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Lessons of leprosy: The emergence of TH17 cytokines during type II reactions (ENL) is teaching us about T-cell plasticity

Frank Martiniuk^{1,a}, Jerome Giovino^{1,b}, Ainah U. Tan^{1,b}, Rozana Shahidullah², Patrick Haslett³, Gilla Kaplan⁴, and William R. Levis^{1,c}

¹New York University School of Medicine, 550 First Ave., N.Y., N.Y. 10016

^aDepartment of Medicine-Pulmonary Division, 550 First Ave., N.Y., N.Y. 10016

^bMedical student, 550 First Ave., N.Y., N.Y. 10016

^cDepartment of Dermatology, 550 First Ave., N.Y., N.Y. 10016

²Brooklyn College, Brooklyn, NY

³Shire Human Genetic Therapies, 700 Main Street, Cambridge, MA

⁴Laboratory of Mycobacterial Immunity and Pathogenesis, Public Health Research Institute at the University of Medicine and Dentistry of New Jersey, Newark, New Jersey.

Abstract

Background—Leprosy was the first disease classified according to the thymus derived T-cell in the 1960s and the first disease classified by the cytokine profile as intact interferon- γ (IFN γ) and interleukin-2 (IL2) or TH1 (tuberculoid) and deficient IFN γ and IL2 or TH2 (lepromatous), in the 1980s.

Objective—In the present study, we set out to explore the T helper 17 (TH17) lymphocyte subset, the hallmark of T-cell plasticity, in skin biopsies from patients with erythema nodosum leprosum (ENL) treated with thalidomide.

Method—RNA was extracted from paraffin embedded tissue before and after thalidomide treatment of ENL and RT-PCR was performed.

Results—*IL17A*, the hallmark of TH17, was consistently seen before and after thalidomide treatment, confirming the TH17 subset to be involved in ENL and potentially up-regulated by thalidomide.

Conclusion—A reduction in *CD70*, *GARP*, *IDO*, *IL17B (IL-20)* and *IL17E (IL-25)* coupled with increases in *ROR γ T*, *ARNT*, *FoxP3* and *IL17C (IL-21)* following thalidomide treatment opens the door for the complex understanding of how the immunomodulatory drug-thalidomide can be anti-inflammatory and simultaneously stimulate cell mediated immunity (CMI). We conclude that TH17 is involved in the immunopathogenesis of ENL and that thalidomide suppresses inflammatory components of TH17, while enhancing other components of TH17 potentially involved in CMI.

Corresponding author: Frank Martiniuk, PhD, NYU School of Medicine, Department of Medicine-Pulmonary New Bellevue 6N5,8 550 First Ave., N.Y., N.Y. 10016; 212 562-3616; martif02@nyumc.org.

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Introduction

Burnet won the Nobel Prize for the clonal selection theory of immunity in 1960.¹ In 1961, Miller published evidence that these lymphocyte “clones” were educated in the thymus.² In 1962, the British classified leprosy according to the thymus with intact cell mediated immunity labeled as “tuberculoid” and the anergic form as “lepomatous.”^{3,4} In the 1980’s, the concept of tuberculoid with intact IFN γ and IL-2⁵ and lepomatous with deficient IFN γ , IL-2 and increased IL-4 and IL-10⁶ led to the concept of the TH1–TH2 paradigm based on cytokine profiles.⁷ This binary concept of the immune system was dominant for 20 years.⁸ In the year 2000, Oppmann and coworkers discovered through bio-informatics the third subset of IL-12, p19 and designated the p40p19 cytokine as IL-23.⁹ In 2005, Steinman, discovered the TH17 lymphocyte subset in the autoimmune disorder EAE and provided a major revision of the TH1–TH2 paradigm.¹⁰ This discovery has led to multiple review articles and further use of the term T-cell plasticity where TH17 is in ying-yang with suppressor T-cell (Tregs) including hybrid T-cells with both ROR γ T and FoxP3.¹¹ Surprisingly, these findings may explain the recent reports that have shown Tregs, as measured by CD4CD25-FoxP3 functions, occur throughout the leprosy spectrum, rather than being dominant at the TH2 or anergic lepomatous form of the disease.^{12,13} Despite the fact that leprosy is the first disease classified according to T-cells, TH17 has not yet been thoroughly studied in the leprosy spectrum. As PMNs are a hallmark of the TH17 subset and type II (ENL) leprosy reactions, we theorized this T-cell subset would be involved in type II (ENL) leprosy reactions.

