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Differential signaling of cmvIL-10 through common variants of the IL-10 receptor 1

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Abstract

Human IL-10 (hIL-10) signaling is mediated by receptors consisting of two subunits, IL-10 receptor 1 (IL-10R1) and IL-10 receptor 2. Two common variants of the IL-10R1 (Ser 138 Gly (single-nucleotide polymorphism 3, SNP3) and Gly 330 Arg (SNP4)) are associated with diverse disease phenotypes. Viral homologs to hIL-10, such as cmvIL-10, utilize the same IL-10 receptor complex as part of viral immune evasion strategies. For the present study we hypothesized that IL-10R1 variants alter the ability of viral IL-10 to utilize the IL-10R1 signaling pathway. HeLa cell clones expressing different IL-10R1 haplotypes (WT or any variant) were incubated with hIL-10 or cmvIL-10. In cells expressing IL-10R1-WT, cmvIL-10 (both non-glycosylated- and HeLa-expressed) resulted in equal or slightly stronger STAT3 phosphorylation compared with hIL-10. In clones expressing IL-10R1-SNP3, IL-10R1-SNP4 or IL-10R1-SNP3+4, the cmvIL-10 showed significantly less STAT3 phosphorylation, especially when HeLa-expressed cytokines were used. Time course experiments demonstrated a slower kinetic of cmvIL-10 STAT3 activation through the variant IL-10R1. Similarly, IL-10R1 variants decreased the cmvIL-10-induced SOCS3 and signaling lymphocytic activation molecule mRNA expression. These data suggest that the IL-10R1 variants differentially reduce the signaling activity of cmvIL-10 and thereby may affect CMV's ability to escape from the host's immune surveillance.

Keywords

cmvIL-10; IL-10; IL-10 receptor 1; Single-nucleotide polymorphism

Introduction

IL-10 is a pleiotropic cytokine widely conserved among higher eukaryotes [1]. Disruption of IL-10 expression in mice leads to spontaneous enterocolitis [2]. Silencing of other components of the IL-10 signaling pathway such as the IL-10 receptor 2 (IL-10R2) or target

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deletion of the IL-10-signal transducer STAT3 also results in chronic intestinal inflammation [3-5]. Thus, IL-10 is considered essential for preservation of immune homeostasis in the gut.

The biological function of IL-10 is mediated through specific cell surface receptors. The functional receptor complex of IL-10 consists of two subunits, IL-10 receptor 1 (IL-10R1) and IL-10R2, which belong to the class II cytokine receptor family [1]. Although IL-10R2 does not bind IL-10, its presence is essential for signal transduction [4, 6]. Mice with a disruption of the IL-10R2 chain lack the ability to respond to IL-10 [4]. IL-10R2 is a shared subunit of receptor complexes for IL-10, IL-22, IL-26, IL-28 and IL-29 [3, 7-11]. The IL-10R2 chain is expressed at variable levels on most somatic cells [9]. In contrast, the ligand-binding chain IL-10R1 is differentially expressed in various tissues, among which are hematopoietic cells such as lymphocytes or cells of the monocytes-macrophage system [12].

The activated IL-10-signaling complex involves several transcription factors (*e.g.* STAT [13]), which are also utilized by other cytokines, chemokines and growth factors. The binding of IL-10 to the extracellular domain of IL-10R1 activates phosphorylation of the receptor-associated Janus tyrosine kinases, JAK1 and Tyk2 [1]. These kinases then phosphorylate specific tyrosine residues on the intracellular domain of the IL-10R1 chain (Y446 and Y496) to allow the binding of the latent transcription factors STAT1, STAT3 and in some cells STAT5 [1]. The STAT then phosphorylate, dimerize and translocate to the nucleus where they bind to STAT-binding elements in the promoters of various IL-10-responsive genes. The primary function of IL-10R2 is to bring Tyk2 to the receptor complex, causing JAK cross-activation and initiation of signal transduction [14]. Besides the JAK-STAT pathway, IL-10 also activates the p38 mitogen-activated protein kinase pathway and phosphatidylinositol 3-kinase [15, 16].

Viral homologs of IL-10 from CMV (cmvIL-10) or EBV (ebvIL-10) are expressed during the lytic phase of the viral life cycle and affect virus–host interaction and viral survival [17, 18]. Several other viral IL-10 homologs are encoded by Orf poxvirus, equine herpesvirus type 2, rhesus lymphocryptovirus and callitrichine herpesvirus 3 [1]. Viral IL-10 homologs are considered to play a major role for the viruses to escape the host's immune surveillance by activation of IL-10 signaling [19, 20]. Both cmvIL-10 and ebvIL-10 interact with IL-10R1. EbvIL-10 shares 85% protein identity with the human IL-10 (hIL-10), whereas cmvIL-10 shows only 27% homology. During latent infection, CMV expresses another IL-10 homolog transcript, LA-cmvIL-10, the role of which is currently unknown [21]. In spite of its lower degree of sequence identity to hIL-10, cmvIL-10 maintains the ability to bind to the specific receptor with affinity similar to that of hIL-10 [22] and even the biological activities of cmvIL-10 are similar to those of IL-10. In contrast, ebvIL-10 exhibits only a subset of functions that are shared with the human homolog [23]. IL-10R1-binding studies have shown that the affinity of hIL-10 is at least 1000-fold higher than ebvIL-10 independent of IL-10R2 [6].

The complexity of hIL-10R signaling is further enhanced by the presence of single-nucleotide polymorphisms (SNP) in the coding sequence of IL-10R1 [24]. SNP3, a Ser 138 Gly (S138G) mutation, is located in the extracellular domain and leads to a loss of a hydrogen bond and to structural changes, which may influence receptor–ligand interactions

and reduce receptor signaling [24]. SNP4, a Gly 330 Arg mutation, is located in the cytoplasmic domain of IL-10R1 close to the membrane and in a region that affects receptor stability [25]. SNP3 is in strong linkage disequilibrium with SNP4 and mostly expressed as SNP3+4 haplotype [24]. These variant receptors are associated with disease phenotypes such as schizophrenia [26] and progression of fibrosis in chronic hepatitis C [27]. A worldwide population analysis has revealed the presence of SNP4 in Sub-Saharan Africa, while SNP3 evolved in Semite and Caucasian populations, which displayed the highest frequency for both variants. Both SNP were almost absent in native populations from East Asia and America [28].

