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Regulation of T cells and cytokines by the interleukin-10 (IL-10)-family cytokines IL-19, IL-20, IL-22, IL-24 and IL-26

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The family of IL-10-related cytokines includes several human members, IL-19, IL-20, IL-22, IL-24 and IL-26, and a series of herpesviral and poxviral paralogs. Some of these cytokines share common receptor subunits. In this study, we investigated the effects of these cytokines on naive T cell differentiation, antigen-specific T cell suppression, survival and expression of surface markers in comparison to IL-10 and cytomegalovirus (CMV)-IL-10. Human CD45RA$^+$ T cells were stimulated in the presence of IL-10-family cytokines in sequential 12-day cycles. After three to four cycles of stimulation, IL-10 and CMV-IL-10 led to increased IFN-γ and IL-10 but decreased IL-4 and IL-13. Interestingly, long-term exposure of T cells to IL-19, IL-20 and IL-22 down-regulated IFN-γ but up-regulated IL-4 and IL-13 in T cells and supported the polarization of naive T cells to Th2-like cells. In contrast, neutralization of endogenous IL-22 activity by IL-22-binding protein decreased IL-4, IL-13 and IFN-γ synthesis. The antigen-specific suppressor activity of IL-10 and CMV-IL-10 was not observed for any of the other IL-10-family cytokines. These data demonstrate that IL-19, IL-20 and IL-22 may participate in T cell-mediated diseases by distinct regulation of T cell cytokine profiles.

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Introduction

IL-10 is a multifunctional cytokine, first described as cytokine synthesis inhibitory factor, that inhibits IFN-γ production by Th1 cells in mice [1, 2]. IL-10 inhibits monocyte/macrophage function during inflammation by down-regulating the production of proinflammatory cytokines, such as IL-12 and TNF-α, and suppressing the surface expression of major histocompatibility class II [3, 4]. It has been shown to activate B cells for immunoglobulin production, induce proliferation of mast cells and increase NK cell cytotoxicity [5–7]. IL-10 can also inhibit CD4$^+$ T cell chemotaxis towards IL-8 [1, 8] and T cell apoptosis [9] by leading to Bcl-2 up-regulation. Interestingly, it can induce the proliferation of CD8$^+$ T cells [10]. The suppressor effect of IL-10 on T cells has recently been shown to be directed toward blocking of the CD28 signaling cascade and subsequent phosphatidylinositol 3-kinase activation in T cells [8].

IL-10-related cytokines were identified in database searches for potential IL-10 homologs, and so far little is known about their biological activities [11]. Cells known

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Abbreviations: **IL-22BP:** IL-22-binding protein · **PLA:** phospholipase A · **Tr1:** adaptive/inducible Treg · **Treg:** regulatory/suppressor T cells

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to secrete IL-19 include monocytes and B cells. It has been demonstrated that LPS strongly induces the production of IL-19 in monocyte cultures [12]. Treatment of monocytes with IL-19 causes release of proinflammatory cytokines, such as IL-1 and TNF-α, and production of reactive oxygen intermediates, which eventually induce apoptosis [13].

IL-20 was initially identified in a human keratinocyte library during a database search to identify sequences encoding for amphipathic α helices [14]. Cells that produce IL-20 are monocytes [15] and keratinocytes [14, 16]. IL-20-transgenic mice showed neonatal lethality as well as severe skin abnormalities similar to those of psoriasis, including hyperkeratosis, hyperproliferation and aberrant epidermal differentiation [14].

IL-22 was originally described as an IL-9-inducible gene and called IL-10-related T cell-derived inducible factor (IL-TIF) [17]. Its gene is located on human chromosome 12q15, near the IFN-γ gene. IL-22 is produced primarily by activated $CD4^+$ T cells, and its target-cell types include mesangial, neuronal and hepatoma cells. IL-22 induces acute-phase responses such as anemia, weight loss, elevated platelet numbers, increased levels of serum amyloid A and fibrinogen and decreased levels of serum albumin [18]. It also upregulates pancreatitis-associated protein 1 (PAP1) and osteopontin, which may have protective or trophic effects during inflammation, in pancreatic acinar cells [17, 19, 20]. In addition to its cellular receptor, IL-22 binds to soluble IL-22-binding protein (IL-22BP), which is a secreted member of the class II cytokine receptor family and appears to act as a natural antagonist for IL-22 [21–24].

IL-24 was initially designated as melanoma differentiation-associated gene-7 (mda-7) expressed by melanocytes [25]. A number of studies reported that the majority of human cancer-derived cell lines, including melanoma, prostate, breast, cervical, lung, fibrosarcoma, pancreatic, colorectal and glioblastoma lines, undergo apoptosis upon exposure to IL-24 [26, 27]. It was also shown to induce IL-6 and TNF-α secretion by monocytes, demonstrating an activity antagonistic to that of IL-10. IL-26 was initially identified in the supernatant of herpes virus saimiri-transformed human T cells. Its gene is located on chromosome 12q15, a susceptibility region for autoimmune and allergic diseases. IL-26 was found to be primarily generated by $CD4^+CD45RO^+$ T cells and NK cells [11].