In 1964, Sheskin prescribed thalidomide as a sedative for a patient with ENL.¹⁴ It resulted in spontaneous resolution of the cutaneous lesions, fever and pain related to ENL.¹⁴ Haslett et al.¹⁵ elucidated the mechanism of action of thalidomide in twenty ENL patients from Nepal who were treated with thalidomide for 21 days. All patients responded to treatment with the majority having complete resolution of cutaneous lesions within seven days.

Despite several studies conducted for over 25 years, thalidomide's mechanism of action in arresting ENL is still unknown despite it being FDA approved. The proven efficacy of thalidomide in the treatment of ENL presents an opportunity to correlate the clinical and immunologic effects of this drug. The dynamic patterns of immunologic changes observed with the effects of thalidomide suggest some new ideas about the possible mechanism of action of the drug against ENL. Results suggest a complex effect of thalidomide that is anti-inflammatory, but also involves a transient boosting of T-cell function. It is hypothesized that thalidomide may actively promote T-cell regulation. In this report, we analyzed skin tissue from seven ENL Nepal patients pre- and post thalidomide treatment and found different relative gene expression patterns for twenty genes in the Th17 pathway.

Materials and Methods

Patient cohort

Our cohort includes seven ENL patients from Nepal treated with thalidomide for 21 days as FFPE skin samples pre- and post treatment with thalidomide (Table 1).

Extraction of RNA and RT-PCR

Three to four 1.0 μ sections of formalin-fixed paraffin-embedded ACD punch biopsy tissues were scraped from glass slides, paraffin removed by addition of 1 ml xylene for 5 min. and tissue precipitated by microcentrifugation for 5 min. The tissue was washed twice with 1 ml 100% ethanol and dried. RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer’s instructions and re-suspended in 50 μ l of DEPC-treated water. RNA was reverse transcribed into ss-cDNA using Superscript II reverse transcriptase

(Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, stored at -80°C and used within two weeks. Primers are listed in Table 2. RNA was equalized to *hGAPDH* using 3 μl ss-cDNA with 0.5 μl Taq polymerase (Promega, Madison, WI); dissociation at 94°C for 1.0 minute, annealing at 58°C for 1.0 minute and extension at 72°C for 1 minute for 45 cycles with a final 10 minute extension. Nested PCR used 3 μl of the first PCR reaction with annealing at 60°C and a second nested PCR used 3 μl of the first nested PCR reaction with annealing at 62°C . Nested and nested-nested PCR was utilized to detect the expression of the following human genes and primers were derived from published sequences for: *hROR γ T* (16); *hCD70*; *hCD27*; *hIL-17A* (*IL-17*); *hIL-17B* (*IL-20*); *hIL-17C* (*IL-21*); *hIL-17D* (*IL-27*); *hIL-17E* (*IL-25*); *hIL-17F* (*IL-24*); *hPLZF-1*; *hCTLA4*, *hAHR*, *hIL22*, *hiNOS2*, *hARNT*, *hIDO*, *hGARP*, *hIL9*, *hCD46* and *hFoxP3* (Table 2). The PCR conditions were: 10 μl ss-cDNA with 0.5 μl Taq polymerase; dissociation at 94°C for 1.0 minute, annealing at 56°C outer for 1.0 minute and extension at 72°C for 1 minute for 45 cycles with a final 10 minute extension. Nested PCR used 3 μl of the first PCR reaction with annealing at 58°C and a second nested PCR used 3 μl of the first nested PCR reaction with annealing at 60°C . Negative reactions (no template DNA) are included in all PCR reactions. The PCR amplicons for *hGAPDH* (20 μl) were electrophoresed in 2–3% agarose containing ethidium bromide (0.4 $\mu\text{g}/\text{ml}$) and visualized under ultra violet light. The PCR amplicons for remaining 20 genes were electrophoresed on agarose gel or 8–12% PAGE-0.5x TBE followed by staining with ethidium bromide. The gels were photographed with a FB-PDC-34 photo-documentation camera (Fisher Scientific-Springfield, NJ).

