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Differential Transcriptional Regulation of CD161 and a Novel Gene, 197/15a, by IL-2, IL-15, and IL-12 in NK and T Cells1

Livio Azzoni, Olga Zatsepina,2 Bekele Abebe, Ian M. Bennett, Palanisamy Kanakaraj,3 and Bice Perussia4

Cytokine-mediated enhancement of spontaneous cytotoxicity depends, at least in part, on modulation of the expression of surface molecules responsible for recognition of target cell structures and triggering or inhibition of the cytotoxic machinery. We previously demonstrated that expression of transcription factors (e.g., Egr-1, JunB, and c-Fos) is differentially regulated by IL-2 and IL-12. Here we show that expression of CD161/NKR-P1A, a molecule involved in triggering cytotoxicity, is specifically up-regulated by IL-12. CD161 transcription, mRNA accumulation, and surface expression are increased by IL-12. Other cytokines sharing the IL-2R β- and/or common γ-chains (i.e., IL-15, IL-4, and IL-7) do not mediate these effects. In an effort to analyze the mechanisms by which IL-2, IL-12, and IL-15 differentially regulate gene transcription, we have isolated a novel gene, 197/15a, the expression of which in NK and T cells is down-regulated by IL-2 and IL-15, up-regulated by IL-12, and not affected by IL-4 and IL-7. IL-2 and IL-12 act, at least in part, repressing 197/15a transcription; their effect on 197/15a mRNA accumulation is partially independent of novel protein synthesis, likely not mediated by JunB, Bcl-2, or Bax, and requires the activity of rapamycin-sensitive molecule(s). The observation that IL-2 and IL-12 differentially modulate CD161 expression suggests the existence of cytokine-specific mechanisms of modulation of spontaneous cytotoxicity based on the regulation of expression of surface molecules involved in target cell recognition and/or triggering of the cytolytic machinery. The Journal of Immunology, 1998, 161: 3493–3500.

Several cytokines enhance cytotoxic activity of NK and Ag-specific T cells. Among these, IFN-αβ, IL-2, IL-15, and IL-12 are qualitatively similar in their ability to enhance target cell recognition and killing (10, 11). IL-2, IL-15, and IL-12 exert similar effects on other NK cell functions (e.g., cytokine production and proliferation) (10–13). However, some of the activation events induced by the different cytokines are distinct. For example, only IL-12 supports the differentiation of CD4+ helper Th0 cells to a Th1 phenotype (14). Proliferation of resting NK cells is induced only by IL-2, although both IL-2 and IL-12 induce cytokine production in the same cells (12), and expression of membrane adhesion molecules (β integrins) is differentially regulated by the two cytokines (15). IL-2 and IL-12 receptors activate distinct signaling pathways. The intermediate affinity IL-2 receptor (IL-2Rβ/CD122) on resting NK cells shares with the IL-15 receptor the β-chain (16), and the common γ (γc)-chain,2 also a component of the IL-4, IL-7, and IL-15 receptors (17); the high affinity IL-12 receptor is composed of at least two chains, β1 and β2, both related to the common gp130 signal-transducing chain of the IL-6, granulocyte-CSF, and leukemia inhibitory factor receptors (18, 19). IL-2 and IL-12 receptors utilize distinct members of the Janus protein tyrosine kinase (Jak) family and activate different molecules of the STAT family (Jak 1 and 3 and STAT 1, 3, and 4 for the IL-2R and Jak 2, Tyk 2, and STAT 1, 3, and 4 for the IL-12R) (20–24). Different STAT complexes bind selective DNA sequences (25), and differential gene expression may be mediated by these complexes after IL-2 and IL-12 stimulation.

We have previously reported that IL-2, but not IL-12, stimulation induces expression of the immediate/early activation genes c-fos, junB, and egr-1, and of bel-2 (26), expression of which correlates with differential resistance of IL-2- or IL-12-treated NK cells to corticosteroid-induced apoptosis (27–29). Most of these

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5 Abbreviations used in this paper: γc, common γ-chain; Jak, Janus kinase; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; NF-AT, nuclear factor of activated T cells; poly(A)1, polyadenylated.

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genes encode transcription factors, and induced AP-1-mediated transcriptional activity has been demonstrated exclusively in IL-2-stimulated cells (26). In addition to transcriptional activation, JunB-containing AP-1 complexes can mediate transcriptional repression (30); moreover, both c-Fos and Jun can inhibit transcription mediated by NF-IL-6 (31), and Bel-2 can inhibit gene transactivation mediated by the nuclear factor of activated T cells (NF-AT) by sequestering calcineurin (32, 33). Both transcriptional activation and repression may play a role to modulate expression of NK cell recognition structures and/or other effector cell molecules involved in triggering cytotoxicity on binding/recognition of different target cells.

