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frontiers in Immunology

# A Fashi lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-1-associated neuroinflammation.

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1 **A Fas$^{hi}$ lymphoproliferative phenotype reveals non-apoptotic Fas signalling in HTLV-**
2 **1-associated neuroinflammation**

3

4 **Running head:** Fas signalling fuels retroviral neuroinflammation

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38 **Keywords:** Fas/CD95; proliferation; HTLV-1-associated myelopathy/tropical spastic

39 paraparesis; lymphoproliferative disease; apoptosis; interferon, NF-kB, multiple sclerosis

40 **Key Points:**

41 • A two-step increase in cell death receptor Fas occurs upon HTLV-1 infection and
42 disease progression

43 • Unexpectedly, higher Fas level was linked to decreased cell death, increased
44 lymphocyte proliferation/activation and early disease onset

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## ABSTRACT

Human T-cell lymphotropic virus (HTLV) -1 was the first human retrovirus to be associated to cancer, namely Adult T-cell Leukemia (ATL), but its pathogenesis remains enigmatic, since only a minority of infected individuals develops either ATL or the neuroinflammatory disorder HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). A functional *FAS -670* polymorphism in an interferon (IFN)-regulated STAT1-binding site has been associated to both ATL and HAM/TSP susceptibility. Fas$^{hi}$ T stem cell memory (Tscm) cells have been identified as the hierarchical apex of ATL, but have not been investigated in HAM/TSP. In addition, both *FAS* and *STAT1* have been identified in an IFN-inducible HAM/TSP gene signature, but its pathobiological significance remains unclear. We comprehensively explored Fas expression (protein/mRNA) and function in lymphocyte activation, apoptosis, proliferation and transcriptome, in PBMC from a total of 47 HAM/TSP patients, 40 asymptomatic HTLV-1-infected individuals (AC) and 58 HTLV-1 -uninfected healthy controls.

Fas surface expression followed a two-step increase from HC to AC and from AC to HAM/TSP. In HAM/TSP, Fas levels correlated positively to lymphocyte activation markers, but negatively to age of onset, linking Fas$^{hi}$ cells to earlier, more aggressive disease. Surprisingly, increased lymphocyte Fas expression in HAM/TSP was linked to decreased apoptosis and increased lymphoproliferation upon *in vitro* culture, but not to proviral load. This Fas$^{hi}$ phenotype is HAM/TSP-specific, since both *ex vivo* and *in vitro* Fas expression was increased as compared to multiple sclerosis another neuroinflammatory disorder. To elucidate the molecular mechanism underlying non-apoptotic Fas signalling in HAM/TSP, we combined transcriptome analysis with functional assays, i.e. blocking vs. triggering Fas receptor *in vitro* with antagonist and agonist- anti-Fas mAb, respectively. Treatment with agonist anti-Fas mAb restored apoptosis, indicating biased but not defective Fas signalling in HAM/TSP. *In silico* analysis revealed biased Fas signalling towards proliferation and inflammation, driven by RelA/NF-kB. Correlation of Fas transcript levels with proliferation (but not apoptosis) was confirmed in HAM/TSP *ex vivo* transcriptomes. In conclusion, we demonstrated a two-step increase in Fas expression, revealing a unique Fas$^{hi}$ lymphocyte phenotype in HAM/TSP, distinguishable from multiple sclerosis. Non-apoptotic Fas signalling might fuel HAM/TSP pathogenesis, through increased lymphoproliferation, inflammation and early age of onset.


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143 **INTRODUCTION**

144 Human T-lymphotropic virus 1 (HTLV-1) is an exogenous human retrovirus infecting 5-10
145 million people worldwide, mostly in HTLV-1 endemic regions.[1] While a majority of HTLV-1
146 carriers remain asymptomatic (AC) lifelong, a minority (0.25-3%) progresses to either adult
147 T-cell leukemia/lymphoma (ATL) or HTLV-1-associated myelopathy/tropical spastic
148 paraparesis (HAM/TSP)[2,3]. Thirty years after its discovery it is still enigmatic how a single
149 retrovirus causes either fatal hematologic malignancy or neuroinflammation in a small subset
150 of infected individuals. Among factors that allow to discriminate between the three clinical
151 groups (AC, ATL, HAM/TSP), humoral immunity,[4] proteome[5,6] have been described. In
152 agreement with a role for immune activation [4,6-9] in HAM/TSP pathogenesis, promising
153 preclinical results were obtained with Jak kinase and NFkB inhibitors.[10,11] Very few drugs, e.g.
154 valproate, have actually overcome the hurdle in transition from preclinical results[12] to clinical trial in
155 HAM/TSP.[13] Taken together, these studies point at a possible clinical benefit of decreasing
156 lymphoproliferation and/or increasing apoptosis in HAM/TSP patients. HTLV-1-infected cells are
157 driven towards spontaneous lymphoproliferation and oligoclonal expansion.[14,15] On the other
158 hand, apoptosis (programmed cell death) is known to play a role in controlling
159 lymphoproliferation in autoimmune diseases.[16,17] Fas (TNFRSF6/CD95/APO-1) is a death-
160 domain containing receptor of the tumor necrosis factor (TNF) receptor superfamily inducing
161 apoptosis[17], when ligated by Fas ligand (FasL) or agonist antibodies.[18] Fas-FasL signalling is
162 proposed to play a role in both autoimmune and infectious diseases.[17] In multiple sclerosis
163 (MS) patients, increased Fas expression has since long been known,[19] while resistance of T
164 cells to Fas-mediated apoptosis has been linked to MS.[20] In HTLV-1 infection, a wealth of
165 data is available on pro- and anti-apoptotic effects of HTLV-1 infection, mainly its proto-
166 oncogene Tax.[21] In the context of HAM/TSP immunopathogenesis, a role for Fas-FasL in the
167 down-regulation of immune response in the CNS has been suggested.[22] Previous studies on
168 Fas in HAM/TSP have shown increased levels of soluble Fas in serum,[23,24] and CSF,[24] as
169 well as surface expression in CD8 cells.[25] A systems biology approach identified *FAS* (but
170 not *FASL*) as part of an IFN-regulated gene signature in HAM/TSP patients.[7] In addition,
171 immunogenetic data revealed that a functional *FAS* -670 gene polymorphism is associated
172 to both ATL[26] and HAM/TSP[27] disease susceptibility. Therefore, we hypothesized that
173 lymphocyte Fas expression and/or apoptosis may reflect clinical status in HAM/TSP patients.

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174 **PATIENTS AND METHODS**

175 A flow chart diagram (Figure 1) provides an overview of the study outline, cohorts, as well as
176 *ex vivo*, *in vitro* and *in silico* experimental approach, while patient information and sample
177 use is summarized in Table 1.

178 HAM/TSP patients (n=47, 66.0% female, mean age 50.2±11.5 years, mean disease duration
179 5.6±4.0 y (range 0.8-14 y), EDSS range 3-7 (mean 5.1±1.2)) were recruited from three
180 endemic regions (Sao Paulo and Salvador-Bahia, Brazil and Lima, Peru) following written
181 informed consent. Age- and gender-matched HTLV-1-infected asymptomatic carriers (AC,
182 n=40) and uninfected healthy controls (HC, n=58) from the same endemic regions were
183 included in the study. The study was approved by the Ethics Committees of University of
184 Sao Paulo and FIOCRUZ-Bahia in Brazil and Universidad Peruana Cayetano Heredia in
185 Lima, Peru. Diagnosis of HAM/TSP was according to WHO criteria[29] Antibodies to HTLV-1/2
186 were investigated by diagnostic ELISA (Murex, Abbott, Germany; Bioelisa HTLV-1+2, Biokit
187 Spain) and confirmed by Western blot capable of discriminating between HTLV-1 and HTLV-
188 2 (HTLV Blot 2.4, Genelab, Singapore). All HTLV-1-infected individuals were seronegative
189 for HTLV-2 and HIV. For comparison with another neuroinflammatory disorder, data from MS
190 patients (recruited during our previous study[30]) was used.

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191 **Isolation of PBMC and *in vitro* cell culture**

192 PBMC isolated from 5-10ml of heparinized venous blood by Ficoll-Hypaque density gradient
193 centrifugation (Sigma-Aldrich) were washed twice with PBS and were plated in 24-well tissue
194 culture plates (Costar, NY) at $4\times10^6$ cells/ml and incubated at 37°C and 5% $CO_2$ in
195 RPMI1640 medium supplemented with 2mM L-glutamine, gentamycin (50µg/ml) and 10%
196 heat-inactivated fetal calf serum (Gibco, NY).

