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Early Divergence in Lymphoid Tissue Apoptosis between Pathogenic and Nonpathogenic Simian Immunodeficiency Virus Infections of Nonhuman Primates

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1
2 Early divergence in lymphoid tissue apoptosis between pathogenic and non-
3 pathogenic SIV infections of non-human primates.

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27
28 * This work is dedicated to Bruno Hurtrel

29

Abstract

31

32 The events that contribute to the progression to AIDS during the acute phase of a
33 primate lentiviral infection are still poorly understood. In this study, we have used pathogenic
34 and non-pathogenic simian models of SIV infection of rhesus macaque (RM) and African
35 green monkeys (AGM), respectively, to investigate the relationship between apoptosis in
36 lymph nodes and its relationship with the extent of viral replication, immune activation and
37 disease outcome.

38 Here we show that, in SIVmac251-infected RMs, a marked increased in lymphocyte
39 apoptosis is evident during primary infection at the level of lymph nodes. Interestingly, the
40 levels of apoptosis correlated with the extent of viral replication and the rate of disease
41 progression to AIDS, with higher apoptosis in RMs of Indian genetic background when
42 compared to those of Chinese origin. In stark contrast, no changes in the levels of lymphocyte
43 apoptosis were observed during primary infection in the non-pathogenic model of SIVagm-
44 sab infection of AGMs despite similarly high viral replication. A further and early divergence
45 between SIV-infected RMs and AGMs was observed in terms of dynamics of T and B cell
46 proliferation in lymph nodes, with RMs showing significantly higher levels of Ki67⁺ cells in
47 the T cell zones in association with relatively low levels of Ki67⁺ in the B cell zones, whereas
48 AGMs displayed a low frequency of Ki67⁺ in the T cell area but a high proportion of Ki67⁺
49 cells in the B cell area. As such, this study suggests that species-specific host factors
50 determine an early immune response to SIV that is predominantly involves either cellular or
51 humoral immunity in RMs and AGMs, respectively.

52 Taken together, these data are consistent with the hypotheses that: (i) high levels of T
53 cell activation and lymphocyte apoptosis are key pathogenic factor during pathogenic SIV
54 infection of RMs, and (ii) low T cell activation and apoptosis are determinants of the AIDS-
55 resistance of SIVagm-infected AGMs despite high levels of SIVagm replication.

56

57

58 **Introduction**

59

60 While rhesus macaques (RMs) infected with simian immunodeficiency virus
61 (SIVmac) usually progress to AIDS in 1-2 years, African non-human primates (NHPs)
62 infected with their species-specific SIV rarely develop disease. Previous studies of natural,
63 non-pathogenic primate models of SIV infection, such as SIVagm infection of African green
64 monkeys (AGMs), SIVsmm or SIVmac infection of sooty mangabeys (SMs), and SIVmnd-1
65 and SIVmnd-2 infection of mandrills (MNDs) have consistently shown that, in these animals,
66 the levels of plasma viral load are similar to those observed in human immunodeficiency virus
67 (HIV)-infected humans and SIVmac-infected rhesus macaques (RMs) (2, 6, 10, 16, 18, 19,
68 29, 32, 35, 39). However, only HIV-infections in humans and SIVmac infections in RMs lead
69 to progressive CD4⁺ T-cell depletion and AIDS. Understanding the basis of pathogenic and
70 nonpathogenic host-virus relationships is likely to provide important clues regarding AIDS
71 pathogenesis.

72

73 The primary acute phase of HIV and SIV infections is characterized by an early burst
74 of viral replication, an exponential increase in viral load, the dissemination and seeding of the
75 virus in all peripheral lymphoid organs, a severe depletion of memory CD4⁺ T cells from the
76 mucosal-associated lymphoid tissue (MALT), and the induction of the host immune response
77 against the virus (21, 24, 27, 41, 45). Several observations indicate that the early induction of
78 an effective immune response against the virus plays a role in determining levels of viral load
79 at the end of the primary phase (i.e., set-point). The levels of viral load in peripheral blood is a
80 strong predictor of disease progression in pathogenic lentiviral infection (21, 31, 36, 37, 45).
81 Moreover, increased level of immune activation in pathogenic lentiviral infection is correlated
82 with disease progression towards AIDS (5, 27, 39). Importantly, the early stages of non-
83 pathogenic SIV infection of AGMs and SMs are also characterized by a peak of virus
84 replication in peripheral blood accompanied by rapid dissemination of the virus and depletion
85 of CD4⁺ T-cells from the MALT (19, 32). However, in these non-progressing hosts, it has
86 been shown that the level of the immune activation remains relatively low when compared to
87 RMs (2, 8, 19, 32, 40).

88

89 An increased lymphocyte susceptibility to apoptosis, that is in turn though to be
90 related to heightened levels of immune activation, has been proposed as one of the main

91 mechanisms responsible for the CD4⁺ T-cell depletion *in vivo* during pathogenic HIV and SIV
92 infections (1, 12, 17). Studies performed in pathogenic and non-pathogenic primate models of
93 SIV-infection during the asymptomatic phase have further suggested the presence of a direct
94 correlation between progression to AIDS and the level of CD4⁺ T cell apoptosis *ex vivo* (8,
95 40, 44). We recently reported that primary SIVmac251 infection of RMs of Chinese origin,
96 that are known to progress to AIDS more slowly than Indian monkeys (22, 34), is also
97 associated with increased numbers of apoptotic cells in the lymph nodes (25, 43). The reasons
98 why the course of SIVmac infection is milder in Chinese RMs (as compared to Indian RMs)
99 are still poorly understood. Since SIVmac strains were propagated either *in vivo* or *in vitro* in
100 cells derived from Indian monkeys, this may have resulted in the selection of viral variants
101 that are adapted for more efficient replication and/or increased pathogenicity for these animals
102 (34).

103

104 Here we reports the results of a comparative study of the initial interactions between
105 SIV and the host immune system in two different species of non-human primates (NHP) that
106 exhibit a divergent outcome of the infection, i.e., non-natural RM hosts, where the infection
107 leads to CD4⁺ T cell depletion and AIDS, and natural AGMs hosts, where the infection is
108 typically non-pathogenic. To investigate the potential immunopathogenic role of lymphocyte
109 apoptosis in lymph nodes occurring during the primary phase of infection, we performed a
110 longitudinal study of 20 experimentally SIV-infected NHPs (6 Indian RMs and 8 Chinese
111 RMs infected with SIVmac251; and 6 African Green Monkeys infected with SIVagm-sab).
112 We examined the frequency of (i) SIV RNA⁺ cells, (ii) apoptotic cells (TUNEL⁺), (iii) TiA-1-
113 positive cells and (iv) cycling Ki67⁺ cells in this diverse array of pathogenic and
114 nonpathogenic primate models that are associated with peculiar rates of progression to AIDS
115 (or lack thereof). Our data demonstrate that the early levels of apoptosis and immune
116 activation are key events associated with progression to AIDS.

117

118 **Animals and virus infection**

119 The following animals were include in this study: (i) Fourteen RMs of either Indian (n=6) or
120 Chinese (n=8) origin were intravenously inoculated with ten 50% animal infectious doses
121 (AID₅₀) of the pathogenic SIVmac251; and (ii) Six AGMs of sabaeus species were
122 experimentally infected with 300 TCID₅₀ of SIVagm.sab92018 strain. As previously
123 described, this virus derives from a naturally infected AGM and has never been cultured *in*
124 *vitro* (6). The pathogenic SIVmac251 isolate was initially provided by R. Desrosiers and
125 titrated in Chinese rhesus macaques (*Macaca mulatta*) by intravenous inoculation. A 1-ml
126 volume of stock virus contained 4×10^4 50% animal infectious doses (AID₅₀) as previously
127 described (25). Animals were demonstrated as being seronegative for STLV-1 (Simian T
128 Leukemia Virus type-1), SRV-1 (type D retrovirus), herpes-B viruses, and SIVmac. Animals
129 were housed and cared for in compliance with existing French regulations
130 (<http://www.pasteur.fr/recherche/unites/animalerie/fichiers/Decret2001-486.pdf>).

