

1 ***Toll* signaling enhances mosquito antiplasmodial immunity by promoting differentiation of**
2 **hemocytes to the Megacyte lineage**

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5 Ana Beatriz Barletta Ferreira¹, Banhisikha Saha¹, Nathanie Trisnadi^{1#}, Gianmarco Raddi^{1#}, and
6 Carolina Barillas-Mury^{1*}.

7 ¹Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases,
8 National Institutes of Health, Rockville, MD 20852.

9 # Present address: Nathanie Trisnadi, Atropos Therapeutics Inc., San Carlos, California, USA.

10 Gianmarco Raddi, School of Clinical Medicine, University of Cambridge, Cambridge CB2 0SP,
11 UK, CRUK Cambridge Institute, Cambridge CB2 0RE, UK.

12 * Correspondence should be addressed to: cbarillas@niaid.nih.gov (CB-M)

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14

15 **Abstract**

16 Activation of Toll signaling in *Anopheles gambiae*, by silencing Cactus, eliminates *Plasmodium*
17 ookinetes by enhancing local release of hemocytes-derived microvesicles that promote activation
18 of the mosquito complement-like system. A new effector hemocyte subpopulation of large
19 granulocytes, the megacytes, was recently identified. We report that Cactus silencing dramatically
20 increases the proportion of megacytes, from 5 to 79% of circulating granulocytes. Transcriptomic
21 and morphological analysis, as well as *in situ* hybridization and expression of cell-specific
22 markers, indicate that Cactus silencing triggers granulocyte differentiation into megacytes.
23 Megacytes are very plastic cells that can extend long filopodia and tend to form clusters *in vivo*.
24 Moreover, megacytes are massively recruited to the basal midgut surface in response to bacterial
25 feeding and *Plasmodium* infection. We propose that Toll signaling promotes differentiation of
26 granulocytes to the megacyte lineage, a major cellular effector of antibacterial and antiplasmodial
27 immunity.

28

29 **Introduction**

30 Ookinete traversal of the *Anopheles gambiae* midgut disrupts the peritrophic matrix, a
31 chitinous network that surrounds the blood meal, and that normally prevents bacteria of the gut
32 microbiota from coming in direct contact with epithelial cells (Kumar et al., 2010). Contact of
33 midgut epithelial cells with bacteria, or their immune elicitors, triggers the release of prostaglandin
34 E2 (PGE2), which attracts hemocytes to the basal surface of the midgut (Barletta et al., 2019).
35 *Plasmodium* ookinete midgut traversal also causes irreversible damage to invaded cells and
36 triggers a strong caspase-mediated nitration response (Han et al., 2000, Oliveira Gde et al., 2012,
37 Trisnadi and Barillas-Mury, 2020). Hemocytes patrolling the midgut undergo apoptosis and

38 release hemocyte-derived microvesicles (HdMv) if they come into contact with an area of the
39 midgut basal lamina that has been nitrated (Castillo et al., 2017). HdMv release promotes local
40 activation of the mosquito complement-like system (Castillo et al., 2017), a major final effector of
41 antiplasmodial immunity that binds to the parasite's surface and forms a complex that ultimately
42 lyses the ookinete (Blandin et al., 2004).