Results

Patient cohort

Our cohort was seven ENL patients from Nepal treated with thalidomide for 21 days. Shown in Table 1 is information on six of seven cases.

Molecular studies on the TH17 pathway

Total RNA from FFPE skin tissue from seven patients ENL pre- and post thalidomide treatment (Table 1) were extracted using xylene deparaffination. Total RNA was reverse transcribed by RT-PCR for *hGAPDH* and genes- *hROR γ T*; *hCD70*; *hCD27*; *hIL-17A*; *hIL-17B* (*IL-20*); *hIL-17C* (*IL-21*); *hIL-17D* (*IL-27*); *hIL-17E* (*IL-25*); *hIL-17F* (*IL-24*); *hPLZF-1*; *hCTLA4*, *hAHR*, *hIL22*, *hiNOS2*, *hARNT*, *hIDO*, *hGARP*, *hIL9*, *hCD46* and *hFoxP3* by nested or nested-nested RT-PCR to detect mRNA expression of human TH17. Initially, mRNA was equalized to *hGAPDH* (Figure 1). Before treatment with thalidomide, we found increased relative expression of *hCD70* (3/7 cases), *hIL-17B*(*IL-20*) (4/7 cases), *hIL-17E*(*IL-25*) (5/7 cases), *hIDO* (5/7 cases) and *hGARP* (3/7 cases) while after treatment, they were not detected. Conversely, we found that after treatment there was an increased relative expression of *hFoxP3* (4/7 cases), *hIL-17C*(*IL-21*) (2/7 cases), *hROR γ T* (2/7 cases) and *hARNT* (3/7 cases) with no expression before treatment. For genes *hCTLA4*, *hIL-17A*(*IL-17*) and *hiNOS2*, we found both expression patterns before and after treatment. No expression of *hAHR*, *hIL22*, *hCD27*, *hPLZF-1*, *hIL-17D*(*IL-27*), *hIL-17F*(*IL-24*), *hIL9* and *hCD46* were observed before or after treatment.

Discussion

Our results are in keeping with the literature that ENL or type 2 leprosy reaction associated with immune up regulation is ameliorated by an immunomodulatory drug (IMiD) thalidomide that is also associated with immune up-regulation. Furthermore, this report extends the original discovery of interleukin-2 and interferon- γ up-regulation of the TH1 T-cell subset by thalidomide^{16, 17} to the TH17 subset which along with TH1 has been

identified in many autoimmune and neoplastic models as the second dominant subset of T-cells involved in cell mediated immunity (CMI). Specifically, IL17A (IL-17), the hallmark cytokine of TH17, was up-regulated both before and after thalidomide treatment. The IL17 pathway is particularly complex as in addition to *IL17A(IL-17)*, there is *IL17B-F(IL-24)*. In this regard *IL17B(IL-20)* and *E(IL-25)* were up before thalidomide treatment of ENL, but not detected afterwards. Conversely, *IL17C(IL-21)* and the TH17 transcription factor-*ROR γ T* along with *ARNT*-a component of the aryl hydrocarbon receptor-implicated in TH17 activation,¹⁸ were only detected after thalidomide treatment of ENL. In keeping with the finding reported by Haslett et al.,¹⁵ we found an increase in *FoxP3*-the transcription factor for CD4/CD25+ Tregs, after thalidomide treatment of ENL. We also found *CD70*, a member of T-cell activation, increased before thalidomide treatment, but not after. These data combined with Haslett's et al.¹⁵ observation of a sustained increase in IL12, suggests the possibility of up-regulation of both TH1 and TH17 immunity during ENL and after thalidomide treatment, yet anti-inflammatory with increased *FoxP3* and decreased *CD70*. While the approach to understanding leprosy type 2 reaction and the effect of thalidomide on the immune system is immunologically interesting, further identification of molecular targets for thalidomide is required. In this regard, indoleamine 2,3-dioxygenase (IDO) needs to be investigated as a potential target of thalidomide as it is up-regulated by INF- γ a hallmark cytokine of ENL and our data show a down-regulation of *IDO* following thalidomide treatment of ENL. As IDO provides kynurenine^{19, 20} which down-regulates TH1 and TH17 lymphocytes via the *AHR* but up-regulates Tregs, a down-regulation of *IDO* would help explain how thalidomide is immune enhancing yet anti-inflammatory via *FoxP3* Tregs. IDO has recently been implicated in the anergy of lepromatous leprosy.²¹ IDO has also been identified as inhibiting TH17 cell responses by non-hematopoietic cells in a mouse model of *M. tb*.²² Our data suggest further investigation of IDO as a direct or indirect target of thalidomide and other IMiDs. Park et al.²³ have identified the macrophage inducible nitric oxide synthetase (iNos) as a potential target of thalidomide in a mouse macrophage cell line-(RAW264.7). In the present study, *iNos2* was found before and after treatment. Recently, Niedbala et al.²⁴ have implicated NO in the regulation of TH17. Lockwood et al.²⁵ have shown iNOS expressed in 78% of 237 leprosy biopsies with reduced levels of non-reactional lepromatous leprosy, indicating this molecule is associated with a well expressed cell-mediated immune response. Furthermore, TGF β was detected in 94% of the 237 leprosy skin biopsies. TGF β has traditionally been looked at as a down-regulator of inflammation, but it also implicated as an up-regulator of FoxP3 positive IL17A-producing Th17 cells.²⁶