131

132 **Determination of viral load and quantitative assessment of productively infected cells**

133 RNA was extracted from plasma of SIV-infected monkeys, using the TRI REAGENT BD Kit
134 (Molecular Research Center Inc., Cincinnati, Ohio). Real-time quantitative reverse
135 transcriptase-polymerase chain reaction (RT-PCR) was used to determine viral loads as
136 previously described (25). The inguinal and axillary lymph nodes (LNs) were sequentially
137 collected from each animal during the course of primary infection. The LNs were frozen in
138 isopentane cooled in liquid nitrogen and then cryostat sectioned at 4 μm intervals. Viral
139 replication in LNs was assessed by *in situ* hybridization using a ³⁵S-labeled RNA probe as
140 previously described (25). Infected cells, detected as spots, were counted in the paracortical
141 zone on a minimum of three sections using a Nikon-FXA microscope. The number of positive
142 cells (counted as spots) was then divided by the surface of the entire LN section, and results
143 were expressed as the number of positive cells per 2mm² section. The mean count obtained
144 for three slides of the same LNs, in a blinded fashion, by two investigators, was calculated.

145

146 **Quantitative assessment of apoptotic cells**

147 Apoptotic cells were identified using the TUNEL assay, which detects DNA fragmentation
148 usually associated with apoptosis as previously described (25). Briefly, cryostat tissue
149 sections were fixed in 2% paraformaldehyde for 10 min, and overlaid with TUNEL reaction
150 mixture containing terminal deoxyribonucleotidyl transferase (Boehringer Mannheim,
151 Mannheim, Germany) and Dig-deoxyuridine triphosphate. The sections were incubated for 1

152 h at 37°C. The sections of LN were washed in phosphate-buffered saline and incubated with a
153 sheep anti-Dig-alcaline phosphatase antibody for 40 min at 37°C and revealed with naphthol
154 AS-MX, fast red, levamisol. LN sections were counterstained using Harris haematoxylin and
155 mounted with gelatin. The number of positive cells in the T-cell areas was then divided by the
156 surface of the entire LN section, and results were expressed as the number of positive cells
157 per 2mm² section. The mean count obtained for three slides of the same LNs, in a blinded
158 fashion, by two investigators, was calculated.

159

160 **Quantitative assessment of cycling cells**

161 Cycling cells were assessed using a monoclonal antibody to the Ki67 nuclear proliferation-
162 associated antigen (MIB-1; Immunotech, Inc., Marseille, France) that is expressed at all
163 phases of the cell cycle except the G0 phase. Alkaline phosphatase was used as the
164 chromogen, and the slides were slightly counterstained with haematoxylin as previously
165 described (25). The numbers of LN-positive cells counted in the T-cell areas were then
166 divided by the surface of the entire LN section, and results were expressed as positive cell
167 numbers/2 mm² section. The proportion of cycling cells (%GCKi67%) in the B-cell areas
168 (Germinal Centers, GCs) was quantified and divided by the surface of the entire LN section.
169 The proportion obtained for three slides of the same LNs, in a blinded fashion, by two
170 investigators, was calculated.

171

172 **Quantitative assessment of TiA1 cells**

173 TiA1 cells were assessed using a monoclonal antibody (clone GMP-17, Beckman Coulter).
174 Alkaline phosphatase was used as the chromogen, and the slides were slightly counterstained
175 with haematoxylin. The proportion of TiA1-positive cells counted in the T-cell areas were
176 then divided by the surface of the entire LN section, and results were expressed as %. These
177 analyses were performed on four different sections, in a blind fashion, by two investigators.

178

179 **Lymphocyte immunophenotyping by flow cytometry**

180 T-cells were stained with the following fluorochrome-labeled monoclonal antibodies: anti-
181 rhesus monkey CD3-conjugated fluorescein-isothiocyanate (FITC) (clone FN18, Biosource
182 International), anti-human CD4 conjugated with phycoerythrin (PE) (clone M-T477, BD
183 Biosciences), anti-human CD8-conjugated with peridinin-chlorophyll protein (PerCp) (BD
184 Biosciences) and anti-human leukocyte antigen (HLA)-DR-conjugated with allophycocyanin
185 (APC) (L243, BD Biosciences). Antibodies were added to 100 µl of 2x10⁵ LN cells. Cells

186 were incubated for 15 min at room temperature. The cells were then washed once in PBA
187 buffer (phosphate-buffered saline-1% bovine serum albumin-10 mM NaN₃) and resuspended
188 in PBS containing 1% paraformaldehyde (PBS-PF) as previously described (reference).

189

190 **Statistical analyses**

191 The Student's t tests and Mann-Whitney U-test (Prism software) were used to determine
192 whether differences were significant if $p < 0.05$. Mann-Whitney U-test was used to compare
193 results between monkey species. Spearman's rank correlation, implemented from Statistica
194 software, was use to evaluate correlations. Best-fit lines are shown.

195

ACCEPTED

196 Results

197

198 1) Viral replication and disease outcomes

199 Rhesus macaques (RMs) of Indian genetic background progressed more rapidly
200 towards AIDS than Chinese RMs despite receiving similar viral stock of SIVmac251 strain
201 that was produced in PBMCs of Chinese monkeys. In this study, all Indians RMs were
202 sacrificed due to the onset of AIDS-related symptoms before one year of infection, while half
203 of the SIV-infected Chinese RMs was alive after 36 months of infection. As expected, all the
204 SIV-infected AGMs were alive at >48 months post infection (**Figure 1A**).

205

206 During acute SIV infection, the initial burst in viral replication causes the exponential
207 increase in plasma viral load that leads to the dissemination of the virus throughout the body
208 and the infection of the CD4⁺ T cells in the MALT (42) as well as in peripheral lymphoid
209 organs, which are the main sites of both virus replication and immune response to the virus.
210 As shown in **Figure 1B**, we first assessed the dynamics of viral load in these different groups
211 of SIV-infected NHPs. In RMs, viremia peaked between days 11 and 14 with values ranging
212 between 7.5×10^6 and 7×10^7 copies/ml in RMs of Indian origin and between 6.7×10^5 and
213 3.2×10^7 copies/ml in Chinese RMs. In AGMs, viremia peaked between days 8 and 14 and the
214 values ranged between 7.9×10^6 and 1.2×10^8 copies/ml. The set-point levels in AGMs ranged
215 between 1.3×10^3 and 2.5×10^5 copies/ml whereas in Indian RMs and Chinese RMs it ranged
216 between 3×10^4 and 1.8×10^7 copies/ml and 3×10^2 and 5.7×10^5 copies/ml, respectively. Thus,
217 these data are consistent with numerous previous observations (13, 19, 21, 25, 27, 32, 41, 45)
218 in indicating that both peak and set-point viremia are similar in pathogenic and non-
219 pathogenic primate models.