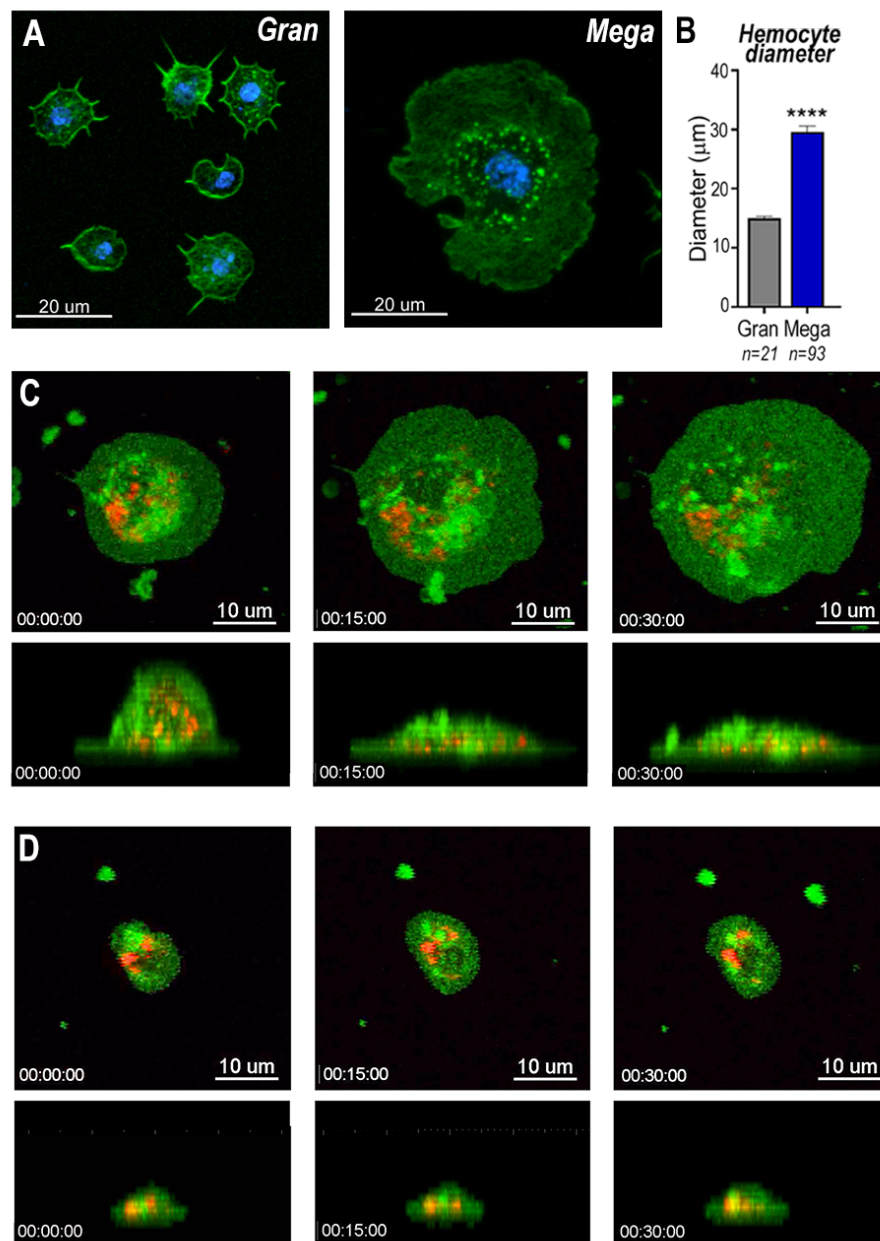
43 Overactivation of *Toll* signaling in *A. gambiae* mosquitoes by silencing *Cactus*, an inhibitor
44 of this pathway (Frolet et al., 2006), elicits a very strong complement-mediated immune response
45 that eliminates *Plasmodium berghei* ookinetes (Frolet et al., 2006). Hemocytes mediate this
46 enhanced immune response, as transfer of *Cactus*-silenced hemocytes into naïve mosquitoes
47 recapitulates the phenotype of systemic *Cactus* silencing (Ramirez et al., 2014). Furthermore,
48 *cactus* silencing increases HdMv release in response to ookinete midgut invasion (Castillo et al.,
49 2017), indicating that hemocytes are more reactive to *Plasmodium* infection. However, the
50 mechanism by which activation of the *Toll* pathway enhances hemocyte responses to *Plasmodium*
51 is not clear.

52 Hemocytes are classified into three cell types, prohemocytes, oenocytoids and
53 granulocytes, based on their morphology. However, single cell RNA sequencing (sc-RNAseq)
54 analysis of *An. gambiae* hemocytes identified several novel subpopulations of granulocytes based
55 on their transcriptional profiles, and defined molecular markers specific for hemocyte
56 subpopulations (Raddi et al., 2020). Furthermore, Lineage analysis revealed that regular
57 granulocytes derive from prohemocytes and can further differentiate into distinct cell types,
58 including dividing granulocytes, and two final effector cells, megacytes and antimicrobial (AM)
59 granulocytes (Raddi et al., 2020). Here explore the role of *Toll* signaling on mosquito hemocyte
60 differentiation and their response to infection with bacteria and *Plasmodium* parasites.