Pharmacologically thalidomide is predominantly hydrolyzed and renally excreted. However, a portion is catabolized via the liver cytochrome P450 system²⁷ and some of the thalidomides catabolites are likely to be biologically active. Thalidomide treatment of metastatic prostate cancer patients, who have a poorly metabolizing form of CYP2C19, had an inferior outcome.²⁸ Furthermore, AHR inhibition can increase TH17 whereas AHR agonists have been shown to reduce the TH17 transcription factor RORC without affecting T-bet, GATA-3, or FoxP3.²⁹ Further study of thalidomide lenalidomide (Revlimid) and catabolites on the regulation of T-cell subsets via IDO and AHR is indicated. While TNF- α has been suggested as a potential target for thalidomide, Haslett et al.¹⁵ were unable to confirm this as important in these patients. As a result, we have emphasized Th-17 transcripts to further our understanding of how thalidomide and other IMiDs function to regulate immunity. It is however likely that additional genes and targets are involved in the complex interplay of small molecular drugs with the human immune system. RNA seq has recently identified a number of new genes involved in the immunopathogenesis of psoriasis³⁰ and offers promise to do the same for understanding the immunopathogenesis of leprosy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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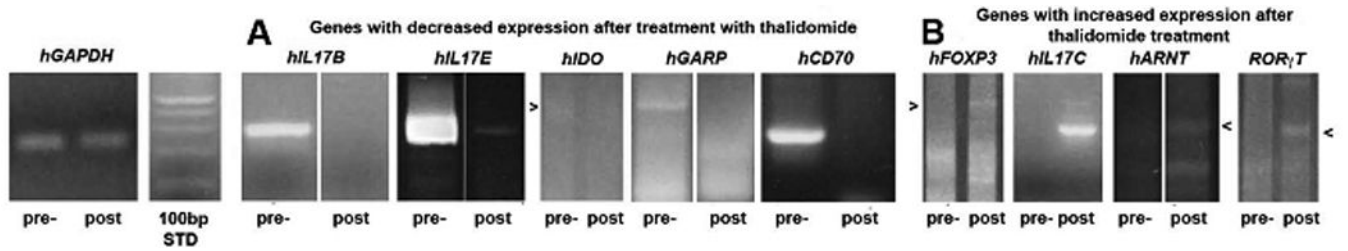


Figure 1.

Composite photograph of agarose gel electrophoresis of representative PCR amplicons for genes indicated pre- and post thalidomide treatment for Nepal ENL patients. *hGAPDH* and 100 bp DNA ladder are in the left three lanes. In A are genes with decreased expression after thalidomide treatment. In B are genes with increased expression after thalidomide treatment.

