Decreased RORC expression and downstream signaling in HTLV-1-associated Adult T-cell Lymphoma/Leukemia uncovers an antiproliferative IL17 link: a potential target for immunotherapy?

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Abbreviations:

HTLV-1: Human T-cell Leukemia Virus-1

ATL: Adult T-cell Leukemia/Lymphoma

WGCNA: Weighted Gene Correlation Network Analysis

ATRA: All-trans retinoic acid

RARα: Retinoic acid receptor alpha

RORC: Retinoic acid orphan receptor C

TBLV: Type B Leukomogenic Virus

AML: Acute Myeloid Leukemia

T-ALL: T-cell acute lymphoblastic leukemia

IL: Interleukin

PCNA: Proliferating cell nuclear antigen

ID: Infectious Dermatitis

Novelty and Impact: A predominant RORC^{lo} phenotype was identified in four independent ATL cohorts, while a minor RORC^{hi} subset displayed lower levels of pathognomonic CADM1 and HbZ biomarkers, pointing at a protective role. RORC expression decreased with age in HTLV-1 infected individuals, but not in healthy controls, hinting at an early step in leukemogenesis. A modular transcriptomic analysis of RORC signaling uncovered a robust antiproliferative link with IL17C, which was shared between ATL, T-ALL and B-ALL. Boosting the RORC/IL17C axis thus represents a novel immunotherapeutic strategy in ATL, and possibly other lymphoid leukemias.

Abstract:

Retinoic acid-related drugs have shown promising pre-clinical activity in Adult T-cell Leukemia/Lymphoma, but RORC signaling has not been explored. Therefore, we investigated transcriptome-wide interactions of the RORC pathway in HTLV-1 and ATL, using our own and publicly available gene expression data for ATL and other leukemias. Gene expression data from ATL patients were analyzed using WGCNA to determine gene modules and their correlation to clinical and molecular data. Both PBMCs and CD4^{+} T-cells showed decreased RORC expression in four different ATL cohorts. A small subset of RORC^{hi} ATL patients was identified with

significantly lower pathognomonic CADM1 and HBZ levels but similar levels of other ATL markers (CD4/CD25/CCR4), hinting at a less aggressive ATL subtype. An age-dependent decrease in RORC expression was found in HTLV-1-infected individuals, but not in healthy controls, suggesting an early molecular event predisposing to leukemogenesis. Genes upstream of RORC signaling were members of a proliferative gene module (containing proliferation markers PCNA/Ki67), whereas downstream members clustered in an anti-proliferative gene module. IL17C transcripts showed the strongest negative correlation to PCNA in both ATL cohorts, which was replicated in two large cohorts of T- and B-cell acute lymphoid leukemia (ALL). Finally, IL17C expression in purified CD4+CCR4+CD26-CD7- 'ATL-like' cells from HTLV-1-infected individuals and ATL patients was negatively correlated with clonality, underscoring a possible antileukemic/antiproliferative role. In conclusion, decreased RORC expression and downstream signaling might represent an early event in ATL pathogenesis. An antiproliferative IL17C/PCNA link is shared between ATL, T-ALL and B-ALL, suggesting (immuno)therapeutic benefit of boosting RORC/IL17 signaling.

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Drugs that affect the retinoic acid pathway are of interest for the treatment of adult T-cell leukemia (ATL). Here, investigation of the role of retinoic acid-related orphan receptor C (RORC), a regulator of the pro-inflammatory Th17/IL-17 axis, reveals a prevailing occurrence of low RORC expression among ATL patients. By comparison, fewer patients exhibited a $RORC^{hi}$ phenotype, which was associated with reduced levels of pathognomonic biomarkers CADM1 and HbZ, indicating a protective role for elevated RORC. An antiproliferative link was identified between RORC and IL17C. The data suggest that strategies to increase RORC/IL17C signaling could be important to improving ATL outcomes.

Introduction:

Human T-Lymphotropic Virus -1 (HTLV-1) is a retrovirus with an estimated prevalence of 10-20 million worldwide[1]. A recent return to the original name of Human T-cell Leukemia Virus-1[2] is in agreement with its exceptional oncogenicity[3]. Although most HTLV-1 infections are asymptomatic, 2-6% of HTLV-1 infected individuals develop a $CD4^+CD25^+$ chemotherapy-resistant and aggressive leukemia known as Adult T-cell Lymphoma/Leukemia (ATL)[4–6]. ATL presents after a long latency period of the virus, commonly several decades[7]. Patients therefore tend to be older individuals with an average age at diagnosis of 40 years in Central and South America and 60 years in Japan. Depending on the subtype (acute, lymphomatous, chronic, and smoldering), survival ranges from 4 months to over 5 years[8].

HTLV-1 has two viral oncoproteins: Tax and HBZ. Tax benefits cell survival in HTLV-1 infected T-cells by interacting with NFκB[9], a key player in immune regulation. However, Tax levels are undetectable in most ATL patients, either due to gene deletion or altered DNA methylation levels, whereas HBZ is expressed consistently in ATL[9]. HBZ modulates Tax expression and induces $CD4^+$ T-cell proliferation[5]. CADM1/TSLC1 is also consistently expressed in ATL cells, such that CADM1 staining overlaps with the $CD4^+CD25^+$ T-cells in ATL and proviral sequences from these leukemic $CD4^+CADM1^+$ cells were consistently positive for the HBZ region[5]. Thus, CADM1 is a sensitive biomarker for ATL and might be used to determine treatment efficacy[5,9].

ATL patients display an increased incidence of opportunistic infections[10], which could be attributed to a deregulation of the Th17 axis, as an intact Th17 response is necessary for the clearance of opportunistic infections[11–14]. IL-17 and its upstream regulator IL-6 were increased in long-term cultured $Tax^{+}CD4^{+}$ T-cell supernatant[15]. IL-17 mRNA was also found to be highly expressed in HTLV-1 infected T-cells and Tax-expressing Jurkat cells[16]. Therefore, we hypothesize Tax-negative ATL cells are unlikely to express IL-17. Induction of the Th17 axis via retinoic acid receptors (RARs) and RAR-like orphan receptors (RORs) could potentially alleviate the increased opportunistic infection frequency caused by Th17 deregulation.

