced pronounced apoptosis in six of seven tested DLBCL cell lines and *de novo* primary tumors that inherently express high levels of IL-21R. Notably, nonmalignant B-cells showed significantly lower levels of IL-21R and were insensitive to IL-21 induced cell death. Furthermore, *in vivo* IL-21 treatment (10 μg for seven consecutive days) led to complete tumor regression in DLBCL xenograft models of RCK8 and MC-116 cells and extended survival of treated animals. All cell lines stimulated with IL-21 showed up-regulation of pSTAT1, pSTAT3, and to a lesser extent of pSTAT5. The mechanism of IL-21 induced apoptosis in DLBCL cells differed from CLL cells, as cell death in DLBCL cells proceeded through the intrinsic pathway of apoptosis and was independent of Bim. Instead, c-Myc, which is a transcriptional target of STAT-3, was found to be a crucial mediator of IL-21-induced apoptosis. IL-21 responsive cells demonstrated prominent upregulation of c-Myc, and its knock down was able to rescue IL-21-induced apoptosis. Down-stream modulation of Bcl-2 family members (down-regulation of Bcl-2 and Bcl-X_L as well as up-regulation of Bax) shifted the balance to the apoptotic phenotype to trigger cell death in DLBCL cells (Figure 2).

Wu et al. showed in the EBV transformed Farage cell line that IL-21 leads to proliferation rather than apoptosis despite up-regulation of c-Myc expression.[58] Inhibition of EBV gene expression reversed this phenotype, revealing that IL-21 might be ineffective in EBV transformed malignancies. However, these findings were based on studies of a single cell line and warranted further investigation.

Mantle cell lymphoma (MCL)

MCL is an aggressive subtype of NHL with relatively short median overall survival. Gelebert et al. carried out the first study examining the effects of IL-21 on two MCL cell lines; Mino and SP-53.[59] They showed that both cell lines contained detectable levels of IL-21R transcripts and IL-21 had anti-proliferative effects on both cell lines. It was shown that IL-21 induced activation of both STAT1 and STAT3, but cell death was mediated by STAT1, since inhibition of STAT1 using siRNA significantly decreased apoptotic responses. Gelebart et al. demonstrated that the apoptotic response induced by IL-21 also correlated with a significant up-regulation of three proapoptotic proteins: BIK, NIP3 and HARAKIRI, and down-regulation of Bcl-2, Bcl-X_L, TNF-a and decreased DNA binding of NF-kappaB, again implicating the intrinsic apoptotic pathway.[59]

A recent study provided further evidence for antitumor activity of IL-21 in MCL.[43] Using established MCL-derived cell lines, it was found that all cell lines expressed IL-21R but unlike other types of lymphomas, only three of the eight tested cell lines showed sensitivity to IL-21 treatment. IL-21-induced direct apoptosis was found to be mediated through a mechanism similar to DLBCL cells: via IL-21R-dependent signaling leading to STAT3-dependent c-Myc upregulation. This is in contrast to the study performed by Gelebart et al., since knock down of STAT1 failed to rescue IL-21 induced cell death. Instead, knock down of STAT3 rescued cells from IL-21-induced apoptosis. This study demonstrates that the level of c-Myc induction after IL-21 treatment can determine cell fate, suggesting that c-Myc expression can be used as a biomarker for response to IL-21. Apart from the direct effects of IL-21 on MCL cells, IL-21 also has the ability to activate the immune compartment and thereby promote indirect killing of malignant cells that were otherwise insensitive to IL-21. The indirect effects of IL-21 treatment are related to NK cell-dependent lysis of tumor cells, and the presented *in vivo* data in a syngeneic mouse transplantation model strongly suggested that antitumor effects are dominated by enhanced activity of $CD4^+$ T and NK cells. These data provide a solid preclinical rationale to consider recombinant IL-21 in MCL therapy, in particular because the indirect effects via immune effector cells in the tumor microenvironment or in the circulation might dominate anti-tumor activity in tumors exhibiting primary resistance to IL-21's proapoptotic effects.