Table 1

Table 1 of six Nepal ENL patients treated with thalidomide

ENL HISTOL#	ENL ID#	Age (Yr)	Sex	Duration of Leprosy Treatment (months)	Ridley-Jopling Classification at Leprosy Diagnosis*	Bacillary Index at Leprosy Diagnosis [†]	Duration of Present ENL episode (days)	Previous Episodes of type II reactions ENL (number)	Previous Episodes of type I lepra reaction (number)
482	1	17	M	17	LL	4.75	24	1	0
483	2	45	M	7	LL	4.5	2	2	0
488	3	32	M	5	BL	1.25	240	1	0
478	4	24	M	17	LL	4.75	8	0	0
517	5	40	M	20	LL	3.25	30	1	1
514	6	37	M	10	LL	4.25	18	0	0

Table 2

Primer sequence and location

Gene and GenBank accession #	Location in cDNA	Primer sequence (actual sequence)
<i>hGAPDH</i> # M17851	outer primers: bp 618-647 bp 997-968 inner primers: bp 681-710 bp 1000-971 inner-inner primers: bp 617-646 bp 936-907	5'-CATCCCTGCCTCTACTGGCGCTGCCAAGGC-3' 5'-AGGCCATGAGGGCCATGAGGTCCACCACCC-3' 5'-TGTGGGCAAGGTTCATCCCTGAGCTG AACGG-3' 5'-TGTGCTGTAGCCAAATTCGTTGTTCATACC-3' 5'-GAAGCTCACTGGCATGGCCTTCCGTGTCCC-3' 5'-AGGAAATGAGCTTGACAAAAGTGGTGGTGA-3'
<i>hRORγT</i> #U16997	outer primers: bp 1231-1260 bp 1590-1561 inner primers: bp 1262-1291 bp 1560-1531 inner-inner primers: bp 1293-1322 bp 1526-1495	5'-TTCGAGCCTTGGGCTGCAGCGAG CTCATC-3' 5'-GCTGAAGAGCTCCTTGTAGAGTGGAGGGAA-3' 5'-GCTCCATCTTTGACTTCTCCCACTCCCTAA-3' 5'-AGCGGCTTGGACCACGATGGGGTGGAGGTC-3' 5'-TGCCTTGCAATTTCCGAGGA TGAGATTGC-3' 5'-AAGATCTGCAGCCTTCCACATGC TGGCTACA-3'
<i>hCD70</i> # BC000725)	outer primers: bp 301-330 bp 660-631 inner primers: bp 331-360 bp 630-601 inner-inner primers: bp 361-390 bp 600-571	5'-CAGGACCCCAGGCTATACTGGCAGGGGGGC-3' 5'-CACTCCAAAGAAGGTCTCATAGTGTTCG-3' 5'-CCAGCACTGGGCCGCTCCTTCTGCATGGA-3' 5'-GGAAGGCAAAAGTGTCCCACTGAGGTTGGT-3' 5'-CCAGAGCTGGACAAGGGGCAGCTACGTATC-3' 5'-GCAGAGTGTGTCCCTCGGGCCAGGGGCGT-3'
<i>HCD27</i> # NM_001242	outer primers: bp 541-570 bp 990-961 inner primers: bp 571-600 bp 960-931 inner-inner primers: bp 601-630 bp 930-901	5'-GCAGTGCAGGGACAAGGAGTGCACCGAGTG-3' 5'-GGGAGCAGGCAGGCTCCGGTTTTTCGGTAA-3' 5'-TGATCCTTCCAACCCTTCGCTGACCGC-3' 5'-CTTCCCTGGATGGGGATGGAGCTGCCCTCC-3' 5'-TCGGTCGTCTCAGGCCCTGAGCCACACCC-3' 5'-TCCTCCCTGGGGCAGCTGTAACGACAAGGC-3'
<i>hIL-17A</i> # NM_002190	outer primers: bp 361-390 bp 780-750 inner primers: bp 391-420 bp 750-721 inner-inner primers: bp 421-450 bp 721-691	5'-GTGGACTACCACATGAACTCTGTCCCATC-3' 5'-CATAAATAACTTAAAGCCCTTAAAAAAGTA-3' 5'-CAGCAAGAGATCCTGGTCTGCGCAGGGAG-3' 5'-AACTTGAAGCAAATGGTACTCAGTCAAAC-3' 5'-CCTCCACTGCCCAACTCCTTCCGGCTG-3' 5'-CTTCTTCTTGAAAGAGGGAAAGGTAATT-3'
<i>hIL-17B</i> # NM_014443	outer primers: bp 121-150 bp 590-561 inner primers: bp 391-420 bp 560-531 inner-inner primers: bp 181-210 bp 530-501	5'-GCCCAAAAGCAAGAGGAAGGGGCAAGGGC-3' 5'-GAAGATGCAGGAGCAGCCACAGCGATGGT-3' 5'-GGCCTGGGCCCTGGCCCTGGCCCTCACCC-3' 5'-CTCCATGACTGCGCGCTGGCGGCAAGGCC-3' 5'-AGGTGCCACTGGACCTGGTGTACGGATGA-3' 5'-TGTGCGGGCGGTGGCGGGCAGAGGCGGGC-3'
<i>hIL-17C</i> # NM_013278	outer primers: bp 241-270 bp 640-611 inner primers: bp 271-300 bp 610-581 inner-inner primers: bp 301-330	5'-AGCCCTGGTGTCCAGCCTGGAGGCAGCAAG-3' 5'-CACTGAACGGGGCAGCACGCAGGTGCAGCC-3' 5'-CCACAGGGGGAGGCACGAGAGGCCCTCAGC-3' 5'-ACGGGACGTGGATGAACTCGGTGTGGAA-3' 5'-TACGACCCAGTGCCTGGTGTGCGGCCGGA-3'