Here we present evidence that CD161 participates in the triggering of polyclonal homogeneous human NK cell populations toward selected human (Daudi) tumor cell lines, as well as the murine P815. Treatment of NK cells with IL-12, but not IL-2, IL-15 or other cytokines sharing the γ-chain, results in increased transcription and surface expression of CD161/NKR-P1A. We also describe a novel gene, 19715a, homologous to the mouse apoptosis-related MA-3 (34) and the human H731 (35) genes, the transcription of which is specifically down-regulated in both NK and T cells by IL-2 and IL-15 via a signaling pathway(s) involving rapamycin-sensitive molecule(s) and relying, in part, on factors constitutively expressed in NK cells. Evidence is presented to indicate that a β-chain-mediated signaling is required, and γ-chain is not sufficient, to transduce signals leading to 19715a down-regulation.

Materials and Methods

Lymphocyte preparations and treatment

Human PBL were separated from venous blood obtained from healthy individuals after density gradient (Histopaque 1077; Sigma, St. Louis, MO) centrifugation and adherence to plastics. NK cells were purified from cocultures of PBL with 50-Gy-irradiated RPMI-8866 B-lymphoblastoid cell line, as described (36), by negative selection with a panel of mAb (OKT3, anti-CD5; B36.1, anti-CD5; B52.1, anti-CD14) and goat anti-mouse Ig-coated sheep. The resulting populations were >95% CD16+/ CD5−, as determined by indirect immunofluorescence (flow cytometry) with a panel of mAb. T cells were prepared from the same cultures by negative selection with anti-CD161 (3G8, produced from cells kindly provided by Dr. J. Unkeless, Mount Sinai Medical School, New York, NY) and anti-CD56 (B159.5) mAb (36). As previously reported, T and NK cells negative selection with anti-CD16 (3G8, produced from cells kindly provided by Dr. J. Unkeless, Mount Sinai Medical School, New York, NY) or IFN-γ or IL-2, IL-12, and IL-15 used were double those required for maximal proliferation of NK cells stimulated for 2 h with IL-2 or IL-12 were used as templates. Heat-denatured RNA was isolated from T cells, T cells with T6 MA, T6 MA, T6 MC, or T6 MG as 3′-primers. cDNA synthesis was performed with random primers in a reaction; kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA), rL-15 (20 ng; sp. act. 2.95 × 10⁶ U/mg of protein; Immunex, Seattle, WA), rL-4 (10 ng; sp. act. 10⁷ U/mg of protein; Genzyme, Cambridge, MA), rL-7 (5 ng; sp. act. 10⁶ U/mg of protein; R&D Systems, Minneapolis, MN) or IFN-β (500 ng; antimurine titer, 1.5 × 10⁶ U/mg of protein, provided by Dr. J. S. Price, Cetus, Emeryville, CA). The concentrations of IL-2, IL-12, and IL-15 used were double those required for maximal proliferation of NK or T cells prepared as described. Where indicated, cells were pretreated (30 min, 37°C) with 50 μM PD098059 (a gift from Dr. A. Saltiel, Parke-Davis Pharmaceutical Research/Warner-Lambert, Ann Arbor, MI), inhibiting IL-2-mediated activation of mitogen-activated protein kinase (MAPK) kinase (MEK) (38), or 30 μM etimibe (Sigma; inhibiting protein synthesis by >70% (37)).

IL-CD161 expression was analyzed on NK cells treated with trepsin (1 mg/ml serum-free medium, 10⁵ cells/ml, 20 min, 37°C) in the presence of DNase A (0.1 mg/ml) (both from Sigma).

Indirect immunofluorescence

This was performed as previously described, using the indicated mAb and human Ig-adsorbed, FITC-conjugated goat F(ab′)2 anti-mouse Ig (Cappel, Durham, NC). Samples were analyzed on an EPICS Profile flow cytometer (Coulter, Hialeah, FL). All mAb used, including the anti-CD161 mAb B199.2 (39), have been produced and characterized in our laboratory (36).

