Activation of the aryl hydrocarbon receptor reveals distinct requirements for IL-22 and IL-17 production by human T helper cells

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Ligands of the aryl hydrocarbon receptor (AHR), a transcription factor mediating the effects of dioxin, favor Th17 differentiation and exacerbate autoimmunity in mice. We investigated how AHR ligands affected human T-cell polarization. We found that the high affinity and stable AHR-ligand dioxin as well as the natural AHR-ligand 6-formylinolo[3,2-b] carbazole induced the downstream AHR-target cytochrome P450A1, and without affecting IFN-γ, they enhanced IL-22 while simultaneously decreasing IL-17A production by CD4⁺ T cells. The specific AHR-inhibitor CH-223191 abolished these effects. Furthermore, blockade of IL-23 and IL-1, important for Th17 expansion, profoundly decreased IL-17A but not IL-22 production. AHR agonists reduced the expression of the Th17 master transcription factor retinoic acid-related orphan receptor C (RORC), without affecting T-bet, GATA-3 and Foxp3. They also decreased the expression of the IL-23 receptor. Importantly, AHR-lation did not only decrease the number of Th17 cells but also primed naïve CD4⁺ T cells to produce IL-22 without IL-17 and IFN-γ. Furthermore, IL-22 single producers did not express CD161, which distinguished them from the CD161⁺ Th17 cells. Hence, our data provide compelling evidence that AHR activation participates in shaping human CD4⁺ T-cell polarization favoring the emergence of a distinct subset of IL-22-producing cells that are independent from the Th17 lineage.

Key words: Aryl hydrocarbon receptor · Dioxin · IL-17 · IL-22 · T cells

See accompanying Commentary by Trifari and Spits

Introduction

Local cytokine milieu during antigen presentation profoundly affects the differentiation program of CD4⁺ T cells [1]. In the mouse, TGF-β, in the presence of IL-6 and pro-inflammatory cytokines, favors the emergence of Th17 cells that produce IL-17 and express the master transcription factor retinoic acid-related orphan receptor (ROR)γt [2–6]. Th17 cells are expanded and terminally differentiated in the presence of IL-23 [2]. Human Th17 cells may be generated in the presence of IL-1 and IL-23; IL-1 and IL-6; or TGF-β and IL-21 [7–10]. Th17 cells express CCR6 and
produce the CCR6-ligand CCL20, thereby amplifying Th17 cell recruitment at sites of inflammation [11]. IL-22, a member of the IL-10-family, is produced by Th17 cells, and to some extent by Th1 cells, NKT cells, NK cells and lymphoid tissue inducer-like cells [12]. IL-22 signals via a receptor consisting of IL-22R and IL-10R2 subunits [11]. Cells of hemapoietic origin do not express IL-22R; instead, IL-2R is highly expressed by epithelial cells of the gastrointestinal tract and the skin. In the mouse, IL-23 was shown to drive preferential expansion of cells that co-express IL-22 and IL-17, that may synergize to augment the expression of genes involved in defense against microbial pathogens [13, 14]. However, several mouse models of infection and autoimmunity suggested distinct roles for IL-17 and IL-22 [15–19].

In addition to cytokines, other mediators may impact CD4⁺ T-cell differentiation. We and others have shown that prostaglandin E2 (PGE2) favors human Th17 expansion [20–22]. Furthermore, ligands of the aryl hydrocarbon receptor (AHR) exert a role. AHR is a ubiquitous transcription factor present in the cytoplasm. Upon ligand-binding, AHR translocates into the nucleus where it regulates the expression of a variety of genes, including the xenobiotic metabolizing enzyme cytochrome P450A1 (CYP1A1) [23]. AHR binds to, and is activated by a range of structurally divergent chemicals including natural dietary, endogenous ligands, and synthetic environmental agents among which dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)) is the most extensively studied pure agonist [23, 24]. In the mouse, AHR is reported to regulate Th17 and Treg differentiation in ligand-specific fashion [25–27] and natural AHR agonists favor Th17 differentiation in vitro and enhanced IL-22 production [27–30]. Human lymphocytes may, however, behave differently and in a recent report AHR agonists appear to favor IL-22 but not IL-17 production [31]. These cells have been named Th22 cells and are thought to be involved in skin immunosurveillance and skin immunopathology [32, 33].