Retinoic acid blocks Th17 differentiation and stimulates regulatory T-cell (Treg) production[17]. Although HTLV-1 proviral integration in the host genome showed greater enrichment of promotor sequence motifs binding p53 and STAT1 instead of the RORC locus[18], downstream effects of p53 and STAT1 downregulate RORC expression by suppressing the transcription factor STAT3[19]. The relevance of RORC in leukemogenesis is further supported by the observed increased proliferation and apoptosis rates in mice deficient in the protein product of the RORC gene RORγ, leading to the development of T-cell lymphoma[20] and lymphoblastic lymphoma[21].

Taken together, deregulation of the RORC/Th17 axis can provide an explanation to both the oncogenic persistence of ATL and to patient susceptibility to opportunistic infections. In this study, we generate a representative consensus gene set for the RORC pathway of the Th17 axis and proceed to a multi-cohort analysis of novel and existing data to test the biological significance of this pathway in ATL.

Results:

Transcriptomic analysis of four independent cohorts reveals a $RORC^{lo}$ *ex vivo* phenotype in ATL

Gene expression profiling of *ex vivo* primary cells from ATL patients showed decreased RORC normalized expression in all four independent cohorts, revealing a common $RORC^{lo}$ phenotype (Figure 1A-B-C-D). Japanese Cohort #3 (n=73) and Caribbean Cohort (n=38) had significant decreases in RORC expression of ATL patients ($p<0.0001$ and $p=0.016$ respectively). Japanese Cohorts #1 (n=18) and #2 (n=50) had borderline significant decreases in RORC expression of ATL patients ($p=0.083$ and $p=0.10$). HAM patients in the Caribbean Cohort did

not have a significant change in RORC expression (p=0.54), however asymptomatic HTLV-1 infected individuals (AC) did display a significant decrease in RORC expression (p=0.016) when compared to healthy controls. ACs in other cohorts were not found to have a significant change in RORC expression, relative to healthy controls. Thus, RORC expression, measured as normalized expression (Figure 1) and percentile rank (Supplementary Figure S1), is consistently lower in ATL than in HC, but varies among cohorts for AC. Since RORC gene expression had been previously shown to decrease in AC[22], we performed a meta-analysis of the fold changes in RORC expression in all four cohorts, using both normalized expression and percentile ranks. Normalized gene expression allows for a comparison of the fold change in absolute RORC levels across cohorts but does not consider the profound perturbation of the cellular transcriptome between healthy vs. leukemic CD4+ cells in AC and ATL patients, respectively. In contrast, percentile ranks are a measure of RORC expression relative to the overall transcriptome for each individual, which is more suitable for a comparison between divergent disease states. This was confirmed by two-way ANOVA, analyzing cohorts and disease status (HC-AC-ATL) as separate variables. For fold-change RORC normalized expression, cohort differences accounted for 21.2% of variation (p=0.34) and disease status for 47.2% of variation (p=0.06). For fold-change RORC percentile ranks, only 1.3% of variation (p=0.48) was explained by cohorts and 95.8% of variation (p<0.0001) was explained by disease status. As shown in Figure 1E, RORC normalized expression was significantly (p<0.01) decreased in ATL patients, but not AC. Figure 1F displays the median RORC percentile rank fold-change, showing a significant 30% decrease in asymptomatic HTLV-1-infected individuals (p<0.0001, vs. HC) and an even further (43%) decrease in ATL patients (p<0.0001, vs. HC; p=0.006 vs. AC). This two-step decrease in RORC gene expression in ATL pathogenesis, first upon HTLV-1 infection and next upon progression to malignant disease, prompted us to investigate the possible influence of age upon RORC expression.

RORC Expression is not influenced by Age in Healthy Controls but decreases with Age in HTLV-1 Infected individuals

Since ATL usually occurs after several decades of HTLV-1 infection[5] and RORγt Tregs were shown to increase with age in mice[23],' we investigated the effect of age upon RORC gene expression in healthy controls and HTLV-1-infected individuals from several cohorts. We found

that RORC expression significantly decreased with age in HTLV-1 infected individuals without ATL, either AC and HAM/TSP patients (r=-0.57, p=0.0002, n=30 from UK Cohort, Figure 2A). We observed a similar tendency of decreased RORC expression with age in our Brazilian cohort (r=-0.62), but this observation did not reach statistical significance levels (p=0.10), most probably due to the small size of this ATL cohort (n=8) (Figure 2B). Unfortunately, the age of ATL patients was not available for the larger Japanese cohort. Next, we examined paired $CD4^+$ T-cells (n=293), $CD8^+$ T-cells (n=283), and PBMC (n=77) microarray results from a cohort of healthy controls with sufficient power to study the effects of age (Healthy Estonian Cohort, Table 1). We found that RORC expression levels did not significantly change with age in $CD4^+$ T-cells (r= 0.002, p= 0.45, Figure 2C), $CD8^+$ T-cells (r=0.0001, p= 0.86, Figure 2D) or PBMC (r=0.0001, p=0.93), nor with gender (data not shown).

A minor $RORC^{hi}$ subgroup of ATL patients displays a unique $CADM1^{lo}HBZ^{lo}$ phenotype

$RORC^{hi}$ outliers (Rout Method[24], Q=0.1%) were observed in the three Japanese cohorts, accounting for a total of 13 out of 108 ATL patients (12.04%), Therefore, we examined this phenotype more closely in the largest examined cohort (Japanese Cohort #2), where 7 outliers with a higher normalized RORC expression were identified (Figure 1B). The patients from this cohort were then split into two groups, according to their RORC levels, as shown in Figure 3. Interestingly, we noted that RORC expression was inversely associated with expression levels of pathognomonic, or unique disease identifying, ATL biomarkers CADM1 and HBZ. Thus, $RORC^{hi}$ patients displayed significantly lower HBZ (p=0.0061) and CADM1 (p=0.045) levels, but similar expression levels of other ATL surface marker genes (*CD4, CD25/IL2RA, CCR4*) suggesting the $RORC^{hi}$ subgroup might represent a distinct, possibly clinically relevant, molecular subgroup of ATL. The lower CADM1 and HBZ expression levels in $RORC^{hi}$ patients may represent the decreased proliferation rate of chronic or less aggressive ATL subtypes. As shown in Supplementary Figure S2, $RORC^{hi}$ patients showed similar expression levels of other ATL driver genes (*STAT3, PLCG1, NFKB1, RELA, FAS*)[25,26], highlighting the specificity of the $RORC^{hi}CADM1^{lo}HBZ^{lo}$ phenotype. Positive expression of IRF4 and c-REL has been associated with resistance to IFN-α + AZT therapy in ATL patients[26,27]. Interestingly, IRF4 and c-REL expression did not differ between $RORC^{hi}$ and $RORC^{lo}$ patients (Supplementary Figure S2). This

finding suggests RORC expression is independent of IFN-α + AZT therapeutic resistance and offers an additional molecular target for patients failing this therapy.

