Published in final edited form as:

Immunol Cell Biol. 2010 August ; 88(6): 624–631. doi:10.1038/icb.2010.53.

Regulation of human Th9 differentiation by type I interferons and IL-21

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Abstract

Interleukin (IL)-9-producing CD4⁺ T cells are a novel subset of T helper (Th) cells that develops independently of the Th1, Th2, Th17 and regulatory T-cell lineages. Similar to the murine model, transforming growth factor (TGF)- β and IL-4 directed human naive CD4⁺ T cells to produce IL-9. Whereas IL-4 suppressed TGF- β -induced Foxp3 expression, TGF- β failed to inhibit IL-4-mediated upregulation of the Th2 transcription factor GATA-3. Addition of IL-1 β , IL-6, IL-10, interferon (IFN)- α , IFN- β or IL-21 to Th9-polarizing conditions augmented Th9 differentiation, while the Th1-associated cytokines IFN- γ and IL-27 partially suppressed IL-9 production. Given that T cells are a primary source of IL-21, IL-21 expression was analyzed under Th9-polarizing conditions in the context of inflammatory cytokines. Surprisingly, type I IFNs induced elevated levels of IL-21, and blockade of IL-21 abrogated their ability to enhance Th9 differentiation. Taken together, these data indicate a complex cytokine network in the regulation of human IL-9-producing CD4⁺ T cells.

Keywords

human; IL-21; Th9; type I interferons

Upon recognition of a foreign antigen displayed by antigen-presenting cells, naive CD4⁺ T cells become activated and, depending on the cellular microenvironment, polarize into distinct lineages of T helper (Th) cells.¹ Th1 and Th2 cells were first described by Mossman and Coffman as subsets that regulate cellular and humoral immune responses, respectively.² Th1 development requires dendritic cell-derived interleukin (IL)-12, which promotes the upregulation of the transcription factor T-bet and the secretion of interferon (IFN)- γ to mediate defense against intracellular pathogens.¹ Disregulation of the Th1 response has been implicated in a number of inflammatory and autoimmune disorders.³ In contrast, Th2 lineage commitment requires IL-4-mediated upregulation of the master regulatory transcription factor GATA-3.^{1,4} Th2 cells secrete IL-4, IL-5 and IL-13 to mount immune

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CONFLICT OF INTEREST

We state the following conflict of interest disclosures over the past 3 years: PJU has served as a consultant to Centocor (Horsham, PA, USA), Biogen Idec (Cambridge, MA, USA), Genentech, Inc. (South San Francisco, CA, USA), Astra Zeneca (London, UK), CoMentis (South San Francisco, CA, USA), Gilead Sciences (Foster City, CA, USA), Regimmune (Mountain View, CA, USA) and UCB (Belgium), and is a co-founder and consultant at Bayhill Therapeutics (San Mateo, CA, USA). EE is on the scientific advisory board of Globeimmune, Inc.

responses against parasitic infections^{1,4} and are frequently linked to the pathogenesis of various allergic disorders.⁵

In addition to the classical Th1 and Th2 lineages, the regulatory T cell (Treg) and Th17 subsets have been described and extensively characterized. Naïve CD4⁺ T cells cultured in the presence of transforming growth factor (TGF)- β upregulate the transcription factor Foxp3 and polarize into Tregs that mediate suppression of immune responses through the secretion of anti-inflammatory cytokines and cell contact-dependent mechanisms.^{6,7} Along with others, we have shown that human Th17 differentiation requires a cocktail of cytokines including IL-1 β , IL-6, IL-21, IL-23 and TGF- β .^{8–13} Th17 cells express the transcriptional regulator ROR γ t and primarily secrete IL-17A, IL-17F and IL-22 to mediate immunity against extracellular bacteria and various fungal infections.¹⁴ Disequilibrium of the Treg/Th17 balance has been associated with numerous autoimmune diseases such as rheumatoid arthritis.^{15,16}

Traditionally associated with the Th2 response, IL-9 is a member of the common gamma chain cytokine family and exerts broad effects on a variety of cell types such as mast cells, eosinophils, T cells and epithelial cells.¹⁷ Two recent reports have identified in the murine system a distinct population of CD4⁺ T cells that produce IL-9 as well as IL-10, but lack the potential to secrete IFN- γ , IL-4 or IL-17,^{18,19} a profile suggesting a distinct lineage of CD4⁺ Th cells. Although the functional relevance of this novel lineage is still unclear, evidence suggests that these cells have an inflammatory role in a mouse model of colitis.¹⁸ Murine Th9 cells require TGF- β in combination with IL-4 to redirect naïve T cells from the Th2 lineage into an IL-9-producing subset,^{18,19} but this has not been confirmed in humans. In this study, we investigated the requirements for the induction and regulation of human Th9 cell differentiation.