220

221 To longitudinally assess the relationship between the level of the early viral
222 dissemination/seeding and the immune system dynamics (i.e., proliferation and cell death),
223 we sequentially collected the inguinal and axillary lymph nodes (LNs) from each animal
224 during the course of primary infection. In all examined tissues, SIV RNA⁺ cells were
225 promptly detected, in the pathogenic as well as in the non-pathogenic models (**Figure 2A**).
226 During the acute phase of SIVmac251 infection of RMs, the peak numbers of productively
227 infected cells per surface unit in the LN T-cell area was reached at days 11-14 post-infection,
228 i.e., coincident with the peak of viral loads, and was then followed by a significant decrease
229 by day 60 (**Figure 2B**). Interestingly, the level of viral replication in LN was significantly

230 higher in RMs of Indian origin when compared to Chinese RMs, with peak numbers of
231 productively infected cells of 204.8 ± 125 SIV RNA⁺ cells/2mm² and 30.8 ± 19.7 SIV RNA⁺
232 cells/2mm², respectively (P=0.007, using Mann-Whitney U-test) (**Figure 2B**). Similarly, the
233 frequency of CD4⁺ T cells harboring proviral DNA at the peak values was higher in Indian
234 than in Chinese RMs (0.043 ± 0.038 and 0.017 ± 0.009 , respectively, data not shown). This
235 difference persisted at day 60, with the numbers of SIV RNA⁺ cells/2mm² higher in Indian
236 than in Chinese RMs (14.2 ± 11.2 and 7.9 ± 8.8 , respectively). Most importantly, we found
237 that the numbers of SIV RNA⁺ cells/2mm² in AGMs at the peak of virus replication ($21 \pm$
238 12.5) were relatively similar to those observed at the peak in SIVmac251-infected Chinese
239 RMs (**Figure 2B**). However, it should be noted that, at day 60, the number of SIV RNA⁺
240 cells/2mm² in LNs was extremely low in AGMs (0.53 ± 0.22), despite similar levels of viral
241 loads in these two species.

242
243 These findings suggest that the level of peak viral replication in the LNs is not
244 discriminative of pathogenic and non-pathogenic primate models of SIV infection (with
245 Indian RMs showing, nevertheless, higher frequencies of infected cells and faster disease
246 progression than Chinese RMs). Interestingly, the level of viral replication in peripheral LNs
247 during the set-point phase of SIV infection was higher in SIVmac251-infected RMs than in
248 SIVagm-sab-infected AGMs, thus suggesting that other anatomic sites (i.e., MALT, spleen
249 etc) are responsible for the high plasma viremia of AGMs.

250 251 2) Lymph node apoptosis is a typical feature of pathogenic SIV-infections

252 We then sought to determine, in the same groups of SIV-infected NHPs, the extent of
253 apoptotic cells in LNs. The numbers of apoptotic cells were quantified in the T-cell areas of
254 tissue sections from the same LN in which numbers of SIV-infected cells were previously
255 assessed (**Figure 3A**). We found that the number of apoptotic cells increased after infection
256 and reached their highest levels at the time of peak viral replication in both Indian and
257 Chinese RMs (Day 14, 138 ± 66 cells/2mm² and 77 ± 16 cells/2mm², respectively, P = 0.04
258 using Mann-Whitney U-test). The numbers of apoptotic cells remained higher at day 60
259 (Indian, 58 ± 59 cells/2mm² *versus* Chinese, 16 ± 7 cells/mm²; P = 0.04) than before infection
260 (8 ± 6 cells/mm²) (**Figure 3B**). Importantly, a significant direct correlation was found in
261 SIVmac251-infected RMs between the level of viral replication within LNs (as measured at
262 the time of peak viremia) and the numbers of apoptotic cells (**Figure 3C**). In SIVagm-
263 infected AGMs the level of T-cell apoptosis in the LNs remained similar to baseline

264 throughout the acute phase of infection (Day 13, 25 ± 28 cells/ 2mm^2 and day 60, 27 ± 9
265 cells/ 2mm^2). The observation that T cell apoptosis does not increase in AGMs during early
266 SIV infection despite levels of viral replication that are similar to those observed in
267 SIVmac251-infected Chinese RMs suggests that indirect (i.e., non virus-related) mechanisms
268 rather than the direct effect of viral-replication are responsible for the increased rate of
269 apoptotic cells in RMs.

270

271 3) Dynamics of cycling cells in the T cell area

272 To investigate the relationship between the extent of LN apoptosis and the level of
273 immune activation (that results in frequent events of activation-induced cell death (17)), we
274 first assessed the level of activated (Ki67⁺) lymphocytes in our group of animals. We found
275 that, in both Indian and Chinese SIVmac251-infected RMs, the paracortical areas (T-cell
276 zone) contained more activated cells than those of uninfected RMs (**Figure 4**). The number of
277 cycling cells peaked at days 11-14 post-infection (Day 14, 2537 ± 1154 cells/ 2mm^2 and 2430
278 ± 1130 cells/ 2mm^2 in Indian and Chinese RMs, respectively) and remained much higher than
279 baseline at day 60 (1655 ± 1099 cells/ 2mm^2 and 1626 ± 502 cells/ 2mm^2 , in Indian and
280 Chinese RMs, respectively, versus Day 0, 45 ± 25 cells/ 2mm^2) (**Figure 4B**). Of note, the
281 differences in Ki67⁺ cells between Indian and Chinese RMs were not statistically significant,
282 thus suggesting that the higher level of apoptotic cells observed during primary SIVmac251
283 infection of Indian RMs is not simply a consequence of greater numbers of cycling T cells.

284

285 We next measured the numbers of cycling lymphocytes in SIVagm-infected AGMs,
286 and found that cycling cells peaked at day 8 (739 ± 173 cells/ 2mm^2 versus day 0, 133 ± 103
287 cells/ 2mm^2 , $P = 0.002$) with a slight decrease thereafter (Day 60, 596 ± 110 cells/ 2mm^2)
288 which was coincident with the decline of viral replication in LNs (**Figure 4B**). While SIV-
289 infected AGMs show a clear increase in the numbers of cycling T cells when compared to
290 baseline (i.e., prior to infection), the absolute levels of T cell activation in the LNs of these
291 animals are approximately one third lower of those observed in SIVmac251-infected RMs
292 (**Figure 4B**). Therefore, this result is consistent with previous reports indicating that non-
293 pathogenic SIV infection of natural hosts is associated with lower levels of immune activation
294 (8, 40).

295

296 To further characterize the dynamics of T cell activation in our group of NHPs, we
297 next assessed by flow cytometry the changes in the levels of CD3⁺CD4⁺ and CD3⁺CD8⁺ T

298 cells expressing HLA-DR in peripheral blood. Interestingly, SIVmac251-infected Indian RMs
299 revealed a profound decrease in the percentage of CD4⁺DR⁺ T cells, while only a transient
300 decline of CD4⁺DR⁺ T cells was observed at the peak of virus replication in Chinese monkeys
301 (**Figure 5**). Moreover, we found an early increase in the fraction of CD8⁺DR⁺ T cells in
302 Chinese RMs that still persists at the time of set-point viremia (**Figure 5**). In SIV-infected
303 AGMs, we found a transitory increase in the activation status of CD8⁺ T cells (but not CD4⁺
304 T cells), with levels of CD8⁺DR⁺ T cells peaking at day 14 but soon returning to levels similar
305 to those observed prior to infection. This pattern of T cell activation in SIVagm-infected
306 AGMs is very similar to that previously reported by our and other laboratories (19, 32).

307

308 While our data failed to identify any significant correlation between the extent of T
309 cell activation and the rate of apoptosis in pathogenic SIVmac251 infection of RMs, it was
310 clearly apparent that, in the non-pathogenic SIVagm infection of AGMs, an attenuated level
311 of T cell activation was associated with lower levels of apoptosis.