The allelic frequency in populations is owing to two factors: natural selection, which is the result of population variation among individual genotypes in their probability to survive and/or reproduce, and random genetic drift, which is due to a finite number of individuals participating in the formation of the next generation [29]. This study addressed the hypothesis that IL-10R1 SNP have evolved in human populations by improving the probability to survive certain viral infections. Adaptive changes in both viral and human genomes may have taken place during co-evolution. IL-10R1 SNP may reduce the ability of viral IL-10 homologous proteins, specifically of cmvIL-10, to utilize the host's IL-10 signaling pathway and thereby reduce the virus's ability to evade the host's immune surveillance. To test this hypothesis we studied the differential signaling of cmvIL-10 through WT and variant IL-10R1.

Results

cmvIL-10 is more active than hIL-10 in phosphorylating STAT3 in IL-10R1-WT cells

The crystal structure of cmvIL-10 displays comparable affinity to the soluble form of WT IL-10R1 [22]. We therefore tested the activity of cmvIL-10 or hIL-10 to induce phosphorylation of STAT3 in IL-10R1-WT-expressing HeLa cells. HeLa clones expressing IL-10R1-WT (WT-53) were stimulated with 10 ng/mL cmvIL-10 or hIL-10 (both non-glycosylated, expressed in bacteria; R&D Systems), and total levels of STAT3 and STAT3 phosphorylation at residue Y705 were analyzed by Western blot (Fig. 1A). Both cytokines were able to induce STAT3 phosphorylation. Only background activation was observed in cells transfected with the control plasmid. Next, the specificity of hIL-10 and cmvIL-10 was analyzed by addition of different concentrations (0–100 µg/mL) of a neutralizing monoclonal anti-IL-10R1 antibody (MAB274), which was added prior to the ligands (Fig. 1B). Almost complete blocking of the STAT3 phosphorylation was observed with hIL-10, confirming IL-10R1 specificity. This neutralizing effect was not observed when cmvIL-10 was added as a ligand.

To better characterize the activity of cmvIL-10, three IL-10R1-WT-expressing HeLa clones (WT-53, WT-41 and WT-38) were stimulated with different concentrations of non-glycosylated cmvIL-10 (0.01, 1 and 10 ng/mL, batch no. EGT0130001) or hIL-10 as control (1 and 10 ng/mL). In all three clones, a significant increase of STAT3 phosphorylation was seen after induction with cmvIL-10 (Fig. 1C), as shown by densitometry (right panel). However, there were major differences between clones. Clone WT-41 showed STAT3 phosphorylation only at 10ng/mL, indicating reduced responsiveness. This correlated

well with a reduction in receptor density (Fig. 1D). With a different batch of cmvIL-10 (EGT0114061), a slightly higher activity was seen in experiments performed later (see below; Figs. 3A and 4A).

To better understand differences of IL-10 signaling in human cells, cmvIL-10 and hIL-10 were expressed in human HeLa cells (and not bacteria as the commercially available cytokines) by transient transfection. Transfection efficiency was comparable in both cmvIL-10 and hIL-10 transfectants as analyzed by enhanced green fluorescent protein (EGFP) expression. Supernatants were collected, concentrated and protein production was analyzed by Western blot with a FLAG-specific antibody (to test for cmvIL-10) or with a specific anti-hIL-10 antibody (Fig. 2A). HeLa cell clones expressing IL-10R1-WT were cultured with various dilutions of concentrated supernatants (cmvIL-10 (1:1, 1:2, 1:20, 1:200, 1:2000) or hIL-10 (1:1, 1:2)) and whole cell lysates were analyzed for pSTAT3 and total STAT3. The HeLa-expressed cmvIL-10 supernatant was about 100-fold more potent in phosphorylating STAT3 at Y705 than the HeLa-expressed hIL-10 supernatant (Fig. 2B); however, a direct comparison of protein activity is not possible as we did not know the exact cytokine concentrations in the respective supernatant. We do not know whether this higher biological activity of HeLa-expressed cmvIL-10 is due to protein glycosylation [18]. Glycosylation of cytokines enforces the stability of proteins and adds to their biological activity [30, 31].

Variant IL-10R1 haplotypes markedly reduce STAT3 phosphorylation by cmvIL-10

IL-10R1-SNP3 but not IL-10R1-SNP4 reduces the hIL-10 signaling both in HeLa and BaF₃ cells by 30–50% [28]. In the present study we asked whether the presence of SNP3 or SNP4 alters signaling of viral IL-10. Identical to the experiment shown for IL-10R1-WT clones (in Fig. 2B), HeLa clones expressing IL-10R1-SNP3, IL-10R1-SNP4 or IL-10R1-SNP3+4 were stimulated with various dilutions of HeLa-expressed cmvIL-10 or hIL-10 and whole cell lysates were analyzed for pSTAT3 and total STAT3. A concentration-dependent reduction of STAT3 phosphorylation was found for cmvIL-10 in WT clones (Fig. 2B). In clones carrying SNP3 (SNP3-28 and SNP3+4-26) cmvIL-10 only induced STAT3 phosphorylation when the supernatant was used as concentrate solution or as 1:2 dilution (Fig. 2C). Thus, SNP3-28- and SNP3+4-26-expressing clones are less sensitive to cmvIL-10 than the WT-53 clones. In contrast, the activity of hIL-10 in these clones was comparable. To control for receptor expression density, clones were analyzed by flow cytometry upon receptor staining (Fig. 2D). Both IL-10-R1 and IL-10-R2 were detected in all clones at similar density.

Similar experiments were performed with various concentrations of non-glycosylated cytokines (R&D Systems). cmvIL-10-induced STAT3 phosphorylation was compared with that induced by hIL-10 in clones expressing IL-10R1-WT, IL-10R1-SNP3, IL-10R1-SNP4 or IL-10R1-SNP3+SNP4. The different blots were quantified by densitometry, ratios of pSTAT3 to total STAT3 expression were calculated and the results of cmvIL-10 stimulation were normalized to hIL-10 for each variant. In this set of experiments (and different to the experiments shown in Fig. 1), cmvIL-10 show a stronger STAT3 phosphorylation than hIL-10 (Fig. 3A). This is most likely due to differences in the non-glycosylated cmvIL-10 batches used. Because the differences between IL-10R1 haplotypes were not as obvious

as with HeLa-expressed cmvIL-10, the experiments were repeated eight times using 16 different clones (four clones each IL-10R1 haplotype). Statistical analyses by Wilcoxon test (corrected for multiple comparisons) revealed a borderline reduction of cmvIL-10-induced STAT3 phosphorylation in IL-10R1 single-mutant clones ($p = 0.016$ for SNP3; $p = 0.016$ for SNP4) and a significant reduction for the double-mutant clone ($p = 0.008$ for SNP3+4; Fig. 3B).