Definition of a consensus RORC pathway and gene set and its relevance to ATL oncogenesis

To facilitate the molecular exploration of the RORC^{hi} phenotype, a RORC gene set was determined based on published literature findings, integrating the intrinsic oncogenic pathway for STAT3 activation, as defined by Yu et al.[28], and RARA/RORC signaling summarized by Muranski and Restifo (2013)[29]. The consensus RORC pathway included IL6, IL23, IL21, IRF4, BATF, STAT1, STAT5, RARα, TGFβ, NFκB, SLC2A1 (GLUT1), BCL6, STAT3, FOXP3, SOCS1, RORC, and IL17A/F. Figure 4A illustrates the interplay between these genes, as detailed in the legend. RNX1, T-bet, RORA, and TGFB1R were not measured by the microarray used for the initial WGCNA analysis on the Brazilian cohort (pilot cohort) and therefore excluded from the gene set. To validate the biological significance of this manually compiled pathway, we applied STRING protein-protein interaction enrichment analysis, which confirmed highly significant interaction for the RORC consensus pathway (expected number of edges: 11, observed number of edges: 83, enrichment $p<10^{-16}$). As displayed in Figure 4B, our compiled RORC pathway was significantly enriched for "Positive regulation of cytokine production" ($p=7.9x10^{-12}$), "Regulation of T-helper cell differentiation" ($p=2.2x10^{-10}$), "Th17 immune response" ($p=1.9x10^{-9}$), "Jak-STAT signaling pathway" ($p=5.1x10^{-9}$), "Pathways in cancer" ($p=1.2x10^{-8}$), "HTLV-1 infection" ($4.4x10^{-5}$) and "Viral carcinogenesis" (p=0.0047), thus validating our approach.

A modular approach reveals a link between the RORC consensus pathway, proliferation and leukemogenesis

Transcriptomic expression levels of RORC pathway members extracted from a UK HTLV-1-infected asymptomatic control dataset (UK Cohort; GSE29312) and ATL cohort (Japanese Cohort #2; EGAD1001411) showed that the majority were expressed at highly variable levels (Supplementary Figure S3). First, prominent STAT1 expression is in line with published findings in AC[22,29] and ATL[26,30,31]. On the other hand, downstream members of the RORC pathway and particularly, IL17 family genes were either undetectable or poorly expressed.

WGCNA analysis of PBMCs from our pilot ATL cohort (n=8, Brazil, Figure 5A) showed overlap of the RORC pathway with a gene module correlated to proliferation, containing bona fide proliferation markers *PCNA* (Proliferating Cell Nuclear Antigen) and *MKI67* (the gene coding for Ki67 antigen, routinely used in flow cytometric quantification of proliferation). As shown in Figure 5B, downstream pathway members RORC and the IL17 family, under-expressed in ATL, were negatively correlated with the proliferative module and positively correlated with the anti-proliferative module. Likewise, upstream and overexpressed gene members of the RORC pathway displayed the reverse trend. This resulted in a significant bifurcation in the RORC pathway, as shown by linear regression of correlation coefficients of member genes with proliferative and antiproliferative modules, respectively (Supplementary Figure S4, $r=-0.97$, $p<0.0001$). Overall, WGCNA analysis suggested that inducing RORC and its downstream signaling, as well as blocking upstream pathway members may decrease the cell proliferation rate in ATL.

To confirm and extend these findings on proliferation, we repeated the WGCNA in the larger cohort of ATL patients (n=44, Japanese cohort #2). We additionally obtained *in silico* estimates of the relative size of 22 immune cell type populations using the CIBERSORT software[32]. As shown in Figure 5B, *RORC* was the only pathway member which was significantly and positively correlated ($r=0.42$) with the presence of resting memory $CD4^+$ T-cells ($p=0.0041$). Downstream pathway members *IL17B* ($r=0.62$, $p= 0.0000054$) and *IL17C* ($r=0.42$, $p=0.04$) were positively correlated with the presence of naïve $CD4^+$ T-cells. STAT3 inducer NFκB subunits 1 and 2 were negatively correlated with naïve $CD4^+$ T-cells ($p=0.02$ and $p=0.052$ respectively) and resting memory $CD4^+$ T-cells (p-0.000073 and $p=0.00084$ respectively). Similar to the observations in the WGCNA of the pilot cohort, a reverse trend was also seen in the CIBERSORT analysis between upstream and downstream members of the RORC pathway and their correlation with naïve and activated memory CD4+ T-cell fractions (Figure 5B). Together, the two WGCNA analyses, combined with CIBERSORT CD4+ subtype quantification, suggest a distinct change in proliferative pathways between upstream and downstream members of the RORC/IL17 pathway with opposite effects in activated memory vs. naïve and resting memory CD4+ T-cells. Among downstream pathway members, *IL17C* showed the strongest antiproliferative gene module membership in both cohorts and was also more frequently detected than other IL17 family members (*IL17A/B/D/F*). Therefore, we classified

ATL patients from the largest cohort (Japanese cohort #2) into *IL17C* expressing, (IL17Cpos, n=17) and *IL17C* negative (IL17Cneg, n=28). As shown in Figure 5C, IL17C-positive patients had significantly lower gene expression levels of proliferative marker *PCNA* (Mann-Whitney p=0.022) and in those patients, *IL17C* was positively correlated to *RORC* gene expression (r=0.54, p=0.026), confirming the findings of our modular analysis.