312

313 4) Dynamics of cycling cells in the B cell area

314 We next assessed the level of activated (Ki67⁺) cells in the B-cell zone by staining the
315 germinal centers (GC). In contrast to the large increase of cycling cells in the T cell area
316 observed in SIVmac251-infected RMs, no changes in the level of Ki67⁺GC⁺ (% Ki67⁺GC⁺)
317 was found in the same animals at the peak of viral replication. Interestingly, the level of B-
318 cell activation within the GC was three- to five-fold higher in SIV-infected AGMs than in
319 RMs at day 60, with 29.3% ± 9.7 of Ki67⁺GC⁺ in AGMs, 11.2% ± 8.1 in Chinese RMs, 6.2%
320 ± 3.1 in Indian RMs (P = 0.008), and 0.155 % ± 0.25 in uninfected animals (**Figure 4C**). We
321 also found that the percentage of CD20⁺CD3⁻ cells (B cells), as analyzed by flow cytometry at
322 day 60 p.i., was increased in LNs of SIV-infected AGMs (28.3% ± 3.5) when compared to
323 preinfection values (15.2% ± 1.7). These dynamics are consistent with the time of detection of
324 SIV-specific antibodies in the plasma that appear between days 30 and 60 (data not shown),
325 and suggest that immune responses to SIV involve predominantly the humoral immunity in
326 AGMs.

327

328 4) Dynamics of TiA-1+ cells

329 To further characterize the early activation of CD8⁺ T cells observed during SIV
330 infection of RMs and AGMs, we examined the expression of cytolytic granules by staining
331 for TiA-1, i.e., a polyadenylate binding protein localized to the granules of cytolytic T cells

332 that is functionally related to perforin. In these experiments we assessed TiA-1 expression *in*
333 *situ* to exclude the possibility that isolated lymphocytes might release their granule contents
334 during isolation procedure (**Figure 6**). We found that, in the LNs of SIVagm-infected AGMs,
335 the fraction of TiA-1 expressing cells showed an early moderate increase (Day 8, $0.5\% \pm 0.3$
336 *versus* Day 0, $0.13\% \pm 0.1$; $P = 0.018$) followed by a slight decline by the time of set-point
337 viremia (Day 60, $0.25\% \pm 0.2$) (**Figure 6B**). As such, the dynamics of TiA-1 expression in
338 AGMs followed the dynamics observed for the proportion of activated CD8⁺ T-cells.

339

340 In SIVmac251-infected RMs, the frequency of TiA-1 expressing T-cells increased
341 earlier in Indian animals (as compared to Chinese). However, at day 60 post-infection the
342 level of TiA-1 reactivity in SIVmac251-infected Chinese RMs was greater than before
343 infection ($6.5\% \pm 1.5$ versus $1.9\% \pm 0.39$; $P = 0.006$) and greater than in Indian RMs ($3.9\% \pm$
344 1.6 ; $P=0.02$) (**Figure 6B**). This dynamics of TiA-1 expression in LNs during SIVmac251
345 infection of RMs was consistent with the observed pattern of CD8⁺DR⁺ T cell dynamics.

346

347 Discussion

348 In this study, we report the results of a detailed longitudinal study of a large number
349 ($n=20$) of non-human primates experimentally infected with SIV, including 6 Indian and 8
350 Chinese RMs infected with SIVmac251, and 6 AGMs infected with SIVagm-sab. The key
351 goal of this study was to assess, in the different non-human primate models of infection, the
352 relationship between virus replication, immune activation, lymphocyte apoptosis, and disease
353 progression. A key premise of this study is the observation that, in striking contrast to HIV-
354 infected humans and SIVmac-infected RMs, natural SIV hosts, such as AGMs, SMs, and
355 mandrills, remain typically asymptomatic despite similarly high levels of virus replication (2,
356 6, 8, 14, 15, 19, 29, 32, 40). It is now widely accepted that understanding the mechanisms
357 underlying the different infection outcomes in natural versus non-natural SIV hosts will
358 provide important clues as to the mechanisms of AIDS pathogenesis.

359

360 SIVmac infection of Chinese RMs is less pathogenic than that of Indian RMs and thus
361 more closely mimics the course of HIV infection in humans (5, 26, 27). As some of this
362 earlier experiments were conducted using virus preparations obtained through repeated serial
363 passage in cells from Indian RMs (or in Indian RMs *in vivo*), it was proposed that increased
364 viral fitness from Indian RM cells may explain the higher replicative capacity and faster
365 disease progression in these animals. However, in our experiments, we used a SIVmac251

366 strain that has been *in vitro* passaged in PBMC derived from Chinese RMs. We still observe
367 an accelerated course of disease in Indian RMs, thus suggesting that host determinants, rather
368 than intrinsic properties of SIV are key determinants of the divergent infection outcomes in
369 RMs.

370

371 Our study also confirmed and extended previous evaluations of viral dynamics in
372 peripheral blood demonstrating that levels of viral replication in RMs and AGMs are quite
373 similar. Importantly, by the end of the acute phase of infection, a clear difference emerged
374 between AGMs and RMs in term of viral replication in LNs, with numbers of SIV RNA⁺ cells
375 that were significantly lower in SIV-infected AGMs compared with those observed in RMs
376 (Indian RMs being higher than Chinese RMs). These data indicate that the level of virus
377 replication in the LNs at the end of the acute phase may be a good predictor of disease
378 progression in RMs (25-27), and that, in AGMs, the level of viral particles in the blood likely
379 reflects virus replication occurring in sites other than peripheral LNs, such as the MALT or
380 spleen.

381

382 The data included in the current study strongly suggests that the extent of apoptotic
383 cell death occurring in the T-cell areas of the peripheral lymph nodes represents an early
384 pathogenic event that seems to determine the subsequent course of disease progression. The
385 fact that SIV_{agm}-infected AGMs do not display any major increase in T-cell apoptosis in the
386 context of significant levels of viral replication suggests that the direct effects of SIV
387 replication alone are unlikely to account for the high levels of T cell apoptosis observed in
388 RMs at the time of peak viremia. Instead, our observation is consistent with a model whereby
389 large numbers of T cells undergo apoptosis as a consequence of a massive event of immune
390 activation. Interestingly, in RMs, we found a positive correlation between the level of viral
391 replication in the LNs and the extent of T cell apoptosis, suggesting either a role, at least
392 partial, for viral replication-mediated cell death in these animals or, alternatively, a role for
393 SIV antigens in determining—through the generation of virus-specific immune responses—
394 the prevailing levels of immune activation and apoptosis. In other studies we have shown that
395 the extent of apoptosis in peripheral LNs was greater in Chinese RMs infected with
396 pathogenic SIV_{mac} than in those infected with a *nef*-deleted SIV_{mac} strain and predicted, in
397 these animals, the risk of progression to AIDS (25, 43). Altogether, these results suggest that
398 in SIV-infected RMs, increased levels of apoptosis represent a key determinant of the
399 progressive immunodeficiency, thus consistent with previous reports performed during the

400 chronic phase of HIV and SIV infection (8, 9, 12, 17, 26, 27). In this context, it is tempting to
401 speculate that an additional deleterious effect of the increased T-cell death observed in
402 SIVmac251-infected RMs is the inability of the immune system to mount a sustained immune
403 response to the virus (43).

404

405 Altogether, these findings suggest that the level of lymphocyte apoptosis in the early
406 stages of SIV infection is a species-specific surrogate marker of AIDS pathogenesis. In this
407 study it was clearly apparent that, in the non-pathogenic SIVagm infection of AGMs, an
408 attenuated T cell activation was associated with lower levels of apoptosis during the acute
409 phase. This new finding is consistent with earlier reports indicating during the chronic phase
410 of SIV infection a relationship between the extent of immune activation, T cell apoptosis and
411 pathogenesis (8, 40). This absence of immune activation in SIV-infected AGMs might be
412 related to an early anti-inflammatory profile characterized by the induction of Treg cells (19).
413 Interestingly, SIV-infected AGMs showed LNs of lower size than RMs (data not shown), thus
414 further suggesting that the "total body" level of immune activation is significantly lower
415 during non-pathogenic infections of natural SIV hosts.

