Elevated IL-17 produced by T_H17 cells promotes myeloma cell growth and inhibits immune function in multiple myeloma

Rao H. Prabhala,[1-3] Dheeraj Pelluru,[3] Mariateresa Fulciniti,[3] Harsha K. Prabhala,[4] Puru Nanjappa,[3] Weihua Song,[3] Christine Pai,[1] Samir Amin,[3] Yu-Tzu Tai,[3] Paul G. Richardson,[3] Irene M. Ghobrial,[3] Steven P. Treon,[3] John F. Daley,[3] Kenneth C. Anderson,[2,3] Jeffery L. Kutok,[2] and Nikhil C. Munshi[1-3]

[1]Veterans Administration Boston Healthcare System, West Roxbury, MA; [2]Brigham & Women's Hospital, Boston, MA; [3]Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA; and [4]MD/PhD Program, University of Virginia Medical School, Charlottesville

Elevated cytokines in bone marrow (BM) micro-environment (interleukin-6 [IL-6], transforming growth factor-beta [TGF-β], and IL-1β) may play an important role in observed immune dysfunction in multiple myeloma (MM). As IL-6 and TGF-β are important for the generation of T-helper 17 (T_H17) cells, we evaluated and observed a significantly elevated baseline and induced frequency of T_H17 cells in peripheral blood mononuclear cells (PBMCs) and BM mononuclear cells (BMMCs) from MM patients compared with healthy donors. We observed significant increase in levels of serum IL-17, IL-21, IL-22, and IL-23 in blood and BM in MM compared with healthy donors. We also observed that myeloma PBMCs after T_H17 polarization significantly induced IL-1α, IL-13, IL-17, and IL-23 production compared with healthy donor PBMCs. We next observed that IL-17 promotes myeloma cell growth and colony formation via IL-17 receptor, adhesion to bone marrow stromal cells (BMSCs) as well as increased growth in vivo in murine xenograft model of human MM. Additionally, we have observed that combination of IL-17 and IL-22 significantly inhibited the production of T_H1-mediated cytokines, including interferon-γ (IFN-γ), by healthy donor PBMCs. In conclusion, IL-17–producing T_H17 cells play an important role in MM pathobiology and may be an important therapeutic target for anti-MM activity and to improve immune function. (*Blood*. 2010;115(26):5385-5392)

Introduction

A significant impairment of T-cell function is observed in patients with monoclonal gammopathy of undetermined significance and multiple myeloma (MM).[1] Both phenotypic and functional aberrations in CD4 and CD8 cells have been described.[2] Although recent studies show that T cells from MM patients contain defective T-cell receptor variable repertoire[3] and impaired viral-specific cytotoxic T lymphocyte, particularly against influenza and Epstein-Barr virus,[4] presence of clonal CD4 and CD8 cells has been observed in MM.[5,6] The significance of the presence of these clonally expanded T cells in MM patients is not well understood; however, this phenomenon is associated with better prognosis. It is presumed that these expanded T cells could play a role in controlling tumor cell growth and survival.[7] Increased hyper-reactive T cells are observed in myeloma that is associated with impaired TCR signaling and increased sensitivity to costimulatory signals.[8]

CD4 helper T lymphocytes are important in both cell-mediated and antibody-mediated immune responses.[9] T-helper 17 (T_H17) cells, a new CD4 subset, are differentiated in the presence of, interleukin-6 (IL-6), IL-1β, IL-21, and IL-23 with or without transforming growth factor-beta (TGF-β) and produce IL-17 and IL-22. These cytokines protect against fungal and parasitic infections; and participates in inflammatory reactions and autoimmunity.[10-18] Activated T_H17 cells produce most of the IL-17 but CD8 cells, γδ T cells, natural killer cells, natural killer T cells, and neutrophils also produce variable amounts of IL-17.[19,20] IL-17 is a structural homologue of cystine knot family of proteins with intrachain disulfide bonds.[21] It is closely related to TGF-β, nerve growth factor, bone morphogenetic protein and platelet-derived growth factor with similar structural motifs. IL-17 induces expression of a number of chemokines and cytokines[22-27] including IL-6, TGF-β, granulocyte-colony stimulating factor or granulocyte, macrophage-colony stimulating factor; matrix metalloproteinase and intercellular adhesion molecule-1 in a variety of cell types, including the BM stromal cells. A number of molecules influence IL-17 production including, prostaglandin E2,[28] granulocyte, macrophage-colony stimulating factor,[29] and aryl hydrocarbon receptor.[13] In addition to c-Jun-N-terminal kinase, and MAP kinase kinase, a number of additional signaling molecules play a role in IL-17 production including, caspase recruitment domain-containing-9,[30] nucleotide-binding oligomerization domain-containing-2,[31] interferon regulatory factor and 4.[32] Even though Janus kinase/signal transducer and activator of transcription (STAT) pathway is critical in most of T_H17-related cytokine-mediated effects, STAT-3 mutations are shown to be detrimental for IL-17 production.[33] suppressor of cytokine signaling-3 inhibits T_H17 cell differentiation by inhibiting IL-23–mediated signaling.[34]

A significant body of information has emerged supporting a critical role for immune cells and associated cytokines in MM pathobiology as well as observed immune dysregulation in MM. IL-6, TGF-β, and IL-1β have been implicated in this process.[35] IL-6 and TGF-β both have been reported to enhance the generation

of T_H17 cells, and are differentiated by number of inflammatory cytokines including, IL-21, IL-22, IL-23, and IL-27. In addition, there is also epidemiologic data that suggest several fold higher incidence of myeloma in patients with autoimmune diseases including ulcerative colitis as well as rheumatoid arthritis.[36] Therefore, we have here evaluated the role of T_H17 cells and associated pro-inflammatory cytokines in myeloma. We demonstrate that T_H17 cells and IL-17 are elevated in myeloma; and IL-17 it promotes myeloma cell growth both in vitro and in vivo via IL-17 receptors (IL17R), and inhibits T_H1 responses.

Methods

Patient samples

Both peripheral blood and BM samples were collected from newly diagnosed myeloma patients, and from patients without treatment for at least 3 months, after informed consent in accordance with the Declaration of Helsinki and approval by the institutional review board (IRB) at Dana-Farber Cancer Institute. Healthy donor samples were obtained from the blood donor center at Children's Hospital, Boston, MA. Normal donor bone marrow samples were obtained from AllCells.

Intracellular IL-17–producing T_H17 cell analysis by flow cytometry

To evaluate baseline frequency of T_H17 cells, PBMCs and BMMCs isolated from healthy donors and myeloma patients were stimulated with phorbol 12-myristate13-acetate (PMA) and ionomycin for 6 hours in the presence of Golgi stop (eBioscience) as described previously.[14,15] After washing, cells were fixed with fix/perm buffer (Beckman Coulter) and stained for CD4 (eBioscience). Intracellular staining for interferon-γ (IFN-γ; BD Biosciences) and IL-17 (eBioscience) were performed using conjugated anti–IFN-γ (BD Biosciences) and anti–IL-17 (eBioscience) antibodies and analyzed using Canto II flow cytometer (BD Biosciences). To evaluate induced T_H17 cells, naive CD4 cells purified from negatively selected CD4 population using CD45RA micro beads (Miltenyi Biotec) were polarized with T_H17-polarizing cocktail consisting of IL-1 (10 ng/mL), IL-6 (20 ng/mL), and IL-23 (10 ng/mL) in addition to anti–IFN-γ (10 µg/mL), anti–IL-4 (10 µg/mL) TGF-β (1 ng/mL; R&D Systems) and anti-CD3 and anti-CD28 antibodies for 6 days in RPMI 1640 supplemented with 10% fetal bovine serum (FBS). Cells were further expanded with IL-2 (20 U/mL; R&D Systems) for additional 6 days prior to restimulation with PMA and ionomycin and staining with anti–IL-17 and anti–IFN-γ antibodies described above in this paragraph. The purified naive CD4 cells were positive for CD45RA in more than 95% cells by flow cytometry.