Validation of IL17C as a potential 'antileukemic' target in independent ATL, T-ALL and B-ALL cohorts

First, we analyzed IL17C expression in an independent UK cohort for which clonality analysis as well as clinical data (including therapeutic response) were available. RNAseq analysis of purified 'ATL-like' cells with a CD4+CCR4+CD26-CD7- immunophenotype demonstrated that *IL17C* transcripts were detectable in all ATL patients, but at significantly lower levels, as compared to AC. As shown in Figure 6A, *IL17C* transcripts were significantly decreased in both indolent and aggressive ATL (One-way ANOVA, Bonferroni's post-test $p<0.05$). No difference in *IL17C* levels was observed between ATL clinical forms or with regard to therapeutic response (chemotherapy and IFN+AZT resistance, not shown). However, *IL17C* expression was negatively correlated to clonality (r=-0.72, p=0.0086, n=12) in AC (fraction of largest clone 0.02-0.34) and patients with ATL (fraction of largest clone 0.68-0.99) (Figure 6B), in support of our hypothesized antiproliferative/antileukemic role for IL17C. Of note, *IL17A* and *IL17F* transcripts were not expressed (data not shown) in 'ATL-like' cells, in agreement with Kagdi et al. (2018)[33], who demonstrated compartmentalized expression of most cytokines in non-leukemic cells.

Second, to explore if the antiproliferative IL17C/PCNA link might be specific to ATL or shared with other leukemias, we analyzed two large cohorts of acute T- and B-cell leukemia (T-ALL, n=138; B-ALL, n=300). Similar to ATL, we found a significant negative correlation between *IL17C* and *PCNA* expression levels in both T-ALL (r=-0.24, p=0.007) and B-ALL (r=-0.28, $p<0.0001$), as shown in Figures 6C and 6D. Unfortunately, no clinical follow-up data (survival or therapeutic response) are available for the T-ALL and B-ALL cohorts.

IFN-α, IFN-β and Ascorbic Acid *in vitro* treatment differentially regulates RORC pathway members in primary ATL cells and HTLV-1 transformed cell lines.

We previously tested the effects of IFN-α and Ascorbic Acid (AA) on HTLV-1-infected transformed cell lines (MT2, MT4, C8166)[34–36]. Although both drugs have shown moderate success in decreasing HTLV-1-induced proliferation[34–37], only the high-dose AA affected the retinoic acid pathway, specifically the shared RORC/Th17 pathway. Reanalysis of our transcriptomic data showed that neither IFN-α nor high-dose AA altered RORC expression levels (log fold-change=0.042, p=0.59 and log fold-change =0.069, p=0.39, respectively). Ascorbic acid stimulated an increase in expression of a key gene in Th17 differentiation, IL23R (log fold-change=0.81, p=0.000024), in support of its possible use in (combination) therapy for ATL. RARα expression was unchanged by IFN-α (log fold change = 0.003, p=0.98) but decreased by ascorbic acid (log fold change=-0.28, p=0.064). Interestingly, our *in vitro* data (Brazilian cohort) demonstrated that RARα levels are upregulated upon *in vitro* treatment of ATL PBMCs with IFN-β (log fold-change=0.31, p=0.017), but not IFN-α. IFN-β also significantly modulated the expression levels of STAT1, IRF4, TGFB1R, IL23R, FOXP3, and IL6, while IFN-α significantly altered BCL6 only, as shown in Supplementary Figure S5. This is in accord with our recently demonstrated differential anti-proliferative and pro-apoptotic effect of both IFN subtypes[38].

Discussion:

Upon transcriptomic meta-analysis of four different cohorts, we found a specific and consistent $RORC^{lo}$ phenotype in primary ATL cells and to a lesser extent in HTLV-1-infected individuals, in contrast to healthy controls. In addition, HTLV-1-infected individuals displayed an age-dependent decrease in RORC expression. The observed two-step decrease of RORC in ACs and ATL patients might thus represent an early event in HTLV-1-driven leukemogenesis. We also identified a small subset (12.0%) of $RORC^{hi}$ ATL patients with significantly lower pathognomonic CADM1 and HBZ levels but similar levels of other ATL markers (CD4, CD25 and CCR4), hinting at a less aggressive ATL subtype.

ATL pathogenesis develops over decades as is seen by patients presenting at least 20 years after HTLV-1 infection; yet not all infected patients develop ATL. Observational studies suggest that ATL, at least in the Caribbean and Brazil, can be triggered by the pediatric cutaneous manifestation known as Infectious Dermatitis[39–42]. ID is a chronic, eczematous condition with scaly, crusted lesions often superimposed by *Staphylococcus aureus* or

Streptococcus pyogenes infections[39,40]. Defects in the Th17 axis increase vulnerability to *S. aureus* and *Candida albicans* infections, whereas *in vivo S. aureus* primed memory Th17 cells inhibited IL-17 production and increased IL-10 production[41,43]. Of interest, two recent papers have demonstrated a role for IL-10 as an unexpected proliferative trigger of infected CD4+ T-cell clones and, possibly, leukemogenesis[44,45]. Corroborating these findings, *IL10* was found to be a significant (r=0.36, p= 0.013) member of the proliferative gene module, together with *PCNA* and *MKI67*, in our WGCNA analysis. In addition, RNAseq analysis of purified cells with a CD4+CCR4+CD26-CD7- leukemic phenotype from an independent UK cohort revealed IL17C is expressed in 'ATL-like' cells. In agreement with our proposed protective role, IL17C was significantly and negatively correlated with clonality (Figure 6B). Therefore, our findings underscore an IL-10 vs. RORC/IL-17 antagonism in HTLV-1-associated pathologies and provide a possible molecular basis for the epidemiological link between ID and ATL, alteration of the RORC/Th17 axis, and subsequent progression to leukemogenesis.