197 **HTLV-1 p19 and Proviral load quantification**

198 HTLV-1 matrix protein p19 was quantified in cell-free supernatant of HAM/TSP patients'
199 PBMC and AC and HC using RetroTek HTLV-1/2 p19 Antigen ELISA kit (ZeptoMetrix) after
200 48h of *in vitro* culture. Proviral load (PVL, i.e. viral DNA integrated into the host genome) in
201 HAM/TSP patients and AC was quantified as published.[30,31]

202 **Quantification of cell surface markers by flow cytometry**

203 For phenotypic analysis, PBMC were resuspended at a density of 200,000 cells in 50μL of
204 1% BSA, 0.1% $NaN_3$ in PBS (+20% human serum to block Fc receptors) and incubated for
205 30min on ice with mAbs specific for CD3, CD4, CD8, , CD80, CD86, CD95/Fas, HLA-DR
206 and corresponding isotype controls (BD Biosciences). For total Fas surface quantification
207 and apoptosis, a minimum of 100,000 events/sample were stained and acquired with
208 FACSort and FACSCanto II flow cytometers (BD Biosciences) and analyzed using CellQuest
209 and Diva software, respectively.

210 **Proliferation and Apoptotic assays**

211 Lymphoproliferation was quantified by [$^3$H]-thymidine incorporation and flow cytometry (as
212 described in[30,32]), the initial stage of apoptosis was analyzed using annexin V staining,
213 whereas cells in the late/final stage of apoptosis were identified as a sub-diploid population
214 by flow cytometry. Nuclear fragmentation was quantified by fluorescence microscopy and
215 ELISA (Cell Death Detection plus, Boehringer-Mannheim, Germany).

216 **Fas triggering and blocking experiments**

217 PBMC were cultured as above for 48h in the presence or absence of agonist or antagonist
218 anti-Fas mAbs (1μg/ml, Alexis Biochemicals) or anti-CD3 mAb (Butantan Institute, Sao
219 Paulo-Brazil) as a positive control for *in vitro* apoptosis.

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220 **Microarray analysis**

221 Total RNA was extracted from PBMC according to manufacturer's protocol (QIAgen, Venlo,
222 The Netherlands). Whole genome microarray was performed at VIB Nucleomics (Leuven,
223 Belgium) using GeneChip® Human Gene1.0 ST Array (Affymetrix, Santa Clara, CA),
224 according to manufacturer's specifications. Data was analyzed using Bioconductor limma
225 package (Smyth, GK, 2005), using a moderated t-test, resulting p-values were corrected for
226 genome-wide testing (5% FDR). All microarray raw data are available at Gene Expression
227 Omnibus database (GEO, http://www.ncbi.nlm.nih.gov/geo/) series accession number
228 GSE82160.

229 **Statistical analysis**

230 The use of parametric (t-test, Pearson correlation) or non-parametric (Mann-Whitney or
231 Spearman rank correlation) tests was based upon normal distribution as determined by
232 Kolmogorov-Smirnov test (all GraphPad Prism v5.0 or v6.0). A p-value of <0.05 was
233 considered significant for all statistical tests. Transcriptome-wide correlation of FAS mRNA
234 expression levels was calculated using Spearman rank correlation test, with stringent
235 correction for multiple testing (5% FDR).

236 **RESULTS**

237 **A two-step increase in *ex vivo* total lymphocyte Fas surface expression, in HTLV-1-**
238 **infected individuals and HAM/TSP patients, distinguishable from MS patients.**

239 In a first cohort, we quantified surface Fas levels as well as apoptosis by flow cytometry, *ex*
240 *vivo* in PBMC from HC (HTLV-1-negative, n=14), AC (HTLV-1-positive, n=30) and HAM/TSP
241 patients (n=18). We observed a significant increase in *ex vivo* levels (%) of $Fas^{+}$ lymphocyte
242 in AC (1.8-fold) as well as in HAM/TSP patients (2.1-fold), when compared to HC (Kruskal-
243 Wallis, Dunn's post-test, p<0.05, p<0.001; respectively, Figure 2A). Moreover, lymphocyte
244 Fas level on a per-cell basis, expressed as mean fluorescence intensity (MFI), revealed an
245 8-fold increase in AC and a striking 19-fold increase in HAM/TSP (Kruskal-Wallis, Dunn's
246 post-test, p<0.001), when compared to HC, but also when compared to AC (p<0.05, Figure
247 2B), indicating that clinical progression to HAM/TSP is characterized by a predominant $Fas^{hi}$
248 lymphocyte population, possibly primed for apoptosis. To confirm the two-step model of Fas
249 increase, we performed a post-hoc test for linear trend, which was highly significant
250 (p<0.001) for both % (slope 18.8) and MFI (slope 64.1).

251 Next, we proceeded to examine Fas expression in CD4, CD8 and B cell subsets in more
252 detail in an independent second cohort of HC (n=7), AC (n=6) and HAM/TSP patients (n=9).
253 There was no difference in the percentage of cells expressing Fas between the three clinical
254 groups for either cellular subset (Figure 2C.). However, we observed a small but significant
255 linear trend in Fas MFI of $CD4^{+}$ T cells with clinical status (ANOVA p=0.067, post-test for
256 linear trend p<0.05, slope=349.2), but not in $CD8^{+}$ T cells or B cells. Thus, the strongest
257 difference between the clinical groups was in total $Fas^{+}$ lymphocytes rather than specific
258 subsets, revealing a $Fas^{hi}$ phenotype in HAM/TSP. To verify if this $Fas^{hi}$ phenotype might be



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259 shared among neuroinflammatory disorders, we compared Fas expression between
260 HAM/TSP and multiple sclerosis (MS) patients. As shown in Figure 2D, we found a
261 significant 1.6-fold increase in % of *ex vivo* $Fas^+$ lymphocytes in HAM/TSP (Mann Whitney,
262 p=0.03), as well as a 2.4-fold increase in Fas MFI, which approached statistical significance
263 (Mann Whitney, p=0.08).

264 Finally, *ex vivo* spontaneous apoptosis in HAM/TSP and AC, as measured by DNA
265 degradation, (quantified as sub-diploid cells in flow cytometry) occurred at very low levels
266 (<0.2% of PBMC, data not shown). Therefore, we questioned if the observed *ex vivo*
267 increase in lymphocyte Fas surface expression in HAM/TSP reflected the immunological,
268 virological or clinical status of HAM/TSP patients, rather than an apoptosis-prone status.

269 ***Ex vivo* lymphocyte Fas surface expression correlates to immune activation markers**
270 **in HAM/TSP**

271 To explore possible clinical relevance of this increased lymphocyte Fas in HAM/TSP
272 patients, we correlated *ex vivo* Fas surface expression to patient demographic and clinical
273 data. We observed that, in HAM/TSP, *ex vivo* lymphocyte Fas (% or MFI) was not correlated
274 to age, gender, disease duration or severity. In addition, *ex vivo* lymphocyte Fas was not
275 significantly correlated to PVL in AC or HAM/TSP (p>0.05). However, *ex vivo* Fas levels (%)
276 correlated significantly to lymphocyte activation markers HLA-DR and CD86 (Figure 3A-B),
277 implying that increased Fas expression may be coupled to immune activation and/or
278 inflammation in HAM/TSP.

279 ***In vitro* $Fas^+$ lymphocyte levels correlate negatively to both age of onset and *in* vitro**
280 **apoptosis: a selective defect in HAM/TSP patients?**

281 Upon quantification of *in vitro* $Fas^+$ lymphocyte expression in HC, AC and HAM/TSP patients
282 by flow cytometry, we again observed a two-step increase in % $Fas^+$ lymphocytes: 2-fold in
283 AC and 3.4-fold in HAM/TSP vs. HC (Post-test for linear trend, p=0.0001, slope=27.0)
284 (Figure 4A). In HAM/TSP, *in vitro* Fas levels per-cell (MFI) were even more pronounced, with
285 an 8-fold increase over HC. Hence, clinical status impacts both *ex vivo* (Figure 2A-B) and *in*
286 *vitro* (Figure 4A) Fas expression. In addition, Fas *in vitro* levels showed a significant negative
287 correlation to age of disease onset in HAM/TSP patients (p=0.019, Pearson's r =-0.69, n=11)

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288 (Figure 4B), but not to age, disease duration and gender, suggesting Fas$^{hi}$ phenotype
289 predisposes to earlier, aggressive disease manifestation. Further, *in vitro* Fas expression
290 neither correlated to viral p19 protein level (p=0.41), nor to PVL (p=0.14) in HTLV-1-infected
291 individuals (data not shown).