Cell-mediated cytotoxicity

This was tested in 4-h ⁵¹Cr release assays with the indicated target cell lines (10⁴ target cells/well) and different numbers of effector NK cells, as previously described (40). When indicated, anti-CD161, or anti-CD56 mAb as control, were present (10 μg/ml) throughout the assay. LU₅₀ was calculated as previously described (40).

mRNA extraction, purification, and Northern blot analysis

Total RNA was extracted from the indicated cells with a guanidinium/phenol/chloroform-based method (Trizol; Life Technologies, Grand Island, NY), following the manufacturer’s specifications. Polyadenylated (poly(A)⁺) mRNA was purified from a minimum of 100 μg of total RNA with the use of biotin-conjugated oligo(dT) and avidin-coated paramagnetic particles (Poly(A)Tract; Promega, Madison, WI). Aliquots of the mRNA were electrophoresed in 16% formaldehyde-agarose gels, transferred to nylon membranes (Hybond; Amersham, Arlington Heights, IL), UV-cross-linked, and analyzed by Northern blotting as described (26). cDNA encoding the bax gene, obtained by RT-PCR (3′ primer, CAAGAGTCAAGAGTGAC; 5′ primer, TCTGCCATGGCACAACAGG) from NK cells; bax cDNA was a kind gift of Dr. E. Alnemri (Kimmel Cancer Center, Philadelphia, PA); the sources of pβGal, β-galactosidase, TCR (β-chain (detecting a nonproductive, truncated 2.0-kb mRNA in NK cells), and IFN-γ cDNA have been previously reported (41). cDNA probes were labeled with [α-³²P]dCTP (sp. act. 3000 Ci/mmol; ICN Pharmaceutical, Costa Mesa, CA) by nick translation (Boehringer Mannheim, Indianapolis, IN), and hybridized to the membrane-bound RNA. Hybridization was detected by autoradiography, and densitometric analysis was performed with a laser scanner (Personal Densitometer; Molecular Dynamics, Sunnyvale, CA) with proprietary software (ImageQuant). Levels of β-galactosidase mRNA served to control for equivalent amounts of total RNA loaded in each lane. Computer-assisted imaging was performed on the scanned autoradiograms. The backgrounds in the figures shown are typical of those in the original films.

RT-PCR differential display

The protocol described by Liang and Pardee (42) was used, modified as follows: 1) reverse transcription: poly(A)⁺ RNA from NK cells stimulated for 2 h with IL-2 or IL-12 were used as templates. Heat-denatured RNA was isolated from T2 MA, T2 MT, T2 MC, or T2 MG as 3′-primers. cDNA synthesis was performed with random primers in a reaction; kindly provided by Dr. S. Wolf, Genetics Institute, Andover, MA), rL-15 (20 ng; sp. act. 2.95 × 10⁶ U/mg of protein; Immunex, Seattle, WA), rL-4 (10 ng; sp. act. 10⁷ U/mg of protein; Genzyme, Cambridge, MA), rL-7 (5 ng; sp. act. 10⁶ U/mg of protein; R&D Systems, Minneapolis, MN) or IFN-β (500 ng; antimurine titer, 1.5 × 10⁶ U/mg of protein, provided by Dr. J. S. Price, Cetus, Emeryville, CA). The concentrations of IL-2, IL-12, and IL-15 used were double those required for maximal proliferation of NK or T cells prepared as described. Where indicated, cells were pretreated (30 min, 37°C) with 50 μM PD098059 (a gift from Dr. A. Saltiel, Parke-Davis Pharmaceutical Research/Warner-Lambert, Ann Arbor, MI), inhibiting IL-2-mediated activation of mitogen-activated protein kinase (MAPK) kinase (MEK) (38), or 30 μM etimibe (Sigma; inhibiting protein synthesis by >70% (37)).

IL-CD161 expression was analyzed on NK cells treated with trepsin (1 mg/ml serum-free medium, 10⁵ cells/ml, 20 min, 37°C) in the presence of DNase A (0.1 mg/ml) (both from Sigma).
FIGURE 1. Involvement of CD161 in spontaneous cytotoxicity. Cytotoxicity of short term culture homogeneous polyclonal NK cell populations was measured in 4-h $^{3}$Cr release assays using the indicated E:T ratios. Medium (●), anti-CD56 mAb B159.5 (Ⅲ), or anti-CD161 mAb B199.2 (+) (10 µg/ml) were added throughout the assay.