RESULTS

Combinatorial addition of TGF- β and IL-4 promotes human Th9 differentiation

Starting with the hypothesis that TGF- β and IL-4 are required for IL-9 production in humans, fluorescence-activated cell sorting (FACS) purified naïve CD4⁺CD45RA⁺CD45RO⁻CD25⁻ T cells (>99.9% purity) were cultured with TGF- β and IL-4 along with anti-CD3/CD28 coated beads as an activating stimulus. As controls, naïve T cells were cultured with beads in the absence of cytokine or in the presence of TGF- β alone or IL-4 alone. After 4 days, analysis of cell-free supernatants revealed that IL-4 alone induced low levels of IL-9 compared with the media alone or TGF- β alone controls. However, addition of TGF- β together with IL-4 potently induced high levels of IL-9 compared with all other conditions while suppressing IL-4-induced secretion of IL-5 and IL-13 (Figure 1a). In contrast to the murine model, naïve T cells cultured with TGF- β and IL-4 produced reduced levels of IL-10 as compared with cells cultured with IL-4 alone (Figure 1a). Intracellular flow cytometric analysis confirmed that IL-9⁺ cells do not co-produce the Th2-associated cytokines IL-4, IL-5, IL-13 and IL-10 (Figure 1b, Supplementary Figure 1 and data not shown). These data indicate that TGF- β and IL-4 potently induce human IL-9 production and, for the remainder of this study, will be referred to as 'Th9-polarizing conditions'.

Transcriptional analysis of human Th9 cells

As TGF- β and IL-4 are required to polarize naïve T cells into the Treg and Th2 lineages, respectively,^{4,6} the intracellular levels of the master regulatory transcription factors Foxp3 and GATA-3 were analyzed on day 3 of culture. TGF- β induced high levels of Foxp3 expression, which was partially suppressed by the addition of IL-4 (Figure 2a). Only a small

fraction of Th9 cells co-expressed Foxp3 (Figure 2a), suggesting that Foxp3 is not absolutely required for Th9 lineage commitment. Conversely, TGF- β did not abrogate IL-4-mediated upregulation of GATA-3 expression (Figure 2b). Analysis of IL-9⁺ and IL-9⁻ cells cultured under Th9-polarizing conditions revealed that GATA-3 was similarly expressed in both populations (Figure 2c), suggesting that GATA-3 may be necessary but not sufficient for human Th9 differentiation.

Positive and negative regulation of human IL-9 production by inflammatory cytokines

Having established the minimum requirements for generating human Th9 cells, we next performed a screen to determine whether additional inflammatory cytokines could regulate IL-9 secretion. Unexpectedly, a group of cytokines added individually to Th9-polarizing conditions upregulated IL-9, including IL-1 β , IL-6, IL-10, IFN- α , IFN- β and IL-21 (Figure 3a). In some cases, IL-12 or IL-23 modestly enhanced IL-9 production in some donors, but this was not statistically significant across all donors (Figure 3a). Addition of either IFN- γ or IL-27 to Th9 conditions significantly inhibited IL-9 production in a dose-dependent manner (Figure 3b). Given that IFN- γ and IL-27 are associated with the Th1 lineage,²⁰ these data suggest that the Th1 response can negatively regulate the development of Th9 cells. However, this does not explain why the Th1-polarizing cytokine IL-12 failed to suppress—and in some cases, actually enhanced—human Th9 differentiation, indicating that IL-12 induces a factor that counteracts the inhibitory effects of IL-12-induced IFN- γ .