292 In agreement with its role as a death receptor in immune homeostasis, Fas surface
293 expression positively correlates with spontaneous *in vitro* apoptosis in HC, while this
294 correlation was lost in AC (data not shown). Surprisingly, *ex vivo* Fas expression correlated
295 negatively (Supplementary Figure 1) to spontaneous *in vitro* apoptosis in HAM/TSP.
296 Furthermore, *in vitro* Fas level (MFI) also correlates negatively to lymphocyte apoptosis in
297 HAM/TSP (Figure 5A). This negative correlation was confirmed by fluorescence microscopy.
298 As shown in Figure 5B, Fas$^{hi}$ cells are negative for annexin V staining and display normal
299 nuclear morphology, whereas Fas$^{lo}$ cells were seen to undergo apoptosis by both annexin V
300 staining and nuclear condensation/fragmentation, occasionally triggering phagocytosis by
301 macrophages, emphasizing their apoptotic nature. Since resistance to Fas induced
302 apoptosis has been observed *in vitro* in lymphocytes from MS patients,[34] we compared *in*
303 *vitro* lymphocyte Fas expression and apoptosis between HAM/TSP and MS patients. As
304 shown in Figure 5C, there was a significant increase (2.4-fold, Mann-Whitney test, p=0.019)
305 in Fas MFI in HAM/TSP as compared to MS patients, but not apoptosis (as measured by
306 annexin V staining, Mann-Whitney test, p=0.84). In contrast to HAM/TSP, no correlation was
307 observed between Fas MFI and apoptotic cells in MS patients (p=0.35, data not shown).
308 Taken together, the significant negative correlations between *ex vivo* and *in vitro* Fas
309 lymphocyte expression and *in vitro* apoptosis observed only in HAM/TSP, suggest a possible
310 selective defect in Fas-mediated apoptosis. Hence, we next aimed to comprehensively
311 explore non-apoptotic Fas signalling in HAM/TSP.

312 **Fas expression positively correlates to lymphoproliferation *in vitro* and *ex vivo* in**
313 **HAM/TSP**

314 We quantified *in vitro* spontaneous lymphoproliferation by [$^{3}$H]-thymidine incorporation in
315 HAM/TSP patients. Surprisingly, we found that Fas expression positively correlates to
316 spontaneous lymphoproliferation *in vitro* (Figure 6A), which might imply that the observed
317 defect in Fas-mediated pro-apoptotic signalling in HAM/TSP might be explained as a bias in



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318 Fas signalling towards proliferation rather than apoptosis. Therefore, we hypothesized that
319 Fas$^{hi}$ cells might be already proliferating *in vivo* in HAM/TSP although at very low level. We
320 thus extended our previously described[27] sensitive flow cytometry assay to quantify Fas$^{+}$
321 diploid vs. tetraploid (proliferating) lymphocytes *ex vivo* in HAM/TSP patients, stained
322 immediately after PBMC isolation, without *in vitro* culture. As shown in Figure 6B, virtually all
323 of the proliferating cells were Fas$^{hi}$ (99.2±0.8%), as compared to non-proliferating
324 lymphocytes (69.4±5.9%, Paired t test, $p=0.0082$).

325 **Stimulation with agonist Fas mAb *in vitro* can trigger apoptotic signalling in HAM/TSP**

326 We then examined if this apparent defect in Fas-mediated apoptosis might be reversible by
327 stimulating with agonist anti-Fas mAb, and if blocking with antagonist anti-Fas mAb could
328 reveal ongoing Fas-FasL signalling in HAM/TSP. Hence, we treated PBMC *in vitro* with anti-
329 Fas mAb (agonist or antagonist) or anti-CD3 mAb as a positive control. No decrease in
330 spontaneous apoptosis was observed upon treatment with antagonist anti-Fas mAb,
331 confirming our hypothesis of inactive Fas-FasL signalling *in vitro* in HAM/TSP. Interestingly,
332 treatment with agonist anti-Fas mAb resulted in significantly increased apoptosis (1.7-fold,
333 $p<0.05$), similar to treatment with anti-CD3 mAb (positive control, 1.8-fold, $p<0.01$) (Figure
334 7A). These results imply that agonist anti-Fas mAb treatment can restore the apparent
335 defect in apoptosis in HAM/TSP, at least *in vitro*.

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336 **Systems analysis of gene expression profiles upon Fas triggering vs. Fas blocking in**
337 **HAM/TSP**

338 Considering the significant correlation between *in vitro* Fas expression to age of onset in
339 HAM/TSP, we resorted to genome-wide transcriptional analysis of PBMC treated *in vitro* with
340 agonist or antagonist Fas mAb, to explore the broad pro/anti-apoptotic, inflammatory,
341 proliferative and immunoregulatory Fas signalling pathways specifically triggered in
342 HAM/TSP. Microarray analysis revealed that *in vitro* treatment with agonist anti-Fas mAb,
343 significantly down-regulated 190 genes and up-regulated 59 genes (Supplementary Table
344 1A and B), while treatment with antagonist anti-Fas mAb down-regulated 38 genes and up-
345 regulated 18 genes (Supplementary Table 1C and D). Thus, triggering Fas signalling effects
346 a broader gene spectrum than inhibiting it. This was also evident from Ingenuity® pathway
347 analysis (IPA), since no biological functions were significantly associated with antagonist

anti-Fas mAb treatment, whereas treatment with agonist anti-Fas mAb resulted in 22 significantly associated biological functions (5% FDR-adjusted and a stringent cut-off of at least five enriched molecules per pathway) (Supplementary Table 2). The top 10 biological functions activated by agonist anti-Fas mAb (Supplementary Table 2), highlight cellular migration, especially of myeloid cells. In addition, IPA network analysis (Figure 7B) of Fas-triggered gene expression reveals a central role for NFkB pro-survival signalling, connecting several up-regulated proliferative and inflammatory molecules (TNF, JNK, RNA Polymerase II, POLR2D, HIST1H3A, HIST1H2AB) as well as down-regulated anti-proliferative genes (L3MBTL2, CARD6). This central role for NFkB signalling was confirmed by Ingenuity upstream regulator analysis, identifying RelA as the top upstream regulatory molecule upon triggering Fas signalling (target genes: BCL2A1, CASR, CXCL3, ICAM1, L3MBTL2, PTGES, TGM2, TNF and TPMT; p= 0.000032). Again, blocking Fas signalling did not yield any significantly enriched upstream regulators (using the same stringent cut-off of five enriched molecules/pathway, data not shown).

**Genome-wide correlation of *ex vivo* Fas RNA levels in HAM/TSP confirms a significant association to proliferation but not apoptosis**

Finally, we used a pathway-based data mining approach, to test our hypothesis of biased Fas signalling, and to possibly extend our findings by including additional pro- and anti-apoptotic genes (e.g. TRAIL, cFlip, etc.). For this purpose, we explored possible interactions of Fas mRNA within the *ex vivo* global gene expression profile in PBMC of HAM/TSP patients (n=6). Using transcriptome-wide correlation, 4554 genes significantly correlated to Fas transcript levels (Supplementary Table 3), after stringent FDR-correction for multiple testing. Using annotated Ingenuity pathways, we found a significant enrichment for proliferation-related genes (159 of 4554 genes, p=0.023). However, apoptosis, as defined by IPA, was not enriched amongst the *ex vivo* Fas-correlating genes (71 genes out of 4554 genes, p=0.10).

## DISCUSSION

In this study, we combined *ex vivo*, *in vitro* and systems analysis of Fas expression with functional apoptosis and proliferation assays, thereby providing an all-inclusive approach of the biological and clinical relevance of Fas signalling in HAM/TSP. We observed a two-step

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increase in *ex vivo* Fas expression: first, a greater percentage of $\text{Fas}^{+}$ lymphocytes upon HTLV-1 infection and second, a strong increase in expression of the death receptor at the single-cell level upon HAM/TSP disease progression. In addition, for the first time, we demonstrate that Fas expression correlates negatively to apoptosis and age of onset, but positively to immune activation and lymphoproliferation.

The most surprising finding of this study is a selective defect in Fas-mediated apoptosis in HAM/TSP patients. First, both *ex vivo* and *in vitro* Fas levels negatively correlated to *in vitro* apoptosis (Figure 5A and Supplementary Figure 1). Second, by fluorescence microscopy (Figure 5B), we document that $\text{Fas}^{lo}$ but not $\text{Fas}^{hi}$ cells preferentially undergo apoptosis *in vitro*. Third, *in vitro* treatment of PBMC with agonist anti-Fas mAb, but not antagonist anti-Fas mAb, was able to trigger apoptosis and restore the selective defect in HAM/TSP patients. Fourth, *in silico* analysis of the HAM/TSP transcriptome revealed a large number of transcripts (>4500) significantly correlating to Fas mRNA level, but are not enriched for apoptotic pathways. Taking together, our data indicate that the death receptor is fully functional in HAM/TSP, and not in a dormant state but skewed towards other biological pathways. Similar to our observation in HAM/TSP, increased Fas[35] and resistance to Fas-triggered apoptosis[36] has been reported in MS, which was also supported by gene expression profiling.[37] Nevertheless, our data reveal that the $\text{Fas}^{hi}$ phenotype is HAM/TSP-specific, since Fas expression was increased both ex vivo and in vitro, as compared to MS patients. Strikingly, the increase in non-apoptotic Fas receptor is also negatively correlated to age of disease onset in HAM/TSP (Figure 4B), rendering Fas as a clinically relevant molecule. It should be stated, however, that formal demonstration of the possible clinical utility of Fas expression or Fas downstream signalling targets as biomarker(s) in HAM/TSP will require confirmation of our findings in prospective cohort studies with a long-term clinical follow-up. In addition, agonist anti-Fas mAb, although restoring the defect in apoptosis in HAM/TSP, would not be a therapeutic option given that anti-Fas mAb therapy caused liver injury and lethality in mice.[38] In the absence of clinical benefit of antiretrovirals in HAM/TSP, immunomodulatory options include IFN-α/β, glucocorticoids, cyclosporine and ascorbic acid.[32,39,40] We previously demonstrated IFN-β can restore defective B cell CD86 up-regulation in HAM/TSP.[30] As in MS, defective Fas-mediated apoptosis in HAM/TSP patients may be overcome by IFN-β therapy.[41,42] In addition to IFN therapy, our *in silico* analysis