FIGURE 2. Surface expression of CD161 on NK cells cultured with IL-2 or IL-12. Left, NK cells were cultured for 1 (a, b, c) or 5 days (d, e) in medium without (a) or with rIL-2 (100 U/ml, b, d) or IL-12 (5 ng/ml, c, e). Indirect immunofluorescence (flow cytometry) was performed with irrelevant Ig (—), anti-CD161 mAb B199.2 (——); anti-CD16 mAb 3G8 (----), and FITC-conjugated goat anti-mouse IgG. Mean fluorescence channel values are indicated above the corresponding peak. Right panels: a. Indirect immunofluorescence was performed on NK cells using anti-CD161 mAb B199.2 before (——) or after (—— —) treatment with trypsin as described in Materials and Methods (——, irrelevant mAb). b to d. Trypsin-treated NK cells were cultured for 4 days with medium alone (b), rIL-2 (100 U/ml, c) or rIL-12 (5 ng/ml, d). Immunofluorescence analysis was performed with irrelevant mAb (——), anti-CD161 mAb B199.2 (1-day culture) (----), or anti-CD161 mAb B199.2 (4-day culture) (——).
The two cytokines combined had only a marginal effect (84.8% effects were maximal at 6 h and persisted up to 18 h of stimulation. The same filter was sequentially hybridized to 32P-labeled CD161 (top) and human β2-microglobulin cDNA for normalization (bottom). The relative mobility of 18S ribosomal RNA is indicated. B. Nuclear run-on analysis was performed on NK cells stimulated for 6 h as in A. Hybridization of 32P-labeled nuclear RNA to CD161 and to TCR β-chain cDNAs was detected by autoradiography (left). not shown) was instead similar in all culture conditions. After trypsin treatment (Fig. 2, right, a), NK cells lost reactivity with the anti-CD161 mAb B199.2. To determine whether IL-2 and IL-12 affect CD161 expression interfering with its de novo synthesis, membrane expression was analyzed in NK cells cultured with either cytokine for 1 or 4 days after trypsin treatment, to allow complete resynthesis of the molecule after cleavage. As shown in Figure 2 (right, b, c, and d), cells cultured with IL-12 expressed levels of CD161 that, although similar to those detected on cells cultured in medium alone, were higher than those on cells cultured with IL-2. In separate experiments, cells cultured with IL-15 expressed levels of CD161 higher than those on cells cultured in medium alone or IL-2 but lower than those on IL-12-treated cells (not shown).

To test the hypothesis, from the above data, that IL-12 allows synthesis and reexpression of the molecule after cleavage, whereas IL-2 inhibits it, we analyzed CD161 mRNA accumulation in NK cells after cytokine treatment. As shown in Figure 3A (experiment representative of six performed), IL-12 induced a significant increase (177 ± 45.7% of unstimulated cells, n = 6, p < 0.05), and IL-2 induced a significant decrease (42.1 ± 28.7% of unstimulated cells, n = 6, p < 0.05) of CD161 mRNA accumulation. Both effects were maximal at 6 h and persisted up to 18 h of stimulation. The two cytokines combined had only a marginal effect (84.8 ± 46.6% of unstimulated cells, n = 6, p > 0.05).

To determine whether the observed effects depend on cytokine-induced modulation of CD161 transcription rate, run-on analysis was performed on nuclei isolated from 6-h IL-2- or IL-12-stimulated NK cells (Fig. 3B). CD161 transcription in nonstimulated cells, normalized to β2-microglobulin, was 100 arbitrary units (average, n = 2). IL-2 and IL-15 had little effect on it (mean 85.4 and 116 arbitrary units, n = 3 and 2, respectively), whereas IL-12 induced a marked increase of its transcription rate (174 arbitrary units, n = 3). Taken together, these findings indicate that whereas the IL-12-mediated increase in CD161 mRNA accumulation depends, at least in part, on its increased transcription, other mechanisms (e.g., increased mRNA degradation) may participate in the IL-2-mediated down-modulation of the same gene.

Isolation of 197/15a, a novel gene the expression of which is differentially regulated upon IL-2 and IL-12 stimulation

To determine whether expression of additional genes is regulated differentially by IL-2 and IL-12, we performed RT-PCR differential display on mRNA obtained from NK cells treated for 2 h with either cytokine. This time point was chosen to maximize the chances of isolating genes the expression of which is regulated directly by signaling events (immediate/early genes), rather than “second wave” genes. An mRNA species expressed in IL-12- but not in IL-2-stimulated cells was isolated. The corresponding cDNA (197/15a) hybridized to mRNA from NK cells. 197/15a mRNA was also detected in the B (Daudi, RPMI-8866, CESS), T (Jurkat, MOLT 4), myeloid (HL-60, ML-3, U937, THP-1), and solid tumor-derived cell lines (A431, MCF7, PC3, LNGAP, SW48) analyzed (not shown). In primary NK and T cells (Fig. 4, A and B, respectively), two mRNA species with different mobility in 1.2% agarose gels were consistently detected in nonstimulated control cells; mRNA accumulation was either maintained or increased after 2 h of IL-12 treatment (131.6 ± 47.9% of unstimulated cells, n = 12; p < 0.05) but decreased after IL-2 stimulation at the same time point (47.1 ± 14.7%, n = 12, p < 0.05) and up to 6 h (not shown). The two cytokines combined induced marginal decrease of 197/15a expression (76.4 ± 20.6%, n = 7, p < 0.05).