The kinetic of STAT3 phosphorylation changes with the type of IL-10R1 ligand and the IL-10R1 haplotype

To examine the kinetic of the cmvIL-10 or hIL-10 signaling, HeLa clones were treated with 2 ng/mL of either non-glycosylated hIL-10 or cmvIL-10 (batch no. EGT0130001) for 0–120 min. Cells were harvested and analyzed for phosphorylation of STAT3 and total STAT3 by Western blot and the signal intensity was quantified and plotted against time (Fig. 4). In IL-10R1-WT clones, cmvIL-10 was more active than hIL-10. Using hIL-10, STAT3 phosphorylation reached a plateau at 15 min, while with cmvIL-10 after 30 min. The difference between non-glycosylated hIL-10 and cmvIL-10 seems to be highest after 30 min. To test whether variant IL-10R1 haplotypes alter the kinetics of hIL-10- or cmvIL-10-dependent STAT3 phosphorylation similar experiments were performed with clones expressing IL-10R1-SNP3, -SNP4 or -SNP3+4. In haplotypes that express SNP4 (IL-10R1-SNP4 and IL-10R1-SNP3+4), the plateau of both hIL-10- and cmvIL-10-induced STAT3 phosphorylation was reached already at 15 min, indicating a different kinetic of receptor activation. In haplotypes expressing SNP3 (IL-10R1-SNP3 and IL-10R1-SNP3+4), the activity of cmvIL-10 was equal to or lower than that of hIL-10 at most time points. Thus, the kinetic of IL-10R1-induced STAT3 phosphorylation confirms our single time-point data from above and is in line with the finding that variant receptors differentially reduce the signal intensity of cmvIL-10 when compared with hIL-10.

cmvIL-10 differentially induces the expression of SOCS3 and signaling lymphocytic activation molecule in IL-10R1-WT and IL-10R1-variant clones

The experiments above demonstrated a differential activity of cmvIL-10 and hIL-10 on phosphorylating STAT3, which changed by utilizing variant IL-10R1 haplotypes for ligand binding. To test whether the changes in STAT3 phosphorylation also affect STAT3 signal transduction activity we investigated the mRNA expression of the STAT3 downstream gene SOCS3, which represents an important negative feedback mechanism of cytokine signaling [32]. In order to test the time-dependent up-regulation of SOCS3 in response to IL-10 (10 ng/mL), we stimulated IL-10R1-WT-expressing clones for 0–960 min. mRNA was extracted, reverse transcribed and real-time PCR with gene-specific primers was performed from total cDNA. SOCS3 expression pattern upon hIL-10 stimulation revealed an early increase at 1 h and a peak at 4 h (Fig. 5A). Next, IL-10R1-WT-, IL-10R1-SNP3-, IL-10R1-SNP4- and IL-10R1-SNP3+4-expressing HeLa clones were stimulated with 0–10 ng/mL of non-glycosylated cmvIL-10 or hIL-10 for 1 and 4 h. Compared with hIL-10, cmvIL-10 (10 ng/mL) displayed a slightly better induction in SOCS3 mRNA expression in the IL-10R1-WT clone after 4 h of incubation. This effect of cmvIL-10 was substantially reduced or absent in cells expressing any of the IL-10R1-variant haplotypes. Experiments analyzed after 1 h of stimulation with the same cytokines revealed similar results (data

not shown). These data indicate that hIL-10-induced STAT3 phosphorylation increases the transcriptional activity of STAT3-dependent genes such as SOCS3 (Fig. 5B). An additional target gene of hIL-10 is the signaling lymphocytic activation molecule (SLAM), which is highly induced upon hIL-10 treatment in macrophages [33]. Real-time PCR was performed from cDNA similar to SOCS3. SLAM mRNA was weakly induced in a dose-dependent fashion. Similar to SOCS3, cmvIL-10-dependent SLAM induction was slightly stronger in IL-10R1-WT clones, whereas it was reduced in all clones expressing IL-10R1 variants.

Structural analysis of the putative cmvIL-10/IL-10R1-SNP3

Comparison of the crystal structures of the complexes of hIL-10/sIL-10R1 [34] and cmvIL-10/sIL-10R1 [22] indicates that the network of hydrogen bonds in the vicinity of S138 is the same in both complexes. S138 hydroxyl group forms a hydrogen bond to carbonyl oxygen of N133, which allows D134 side chain belonging to C-terminal fibronectin type III domain of IL-10R1 to make an inter-domain hydrogen bond with the side chain of H71, which belongs to N-terminal domain. Therefore, S138 does not have any direct contact with bound ligand molecules. However, it is clear that mutation S138G (SNP3) leads to disruption of the above hydrogen bond network, which will inevitably affect the conformation of the inter-domain junction. In other words, the S138G (SNP3) mutation will very likely affect the formation of the ligand/receptor complex through the change in the mutual positions of the N- and C-terminal domains. The main difference in the overall structures of hIL-10 and cmvIL-10 dimers is that while inter-domain linkages in human protein are quite flexible, that of the cmvIL-10 are much more rigid due to an additional relatively short disulfide bridge (C59–C59') connecting monomers. Because of that it seems plausible that any rearrangement of the domains of the receptor invoked by S138G mutation could be easily compensated by flexible hIL-10, while cmvIL-10 may have a problem to adapt due to its rigidity.

Discussion

IL-10 is considered a key signaling pathway for various immune cells, specifically those that recognize antigens and trigger immune response. Disruption of IL-10 signaling is associated with enterocolitis indicating its relevance to immune homeostasis [2]. In fact, one of the first observations with IL-10 was its inhibitory effect on TNF production in activated macrophages thereby originating the concept of IL-10 as an anti-inflammatory cytokine [35]. Since then, several cytokines have been identified through homology with IL-10 that also share parts of the receptor complex (mostly IL-10R2) and function, but seem to act organ specific. The importance of IL-10 signaling for restraining the host's immune response to viral infections is emphasized by the existence of virus DNA that encodes for homologs of this cytokine in certain herpesviruses such as CMV and EBV. More recently, high IL-10 levels were associated with viral persistence in an animal model of chronic viral infection and blockade of the IL-10R1 prevented viral persistence in the early phase of infection [36] and enhanced the antiviral responses in mice with persistent infection [37].