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409 might reveal novel treatment options. As shown in Figure 7B, a molecular network elegantly
410 describes the interplay between the molecular players of apoptosis (CARD6, caspases),
411 proliferation (POLR2D, L3MBTL2) and inflammation (TNF, JNK), with a central role for
412 NFkB. Therefore, our data confirm and extend the findings of Oh et al.[11] and Talledo et al.,[9]
413 who pointed at the importance of NFkB signalling in HAM/TSP from a pharmacological and
414 immunogenetic perspective. Furthermore, our Fas-triggered gene expression in HAM/TSP
415 reveals the same upstream regulator (Rel A), which is associated to active disease in MS.[37]
416 Thus, transcriptomics can reveal neuroinflammatory disorders sharing analogous biological
417 pathways, indicating approved MS drugs to be considered in HAM/TSP, but also allow the
418 identification of possible novel therapeutic targets, e.g. TGM2 or L3MBTL2 (Figure 7B).

419 Regarding HAM/TSP pathogenesis, both genetic and environmental triggers have been
420 suggested.[43] Interestingly, in a large cohort in the same endemic area (Salvador-Bahia), a
421 city with Afro-descendent demography, probable (but not definite) HAM/TSP occurred in
422 31% of AC during 8-year follow-up,[44] which suggests lifetime risk in this population is 10-fold
423 higher than previously reported.[43] As for environmental factors, co-infection with Gram-
424 positive bacteria, as in infective dermatitis, has been shown to trigger early HAM/TSP in
425 children from the same endemic area.[45,46] Concerning genetics, a single *FAS* -670
426 polymorphism has been associated to both ATL[26] and HAM/TSP[27] susceptibility. Since this
427 polymorphism also determined CD4 Tscm levels in a genome-wide twin study (Khouri et al,
428 submitted), the proliferative, non-apoptotic Fas$^{hi}$ cells in HAM/TSP are reminiscent of a Tscm
429 phenotype,[47] as outlined in Figure 8. However, since CD4 or CD8 Tscm represent only a
430 minor subset of Fas$^{+}$ lymphocytes[28], a Tscm origin of Fas$^{hi}$ cells is not likely, considering the
431 two-step increase we observed both *ex vivo* and *in vitro* (Figures 2A-B and 4A), first in AC
432 and second in HAM/TSP.

433 Non-apoptotic Fas signalling towards proliferation has been previously demonstrated,[48,49]
434 while Tax gene expression and cell cycling but not cell death are selected during HTLV-1
435 infection *in vivo*.[50] Tax mediates its anti-apoptotic activity by activating the NFkB pathway,[51]
436 associating NFkB to cell survival and inflammation, similar to our *in silico* findings. In
437 addition, Tax-deregulated autophagy and cFLIP expression are responsible for resistance to
438 apoptosis *in vitro*,[52] in agreement with our *ex vivo* and *in vitro* results. In contrast, many viral
439 infections are associated with heightened apoptosis. The most striking example is HIV,[53]

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440 which manipulates apoptotic pathways to enable efficient viral replication.[54] In the case of
441 HTLV-1, *in vitro* culture triggers viral protein synthesis and subsequent cytokine-driven
442 lymphoproliferation.[14] However, Fas did not correlate to PVL, similar to[25] and two other
443 published cohorts (p>0.5 for test and training sets).[7] Interestingly, PVL also did not correlate
444 to apoptosis or age of disease onset, in contrast to Fas. A previous larger study with
445 sufficient statistical power also demonstrated PVL does not correlate to age of onset in
446 HAM/TSP.[55] Furthermore, viral p19 protein levels did not correlate to Fas in our cohort.
447 Taken together, increased Fas levels in HAM/TSP appear to be driven by a IFN/STAT1 axis,
448 either genetically[27] or environmentally[45] linked, rather than by the virus itself, suggesting the
449 role of Fas in HAM/TSP pathogenesis is independent of PVL. Therefore, it is tempting to
450 speculate that a similar IFN/STAT1 signalling pathway might underlie the suggested
451 deleterious role of $CD80^+$ B cells, correlating positively to disease severity, also independent
452 of PVL.[30]

453 In conclusion, our results suggest defective Fas-mediated apoptosis is linked to early
454 disease onset and might be an additional factor in HAM/TSP pathogenesis, independent of
455 PVL. Triggering Fas signalling, rather than inhibiting it, induces a specific gene set with a
456 central role for NFkB pro-survival signalling. Thus, our integrated *ex vivo*, *in vitro*, *in silico*
457 approach identifies biased pro-inflammatory and proliferative Fas signalling in HAM/TSP,
458 revealing possible novel therapeutic targets.

459 Supplementary data

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**Figure legends**

Figure 1. Schematic representation of the methodology (ex vivo, in vitro and in silico approaches).

Figure 2. *Ex vivo* lymphocyte Fas surface expression in HTLV-1-infected individuals, HAM/TSP and MS patients. Using flow cytometry, Fas levels as % (A) and MFI (mean fluorescence intensity on a per cell basis) (B) were quantified in HC, AC and HAM/TSP patients. (*p<0.05, **p<0.01, ***p<0.001; Kruskal-Wallis, with Dunn's multiple comparison post-test). (C) Fas expression in CD4, CD8 and B cells was quantified in *ex vivo* PBMCs in HC, AC and HAM/TSP patients (ANOVA, p=0.067, post-test for linear trend p<0.05). (D) *Ex vivo* Fas levels (% and MFI) are compared between neuroinflammatory diseases HAM/TSP and MS (Mann Whitney test, *p<0.05).

Figure 3. Increased *ex vivo* lymphocyte Fas surface expression in HAM/TSP patients correlates with activation markers. Positive correlation between the percentage of Fas$^{+}$ lymphocytes and (A) HLA-DR$^{+}$ (*p=0.039, Spearman's r= 0.56, n=14) and (B) CD86$^{+}$ (*p=0.031, Spearman's r=0.60, n=13) lymphocytes in HAM/TSP patients.

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Figure 4. Significant linear trend in Fas$^{+}$ lymphocyte levels in PBMCs of HC, AC and HAM/TSP patients upon *in vitro* culture, and negative correlation with age of onset of HAM/TSP. (A) Fas levels were quantified by flow cytometry after 48h of *in vitro* culture. Fas$^{+}$ lymphocytes (%) gradually increase (HC n=12 AC n=4 HAM n=12) upon infection (AC) and further upon disease progression to HAM/TSP (ANOVA, p=0.0005; post-test for linear trend, p<0.0001). (B) Lymphocyte Fas levels (after 48h of *in vitro* culture) quantified by flow cytometry (MFI) correlate negatively to age of onset in HAM/TSP patients (*p=0.019, Pearson's r= - 0.69, n=11).

Figure 5. Fas$^{hi}$ cells are apoptosis-resistant in HAM/TSP patients. (A) Fas MFI (mean fluorescence intensity on a per-cell basis) negatively correlates to apoptosis (quantified as % annexin V$^{+}$ cells) in lymphocytes of HAM/TSP patients (*p=0.012, Spearman's r= - 0.63, n=15). (B) In the middle panel is a representative image of a non-apoptotic Fas$^{hi}$ cell