416

417 The increase of TiA1-expressing T cells in LNs concomitant with the observation of a
418 transient increase of activated CD8⁺ T cells (DR⁺) during the acute viral replication period in
419 AGMs is consistent with recent reports (13, 32). This observation supports the hypothesis that
420 the post-peak decline in virus replication observed in the LNs of SIVagm-infected AGMs
421 might be, at least in part, related to the emergence of cellular immune responses to the virus.
422 On the other hand, however, it should be noted that, to this date, there is no experimental
423 evidence indicating that in SIV-infected AGMs (or any other natural SIV hosts), the cellular
424 immune responses to the virus are broader or more potent/effective than those described in
425 pathogenic HIV-1 and SIV infections (7). Alternatively, the observed low levels of activated
426 CD4⁺ T cells (DR⁺) in SIV-infected AGMs may suggest that the post-peak decrease of viral
427 load in the LNs of SIVagm-infected AGMs is related to the rapid exhaustion of target cells
428 due to the attenuated levels of local T cell activation.

429

430 Interestingly, we found a clear difference in the dynamics of T and B cells between
431 AGMs and RMs. SIVmac251-infected RMs showed more extensive and persistent
432 proliferation of T cells than SIVagm-infected AGMs. On the contrary, SIVagm-infected
433 AGMs showed a more prominent B cell activation than SIVmac251-infected RMs, as

434 manifested by the level of Ki67⁺ cells in the GCs at the set point than RMs. This observation
435 is also consistent with two recent reports indicating that SIV infection of AGMs is associated
436 with high levels of Ki67⁺ cells in the GCs and rapid expansion of B cells (13, 32). While
437 these results seem at odd with the observation that the titer of SIV-specific antibodies are
438 lower in AGMs than RMs (28), it should be noted that high fractions of Ki67⁺ GC cells do not
439 necessarily translate in high titers of SIV-specific antibody. In any event, our intriguing
440 observation of an early and selective B cell response during non-pathogenic SIV_{agm} infection
441 of AGMs suggest that the role of SIV-specific humoral responses in primate lentiviral
442 infections should be further explored in future studies, particularly with respect to the impact
443 of SIV-specific antibodies in the control and/or compartmentalization of viral replication at
444 the end of the acute phase.

445

446 A question that remains to be answered is how host genetic factors may influence the
447 strikingly different level of lymphocyte apoptosis in LNs that we observed during early SIV
448 infection in the various NHP models studied. A first possibility is that individual differences
449 in the signaling pathways that participate in immune activation and apoptosis regulation may
450 lead to different sensitivity to death signal transduction induced either by viral proteins or by
451 death ligands and receptors (such as CD95 ligand and CD95) (9, 11). It has been recently
452 proposed a role for CD95/CD95L in the depletion of uninfected CD4⁺ T cells occurring
453 during primary infection in the lamina propria of SIV-infected RMs (20). As the extent of T-
454 cell activation is typically lower in AGMs than in RMs, species-specific differences in the
455 immunomodulatory activity of Treg cells might be beneficial in controlling T-cell activation
456 and activation-induced cell death (AICD) (19). A second possibility is that differences in the
457 induction of apoptosis are related to quantitative differences in the initial levels of co-
458 receptors expression. Several studies have suggested that co-receptor expression affect disease
459 progression in HIV-infected individuals (4, 23, 38) as the proportion of CD4⁺CCR5⁺ and/or
460 the density of cell-surface CCR5 correlate with disease progression (3, 30). Moreover, it has
461 been recently reported a difference in the level of CCR5 between pathogenic and non-
462 pathogenic primate models of AIDS (33). Finally, we recently demonstrated that in addition
463 to CCR5, ligation of GPR15 (BOB), an additional co-receptor utilized by the SIV_{mac251}
464 strain, mediates CD4⁺ T cell death (43). However, dynamics of CD4⁺ T cells expressing this
465 latter co-receptor are still unknown.

466

467 In conclusion, the current set of data is clearly consistent with the hypothesis that the
468 prevailing levels of immune activation and T cell apoptosis are key marker of disease
469 progression during pathogenic primate lentiviral infections. Similarly, this work supports the
470 concept that limited immune activation and low levels of T cell apoptosis are instrumental to
471 avoid progression to AIDS in AGMs and other natural SIV hosts as well.

472

473

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662 Legends:

663 Figure 1: (A) Month survival. The percentage of survival in three different groups of SIV-
664 infected monkeys (Indian rhesus macaques (RMs) and Chinese RMs infected with
665 SIVmac251; AGMs infected with SIVagm.sab92018) during the follow-up period of this
666 study is shown. Clinical progression towards disease was continuously evaluated. The
667 animals were killed for a wasting syndrome with cachexia and opportunistic infections. (B)
668 Viremia. Kinetic analysis of viremia in SIV-infected Indian and Chinese RMs, and AGMs.

669
670 Figure 2: Productive SIV infection in LNs from Indian and Chinese SIVmac251-infected
671 RMs, and SIVagm-infected AGMs at different time point post-infection. (A) SIV RNA⁺ cells
672 were detected in LNs of SIV-infected monkeys (magnification, x100). (B) Quantitative
673 assessment of SIV RNA⁺ cells. Statistical significance was assessed using paired Student t
674 test.

675
676 Figure 3: Immunohistochemical analysis of apoptotic cells in LNs from Indian and Chinese
677 SIVmac251-infected RMs, and SIVagm-infected AGM at different time points after infection.
678 (A) Apoptotic cells measured by Tunel method (magnification, x100). (B) Quantitative
679 assessment of apoptotic cells. Statistical significance was assessed using paired Student t test.
680 (C) Correlation between the extent of viral replication (LogSIV RNA⁺) and the extent of
681 apoptosis at the peak. Each symbol represents one individual RMs either from Indian (○) or
682 Chinese (●) RMs.

683
684 Figure 4: Immunohistochemical analyses of cycling cells in LNs from Indian and Chinese
685 SIVmac251-infected RMs, and SIV-infected AGMs at different time points post-infection.
686 (A) Ki67 staining was performed in LNs of SIV-infected monkeys (magnification, x100). (B)
687 Quantitative assessment of Ki67 in T-cell area, and (C) B-cell area (%GCKi67⁺). Statistical
688 significance was assessed using paired Student t test.

689
690 Figure 5: Dynamics of HLA-DR on CD4⁺ and CD8⁺ T cells in LNs. Flow cytometry was used
691 to quantify the percentage of T cells expressing HLA-DR gating on CD3⁺ cells of Indian and
692 Chinese SIVmac251-infected RMs, and SIV-infected AGMs at different time points post-
693 infection. Data shown were calculated as follow: $\text{DR}^+\text{CD4}/\text{CD8}^+ / (\text{DR}^+\text{CD4}/\text{CD8}^+ + \text{DR}^-$
694 $\text{CD4}/\text{CD8}^+) \times 100$. Statistical significance was assessed using paired Student t test.

695

696 Figure 6: Immunohistochemical analyses of TiA-1 from Indian and Chinese SIVmac251-
697 infected RMs, and SIV-infected AGMs at different time point post-infection. (A) TiA-1
698 staining was performed in LNs of SIV-infected monkeys (magnification, x100). (B)
699 Quantitative assessment of TiA-1 cells. Statistical significance was assessed using paired
700 Student t test.