In this study we tested the signaling of cmvIL-10 through IL-10R1 and its variants. cmvIL-10 was chosen over ebvIL-10 on the basis of its higher receptor affinity (cmvIL-10's

affinity is comparable to hIL-10, and about 1000-fold higher than that of ebvIL-10) [6, 22] and by experimental differences in the neutralization of STAT3 signaling by the IL-10R1 mAb (for EBV data not shown). The activity of cmvIL-10 (and hIL-10 as control) on STAT3 phosphorylation, SOCS3 and SLAM transcription was compared in cells that expressed IL-10R1 WT or IL-10R1 variants. The best model for mimicking the *in vivo* situation of human CMV infection is to express cmvIL-10 in human cells. Therefore, besides the commercially available cytokines (expressed in bacteria, quantified in ng/mL and showing quite some batch variation) we also expressed both proteins in HeLa cells and collected the concentrated supernatants as a source of cytokines. By using the same expression vectors and experimental transfection methods we equalized the supernatants for the level of impurities. Comparable transfection efficiency was checked by co-transfection of EGFP. For the purpose of clarity we used the term “non-glycosylated” when bacteria-expressed proteins were tested and “HeLa-expressed” when HeLa supernatants were used. Both IL-10R1-variant alleles displayed a loss of cmvIL-10 signaling by a decrease in ligand-induced STAT3 phosphorylation and SOCS3 expression. The importance of testing cmvIL-10 that is expressed in human cells is underlined by the larger difference that was seen with this kind of cytokine preparation (Fig. 2B and C). HeLa-expressed cytokines are likely glycosylated (as judged by their band pattern on Fig. 2A) [18] and commercially available cytokines are not as they are expressed in *Escherichia coli*. Glycosylation may be required for stability, secretion, transport and receptor binding as described in [38-40]. In contrast, Poxviruses express IL-10 homolog Y134R (134R protein of Yaba-like disease virus) and deployed its activity whether it is glycosylated or not [38].

We conclude that IL-10R1 SNP impair cmvIL-10 signal transduction. Individuals carrying these SNP may be more resistant to the immune evasion strategy by this virus. We like to speculate that the IL-10R1 SNP have improved host survival in ancient times, led to natural selection in virus exposed populations and may serve as example of virus-driven human evolution. The evolutionary pressure of the host's immune response on viral survival is outstanding. CMV developed sophisticated strategies to evade human immune surveillance [19]. Besides production of cmvIL-10, other CMV-encoded proteins interfere with MHC expression, interferon-stimulated antiviral response, NK-cell mediated killing and chemotaxis. Although, it seems that viruses and especially CMV get more and more successful after a period of time under evolutionary pressure, it is reasonable that this coexistence may affect the host's defence as well. From the perspective of human evolution, an ideal IL-10R1 mutation would maintain a full utilization of the IL-10R complex for hIL-10 but block viral IL-10 signaling. Modeling of the structural changes of the IL-10R1 that are induced by the S138G (SNP3) mutation revealed a change in the ternary organization of the extracellular receptor domain that is likely to cause a change in the conformation of the inter-domain junction. The more rigid dimer structure of cmvIL-10 seems to be more sensitive to the changes in the mutual positions of N- and C-terminal domains of the receptor than the more flexible hIL-10 dimers.

In another study we have tested an association between IL-10R1 SNP and ulcerative colitis without finding major differences [28]. An interesting parallel between the current and the previous findings is that reactivation of intestinal CMV infection often complicates ulcerative colitis by a yet unknown mechanism [41, 42]. cmvIL-10 possesses potent

immunosuppressive properties, such as the inhibition of proliferation of mitogen-stimulated mononuclear cells, the inhibition of proinflammatory cytokine synthesis and decrease in MHC classes I and II expression [43]. Besides expression of cmvIL-10 during the productive CMV infection, a recent study has pointed to another IL-10 homolog transcript (LA-cmvIL-10) that is expressed during latent infection [21]. Expression of LA-cmvIL-10 may enhance the virus's ability to avoid immune recognition and clearance during the latent phase of infection. We did not have the ability to study the differential effects of LA-cmvIL-10 with the variant receptors, but as the SNP3 changes the extracellular ternary receptor structure it may potentially also interfere with LA-cmvIL-10. The mechanisms of viral reactivation are poorly understood but they typically occur in immunocompromised patients. In fact, CMV reactivation in ulcerative colitis occurs almost exclusively in patients on corticosteroid therapy. However, CMV reactivation is rare in patients with Crohn's disease, another type of inflammatory bowel disease with similar immunosuppressive therapy. Thus, host factors such as the IL-10R1 variants may influence the course of CMV infection, both the primary infection and viral reactivation.

Previous studies demonstrated that cmvIL-10 and hIL-10 display similar affinity to IL-10R1 [22]. However, information about its intracellular signaling activity is incomplete [18]. In the present work, we first compared the differential STAT3 phosphorylation of cmvIL-10 relative to hIL-10 using HeLa cells stably expressing IL-10R1-WT. Receptor specificity was demonstrated by using a certain neutralizing antibody against IL-10R1. Despite similar receptor affinity and cytokine activity, STAT3 phosphorylation by cmvIL-10 was incompletely blocked even at high antibody concentrations. This effect is likely due to differences in the interaction of cmvIL-10 and hIL-10 with IL-10R1 and IL-10R2 [22]. The epitope to which MAB274 binds includes only two parts (amino acids 70–76 and 138–147) of the real IL-10/IL-10R1-binding site [44]. One can speculate that cmvIL-10 may use only part of the IL-10-binding site or it may compete for binding with MAB274 because of its higher affinity than IL-10 affinity towards IL-10R1 [22]. A certain degree of variation was observed in between several HeLa clones. However, this variation was seen for both hIL-10 and cmvIL-10.

It has been shown in a previous publication that cmvIL-10, ebvIL-10 and hIL-10 bound equivalent amounts of sIL-10R1, but the cmvIL-10/sIL-10R1 complex bound approximately three to five times as much IL-10R2 as hIL-10/sIL-10R1 and ebvIL-10/sIL-10R1 [13, 45]. These findings may provide additional explanations as to why cmvIL-10 shows stronger STAT3 signaling in IL-10R1-WT-expressing HeLa clones. However, the IL-10R2 expression may be a limiting factor for the hIL-10 or cmvIL-10 signal transduction. In our experiments, IL-10R2 expression was comparable in all clones used as demonstrated by flow cytometry. Therefore, it is unlikely that IL-10R2 variations may explain the observed differences in IL-10 signaling due to the IL-10R1 variants and the WT.

Recent data showed that IL-10R2 interactions are not influenced by the different inter-domain angles of hIL-10 and viral IL-10 but it appears that weak IL-10R2 interactions initiate cell signaling of the hIL-10/IL-10R1 complex [45]. Different inter-domain angles of the cytokines and the presence of SNP3 in the IL-10R1 might change the ternary complex formation. Therefore, the different ternary complex formation of IL-10R1-WT or IL-10R1

variants and IL-10R2 with hIL-10 or cmvIL-10 could be one of the explanations for an altered signaling response. Especially the structural change by SNP3 in the IL-10R1 may explain why cmvIL-10-dependent signaling is reduced in these clones.