Type I IFNs induce IL-21 expression

As IL-21 potentially augmented human Th9 differentiation (Figure 3a) and CD4⁺ T cells produce IL-21, we analyzed IL-21 expression in the context of Th9-polarizing conditions and inflammatory cytokines. In concordance with two recent reports, IL-12 alone induced robust intracellular expression of IL-21 compared with naïve T cells cultured in the absence of cytokine.^{21,22} Addition of Th9 polarizing conditions only partially inhibited IL-21 expression but induced a population of cells that co-produced IL-9 and IL-21 (Figure 4a). Although IL-1 β or IL-6 alone modestly promoted elevated levels of IL-21, addition of Th9 conditions almost completely suppressed IL-21 (Figure 4a). Strikingly, data presented in Figure 4 show that IFN- α or IFN- β alone induced a substantial population of IL-21-producing T cells, which was only partially suppressed by adding Th9-polarizing cytokines. IL-21 promoted its own expression, whereas IL-10 alone did not alter IL-21 levels (Figure 4a). However, in both cases a small proportion of IL-21-producing cells remained in spite of the presence of Th9 conditions. These trends were observed across several donors in spite of the variability in the absolute percentages of IL-9⁺ and IL-21⁺ cells (Figure 4b). Although IL-21 has also been shown to promote human Th17 differentiation,¹¹ Th9 cells did not produce IL-17 under the aforementioned culture conditions (Supplementary Figure 2).

Blockade of IL-21 inhibits type I IFN enhancement of human Th9 differentiation

The finding that type I IFNs could promote IL-21 expression led us to test the hypothesis that IFN- α and IFN- β augment human Th9 differentiation through the induction of IL-21. Naïve CD4⁺ T cells were cultured with Th9 conditions and IFN- α , IFN- β , IL-12 or IL-21 in the presence of either an IL-21R/Fc chimera that has been shown to specifically inhibit IL-21^{21,23} or an isotype control. Analysis of IL-9 secretion by enzyme-linked immunosorbent assay (ELISA) revealed that inhibiting IL-21 in the context of Th9 conditions and either IFN- α or IFN- β potentially inhibited Th9 differentiation as compared with the isotype control (Figure 5a). Addition of IL-21R/Fc to the Th9 condition alone only modestly downregulated IL-9 production (Figure 5a), which is consistent with the low levels of IL-21 expression observed under Th9-polarizing cytokines (Figure 4). Naïve T cells cultured in the presence of Th9 conditions, IL-21 and IL-21R/Fc downregulated IL-9 to

levels approaching those of cells cultured with Th9 conditions and isotype (Figure 5a), confirming the specificity and efficacy of inhibiting IL-21.

Although IL-12 does not consistently enhance Th9 differentiation, blocking IL-21 in the presence of Th9 conditions and IL-12 potentially suppressed IL-9 secretion to levels consistently below those cells cultured with Th9 conditions and isotype (Figure 5a). However, blockade of IFN- γ in the presence of Th9 conditions and IL-12 did not substantially promote IL-9 production, suggesting that additional signals are countering the effect of IL-12-induced IL-21 to enhance Th9 differentiation (Supplementary Figure 3). Further analysis of intracellular IL-9 and IL-21 percentages by flow cytometry showed the same trends observed with ELISA (Figure 5b), confirming that blocking IL-21 specifically inhibits Th9 differentiation as opposed to suppressing cell proliferation. IL-21R/Fc also downregulated intracellular IL-21 levels (Figure 5b), which is consistent with the observation that IL-21 induces its own expression (Figure 4a). In summary, these data strongly indicate that IL-21 is a critical enhancer of IL-9 production in humans.

Memory Th9 cells are found within the CRTH2+ population

To determine whether Th9 cells are present within the memory T-cell compartment, CD4⁺CD45RO⁺CD45RA⁻ T cells were freshly isolated from the peripheral blood of healthy donors and stained for intracellular expression of IL-4, IL-5, IL-13 and IL-9. Although memory T cells expressed detectable levels of intracellular IL-4, IL-5 and IL-13 (>1% of memory cells), IL-9 comprised <0.1% of memory T cells (Figure 6a). This low level of IL-9 expression was confirmed by ELISA after stimulation of unfractionated CD4⁺ memory cells with PMA and ionomycin (Figure 6b). As Th2 cells have been shown to express the surface marker CRTH2,^{24,25} we examined whether IL-9 producers were enriched within the CRTH2⁺ or CRTH2⁻ populations. As expected, FACS-purified memory CD4⁺CD45RA⁻CRTH2⁺ cells expressed elevated levels of the Th2 cytokines IL-4, IL-5 and IL-13 compared with CRTH2⁻ and unfractionated memory T cells (Figure 6b and Supplementary Figure 4). Sorting on CRTH2⁺ cells resulted in approximately threefold greater levels of IL-9 by ELISA (Figure 6b), suggesting an association with the Th2 response.