Cytomegalovirus (CMV) is a member of the β-herpes virus family and shows species specificity. Human CMV has been demonstrated to produce a viral homolog of IL-10 that can bind to and signal through the human IL-10 receptor (IL-10R) despite having only 27% homology with human cellular IL-10 [28]. As they share several receptor subunits, these novel IL-10-family cytokines are expected to display similar as well as distinct functions. Accordingly, we investigated the potential roles of IL-19, IL-20, IL-22, IL-24 and IL-26 on naive T cell differentiation, suppression of antigen-specific T cell proliferation and expression of surface markers related to regulatory/suppressor T cells (Treg) and compared the results to those for IL-10.

Results

IL-10 and CMV-IL-10 induce the polarization of naive T cells into IFN-γ- and IL-10-secreting T cells

The redundancy and similarities of IL-10-family cytokines in receptor subunits and signal transduction pathways with cytokines that are known to induce

Table 1. Effect of IL-10-family cytokines on naive T cell differentiation

	$IFN\text{-}\gamma^+IL\text{-}13^-$	$IFN\text{-}\gamma^-IL\text{-}13^+$	$IL\text{-}4^+IL\text{-}10^-$	$IL\text{-}4^-IL\text{-}10^+$
Control	18.8 ± 7.4[b)]	13.9 ± 5.1	14.1 ± 5.2	4.1 ± 3.9
IL-10[a)]	56.7 ± 11.4*[c)]	3.7 ± 2.1*	4.9 ± 4.6*	11.8 ± 4.1*
CMV-IL-10	48.9 ± 14.7*	4.4 ± 2.6*	4.7 ± 3.3*	10.9 ± 3.6*
IL-19	9.4 ± 3.6*	25.6 ± 10.7*	22.8 ± 8.2*	4.1 ± 4.4
IL-20	5.8 ± 4.7*	35.2 ± 9.3*	25.9 ± 6.8*	4.7 ± 2.9
IL-22	4.6 ± 3.0*	39.2 ± 7.6*	24.7 ± 7.1*	5.4 ± 3.6
IL-24	21.6 ± 7.1	15.8 ± 5.6	11.9 ± 4.6	7.4 ± 3.8
IL-26	17.9 ± 6.6	20.7 ± 8.1	12.7 ± 5.1	7.8 ± 5.2

a) $CD45RA^+$ T cells from four different healthy donors were stimulated with anti-CD2, anti-CD3 and anti-CD28 mAb in the presence of IL-2 and different IL-10-family cytokines for four consecutive 12-day cycles.
b) Intracytoplasmic cytokines were determined by flow cytometry 6 h after PMA and ionomycin stimulation after the fourth cycle.
c) $*p<0.01$

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naive T cell differentiation are well documented [11]. Accordingly, we investigated the roles of the IL-10-family cytokines in determining T cell cytokine profiles by stimulation of naive T cells in sequential 12-day proliferation cycles (Table 1). Activation of naive T cells with anti-CD2, anti-CD3 and anti-CD28 in the presence of IL-10 or CMV-IL-10 decreased the frequency of IL-13- and IL-4-producing T cells after four rounds of identical stimulation (Fig. 1A). There was a significant increase in the frequency of IFN-γ^+IL-13$^-$ cells and IL-10$^+$IL-4$^-$ cells. After four stimulation cycles, IL-10 and CMV-IL-10 merely induced IFN-γ^+ and IL-10$^+$ T cells. Control cells stimulated with only IL-2 expressed all four cytokines and showed a typical Th0 profile. IL-10 and CMV-IL-10 significantly increased IFN-γ secretion and CMV-IL-10 suppressed IL-4 secretion in cell cultures (Fig. 1B).

Repeated IL-19, IL-20 or IL-22 stimulation induces naive T cell polarization toward a Th2-like profile

Because of their common receptor subunit expression, we determined whether IL-19, IL-20 and IL-22 have a direct effect on T helper cell subset development. As shown in Fig. 2A and Table 1, when naive T cells were cultured with IL-19, IL-20 and IL-22, the frequency of IL-4- and IL-13-producing T cells significantly increased, whereas the frequency of IFN-γ-producing cells decreased. Analysis of T cell differentiation after each round of stimulation by IL-20 revealed an increase in IL-4-producing T cells following each stimulation cycle (Fig. 2B). Similarly, IL-19 and IL-22 induced increased IL-4 or IL-13 production after each cycle, leading to a