In the current study, we investigated the effect of synthetic and natural AHR ligands on the production of the human T helper signature-cytokines IL-17A, IFN-γ and IL-22. We found that AHR ligands decrease Th17 polarization and favor the differentiation of a subset of CD4⁺ T cells that produce IL-22 independently from IL-17A or IFN-γ. Importantly, we found that IL-22 production by CD4⁺ T cells did not require IL-1 or IL-23 and was independent from RORC expression, thus being distinctly different from IL-17 production. Finally, Th22 cells did not express CD161 as opposed to Th17 cells. Thus, our data contribute to the identification and characterization of IL-22 production by human T helper cells and reinforce the contention that human Th22 cells exist as a distinct subset.

**Figure 1.** TCDD and PGE2 exert opposite effects on the production of IL-22 and IL-17 by human PBMC. PBMC were activated by CD3-crosslinking and cultured in the presence of IL-23 (10 ng/mL) and IL-2 (20 U/mL). Cytokine levels were assessed in the supernatants at day 7 of culture. (A, B) Box plots show the median (50th percentile) and 25th and 75th percentiles in cytokine production of nine healthy individuals; whiskers show the highest and the lowest values. Significant differences between cultures without and with TCDD (10 nM) or PGE2 (50 ng/mL) are indicated based on paired Student’s t-test. (C) Bars show one representative individual out of three. FICZ (0.3 μM) and β-NF (5 μM) (ctr, control).
Results

TCDD and PGE2 exert opposite effects on IL-22 and IL-17A production

To assess the capacity of the stable, high-affinity AHR-ligand TCDD [30] to impact on the production of T-cell cytokines, particularly IL-17, we decided to compare the effect on cytokine production by human PBMC of TCDD to that of PGE2, previously shown to enhance IL-17A and decrease IL-22 [20]. To favor the expansion of Th17 cells, we added exogenous IL-23 to the culture medium. TCDD dramatically decreased IL-17A and significantly increased IL-22 but did not modify IFN-γ production. By contrast, PGE2 enhanced IL-17A, while decreasing the production of IL-22 and IFN-γ (Fig. 1A). Simultaneously, we found that TCDD significantly decreased and PGE2 tended to decrease the production of IL-10 while no significant effects were observed on TGF-β production (Fig. 1B). Of interest, also the natural AHR-ligands β-naphthoflavone (β-NP) and the high-affinity ligand 6-formylinolo[3,2-b] carbazole (FICZ), a tryptophan-derived photoproduct [23], significantly enhanced IL-22 and decreased IL-17A when added to PBMC cultures, thus extending the observation beyond the TCDD effect (Fig. 1C).

CD4+ T cells were responsible for the bulk of IL-22 production under all culture conditions (control, TCDD and PGE2) as assessed by intracellular staining (respectively Fig. 2A–C, left panels, for the results in nine individuals). Interestingly, CD4+ T cells could be subdivided into IL-2-single, IL-17A-single and IL-22/IL-17A double-positive cells. TCDD induced a marked decrease of both IL-17A single and IL-22/IL-17A double-positive cells, while PGE2 increased the percentage of IL-17A-producing cells (Fig. 2A–C, middle panels). Inclusion in the analysis of IFN-γ production revealed a population of cells producing IL-22 but not IFN-γ or IL-17A (Fig. 2A–C, right panels-lower/left quadrant), the frequency of which was increased by TCDD.