61 **Results**

62 **Cell dynamics of mosquito granulocytes**

63 Megacytes are present in low abundance (~1-2% of hemocytes) in sugar fed females. They
64 are large cells, five times larger than regular granulocytes, and express a specific transmembrane
65 protein marker (TM7318) (Raddi et al., 2020). Regular granulocytes (Fig. 1A) reach an average
66 diameter of 14.2 μm (Fig. 1B) when they spread over a glass surface, while the average diameter
67 of megacytes is 28.6 μm ($p < 0.0001$, Unpaired T-test) (Fig. 1A and 1B). Granulocyte cellular
68 dynamics were evaluated by live imaging of perfused hemocytes *in vitro* as they adhered and
69 spread on a glass surface. Hemocytes were labeled *in vivo*, through systemic injection of adult
70 females with a red lipophilic dye (Vybrant CM-DiI) that accumulates on intracellular vesicles. A
71 green, fluorescent probe (Cell Mask) was added after perfusion to label the plasma membrane.
72 Both regular granulocytes and megacytes attached to the glass surface and spread fully within one
73 hour (Videos S1-S4). Megacytes already have a larger cell diameter when they first attach to glass
74 (Fig. 1C, upper panel and Video S3), and exhibit a peripheral “halo”, corresponding to an area of
75 extended thin cytoplasm cell, almost devoid of vesicles (Fig. 1C, upper panel and Video S3).
76 Lateral views revealed that, initially, megacytes have a large nucleus and a voluminous cytoplasm
77 in the central region of the cell that flattens dramatically as the cell “spreads” over the glass surface
78 (Fig. 1C, lower panel and Video S4). In contrast, the central region of regular granulocytes remains
79 unchanged in (Fig. 1D, lower panel and Video S2) and the periphery of the cell exhibits a modest
80 increase in diameter as the cell spreads along the surface (Fig. 1D, upper panel and Video S1).



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82 **Fig.1: Megacyte and granulocyte cell dynamics.** (A) Regular granulocytes and megacytes from
83 *An. gambiae* females spread on a glass surface. Actin, green (phalloidin) and nuclei, blue
84 (Hoechst). Scale bar: 20 μm . (B) Granulocyte diameter of sugar-fed mosquitoes after spreading on
85 a glass surface. Error bars represent mean \pm SEM. Unpaired t-test. **** $P \leq 0.0001$. (C) Live imaging
86 time-lapse of a megacyte spreading in a glass surface for 30 minutes. Plasma membrane stained in
87 green and microvesicles in red. Top (XY) and lateral view (XZ) of a megacyte. Scale Bars: 10 μm
88 and 5 μm , respectively. (D) Live imaging time-lapse of a megacyte spreading on a glass surface for
89 30 minutes. Top (XY) and lateral view (XZ) of a regular granulocyte. Scale Bars: 10 μm ,
90 respectively.

91 **Effect of *Toll* signaling activation on mRNA markers of granulocyte populations.**

92 The effect of *Toll* signaling on hemocyte differentiation was explored by silencing *Cactus*,
93 an inhibitor of the *Toll* pathway. Hemocytes that adhered to glass (mostly granulocytes) or that
94 remain in suspension (mostly prohemocytes and oenocytoids) were collected 4 days post-injection
95 from *dsLacZ* control and *dsCactus*-injected females. Bulk sequencing of cDNA libraries generated
96 between 16.2 and 25.3 million fragments that mapped to the *Anopheles gambiae* AgamP4.9
97 transcriptome. Only transcripts with 10 or more reads were included in the analysis, resulting in a
98 total of 9,421 unique transcripts (Accession E-MTAB-11252). Glass-bound and unbound
99 hemocyte samples were analyzed together, because the differences in expression between *dsLacZ*
100 vs *dsCactus*-silenced hemocytes explained 81% of the variance between the four experimental
101 groups (Fig. S1A and S1B). Differential expression (DE) analysis of *Cactus*-silenced hemocytes
102 using the DESeq2 software, identified 1071 differentially expressed genes (Q-value < 0.001), of
103 which 407 were upregulated (log₂ fold change >2), while 664 were downregulated (log₂ fold
104 change < -2) (Fig 2A).

105 The effect of *Cactus* silencing on expression of the transcripts that define the different
106 hemocyte clusters established by (sc-RNAseq) (Raddi et al., 2020) was analyzed (Tables S1 and
107 S2), to establish whether there was a significant effect on the relative abundance of specific
108 hemocytes subpopulations. Overall, 23 oenocytoid markers, 2 from prohemocytes and 57 from
109 granulocytes were differentially expressed between *dsLacZ* and *dsCactus* hemocytes (Tables S1
110 and S2). Most differentially expressed oenocytoid markers 22/23 (95%) were down-regulated,
111 while one of the prohemocyte markers was up-regulated and the second one down-regulated (Fig.
112 2B). The number of down-regulated granulocyte markers 28/57 (49%) was very similar to that of
113 up-regulated ones 29/57 (51%). However, detailed analysis of granulocyte subpopulations

114 revealed that most up-regulated markers 18/29 (62%) correspond to megacytes, while most down-
115 regulated markers correspond to regular granulocytes 21/28 (75%). This suggests that *dsCactus*
116 silencing increases the proportion of circulating megacytes, at the expense of a reduction in regular
117 granulocytes.