To evaluate induced T_H1 cells, healthy donor PBMCs were polarized with T_H1-polarizing cocktail consisting of IL-12 (10 ng/mL; R&D Systems) and anti-CD3 antibodies for 6 days in RPMI 1640 supplemented with 10% FBS. Cells further expanded with IL-2 (20 U/mL; R&D Systems) for additional 6 days prior to restimulation with PMA and ionomycin and staining with anti–IFN-γ antibodies described in the previous paragraph. In these experiments activated cells were stained with CD4 and CD69 prior to intracellular staining of IFN-γ.

Quantitative PCR for IL-17 and IL-17R expression

After polarization, RNA from CD4 cells in case of IL-17 and from primary myeloma cells purified with CD138 micro-beads (Miltenyi Biotec) in case of IL-17R was isolated using the RNeasy kit according to the manufacturer's instructions (QIAGEN). Reverse transcription of RNA to cDNA was prepared using the RETROscript kit (Ambion) according to the manufacturer's protocol. Quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed using the Applied Biosystems 7500 apparatus with the SYBR Green PCR master mix (Applied Biosystems) according to manufacturer's suggestions. Primer pairs were used as previously described.[15]

Myeloma cell-proliferation assays

Seven different myeloma cell lines (RPMI 8226, KMS-12BM, OPM-1, OPM-2, INA-6, U226, and H929) were cultured in RPMI 1640 supplemented with 10% FBS for 3 days in the presence or absence of IL-17. Proliferation was assessed by 3_H-thymidine incorporation over 6 hours. Colony forming assay was performed using MethoCult agar media (StemCell Technologies) in the presence or absence of IL-17 for 3 weeks. Primary MM cells were purified from bone marrow mononuclear cells by positive selection with CD138 micro-beads (Miltenyi Biotec), according to the manufacturer's instructions. BMMCs were isolated using Ficoll-Hypaque density gradient sedimentation from BM aspirates from MM patients after informed consent and IRB (Dana-Farber Cancer Institute) approval. BMMCs were cultured in RPMI 1640 supplemented with 20% FBS (4-8 weeks) to establish bone marrow stromal cells. MM cell lines were cultured in RPMI 1640 supplemented with 10% FBS. After overnight culturing of BMMCs, MM cell lines were cocultured in the presence or absence of IL-17. These experiments were also performed with or without anti–IL-17 receptor antibody.

Adhesion assay

Cell adhesion assay was done as previously described.[37] In brief, serum-starved MM cells (5×10^6/mL) were labeled with calcein (Molecular Probes) for 30 minutes at 37°C, washed, and resuspended in culture medium. Cells were added to bone marrow stromal cell (BMSC)–coated 96-well plates, treated with or without IL-17, and incubated at 37°C for 4 hours. Unbound cells were removed by 4 washes with RPMI 1640 complete medium. The absorbance of each well was measured using 492/520 nm filter set with a fluorescence plate reader (Wallac VICTOR2; Perkin-Elmer).

Myeloma murine xenograft model

Six- to 8-week-old male CB-17 severe combined immunodeficient (SCID) mice (Taconic) were housed and monitored in the VA Boston Healthcare System Animal Research Facility. All experimental procedures and protocols had been approved by the Institutional Animal Care and Use Committee. Five million OPM-1 MM cells treated with or without IL-17 (R&D Systems) at 100 ng/mL concentration were injected subcutaneously into SCID mice and tumor volumes were measured at 3 weeks as described previously.[38]

Cytokine measurements using ELISA

Serum samples were collected from blood and bone marrow after informed consent approved by the IRB from Dana-Farber Cancer Institute and stored at −80°C until used. T_H17-associated cytokines were measured using multiplex luminex assay (Luminex Corp) for IL-17 and standard enzyme-linked immunosorbent assay (ELISA) kits for IL-22 (R&D Systems) and IL-21 and IL-23 (eBiosciences). Supernatants collected after T_H17 polarization were analyzed using Quansys multiplex ELISA assay. Measurements for IFN-γ were performed using standard commercial ELISA kits (R&D Systems).

Western blotting

Myeloma cell lines were grown in RPMI-1640 supplemented with 10% FCS (FBS; Sigma Chemical), 2µM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (GIBCO). Cells were pelleted at 500*g* for 5 minutes at 4°C, washed with ice-cold phosphate-buffered saline twice, and resuspended in 50 µl CelLytic M (Sigma-Aldrich) supplemented with complete mini ethylenediaminetetraacetic acid-free protease inhibitor cocktail (Roche Diagnostics) and 25mM N-Ethylmaleimide (Sigma-Aldrich). Cells were pipetted up and down thrice, rotated for 15 minutes at 4°C, centrifuged at 12 000*g* for 15 minutes and supernatant was then removed and used as total cell lysate.

Samples were separated by electrophoresis on Supersep Ace 5%-20% gradient gels (Wako). Gels were transferred to nitrocellulose membranes (Invitrogen), blocked with 5% carnation nonfat dry milk (Nestle) in tri-buffered saline supplemented with 0.5% Tween 20 (Sigma-Aldrich) for
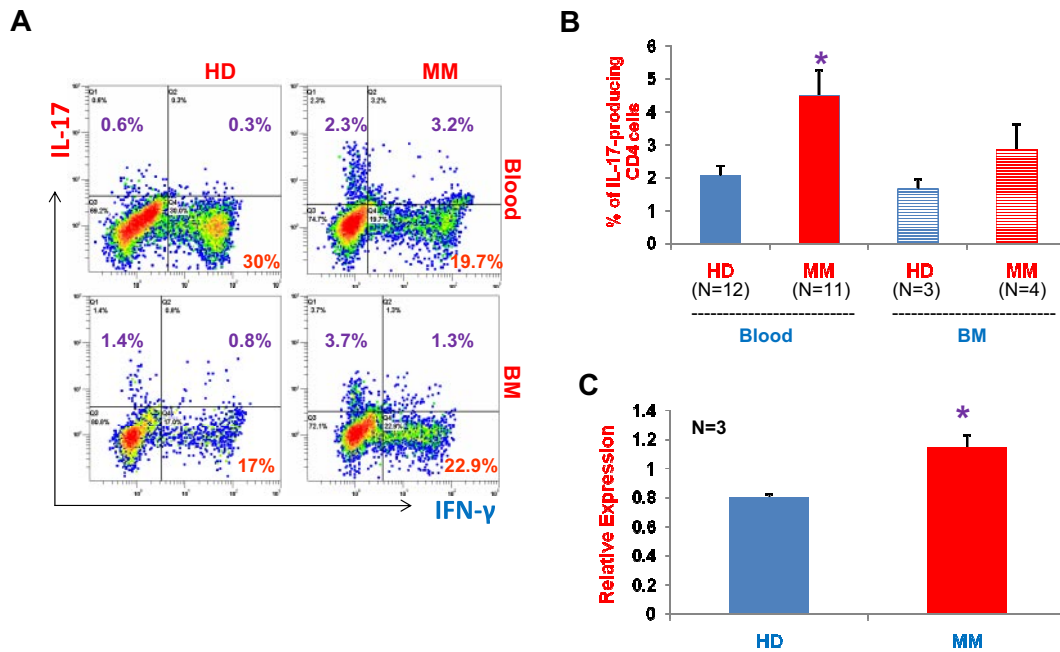
Figure 1. Increased frequency of T$_H$17 cells in freshly isolated mononuclear cells in myeloma. (A) Mononuclear cells were isolated from multiple myeloma (MM) patients (blood, N = 11, bone marrow [BM], N = 4) and from healthy donors (HDs; blood, N = 12; BM, N = 3) and stimulated for 6 hours with PMA and ionomycin and stained for intracellular interleukin-17 (IL-17) and interferon-γ (IFN-γ). Proportion of IL-17–producing T$_H$17 cells was determined in CD4 population by flow cytometry. A representative dot plot analysis showing percentage of cells that are positive for intracellular IL-17 and IFN-γ within gated CD4 population using matching peripheral blood and BM samples from MM and HDs is shown. (B) Composite results presented as mean value with SEM for T$_H$17 with in CD4 population. (C) RNA was isolated from CD4 cells from MM patients (N = 3) and healthy donors (N = 3) using QIAGEN kits and quantitative PCR was performed. *P < .05.