TGF- β alone is required to expand memory Th9 cells

Given the low frequency of Th9 cells observed in memory CD4⁺ T cells, we next analyzed the requirements for expanding memory Th9 cells. Similar to the culture conditions described in Figure 1, memory CD4⁺CD45RO⁺CD45RA⁻ T cells were cultured with various combinations of TGF- β and IL-4. Although IL-4 alone did not promote expansion of Th9 cells, TGF- β alone induced a substantial population of IL-9-producing cells (Figure 6c). Addition of IL-4 in combination with TGF- β further expanded Th9 cells (Figure 6c), suggesting that IL-4 acts as an enhancer of Th9 polarization from memory cells. Furthermore, Th9 cells generated from memory cells did not co-produce IL-4 or IL-10 (Figure 6c and Supplementary Figure 1). These data, along with the results generated with naive T cells, indicate that TGF- β is absolutely required for promoting the Th9 response in humans.

DISCUSSION

In this study, we have investigated the requirements for generating Th9 cells in humans. In contrast to the differences found between mice and humans with regard to Th17 polarization,²⁶⁻²⁸ potent Th9 differentiation in humans seems to require the same cocktail of cytokines as observed in mice,^{18,19} TGF- β and IL-4. We performed our *in vitro* studies on highly purified naive T cells from the peripheral blood; however, it is critical that future

studies use umbilical cord blood to definitely establish whether human Th9 cells derive from naive precursors. Furthermore, several inflammatory cytokines have the capacity to increase the frequency of IL-9-producing cells, most notably IFN- α , IFN- β and IL-21. Although Th9 cells are present at extremely low frequencies in the peripheral blood, these cells express the Th2 marker CRTH2 and require TGF- β for expansion from memory CD4⁺ T cells.

One question raised by this study is the complex signaling and transcriptional networks that lead to the production of IL-9 in humans. In spite of the downregulation of Foxp3 under Th9-polarizing conditions, GATA-3 expression is maintained, suggesting that GATA-3 is required for IL-9 production. However, these data do not rule out an unidentified transcription factor that can act alone or in combination with GATA-3 to promote the transcription of IL-9. This is supported by our data showing that Th9 cells do not express the Th2 cytokines IL-4, IL-5 and IL-13, suggesting that Th2 and Th9 cells develop along a reciprocal developmental pathway. The fact that several inflammatory cytokines, including IL-1 β , IL-6, IL-10, IL-21 and type I IFNs, augment IL-9 production adds an additional layer of complexity to the regulation of Th9 differentiation. Several of these cytokines induce the phosphorylation of both STAT1 and STAT3 (data not shown), including IL-6, IL-10 and IL-21. Whether enhancement of IL-9 production is primarily mediated by one or both of these STAT proteins likely requires *in vivo* mouse models or studies using selective JAK or STAT inhibitors. Alternatively, human CD4⁺ T cells obtained from patients with either STAT1 or STAT3 mutations^{29,30} could be used to analyze the role of STAT proteins in regulating human Th9 differentiation.

The finding that type I IFNs induce IL-21 expression supports a recent study implicating a role for type I IFN signaling in the development of IL-21-producing T follicular helper cells.³¹ These data also corroborate a study reporting that IFN- α and IFN- β upregulate IL-21 mRNA levels in activated human T cells.³² Although we have also observed that IL-12 potently induces high levels of IL-21, our findings are inconsistent with a study that failed to identify a role for IFN- α in promoting IL-21 expression,²¹ likely reflecting differences in *in vitro* culture systems. Thus, whether IL-21-producing T cells generated by type I IFNs demonstrate the properties of T follicular helper cells and whether T follicular helper cells regulate Th9 cells *in vivo* certainly requires further study. Another question that arises from these experiments is the functional relevance of Th9 cells, especially with regard to disease pathogenesis. Given that type I IFNs and IL-21 have both been shown to have a role in systemic lupus erythematosus,^{33–37} analysis of IL-9-producing T cells as a possible mediator of autoantibody production in systemic lupus erythematosus warrants further investigation.