The SNP3 mutation causes a moderate (about 50%) loss-of-function for STAT3 signal transduction and maybe more for STAT1 [28]. When relating this to the strong relative loss of cmvIL-10 activity (as shown for glycosylated cytokines in cells expressing IL-10R1 SNP3+4 haplotype; Fig. 2C), this seems to be a minor drawback without major consequence, specifically for heterozygote carriers. In fact, from various disease models, which have been studied so far, associations of IL-10R1 SNP were found only with progression of liver fibrosis and schizophrenia [26, 27]. On the other hand, no association of these alleles was found in inflammatory bowel diseases, type 1 diabetes and psoriasis (unpublished data). Future studies should evaluate the effect of IL-10R1 variants on susceptibility and clinical course of primary CMV infections and CMV reactivation in immunocompromised individuals.

Materials and methods

Cloning and expression of IL-10R1

The various combinations of SNP3 and SNP4 result in four IL-10R1 haplotypes [24]: WT nucleotides at both SNP3 and SNP4 positions (haplotype-1), alleles carrying the SNP3 variant only (haplotype-3, which are extremely rare), alleles carrying the SNP4 variant only (haplotype-4) and alleles carrying both SNP3 and SNP4 variants (haplotype-3+4). The coding sequence of IL-10R1 haplotypes-1 (WT nucleotides at both SNP3 and SNP4 positions), haplotypes-4 (alleles carrying the SNP4 variant only) and haplotypes-3+4 (alleles carrying both SNP3 and SNP4 variants) were obtained by the 5'-RACE method from two individuals [28]. A FLAG-tag was introduced behind the signal peptide by PCR mutagenesis. The haplotype-3 (SNP3 variant only) was generated from IL-10R1-haplotype-1 by PCR mutagenesis. The various FLAG-tagged receptor haplotypes or the EGFP were cloned into vector pIRESpuro3 (Clontech) resulting in the plasmid pIRESpuro3-IL-10R1-WT, pIRESpuro3-IL-10R1-SNP3, pIRESpuro3-IL-10R1-SNP4, pIRESpuro3-IL-10R1-SNP3+4 or pIRESpuro3-EGFP [28]. HeLa cervical carcinoma cell line was obtained from American-Type Culture Collection (ATCC), grown as monolayers in RPMI (GIBCO/Invitrogen) containing 2 mM glutamine and 10% FBS at 5% CO₂ and transfected. Single-cell clones were prepared by limiting dilution, identified by inverse microscopy and analyzed for IL-10R1 expression by flow cytometry (FACScan, Becton Dickinson) using a PE-conjugated anti-IL-10R1 antibody (Pharmingen). For expression of IL-10R2, cells were stained with a primary monoclonal antibody (MAB874; R&D Systems) and visualized by using a secondary goat antimouse Ab conjugated to a fluorochrome (PE) (Pharmingen). If not otherwise indicated, stimulation with cytokines was performed for 30 min to test for STAT3 phosphorylation or 4 h to test for SOCS3 mRNA expression.

Cloning and expression of hIL-10 and cmvIL-10

cDNA coding for cmvIL-10 (a kind gift of Sergei Kotenko – UMDNJ-New Jersey Medical School, NJ, USA) was cloned into pCEP' expression vector (Invitrogen) and expressed in HeLa cells as FLAG-tagged protein. cDNA coding for hIL-10 was obtained from ATCC (ATCC number: 68191; pH5C); 5×10^6 HeLa cells were transfected with 30 μ g DNA and 1.5 μ g of an EGFP expressing plasmid (pIRESpuo3-EGFP) and transfection efficiency was determined by flow cytometry (% EGFP expression *per* cell) after 24 h. For collection of expressed cytokines the culture medium was removed and replaced by RPMI only 6 h post transfection. Cells were incubated for another 48 h, supernatant was collected, concentrated tenfold by filtration-centrifugation using Macrosep centrifugal devices (Pall Life Sciences, Ann Arbor, MI, USA) and stored at -80°C until use. Expression of FLAG-tagged cytokines and hIL-10 was tested by Western blot. Non-glycosylated hIL-10 and cmvIL-10 were purchased from R&D Systems.

Western blot

Whole protein extracts were prepared using extraction buffer (20 mM Hepes pH 7.5, 100 mM NaCl, 0.2% NP-40, 10% glycerol, 1 mM DTT) containing protease inhibitors (1 mM Pefabloc–Promega, 1 mM aprotinin and complete protease inhibitor mix – Roche) and phosphatase inhibitors (50 mM β -glycerophosphate, 50mM NaF and 1 mM Na_3VO_4 – all Sigma). Equal amounts of proteins were separated on pre-cast 4–12% polyacrylamide SDS-PAGE gels (Invitrogen) and transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Biosciences) in methanol-containing Tris-glycine buffer (0.25 M Tris base, 1.92 M glycine, pH 8.3, 20% methanol). Membranes were blocked with 5% non-fat dried milk in PBS-Tween (1%) and incubated with the primary antibody at 4°C overnight followed by incubation with HRP-conjugated secondary antibody (Amersham Biosciences) prior to development with ECL (coumaric acid: 13 mg/mL in DMSO, luminol: 44 ng/mL in DMSO, in 100 mM Tris-HCl, pH 8.5; 3% H_2O_2). The following primary antibodies were used: anti-phospho-STAT3 Tyr 705, anti-STAT3 (both from Cell Signaling Technology), monoclonal anti-flag Ab M2 F1804 (Sigma), anti-hIL-10 (MAB217; R&D Systems), and anti- β -tubulin (Abcam). Films were quantified using the ImageJ software (<http://rsb.info.nih.gov/ij>).

Real-time PCR

Total RNA was isolated from HeLa cells using TRIzol reagent (Invitrogen) and reverse transcribed with oligo (dT) primers (ThermoScript, Invitrogen). Real-time PCR was performed from cDNA with SOCS3 or SLAM and GAPDH-specific primers and a fluorogenic probe on an ABI Prism 7700 sequence detection system (Perkin Elmer). Sequences of primers and probes were the following: GAPDH (FW-5'-CCTGAGCTGAACGGGAAGC-3'; RV-5'-AGGTCCACCACTGAGACGTTG-3'; probe: 6-FAM-CATGGCCTTCCGTGTCCTCCACTTAMRA); SOCS3 (FW-5'-TTCTGATCCGCGACAGCTC-3'; RV-5'-TCCCAGACTGGGTCTTGACG-3'; probe: 6-FAM-ACCAGCGCCACTTCTTACGCTCATAMRA); SLAM (FW-5'-CCATGTGGCTTACAGCTGGAG-3'; SLAM-RV-5'-GGAGCTGTTGGCTGGGTTTC-3'; probe: 6-FAM-AAAAGGCGGGCACCCACCCATAMRA). Amplification was performed in a total volume of 25 μ L for 45 cycles with denaturation at 95°C for 15 s and annealing/

extension at 60°C for 1 min. Samples were run in triplicate and the relative expression was determined by normalizing to GAPDH (delta CT) [46].