By differentially impacting on the proportion of cells producing IL-22, IL-17A, and IFN-γ, TCDD and PGE2 dramatically modified the ratio of the cytokines produced. TCDD increased the ratio of IL-22/IL-17A-producing cells (Fig. 2D, middle panel), while the ratio IL-22/IFN-γ remained constant (Fig. 2D, right panel). By contrast,

Figure 2. TCDD downregulates IL-17A and enhances IL-22 production by CD4+ T cells. PBMC were activated by CD3-crosslinking in the presence of IL-23. Cells were harvested at day 7 and stained for FACS analysis upon PMA/ionomycin activation. Representative surface/intracellular cytokine staining of PBMC cultured in (A) control condition, or (B) in the presence of TCDD, or (C) PGE2, for CD4, IL-22, IL-17A and IFN-γ. Dot plots show CD4 and IL-22 expression gated on live lymphocytes (left panels), for IL-22 and IL-17 gated on CD4+ cells (middle panels) and for IFN-γ and IL-17 gated on CD4+IL-22- cells (right panels). Areas containing IL-22/IL-17A IFN-γ– CD4+ T cells are highlighted in gray. Numbers in plots indicate the percentage of cells in each quadrant. Data are representative of nine individuals tested. (D) The percentage of IL-17A+ cells and the ratio of total IL-22+ cells over total IL-17A+ and IFN-γ+ cells as assessed by FACS analysis. Bars represent the mean ± SD of nine healthy individuals. (E) PBMC, purified CD4+ T cells (purity > 99%) were cultured for 7 days in the presence or absence TCDD (10 nM) after activation by CD3-crosslinking (PBMC) or by CD3- and CD28-crosslinking (CD4+ T cells). Bars represent the mean ± SD of four individuals. p-values shown are significant differences compared to the cultures without TCDD as assessed with the Student’s t-test for paired samples. Culture conditions were the same as that described in Fig. 1.
PGE2 increased the ratio of IL-22/IFN-γ and decreased the ratio of IL-22/IL-17A-producing cells (Fig. 2D, middle and right panel).

Of interest, TCDD enhanced IL-22 and decreased IL-17A production (without affecting IFN-γ) also in purified CD4+ T cells activated by CD3 and CD28 cross-linking in the absence of accessory cells (Fig. 2E). As expected, IL-17 and IL-22 were found mainly in the supernatants of CD4-positive cells.

Hence, TCDD affects IL-22 and IL-17A production by CD4+ T cells and favors the outgrowth of a cell subset that produces only IL-22 (IL-22+IL-17A−IFN-γ−) while inhibiting Th17 cells (IL-22−IL-17A+ as well as IL-22−IL-17A−cells).

Involvement of AHR in upregulation of IL-22 and downregulation of IL-17A

Both TCDD and FICZ upregulated the expression of CYP1A1 as typically observed upon AHR ligation in purified CD4+ T cells (Fig. 3A). Furthermore, the specific AHR antagonist CH-223191 [34] completely reversed the inhibitory activity of TCDD on IL-17A as well as the enhancing activity on IL-22 (Fig. 3B).

Interestingly, when the AHR antagonist was added to the cultures in the absence of TCDD, we observed a significantly reduced production of IL-22 and a trend toward an increased production of IL-17A. These results not only show that AHR is involved in TCDD-induced regulation of IL-22 and IL-17A but also, consistent with previous observations [28], suggest that natural AHR ligands present in the cells and/or in the external milieu exert an important biological activity capable of regulating the production of IL-22 and IL-17A.

IL-23 and IL-1 are not required for TCDD-induced increase in IL-22 production

IL-23 is reported to favor Th17 cell terminal differentiation and coexpression of IL-17A and IL-22 [13, 17]. To determine whether IL-23 was required for the production of IL-22, we activated purified CD4+ T cells by CD3 and CD28 cross-linking in the presence of a neutralizing anti-IL-23 mAb. Although neutralization of IL-23 significantly decreased IL-17A, we observed no change in IL-22 production (Fig. 4). Furthermore, IL-23 neutralization did not affect the TCDD-induced increase in IL-22 production but did synergize with TCDD in reducing IL-17A. Of interest, the production of IFN-γ and IL-10 was not modified whether IL-23 was neutralized or not (Fig. 4). Thus, IL-23 favors IL-17A but does not affect IL-22 production and does not mediate the effect of TCDD. Pro-inflammatory IL-1 is critically needed for Th17 priming and expansion [5, 7, 8, 20, 35]. When purified CD4+ T cells were activated in the presence of IL-1 receptor agonist (IL-1Ra), to block the membrane-bound IL-1α expressed by activated T cells, IL-17A production was practically abolished. However, IL-22 production and the enhancing effect of TCDD were not affected (Fig. 4). Furthermore, IL-1 neutralization significantly decreased IFN-γ production while IL-10 production was increased. However, the effect of TCDD on IFN-γ and IL-10 production was similar to that observed without IL-1 neutralization (Fig. 4). Thus, IL-1 blockade impacts the production of several CD4+ T-cell cytokines but does not affect the production of IL-22. Interestingly, the inhibitory effect of IL-23 and IL-1 blockade on CCL20 was similar to that observed on IL-17A indicating similar regulation, with TCDD acting as further inhibitor. Finally, none of the culture conditions impacted TGF-β production (Fig. 4). These data demonstrate that IL-23 and IL-1 both regulate IL-17A (and CCL20) but have no effects on IL-22 production and, importantly, do not mediate the effects of TCDD on IL-22.

