IL-21 Stimulates Human Myeloma Cell Growth through an Autocrine IGF-1 Loop

Emmanuelle Ménoret, Sophie Maïga, Géraldine Descamps, Catherine Pellat-Deceunynck, Caroline Fraslon, Melania Cappellano, Philippe Moreau, Régis Bataille and Martine Amiot

*J Immunol* 2008; 181:6837-6842; doi: 10.4049/jimmunol.181.10.6837
http://www.jimmunol.org/content/181/10/6837

---

**References** This article cites 23 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/181/10/6837.full#ref-list-1

**Subscription** Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions** Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts** Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
IL-21 Stimulates Human Myeloma Cell Growth through an Autocrine IGF-1 Loop

Emmanuelle Ménoret,* Sophie Maïga,* Géraldine Descamps,* Catherine Pellat-Deceunynck,* Caroline Fraslon,§ Melania Cappellano,§ Philippe Moreau,§ Régis Bataille,* and Martine Amiot1®†

IL-21 is a member of the type I cytokine family related most closely to IL-2 and IL-15. IL-21 is a pleiotropic cytokine, produced by T, NK, and dendritic cells, which modulates lymphoid and myeloid cell functions. Besides its activities on normal lymphoid cells, it has been shown that IL-21 is a growth factor for myeloma cells. In the present study, we demonstrate that IL-21 generated myeloma colonies from 9 of 24 human myeloma cell lines (HMCL) in a collagen-based assay. Of major interest, the capacity of IL-21 to stimulate clonogenicity was restricted to CD45+ HMCL. We found that IL-21 induced tyrosine phosphorylation of STAT-3, STAT-1, and Erk1/2. Interestingly, an Akt activation was observed lately after 30 min to 1 h of IL-21 stimulation, indicating that this Akt phosphorylation could be due to an IGF-1 autocrine loop. This hypothesis was sustained by the fact that IL-21 treatment induced an IGF-1 mRNA synthesis and that an antagonistic anti-IGF-1 receptor mAb (AVE1642) strongly inhibits the IL-21-induced clonogenicity. Thus, we demonstrated by quantitative PCR that IL-21 induced clonogenicity through an autocrine IGF-1 secretion in HMCL and primary myeloma cells. Because we have previously demonstrated that CD45 phosphatase inhibits the IGF-1 signaling, this inhibitory effect of CD45 explains why the IL-21-induced clonogenicity was restricted to CD45+ HMCL. These results support that therapy against IGF-1R, which are presently under investigation in multiple myeloma, could be beneficial, not only to suppress IGF-1-mediated myeloma cell growth, but also IL-21-mediated myeloma cell growth. The Journal of Immunology, 2008, 181: 6837–6842.

Multiple myeloma (MM) is a fatal neoplasia characterized by the accumulation of malignant long-lived plasma cells. MM presents as a heterogeneous disease with very different clinical outcome. Differences in survival are linked to biological heterogeneity including morphology, phenotype, and genotype (1). Among the different prognostic markers, CD45 expression appears as a powerful one (2). Indeed, CD45+ patients have a very poor survival compared with CD45− patients. This phenotypic heterogeneity can be explained by the fact that CD45 is essential to discriminate signaling and proliferation of human myeloma cells in response to either IL-6 or IGF-1, the two major growth factors for MM cells. Thus, the CD45 phosphatase appears as a positive regulator of IL-6 response by controlling the lyn src activity and a negative regulator of IGF-1 response (3–6). Consequently, CD45+ and CD45− human myeloma cell lines (HMCL) proliferate primarily in response to IL-6 and IGF-1, respectively. In a recent study, we demonstrated that IGF-1 stimulates the clonogenicity of CD45− HMCL only (7). Beside the cytokines of the IL-6 family and IGF-1, it was recently shown that IL-21 is a growth and survival factor for MM cells (8). IL-21 is produced by activated CD4+ T and NKT cells and its receptor is expressed on T, B, NK, and dendritic cells (9–11). IL-21R contains the common cytokine-receptor γ-chain, which is also shared by the receptors for IL-2, IL-4, IL-7, IL-9, and IL-15 (12). IL-21R has highest homology to the IL-2Rβ-chain, which is a component of IL-2 and IL-15 receptors (13). IL-21 has a wide range of effect and modulate the function of T, B, and NK cells (9). On B cells, the effects of IL-21 are different either proapoptotic on naive B cells or promoting their differentiation into plasma cells after co-stimulation with signal from T cells (14). Indeed, IL-21 induces the differentiation of B cells into plasma cells through up-regulation of BLIMP1 (15). Moreover, a recent study demonstrated the requirement of IL-21 in B cell activation expansion and plasma cell differentiation induced by direct interaction of B cells with activated T cells (16). The role of IL-21 in regulating Ig production is supported by knockout mouse models. IL-21R−/− mice have diminished IgG1, but elevated IgE level in response to Ag (17).