Follicular lymphoma (FL)

FL is the most common type of indolent NHL that may transform into DLBCL. Two independent studies examining the effects of IL-21 on FL showed that IL-21R was detectable on 17 of 17 primary FL cases and IL-21 treatment induced apoptosis of all IL-21R positive FL

tumors.[60,61] However, patients who progressed from FL to DLBCL had lower IL-21R expression and also were refractory to IL-21 treatment.[60] In contrast to studies in CLL and DLBCL, IL-21 induced apoptosis was independent of IL-21R expression. Only SUDHL-4 out of seven cell lines with t(14;18) showed increased cell death in response to IL-21, while other cell lines remained unresponsive despite IL-21R expression.[61] Susceptibility of primary FL cells to spontaneous apoptosis in the absence of stroma cells *in vitro* may have contributed to differing sensitivities of IL-21 in primary tumors and cell lines. In agreement with findings from other types of lymphoma, induction of apoptosis was associated with caspase 3, 8, and 9 activation. In addition, IL-21 decreased Bcl-2 and increased Bax expression in only the IL-21 responsive cell line SUDHL-4, whereas JAK-STAT signaling was activated irrespective of sensitivity to IL-21. Of note, SUDHL-4 cells represent DLBCL originating from FL and are not representative of primary FL.

However, emerging studies have suggested that *in vivo* access to IL-21 from T_{FH} cells within follicle can actually stimulate the growth of FL cells.[62] In a separate study it was found that higher number of follicular $CD4^+$ T cells had a worse prognosis compared to other subpopulation.[63] These later studies warn a caution on IL-21 therapy in FL and suggest that IL-21 blocking antibody may need to be tested to further confirm these findings.

Stimulation of lymphoid proliferation by IL-21 in NHL

Burkitt lymphoma (BL) is an EBV associated malignancy characterized by high proliferation rate. Akamatsu et al. found that IL-21R was not expressed in primary BL tumors but was detectable in three of four BL cell lines (Ramos, Daudi and CA46) at expression levels lower than usually observed in FL or DLBCL cell lines.[60] Jahrsdorfer et al. showed that in response to IL-21 and anti-BCR stimulation, EBV transformed lymphoblasts and BL lines produced high levels of granzyme B, suggesting that IL-21 could promote BL apoptosis.[55] However, Akamatsu et al. detected proliferation of BL cell lines upon treatment with IL-21 despite pSTAT1 and pSTAT3 activation.[60]

Adult T-cell leukemia lymphoma (ATLL) is an aggressive T-cell malignancy caused by human T-cell leukemia virus type-I (HTLV-1). Studies from Ueda et al. provided the first evidence that ATLL primary tumors (nine of 11) and cell lines (seven of seven) express functional cell surface IL-21R via flow-cytometry.[64] A subsequent study led by Akamatsu et al. demonstrated that IL-21R was present on only one of eight ATLL primary tumors.[60] Both studies showed increased proliferation of ATLL cell lines in response to IL-21 stimulation. IL-21 treatment activated STAT-3 and STAT-5, suggesting that these cells are responsive to IL-21.

ALK positive anaplastic large cell lymphoma (ALCL), an uncommon type of T-cell NHL, also expresses IL-21R and undergoes cell proliferation upon IL-21 stimulation.[65] Multiple myeloma is characterized by the accumulation of long-lived malignant plasma cells. IL-21 was reported to be a growth factor for multiple myeloma cells,[15] but subsequent studies have shown that this effect of IL-21 is restricted to the $CD45^-$ subset of these cells.[66] The IL-21-mediated growth-promoting effect results from the induction of insulin-like growth factor 1 (IGF1), which has autocrine growth effects that are additive upon costimulation with IL-21.[66]

Reed–Sternberg cells of Hodgkin lymphomas (HL) express IL-21R as well as IL-21. Blocking of IL-21 signaling was shown to decrease the proliferation of a Hodgkin lymphoma cell line.[67]

The growth-promoting effects of IL-21 in multiple myeloma, Hodgkin lymphoma and other hematological malignancies suggest that blocking the IL-21 signaling pathway might be of therapeutic value in these settings. Blockade of IL-21 signaling could be achieved by targeting the extracellular interaction with IL-21R-specific antibodies, IL-21R–Fc fusion protein or via inhibition of the JAK–STAT pathway.

Results from clinical trials of IL-21

Recombinant IL-21 (rIL-21) has been evaluated in phase I/II clinical trial against metastatic melanoma, renal cell carcinoma (RCC), colorectal cancer and NHL. IL-21 used during human clinical trials was manufactured by Bristol-Myers Squibb (Denenicokin; Other Names: BMS-982470, rIL-21 (recombinant interleukin 21)). So far, IL-21 has been tested in six phase I/II clinical trials as a single agent and in five trials in combination with targeted agents (Table 1). However, it has not been approved by FDA for clinical treatment. Detailed discussion on the clinical trial results is described in earlier reviews.[68]

Clinical trials in solid tumors

To evaluate the potential of IL-21 as an immunotherapeutic cytokine, three phase I dose-escalation studies have been completed along with one phase IIa study. These studies have reported activity of rIL-21 in

Table 1. Summary of clinical trials.