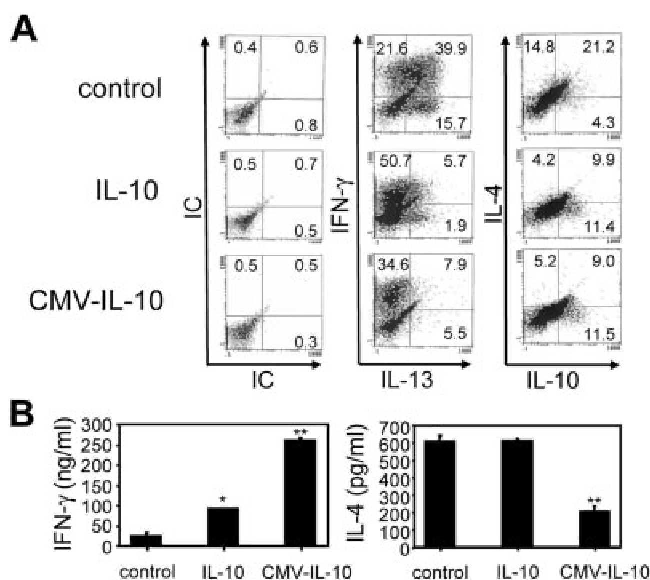

Figure 1. IL-10 and CMV-IL-10 induce the development of IFN-γ- and IL-10-producing T cells. Purified human CD45RA$^+$ T cells were activated by anti-CD2, anti-CD3 and anti-CD28 in the absence (control) or presence of IL-10 or CMV-IL-10. IL-2 was used as a T cell growth factor in all experiments. Following four rounds of identical stimulations, cells were characterized for cytokine production by intracellular flow cytometric analysis (A) as well as by ELISA (B). IC indicates staining with isotype control antibody. Values represent mean ± SD of triplicate determinations (**$p<0.02$, *$p<0.01$ *versus* negative control). Similar results were obtained in four different healthy donors.

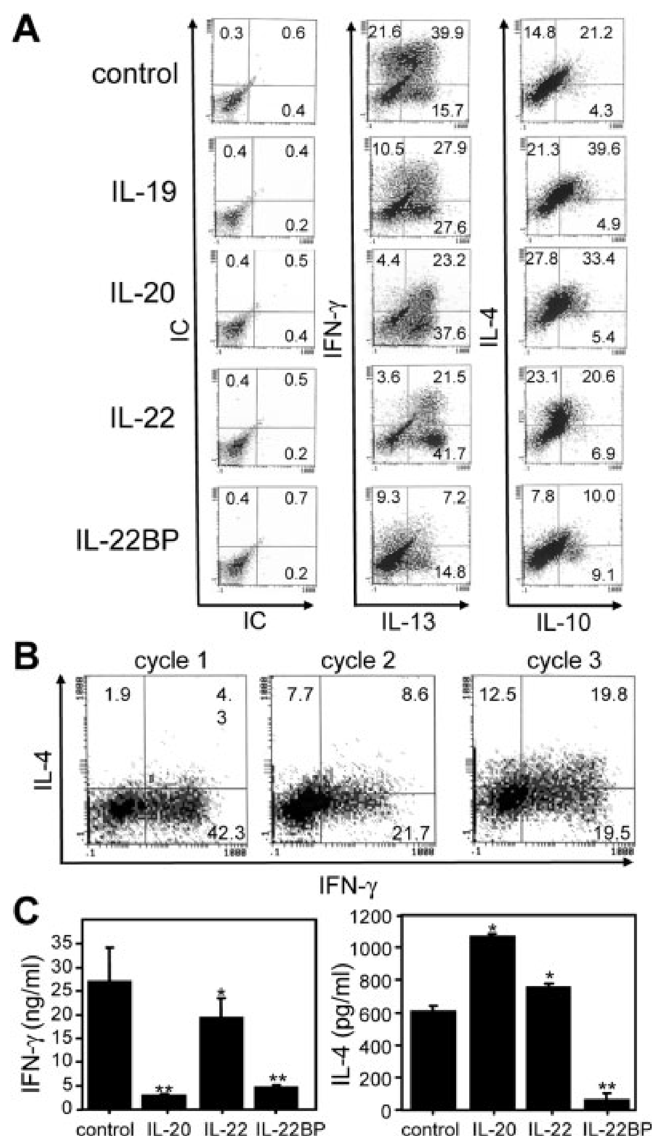

Figure 2. IL-19, IL-20 and IL-22 induce the development of Th2-like cells, whereas IL-22BP suppresses both Th1 and Th2 cytokines. Purified human CD45RA$^+$ T cells were activated as in Fig. 1 in the absence (control) or presence of IL-19, IL-20, IL-22 or IL-22BP. (A) Cells were characterized for cytokine production by intracellular flow cytometric analysis following four rounds of identical stimulation. IC indicates staining with isotype control antibody. (B) Increased IL-4 production in a representative experiment for IL-20 is shown after each cycle of stimulation. (C) IL-4 and IFN-γ levels were also determined by ELISA. Values represent mean ± SD of triplicate determinations (*$p<0.05$, **$p<0.01$ *versus* negative control). Similar results were obtained in four different healthy donors for IL-20, IL-22 and IL-22BP and six different donors for IL-19.