1 hour and then incubated with anti–IL-17 receptor antibody used at final concentration of 1:1000 and anti-glyceraldehydes 3-phosphate dehydrogenase antibody used at final concentration of 1:5000, (Santa Cruz Biotechnology) for overnight at 4°C. Secondary antibodies were goat anti–rabbit IgG-horseradish peroxidase and goat anti–mouse IgG-horseradish peroxidase (Santa Cruz Biotechnology) were used at a final concentration of 1:2000 for 1 hour at room temperature. Immunoblots were developed using the enhanced chemiluminescence (ECL) reagent system from GE Healthcare Bio-Sciences and Kodak BioMax MR Film.

Immunohistochemistry

Immunohistochemistry was performed using 5m-thick zenker-fixed, paraffin-embedded tissue sections. Briefly, slides were soaked in xylene, passed through graded alcohols, and put in distilled water. Slides were then pretreated with 10-mM citrate, pH 6.0 (Zymed) in a steam pressure cooker (Decloaking Chamber; BioCare Medical) per manufacturer's instructions, followed by washing in distilled water. All further steps were performed at room temperature in a hydrated chamber. Slides were pretreated with Peroxidase Block (DAKO USA) for 5 minutes to quench endogenous peroxidase activity. Primary rabbit anti–IL-17R antibody (Santa Cruz Biotechnology) was applied at a 1:50 dilution in antibody diluent (DAKO) for 1 hour. Slides were washed in 50mM Tris-Cl, pH 7.4, and anti–rabbit horseradish peroxidase–conjugated antibody (Envision detection kit; DAKO) was applied for 30 minutes. After further washing, immunoperoxidase staining was developed using a DAB chromogen kit (DAKO) per the manufacturer and counterstained with Harris hematoxylin. Sections were observed and photographed with an Olympus BX41 microscope with 40× objective lens and 0.75 numeric aperture at room temperature (total magnification 200×) and an Olympus-QColor 5 camera, and analyzed with acquisition software QCapture (QImaging) and Adobe Photoshop (Adobe).

Confocal microscopy

Myeloma cell lines were stained with anti–IL-17 receptor antibodies in addition to isotype antibody controls (phycoerythrin) and then analyzed using multiphoton microscopy at room temperature (BioRad MRC 1024ES multiphoton system; Bio-Rad). A Zeiss Axiovert S 100 inverted microscope

equipped with a high-quality water-immersion 40×/1.2 numeric aperture C-Apochromat objective was used to obtain images (total magnification, 640×). Images were reconstructed using the Bio-Rad LaserSharp and/or MetaMorph software (MetaMorph Imaging Series; Universal Imaging).

Statistical analysis

Statistical analyses were performed by Student t test as well as Mann-Whitney test. P values less than .05 were considered statistically significant.

Results

Presence of elevated T$_H$17 cells in freshly isolated mononuclear cells from myeloma

Significant dysregulation in T-helper cell subsets, T$_H$1, T$_H$2, and T regulatory cells, have been reported in myeloma. Some of these effects are mediated by the cytokines produced by myeloma cells and/or bone marrow stromal cells. Because these cytokines, especially IL-6, TGF-β, and IL-1 also impact generation of T$_H$17 cells, we first evaluated the baseline frequency of T$_H$17 cells in freshly isolated PBMCs and BMMCs from myeloma patients and compared it with its frequency in healthy donor PBMCs and BMMCs. Freshly isolated PBMCs and BMMCs were stimulated for 6 hours with PMA and ionomycin and stained for intracellular IL-17 and IFN-γ and analyzed by flow cytometry. The mean frequency of T$_H$17 cells was 4.49% (± 0.78%) in MM PBMCs compared with 2.05% (± 0.3%) in healthy donor (HD) PBMCs (P < .05) and 2.85% (± 0.78%) in MM BMMCs compared with 1.67% (± 0.29%) in HD BMMCs (Figure 1B). As seen in Figure 1A and B, significantly increased frequency of T$_H$17 cells was observed in PBMCs (N = 11) from MM patients compared with HD PBMCs (N = 12); however, the increase of T$_H$17 cells seen in BMMCs (N = 4) from MM patients compared with HD BMMCs
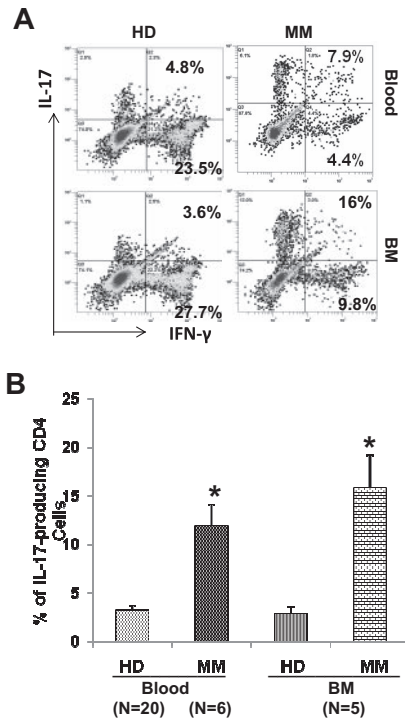
Figure 2. Increased frequency of induced T_H17 cells in myeloma. Purified naive CD4 cells from peripheral blood mononuclear cells (PBMCs) and bone marrow mononuclear cells (BMMCs) were polarized with T$_H$17 cocktail for 12 days. After restimulation with PMA and ionomycin, IL-17–expressing cells were measured by intracellular IL-17 staining using flow cytometry. (A) A representative dot plot analysis shows IL-17– and IFN-γ–expressing cells as percentage of CD4 cells in peripheral blood and BM samples from HD and myeloma (MM) patients. (B) Composite results presented as mean values with SEM for IL-17–expressing cells within CD4 population. *$P < .05$.
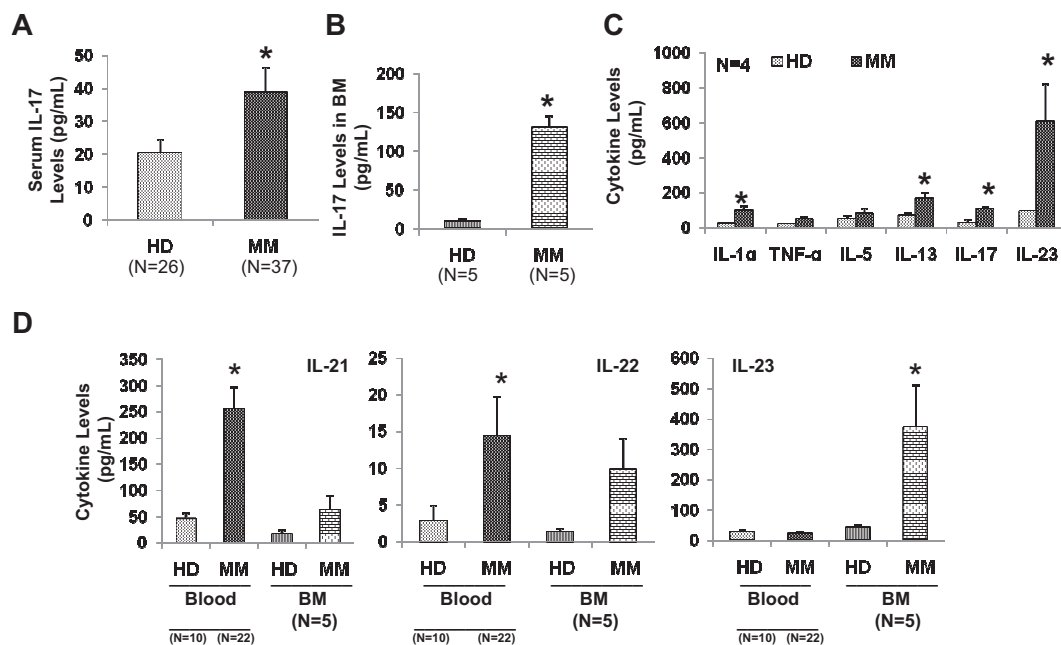
compared with healthy donor PBMCs (data not shown). We have also confirmed the increased frequency of IL-17–producing cells in CD4 cells from MM compared with HD by qPCR (Figure 1C).