To isolate the full length cDNA sequence of this gene, a AZAPII cDNA library obtained from the T cell line Jurkat, in which relatively high RNA levels are detected, was screened using 197/15a cDNA under high stringency conditions. Two positive clones of ~2500 and ~2000 bp, respectively, were isolated, and their sequence was determined using the dyeoxy-NTP termination method. The sequence (GenBank accession number U96628) revealed a high degree of homology (88% identity) with a mouse gene independently isolated by Shibahara et al. (34), and by Onishi and Kizaki (44). Expression of this gene is increased in thymocytes and RVC lymphoma cells, respectively, after corticosteroid- or topoisomerase inhibitor-induced apoptosis. With the exception of an additional 87 bp at position 126, resulting in a different predicted start codon, and the lack of the first 15 amino acids (MDVENEQILNVPAD), the 197/15a sequence is 98% identical with the cDNA sequence of the human H731 gene, encoding a protein the expression of which is modulated during the cell cycle in chick embryo cells and mouse fibroblasts (35, 45). The accuracy of our sequence was confirmed sequencing a cDNA product obtained from NK cell-derived mRNA with the use of RT-PCR with primers encompassing the region of interest (5′ = 87′ – 106; 3′ = 217′ – 234). The differences between 197/15a and H731 in the predicted protein sequences do not encompass areas previously identified as putative casein kinase (amino acids 25–29 and 33–35) or protein kinase (amino acids 64–74; 102–109; 211–223; 237–243; 367–393) phosphorylation site motifs or the location of basic and acidic domains.

Regulation of 197/15a expression

To define the molecular mechanisms of IL-2-mediated down-modulation of 197/15a mRNA accumulation, run-on analysis was performed on nuclear RNA extracted from NK cells stimulated for 2 h.
with either IL-2 or IL-12. As shown in Figure 4C (experiment representative of two performed), IL-2 treatment, compared with that with IL-12, resulted in lower levels (47%) of 197/15α transcription; in separate experiments, transcription levels of 197/15α in IL-2-stimulated cells were 56% of those in unstimulated cells. These data indicate that the IL-2 controls expression of this gene, at least in part, via negative transcriptional regulation.

To determine whether down-modulation of 197/15α depends on expression of repressor molecules induced by IL-2, but not by IL-12, we analyzed the effect of inhibitors of different biochemical pathways known to lead to expression of these genes. Similar studies on CD161 expression could not be performed because of poor cell viability after lengthy treatment with the various inhibitors. IL-2, but not IL-12, induces expression of AP-1 family genes (c-fos and junB) and bcl-2 in NK and T cells (26). JunB has been demonstrated to act as a transcriptional repressor (30, 31). Treatment of NK cells with the MEKK inhibitor PD098059 resulted in >65% inhibition of IL-2-induced junB mRNA accumulation (not shown), indicating that activation of the MAPK pathway is a required signaling event. However, as reported in Table I, the IL-2-mediated down-modulation of 197/15α mRNA accumulation was not affected under the same conditions. Bcl-2 acts as a transcriptional repressor interfering with NF-AT-mediated transcription (33), and its induced expression in B cells is sensitive to rapamycin (46). Rapamycin treatment of NK cells inhibited, as expected, IL-2-induced activation of p70s6 kinase (not shown) and reduced significantly the down-modulatory effect of IL-2 on 197/15α expression (Table I) without affecting IL-2-induced bcl-2 expression (not shown).

In the IL-2-dependent T lymphoma cell line 2780a, overexpression of Gfi-1 results in repression of bax and bak transcription, and protection from IL-2 withdrawal-induced apoptosis, suggesting that this transcriptional repressor may mediate the inhibitory effects of IL-2 (47). NK cells were stimulated for 2 or 6 h with IL-2, IL-15, or IL-12, alone or in combination, and bax mRNA accumulation was detected in Northern blotting. In two separate experiments, cytokine treatment did not affect significantly bax expression, although inducing, as expected, IFN-γ mRNA accumulation (not shown), suggesting that Gfi-1 does not participate in the IL-2-mediated repression of CD161 or 197/15α transcription at the time points analyzed.