Because a recent report indicates that IL-21 is a growth and survival factor for MM, we currently evaluated the capacity of IL-21 to induce myeloma colonies in a collagen-based assay. We show that the capacity of IL-21 to generate myeloma colonies is restricted to CD45− HMCL. Furthermore, we demonstrate that IL-21 essentially acts through an autocrine IGF-1 secretion loop.

Materials and Methods

Human myeloma cell lines and culture conditions

LP-1, L363, NCI-H929, and OPM-2 HMCL were purchased from DSMZ and RPMI 8226 and U266 were purchased from the American Type Culture Collection. JIM-3 and JJN-3 were provided by Prof. L. Bergsagel
(Mayo Clinic, Scottsdale, AZ) and Prof. B. Van Camp (Brussels, Belgium), respectively. MM.1S and Karpas 620 were a gift from Dr. S. T. Rosen (Northwestern University, Chicago, IL) and Dr. A. Karpas (Cambridge University, Cambridge, U.K.), respectively. AMO1 was provided by Dr. Shimizu (Kanazawa Medical University, Ishikawa). KMS-11, KMS-18 and KMS-12-PE were provided by Dr. T. Ohtsuki (Kawasaki Medical School, Okayama, Japan). The XG-1, XG-2, XG-5, XG-6, XG-7, NAN-1, NAN-3, NAN-6, SBN, and BCN HMCLs have been previously established in our laboratory from peripheral blood samples or pleural effusion of patients with MM (1) and are cultured in the presence of 3 ng/ml of r-IL-6 (Novartis). Cell lines were maintained in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine, antibiotics, and 5 × 10⁻⁵ M 2-βME.

**Reagents and Abs**

The antagonist Ab anti-IGF-R (AVE1642) was provided by sanofi-aventis (6). Anti-phospho-Akt (Ser473), anti-Akt, anti-phospho-STAT-3 (Tyr705), anti-phospho-p44/42MAPK (Thr202/Tyr204), and anti-phospho-STAT-1 (Tyr701) were purchased from Cell Signaling Technology, anti-STAT-1 was from Biolabs and anti-STAT-3 from BD PharminGen. Human recombinant IL-21 was purchased from R&D Systems.

**Immunofluorescence analysis**

Cells (0.5 × 10⁶) were incubated with anti-IL-21R-PE (R&D Systems) for 20 min. After two washes, cells were fixed in 1% formaldehyde. Flow

**FIGURE 1.** IL-21 is a clonogenic factor for CD45⁻ HMCL. For myeloma colony assays, 10³ myeloma cells were seeded per ml of serum-free collagen-based semisolid stem III medium with or without IL-21 (50 ng/ml) and grown for 15 days. Then gels were dried and stained with MGG then myeloma colony formation (MCF) was scored.

**FIGURE 2.** A, Quantitative PCR analysis of IL-21R on HMCL and B, primary CD138⁺ myeloma cells. Plasma cells were obtained after purification by CD138-immuno-magnetic beads (Miltenyi Biotec) as previously described (23). C, IL-21R surface expression on HMCL by FACS analysis. Cells were stained with isotype-matched control-PE mAb or with anti-IL-21R PE. MFR, mean fluorescence ratio.