Increased number of induced-T$_H$17 cells in myeloma

Next, we evaluated the frequency of induced T$_H$17 cells under T$_H$17 polarizing conditions. Cultures in polarizing condition determine the potential for further increase in T$_H$17 cells. To determine the frequency of induced T$_H$17 cells, we stimulated naive CD4 cells with anti-CD3 and anti-CD28 antibodies, in the presence of IL-6, IL-1, IL-23, and TGF-β for 6 days. Cells were further expanded with IL-2 for 6 additional days, restimulated with PMA and Ionomycin, and analyzed by flow cytometry for percentage of cells expressing intracellular IL-17 in the CD4 population. As seen in Figure 2, there was significant increase in induced T$_H$17 cells after culture in polarizing condition from MM compared with healthy donor PBMCs (11.9% ± 2.2% and 3.2% ± 0.5%, respectively; $P < .05$, N = 6) and BMMCs (15.8% ± 3.4% and 2.9% ± 0.7%, respectively; $P < .05$, N = 5). The starting frequency of T$_H$17 cells in purified naive CD45RA positive cells is very low and similar in all groups of samples.

Elevated T$_H$17-associated cytokines in myeloma microenvironment

We next analyzed the pro-inflammatory cytokine network supporting the generation of T$_H$17 cells in myeloma. We observed significantly elevated levels of serum IL-17 in myeloma patients (N = 37) compared with healthy donor sera (N = 26; 39 + 7 vs 20.5 ± 3.9, respectively; $P < .05$) as measured by multiplex luminex assay (Figure 3A). We also observed significant elevation of IL-17 in sera from BM from MM compared with healthy donors (Figure 3B). Furthermore, we evaluated the supernatants from PBMCs cultured in presence of T$_H$17 polarizing conditions from myeloma patients and healthy donors (N = 4) using multiplex

(N = 3), although higher, is not statistically significant. Furthermore, we observed that both IL-17+/IFN-γ+ as well as IL-17$^+$/IFN-γ$^-$ cell ratios are significantly increased in MM PBMCs

Figure 3. Elevated levels of T$_H$17-related cytokines in myeloma. Sera samples from myeloma patients (MM) and from healthy donors (HD) were analyzed by ELISA for the presence of IL-17 in (A) peripheral blood, (B) IL-17 in BM, and (D) IL-21, IL-22, and IL-23 in blood and BM by ELISA. (C) PBMCs isolated from myeloma patients (N = 4) and healthy donors (N = 4) were stimulated with anti-CD3 antibody in the presence of IL-6 and TGF-β for 6 days and cell supernatant was evaluated for various cytokines by multiplex ELISA assay. All the values presented in the bar graphs consist of mean plus or minus SEM. *$P < .05$.
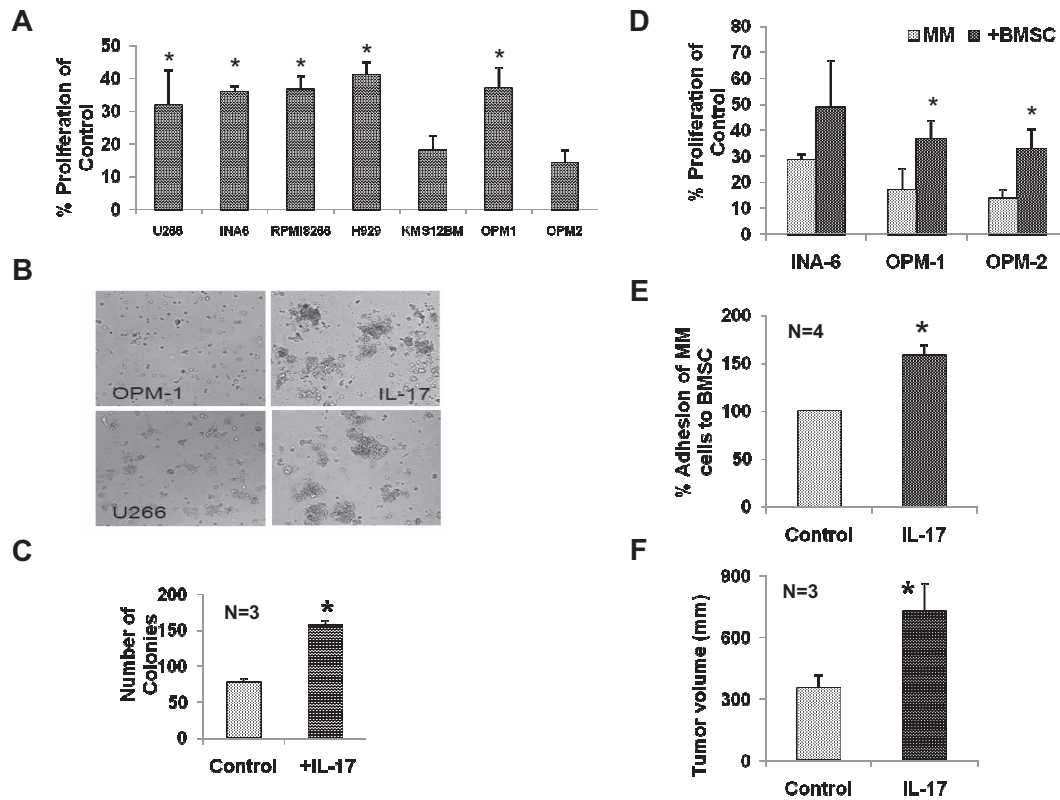
Figure 4. IL-17 promotes myeloma cell growth in vitro and in vivo. (A) Myeloma cell lines (N = 7) were incubated with or without IL-17 and proliferation was measured by ^{3}H-thymidine incorporation after 3 days. Data are presented as percentage increase in proliferation in presence of IL-17 compared with control and showed as mean plus or minus SEM. (B) Myeloma cell lines (OPM-1 and U266) were cultured in methocult agar plates in the presence or absence of IL-17. Representative photomicrograph is presented. Photographs were obtained using a Nikon TE200 microscope (40× objective) with attached camera (Nikon) at room temperature (total magnification 200×) and analyzed with Metafluor software (Molecular Devices). (C) Primary MM cells (N = 3) were cultured in methocult agar plates in the presence or absence of IL-17 and number of colonies was counted in unit area and presented as mean plus or minus SEM. (D) MM cell lines were cultured with or without BMSCs in the presence or absence of IL-17 and proliferation was increased as measured by ^{3}H-thymidine incorporation after 3 days and presented as percentage of proliferation of control. (E) Serum-starved MM cells were labeled with calcein, washed, and added to BMSC-coated plates for 4 hours and nonadherent cells were removed by washing. Adhesion was measured by measuring the absorbance using 492/520 nm filter set with a fluorescence plate reader. Results represent mean plus or minus SEM of 4 independent experiments performed in triplicate. (F) Myeloma cells suspended in medium with or without IL-17 were injected subcutaneously in SCID mice (3 mice per group), and tumor size was measured after 3 weeks after MM cell injection. *$P < .05$.