The requirement for de novo protein synthesis in the IL-2-mediated repression of 197/15α was tested in Northern blot analysis of total RNA from NK cells cultured for 2 h with or without IL-2, IL-12, and emetine. As summarized in Table I, emetine treatment

Table I. Effect of rapamycin, MEKK, and protein synthesis inhibitors on IL-2 and IL-12-mediated modulation of 197/15α

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Treatment</th>
<th>Stimulus</th>
<th>Treatment</th>
</tr>
</thead>
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<tr>
<td>None (2)</td>
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<td>PD098059 (4)</td>
<td>None (3)</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>100</td>
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</tr>
<tr>
<td>IL-2</td>
<td>52.5 ± 10.6</td>
<td>92.0 ± 4.3</td>
<td>61.2 ± 15.0</td>
</tr>
<tr>
<td>IL-12</td>
<td>96.3 ± 12.0</td>
<td>117.2 ± 12.3</td>
<td>132.2 ± 38.9</td>
</tr>
</tbody>
</table>

* Northern blot analysis was performed on total RNA from NK cells incubated (5 × 10⁶/ml, 2 h, 37°C) in medium without (none) or with IL-2, 100 U/ml, or IL-12, 5 ng/ml.
* Cells were pretreated with the indicated inhibitors for 30 min, as described in Materials and Methods; the inhibitors were present throughout incubation.
* Number of experiments performed under each condition.
* Percentage expression (mean ± SD) of 197/15α mRNA in stimulated compared with control nonstimulated cells (100%). These were calculated based on densitometric analysis of Northern blots. Levels of mRNA accumulation in control cells incubated in medium with or without inhibitors only were not significantly different from each other.
* Rapamycin-treated, IL-2-stimulated vs nontreated, IL-2-stimulated cells, p < 0.05. Nontreated or PD098059-treated, IL-2-stimulated vs nonstimulated cells, p < 0.05. All other values are not significantly different from each other or controls.
* Nontreated, IL-2-stimulated vs nonstimulated cells, p < 0.05; emetine-treated, IL-2 stimulated vs nonstimulated cells, p < 0.05; emetine-treated IL-2 stimulated vs nontreated, IL-2 stimulated, p < 0.05.
reduced significantly but did not abolish the IL-2-induced down-modulation of 197/15a mRNA accumulation, indicating that the effect is in part independent from de novo protein synthesis; the effect of IL-12 on 197/15a expression was not significantly affected under the same conditions.

Modulation of 197/15a and CD161 expression in NK cells by cytokines utilizing receptors that share the γc-chain

The IL-2 but not the IL-12 receptor shares a γc-chain with the IL-4, IL-7, and IL-15 receptors (reviewed in Ref. 17), all expressed in NK cells, and stimulation of the latter receptors results in functional effects similar to those induced by IL-2 (28, 29, 48, 49). Additionally, IL-2 and IL-15 receptors share the IL-2R β-chain as a signal transducing moiety. To start analyzing the role of β- and γc-chains in transducing events leading to CD161 and 197/15a down-modulation and to define the cytokine specificity of this effect, CD161 and 197/15a mRNA accumulation were analyzed in NK cells stimulated with different cytokines, with or without IL-12 added. IL-15, like IL-2, but unlike IL-4, IL-7 (Table II), or IFN-β (not shown), induced significant decrease in the expression of 197/15a, whereas IL-12, alone or in combination with IL-4 and with IL-7, increased it significantly. Only IL-2 down-modulated significantly CD161 mRNA accumulation, whereas IL-12, alone or in combination with IL-4 and IL-15, enhanced it significantly. These results indicate that γc-chain usage is insufficient, per se, to transduce signals resulting in transcriptional repression or activation of the genes studied. Moreover, the differential sensitivity of CD161 and 197/15a to IL-2 and IL-15 treatment suggests that different biochemical pathways control the expression of the two genes.

Discussion

Cytokine stimulation of NK cells, inducing expression of genes encoding cytokines, transcription factors, cytotoxic mediators, and surface molecules responsible for their biologic functions, represents an important mechanism for the regulation of immune responses. IL-2, IL-12, and IL-15 induce similar, yet distinct, functional effects on NK cells (50). Here we report evidence that the three cytokines differentially regulate expression of at least two genes, CD161 (NKR-P1A) and 197/15a, and that this differential regulation occurs, at least in part, at the transcriptional level. This, together with the novel observation that CD161 is indeed involved in cytotoxicity of human polyclonal NK cell populations against selected human target cells, supports the possibility that cytokotoxic activity of human NK cells against some target cells may be preferentially regulated by different cytokines.