652 (indicated by a red horizontal arrow). This $Fas^{hi}$ cell is annexin V negative as visualized in
653 the first panel and displays a normal nuclear morphology seen in the third panel. On the
654 contrary, a $Fas^{lo}$ cell in panel 1 (black vertical arrow), displays pronounced annexin V
655 staining (panel 1) and is undergoing apoptosis, as evidenced by nuclear condensation, and
656 is being engulfed by a macrophage. (C) *In vitro* Fas levels (MFI) and apoptosis (% of
657 Annexin $V^+$ cells) are compared between neuroinflammatory diseases HAM/TSP and MS
658 (Mann Whitney test, *$p<0.05$).
659
660 Figure 6. Fas surface expression correlates positively with *in vitro* and *ex vivo*
661 lymphoproliferation in HAM/TSP patients. (A) *In vitro* Fas expression as measured by flow
662 cytometry (MFI) correlates positively to lymphoproliferation quantified by [3H]-thymidine
663 incorporation (*p=0.018, Pearson's r=0.62, n=14). (B) *Ex vivo* Fas surface expression
664 measured by flow cytometry (% and MFI) is significantly higher in proliferating (tetraploid, 4n)
665 cells vs. diploid (2n) cells in HAM/TSP patients (Paired t test, p=0.0082 and p=0.0023
666 respectively, n=5)
667
668 Figure 7. *In vitro* Fas triggering with agonist anti-Fas mAb induces apoptosis in HAM/TSP
669 and activates a molecular network linking apoptosis, proliferation and inflammation. (A)
670 Agonist (ago) anti-Fas mAb but not antagonist (ant) anti-Fas mAb increased apoptosis
671 (quantified by CellDeathPlus ELISA) in PBMCs upon *in vitro* treatment for 24h when
672 compared to control (untreated) PBMCs. Treatment with anti-CD3 mAb was used as a
673 positive control. (ANOVA, with Bonferroni's post test *$p<0.05$, **$p<0.01$). (B) Top molecular
674 network (score=34, linking cell-to-cell signalling, interaction, and cellular growth and
675 proliferation) identified by Ingenuity pathway analysis (IPA) among 249 genes significantly
676 up- and down-regulated (red and green, respectively) in PBMCs of HAM/TSP patients by *in*
677 *vitro* treatment with agonist anti-Fas mAb.
678
679 Figure 8. Model indicating the two-step increase in *ex vivo* lymphocyte Fas surface
680 expression. First, following HTLV-1 infection, there is an increase in lymphocyte Fas
681 expression (%) in AC. Second, upon progression to HAM/TSP, Fas expression is increased
682 on a per-cell basis as Mean Fluorescence Intensity (MFI), (Figure 2A-B). In agreement with
683 its role as a death receptor, $Fas^+$ cells in HC are primed to follow the apoptotic pathway,

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684 depicting nuclear condensation and cell blebbing, which is lost upon HTLV-1 infection (AC).
685 In contrast, in HAM/TSP patients, $Fas^{hi}$ cells are driven towards proliferation (Figure 7A-B).
686 We recently discovered a genotype/phenotype interaction for the *FAS* -670 polymorphism
687 with both apoptosis and proliferation in ATL patients and healthy controls (Khouri et al,
688 submitted). This $Fas^{hi}$ proliferating and chemotherapy-resistant leukemic phenotype is in
689 agreement with the recently discovered CD4 Tscm hierarchical apex of ATL. The same *FAS*
690 -670 polymorphism also determined CD4 Tscm levels in a genome-wide twin study,
691 confirming our hypothesis (Khouri et al, submitted). Therefore, a genetically determined
692 IFN/STAT1/FAS axis might help explain the proliferative, non-apoptotic phenotype in
693 HAM/TSP suggesting CD4 Tscm as a pivotal factor not only in ATL but also in HAM/TSP
694 pathogenesis. Considering STAT1 and FAS are in the HAM/TSP gene signature, our data
695 further refine the data of Tattermusch et al.[7] It is not unexpected that a Tscm phenotype is
696 absent from the disease signature, since Tscm are rare (2-3%)[47] and their genome-wide
697 expression profile is intermediate between naïve and central memory T cells. However,
698 Tscm cells have a $Fas^{hi}$, apoptosis-resistant and drug-resistant, proliferative phenotype, in
699 agreement with their stem cell-like nature. Interestingly, the proliferating cells in HAM/TSP
700 patients were almost exclusively $Fas^{hi}$, (Figure 6B), compatible with a Tscm phenotype.
701
702
703 Table 1.
704 Patient information and sample use

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Table 1. Patient information and sample use

| Patient | Age | Gender | Cohort | Analysis |
|---|---|---|---|---|
| 1 | NA | F | BA | Ex vivo flow cytometry |
| 2 | NA | M | BA | Ex vivo flow cytometry |
| 3 | NA | F | BA | Ex vivo flow cytometry |
| 4 | NA | F | BA | Ex vivo flow cytometry |
| 6 | NA | F | BA | Ex vivo flow cytometry |
| 7 | 51 | M | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 8 | 40 | M | BA | Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 9 | 40 | F | BA | Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 10 | 63 | F | BA | Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 11 | 51 | F | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 12 | 36 | M | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 13 | 40 | F | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 14 | 60 | F | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 15 | 44 | M | BA | Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 16 | NA | F | BA | Ex vivo flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 17 | 53 | M | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray |
| 18 | 45 | F | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray |
| 20 | 59 | M | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 21 | 60 | F | BA | Ex vivo and in vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 22 | 38 | M | BA | In vitro lymphoproliferation |
| 23 | 59 | F | BA | In vitro lymphoproliferation |
| 24 | 56 | F | BA | In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray |
| 25 | 49 | F | BA | In vitro apoptosis |
| 26 | 57 | M | BA | In vitro apoptosis |
| 27 | 49 | F | BA | In vitro flow cytometry, In vitro apoptosis In vitro lymphoproliferation |
| 28 | 60 | M | BA | In vitro flow cytometry, In vitro apoptosis In vitro lymphoproliferation, Microarray |
| 29 | 46 | M | BA | In vitro apoptosis, In vitro lymphoproliferation, Microarray |
| 31 | 50 | M | BA | In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray |
| 32 | 50 | F | BA | In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation, Microarray |
| 33 | 62 | F | BA | In vitro flow cytometry, In vitro apoptosis, In vitro lymphoproliferation |
| 2569 | 27 | F | LI | In vitro apoptosis |
| 2570 | 50 | F | LI | In vitro apoptosis |
| 2574 | 35 | F | LI | In vitro apoptosis |
| 2817 | 64 | F | LI | Ex vivo flow cytometry |
| 2819 | 32 | F | LI | Ex vivo flow cytometry |
| 2821 | 63 | F | LI | Ex vivo flow cytometry |
| 2822 | 50 | F | LI | Ex vivo flow cytometry |
| 2823 | 64 | M | LI | Ex vivo flow cytometry |
| SP5 | 32 | F | SP | Ex vivo flow cytometry |
| SP6 | 65 | F | SP | Ex vivo flow cytometry |
| SP7 | 62 | F | SP | Ex vivo flow cytometry |
| SP8 | 47 | F | SP | Ex vivo flow cytometry |
| SP26 | 35 | M | SP | Ex vivo flow cytometry |
| SP30 | 72 | M | SP | Ex vivo flow cytometry |
| SP32 | 27 | M | SP | Ex vivo flow cytometry |
| SP36 | 52 | F | SP | Ex vivo flow cytometry |
| SP46 | 61 | F | SP | Ex vivo flow cytometry |

Cohorts: BA Bahia, LI Lima, SP Sao Paulo
NA: Not available

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706
707
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709 **Footnote page:**

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714 Leuven), Belgium.

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716 **Authorship:** JVW designed research; SMM, FEL, TD, RicardoK, DD, GSS, GL and JVW
717 performed research; SVS, DFN, JV and AMV contributed to data analysis; FEL, RamonK,
718 CA, MT, EG, DB, RL and BGC provided patient samples; SMM and JVW analyzed data and
719 wrote the paper.

720 **Meetings presented at:**

721 16th International Conference on Human Retrovirology: HTLV and Related Retroviruses, 26-
722 30 June 2013, Montreal, Canada.