ELISA assay (Quansys). As shown in Figure 3C, IL-1α, IL-13, IL-17, and IL-23 were significantly elevated in supernatants from PBMCs from myeloma patients compared with healthy donor samples. In addition, we analyzed the levels of other pro-inflammatory cytokines that are important in relationship to T$_H$17 cells in sera of myeloma patients. We observed significant elevation of IL-21, IL-22, and IL-23 in sera from both blood and BM from MM compared with healthy donors. These results suggest that T$_H$17-associated pro-inflammatory cytokines may be present in the myeloma BM microenvironment and may modulate MM cell growth as well as immune responses.

IL-17 promotes myeloma cell growth both in vitro and in vivo

We next evaluated effect of IL-17 on myeloma cell growth and survival in vitro. MM cell lines (N = 7) were incubated in the presence or absence of IL-17 and cell proliferation was measured by ^{3}H-thymidine incorporation. As seen in Figure 4A, IL-17 significantly induced proliferation of all myeloma cell lines tested (30.7% ± 2.8%; Figure 4A). In addition, we also observed that in both myeloma cell lines and primary patient MM cells, IL-17 significantly induced colony size and number as observed by methoCult colony assay (Figure 4B and C, respectively). We also observed increased number of MM cells in the S phase and reduced number of cells in G$_1$ phase (data not shown). As seen in Figure 4D, we showed the induction of proliferation in

3 myeloma cell lines by IL-17 even in presence of BMSCs, as measured by ^{3}H-thymidine incorporation (range, 32.6% to 48.6%). We also observed that IL-17 significantly increased adhesion of myeloma cells to BMSCs (Figure 4E). We next evaluated whether IL-17 promotes myeloma cell growth in vivo in a murine xenograft model of MM. We subcutaneously injected OPM-1 myeloma cells with and without IL-17 pretreatment and evaluated the tumor growth after 3 weeks after MM cell injection. As seen in Figure 4F, IL-17 pretreatment led to development of significantly larger tumors compared with control ($P < .05$).

Significant expression of IL-17 receptor in myeloma cells

We have observed significant up-regulation of myeloma cell growth by IL-17, both in vitro and in vivo. Therefore, we further investigated whether MM cells expressed IL-17R. As seen in Figure 5A, we observed by Western blot analysis the expression of IL-17R in all 3 myeloma cell lines tested. We confirmed this observation in primary myeloma cells by quantitative PCR as shown in Figure 5B. Three of 4 primary CD138$^+$ myeloma cells showed significantly higher expression of IL-17R than normal plasma cells. We have further validated IL-17R expression on primary MM cells by evaluating the paraffin-embedded bone marrow biopsy sections from myeloma patients with immune-histochemistry using anti–IL-17R antibody. As seen in Figure 5C, a majority of myeloma cells express IL-17R in 7 of 10 patient
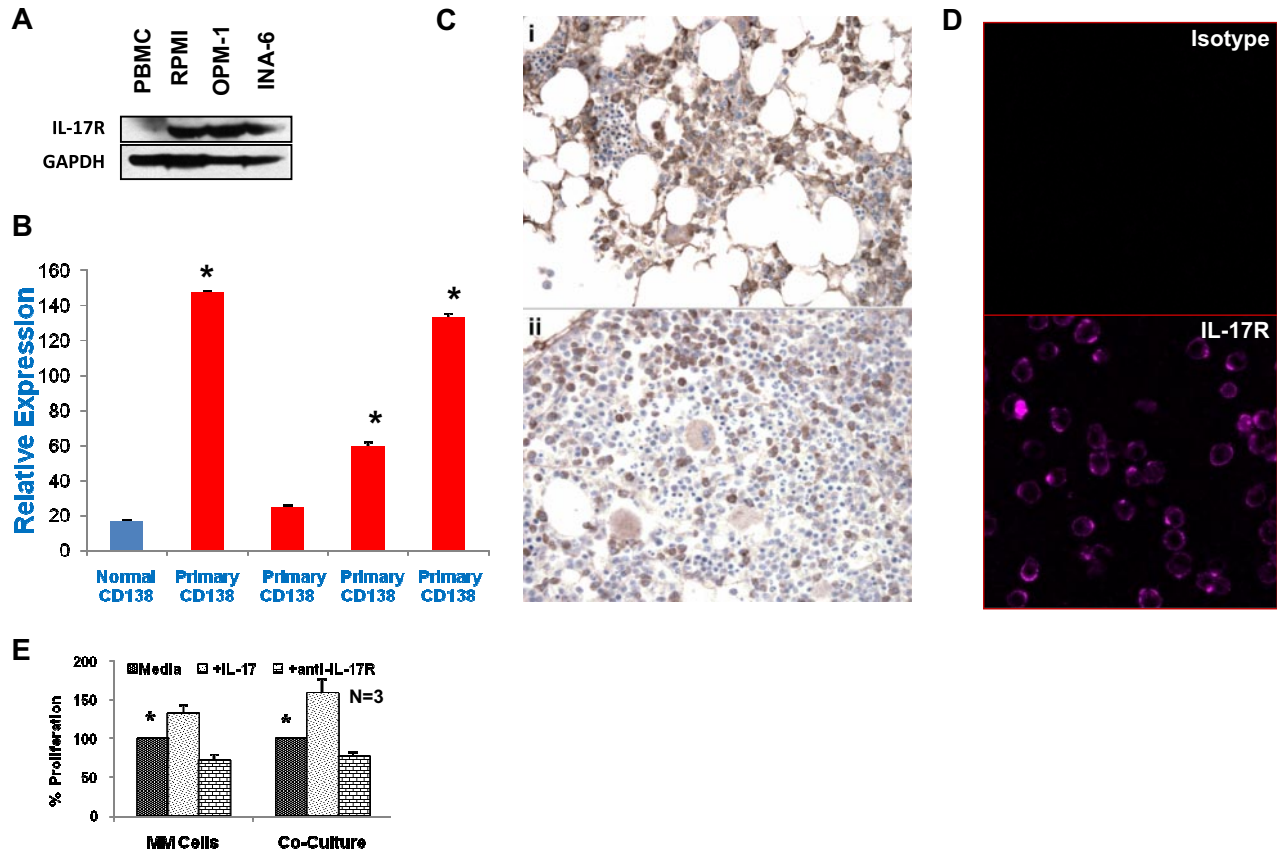
Figure 5. Significant expression of IL-17 receptor in myeloma cells. (A) Total cell lysates were prepared from MM cells, and separated by electrophoresis on 5% to 20% polyacrylamide gradient gels. Samples were probed with antisera to IL-17 receptor and GAPDH as indicated. (B) RNA was isolated from purified primary MM cells and qPCR was performed as described in "Quantitative PCR for IL-17 and IL-17R expression." Results are presented as relative expression value. *$P < .05$. (C) Paraffin-embedded tissue sections from MM patients (N = 10) were stained using anti–IL-17R antibody as described in "Immunohistochemistry" and evaluated using a Nikon transmitted light microscope. The majority of the MM cells are positively stained with IL-17R receptor antibody in 7 of 10 patients. Two representative stained sections are shown at 200× magnification. (D) Myeloma cell lines were stained with isotype control antibody (top panel) or anti–IL-17R antibody (bottom panel) and analyzed by confocal microscopy. One representative cell line of 4 experiments is shown at 640× magnification. (E) MM cell lines (N = 3) were cultured alone or cocultured with BMSCs with or without IL-17 in the presence or absence of anti–IL-17R antibody. Proliferation was measured by ^{3}H-thymidine incorporation after 3 days. Data are presented as percentage proliferation in the presence of IL-17 or IL-17R antibody compared with control and shown as mean plus or minus SEM. *$P < .05$.