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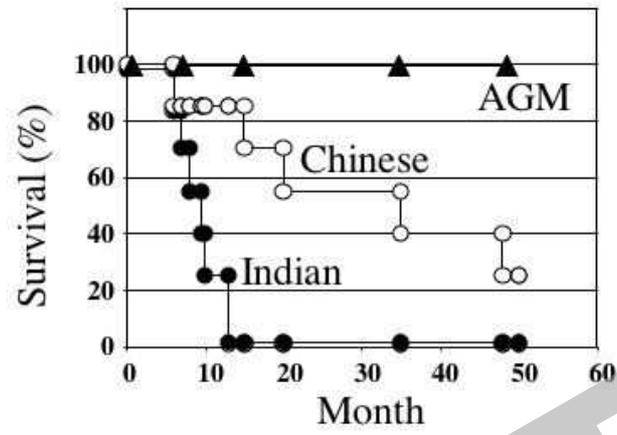
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ACCEPTED

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A



B

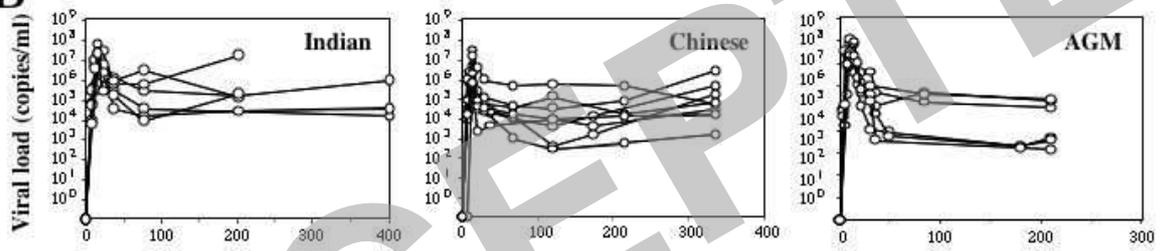


Figure 1

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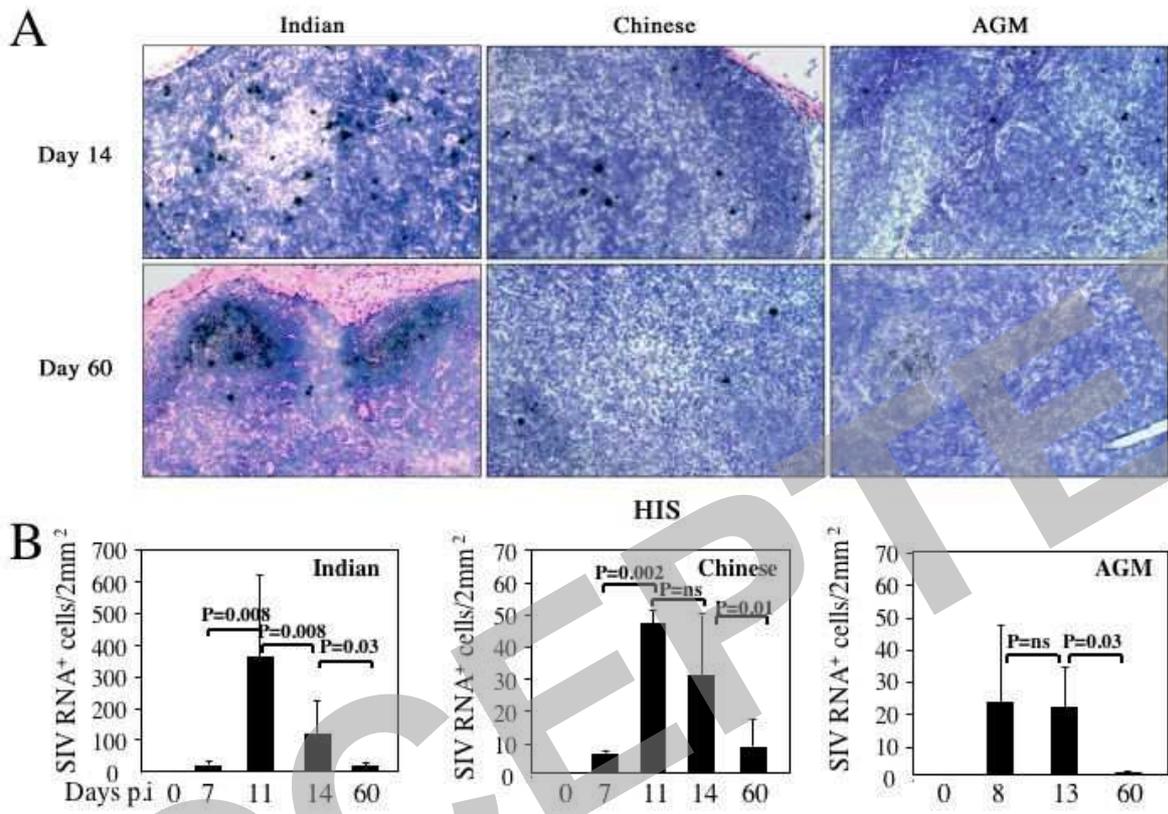
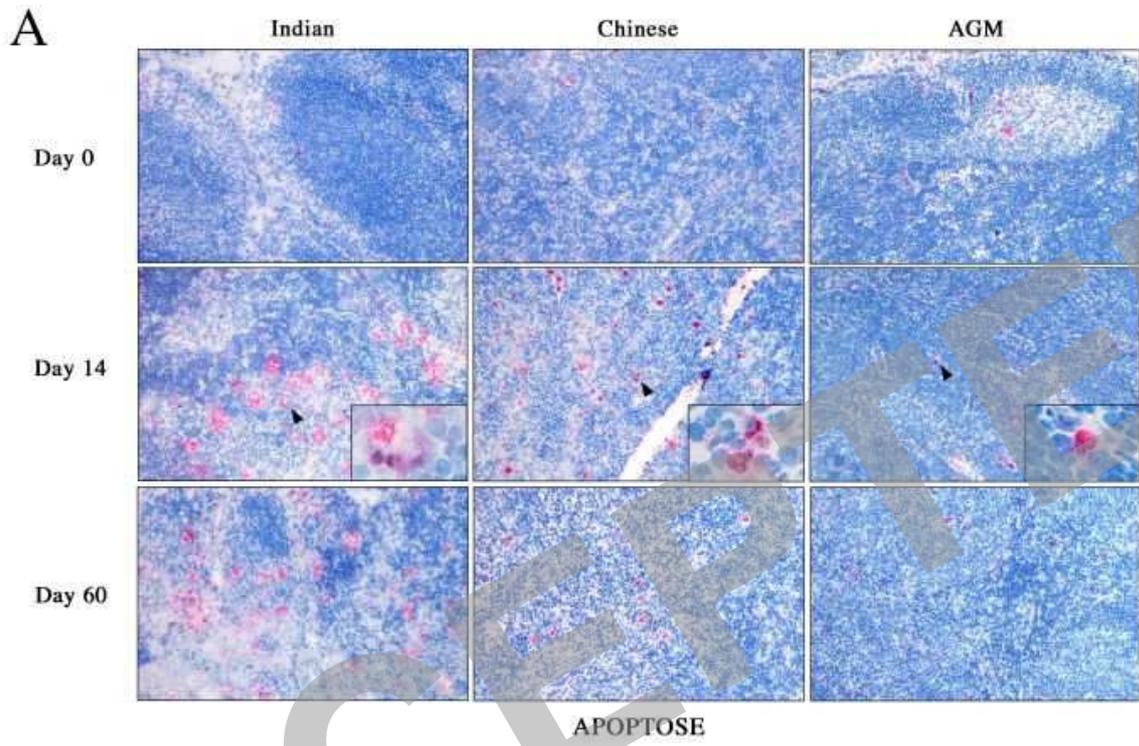