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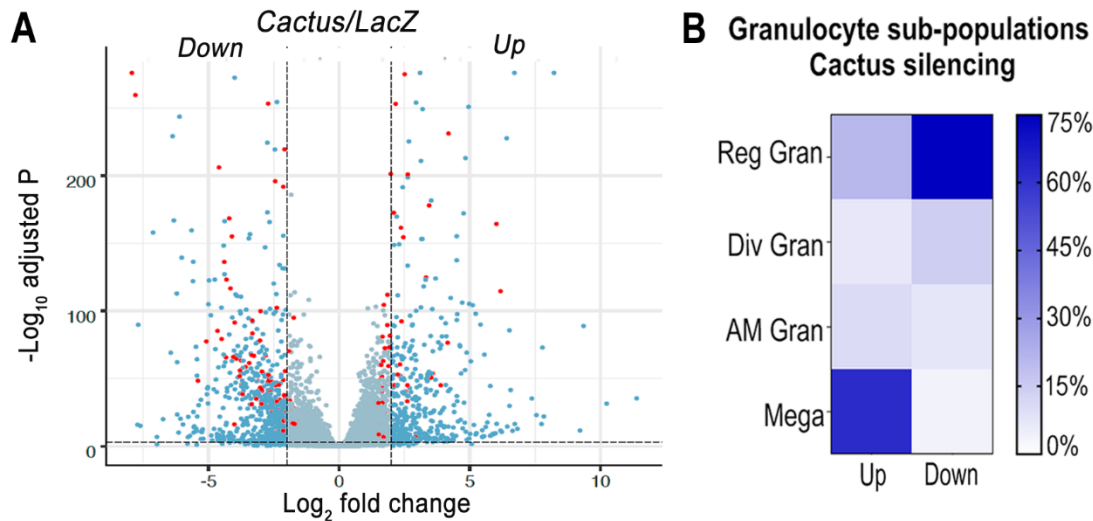
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138 **Fig.2: Effect of *Toll* pathway activation on mRNA markers of granulocyte populations.** (A)
139 Differential expression of Cactus dsRNA knockdown. From a total of 9421 filtered genes. Volcano
140 plot of DE genes in Cactus silenced hemocytes compared to LacZ control filtered for log2 fold
141 change > 2 and Q-value <0.001. Dark blue dots on the right represent upregulated DE genes and
142 on the left the downregulated ones. Red dots show genes that are hemocytes specific markers.
143 Complete list of up and down regulated genes is listed in Tables S1 and S2. (B) Percentage of
144 granulocyte sub-population markers up and downregulated in Cactus silenced hemocytes.
145 Complete list of up and down regulated genes for each hemocyte subpopulation is in Tables S1
146 and S2.

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158 **Cactus silencing promotes granulocyte differentiation into megacytes**

159 Cactus silencing did not significantly increase the proportion of total circulating
160 granulocytes, based on hemocyte counts by light microscopy (Ramirez et al., 2014), suggesting
161 that the observed enhanced immune response could be due to functional changes in hemocytes.
162 The morphology of hemocytes perfused from *Cactus*-silenced females was analyzed using
163 fluorescent probes to stain the actin cytoskeleton and the nucleus. *Cactus* silencing dramatically
164 increased the proportion of large granulocytes (diameter > 40 μm), presumably megacytes, from
165 5.3% to 79.2% ($p < 0.0001$, X^2 test) (Fig. 3A and B), in agreement with the observed increase in
166 up-regulated megacyte-specific markers in the transcriptomic analysis of *Cactus*-silenced
167 hemocytes (Fig. 2B). Interestingly, megacytes from *Cactus*-silenced mosquitoes (Fig. 3A) and are
168 even larger (average diameter of 47 μm after spreading in a glass surface) than megacytes from
169 dsLacZ controls (average diameter of 30 μm) (Figure 3C and D). *In situ* RNA hybridization of
170 dsCactus granulocytes with a fluorescent probe for the megacyte-specific marker TM7318,
171 confirmed that the proportion of TM7318-positive granulocytes was much higher (80%) in *Cactus*-
172 silenced females than in dsLacZ controls (4%) ($p < 0.0001$, X^2 test) (Fig. 3E and F), providing direct
173 evidence that overactivation of *Toll* signaling triggers a dramatic increase in the proportion of
174 circulating megacytes. Expression analysis of the TM7318 marker in perfused hemocyte samples
175 confirmed that mRNA levels were 42-fold higher in dsCactus hemocytes than the dsLacZ control
176 group ($p < 0.001$, T-test) (Fig. 3G), while a modest increase (2.8-fold) in FBN50 mRNA, a marker
177 of antimicrobial (AM) effector granulocytes, was observed ($p < 0.0001$, T-test). Conversely,
178 expression of FBN11228, a marker of regular granulocytes, decreased by 30-fold in circulating
179 hemocytes of *Cactus*-silenced mosquitoes (Fig. 3G). The changes in the relative abundance of