samples tested; while 2 patients showed less frequent staining of plasma cells and 1 sample was negative for IL-17R. This finding is further confirmed with confocal microscopy using myeloma cell lines as shown in Figure 5D. We have further evaluated the role of IL-17R in IL-17–mediated MM cell proliferation using anti–IL-17R antibody. As seen in Figure 5E, presence of anti–IL-17R antibody significantly suppresses MM cell proliferation in presence of IL-17. Similarly, anti–IL-17R antibody was also able to significantly block MM cell growth in the presence of BMSCs and IL-17.

Down-regulation of TH1 cell responses by T$_H$17-secreted cytokines in myeloma

To determine the effect of cytokines produced by T$_H$17 cells (IL-17 and IL-22) on T$_H$1 cells, we incubated PBMCs isolated from healthy donors under T$_H$1 polarizing conditions for 12 days in the presence or absence of IL-17 and/or IL-22. Cells were then restimulated with PMA and ionomycin for 6 hours and intracellular IFN-γ was measured in the CD4 population. Although IL-17 and IL-22 by themselves had little effect on IFN-γ–producing cells (data not shown), significant inhibition of IFN-γ–producing cells (Figure 6A-B), as well as IFN-γ production (Figure 6C), was observed in the presence of combination of IL-17 and IL-22.
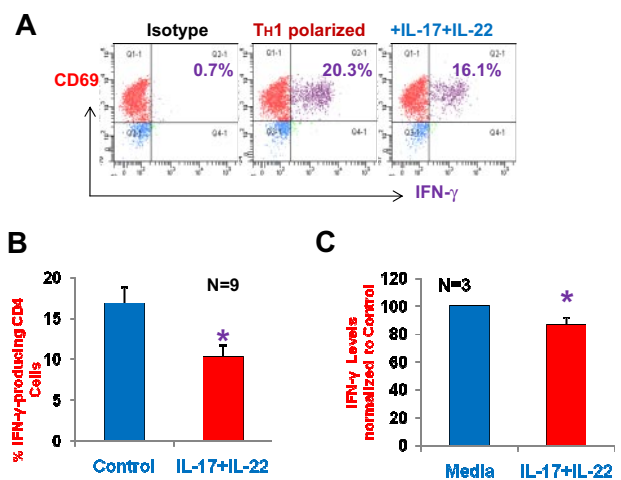
Figure 6. Down-regulation of protective T$_H$1 response by T$_H$17-related cytokines. (A) Healthy donor PBMCs were activated with T$_H$1 polarizing cytokines as described in "Intracellular IL-17–producing T$_H$17 cell analysis by flow cytometry" in presence or absence of IL-17 and IL-22 for 12 days. Cells were treated with PMA and ionomycin, stained for intracellular IFN-γ, and evaluated by flow cytometry. IFN-γ–producing cell number was evaluated in CD69+ cell population in CD4 gated cells. A representative dot plot analysis showing percentage of cells that are positive for intracellular IFN-γ within gated CD4 population. (B) Composite results of 9 experiments presented in a bar graph. Results are mean plus or minus SEM in healthy donors. (C) Healthy donor PBMCs were activated with TH1 polarizing cytokines in the presence or absence of IL-17 and IL-22 for 6 days, and supernatants were analyzed for IFN-γ by ELISA. *$P < .05$.

Discussion

T$_H$17 cells induced by IL-6, IL-1β, IL-21, and IL-23 participate in protection against fungal and parasitic infections and their levels are elevated in a number of inflammatory and autoimmune diseases.[39,40] We have shown here that T$_H$17 cells are significantly elevated in peripheral blood and bone marrow of myeloma patients compared with healthy donors. Interestingly, interactions between MM cells and the BM microenvironment lead to production of a number of cytokines and chemokines[35,41] with immuo-modulatory activity that may skew T$_H$ subsets toward T$_H$17 cells. The interplay of TGF-β and IL-6, which are both expressed at high levels in MM bone marrow,[35,41] may affect generation of T$_H$17 cells both directly and via other pro-inflammatory cytokines and thereby modulate antitumor immune responses. T$_H$17 cells and the ratios of IL-17+/IFN-γ+ cells were significantly higher in MM than in healthy donor samples. These results suggest increased frequency of T$_H$17 cells present in the total CD4 cell population consisting of both naive and memory cell pools. Furthermore, when purified naive CD4 cells were cultured under T$_H$17 polarizing conditions, T$_H$17 cells were induced in significantly higher numbers in myeloma compared with healthy donors. These results are consistent with a recent report showing increased T$_H$17 cells polarized by DCs in BM compared with peripheral blood in MM patients.[42] Increased frequency of T$_H$17 cells is also observed in tumor microenvironment in a number of human tumors, including ovarian, prostate, renal, and pancreatic carcinomas.[43-44] Some animal studies have shown that T$_H$17 cells are important in antitumor activity.[45] Interestingly, a human study has also shown that IL-17–producing T$_H$17 cells facilitate antitumor activity via enhancing the presence of T$_H$1 effectors in the tumor microenvironment of ovarian cancer patients.[46] Elevated levels of T$_H$17 cells and associated cytokines have also been documented in rheumatoid arthritis, and these pro-inflammatory cytokines also participate in bone damage observed in this disease. Interestingly, there have been reports of increased incidence of myeloma in patients with autoimmune disorders,[36] raising question regarding relation between the T$_H$17 cells and pro-inflammatory cytokines and the development of MM in patients with autoimmune disorders.