TCDD does not affect AHR-expression but decreases the expression of RORC

Polarization of CD4+ T-cell subsets is regulated by master transcription factors [1]. TCDD did not modify the expression of Tbx21, Gata3 and Foxp3 after PMA/ionomycin reactivation of CD4+ T cells after 7 days of culture. Only RORC expression was significantly decreased (Fig. 5A, left panel). Under the same conditions, steady-state levels of IL-22 mRNA increased and those
of IL-17A decreased (Fig. 5A, right panel) without a concomitant change in AHR transcription (Fig. 5A, middle panel and Fig. 5B). Changes in RORC expression occurred already before the restimulation after the 7-day culture (Fig. 5B). Hence, RORC may not be implicated in the regulation of IL-22 production in human T cells. Interestingly, we also observed a significant decrease in IL-23 receptor transcription indicating that TCDD interferes with Th17 polarization at different levels (Fig. 5B).

TCDD favors the expansion of IL-22 single-producing cells from naïve CD4+ T cells

To test the hypothesis that AHR-ligands favor the outgrowth of T cells prone to IL-22 production, we activated highly purified naïve human CD4+ T cells (CD4+CD45RO−CD25−) under non-polarizing, Th1- or Th17-polarizing conditions in the presence or absence of TCDD. Th17 polarization was obtained by supplementing the serum-containing medium with IL-1β and IL-23. Under all conditions, IL-22 + IL-17A + IFN-γ single-positive T cells were present, and importantly, TCDD significantly enhanced their frequency without much impact of the exogenous cytokines used for priming (Fig. 6A). By contrast, apart from the tendency of TCDD to reduce the frequency of IL-22 + IL-17A + IFN-γ single-positive T cells under Th17 priming conditions (Fig. 6B), no other effects of TCDD were observed (Fig. 6B and C). Thus, IL-22 single producing cells can be generated from human naïve T cells and AHR occupancy by TCDD favors the differentiation of this T-cell subset. Similar data were obtained when highly purified memory T cells were cultured in the presence of TCDD resulting in a significant increase in IL-22 + IL-17A + IFN-γ single-positive and a significant decrease in IL-22 + IL-17A + IFN-γ single-positive T cells compared to controls (Fig. 6D).

Finally, we assessed whether IL-22 single-positive cells generated in vitro from naïve T cells expressed CD161, a marker reported to be specific of Th17 cells [9, 36]. Our results confirm the strong expression of CD161 by most IL-17+ IL-22 + IFN-γ− cells but also show that CD161 is virtually absent from IL-22 + IL-17A + IFN-γ− single-positive T cells (Fig. 6E). This not only stresses the phenotypic differences between T cells with polarized production of IL-22 or IL-17A but also suggests further that these cells may belong to different subsets.

Discussion

The cytokine production of individual cells within a population of Th17 cells is heterogeneous. Whether this reflects different states of maturation in a single T-cell subset with intrinsic plasticity or the presence of cells belonging to distinct subsets is still matter of debate [37–39]. In the current work, we documented profound modifications occurring in the capacity of human CD4+ T cells to produce IL-17A and IL-22 when activated under the influence of both synthetic as well as natural AHR ligands. We observed reciprocal differences when CD4+ T cells were activated in the presence of PGE2. These modifications imply that the regulation of IL-22 and
IL-17A gene expression are distinct and provide evidence that the differentiation program of T helper cells, at least in humans, may consist of layers of higher complexity than previously considered.