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predominant Th2-like cytokine profile after three to four cycles (data not shown). This effect on cytokine secretion and Th2-like cytokine development, with reduced IFN-γ and elevated IL-4 secretion, was also observed in culture supernatants (Fig. 2C). The frequencies of T cells producing IL-4, IL-10, IL-13 and IFN-γ following activation of naive T cells in the presence of IL-24 or IL-26 was found to be similar to those observed for control T cells (Fig. 3).

IL-22BP suppresses both Th1- and Th2-type cytokine production

To determine the effect of continuous neutralization of endogenous IL-22 activity by IL-22BP on the differentiation of T cells, naive T cells were stimulated with anti-CD2, anti-CD3 and anti-CD28 in the presence or absence of IL-22BP. The frequency of the T cells producing IL-4, IL-13 and IFN-γ was reduced after four rounds of stimulation in the presence of IL-22BP (Fig. 2A). The effect of IL-22BP on cytokine secretion also reflects strong immune suppression, with decreased IL-4 and IFN-γ production (Fig. 2C), which correlates with the cytokine profiles observed in the flow cytometric analysis. The percentage of total IFN-γ-producing cells was 61.5% in the control group, which decreased to 16.5% in the presence of IL-22BP, and the percentage of total IL-13-producing cells decreased from 55.6% to 22.0% by the use of IL-22BP during naive T cell differentiation.

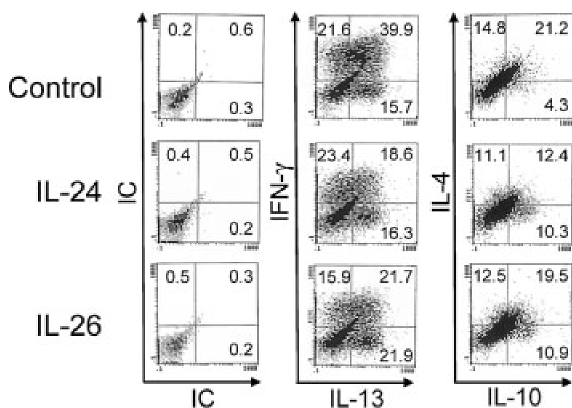

Figure 3. IL-24 and IL-26 do not have any significant effects on naive T cell differentiation. Purified human CD45RA$^+$ T cells were activated as in Fig. 1 in the absence (control) or presence of IL-24 or IL-26. Following four rounds of identical stimulations, cells were characterized for cytokine production by intracellular flow cytometric analysis. IC indicates staining with isotype control antibody. Similar results were obtained in four different healthy donors.

Effect of IL-10-family cytokines on surface expression of CD25, CD69, HLA-DR, GITR, CTLA-4, PD-1

Several studies have suggested that IL-10 plays an important role in the generation of Treg cells [29–33]. Treg express certain surface molecules that are related to the activation status as well as the suppressive functions of T cells. For example CD25, CD69 and HLA-DR are known to be up-regulated on the surface of activated T cells. In addition, CD25, GITR, PD-1 and CTLA-4 are highly expressed on Treg [34]. Accordingly, we investigated the effects of IL-10-related cytokines on surface expression of the above molecules. CD45RA$^+$CD4$^+$ T cells were purified and stimulated with anti-CD2, anti-CD3 anti-CD28 mAb in the presence of IL-2 and recombinant IL-10, IL-19, IL-20, IL-22 and IL-24 for two 12-day cycles. Surface expression of CD25, CD69, HLA-DR, GITR, CTLA-4 and PD-1 was determined on T cells 72 h after stimulation (Fig. 4). T cells activated by anti-CD2, anti-CD3 and anti-CD28 mAb highly up-regulated CD25 (>99.2% of cells) and GITR (>98.0% of