Investigators	Treatment	Disease setting	Developmental status	Study status	Overall response rate	Additional results
Davis et al. [70]	IL-21 (intravenous)	Metastatic melanoma	Phase I	Completed	3%	29 total 1 CR 9 SD 14 months PFS
Davis et al. [69]	IL-21 (intravenous)	Malignant melanoma (stage IV)	Phase IIa	Completed	8.30%	24 total 1 CR 1 PR 56-77 days DR
Thompson et al. [71]	IL-21 (intravenous)	Metastatic melanoma and renal cell carcinoma	Phase I	Completed	• Metastatic melanoma: 4%	24 total 11 SD 1 CR 8 months DR
					• Renal cell carcinoma: 21%	19 total 13 SD 4 PR 5-18 months DR
Schmidt et al. [72]	IL-21 (subcutaneous)	Metastatic melanoma and renal cell carcinoma	Phase I	Completed	• Metastatic melanoma: 7.6%	13 total 1 PR 6-15 months DR
					• Renal cell carcinoma: 15.4%	13 total 2 PR 6–15 months DR
Petrella et al. [74]	IL-21 (intravenous)	Metastatic melanoma	Phase II	Completed	22.5%	37 total 9 PR 16 SD 3-23 months DR
Petrella et al. [75]	IL-21 or dacarbazine	Metastatic melanoma	Randomized phase II	Completed	• IL-21 therapy: 13.3%	30 total 4CR + PR 1. 8 months PFS
					• Dacarbazine therapy: ORR: 14.3%	28 total 4CR + PR 2. 0 months PFS
Grunwald et al. [76]	IL-21 plus sunitinib	Renal cell carcinoma (stage IV)	Phase I	Study terminated		Therapeutic dose not tolerated
Timmerman et al. [47]	IL-21 plus rituximab	Non-Hodgkin lymphoma	Phase I	Completed	42%	19 total 3 CR/Cru 5 PR 25-28 months PFS
Bhatia et al. [77]	IL-21 plus sorafenib	Metastatic renal cell carcinoma	Phase I/II	Completed	21%	33 total 6 PR 22 SD 5. 6 months median PFS
Steele et al. [79]	IL-21 plus cetuximab	Colorectal cancer (stage IV)	Phase I	Study terminated		No MTD determined
NCT01629758	IL-21 plus FD1-specific antibody (nivolumab)	Solid tumors	Phase I	Completed	Results pending	
NCT01489059	IL-21 plus anti-CTLA-4-specific antibody (ipilimumab)	Melanoma	Phase I	Completed	Results pending	

IL-21: interleukin-21; CR: complete response; PR: partial response; SD: stable disease; PFS: progression free survival: DR: duration of response; MTD: maximum tolerated dose; PD-1: programed cell death-1 gene; CTLA-4: cytotoxic T-lymphocyte associated protein-4.

metastatic melanoma without inducing vascular-leak syndrome.[69–71] IL-21 treatment was generally well tolerated with no dose limiting toxicity at 1, 2, and 10 μg/kg dose levels. Although IL-21 was biologically active at all dose levels, the phase IIa studies were carried out at 30 μg/kg/day (maximal tolerated dose (MTD) for daily IV infusion). In a phase IIa trial, 24 patients with stage IV previously untreated malignant melanoma received IL-21 therapy.[69] Complete response (CR) was observed in one patient and one patient achieved a partial response (PR). Another phase I study in patients with metastatic melanoma and RCC also reported similar MTD dose and toxicity profiles.[71] Anti-tumor activity was observed in both metastatic melanoma (one CR and 11 stable disease (SD)) and RCC (4-PR and 13-SD). A phase I study

evaluating the efficacy of subcutaneous treatment of rIL-21 (3 days per week for 8–16 weeks) in metastatic melanoma and RCC was performed.[72] rIL-21 was generally well tolerated with dose-limiting toxicities at 200–300 μg/kg and MTD of 200 μg/kg. Similar to earlier studies, subcutaneous IL-21 also induced immune activation and showed anti-tumor activity (one patient with MM and two with RCC had PR out of 26 patients). To investigate the immunostimulatory effects of IL-21, Dodds et al. evaluated the serum samples of treated patients from two phase I studies.[73] As expected, patients receiving IL-21 therapy showed significant modulation of biomarkers indicative of lymphoid and myeloid activation (increase in IL-16, macrophage derived chemokine, macrophage inflammatory protein), and leukocyte trafficking (soluble cell adhesion molecule and monocyte chemoattractant protein). To further evaluate IL-21 efficacy, multicenter, open label phase II study was carried out in metastatic melanoma.[74] Twenty-five (62.5%) of 40 patients had either a PR ($n = 9$) or SD ($n = 16$). This encouraging activity met the criteria for positive response and led to the initiation of a randomized phase II trial in metastatic melanoma. This study enrolled 64 patients that were randomized to rIL-21 arm ($n = 32$) and dacarbazine arm ($n = 32$). In this study, IL-21 activity was found to be comparable to that of current standard treatment-dacarbazine.[75]