180 mRNAs from cell-specific markers in *dsCactus*-hemocytes coincides with the observed changes
181 in hemocyte morphology and the *in situ* hybridization and transcriptomic data (Fig. 3G).

182 The relative increase in megacytes in *Cactus*-silenced *An. gambiae* females could be due
183 to enhanced megacyte proliferation or to increased differentiation of regular granulocytes into
184 megacytes. The effect of *Cactus*-silencing on granulocyte proliferation was evaluated by
185 quantitating the proportion of hemocytes that incorporated Bromodeoxyuridine /5-bromo-2'-
186 deoxyuridine (BrdU), a thymidine analog. The proportion of BrdU+ hemocytes that adhered to
187 glass (mostly granulocytes) in *dsCactus* mosquitoes (51%, n=694 cells) is not significantly
188 different from *dsLacZ* controls (52%, n=410 cells) (Fig. S2A and B). BrdU fluorescence intensity
189 (RFU) is also not significantly different between *dsLacZ* and *dsCactus* hemocytes (Fig. S2C).
190 However, the ratio of BrdU fluorescence intensity to nuclear volume is significantly lower in
191 *dsCactus* hemocytes (Fig. S2D). This indicates that the increase in nuclear volume in megacytes
192 does not involve DNA replication. These observations, together with the increase in the proportion
193 of megacytes in *dsCactus* females, at the expense of other regular granulocytes (Fig. 2B and 3G),
194 indicate that activation of *Toll* signaling promotes differentiation of granulocytes towards the
195 megacyte lineage.

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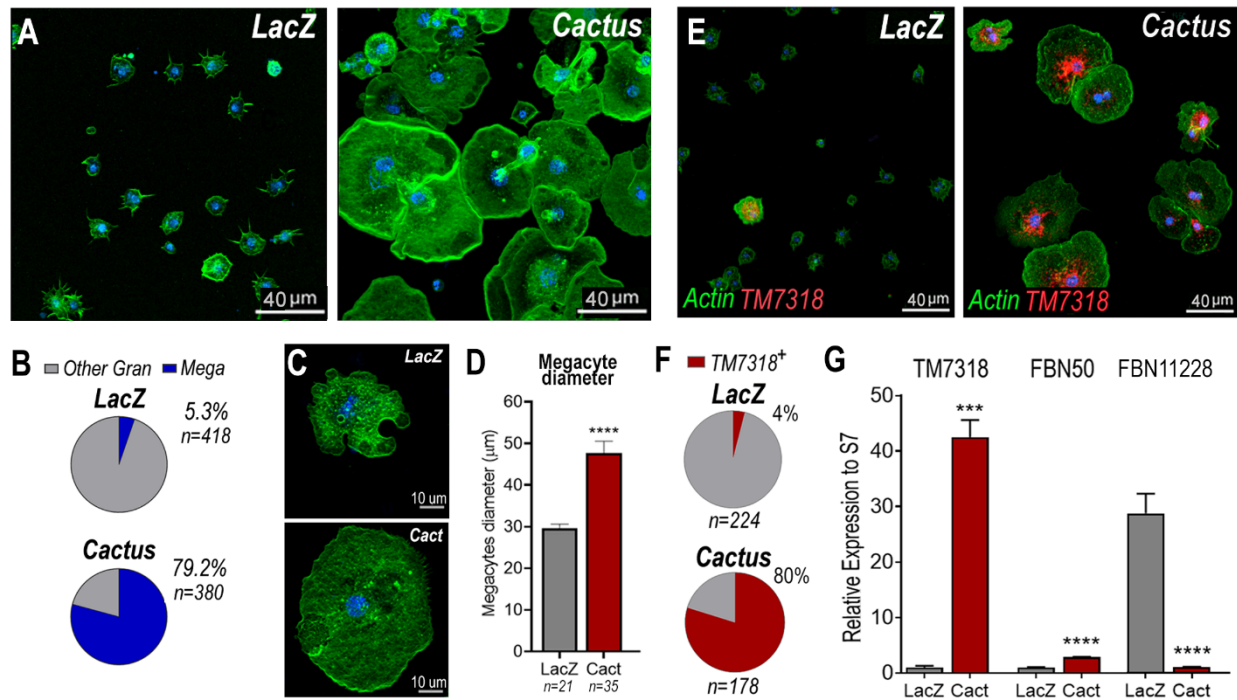
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204 **Fig.3: Cactus silencing promotes granulocyte differentiation into megacytes.** (A) *An.gambiae*
 205 hemocytes in LacZ control and Cactus attached to a glass surface. Actin is showing in green and
 206 nuclei in blue. Scale Bar: 40um. (B) Percentage of megacytes among all granulocytes in dsLacZ
 207 and dsCactus mosquitoes. Percentages were compared using X² test. ****P≤0.0001. (C) Megacyte
 208 in control LacZ mosquitoes (upper) and in Cactus-silenced mosquitoes (lower). Actin is showing
 209 in green, and nuclei is in blue. Scale Bar: 10um. (D) Diameter of megacytes from LacZ control
 210 and Cactus-silenced mosquitoes. Error bars represent mean± SEM. Unpaired t-test. ****P≤0.0001.
 211 (E) RNA *in situ* hybridization for megacyte specific marker TM7318. Actin is showing in green
 212 (phalloidin), TM7318 mRNA in red and the nuclei in blue (Hoechst). Scale bar: 40um. (F)
 213 Percentage of TM7318 positive cells in LacZ and Cactus silenced granulocytes. Percentages were
 214 compared using X² test. ****P≤0.0001. (G) Relative mRNA expression of hemocyte specific
 215 markers in LacZ control and Cactus hemocytes for transcriptome validation. Megacyte marker
 216 (TM7318), antimicrobial granulocytes (FBN50) and regular granulocytes (FBN11228). Gene
 217 expression was normalized using RpS7 expression. Error bars represent mean ± SEM. Unpaired t-
 218 test, ****P≤0.0001.