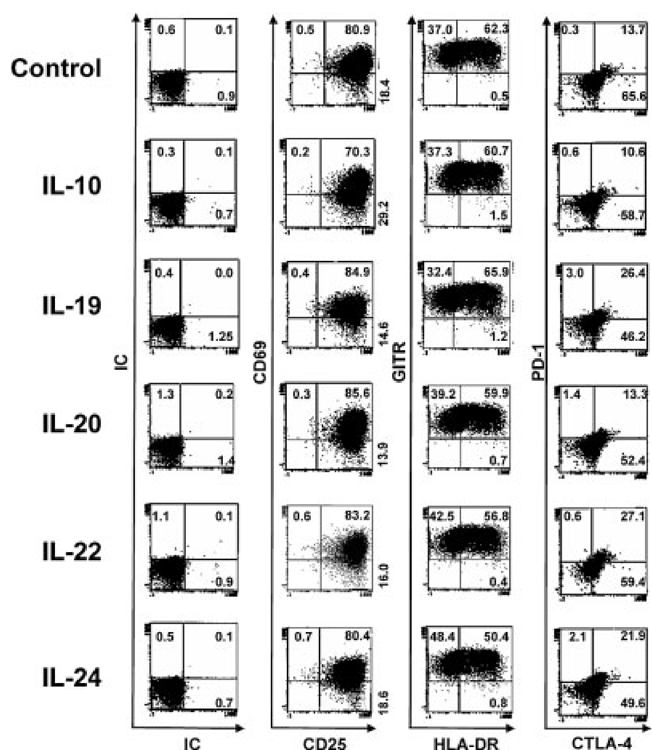

Figure 4. IL-10-family cytokines do not affect the expression capacity of surface CD25, CD69, HLA-DR, GITR, CTLA-4 or PD-1. Purified human CD45RA$^+$ T cells were activated by anti-CD2, anti-CD3 and anti-CD28 in the absence (control) or presence of IL-10, IL-19, IL-20, IL-22 or IL-24 for two cycles. Surface expression of CD25, CD69, HLA-DR, GITR, CTLA-4 and PD-1 was measured by flow cytometry 72 h after stimulation. IC indicates staining with isotype control antibody. Similar results were obtained in four different experiments.

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cells) in all conditions. CD69 and HLA-DR were partially up-regulated (in the range of 70.5–85.9% and 51.2–67.1% of cells, respectively), and CTLA-4 and PD-1 also showed up-regulation (69.3–79.3% and 11.2–29.4% of cells, respectively). There was no statistically significant difference in the expression of surface receptors between treatments with the different IL-10-family cytokines.

Effect of IL-10-family cytokines on T cell proliferation, survival and viability

After demonstration of distinct polarization of naive T cells by IL-10, CMV-IL-10, IL-19, IL-20 and IL-22, we investigated whether this effect is mediated by predominant apoptosis or increased proliferation of one T cell subset over the others. Accordingly, we investigated the suppressive activity of the IL-10-family cytokines under three different stimulation conditions. We used phospholipase A_2 (PLA), the major allergen of honeybee venom (*Apis mellifera*), for antigen-specific stimulation. Bee venom-allergic individuals and bee venom-hyperimmune beekeepers show a proliferative response of PBMC to PLA [8]. We used PBMC from bee venom-sensitized individuals to analyze the roles of IL-19, IL-20, IL-22 and IL-24 on PLA-specific T cell proliferation and used the same doses of IL-10 as a control. IL-10 and CMV-IL-10 significantly suppressed PLA-stimulated T cell proliferation in PBMC, however none of the IL-10-family cytokines showed any suppressive effect (Fig. 5A).

We also investigated the role of IL-10-family cytokines on anti-CD3-induced T cell proliferation. Culture plates were coated with anti-CD3 mAb, PBMC were cultured for 5 days and [^{3}H]-thymidine incorporation was determined (Fig. 5B). Neither IL-10 nor other IL-10-family cytokines showed any suppressive effect on anti-CD3-induced T cell proliferation.

In the third set of proliferation experiments, we coated the plates with anti-CD28 mAb, and [^{3}H]-thymidine incorporation was determined (Fig. 5C). Doses of 10 ng/mL IL-10 or CMV-IL-10 significantly suppressed anti-CD28-induced proliferation. Again, none of the other IL-10-family cytokines (IL-19, IL-20, IL-22, IL-24) showed any suppressive effect on anti-CD28-induced T cell proliferation. Interestingly, IL-19, IL-20 and IL-24 increased T cell proliferation in some of the cultures. In addition, incubation of naive T cells with or without anti-CD2, anti-CD3 and anti-CD28 mAb in the absence or presence of IL-10, CMV-IL-10, IL-19, IL-20, IL-22, IL-24 or IL-26 did not lead to any significant toxic or cell death-inducing effect (Fig. 5D).