**FIGURE 3.** IL-21 activates STAT-3, STAT-1, and Erk-1/2 in HMCL able to clone in presence of IL-21. HMCL were grown in serum-free medium for 18 h and then stimulated with IL-6 (10 ng/ml) or IL-21 (20 ng/ml) for 30 min. Equivalent amount of cell lysates (100 μg) were separated by SDS-PAGE, then immunoblotted either by anti-phospho-STAT-3, -STAT-1, -Akt, and -p44/42MAPK. Anti-STAT-3, anti-STAT-1, and anti-p44/42MAPK were used as loaded control.

in RPMI 1640 medium supplemented with 5% FCS, 2 mM glutamine, antibiotics, and 5 × 10⁻⁵ M 2-βME.
Cytometry analysis was performed on a FACSCalibur using the CellQuest program (BD Biosciences). The ratio of fluorescence was determined by dividing the mean fluorescence intensity by the mean fluorescence intensity of the respective control.

**Myeloma cell colony-forming assay**

Myeloma cells (10^3 cells) were plated in 1 ml serum-free, cytokine-free, collagen-based semisolid medium (stemIII, StemAlpha SA) in triplicates (330 μl/well) in 4-well plates and grown for 15 days. The gels were harvested on glass slides, dried and stained with May-Grunwald-Giemsa. Colonies were counted on triplicate gels. The number of colonies was expressed as average per 10^3 cells.

**Quantitative real-time PCR**

Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). Two micrograms of the total RNA was reverse transcribed using the Maloney murine leukemia virus reverse transcriptase (Invitrogen) and random hexamers (Amersham Bioscience). Quantitative PCR was performed using the TaqMan Universal PCR Master Mix (Applied Biosystems) and the MX4000 instrument (Stratagene). IL-21R (Hs00222310_m1), IGF-1 (Hs00153126_m1), and RPL37a (Hs01102345_m1) TaqMan gene expression assays were from Applied Biosystems. The thermal cycling parameters used were 50°C for 2 min for optimal AmpErase UNG activity, then 40 cycles at 95°C for 30 s and 60°C for 1 min. To control specificity of the amplified product, a melting curve analysis was performed. No amplification of unspecific product was observed. Amplification of RPL37a was conducted for each sample as endogenous control. The relative expression of IL-21R or IGF-1 mRNA was calculated according to the equation of Pfaffl.

**FIGURE 4.** IL-21 activates first STAT-3, STAT-1, and Erk-1/2 and then lately Akt. HMCL were grown in serum-free medium for 18 h and then stimulated with IL-21 (20 ng/ml) for 5, 15, 30, 60, or 180 min. Equivalent amount of cell lysates (100 μg) were separated by SDS-PAGE, then immunoblotted with anti-phospho Abs. Protein loading was controlled with total Abs.

**FIGURE 5.** IL-21 triggers myeloma cell growth through an IGF-1 secretion in CD45+ HMCL. A. 10^3 myeloma cells were seeded per ml of serum-free collagen-based semisolid stemo III medium with IL-21 (50 ng/ml) in the presence or not of AVE1642 (6 μg/ml) and grown for 15 days. Then gels were dried and stained with MGG and then MCF was scored. Values represent the mean ± SD of the percentage of inhibition of three experiments. B. HMCL were grown in serum-free medium for 18 h and then stimulated either with IL-21 or with the combination of IL-21 and AVE1642 for 3 h. Equivalent amount of cell lysates (100 μg) were separated by SDS-PAGE, then immunoblotted by the indicated Ab. C. 10^3 myeloma cells were seeded per ml of serum-free collagen-based semisolid stemo III medium with IL-21 and with or without wortmaninn and grown for 15 days. Then gels were dried and stained with MGG and then MCF was scored. Values represent the mean ± SD of the percentage of inhibition of three experiments. D. IGF-1 mRNA was measured by quantitative PCR in HMCL or primary myeloma cells stimulated by IL-21 for 3 h. Means ± SD of three experiments is expressed as a ratio to RPL37A mRNA.
Immunoblot analysis

Cells (4 × 10^6) were resuspended in 0.5% NP40 lysis buffer (10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 5 mM EDTA, and protease inhibitors). After 40 min on ice, lysates were cleared by centrifugation at 12000 × g for 30 min at 4°C. Protein concentration was measured using bichinchoninic acid protein assay (Pierce). One hundred micrograms of proteins were loaded for each lane. The proteins were separated by 10% SDS-PAGE and then electrotransferred to PVDF membranes. Western blot analysis was performed by standard techniques with ECL detection (Roche).