Engagement of the C-type lectin CD161 triggers the cytotoxicity activity of rodent NK and dendritic cells (6, 51). Here we demonstrate that blocking this molecule with nonstimulatory anti-CD161 mAb reduces significantly NK cell cytotoxicity of NK cell populations toward at least one human target cell line (Daudi, B-lymphoblastoid). This observation extends to a human target cell and to polyclonal NK cells previous reports (7) on murine target cells (insensitive to resting human NK cells) and NK cell clones (i.e., cells stimulated with large doses of IL-2 in long term cultures) and supports the hypothesis that CD161 may play a relevant role in NK cell-mediated cytotoxicity in vivo. Of the human hematopoietic cell lines tested, only Daudi appears capable to trigger cytotoxic activity in a CD161-dependent fashion, suggesting that the use of CD161 for recognition/activation by NK cells is selective for specific target cells. Alternatively, CD161 may behave like a killer-inhibitory receptor, and the inhibition of cytotoxicity against the FcγRII+ Daudi in the presence of the IgG2b anti-CD161 mAb may depend on induced cross-linking of this molecule at the NK cell membrane, with consequent triggering of its inhibitory activity. The observation that the same mAb does not inhibit killing of other FcγRII+ cell lines (specifically K562, Raji, U937, THP-1, and HL-60) that can function as Daudi cells to cross-link CD161 on the NK cells is against this hypothesis. Studies in murine NK cells (6, 51) and in human dendritic cells (52) also support an activatory role for CD161.

Regulation of the surface expression of CD161 may be expected to affect the cytotoxic activity of NK cells, at least against selected (i.e., CD161 ligand expressing) target cells. Our observation that only IL-2, among several cytokines tested, inhibits CD161 expression suggests the possibility that this cytokine alters preferentially cytokine responses of NK cells to CD161-engaging target cells, therefore contributing to diversify NK cell responses to the different cytokines and target cells. Although it is possible that increased expression of a cytotoxicity-triggering receptor (like that specifically induced by IL-12) contributes to more efficient killing of distinct target cells, direct proof of this prediction cannot be obtained with primary NK cells. In these cells, cytokine activation induces expression of several molecules involved in cytotoxic activity (e.g., adhesion molecules (15), perforin, granzymes (53), and possibly additional molecules yet to be identified). This makes it impossible to distinguish effects that depend directly on cytokine-induced altered expression of CD161 from those related to the other effects. Studies of the effect of CD161 gene inactivation or overexpression in cytotoxic cell lines, beyond the scope of the present study, are needed to define the functional consequence specifically of the modulation of CD161 expression human NK cell-mediated cytotoxicity.

Although IL-2 and IL-15 bind to receptors that share both β- and γc-chains (16), a combination considered sufficient for complete signaling (46), only IL-2 affects significantly CD161 expression. Because the cultured NK cells we used do not express detectable levels of IL-2R α-chain and stimulation of cytokine receptors utilizing only the γc-chain (IL-4R and IL-7R) does not affect CD161 mRNA accumulation, our data suggest that engagement of the IL-15R α-chain transduces signals that serve to maintain expression of CD161 mRNA and that IL-2R β-chain-mediated signaling is required to induce down-regulation of this gene, whereas the γc-chain is not sufficient. Requirement for β-chain signaling may not be restricted to CD161 modulation, as suggested by the observation that mRNA accumulation of another gene, 197/15a, in NK and T cells is specifically down-modulated by IL-2 and
IL-15, but not by IL-12, IL-4, or IL-7. IL-2-mediated down-regulation of 197/15a is, at least in part, transcriptional, as demonstrated by the reduced transcription rate of this gene in IL-2-treated cells, compared with untreated or IL-12-stimulated cells.

IL-2 significantly inhibits expression of both 197/15a and CD161 mRNA, but the molecular mechanisms involved likely differ, because: 1) only 197/15a transcription is significantly down-regulated by IL-2 treatment and 2) down-regulation of 197/15a and CD161 mRNA expression occurs with different kinetics (detectable after 2 and 6 h of stimulation, respectively). Although IL-2 markedly reduces CD161 mRNA expression, it only marginally affects its transcription rate (84% of unstimulated control). Therefore, it is likely that posttranscriptional mechanisms, e.g., heterogeneous RNA processing and reduced mRNA stability, are involved in the observed IL-2-induced down-modulation of CD161 mRNA accumulation. On the contrary, only IL-12, of the cytokines tested, induces increased transcription of CD161; this is likely to contribute to the IL-12-specific positive regulation observed at both mRNA and protein level.

The 197/15a cDNA sequence is highly homologous to that of the murine MA-3 gene, isolated by separate groups from lymphoid cells induced to undergo apoptosis with a variety of stimuli, including toposoisomerase inhibitors, corticosteroids, and cytokine deprivation (34, 44). Because 197/15a mRNA is abundant both in resting primary cells and in proliferating cell lines, we did not analyze its expression in apoptosis-inducing conditions. However, it is tempting to speculate that low levels of expression of this gene may correlate with cell survival and protection from apoptosis. Such an effect, if proved, is unlikely to depend directly on expression of this gene, since IL-12, IL-4, and IL-7 protect NK cells from IL-2 withdrawal-induced apoptosis (28) without inducing 197/15a down-modulation (our data). Also, IL-2, IL-15, and IL-12 all prime NK cells for activation-induced apoptosis via the FcγRIIA (Ref. 54; our unpublished results) irrespective of their effect on 197/15a expression, indicating that protection from apoptosis, if related to expression of 197/15a, may be limited to specific conditions exclusive of activation-mediated apoptosis. Experiments using antisense oligodeoxyribonucleotide to down-modulate 197/15a expression (not shown) did not yield significant results, and the possible role of this gene in cell division/survival remains to be determined.