Structural analysis of cmvIL-10/IL-10R1 complex

Atomic co-ordinates of the crystal structures of the hIL-10/IL-10R1 complex [34] and the cmvIL-10/IL-10R1 [22] have been used to generate a tentative structural model of the IL-10R1 S138G-variant interaction with the two ligands.

Statistical analyses

Densitometric data of Western blots were compared by Wilcoxon test and corrected for multiple (three) comparisons (using Bonferroni). Statistical significance was then considered at a *p*-value below 0.017.

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Abbreviations:

ATCC	American-type culture collection
EGFP	enhanced green fluorescent protein
hIL-10	human IL-10
IL-10R1	IL-10 receptor 1
IL-10R2	IL-10 receptor 2
SLAM	signaling lymphocytic activation molecule
SNP	single-nucleotide polymorphism
S138G	Ser 138 Gly

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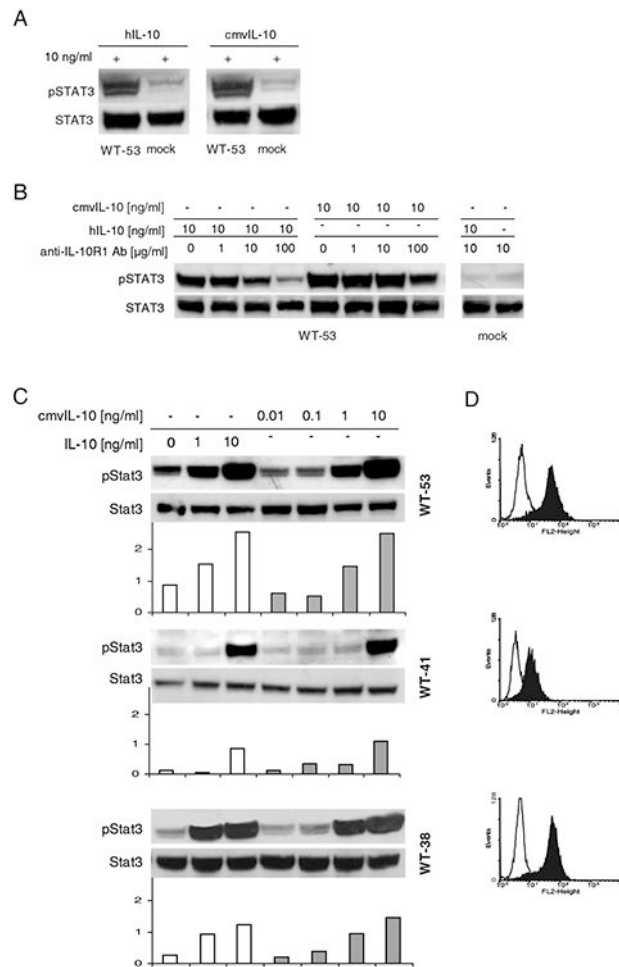


Figure 1.

Viral and hIL-10-dependent activation of STAT3 in IL-10R1-WT-transfected HeLa cells. (A) IL-10R1-WT (WT-53) or EGFP-plasmid transfected (mock) HeLa clones were stimulated with 10 ng/mL of non-glycosylated hIL-10 or cmvIL-10 (R&D Systems). Whole cell lysates were analyzed for total STAT3 and phosphorylation of STAT3 (Y705) by Western blot. All cytokines induced STAT3 phosphorylation in cells expressing IL-10R1-WT. Some background was observed in cells transfected with the EGFP construct (mock). The experiment was repeated at least three times. (B) WT-53 cells were incubated with 0, 1, 10, 100 µg/mL of neutralizing monoclonal anti-IL-10R1 Ab (MAB274; R&D Systems) 60min prior to a 30 min incubation with cmvIL-10 or hIL-10. At the highest antibody concentration hIL-10-induced phosphorylation of STAT3 was almost completely blocked. Minor neutralization was achieved for cmvIL-10. No STAT3 phosphorylation was seen in EGFP-plasmid-transfected cells (mock) upon anti-IL-10R1 Ab or hIL-10 incubation. The experiment was repeated twice. (C) Three different 10R1-WT-expressing HeLa clones (WT-53, WT-38 and WT-41) were stimulated with serial dilutions of non-glycosylated cmvIL-10 or hIL-10, respectively (0–10 ng/mL). Western blot showed a similar phosphorylation of STAT3 upon stimulation with cmvIL-10 and hIL-10 in all clones. Signals were analyzed by densitometry (relative od pSTAT3/STAT3) and blotted below (white columns hIL-10, grey columns cmvIL-10). All clones were blotted three times.

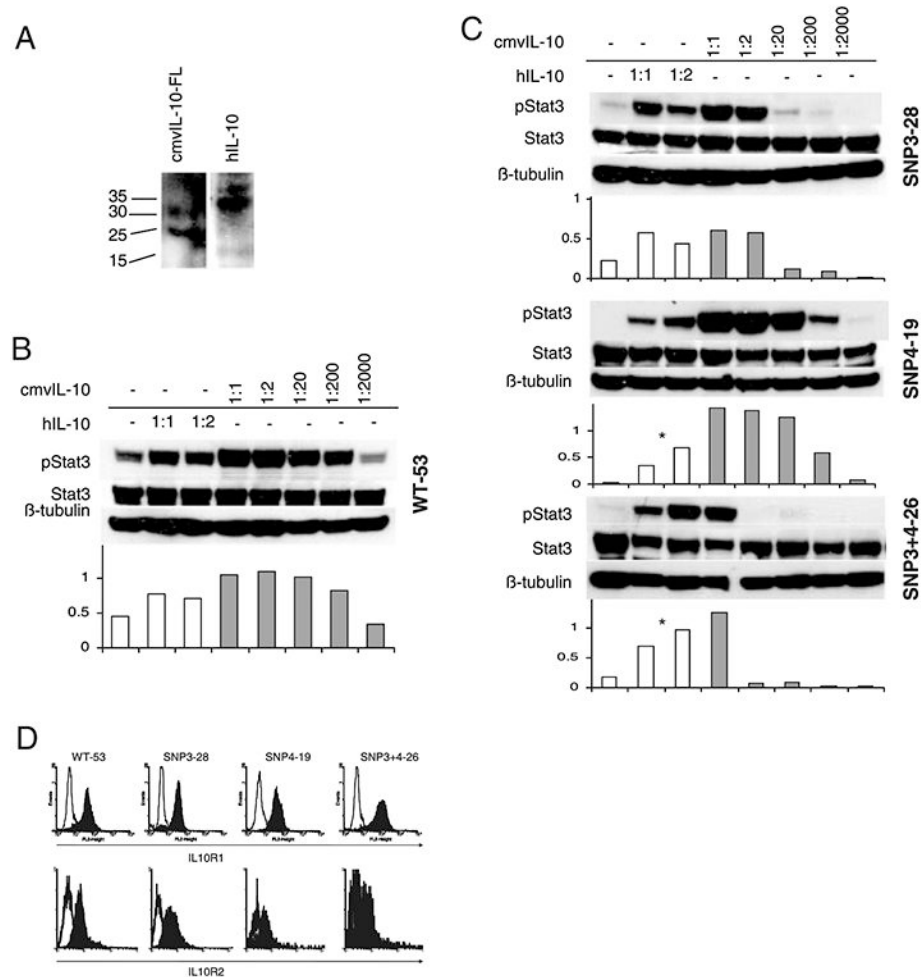
(D) IL-10R1 surface expression was assessed by flow cytometry using a PE-labeled anti-IL-10R1 antibody. Histograms display receptor density (black) and isotype control (white). All experiments were performed with non-glycosylated cmvIL-10 batch no. EGT0114061.