Modular transcriptomic analysis in ATL shows a strong correlation of the RORC pathway with cell proliferation and possibly oncogenesis, which supports its therapeutic potential. WGCNA analysis combined with CIBERSORT suggested the involvement of RORC pathway members in the homeostasis of resting memory and naïve CD4+ T-cells. Combining the $RORC^{lo}$ observation in ATL cohorts with our WGCNA analysis, we find that decreased RORC expression is correlated with proliferation and ATL driver genes (STAT3, NF-kB). Thus, inducing RORC and switching to a $RORC^{hi}$ phenotype may convert ATL cells to a less aggressive subtype, suggested by the lower CADM1 and HBZ levels seen in the $RORC^{hi}$ subset (Figure 3). However, no overlap was found between the module memberships of RORC, HBZ, and Tax in the WGCNA of Japanese Cohort #2 (data not shown). In addition, no RORC gene module members were significantly correlated to HBZ or Tax transcript levels, suggesting decreased RORC levels and signaling in ATL are not a direct consequence of retroviral transcription. Therefore, we hypothesized the RORC/IL17 axis might be linked to proliferation in other (lymphoid) leukemias. Indeed, the strongest negative correlation observed in both ATL cohorts, between IL17C and proliferation marker PCNA, was replicated in two large cohorts of other acute lymphoid leukemias, namely T-ALL and B-ALL (Figures 6C-D).

Thus, our data reveal a widely prevalent antagonistic regulation between Th17 cells, usually considered as pro-inflammatory, and leukemic cell proliferation. Regarding the clinical

translation of these results, antitumor immunotherapy using Th17 cells has recently shown promising results in animal models. Adoptive cell therapy using *ex vivo* Th17 cell selection enhanced antitumor activity[44,46], to a greater extent than Th1 cells and other $CD4^+$ T-cells[44,46]. In addition, inducing IL17 expression via RORC stimulation would also subsequently alter the host immune response to reduce the risk of opportunistic infections by increasing Th17 cell count[10-14].

Although most often believed to antagonize IL17 production, IFN-β can trigger and even exacerbate IL17 production, especially in Th17-mediated inflammatory diseases[45,47]. This becomes problematic in cases of multiple sclerosis, where 30-50% of patients are resistant to IFN-β therapy[45]. However, this same exacerbation could be useful in ATL as a means of increasing Th17 cell production and decreasing proliferation of leukemic clones. IFN-β significantly alters the expression of more RORC pathway members than IFN-α (Supplementary Figure S5), a common therapeutic adjuvant to zidovudine in ATL treatment. This finding, along with the observation that IFN-β has superior anti-proliferative and pro-apoptotic properties compared to IFN-α[38], makes IFN-β a novel, valuable option for combination therapy in ATL.

Recently, immune checkpoint inhibitors have come to the forefront of anticancer immunotherapy[46,47]. Immunotherapeutic targeting of Programmed death ligand -1 (PD-L1) can increase Th17 cell count, restoring IL-17A protein levels in naïve T-cells of patients with a loss-of-function STAT3 mutation[46–48]. Conversely, inducing Th17 cell differentiation by RORγ agonist LYC-54143 simultaneously reduced $PD\text{-}1^+$ cell numbers and PD-1 expression *in vitro*, and resulted in tumor growth inhibition *in vivo* in two murine models[49]. In ATL, *PDL1* gene amplifications have been associated with worse prognosis, especially in aggressive subtypes[50]. For *PDL1* transcript levels, we observed a trend for positive correlation to CD4+ cells ($r=0.66$, $p=0.091$) as well as proliferation ($r=0.66$, $p=0.075$) in our Brazilian ATL cohort (Subramanian et al. unpublished), in agreement with a deleterious role for PD-L1. Again, combination immunotherapy by RORγ agonists and PD-L1 blockade might be a more effective option in ATL, similar to the superior response rate to dual therapy with PD-1 and CTLA-4 blocking antibodies in advanced melanoma, as compared to monotherapy[51].

In conclusion, we describe a predominant $RORC^{lo}$ phenotype observed in four cohorts of ATL patients and a minor $RORC^{hi}$ molecular subgroup with significantly lower mRNA levels of pathognomonic ATL biomarkers CADM1 and HBZ mRNA levels. An age-dependent decline in RORC level indicates a possible early event in HTLV-1-driven leukemogenesis, supported by

modular transcriptomic analysis of ATL patients, revealing a strong negative correlation of the RORC/IL17 pathway with proliferation, which was shared with T-ALL and B-ALL patients. Thus, inducing RORC levels and/or downstream signaling might represent (immuno)therapeutic benefit in ATL and possibly other acute lymphoid leukemias, which awaits further testing in clinical settings.

Methods:

In silico analysis

RORC expression levels were examined in publicly available transcriptomic data sets from patients with ATL, HTLV-1 infected asymptomatic controls, and healthy controls. A total of 135 untreated ATL patients, 12 HAM patients, 40 asymptomatic controls (AC), and 242 healthy controls (HC) from the Gene Expression Omnibus datasets GSE55851, GSE33615, GSE19080, GSE85487, and the European Genome-phenome archive EGAD1001411 dataset were used in this study (Table 1). EGAD1001411 initially contained 45 ATL patients, but one outlier with an overall strongly divergent transcriptome was removed. The effect of age on RORC expression was investigated in the Healthy Estonian Cohort for healthy controls (n=293) and the UK Cohort for HTLV-1 infected individuals (n=30).

The Japanese Cohort #2 (EGAD1001411) RNA-Seq data was quality- and adapter-cleaned using trimmomatic[52] and cutadapt[53] and quantified with kallisto[54] using an index built on the transcriptome obtained from the Genome Reference Consortium GRCh38, rel79. CIBERSORT was used to generate an *in silico* approximation of the relative composition of 22 immune cell types in the samples[32].

To facilitate consistent analysis of both the microarray and RNA-Seq data, the ensemble and/or Agilent IDs of the datasets were matched with corresponding Entrez IDs using the biomaRt package[55,56] in R. The Entrez IDs were verified with the associated GPL files on GEO where available. Considering transcriptomic analysis of the Caribbean Cohort was performed on a limited (non-genome-wide) microarray platform, 2134 Entrez IDs were common to all examined microarrays and comprised the list of genes examined in this study. To address the bias in the measurements inherent to each platform, we adapted the quantile discretization method proposed by Warnat et al.[57] and transformed gene expression levels into percentile ranks among the surveyed 2134 genes for the meta-analysis. To further exclude the possibility of

biasing our results, we refrain from making direct statistical comparisons of gene expression levels between datasets.

Published literature on *RORC* and RORγ, as cited and detailed in the results section, was used to develop a consensus molecular pathway, which was validated using STRING (version 10.5) protein-protein interaction enrichment analysis (www.string-db.org) using the whole genome as background.