A very robust finding here reported is the decreased production of IL-17A and increased production of IL-22 when T cells were activated in the presence of TCDD, results replicated in the presence of FICZ and β-NP. Our findings with TCDD corroborate the results of Trifari and colleagues who found in humans an enhanced frequency of IL-22 and reduced frequency of IL-17A producing cells after the priming of T cells under Th17 conditions.

Figure 5. RORC and IL-23R expression is decreased in the presence of TCDD. (A) mRNA levels of FOXP3, GATA3, RORC, TBX21, AHR, IL-22 and IL17A in purified CD4⁺ T cells as quantified by real-time PCR after 7 days of culture and a subsequent 4.5 h activation by PMA/ionomycin. Expression levels were normalized against the geometric mean of three house-keeping genes (GUSB, EEF1A1 and TBP). (B) Changes in mRNA levels in purified CD4⁺ T cells of RORC, IL23R and AHR quantified by real-time PCR. Data are expressed as the normalized level of mRNA at day 7 divided by the normalized level of mRNA at day 0. Bars represent the mean ± SD of three (FOXP3, GATA, TBX21, IL17A, IL22) or four (RORC, IL23R, AHR) individuals. Statistically significant differences according to Student's t-test for paired samples.

Figure 6. TCDD favors the outgrowth of IL-22-producing T cells from the naïve T-cell pool. Frequency of (A) IL-22⁺ IL-17A⁻ IFN-γ⁺, (B) IL-22⁺ IL-17⁺ IFN-γ⁺ and (C) IL-22⁺ IL-17⁻ IFN-γ⁺ single-positive cells after culture of highly purified (>98%) naïve CD4⁺ CD45RO⁻ CD25⁻ T cells activated by CD3 and CD28-crosslinking in the presence or absence of TCDD (10 nM), under non-polarizing, Th1 (10 ng/mL IL-12, 10 ng/mL anti-IL-4) or Th17 (1 ng/mL IL-1β, 10 ng/mL IL-23) conditions. IL-2 (20 U/mL) was added 48 h after culture initiation. Cells were harvested at day 11, activated by PMA/ionomycin for 4.5 h and processed for intracellular staining. (D) Frequency of IL-22⁺ IL-17A⁻ IFN-γ⁺, IL-22⁺ IL-17⁺ IFN-γ⁺ and IL-22⁺ IL-17⁻ IFN-γ⁺ single-positive cells after culture under non-polarizing condition of highly purified (>98%) memory CD4⁺CD45RO⁺ T cells. Culture conditions were the same as in A–C. Each symbol represents one individual; horizontal line indicates the mean. Statistically significant differences according to Student's t-test for paired samples. (E) CD161 expression by IL-22⁺ IL-17A⁻ IFN-γ⁺ and IL-17⁺ T cells. A representative result of three experiments is shown.
and natural AHR ligands [31]. They are at variance with the enhanced IL-22 production and concomitant enhanced IL-17A production observed in the presence of FICZ and TCDD in the mouse [27–29]. TCDD was, in addition, shown to induce Treg in the mouse [25, 26], whereas in our in vitro system we could not trace the emergence of Treg as far as TCDD did not upregulate the expression of FOXP3, nor the production of IL-10 and TGF-β. All together, these data stress a fundamental function of AHR in modulating the adaptive immune response with subtle but interesting differences between human and mouse T cells. These discrepancies demonstrate that the response to AHR activation may vary with the cell type and during differentiation and maturation processes [40].