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Figure 01.TIF

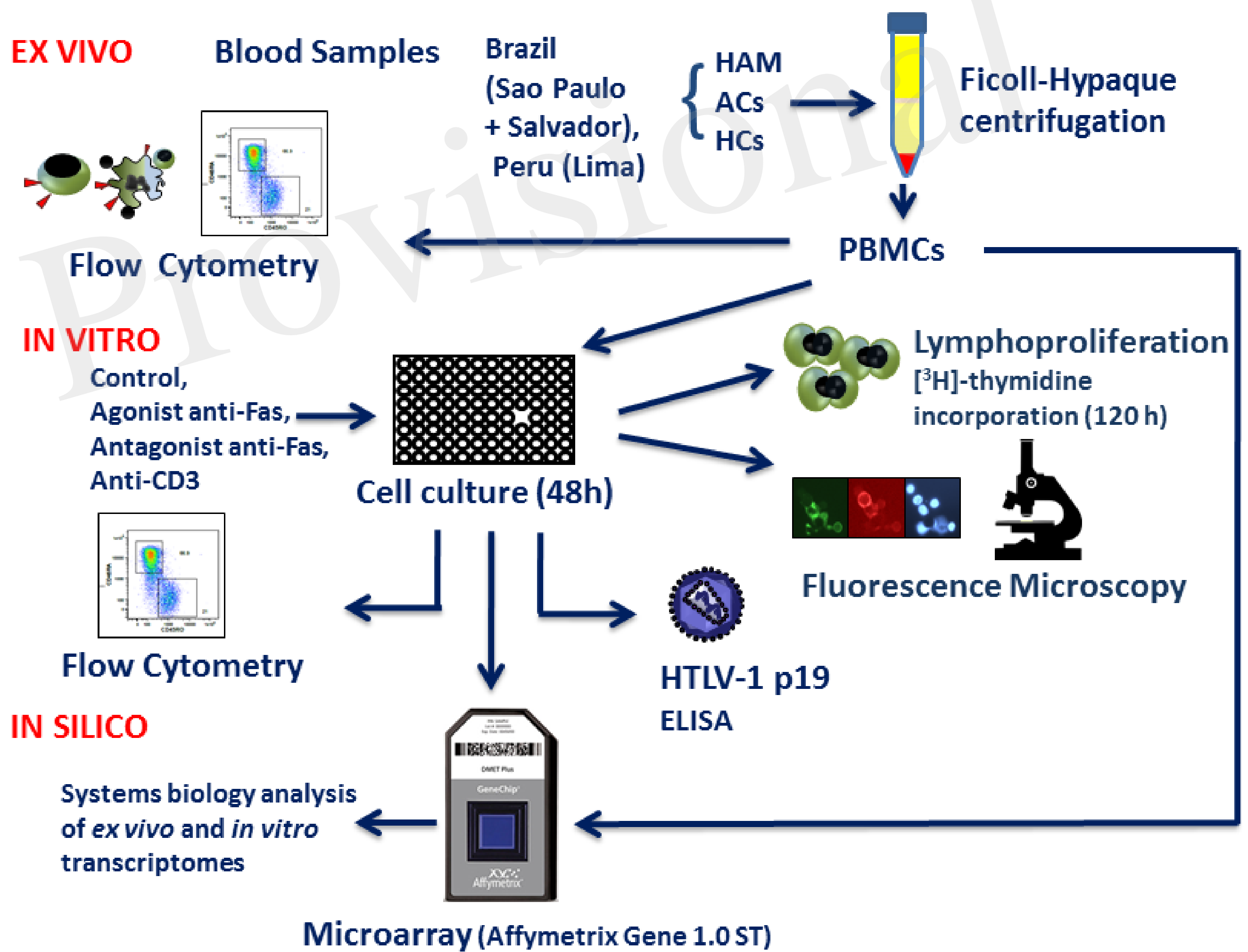

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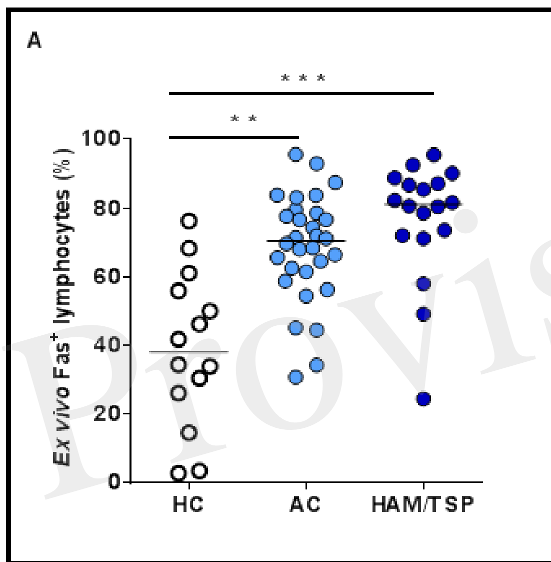
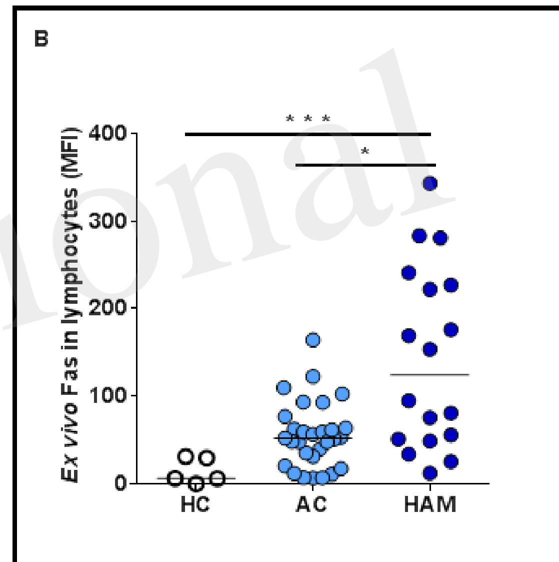
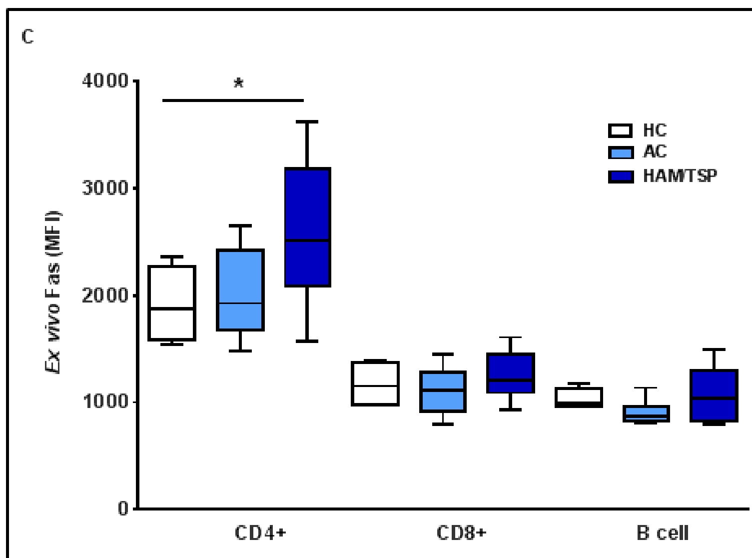
Figure 02.TIF