Clinical trials of IL-21 in combination with other therapies

Although IL-21 has shown clinical activity as a single agent, the observed efficacy was not favorable enough to obtain approval as a standalone therapy, suggesting a need for combination approaches. rIL-21 (s.c.) was tested in combination with sunitinib in a phase I trial in naïve RCC patients.[76] However this combination led to significant hematological toxicity and the study was discontinued. Another phase I/II trial of IL-21 (iv) in combination with oral sorafenib in metastatic RCC showed objective response rate of 21%, disease control rate of 82% with two persistent responses (41 and 30 months) that continued after therapy withdrawal, and median progression free survival of 5.6 months in phase II trial.[77] The combination was well tolerated even in previously-treated patients with grade 3 skin rash as the only dose-limiting toxicity. This combination showed potential therapeutic benefit and should be explored further.

A number of preclinical studies have indicated a potential role for IL-21 in enhancing NK cell mediated ADCC activity of mAbs. To test this in the clinical setting, rIL-21 was combined with cetuximab (anti-EGFR antibody) in chemo-naïve stage IV colorectal cancer patients. This phase I study enrolled 15 patients and 60% of them maintained stable disease post therapy and immune activation was confirmed by various T- and NK-cell activation markers. Due to premature study closure, MTD was not determined, but maximum administered dose for rIL-21 was 100 μg/kg/weekly.[78]

To address possible cooperative anti-tumor effects of IL-21 with immune checkpoint inhibitors, two clinical trials have been recently completed and analysis is undergoing. A safety study of IL-21 in combination with the anti-PD1 mAb (nivolumab) was performed in advanced metastatic solid tumors (NCT01629758). Finally, a phase I study of IL-21 combined with the anti-CTLA-4 mAb (ipilimumab) in un-resectable stage II or stage IV melanoma was carried out to determine the safety and clinical benefit of this combination over single agent (NCT01489059). Results from these trials will hopefully reveal the potential of IL-21 as an adjuvant to immunotherapy.

Clinical trial of IL-21 in NHL

Timmerman et al. conducted a phase I study of rIL-21 in combination with rituximab in patients with low-grade, relapsed and refractory NHL.[47] Twenty-one patients with relapsed CLL/SLL ($n = 11$), FL ($n = 9$), or marginal zone lymphoma ($n = 1$) were enrolled, with 19 patients completing at least one course of therapy. IL-21 was given as a weekly bolus at one of three dose levels (30, 100 or 150 μg/kg/week) for four weeks along with standard-dose rituximab (375 mg/m^2). Patients with stable disease or better response were eligible to receive a second cycle of therapy. In the dose-escalation phase, three patients were treated at each dose level. During the first cycle, no dose-limiting toxicities were observed. Retreatment at the rIL-21 at dose of 150 μg/kg was associated with grade 3 toxicity in one patient and therefore MTD was fixed at 100 μg/kg/week. Of 19 patients with evaluable response, a decrease in the size of target lesions was seen in 16 (84%), and objective clinical responses were seen in eight (42%) patients, including three CR/CRu and five PR. Strikingly, four of these eight responders were in remission for longer duration than their previous response to rituximab-based treatment (median 9 months vs. 3 months). Among the 15 patients with rituximab-resistant disease, the objective response rate was 33%. The authors concluded that this treatment is safe, clinically active and deserves further investigation.

Concluding remarks and perspective

IL-21 has shown potent anti-tumor activity against many tumor types in a number of preclinical studies using *in vivo* animal models as well as in human clinical trials. There are two major areas where IL-21 holds substantial promise as an anti-cancer agent.