Weighted Gene Correlation Network Analysis (WGCNA)[58] clusters genes into modules according to their topological overlap measure which quantifies how many gene-correlates were common to both members of each gene pair. To determine coherent gene modules and their correlation to clinical and molecular data, we performed WGCNA on each of the transcriptomic datasets from two independent ATL cohorts recently published by our group: *in vitro* gene expression data from short-term cultured ATL patient PBMCs (n=8, Brazilian Cohort) performed in parallel with lymphoproliferation, and *ex vivo* expression data from ATL patient PBMCs (n=44) of Japanese Cohort #2[25]. Module membership of the RORC gene set and the ATL signature genes were determined and correlated to demographic, clinical, and *in vitro* data.

In vitro analysis

Spontaneous lymphoproliferation of primary cells (PBMC) from ATL patients (n=8, Brazilian Cohort) was measured by [^{3}H]-thymidine incorporation, as described previously[38].

Statistical analysis

Statistical analysis was performed using GraphPad Prism 7.0. Differences in RORC gene expression were analyzed by Kruskall-Wallis test for Japanese Cohorts #1 and #2, and the Caribbean Cohort. For Japanese Cohort #3, where ACs were not included, Mann-Whitney was used to compare HC and ATL patients. The false discovery rate two-stage method of Benjamini, Krieger, and Yekutieli was used to correct for multiple comparisons. Spearman's Rho was used to correlate gene expression (either per gene or per WGCNA module using their eigengene expression) to demographic (age), clinical data (patient survival) and *in vitro* data (proliferation and apoptosis).

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Table 1. Transcriptomic (microarray and RNAseq) datasets used in RORC expression analyses

Data set	Source	Cell Type	Disease Status	Sample Size
GSE55851 Japanese Cohort #1	Kobayashi et al. (2014)	$CD4^+$ T-cells	HC	3
			AC	6
			ATL	12
EGAD1001411 Japanese Cohort #2	Kataoka et al. (2015)	$CD4^+$ T-cells	HC	3
		PBMCs	AC	3
			ATL	44
GSE33615 Japanese Cohort #3	Yamagishi et al. (2012)	$CD4^+$ T-cells	HC	21
		PBMCs	ATL	52
GSE19080 Caribbean Cohort	Oliere et al. (2010)	$CD4^+$ T-cells (Immunoarray)	HC	8
			AC	11
			ATL	7
			HAM	12
GSE85487 Brazilian Cohort	Dierckx et al. (2017)	PBMCs*	HC	5
			ATL – Untreated	8
			ATL - IFN-α	6
			ATL - IFN-β	6
GSE29312 UK Cohort	Tattermusch et al. (2012)	Whole Blood	HC	9
			AC	20
			HAM	10
GSE78840 Healthy Estonian Cohort	Kasela et al. (2017)	$CD4^+$ T-cells	HC	293
		$CD8^+$ T-cells	HC	283
		PBMCs	HC	77
ImmuCo	Wang et al. (2015)	$CD4^+$ T-cells	HC	551
		$CD8^+$ T-cells	HC	149
		Bone marrow Mononuclear Cells	AML	814
		Acute T-cell Leukemia	T-ALL	138
		Acute B-cell Leukemia	B-ALL	300

HC = Healthy Control. AC = Asymptomatic HTLV-1 Infected Control. ATL = Adult T-cell Lymphoma/Leukemia patients. HAM = HTLV-1 Associated Myopathy. AML = Acute Myeloid Leukemia. ALL = Acute Lymphoblastic Leukemia. *Short-term in vitro cultured PBMCs.

Figure Legends:

Figure 1. Normalized RORC expression levels for four independent cohorts consisting of ATL patients and healthy uninfected (HC) and/or HTLV-1 infected healthy controls (AC) showed a consistent decreased expression in ATL (A-D). (E) Meta-analysis of RORC normalized expression fold-change by disease status shows a significant decrease in ATL patients but not ACs. (F) Meta-analysis of RORC percentile rank fold-change by disease status shows a significant two-step decrease for ACs and ATL. HCs = Healthy Controls, ACs = Asymptomatic Controls, ATL = Adult T-cell Lymphoma/Leukemia Patients, HAM = HTLV-1-Associated Myelopathy patients. *p<0.05, **p<0.01, ****p<0.0001.

Figure 2. HTLV-1-infected Individuals from UK Cohort (A) and ATL patients from Brazilian Cohort (B) showed a decrease in RORC expression as age increased. This decrease was absent in healthy controls, in either CD4+ cells (C), CD8+ cells-(D) or PBMCs (not shown).