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225 **Toll signaling-mediated megacyte differentiation is partially dependent on LL3**

226 Single cell sequencing revealed high expression of the LPS-induced TNF α transcription
227 factor (LITAF)-like 3 (LL3) mRNA in megacytes, and LL3-silencing abrogated the ability of
228 hemocytes to respond to systemic injection of HDF-containing hemolymph (Raddi et al., 2020).
229 The potential interaction between of LL3 and Toll-mediated megacyte differentiation was explored
230 by co-silencing Cactus and LL3 by dsRNA injection. LL3 silencing alone had no significant effect
231 in the proportion of megacytes (Fig. S3). As expected, Cactus-silencing greatly increased the
232 proportion of megacytes from 1% in the dsLacZ control to 68% ($p < 0.0001$, X^2 test); while co-
233 silencing LL3 and Cactus resulted in an intermediate phenotype, with a partial, but significantly,
234 reduction in the proportion of megacytes from 68% to 34.3% ($p < 0.0001$, X^2 test) (Fig. S3B). This
235 indicates that Toll-mediated differentiation of regular granulocytes into megacytes is partially
236 dependent on LL3, and suggests that there may be an alternative LL3-independent pathway that
237 remains to be identified.

238 **Characterization of megacyte *in vivo* dynamics and ultrastructure**

239 The effect of *Cactus* silencing on granulocyte dynamics was evaluated *in vivo*, through live
240 imaging within adult female mosquitoes. Female mosquitoes were imaged for 2h, one day after
241 blood feeding on a healthy mouse. Hemocytes were visualized by systemic injection of Vybrant
242 CM-DiI, a fluorescent lipophilic dye that is preferentially taken up by granulocytes. Circulating
243 hemocytes in dsLacZ females (presumably normal granulocytes) have a smaller diameter than
244 those of dsCactus females (Videos S5 and S6) (Fig. 1A and B) and they seldom come in contact
245 with each other as they patrol the basal surface of the midgut (Video S5). Hemocytes from
246 dsCactus females (presumably megacytes) are larger and have a spindle shape (Fig.1A and B,

247 Videos S3 and S4). They appear to have higher plasticity, as they can readily stretch their
248 cytoplasm and they often come into contact with each other (Videos S6). The plasticity of
249 dsCactus megacytes was confirmed by *in vitro* live imaging of perfused hemocytes labeled by
250 systemic injection of Vybrant CM-DiI and green Cell Mask. Some megacytes from dsCactus
251 mosquitoes projected long thin filopodia towards other megacytes (Video S7). This process was
252 not observed in regular granulocytes or in megacytes from the dsLacZ controls. Taken together,
253 our live imaging data indicates that, in addition to their larger diameter (Fig. 3C and D), dsCactus
254 megacytes are also more active, have increased plasticity as they patrol the midgut (Video S6),
255 and greater tendency to interact with each other and form clusters (Videos S6 and S7).