Several lines of circumstantial evidence argue in favor of an effect of AHR-ligands on a subset of CD4+ T cells prone to IL-22 production distinct from Th17, Th1 and Treg cells. First, enhanced IL-22 was observed concomitantly to reduced IL-17A with no changes in IFN-γ production. Second, IL-23 blockade selectively reduced IL-17A and CCL20 without affecting IL-22 production. Third, IL-1 blockade abrogated IL-17A, decreased CCL20 and IFN-γ, did not modify TGF-β, and actually increased IL-10 without affecting IL-22 production. Fourth, TCDD decreased RORC without affecting the mRNA steady-state levels of other master transcription factors including TBX21, GATA3 and FOXP3. Fifth, priming of CD4+ naïve T cells in the presence of TCDD resulted in increased number of IL-22+IL-17−IFN-γ+ single-positive T cells. Finally, CD161 selectively expressed in Th17 cells [9, 36] was absent in IL-22+IL-17A−IFN-γ− single-positive T cells. Furthermore, our data regarding the dissociated IL-22 and IL-17 production are consistent with recent reports showing that skin-homing memory T cells [31, 32], T cells present in the skin of individuals affected by atopic dermatitis [41], plaque psoriasis, allergic contact dermatitis [33] and T cells responding to mycobacteria [42] or Candida albicans [43] produce IL-22 but not IL-17A or IFN-γ. Of interest, human Langerhans cells appear to favor the induction of CD4+ T cells producing IL-22 but not IL-17, thus stressing a preferential role of these cells in skin immunosurveillance [44]. Finally, Notch signaling has been shown to selectively drive IL-22 but not IL-17A production in murine CD4+ T cells by inducing endogenous AHR ligands [45].

We showed a decreased expression of mRNA for IL-23R in CD4+ T cells cultured in the presence of TCDD. It is tempting to speculate that the decreased production of IL-17A and reduced frequency of Th17 cells observed in the presence of TCDD was indeed due to the reduced capacity of cells cultured in the presence of TCDD to respond to IL-23.

IL-22 production by CD4+ T cells induced by TCDD was accompanied by enhanced IL-22 mRNA levels and was not directly regulated by the level of RORC expression. These data are at variance with the reduction of IL-22 reported in CD4+ memory T cells in which RORC was silenced [31], but in agreement with the finding that Th22 clones express lower RORC levels than Th17 clones [32, 33] and with the fact that lentivirus-induced expression of RORγt in human naïve T cells results in IL-17 without IL-22 production [5]. In this respect, it is important to note that whole genome transcriptome analysis of T-cell clones with preferential IL-22 production pointed to the preferential expression of BNC2 and FOXO4 transcription factors when compared to Th1, Th2 and Th17 cell clones [33]. It remains to be demonstrated that these transcription factors are involved in the polarization process of cells committed to IL-22 production.

Of further interest, AHR ligation induced a greater increase of IL-22 production in PBMC than in purified CD4+ T cells. The likeliest explanation is that accessory cells in PBMC are a source of cytokines favoring IL-22 production by CD4+ T cells, consistent with the work of Duhen and colleagues demonstrating the capacity of IL-6 and TNF to cooperatively enhance IL-22 production by T cells [32, 33].

Overall, our data indicate that AHR agonists profoundly change the balance between the number of human CD4+ T cells producing IL-17A or IL-22 by impairing Th17 responses but not IL-22 production in an IL-23 and IL-1 independent manner. This is consistent with the recent finding that IL-22 production by T cells is, at least partially, AHR dependent [31] and reinforce the claim that T cells selectively producing IL-22 exist in humans as a separate subset named Th22 [32, 33, 44].

### Material and methods

#### Reagents

PMA, β-mercaptoethanol, brefeldin A and PGE2 were from Sigma Chemicals; TCDD from Cambridge Isotope Laboratories; β-NP and FICZ from Enzo Life Sciences; ionomycin from Calbiochem; RPMI 1640 medium, PBS, penicillin, streptomycin, l-glutamine, non-essential amino acids, sodium pyruvate from Life Technologies; FBS from Amimed; CD4 Positive and Negative Isolation Kits and CD3/CD28 T-cell Expander from Invitrogen; human rIL-2 and IL-1β from Biogen; human rIL-12, anti-IL-22-PE and anti-IL-4 were from R & D; anti-CD4-PE, anti-CD4-PE-Cy5, anti-IFN-γ-PE-Cy7, anti-CD25-FITC, anti-CD161-APC and anti-CD45RO-FITC were kindly provided by Dr. J. E. Sims (Amen, Seattle, WA, USA); anti-IL-17A from eBioscience; IL-1 Ra from Amgen; anti-CD45RO-FITC were from BD; OKT3 from ATCC (Manassas, VA, USA); anti-IL-17A from ebiosciences; IL-1 Ra from Amgen; anti-CD22p19 (M56) and anti-IFN-γ were kindly provided by Dr. J. E. Sims (Amgen, Seattle, WA, USA) and Dr. W. Ferlin (Novimmune, Geneva, Switzerland), respectively.