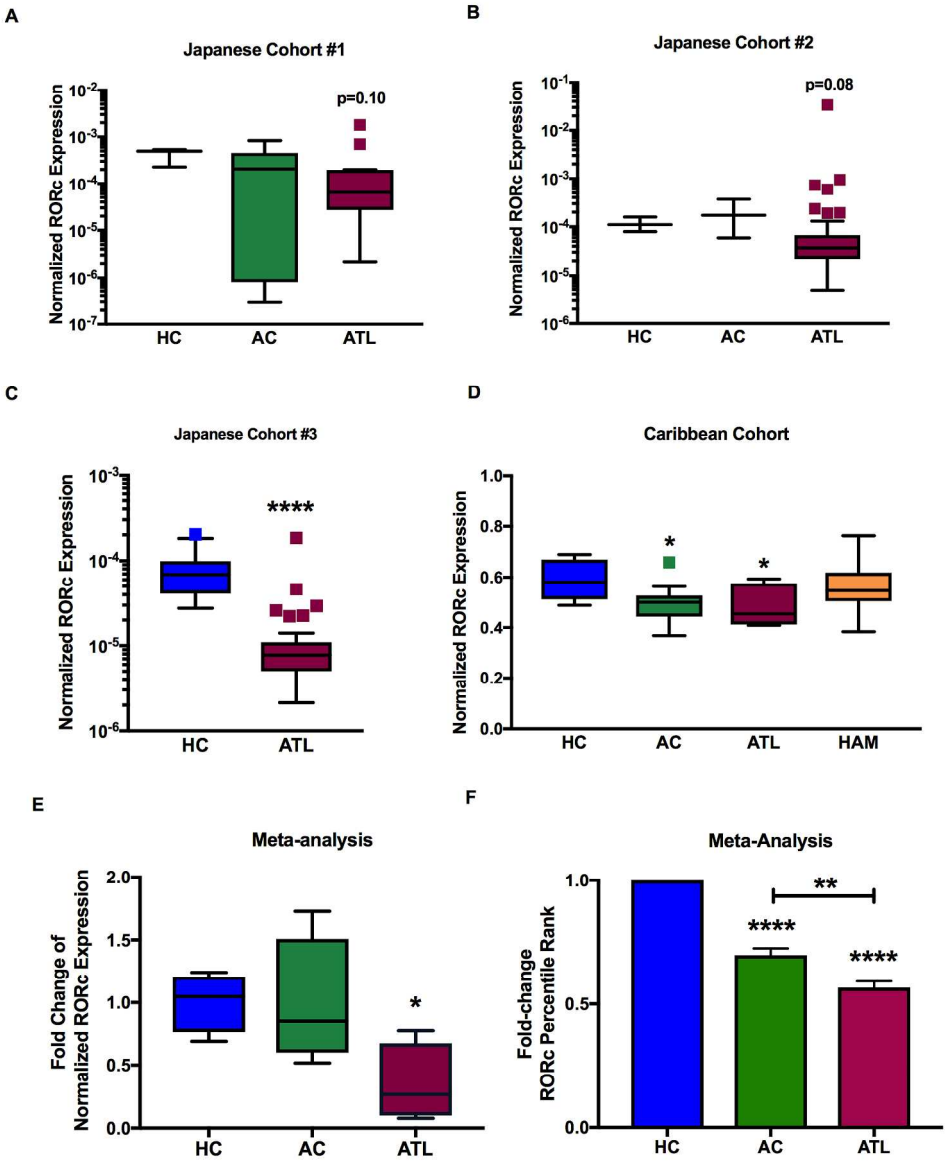
Figure 3. RORC expression levels of ATL patients from Japanese Cohort #2 separated into two groups: $RORC^{hi}$ (Green) and $RORC^{lo}$ (Red) show $RORC^{hi}$ levels were associated with lower HBZ and CADM1 expression levels. $RORC^{hi}$ (7 outliers) and $RORC^{lo}$ groups were compared with expression levels for ATL driver/mutated genes. *p<0.05 **p<0.01 ****p<0.0001

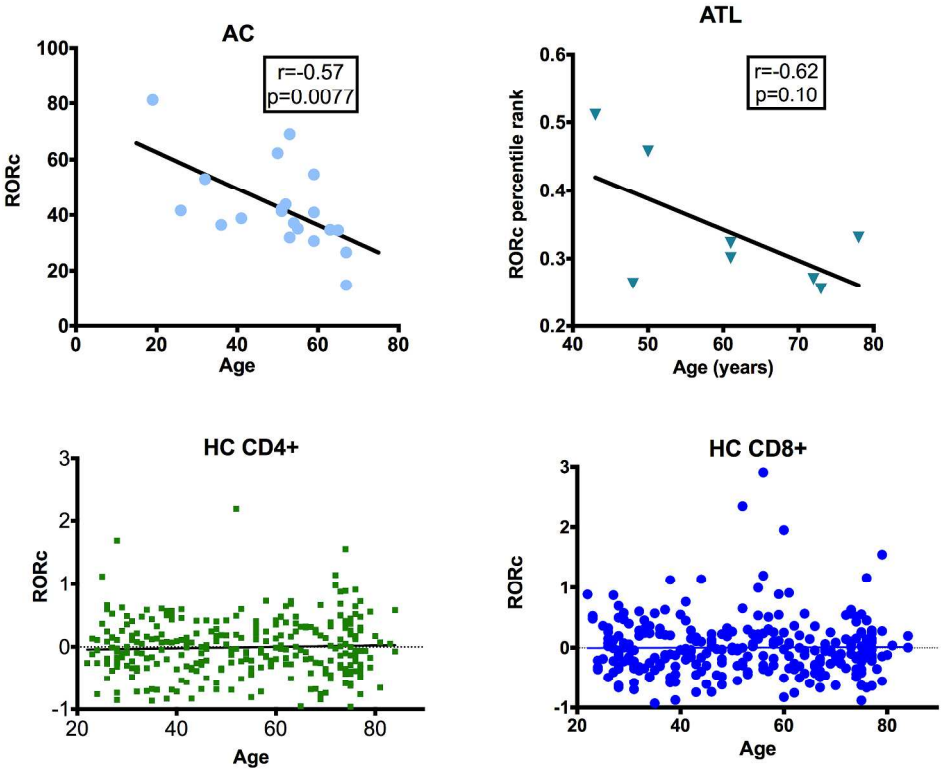
Figure 4. A) Simplified figure depicting the roles of RORC consensus pathway members, adapted from Yu et al. (2009)[36] and Muranski and Restifo (2013)[37]. The figure was produced using Servier Medical Art (http://www.servier.com) and edited using Inkscape software. B) The RORC consensus pathway was validated using STRING protein-protein interaction, GO biological process and KEGG pathway enrichment analysis. Significant enrichment (genome-wide FDR<0.05) is shown for "Positive regulation of cytokine production" (red), "Regulation of T-helper cell differentiation" (purple), "Th17 immune response" (green), "Jak-STAT signaling pathway" (yellow), "Pathways in cancer" (magenta), "HTLV-1 infection" (dark green) and "Viral carcinogenesis" (turquoise).