We have evaluated the serum levels of T$_H$17-associated cytokines in peripheral blood and the bone marrow of healthy donors and MM patients. Our results demonstrate that a number of T$_H$17-associated cytokines, including IL-17, are significantly elevated in myeloma compared with healthy donors. Significantly elevated IL-17 levels have been previously reported in stage II and III MM.[47] Interestingly, reduced levels of serum IL-17 were reported after bis-phophonate therapy.[48] Serum levels of IL-23, a T$_H$17-associated cytokine, observed to be elevated in MM bone marrow, is also elevated in colon, ovarian, head/neck, lung, breast, and stomach cancer as well as melanoma. This has been associated with reduced CD8 T-cell infiltration into the tumor micro-environment.[49] Consistent with these observations, we report that combination of these T$_H$17-associated pro-inflammatory cytokines suppresses T-cell responses. In addition, we have shown that a number of other T$_H$17-associated pro-inflammatory cytokines, including IL-1, IL-13, IL-17, and IL-23, are elevated after T$_H$17 polarization in myeloma compared with healthy donors. Of course, these results will now excite us to conduct larger studies to understand their role in the progression of myeloma and the relationship between disease stage and response to therapy as well as survival.

We have further demonstrated by both ^{3}H-thymidine incorporation and clonogenic assay that IL-17 increases myeloma cell proliferation. In addition, we have observed that IL-17 promotes myeloma tumor cell growth in SCID mouse model. We have further shown, using various techniques, that MM cell lines and primary cells express IL-17 receptor, providing the biologic mechanism for IL-17 effects on MM cells. As predicted, blocking IL-17R by antibody abolishes IL-17 effects on MM cells. This provides a rationale for IL-17 and IL-17R blockade as a potential therapy in MM. We are in the process of evaluating IL-17 blockade experiments in vivo. A recent study[50] shows that IL-21, a T$_H$17-associated pro-inflammatory, is also capable of inducing STAT-3–mediated myeloma growth-promoting effects in synergism with IGF-1. The biologic basis for this growth-stimulating effect remains unclear. However, based on observation in rheumatoid arthritis, we postulate that IL-17 may induce production of IL-6 in the bone marrow milieu. It will be important to evaluate the effect of these cytokines not only on MM cell growth but also on BMSCs, as well as on production of other growth-promoting cytokines and chemokines in MM. In addition, we also report that IFN-γ–producing cells are reduced when PBMCs from healthy donors are polarized in the presence of IL-17 and IL-22, suggesting immune suppressive activity of these cytokines. Because most T$_H$17 cells produce IL-17 and IL-22, we believe that the observed immune-suppression in MM may be partly induced by this pathway.

In conclusion, we observed significantly increased numbers of T$_H$17 cells in MM along with increased levels of IL-17 and other pro-inflammatory cytokines supporting MM cell growth as well as suppressing immune responses. These results suggest T$_H$17 cells and IL-17 as an important therapeutic target in MM for both anti-MM responses as well as to improve immune function.

Authorship

Contribution: R.H.P. conceived and developed the experimental plan, performed experiments, analyzed the data, and prepared the manuscript; D.P., M.F., H.K.P., P.N., W.S., C.P., S.A., J.F.D., and J.L.K. assisted in experiments; Y.-T.T., P.G.R., I.M.G., and S.P.T. provided patient samples; K.C.A. helped in data analysis and patient samples; N.C.M. participated in study design and coordination, data analysis, patient samples, and manuscript preparation; and all authors read and approved the final manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.

Correspondence: Nikhil C. Munshi, MD, Dana-Farber Cancer Institute, 44 Binney St, Boston, MA 02115; e-mail: nikhil_munshi @dfci.harvard.edu; or Rao H. Prabhala, PhD, VA Boston Health-care System/Harvard Medical School, 1400 VFW Pkwy, West Roxbury, MA 02132, e-mail: rao_prabhala@dfci.harvard.edu.

References

1. Munshi NC. Immunoregulatory mechanisms in multiple myeloma. *Hematol Oncol Clin North Am.* 1997;11(1):51-69.

2. Raitakari M, Brown RD, Gibson J, Joshua DE. T cells in myeloma. *Hematol Oncol.* 2003;21(1):33-42.

3. Mariani S, Coscia M, Even J, et al. Severe and long-lasting disruption of T-cell receptor diversity in human myeloma after high-dose chemotherapy and autologous peripheral blood progenitor cell infusion. *Br J Haematol.* 2001;113(4):1051-1059.

4. Maecker B, Anderson KS, von Bergwelt-Baildon MS, et al. Viral antigen-specific CD8+ T-cell responses are impaired in multiple myeloma. *Br J Haematol.* 2003;121(6):842-848.

5. Moss P, Gillespie G, Frodsham P, Bell J, Reyburn H. Clonal populations of CD4+ and CD8+ T cells in patients with multiple myeloma and paraproteinemia. *Blood.* 1996;87(8):3297-3306.

6. Sze DM, Giesajtis G, Brown RD, et al. Clonal cytotoxic T cells are expanded in myeloma and reside in the CD8(+)CD57(+)CD28(-) compartment. *Blood.* 2001;98(9):2817-2827.

7. Hayashi T, Hideshima T, Akiyama M, et al. Ex vivo induction of multiple myeloma-specific cytotoxic T lymphocytes. *Blood.* 2003;102(4):1435-1442.

8. Massaia M, Bianchi A, Attisano C, et al. Detection of hyperreactive T cells in multiple myeloma by multivalent cross-linking of the CD3/TCR complex. *Blood.* 1991;78(7):1770-1780.

9. Mosmann TR, Cherwinski H, Bond MW, Giedlin MA, Coffman RL. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J Immunol.* 1986;136(7):2348-2357.

10. Mangan PR, Harrington LE, O'Quinn DB, et al. Transforming growth factor-beta induces development of the T(H)17 lineage. *Nature.* 2006;441(7090):231-234.

11. Ivanov II, McKenzie BS, Zhou L, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell.* 2006;126(6):1121-1133.

12. Bettelli E, Carrier Y, Gao W, et al. Reciprocal developmental pathways for the generation of pathogenic effector T$_H$17 and regulatory T cells. *Nature.* 2006;441(7090):235-238.

13. Veldhoen M, Hirota K, Westendorf AM, et al. The aryl hydrocarbon receptor links T$_H$17-cell-mediated autoimmunity to environmental toxins. *Nature.* 2008;453(7191):106-109.

14. Acosta-Rodriguez EV, Napolitani G, Lanzavecchia A, Sallusto F. Interleukins 1beta and 6 but not transforming growth factor-beta are essential for the differentiation of interleukin 17-producing human T helper cells. *Nat Immunol.* 2007;8(9):942-949.

15. Wilson NJ, Boniface K, Chan JR, et al. Development, cytokine profile and function of human interleukin 17-producing helper T cells. *Nat Immunol.* 2007;8(9):950-957.

16. Wolk K, Kunz S, Witte E, Friedrich M, Asadullah K, Sabat R. IL-22 increases the innate immunity of tissues. *Immunity.* 2004;21(2):241-254.

17. Zheng Y, Valdez PA, Danilenko DM, et al. Interleukin-22 mediates early host defense against attaching and effacing bacterial pathogens. *Nat Med.* 2008;14(3):282-289.