First, patients with certain solid tumors (metastatic melanoma, metastatic RCC and colorectal cancer) may benefit due to ability of IL-21 to activate the cytotoxic potential of $CD8^+$ T cells, NK cells and NKT cells. In phase I/II clinical studies, rIL-21 was proven to be safe and also demonstrated therapeutic activity by leading to objective responses or disease stabilization in a fraction of metastatic melanoma and RCC patients. However, the observed anti-tumor activity failed to exceed responses achieved by standard therapy with dacarbazine in a randomized phase-III trial, indicating that monotherapy with IL-21 may not suffice. Of note, IL-21 appears to be well tolerated with acceptable toxicity at the MTD with no grade 3 toxicity when used as monotherapy. In contrast to IL-21, front line treatment of melanoma and RCC with IL-2 and IFN-α is associated with severe toxicities. This is especially true for IL-2 therapy, which requires hospitalization. Therefore, in view of IL-21's acceptable toxicity profile and good clinical activity, it is a suitable candidate for combination trials with other agents. Although challenging, the identification of optimal combination treatments may help to achieve superior anti-tumor activity.

Second, although IL-21 has diverse effects in hematological malignancies depending on cell type, preclinical evidence demonstrates potent anti-tumor activity in CLL, MCL, DLBCL and FL. Despite the striking anti-tumor activity of IL-21 in various types of NHLs, clinical evaluation of IL-21 in this disease still remains in its infancy. A phase I trial of rIL-21 in combination with rituximab in relapsed refractory NHLs showed encouraging results, with clinical responses being observed in eight of 19 patients. In this trial, patients were previously treated with rituximab (with 75% of patients having rituximab-resistant disease at treatment initiation), raising the possibility that the therapeutic activity observed may be driven mostly by IL-21. It would be of interest to examine the effect of IL-21 monotherapy against rituximab-refractory cases. Although preclinical studies have demonstrated the most impressive activity against DLBCL and MCL, no patients with these diseases were enrolled in this study.

The favorable safety profile of IL-21 suggests that it may be used or tested broadly in DLBCL and MCL, yet identifying the patients that are most likely to respond favorably would undoubtedly improve chances for successful use and eventual approval. Most importantly, the detection of cell surface IL-21R on the hematological malignancy to be treated would be required, yet this is insufficient to predict a robust clinical response on its own. In preclinical studies, favorable responses in lymphomas were dependent on strong STAT3 activation as well as upregulation of cMyc. It would be possible to develop and deploy an *ex vivo* test on primary lymphomas/leukemias to determine if cMyc expression is induced by IL-21 treatment, which could predict which patients are most likely to respond to systemic IL-21 therapy. Furthermore, a rapid *ex vivo* test such as Dynamic BH3 Profiling could be used to detect early apoptotic signaling resulting from IL-21 treatment, again suggesting that a patient may benefit.[79]

As IL-21 has dual targeting potential whereby both direct apoptosis and immune activation pathways contribute to tumor cell death, this becomes an advantage to the IL-21 based therapies. IL-21 has the potential to be a versatile therapeutic. It could be used in combination with existing chemotherapy or immunotherapy to augment antitumor activity. Indeed, the combination of IL-21 with rituximab has shown higher ADCC activity against MCL and CLL cells. In several other studies,[24,48,79] IL-21 augmented NK-cell and T-cell responses to therapeutic antibody-coated target cells in culture and increased IFN-γ production. Based on the versatile actions of IL-21 on tumor cells and host immune cells, we believe that IL-21 may find a use in combination with a variety of other immunotherapeutic agents, including antibodies, other cytokines and tumor antigen vaccines. Hence, combination therapies that can enhance IL-21's therapeutic potential should be a future area of investigation.

Investigation of additional mechanisms of resistance to IL-21 treatment is crucial for therapeutic success of IL-21 in clinic. Identification of other molecular targets in addition to cMyc may serve as a prognostic factor to determine the therapeutic response to IL-21. IL-21 resistance could also arise by a block of IL-21 pro-apoptotic signaling at other points in the STAT3–cMyc–Bcl-2/Bcl-X_L cascade. For instance, further upregulation of Bcl-X_L could shift the Bcl-2 rheostat within DLBCL/MCL cells further away from apoptosis and prevent the IL-21-mediated downregulation of Bcl-X_L from releasing sufficient pro-apoptotic factors to induce mitochondrial membrane permeabilization. Whether these tumors have the ability to evade IL-21-induced apoptosis by downregulating receptor levels or increasing expression of anti-apoptotic proteins needs to be studied in order to determine the

likelihood and importance of these potential mechanisms of IL-21 resistance.

In summary, IL-21 has shown impressive preclinical anti-tumor activity in certain cancer types, especially DLBCL and MCL. Furthermore, IL-21 treatment is extremely well-tolerated in humans, setting the stage for potentially successful clinical development.

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