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**Figure 2.**

IL-10R1 variants reduce differential activity of cmvIL-10 to induce STAT3 phosphorylation. (A) HeLa cells were transiently transfected with plasmids encoding for C-terminally FLAG-tagged cmvIL-10 and for hIL-10. Concentrate culture supernatants (SN) were tested by Western blot with an anti-flag antibody (mAb M2 F1804; Sigma) and with a specific anti-hIL-10 antibody (MCA926; Serotec). The cmvIL-10 supernatant displayed a heterogeneous MW of 25–35 kDa indicative of glycosylation (as previously shown [18]) while hIL-10 revealed a molecular mass of around 35–37 kDa corresponding to the glycosylated homodimer [1]. (B and C) Cells expressing IL-10R1-WT (WT-53), IL-10R1-SNP3 (SNP3-28), IL-10R1-SNP4 (SNP4-19) or IL-10R1-SNP3+4 (SNP3+4-26) were treated with serial dilutions of HeLa-expressed cmvIL-10 (1:1, 1:2, 1:20, 1:200, 1:2000) or hIL-10 (1:1, 1:2) and RPMI as control. Total cell lysates were analyzed for STAT3 phosphorylation at site Y705 and total STAT3 by Western blotting. In addition, β-tubulin was analyzed as loading control (*concentrations of hIL-10 were loaded in reversed order, 1:2 and 1:1, in blots of clones SNP4-19 and SNP3+4-26). Densitometric quantification of STAT3 phosphorylation is shown in the bar graphs as relative od (pSTAT3/STAT3; white columns hIL-10, grey columns cmvIL-10). In IL-10R1-WT-53 cells (B), cmvIL-10 caused a concentration-dependent increase of STAT3 phosphorylation with a maximum effect in

dilutions equal to or above 1:200. In IL-10R1-SNP3-28 and IL-10R1-SNP3+4-26 clones (C), cmvIL-10-dependent STAT3 phosphorylation was seen only at dilutions equal to or above 1:2. hIL-10 (positive control) did not show such differences between clones. The experiment was repeated once. (D) To control for receptor expression, IL-10R1 and IL-10R2 expression was assessed by flow cytometry for each clone demonstrating comparable receptor surface levels.

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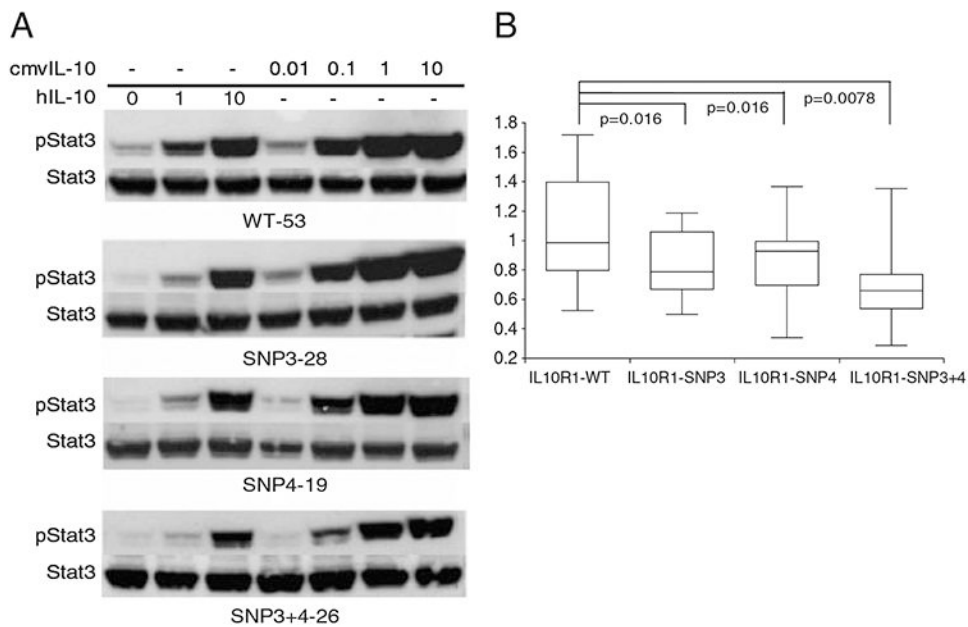


Figure 3. Differential activation of variant IL-10R1 by non-glycosylated cmvIL-10 and hIL-10. (A) HeLa clones (as in Fig. 2) were treated with serial dilutions of non-glycosylated hIL-10 or cmvIL-10 (batch no. EGT0114061, R&D Systems) at a range of concentrations (0–10 ng/mL) for 30 min. Total cell lysates were analyzed for STAT3 and phosphorylation of STAT3 at Y705 by Western blot. Differences were not as dramatic as seen with HeLa-expressed cytokine supernatants (Fig. 2C). (B) Quantification of eight independent experiments was done by densitometry, ratios of pSTAT3 to STAT3 expression levels were calculated and the signal intensity of cmvIL-10 (at 0.1 ng/mL) was normalized to hIL-10 (at 10 ng/mL) (relative od: (pSTAT3 cmvIL-10/STAT3 cmvIL-10)/(hIL-10 pSTAT3/hIL-10 STAT3)). Statistical analyses (Wilcoxon test corrected for multiple comparisons) revealed that the single-mutant IL-10R1-haplotypes (IL-10R1-SNP3 and IL-10R1-SNP4) showed borderline reduction and the double-mutant IL-10R1-SNP3+4 showed significant reduction of STAT3 phosphorylation.