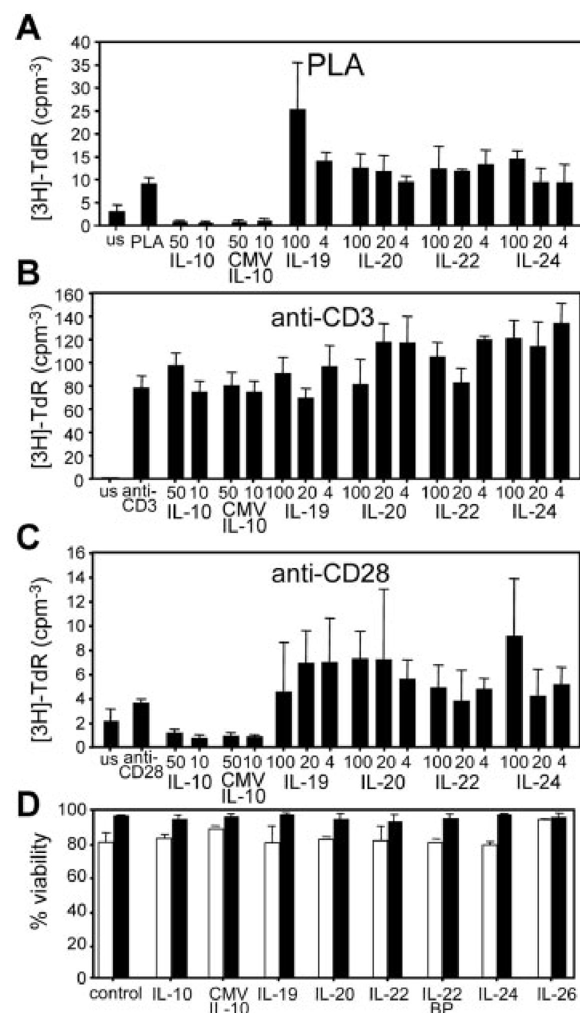

Figure 5. Effect of IL-10-family cytokines on T cell proliferation and viability. PBMC from a bee venom-sensitized donor were stimulated with (A) 0.3 μM PLA, (B) 10 μg/mL plate-bound anti-CD3 or (C) 10 μg/mL plate-bound anti-CD28 mAb for 5 days in presence of recombinant IL-10, CMV-IL-10, IL-19, IL-20, IL-22 or IL-24. [^{3}H]-thymidine incorporation was determined 8 h after its addition to the culture. (D) Incubation of naive T cells with IL-10, CMV-IL-10, IL-19, IL-20, IL-22, IL-24 or IL-26 did not lead to any significant toxic or cell death-inducing effects. Purified human CD45RA$^+$ T cells were stimulated with (empty bars) or without (filled bars) anti-CD2, anti-CD3 and anti-CD28 mAb in the absence or presence of IL-10, CMV-IL-10, IL-19, IL-20, IL-22, IL-24 or IL-26. Cell viability was measured by ethidium bromide staining after 6 days. Six experiments performed during different cycles gave similar results.

Discussion

The present study elucidates the effects of human IL-10-family cytokines on naive T cell differentiation, antigen-specific suppression and expression of T cell surface markers related to T cell activation and regulation in comparison to each other and IL-10. Different roles of these cytokines in the generation of distinct cytokine-secreting T cell populations were observed, which

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implies the involvement of certain shared receptor subunits in the specific effect. Continuous stimulation of naive T cells with IL-10 or CMV-IL-10 induced a distinct cytokine response characterized by increased IFN-γ and IL-10 but decreased IL-4 and IL-13 expression. Treg cells with immunosuppressive function and a cytokine profile distinct from that of Th1 or Th2 cells have been described [35, 36]. The adaptive, or inducible, Treg subset (Tr1) can develop in the presence of IL-10. Upon T cell receptor-mediated activation, human Tr1 cells have been shown to produce high amounts of IL-10, considerable levels of IFN-γ, TGF-β and IL-5 but no IL-4 and very low or no IL-2 [32, 37]. Thymus-derived natural Treg express Foxp3 and suppress immune responses in a contact-dependent manner [38]. Induction of IL-10 by IL-10 and CMV-IL-10 implies the generation of adaptive Tr1-like cells. On the other hand, CMV-IL-10-induced IFN-γ production may act as a counter-defense mechanism developed against viral evasion by the host [39]. The other possibility may be that IL-2, which is used as a growth factor for T cells in cultures [40], causes the stimulation of IFN-γ production in the presence of IL-10. However, this did not appear to be the case, as other cytokines, particularly IL-20 and IL-22, induced a different cytokine profile under the same conditions.

Our study provides strong evidence that IL-19, IL-20 and IL-22 affect the Th1/Th2 balance by inhibiting IFN-γ (Th1 cytokine) and augmenting IL-4 and IL-13 (Th2 cytokines) production. Following the first observation that induction of IL-19 synthesis by LPS in monocytes is down-regulated by IFN-γ and up-regulated by IL-4 [41], it was shown that IL-19 dose-dependently promotes the Th2 response by up-regulating IL-4 and down-regulating IFN-γ in T cells [42]. The target cell for IL-20 was found to be keratinocytes, which express the specific heterodimeric receptor composed of IL-20R1 and IL-20R2. This binding causes signal transducer and activator of transcription 3 (STAT3) phosphorylation and activation [41]. In contrast to IL-22, IL-22BP suppressed both Th1 and Th2 cytokines without affecting T cell viability and proliferation. Further studies are needed to elucidate the general suppressive and redundant effects of IL-22BP (in addition to its neutralizing effect on IL-22), because the complexity of the cytokine network may also enable several other cytokines to bind to it.