Results

IL-21 is a clonogenic factor for 9 out of 24 HMCL being all CD45^− HMCL

We evaluated the capacity of IL-21 to induce myeloma colonies in a collagen-based assay as previously described (7). We investigated the effect of IL-21 on 24 HMCL and we showed that IL-21 generated myeloma colonies in 9 out of 24 HMCL. The clonogenicity ranged from 1 to 26% (Fig. 1). Of major interest, the capacity of IL-21 to stimulate clonogenicity was exclusively restricted to CD45^− HMCL; indeed, IL-21 stimulated clonogenicity of 9 of 17 CD45^− HMCL vs 0/7 CD45^+ HMCL (Fisher exact test p < 0.022). We next examined whether the absence of clonogenicity could be related to an absence of IL-21R expression. The expression of IL-21R transcripts was examined by both quantitative real-time PCR analysis and confirmed by flow cytometry. Most of the HMCL including both CD45^− and CD45^+ HMCL expressed IL-21R (19 out of 24 HMCL) as shown in Fig. 2A. It is worth noting that the level of expression of IL-21R is weak for most of the HMCL (as illustrated in Fig. 2C for LP1), except for XG-5, which expressed very high level of IL-21R detected either by both methods (Fig. 2, A and C). Thus, the level of IL-21R is not directly correlated to the IL-21 induced clonogenicity. To investigate the biological significance of the IL-21/IL21R system in MM patients we examined the expression of IL-21R transcripts by quantitative real-time PCR in primary myeloma cells. We showed that 9 out of 18 purified CD138^+ myeloma cells express IL-21R transcripts (Fig. 2B), demonstrating that IL-21/IL-21R could be potentially important in MM development.

IL-21 activates first STAT-3, STAT-1, and Erk then lately Akt in CD45^− HMCL

It was previously shown that IL-21 mediated its effects through STAT-3 and Erk1/2 activation in myeloma cells (8). We found that IL-21 induced tyrosine phosphorylation of STAT-3, STAT-1, and Erk1/2 and thus only in HMCL able to clone in the presence of IL-21 as shown for LP-1, L363, and KMS-12-PE HMCL (Fig. 3). Indeed, IL-21 neither induced STAT-3, STAT-1, or Erk1/2 phosphorylation in JIM-3, a HMCL that did not clone in the presence of IL-21. Of note, we have previously shown that IL-6 induced the clonogenicity of these cell lines (7) and, as expected, IL-6 induced STAT-3 and Erk 1/2 activation in all HMCL tested (Fig. 3). We next examined the kinetics of activation of these proteins in the presence of IL-21. IL-21 induced a rapid (after a 5-min stimulation), strong, and sustained STAT-3 phosphorylation up to 3 h, whereas the rapid STAT-1 activation was more transient and started to decrease after 1-h stimulation. Surprisingly, an Akt activation was observed after 30 min to 1 h of IL-21 stimulation (Fig. 4). Thus, we hypothesized that Akt phosphorylation could be due to an autocrine loop secondary to IL-21 stimulation in CD45^− HMCL.