256 The detailed ultrastructure of megacytes was explored using Transmission Electron Microscopy
257 (TEM). Hemocytes from Cactus silenced females were collected by perfusion, allowed to settle
258 and fixed in suspension. As expected, the maximum diameter of hemocytes fixed while in
259 suspension was smaller than when they were allowed to spread on a glass surface. Regular
260 granulocytes were still significantly smaller (6-10 μm) than megacytes (15-20 μm), with nuclei
261 that are also proportionally smaller (Fig. 4A and B). Extensive electron-dense areas are observed in
262 the nuclei of megacytes, probably corresponding to the nucleolus. Large numbers of cytoplasmic
263 vacuoles that contain abundant amorphous material are observed, as well as an extensive
264 mitochondrial network (Fig. 4A and B). Mitochondrial organization of perfused hemocytes was
265 further investigated using Mitotracker staining. Mitochondria of regular granulocytes have a
266 punctate pattern with strong staining on individual organelles (Fig. 4D). In contrast, megacytes
267 exhibit a more diffuse and extensive mitochondrial network (Fig. 4E). It is noteworthy that large
268 membrane-bound mitochondria-like extracellular structures and small vesicles are often observed

269 “budding off” from the surface of dsCactus megacytes (Fig. 4B-C), but not from regular
270 granulocytes (Fig. 4A).

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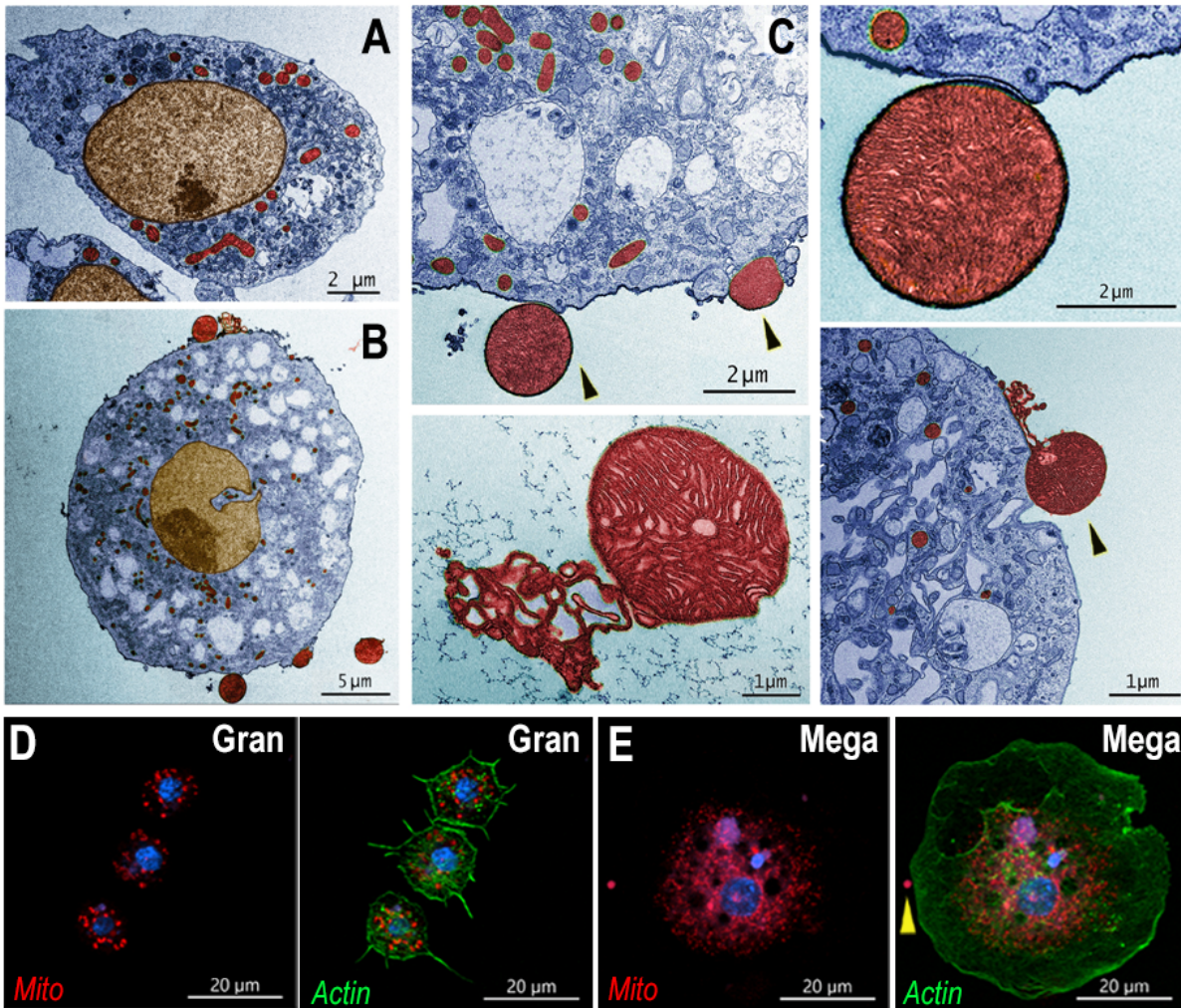
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286 **Fig.4: Ultrastructure of megacytes in Cactus-silenced mosquitoes.** (A) Transmission Electron
287 Microscopy (TEM) of regular granulocytes from Cactus-silenced mosquitoes. Scale Bar: 2um. (B)
288 TEM of megacytes from Cactus-silenced mosquitoes. Scale Bar: 5um. (C) Extracellular giant
289 mitochondria-like structures (black arrows). Close-up of a mitochondria-like structure (lower
290 center). Scale Bars: 2um and 1um. TEM images were digitally colorized, cytoplasm is shown in
291 blue, mitochondria in red and nuclei in golden yellow. (D) Mitotracker staining in regular
292 granulocytes. Scale Bar: 20um. (E) Mitotracker staining in Cactus-megacytes. Actin is stained in
293 green (phalloidin), mitochondria is in red (mitotracker) and nuclei in blue (Hoechst). Yellow arrow
294 indicates an extracellular mitochondrion like structure outside of a megacyte. Scale bar: 20um.