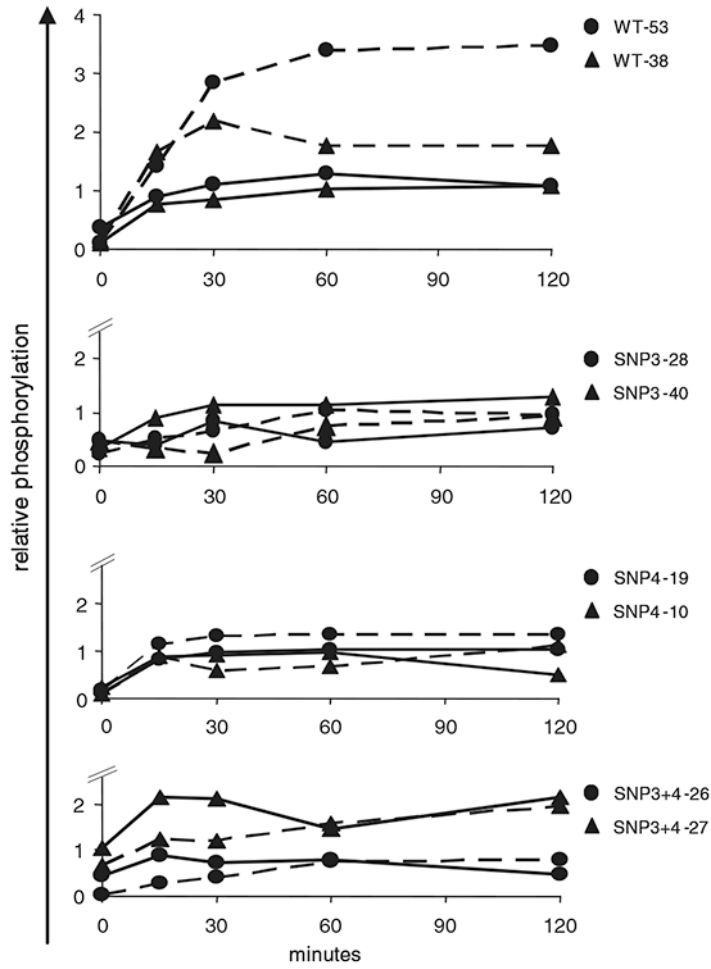


Figure 4. Differential kinetics of cmvIL-10- and hIL-10-induced STAT3 phosphorylation through IL-10R1 variants. Two of each IL-10R1-expressing clones (WT-53 and -38, SNP3-28 and -40, SNP4-19 and -10, SNP3+4-26 and -27) were stimulated with 2 ng/mL of non-glycosylated cmvIL-10 (broken lines; batch no. EGT0130001) or non-glycosylated hIL-10 (solid lines) for 0–120 min. Protein extracts were harvested and analyzed by Western blot. Signals were quantified by densitometry, pSTAT3 levels were normalized to STAT3 and plotted against time (min; x-axis). cmvIL-10 demonstrate a faster and stronger activation of STAT3 than hIL-10 in both IL-10R1-WT clones reaching a maximum at 30–60 min. In cells expressing the IL-10R1-variant receptors (IL-10R1-SNP3, IL-10R1-SNP4 and IL-10R1-SNP3+4), the relative activity of cmvIL-10 to hIL-10 was equal or lower at most time points. These data show that the variant IL-10R1 reduces the ability of cmvIL-10 to activate STAT3 in the host. The experiment was repeated once.

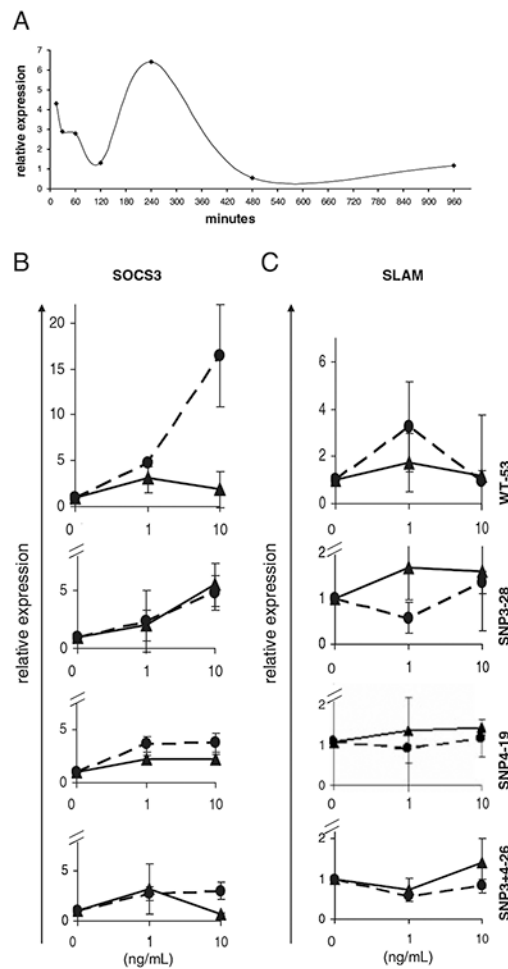


Figure 5. Differential induction of SOCS3 mRNA in IL-10R1-expressing clones. (A) A time course experiment of IL-10-dependent up-regulation of SOCS3 is shown. Cells were incubated with 10 ng hIL-10 for 0–960 min. The relative expression of SOCS (y -axis) was calculated by the delta–delta CT method. (B) HeLa single-cell clones expressing either IL-10R1-WT or its variants were treated with either non-glycosylated hIL-10 (solid lines) or cmvIL-10 (broken lines 0.1, 1, 10 ng/mL, batch no. EGT0130001) for 4 h (x -axis). mRNA was isolated and reverse transcribed. SOCS3 expression was analyzed by real-time PCR and levels were normalized to GAPDH. The relative expression (y -axis) was calculated by the delta–delta CT method. Compared with hIL-10, cmvIL-10-induced SOCS3 expression was increased in the IL-10R1-WT clone (WT-53). cmvIL-10-dependent (but not hIL-10-dependent) induction of SOCS3 mRNA was diminished in SNP3-18, SNP4-19 and –SNP3+4-26 clones. (C) Similarly, SLAM, another hIL-10-responsive gene, was analyzed by real-time PCR at 4 h. When compared with the IL-10R1-WT cells, the variant IL-10R1 clones demonstrate less cmvIL-10-induced SLAM expression. Both experiments were done in triplicate.