IL-21 response corresponds to an IGF-1 autocrine loop in CD45^− HMCL

IGF-1 is a well-known growth factor for HMCL which induced a strong Akt phosphorylation, but the response to IGF-1 is related to the presence or not to the CD45 phosphatase (5). Thus, we hypothesized that IL-21 could induce an IGF-1 autocrine loop in CD45^− HMCL leading to Akt phosphorylation. Also, we first analyzed the effects of the antagonistic mAb anti-IGF-1R (AVE1642) on the clonogenicity induced by IL-21. This Ab triggers a total inhibition of the IL-21-induced clonogenicity of LP-1, KMS-12-PE, Karpas 620, NCI-H929, KMS-11, and RPMI 8226 and a partial inhibition of that of L363 (44%) (Fig. 5A). These results indicate that an autocrine IGF-1 loop was either totally or partially responsible for the IL-21-observed clonogenicity. According to this concept, we observed that AVE1642 mAb induced...
a total inhibition of Akt phosphorylation induced by IL-21 as shown for LP-1 and KMS-12-PE (Fig. 5B) whereas it had no effect on Stat-1, 3, and Erk1/2 phosphorylation. These results indicate that the Akt phosphorylation is necessary to the clonogenicity induced by IL-21. We confirm this hypothesis showing that wortmannin totally inhibits the IL-21-induced clonogenicity (Fig. 5C). Furthermore, quantitative PCR analysis was performed showing that a 3-h IL-21 treatment triggered an IGF-1 mRNA level increase in all the cell lines tested \(n = 6\) and in a MM patient, confirming the hypothesis of IGF-1 autocrine loop (Fig. 5D).

**IL-21 and IGF-1 have an additive effect on clonogenicity**

We have previously shown that IGF-1 induced clonogenicity of CD45⁻ HMCL only \(7\). We show in this study that all the HMCL that cloned with IL-21 were also able to clone with IGF-1 (Fig. 6A). Whereas IGF-1 is usually a more powerful clonogenic factor than IL-21, it induced fewer colonies than IL-21 in L363. We next analyzed the effects of IL-21 in combination with IGF-1. We found that this combination triggered an additive effect on clonogenicity in three of four HMCL tested (Fig. 6A). When the signal transduction was analyzed (Fig. 6B), we observed an increase of the phosphorylation of Akt and Erk with the combination of IL-21 and IGF-1 compared with either IL-21 or IGF-1 alone, which can explain this additive effect on clonogenicity.

**Discussion**

Numerous studies have documented that IL-21 stimulates B cell differentiation and IgG production, and a recent study demonstrated that IL-21 is required in B cell activation expansion and plasma cell generation during CD4⁺ T cell-B cell interaction \(15–17\). Although, IL-21 has been shown to be a growth and survival factor in MM several years ago, no subsequent study has been devoted to the biological significance of the IL-21/IL-21R system in MM. In the present study, we have demonstrated that a majority of HMCL expressed IL-21R, and this expression is also found in \(50\%\) of primary myeloma cells. In contrast to the expression of IL-21R on malignant plasma cells, it was recently shown that normal plasma cells isolated from the spleen lacked expression of IL-21R \(18\). Thus, sustained expression of IL-21R on malignant plasma cells supports the notion that sustained IL-21 signaling may be involved in the development of MM either by increasing proliferation or giving a survival advantage to the malignant cells.

To evaluate the biological significance of IL-21R expression, we have analyzed the capacity of IL-21 to induce self-renew of isolated myeloma cells in a serum-free collagen-based assay. In the absence of cytokines, most of the HMCL do not retain the capacity to self-renew (with the exception of RPMI 8226, KMS-18, and AMO1, and to a less extend NCI-H929 and JIN-3). Using a large panel of HMCL, we show that IL-21 generates myeloma colonies in \(37\%\) of the cases, indicating that IL-21 is an important clonogenic factor for MM. Indeed, we have previously shown that besides IL-6 and IGF-1, which are very potent clonogenic factors for myeloma cells, the other documented growth factors for MM as fibroblast growth factor, hepatocyte growth factor, and heparin-binding-epidermal-like growth factor were very poor clonogenic factors \(7\). IL-21, as IGF-1, but in contrast to IL-6, generates myeloma colonies depending on their CD45 phenotype since only CD45⁺ HMCL cloned with IL-21.