METHODS

Cell isolation and cultures

Enriched CD4⁺ T cells were prepared from buffy coats obtained from healthy donors (Stanford Blood Center after informed consent and Institutional Review Board approval) using a RosetteSep Human CD4⁺ T-Cell Enrichment (Stem Cell Technologies, Vancouver, BC, Canada) before density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare, Uppsala, Sweden). Naïve CD4⁺CD45RA⁺CD45RO[−]CD25[−] or memory CD4⁺CD45RO⁺ T cells were either magnetically sorted with commercially available kits (Miltenyi Biotec, Bergisch Gladbach, Germany) or FACS purified. For FACS purification of CRTH2⁺ and CRTH2[−] cells, magnetically sorted memory T cells were labeled with fluorescently labeled Abs against CD4, CD45RA (Invitrogen, Carlsbad, CA, USA) and CRTH2 (BD Bioscience, San Jose, CA, USA). In addition, cells were labeled with CD25 and CD127 (Biolegend, San Diego, CA, USA) to remove Tregs. Magnetically sorted cells were always 97–99% pure, while FACS purified cells were always >99% pure. It is

noteworthy that similar results were obtained with magnetically sorted cells compared with FACS sorted cells. All cells were cultured at 37 °C in 48-well flat-bottomed plates (BD Falcon, San Jose, CA, USA) in X-VIVO 15 media (Lonza, Basel, Switzerland) supplemented with 10% human serum type AB (Lonza), 100 units ml⁻¹ penicillin/streptomycin, L-glutamine (Invitrogen) and 50 µM β-mercaptoethanol (Sigma-Aldrich, St Louis, MO, USA). Naïve or memory CD4⁺ T cells were cultured at 2.5×10⁵ cells ml⁻¹ with anti-CD3/CD28 coated beads (Invitrogen) at a 1:1 bead-to-cell ratio in the presence or absence of the indicated cytokines. IL-1β, IL-4, IL-6, IL-12, IFN-α (Humanzyme, Chicago, IL, USA), IL-10 and IL-21 (eBioscience, San Diego, CA, USA) were all added at 10 ng ml⁻¹; IFN-β (Preprotech, Rocky Hill, NJ, USA) was added at 100 Uml⁻¹ and TGF-β (Humanzyme) was added at 5 ng ml⁻¹. Recombinant human IL-21R/Fc chimera and a mouse IgG1/Fc isotype control (R&D Systems, Minneapolis, MN, USA) were added at 5 µg ml⁻¹. After 4 days, supernatants were collected for measurement of IL-5, IL-9, IL-10 and IL-13 by ELISA, performed according to the manufacturer's instructions.

Intracellular cytokine staining

After 4-day cultures, beads were magnetically removed and cells were re-stimulated with 25 ng ml⁻¹ PMA and 750 ng ml⁻¹ Ionomycin (Invitrogen) in the presence of Brefeldin A (eBioscience) for 4 h. Cells were then stained with a LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) according to the manufacturer's protocol, fixed with 1.6% paraformaldehyde and permeabilized with BD Perm/Wash buffer (BD Bioscience). Subsequently, cells were stained with fluorescently labeled Abs against IL-4, IL-5, IL-13, IL-9, IL-17 or IL-21 (eBioscience). For intracellular staining of transcription factors, beads were magnetically removed after 3 days of culture, and stained for viability as described above. Cells were then fixed and permeabilized (eBioscience) and stained with fluorescently labeled antibodies against GATA-3 (BD Bioscience) or Foxp3 (eBioscience). Labeled cells were analyzed on the BD LSRII (BD Bioscience), and FACS plots were further analyzed by FlowJo (Treestar, Ashland, OR, USA). All FACS plots shown are gated on singlets and live cells.

ELISA and statistical analysis

ELISA antibody pairs for IL-5 and IL-10 were obtained from eBioscience, while IL-9 and IL-13 antibody pairs were obtained from BD Bioscience. Samples were run in duplicate, and data and statistics were analyzed with GraphPad Prism (La Jolla, CA, USA). The Wilcoxon rank-sum test was used to analyze pairwise comparisons between conditions. For analysis of decreasing linear trends, repeated measures one-way analysis of variance was used with a post-test for linear trend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by 5 U19 AI050864, 1 U19 AI082719, NHLBI Proteomics contract N01-HV-28183, NIH grant DK082537, and a gift from the Floren Family Foundation and the Ben May Trust. MTW was funded by a Mason Case Fellowship. JJY was funded by the SIMR Summer Research Program. AL and RKC were funded by fellowships from the National Science Foundation. MNA was funded by an NIH Fellowship (NRSA) F31 CA142272. We would like to thank members of the Utz laboratory for helpful discussions and Nicole Hanick Kattah and Mike Kattah for thoughtful review of the paper. We would also like to thank Chris Heid (Humanzyme) for providing many of the cytokines used in this study.