#### Cell culture

Peripheral blood was obtained from healthy members of the laboratory. Permission to perform this investigation was granted by the ethical committee of our institution. Informed consent was obtained in accordance with the declaration of Helsinki. PBMC were purified by Lymphoprep gradient centrifugation. CD4+ T cells (purity >99%) were obtained by positive selection using Dynal® CD4 Positive Isolation Kit. Naïve and memory CD4 T cells...
Flow cytometry

Intracellular cytokine accumulation and cell surface staining was assessed by FACS analysis using FACS 500 (Beckman Coulter) or FACS Canto (BD) and data were analyzed by FBS express (Denovo software) or FlowJo software 7.5 (Tree Star) using FACSCanto (BD) and data were analyzed by FBS express and FACS 500 (Beckman Coulter) or FACS Canto (BD). Flow cytometry analysis was used to assess the expression of intracellular cytokines and cell surface markers. Cells were harvested and used for FACS analysis or mRNA determination. Flow cytometry analysis was performed on a BD FACSAria and data were analyzed using FacSDiva software.

Cytokine assays

IL-17, IFN-γ and IL-10 were quantified in culture supernatants by Luminex xMAP Technology using multiplex bead immunoassay (Fluorokine MAP Multiplex Human Cytokine Panel, R&D). IL-22, CCL20 and TGF-β were assessed by ELISA (R&D Systems).

Real-time quantitative PCR

Cells were resting or activated for 4.5 h in the presence of PMA/ionomycin. Total RNA (0.5 μg) was extracted using the RNeasy micro kit (Qiagen) and cDNA synthesized using random hexamers and SuperScript III reverse transcriptase (Invitrogen). Oligonucleotides were obtained from Qiagen: IL-17A, QT00009233; IL-22, QT00034853; GATA3, QT00095501; TBX21, QT00042217; FOXP3, QT00048286; RORC, QT00097888; IL23R, QT00032914; CYP1A1, QT00012341; GUSB, QT00046046; TBPI, QT00000721; and EF1A1: QT01669934 from Invitrogen: AHR forward (5'-GCCAGGCAACAGCCAT-3′) and reverse (5′-TCATTAAAAACTCCATTCT GAAACITGT-3′). SYBR Green assays were performed on a SDS 7900 HT instrument (Applied Biosystems). Each reaction was performed in triplicates. Raw Ct values obtained with SDS 2.2.2.2 software (Applied Biosystems) were analyzed and the more stable housekeeping genes (GUSB (glucuronidase), TBP (TATA-box-binding protein) and EF1A1 (eukaryotic translation elongation factor 1-alpha 1) selected for normalization. Separate experiments were performed for CYP1A1 using LightCycler instrument (Roche) and QuantiTect SYBR Green PCR Master Mix (Qiagen). Each sample was run in triplicate and results were normalized to GUSB.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software). Box plots were generated using GraphPad. Two-tailed Student's t-test for paired samples was used to compare the different experimental conditions. p values less than 0.05 were considered significant.

Acknowledgements:
Work supported in part by grant 31003A_124941/1 from the Swiss National Science Foundation to C. C.

Conflict of interest: The authors declare no financial or commercial conflict of interest.

References


Abbreviations: AHR: Aryl hydrocarbon receptor · β-NP: beta naphthoflavone · CYP1A1: cytochrome P450A1 · FICZ: 6-formylindolo[3,2-b] carbazole · PGE2: prostaglandin E2 · RORγT: retinoic acid-related orphan receptor γT · TCDD: 2,3,7,8-tetrachlorodibenzo-p-dioxin

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See accompanying Commentary: http://dx.doi.org/10.1002/201040848

Received: 4/3/2010 Revised: 17/5/2010 Accepted: 15/6/2010 Accepted article online: 13/7/2010