We searched for a correlation between clonogenicity and signaling pathway activated by IL-21, showing that only HMCL able to clone are also able to activate all the downstream molecules of IL-21 signaling pathways. Similar to IL-2, IL-4, IL-7, and IL-15 it has been shown that IL-21 activates JAK1 and JAK3 with JAK1 binding IL-21R \(13\) and JAK3 binding \(\gamma_c\) \(19\). According to JAK1 and JAK3 activation, we found that IL-21 induced activation of both STAT-1 and STAT-3 in HMCL but not of STAT-5 (result not shown). IL-21 is thus distinct from IL-2, IL-7, and IL-15, which mainly activate STAT-5 \(19\). Although, the activation of STAT-1 by IL-21 was not previously detected by Brenne et al. \(8\) in myeloma cells, we found it in the current study that is in agreement with numerous other studies on other cell types \(12,20\).

Kinetics study indicates that Stat-1 activation is more transient than Stat-3 activation showing that STAT-3 might play a greater role in IL-21 induced clonogenicity. This result is consistent with the notion that STAT-1 is a negative regulator of cell growth and survival \(21\) whereas Stat-3 is a positive regulator of survival and promotes oncogenesis \(22\). In addition to the JAK/STAT pathway, certain \(\gamma_c\) dependent cytokines can activate MAPK and PI3K/Akt pathways \(20\). In myeloma cells, we found that IL-21 triggers a rapid Erk 1/2 phosphorylation, a pathway known to contribute to proliferation. Because IL-21 activates strongly STAT-3 but also Erk1/2, we show an important similitude in the downstream signaling molecule activated by IL-21 and IL-6. Finally, we found that IL-21 induced a delayed Akt phosphorylation that starts 30 min to 1 h after IL-21 stimulation. Thus, in CD45⁻ HMCL, IL-21-mediated clonogenicity required cooperative effects of the three pathways: JAK/STAT, Erk/MAPK, and Akt/PI3K pathways. We can assume that the JAK/STAT pathway is involved in survival effect whereas both Erk/MAPK and Akt/PI3K pathways are involved in the proliferation effect. Because in myeloma cells, IGF-1 is the major growth factor known to induce a strong Akt phosphorylation and since the effects of IGF-1 as those of IL-21, are restricted to CD45⁻ HMCL, we have investigated the effect of an antagonistic anti-IGF-1R Ab (AVE1642) on the IL-21 induced clonogenicity. AVE1642 induced a strong inhibition of the IL-21-induced clonogenicity indicating that an autocrine IGF-1 loop was involved in the IL-21 clonogenicity sustained by the result of quantitative PCR. Thus, this result explains that the clonogenicity of IL-21 was restricted to CD45⁻ HMCL as it was already demonstrated for IGF-1 induced clonogenicity \(7\). Indeed, we have previously demonstrated that the presence of CD45 phosphate inhibits IGF-1 signaling by a direct interaction between CD45 and IGF-1R, which probably dephosphorylates the IGF-1R and results in an inhibition of IGF-1 signaling \(5\). Thus, in CD45⁺ HMCL, the clonogenic effect of autocrine IGF-1 loop induced by IL-21 was inhibited by the presence of the CD45 phosphate. Although, the proliferative effects of IL-21 seem to be due to an autocrine IGF-1 loop, we still observed an additive effect of IL-21 and IGF-1 on clonogenicity. We can hypothesize that Stat-3 phosphorylation induced by IL-21 will give a survival advantage which finally result in increased clonogenicity after exposure to both IL-21 and IGF-1. Moreover, we observed that Akt phosphorylation induced by IL-21 can be increased by the combination of IL-21 and IGF-1 explaining in part the additive effect of the IL-21/IGF-1 combination on clonogenicity. In conclusion, our data confirm that IL-21 is a potent MM cell growth factor as previously published \(8\). However, our results demonstrate that IL-21 is not a really new independent growth factor for MM but that it is dependent on IGF-1 to act, reinforcing the major role of IGF-1 as a growth factor of MM. These results support the notion that therapy against IGF-1R, which is presently under investigation in MM, could be beneficial, not only to suppress IGF-1-mediated MM cell growth, but also IL-21-mediated MM cell growth.

**Acknowledgments**

This work was supported by The Ligue Nationale contre le Cancer (équipe labelisée 2008) and by Institut National du Cancer (PL06_070).
Disclosures
The authors have no financial conflict of interest.

References