Gene and GenBank accession #	Location in cDNA	Primer sequence (actual sequence)
	bp 580-551	5'-GGCAAAGGCCCCAGGTGTGGGGAGCCCCGA-3'
<i>hIL-17D</i> # NM_138284	<u>outer primers:</u> bp 241-270 bp 810-781 <u>inner primers:</u> bp 271-300 bp 780-751 <u>inner-inner primers:</u> bp 301-339 bp 750-721	5'-CGCCTGGCGGCCGGCGTGTCTCAGTGCCTTC-3' 5'-CCAGCGGGCGCGTGTGGGGCCAGCAGG-3' 5'-CACCACACGCTGCAGCTGGGGCCGCGTGAG-3' 5'-AGCTTGGCGCCCTGTTTGTGATGCTGGAG-3' 5'-CAGGCGCGCAACGCGAGCTGCCCGCAGGG-3' 5'-TTGATGCTGTCTGCGTCTTCTCCGGCTCG-3'
<i>hIL-17E</i> # NM_022789	<u>outer primers:</u> bp 421-450 bp 840-810 <u>inner primers:</u> bp 451-480 bp 810-781 <u>inner-inner primers:</u> bp 481-510 bp 780-751	5'-CAGCTGTGCCCCAGCAAAGGGCAGGACAC-3' 5'-CCCATCACACGGGGCCGCACACACACAAA-3' 5'-CTCTGAGGAGCTGCTGAGGTGGAGCACTGT-3' 5'-GCTAAGGAAACACGGTACAGCCTGCGCTCC-3' 5'-GCCTGTGCTCCCTAGAGCTGCTAGGCC-3' 5'-AGGCAGTAGCCCTTGTGGGTGCCCTTCTCG-3'
<i>hIL-17F</i> # BC070124	<u>outer primers:</u> bp 81-110 bp 540-511 <u>inner primers:</u> bp 111-140 bp 510-481 <u>inner-inner primers:</u> bp 141-170 bp 480-451	5'-AAGACCCTGCATGGCCAGCCATGGTCAAG-3' 5'-GGGTGACGCAGGTGCAGCCAACAGTCACCA-3' 5'-TACTTGTCTGTCTGATATTGGGGCTTGCC-3' 5'-GCACCTTCTCCAAGTGGAAAGAAACAGAGC-3' 5'-TTTCTGAGTGAGGCGGCAGCTCGGAAAATC-3' 5'-AGCCTTGGTGCTTCTCCGGACGACCAGGG-3'
<i>HPLZF-1</i> # BC029812	<u>outer primers:</u> bp 1891-1920 bp 2220-2161 <u>inner primers:</u> bp 1921-1950 bp 2160-2131 <u>inner-inner primers:</u> bp 1951-1980 bp 2160-2131	5'-GAGTTCTGTGGCAGCTGCTTCCGGGATGAG-3' 5'-CGGGATCTCCTCGGGCTTGTGGCCCTTCAT-3' 5'-AGCACACTCAAGAGCCACAAACGCATCCAC-3' 5'-GTGCTTCTGCATGGAGGAGAGGCTGGGGCA-3' 5'-ACGGGTGAGAAACCCTACGAGTGAATGGC-3' 5'-GTACTCTGTGCAGATGGTGCA CTGGTAGGG-3'