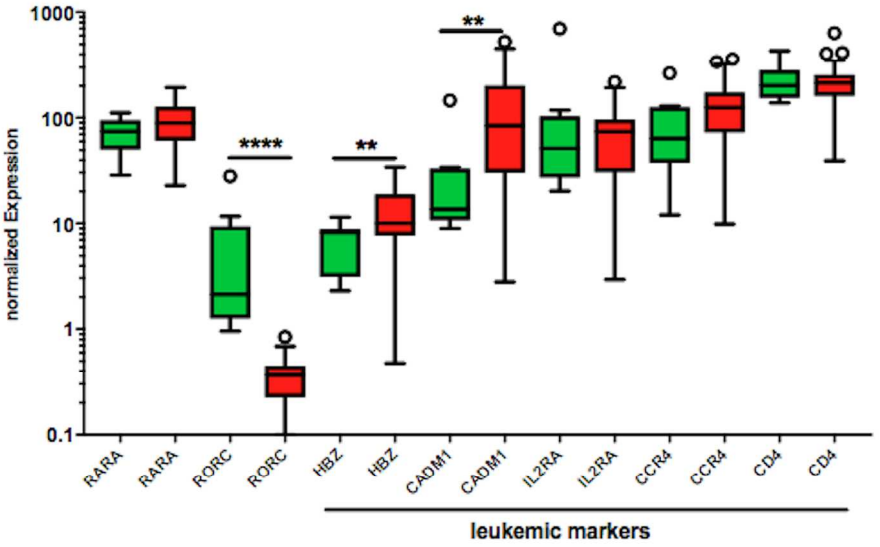
Figure 5. Modular transcriptomic analysis of primary ATL cells reveals a strong association of the RORC/IL17 pathway with proliferation. A) WGCNA findings for the Turquoise and Blue modules are shown for selected molecular and clinical correlations. R-value and p-values (in

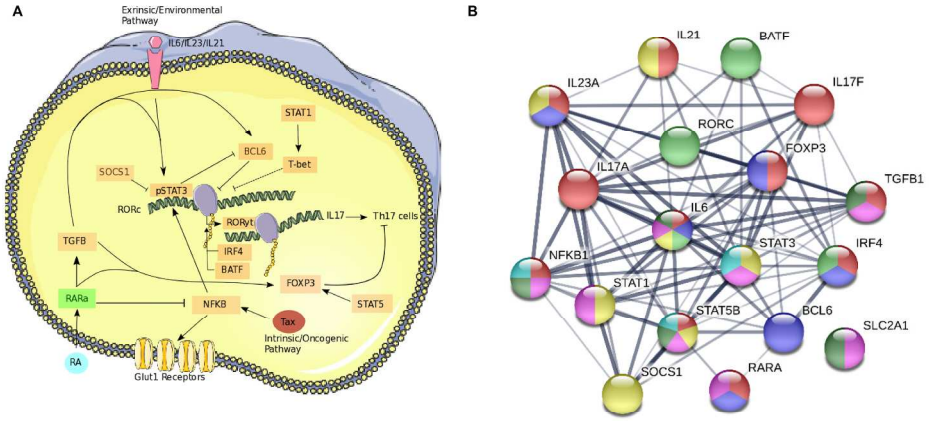
parenthesis) are shown *p<0.05 **p<0.01. B) Most RORC pathway members were positively correlated with the turquoise ("antiproliferative") gene module in primary ATL cells and negatively correlated with the blue ("proliferative") modules, such that most downstream members were found to be associated with the antiproliferative module (Brazilian cohort, n=8, left panel). WGCNA of Japanese Cohort #2 (right panel, n=44) shows RORC has a positive correlation with $CD4^+$ resting memory T-cells (Royal Blue module, r=0.47, p=0.001). Yellow module is positively correlated with $CD4^+$ memory active T-cells (r=0.39, p=0.007), as well proliferation markers *PCNA* (r=0.92, $p=10^{-15}$) and *MKI67* (r=0.87, $p=10^{-12}$). Brown module is positively correlated with $CD4^+$ naïve T-cells (r=0.49, p=0.0006). Genes which were validated in both ATL cohorts for proliferative modules are colored according to their R-values. C) ATL patients expressing *IL17C* (IL17pos, n=17, Japanese Cohort #2) showed decreased *PCNA* expression as compared to patients with undetectable *IL17C* (IL17neg, n=28), and *IL17C* levels were positively correlated with *RORC* levels.

Figure 6: Validation of IL17C as a potential 'antileukemic' target in independent ATL, T-ALL and B-ALL cohorts. A) In an independent UK cohort of HTLV-1-infected individuals, *IL17C* transcripts were significantly decreased in purified CD4+CCR4+CD26-CD7- cells from both indolent and aggressive ATL patients, as compared to AC (One-way ANOVA, Bonferroni's post-test p<0.05), with no difference in *IL17C* levels between ATL clinical forms. B) *IL17C* expression was negatively correlated to clonality in AC and ATL patients (fraction of largest clone 0.02-0.34 and 0.68-0.99, respectively). A negative correlation between *IL17C* and *PCNA* transcript levels was replicated in C) T-cell Acute Lymphoid Leukemia (ALL) (n=138) and B-cell ALL cohorts (n=300).





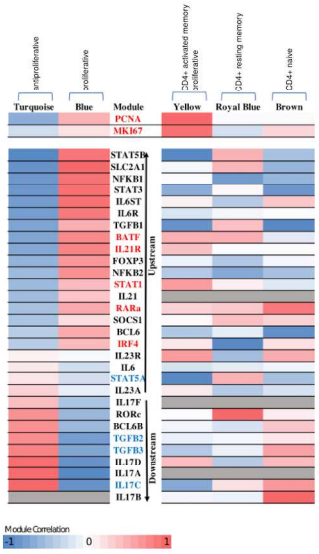




A

Module	% CD4 cells	% CD8 cells	in vitro proliferation	Age at sampling	Survival at followup
Turquoise "antiproliferative"	-0.70 (0.06)	0.33 (0.4)	-0.92 (0.001) **	0.44 (0.3)	-0.34 (0.4)
IL17C	-0.68 (0.06)	0.36 (0.4)	-0.94 (0.0005)***	-0.39 (0.3)	-0.21 (0.6)
IL17D	-0.76 (0.03)*	0.46 (0.25)	-0.93 (0.0007)***	-0.49 (0.2)	-0.25 (0.5)
IL17A	-0.64 (0.09)	0.32 (0.43)	-0.89 (0.003)**	-0.12 (0.8)	-0.51 (0.2)
IL17B	-0.72 (0.04)*	0.50 (0.2)	-0.58 (0.1)	-0.7 (0.1)	-0.27 (0.5)
Blue "proliferative"	0.60 (0.1)	-0.21 (0.6)	0.87 (0.006) **	0.19 (0.6)	0.56 (0.1)
MKI67	0.39 (0.35)	-0.07 (0.87)	0.86 (0.01)*	0.33 (0.43)	-0.51 (0.20)
PCNA	0.69 (0.06)	-0.32 (0.44)	0.87 (0.004)**	0.69 (0.06)	-0.38 (0.36)

B



C