Investigation of the receptors of IL-10-related cytokines has contributed to better understanding of their effects [43]. These cytokines bind to heterodimeric receptor complexes containing subunits of IL-10R, IL-20R and/or IL-22R. The IL-20R has two subtypes, IL-20R1 and IL-20R2. IL-19 signals through a heterodimeric receptor complex composed of IL-20R1 and IL-20R2 lead to STAT3 activation [44, 45]. IL-20 and IL-22 share a common receptor subunit, the IL-22R1 [14]. In addition, IL-22R contains IL-10R2 as the second chain of the heterodimer, whereas IL-20R contains IL-20R2 chain [46]. The activities of IL-22 are mediated through a heterodimeric receptor composed of the long chain IL-22R (CRF2-9) and the β-chain of IL-10R (IL-10R2) [18, 46, 47]. IL-24 binds to IL-20R1/IL-20R2 and IL-22R/IL-20R2 receptor complexes [13, 44] and activates JAK/STAT signaling pathways, especially STAT3 [13, 44]. IL-26 signals through a specific and unique receptor complex composed of IL-20R1 and IL-10R2 and activates STAT1 and STAT3 [48]. There is controversial data about the expression of the receptors for IL-10-related cytokines in primary blood cells. Some studies reported the detection of activity of several IL-10-related cytokines on blood cells [15, 49]. However, according to one study, T cells express only IL-20R2 under resting conditions, which suggests that in certain settings either IL-22R1 or IL-20R1 – or another novel receptor combination – can be expressed, resulting in a functional receptor complex for other IL-10 family members, such as IL-20 and IL-22 [15]. Another explanation is that the level of receptor expression is below the level of detection. Although the study by Wolk *et al.* did not detect any IL-20R1 mRNA in blood cells [15], the study by Nakagalishmi *et al.* was able to detect it in certain cell populations [43]. It is important to note that reproducible biological activities for the cytokines were detected in our experiments, which suggests that under the utilized experimental conditions, the low level of receptors might be sufficient to enable cytokines to induce the reported activities. Alternatively, the level of receptors might increase during the experiment or an alternative, as-yet-unknown, combination of receptor subunits might exist. Efficient expression of complex receptors on certain cells can only be understood by functional assays. In the present study, stimulation of naive T cells with anti-CD2, anti-CD3 and anti-CD28 mAb in the presence of IL-2 in each stimulation cycle may lead to changes in expression levels and profiles of the respective cytokine receptor subunits. These important questions remain to be elucidated by further studies.

Treg express certain surface molecules that are related to the activation status as well as the suppressive functions of these T cells. For example CD25, CD69 and HLA-DR are known to be up-regulated on the surface of activated T cells. In addition, CD25, GITR and CTLA-4 are highly expressed by resting natural Treg but not by IL-10-secreting Tr1 cells, whereas in activated T cells, none of these markers can be used to distinguish T cells with effector or regulatory functions [38]. We investigated whether the IL-10-family of cytokines induce a Treg-like phenotype; in the present study, the expression of Treg cell or T cell activation-related receptors was not influenced by any of these cytokines. In our experiments, with the exception of IL-10 and CMV-IL-10, none

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of the IL-10-family cytokines suppressed PLA-stimulated T cell proliferation in PBMC from bee venom-sensitized individuals. Several studies have shown that IL-10 does not suppress anti-CD3 or T cell receptor-mediated signals in T cells, whereas IL-10 specifically blocks CD28 signal transduction [8, 50]. In our experiments, neither IL-10 nor other IL-10-family cytokines suppressed anti-CD3-induced T cell proliferation. On the other hand, IL-10 and CMV-IL-10 significantly suppressed anti-CD28 mAb-induced proliferation, whereas the IL-10-family cytokines IL-19, IL-20, IL-22 and IL-24 did not show any suppressive effect. The reason for increased T cell proliferation in the presence of IL-19, IL-20 and IL-24 in some experiments remains to be further investigated.

The search for translated human genomic sequences has enabled the discovery of several families of cytokines with homology/identity in certain regions. Some of these cytokine families show similar effects, such as the IL-12 family, which involves IL-23 and IL-27 [51], whereas some have unrelated effects, as demonstrated for the IL-10 family. In the present study, the effects of IL-19, IL-20 and IL-22 on T cell suppression and differentiation were completely different compared to IL-10 and CMV-IL-10 or IL-24 and IL-26, suggesting different and inducible receptor expression on human T cells. Together these findings demonstrate the diversity of the IL-10 family of cytokines in the regulation of naive T cell differentiation and antigen-specific T cell suppression.