197/15a cDNA sequence is identical, with the exception of 87 bp in its 5′-region, with the human H731 gene, encoding a protein of ~56 kDa, expression of which is modulated during the cell cycle (35, 45). Abs to an epitope contained within the first 147 amino acids of this protein did not detect IL-2-down-modulated products in NK cells, as analyzed in Western blotting or immunocytochemistry (not shown). It is unlikely that sequencing errors account for the differences observed between 197/15a and H731 because two identical 197/15a sequences, with high homology to the 5′-region of the murine gene, have been cloned independently from the Jurkat cell line and from primary NK cells. However, differential splicing or gene duplication cannot be excluded. Although the protein encoded by this gene remains to be identified and its function defined, the observation that IL-2, but not IL-12, negatively regulates its expression serves to underscore major differences in the biologic effects of these cytokines.

Negative gene regulation may result either from direct activation of repressor molecules and/or inactivation of transcriptional activators or from de novo expression of “first wave” genes encoding them. A few genes encoding molecules acting as transcriptional repressors in specific circumstances are expressed in NK cells after IL-2 but not IL-12 stimulation. We have started to investigate their possible role in the IL-2-mediated 197/15a down-regulation using an indirect approach. The observation that the MEKK inhibitor PD098059, while inhibiting IL-2-mediated JunB induction (data not shown), has no effect on 197/15a regulation indicates that neither the former nor other molecules activated in a MAPK-dependent manner contribute to regulation of its expression. Several lines of evidence make Bcl-2 involvement unlikely: 1) Bcl-2 can exert transcriptional repression by sequestering calcineurin, thereby inhibiting NF-AT activation and nuclear translocation; however, NF-AT activity is not induced in NK cells upon IL-2 stimulation (our unpublished observations); 2) bcl-2 expression is induced late (6 h) in response to IL-2, whereas 197/15a transcription is down-modulated within 2 h and is, at least in part, independent of de novo protein synthesis; 3) the IL-2-induced bcl-2 expression in NK cells, unlike 197/15a down-modulation, is rapamycin resistant; and 4) IL-7 induces bcl-2 (29) without affecting 197/15a expression. Expression of bax, the negative regulation of which may reflect indirectly Gfi-1 repressor activity (47), is not modulated by IL-2 in NK cells. This observation, although it suggests that Gfi-1 does not play a major role in controlling 197/15a expression, does not rule this out completely: bax transcriptional regulation likely depends on a balance between the activity of both trans-activating and repressor molecules, and the effects of each single molecule might be difficult to isolate in intact cells. Taken together, our data support the conclusion that a yet to be defined molecule(s), target of IL-2-activated signaling elements and sensitive to rapamycin, controls 197/15a expression. It is also possible that the inhibitory effect of rapamycin depends, at least in part, on inhibition of p70S6 kinase or other target molecules, with consequent lack of activation of a repressor (55). Protein synthesis inhibition reverts only partially the IL-2-induced 197/15a down-regulation, supporting the hypothesis that the latter effect is, at least in part, independent from de novo expression of repressors or molecules that control transcriptional activators, and relies on activation of preexisting repressor molecules; alternatively, transcriptional activators operating in resting cells may be specifically inactivated upon IL-2 stimulation.

Although IL-2 treatment inhibits both CD161 and 197/15a expression, the experimental evidence reported here (different kinetics of gene modulation, distinct effects of IL-15 on the two genes, and enhanced expression of CD161 exclusively by IL-12) indicates that transcriptional modulation of these two genes involves different molecules. Moreover, our data present the first experimental evidence, to our knowledge, of positive regulation of gene transcription mediated specifically via IL-12R stimulation. This makes CD161 an especially interesting target for studies of the molecular mechanisms involved in its regulation. Definition, via genomic cloning, of the promoter structure of both genes, presently under way in our laboratory, will allow the characterization of IL-12-, IL-2-, and IL-15-responsive element(s) and the identification of DNA-binding complexes specifically induced by each cytokine, involved in transcriptional regulation of 197/15a and CD161, and possibly other genes relevant to NK cell biology.

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