<i>HCTLA4</i> # AF414120	<u>outer primers:</u> bp 211-240 bp 480-461 <u>inner primers:</u> bp 245-275 bp 460-431 <u>inner-inner primers:</u> bp 281-306 bp 425-396	5'-CCTGGCCCTGCACTCTCCTGTTTTTCTTC-3' 5'-ATCCCTGTCTCTGCAAAGCAATGCACGTG-3' 5'-CATGTAGGTTGCGCACAGACTTCAGTCAC-3' 5'-ATGGAATCATCTAGGAAGG TCAACTATTC-3' 5'-GCCTGCTGTGGTACTGGCCAGCAGCC -3' 5'-ACTTGATTTCCACTGGAGGTGCCCGTGCAG-3'
<i>hFoxP3</i> # AF277993	<u>outer primers:</u> bp 961-990 bp 1440-1471 <u>inner primers:</u> bp 991-1020 bp 1410-1381 <u>inner-inner primers:</u> bp 1021-1050 bp 1380-1351	5'-CCCACCTGGCTGGGAAAATGGCACTGACCA-3' 5'-AGGCTTCATCTGTGGCATCATCCGACAAGG-3' 5'-GCTCCTGCTGCATCGTAGCTGCTGGCAGCC-3' 5'-CCCGCACAAAGCACTTGTGCAGACTCAGGT-3' 5'-CGGTCCACACAGCCCCCTTCTCGCTCTCCA-3' 5'-TCCGTTTCTTGGGAACTCCAGCTCATCCA-3'
<i>hIL22 #</i> NM_020525	<u>outer primers:</u> bp 121-150 bp 590-561 <u>inner primers:</u> bp 151-180 bp 560-531 <u>inner-inner primers:</u> bp 181-210 bp 530-501	5'-TCTTGGCCCTCTGGTACAGGGAGGAGCAG-3' 5'-AATGCAGGCAATTTCTCAGAGACATAAACAG-3' 5'-CTGCGCCATCAGCTCCCACTGCAGGCTTG-3' 5'-CAAATCCAGTTCTCCAATTGCTTTGATCTC-3' 5'-ACAAGTCCAAGTCCAGCAGCCCTATATCA-3' 5'-TCCACTCTCTCCAAGCTTTTTACTGAGAC-3'
<i>hINOS2 #</i> NM_000625.4	<u>outer primers:</u> bp 3121-3150	5'-CCCCAAGACCCAGTGCCTGCTTTGTGCGG-3' 5'-CGTGACATCTCCAGGCTGCTGGGCTGCAC-3'