Figure 2



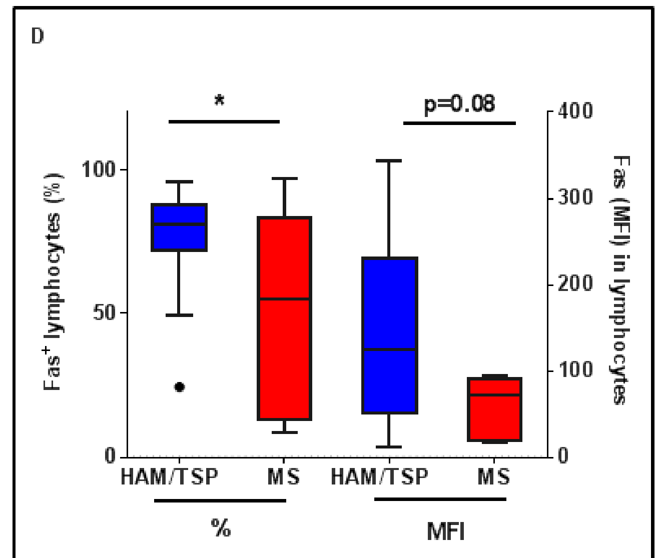




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Figure 03.TIF

Figure 3

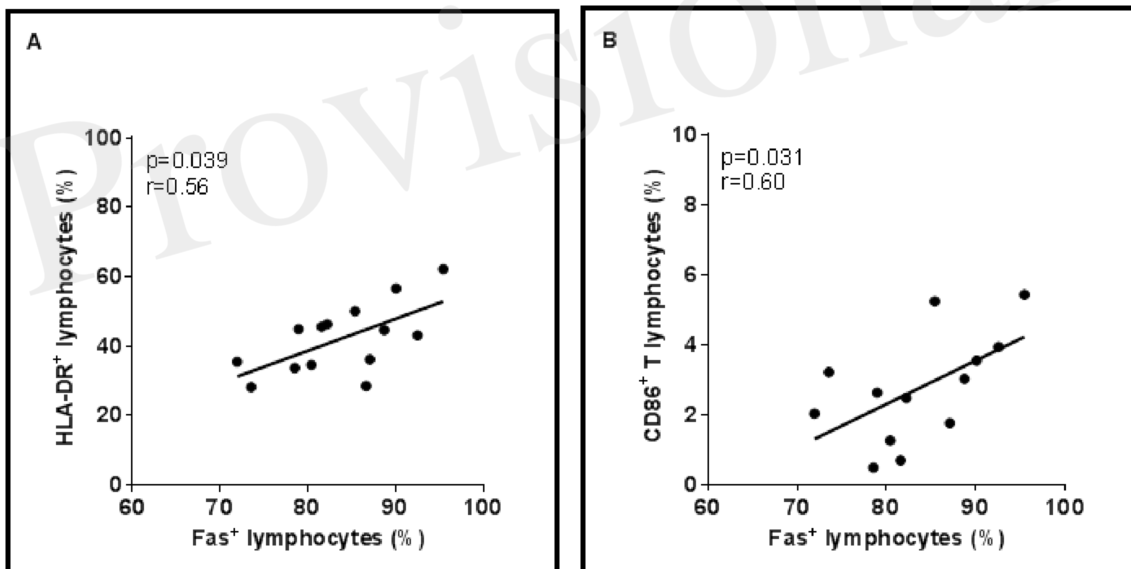


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Figure 04.TIF

Figure 4

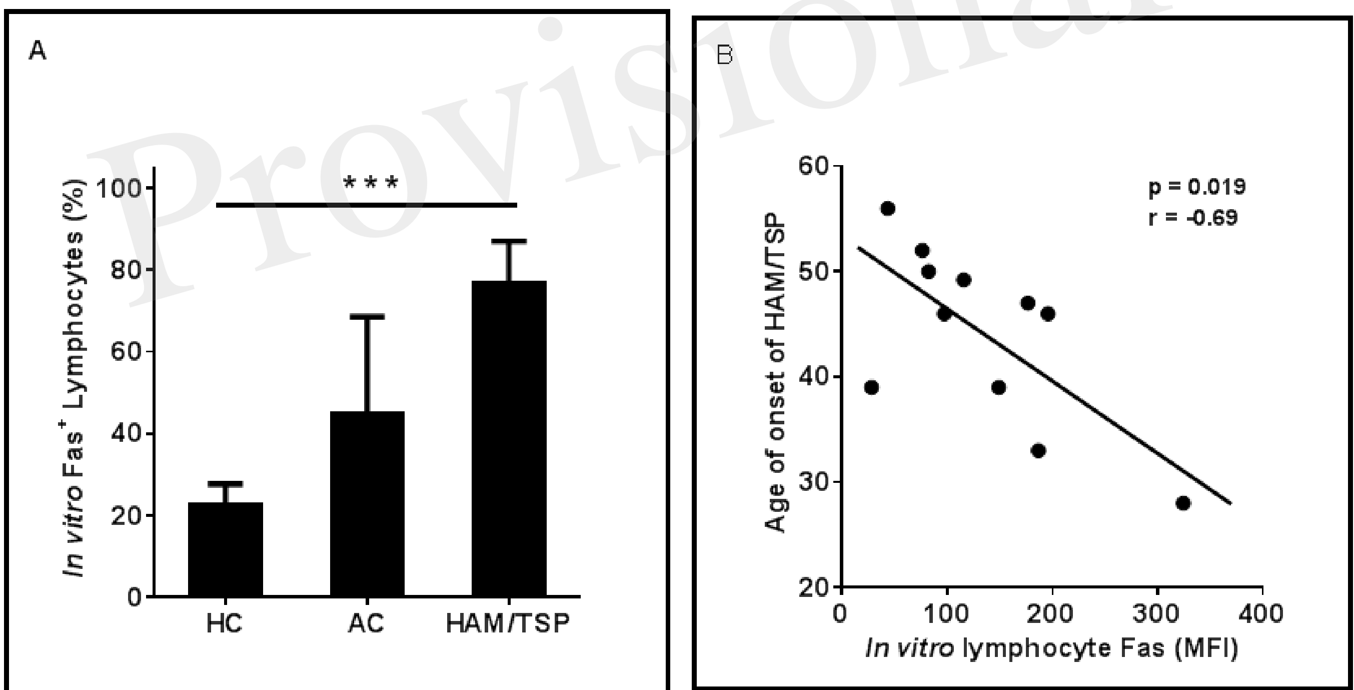


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Figure 05.TIF

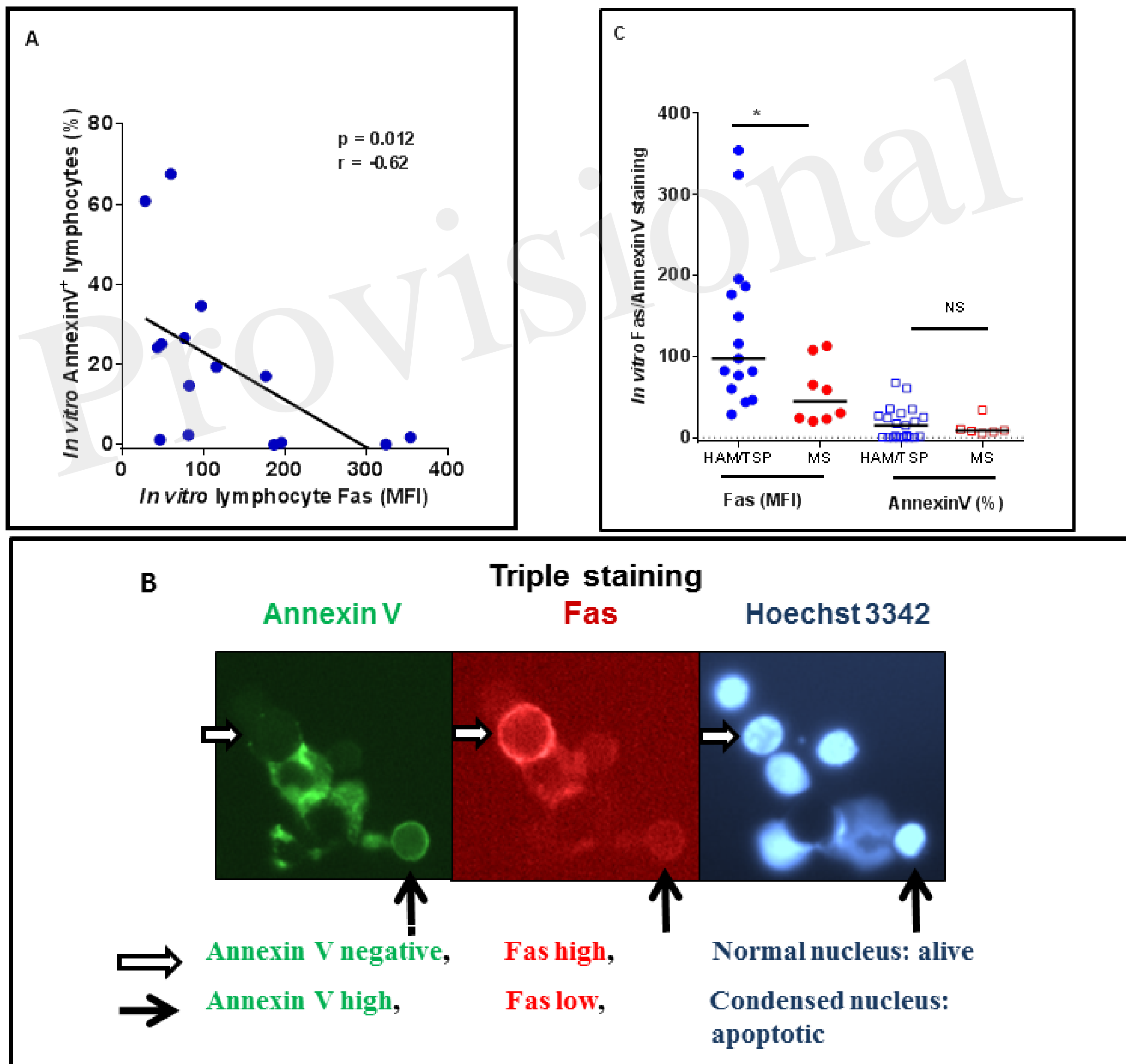


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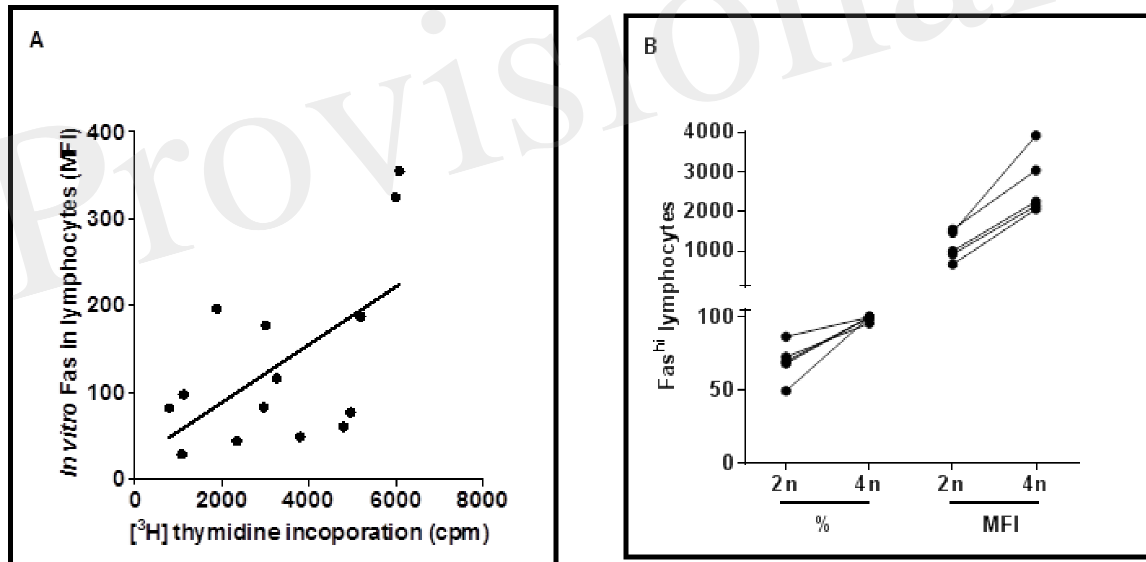
Figure 06.TIF