18. Aujla SJ, Chan YR, Zheng M, et al. IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nat Med.* 2008;14(3):275-281.

19. Stark MA, Huo Y, Burcin TL, Morris MA, Olson TS, Ley K. Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17. *Immunity.* 2005;22(3):285-294.

20. Michel ML, Mendes-da-Cruz D, Keller AC, et al. Critical role of ROR-gammat in a new thymic pathway leading to IL-17-producing invariant NKT cell differentiation. *Proc Natl Acad Sci U S A.* 2008;105(50):19845-19850.

21. Hymowitz SG, Filvaroff EH, Yin JP, et al. IL-17s adopt a cystine knot fold: structure and activity of a novel cytokine, IL-17F, and implications for receptor binding. *EMBO J.* 2001;20(19):5332-5341.

22. Kolls JK, Linden A. Interleukin-17 family members and inflammation. *Immunity.* 2004;21(4):467-476.

23. Yao Z, Fanslow WC, Seldin MF, et al. Herpesvirus Saimiri encodes a new cytokine, IL-17, which binds to a novel cytokine receptor. *Immunity.* 1995;3(6):811-821.

24. Fossiez F, Djossou O, Chomarat P, et al. T cell interleukin-17 induces stromal cells to produce proinflammatory and hematopoietic cytokines. *J Exp Med.* 1996;183(6):2593-2603.

25. Kao CY, Huang F, Chen Y, et al. Up-regulation of CC chemokine ligand 20 expression in human airway epithelium by IL-17 through a JAK-independent but MEK/NF-kappaB-dependent signaling pathway. *J Immunol.* 2005;175(10):6676-6685.

26. Khader SA, Bell GK, Pearl JE, et al. IL-23 and IL-17 in the establishment of protective pulmonary CD4+ T cell responses after vaccination and during Mycobacterium tuberculosis challenge. *Nat Immunol.* 2007;8(4):369-377.

27. Rifas L, Arackal S. T cells regulate the expression of matrix metalloproteinase in human osteoblasts via a dual mitogen-activated protein kinase mechanism. *Arthritis Rheum.* 2003;48(4):993-1001.

28. Chizzolini C, Chicheportiche R, Alvarez M, et al. Prostaglandin E2 synergistically with interleukin-23 favors human Th17 expansion. *Blood.* 2008;112(9):3696-3703.

29. Sonderegger I, Iezzi G, Maier R, Schmitz N, Kurrer M, Kopf M. GM-CSF mediates autoimmunity by enhancing IL-6-dependent Th17 cell development and survival. *J Exp Med.* 2008;205(10):2281-2294.

30. LeibundGut-Landmann S, Gross O, Robinson MJ, et al. Syk- and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce interleukin 17. *Nat Immunol.* 2007;8(6):630-638.

31. van Beelen AJ, Zelinkova Z, Taanman-Kueter EW, et al. Stimulation of the intracellular bacterial sensor NOD2 programs dendritic cells to promote interleukin-17 production in human memory T cells. *Immunity.* 2007;27(4):660-669.

32. Kano S, Sato K, Morishita Y, et al. The contribution of transcription factor IRF1 to the interferon-gamma-interleukin 12 signaling axis and TH1 versus TH-17 differentiation of CD4+ T cells. *Nat Immunol.* 2008;9(1):34-41.

33. de Beaucoudrey L, Puel A, Filipe-Santos O, et al. Mutations in STAT3 and IL12RB1 impair the development of human IL-17-producing T cells. *J Exp Med.* 2008;205(7):1543-1550.

34. Chen Z, Laurence A, Kanno Y, et al. Selective regulatory function of Socs3 in the formation of IL-17-secreting T cells. *Proc Natl Acad Sci U S A.* 2006;103(21):8137-8142.

35. Hideshima T, Mitsiades C, Tonon G, Richardson PG, Anderson KC. Understanding multiple myeloma pathogenesis in the bone marrow to identify new therapeutic targets. *Nat Rev Cancer.* 2007;7(8):585-598.

36. Brown LM, Gridley G, Check D, Landgren O. Risk of multiple myeloma and monoclonal gammopathy of undetermined significance among white and black male United States veterans with prior autoimmune, infectious, inflammatory, and allergic disorders. *Blood.* 2008;111(7):3388-3394.

37. Tai YT, Podar K, Catley L, et al. Insulin-like growth factor-1 induces adhesion and migration in human multiple myeloma cells via activation of beta1-integrin and phosphatidylinositol 3′-kinase/AKT signaling. *Cancer Res.* 2003;63(18):5850-5858.

38. Ikeda H, Hideshima T, Fulciniti M, et al. The monoclonal antibody nBT062 conjugated to cytotoxic Maytansinoids has selective cytotoxicity against CD138-positive multiple myeloma cells in vitro and in vivo. *Clin Cancer Res.* 2009;15(12):4028-4037.

39. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. Type 17 T helper cells-origins, features and possible roles in rheumatic disease. *Nat Rev Rheumatol.* 2009;5(6):325-331.

40. Pernis AB. Th17 cells in rheumatoid arthritis and systemic lupus erythematosus. *J Intern Med.* 2009;265(6):644-652.

41. Munshi NC, Mitsiades CS, Richardson PG, Anderson KC. Does maintenance therapy with thalidomide benefit patients with multiple myeloma? *Nat Clin Pract Oncol.* 2007;4(7):394-395.

42. Dhodapkar KM, Barbuto S, Matthews P, et al. Dendritic cells mediate the induction of polyfunctional human IL17-producing cells (Th17-1 cells) enriched in the bone marrow of patients with myeloma. *Blood.* 2008;112(7):2878-2885.

43. Miyahara Y, Odunsi K, Chen W, Peng G, Matsuzaki J, Wang RF. Generation and regulation of human CD4+ IL-17-producing T cells in ovarian cancer. *Proc Natl Acad Sci U S A.* 2008;105(40):15505-15510.

44. Kottke T, Sanchez-Perez L, Diaz RM, et al. Induction of hsp70-mediated Th17 autoimmunity can be exploited as immunotherapy for metastatic prostate cancer. *Cancer Res.* 2007;67(24):11970-11979.

45. Martin-Orozco N, Muranski P, Chung Y, et al. T helper 17 cells promote cytotoxic T cell activation in tumor immunity. *Immunity.* 2009;31(5):787-98.

46. Kryczek I, Banerjee M, Cheng P, et al. Phenotype, distribution, generation, and functional and clinical relevance of Th17 cells in the human tumor environments. *Blood.* 2009;114(6):1141-9.

47. Alexandrakis MG, Pappa CA, Miyakis S, et al. Serum interleukin-17 and its relationship to angiogenic factors in multiple myeloma. *Eur J Intern Med.* 2006;17(6):412-416.

48. Oteri G, Allegra A, Bellomo G, et al. Reduced serum levels of Interleukin 17 in patients with osteonecrosis of the jaw and in multiple myeloma subjects after bis-phosphonates administration. *Cytokine.* 2008;43(2):103-104.

49. Langowski JL, Zhang X, Wu L, et al. IL-23 promotes tumor incidence and growth. *Nature.* 2006;442(7101):461-465.

50. Brenne AT, Ro TB, Waage A, Sundan A, Borset M, Hjorth-Hansen H. Interleukin-21 is a growth and survival factor for human myeloma cells. *Blood.* 2002;99(10):3756-3762.