Gene and GenBank accession #	Location in cDNA	Primer sequence (actual sequence)
	bp 3720-3691 <u>inner primers:</u> bp 3151-3180 bp 3690-3661 <u>inner-inner primers:</u> bp 3181-3210 bp 3660-3631	5'-AATGCCAGCGGCTTCCACCTCCCCGAGGAT-3' 5'-CGCCACCCTGTCCTTCTTCGCCTCGTAAGG-3' 5'-CCCTCCCATCCTTGCATCCTCATCGGGCCT-3' 5'-AAATTCTGCACCAAAGATATCTTCGTGATA-3'
<i>hARNT</i> # NM_001668	<u>outer primers:</u> bp 2101-2130 bp 2490-2461 <u>inner primers:</u> bp 2131-2160 bp 2460-2431 <u>inner-inner primers:</u> bp 2161-2190 bp 2430-2401	5'-CTACCCAGGCTACTGCTAAGACTCGTACTT-3' 5'-TAGTTAGATCAGGGAATTCTTCATTGTTGT-3' 5'-CCCAGTTTGGTGTGGGCAGCTTTCAGACTC-3' 5'-AGCTGTTGCTCTGATCTCCAGCATGGACA-3' 5'-CATCCTCCTTACGTCCATGTCCCTCCCTG-3' 5'-GCATCTCTGGAAGACCTCAGGCTGGCCAG-3'
<i>hIDO</i> # NM_002164.4	<u>outer primers:</u> bp 871-900 bp 1320-1291 <u>inner primers:</u> bp 901-930 bp 1290-1261 <u>inner-inner primers:</u> bp 931-960 bp 1260-1231	5'-TGGGAAGACCCAAAGGAGTTTGCAGGGGGC-3' 5'-TTCCTTCAAAAAGGATTTCTCAGTTGTACT-3' 5'-AGTGCAGGCCAAAGCAGCGTCTTTCAGTGC-3' 5'-TCTTACAGTCTTCAGGAAAATTCATTAATC-3' 5'-TTGACGTCCTGCTGGGCATCCAGCAGACT-3' 5'-AGTGCCTCCAGTTCCTTTGGCTTCCAGTTT-3'
<i>hGARP</i> # Z24680.1	<u>outer primers:</u> bp 1591-1620 bp 2070-2041 <u>inner primers:</u> bp 1621-1650 bp 2040-2011 <u>inner-inner primers:</u> bp 1651-1680 bp 2010-1981	5'-GGGCAACGGGCTGATGGTCTGCAGGTGGA-3' 5'-TGTTGGTTAAACTTCTGCCGGCGGACGCAG-3' 5'-CCTGCCCTGCTTCATCTGCCTCAAGCGGT-3' 5'-CAGCAGGCGCCAGCGTGGTGCAGGATG-3' 5'-CAATCTGCCGAGAACCCCTGAGCCACCT-3' 5'-GCAGAGACCAGTATGAAGGTGAGGATGATG-3'
<i>hIL9</i> # NM_000590	<u>outer primers:</u> bp 21-50 bp 440-411 <u>inner primers:</u> bp 51-80 bp 410-381 <u>inner-inner primers:</u> bp 81-110 bp 380-351	5'-GCCATGGTCTTACTCTGCCCTGCTCCTG-3' 5'-CTTGCCTCTCATCCCTCTCATCTTTTCTTT-3' 5'-TGCTCCGTGGCAGGCCAGGGGTGTCCAACC-3' 5'-CTGAAAAATTTCCAGAAGACTCTTCAGAAA-3' 5'-TTGGCGGGGATCCTGGACATCAACTTCCTC-3' 5'-TGTCAGCGGCTTGCTGCCGTGTTTGGTT-3'
<i>hCD46</i> # NM_002389.4	<u>outer primers:</u> bp 691-720 bp 1260-1231 <u>inner primers:</u> bp 721-750 bp 1230-1201 <u>inner-inner primers:</u> bp 751-780 bp 1200-1271	5'-ACACACCTTTAGTGAAGTAGAAGTATTTGA-3' 5'-CAAATTAAGTCAACTCCAACAATATGGCA-3' 5'-GTATCTTGATGCAGTAAGTATAGTTGTGA-3' 5'-ATAACAATCAGCAATGACCCAAACATCC-3' 5'-TCCTGCACCTGGACCAGATCCATTTTCACT-3' 5'-AAACTGTCAAGTATTCCTTCCTCAGTTTA-3'
<i>hAHR</i> # BC070080.1	<u>outer primers:</u> bp 2611-2640 bp 3060-3031 <u>inner primers:</u> bp 2641-2670 bp 3030-3001 <u>inner-inner primers:</u> bp 2671-2700 bp 3000-2971	5'-ATGCCTTATACACAGAAGTATTTCTCTGT-3' 5'-TGTCAAATCAGGAAAAGGTCTGGCTTCTGA-3' 5'-AATCAGCTGTATTACCACAACATTCCAAA-3' 5'-CGGATGATGAAGTGGCTGAAGATGTGTGGT-3' 5'-TGACAGAGCTGGACTACCCATGGGGAGT-3' 5'-AGTCTGAGTGTTATTTATGTTATTTAATTC-3'