Figure 6

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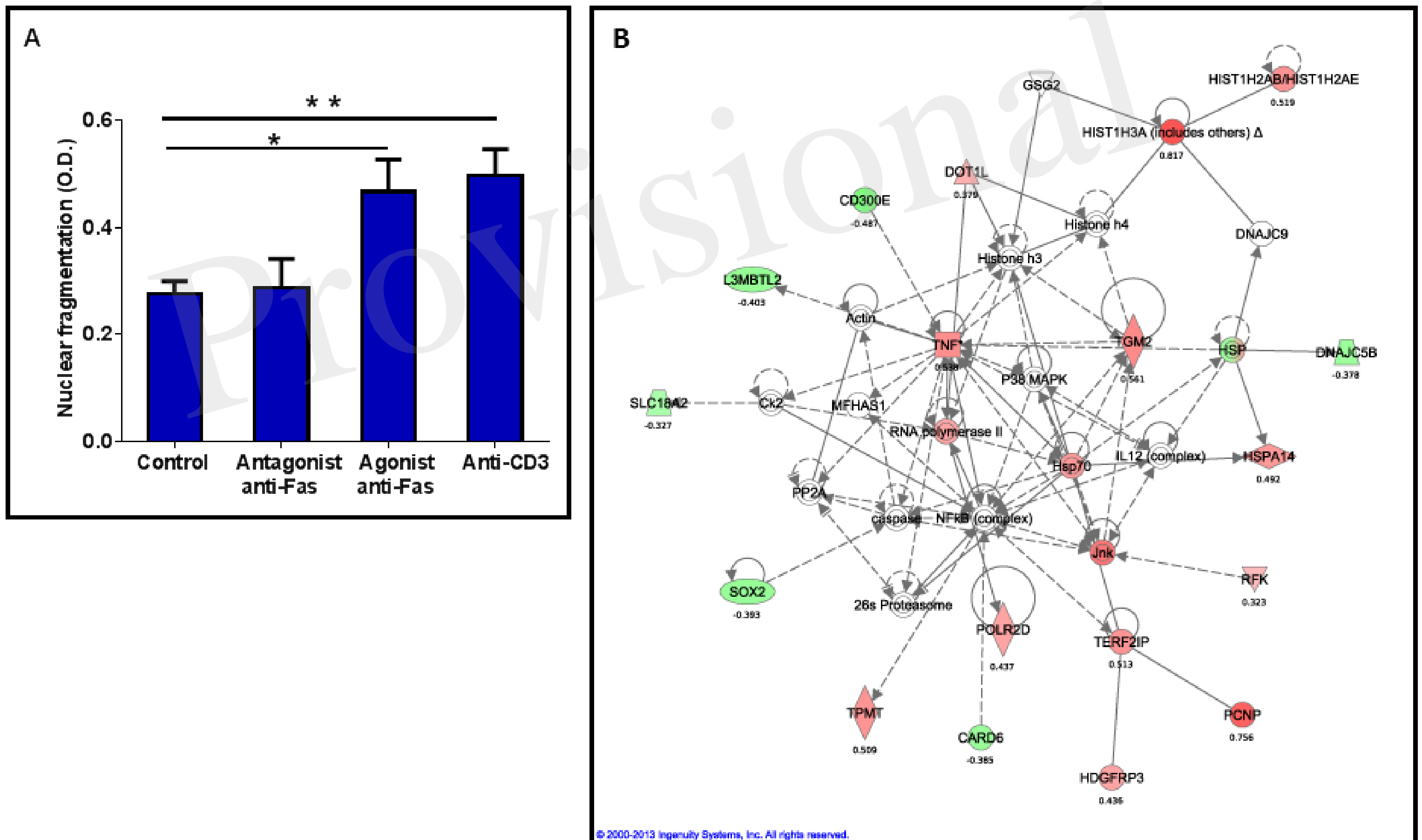

Figure 7
A
Nuclear fragmentation (O.D.)
0.0
0.2
0.4
0.6
* *
*
Control
Antagonist anti-Fas
Agonist anti-Fas
Anti-CD3
B
GSG2
HIST1H2AB/HIST1H2AE
0.519
HIST1H3A (includes others) Δ
0.817
DOT1L
0.379
CD300E
-0.487
Histone h4
DNAJC9
Histone h3
L3MBTL2
-0.403
Actin
TNF
0.538
TGM2
0.561
HSP
DNAJC5B
-0.378
P38 MAPK
SLC18A2
-0.327
Ck2
MFHAS1
RNA polymerase II
Hsp70
IL12 (complex)
HSPA14
0.492
PP2A
caspase
NFkB (complex)
Jnk
RFK
0.323
SOX2
-0.393
26s Proteasome
POLR2D
0.437
TERF2IP
0.513
TPMT
0.509
CARD6
-0.385
PCNP
0.756
HDGFRP3
0.436
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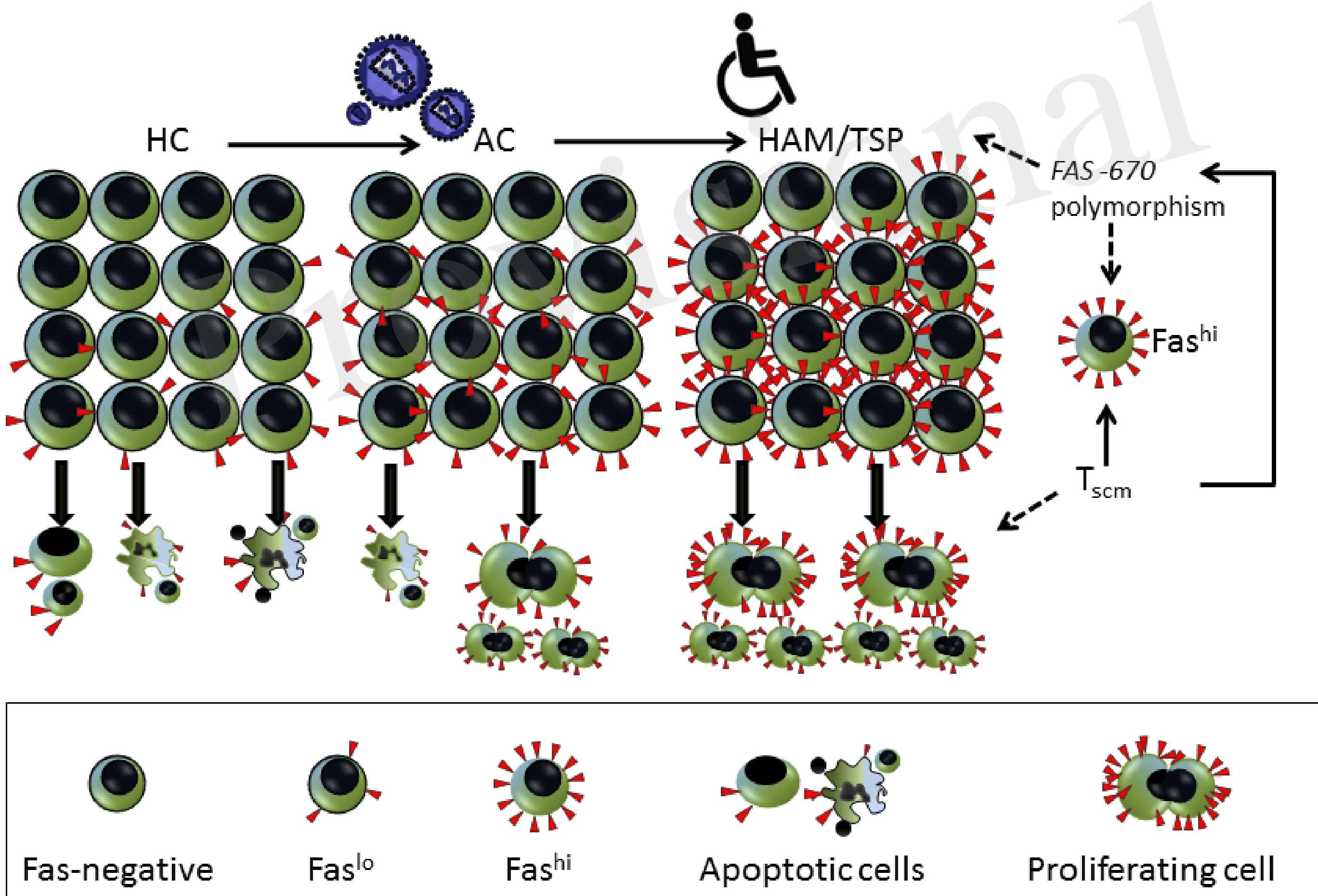
Figure 08.TIF



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