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Figure 1. TGF- β in combination with IL-4 induces IL-9 production from human naïve CD4⁺ T cells. Naïve CD4⁺ T cells were activated with anti-CD3/CD28 coated beads in the presence or absence of the indicated cytokines for 4 days. **(a)** ELISA of IL-5, IL-10, IL-13 and IL-9 in cell-free supernatants. Data (mean and s.e.m.) are from four independent experiments with six donors, * $P < 0.05$, ** $P < 0.01$ as compared with all other conditions. **(b)** Intracellular expression of IL-4 and IL-9 after restimulation with PMA and ionomycin in the presence of Brefeldin A (BFA) for an additional 4 h. Data are representative of three independent experiments.

Figure 2.

Transcriptional analysis of IL-9-producing cells. Naïve CD4⁺ T cells were activated with anti-CD3/CD28 coated beads in the presence or absence of the indicated cytokines for 3 days. **(a, b)** Intracellular expression of Foxp3, IL-9 **(a)** and GATA-3 **(b)** after restimulation with PMA and ionomycin in the presence of Brefeldin A (BFA) for an additional 4 h. **(c)** Cells cultured under Th9-polarizing conditions (TGF- β +IL-4) were analyzed for GATA-3 expression (right panel) after gating on IL-9⁻ and IL-9⁺ cells (left panel). Data are representative of three independent experiments.

Figure 3.

Regulation of human Th9 differentiation by inflammatory cytokines. Naïve CD4⁺ T cells were cultured under Th9-polarizing conditions and the indicated cytokines for 4 days. **(a)** ELISA of IL-9 in cell free supernatants. Data (mean and s.e.m.) are from five independent experiments with seven donors, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with the Th9 condition. **(b)** Cells were cultured under Th9-polarizing conditions in the presence or absence of increasing concentrations of IFN- γ (left panel) or IL-27 (right panel). ELISA of IL-9 (mean and s.e.m.) are from three independent experiments with three donors, ** $P < 0.01$ for decreasing linear trend by repeated-measures one-way analysis of variance (ANOVA) with post-test for linear trend.

Figure 4.

Type I IFNs induce elevated levels of intracellular IL-21. Naïve CD4⁺ T cells were cultured under Th9-polarizing conditions and the indicated cytokines. **(a)** After 4 days, cells were restimulated with PMA and ionomycin in the presence of Brefeldin A (BFA) for an additional 4 h and stained for intracellular expression of IL-9 and IL-21. Data are representative of five independent experiments. **(b)** Percentage of IL-9+ (left panel) or IL-21+ (right panel) cells as measured by FACS. Data (mean and s.e.m.) are from five independent experiments with five donors.

Figure 5.

IL-21 is required for type 1 IFN enhancement of human Th9 differentiation. Naïve CD4⁺ T cells were cultured under Th9-polarizing conditions and the indicated cytokines in the presence of an isotype control (white bars) or an IL-21 receptor-Fc fusion protein (black bars) for 4 days. **(a)** ELISA measuring IL-9 from cell-free supernatants. Data (mean and s.e.m.) are from three independent experiments with four donors. **(b)** Intracellular expression of IL-9 and IL-21 after restimulation with PMA and ionomycin in the presence of Brefeldin A (BFA) for an additional 4 h. Data are representative of three independent experiments.

Figure 6.

Characterization of IL-9 production in memory CD4⁺ T cells. **(a)** Freshly isolated memory CD4⁺ T cells were stimulated with PMA and ionomycin in the presence of Brefeldin A (BFA) for 4 h and stained for intracellular expression of IL-4, IL-5, IL-13 and IL-9. Data are representative of three independent experiments. **(b)** Unfractionated memory CD4⁺ T cells (bulk memory) or memory T cells sorted according to CRTH2 expression were stimulated with PMA and ionomycin for 18 h. Cell-free supernatants were analyzed for expression of IL-4, IL-5, IL-13 and IL-9 by ELISA. Data (mean and s.e.m.) are from three independent experiments with three donors. **(c)** Memory CD4⁺ T cells were activated with anti-CD3/CD28 coated beads and the indicated cytokines for 4 days. Cells were restimulated with PMA and ionomycin in the presence of Brefeldin A (BFA) for an additional 4 h and stained for intracellular expression of IL-4 and IL-9. Data are representative of three independent experiments.