Figure 2

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Figure 3

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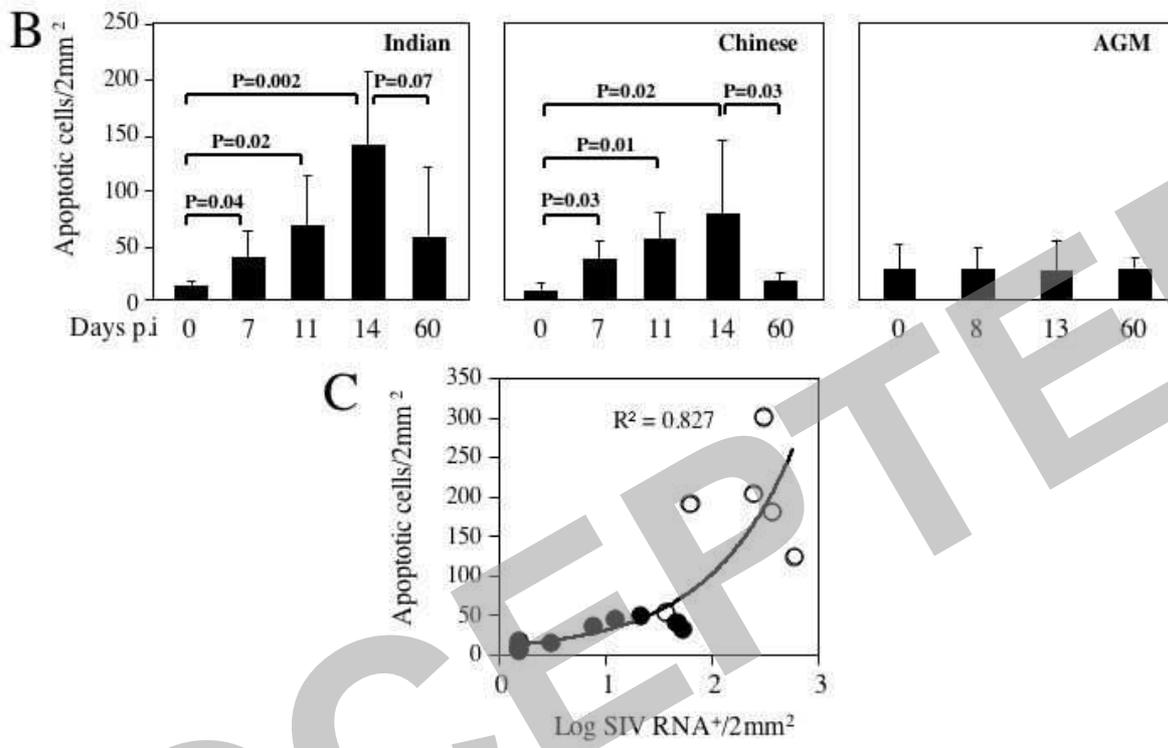


Figure 3

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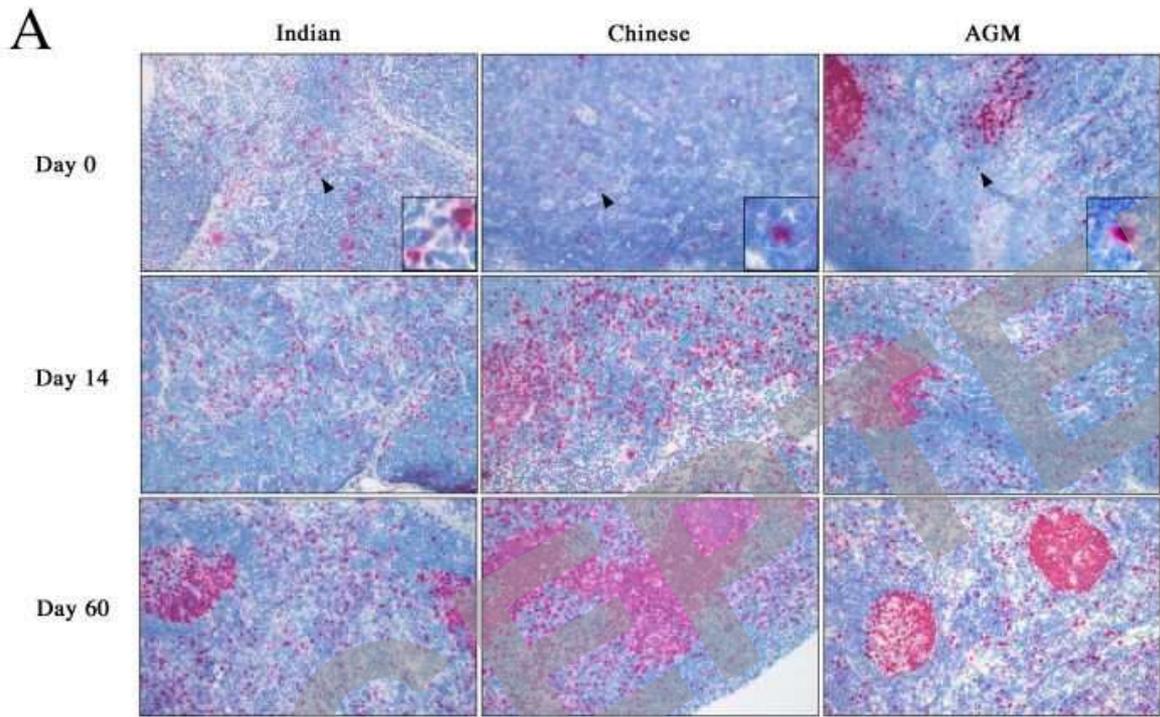


Figure 4

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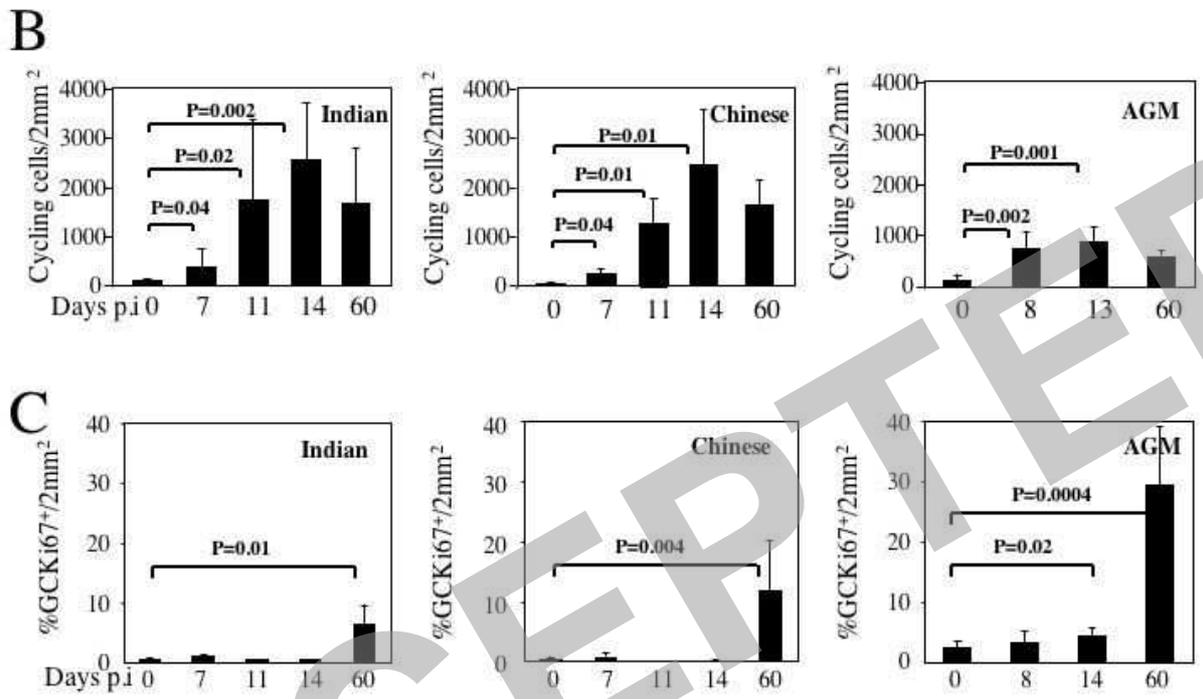