Materials and methods

Antibodies and reagents

All fluorescence- or biotin-labeled mAb for cell purification or flow cytometry analysis were purchased from Pharmingen (San Diego, CA). Anti-CD14-, anti-CD16-, anti-CD19- and anti-CD45RO-conjugated magnetic microbeads for magnetically activated cell sorting (MACS) were obtained from Miltenyi Biotec (Marburg, Germany). Human IL-2 was provided by Novartis (Basel, Switzerland). IL-10 and IL-19 were purchased from Peprotech (UK). Other cytokines, which include hCMV-IL-10, IL-20, IL-22, IL-22BP, IL-24 and IL-26, were isolated, characterized and produced as described before [28, 44, 52, 46]. The supernatants from cultures of transfected cells were kept at –80°C before use in experiments.

Lymphocyte cultures

PBMC from normal healthy individuals were isolated by Ficoll (Biochrom, Berlin, Germany) density gradient centrifugation of peripheral venous blood. Purification of naive T cells was performed by MACS (Miltenyi Biotec) as described [53]. Briefly, $CD45RA^+$ T cells were negatively selected from PBMC by using microbead-labeled anti-CD14, anti-CD16 and anti-CD19 mAb (AutoMACS®). Naive T cells (1.5×10^5/mL) were stimulated in a 48-well plate with 50 μL supernatant from transfected COS cells (hCMV-IL-10, IL-20, IL-22, IL-22BP, IL-24, IL-26) or 10 ng/mL IL-10 or IL-19 for a 12-day stimulation cycle. Purified cytokines were used at a concentration of 50 ng/mL in some experiments. These stimulation cycles were carried out four times during a 6- to 7-week period [54]. Culture supernatant from non-transfected cells was used as a control. The antigen-specific T cell proliferative response was determined by stimulation of 2×10^5 PBMC for 5 days with 0.3 μM bee venom PLA (Sigma Chem Co.) in 200 μL medium in 96-well flat-bottom tissue culture plates in triplicates [53]. PBMC were stimulated with 10 μg/mL plate-bound anti-CD3 and anti-CD28 mAb in triplicates. The 96-well flat-bottom plates (Costar, Corning, NY) were coated with the mAb for 2 h at 37°C in PBS pH 7.4 [55]. Cells were pulsed with 1 μCi/well [^{3}H]-thymidine (Du Pont/New England Nuclear, Boston, MA), and incorporation of labeled nucleotide was determined after 8 h in an LKB beta plate reader (Wallax, Pharmacia Turku, Finland). Viability of T cells was assessed by uptake of 1 μM ethidium bromide and flow cytometric analysis.

Detection of cytokines

For intracytoplasmic cytokine detection, cells were stimulated with 500 ng/mL PMA and 50 ng/mL Ca^{+2} ionophore (ionomycin) for 6 h. Brefeldin A was added at a final concentration of 10 μM (all from Sigma, St. Louis, MO). The cells were fixed and permeabilized with paraformaldehyde/saponin solution (Ortho Permeafix, Ortho Diagnostic Systems Inc., Raritan, NJ), stained with PE- or FITC-conjugated isotype control antibodies (rat IgG1 and rat-IgG2a), anti-IL-4, anti-IL-10, anti-IL-13 or anti-IFN-γ mAb (all from Pharmingen) for 30 min at 4°C and analyzed by flow cytometry. Cells were sub-cultured with anti-CD3 and anti-CD28 and harvested at day 3 for cytokine detection. The solid-phase sandwich ELISA for IFN-γ and IL-4 have been described [53]. The sensitivity of the IFN-γ ELISA was $<$10 pg/mL, and the sensitivity of the IL-4 ELISA was $<$50 pg/mL (kindly provided by Dr. C. H. Heusser, Novartis, Basel).

Analysis of surface molecule expression by flow cytometry

Cells (10^6) were stimulated in a 24-well plate with mAb specific for CD2, CD3 and CD28 (0.5 μg/mL each). After 72 h at 37°C and 5% CO_2, cells were washed at $300 \times g$ for 10 min at 4°C and stained with FITC- or PE-labeled mAb to CD25, CD69, HLA-DR, GITR, CTLA-4 or PD-1 for 30 min at room temperature and in the dark. Control antibodies were FITC- or PE-labeled mouse IgG1 or IgG2a.

Statistical analysis

Student's *t*-test was used for all paired comparisons between the IL-10-family cytokine-stimulated cells and negative (unstimulated) controls.

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