Figure 4

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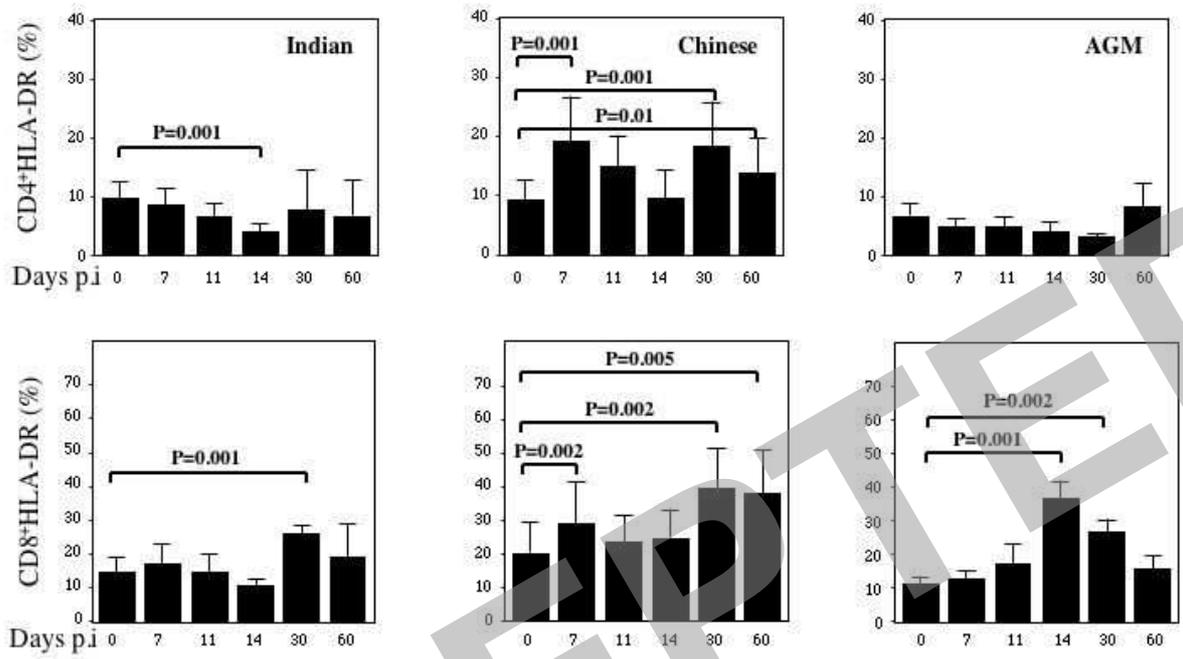


Figure 5

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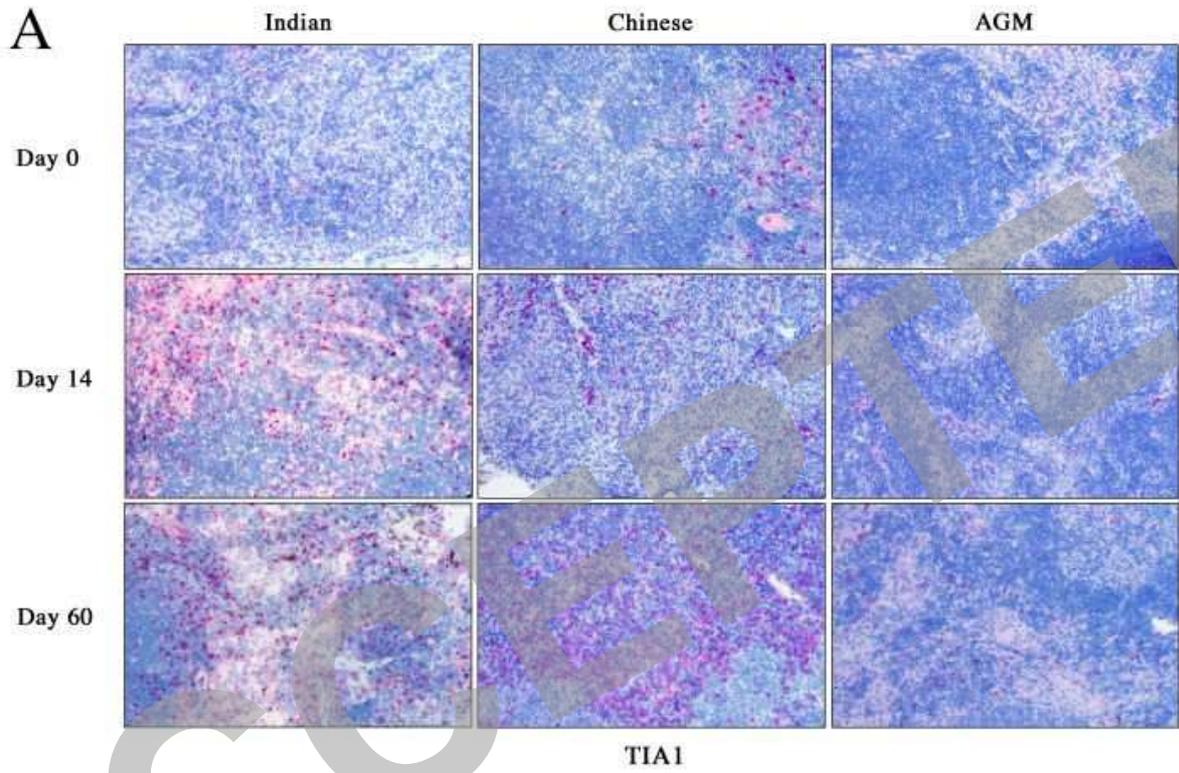


Figure 6

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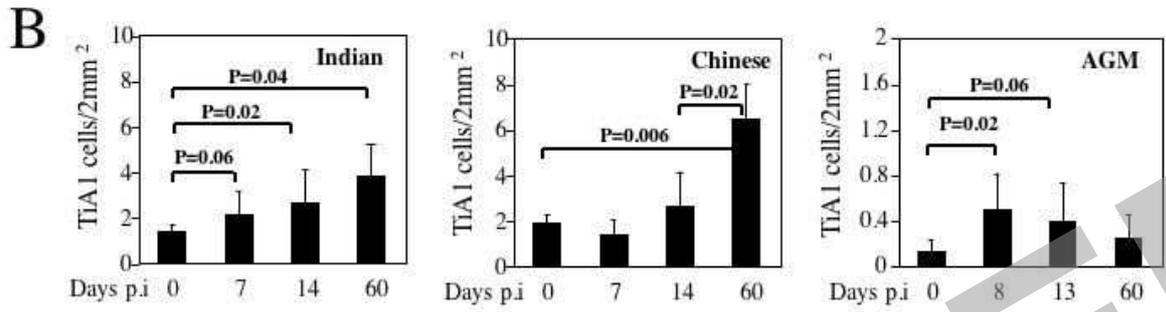


Figure 6

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