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298 **Megacytes associate with the basal surface of the midgut in response to bacterial feeding and**
299 ***Plasmodium berghei* infection**

300 We have shown that the direct contact of bacteria with epithelial cells, before the peritrophic matrix
301 is formed, triggers PGE2 release and attracts hemocytes to the basal surface of the midgut (Barletta
302 et al., 2019). Hemocyte recruitment to the midgut in dsCactus females was explored by feeding
303 them a BSA protein meal containing bacteria. As expected, bacterial feeding attracted hemocytes
304 to the midgut in both dsCactus and dsLacZ control females (Fig. 5A and B). However, there are
305 important differences in hemocyte recruitment. In dsLacZ females, hemocytes attach to the midgut
306 basal lamina individually or in doublets (Fig.5A), while hemocytes from dsCactus females form
307 large clusters on the basal midgut surface, with multiple hemocytes in very close association
308 (Fig.5B and C). dsCactus hemocytes on the midgut surface have the characteristic morphology
309 of megacytes, with a larger cytoplasm and nuclei than those from dsLacZ females (Fig. 5B and C).
310 Accumulation of actin was often observed in the boundaries where hemocytes from dsCactus
311 females come in direct contact as they form clusters (Fig. 5D).

312 The recruitment of granulocyte subpopulations to the midgut of dsCactus females, in
313 response to both bacterial feeding and *P. berghei* infection, was analyzed by quantitation of mRNA
314 transcripts of cell-specific hemocyte markers in dissected mosquito midguts. TM7318 mRNA
315 levels increased dramatically in dsCactus midguts after bacterial feeding (250-fold increase)
316 relative to dsLacZ control (p=0.0022, Mann-Whitney test) (Fig. 5E), indicative of extensive
317 megacyte recruitment. A significant, but more modest increase in FBN50 (5-fold) (p=0.0152,
318 Mann-Whitney test) a marker of antimicrobial granulocytes, was also observed (Fig. 5E).
319 Although hemocytes undergo apoptosis as they release HdMv in response to *P. berghei* midgut
320 invasion, a strong increase in TM7318 mRNA associated with the midgut of dsCactus infected

321 females (50-fold increase) ($p=0.0022$, Mann-Whitney test), relative to dsLacZ controls (Fig. 5F)
322 was still detected. FBN50 levels were also significantly higher (10-fold increase) ($p=0.0043$,
323 Mann-Whitney test), although the increase was not as robust as that of the megacyte-specific
324 marker (Fig. 5F).

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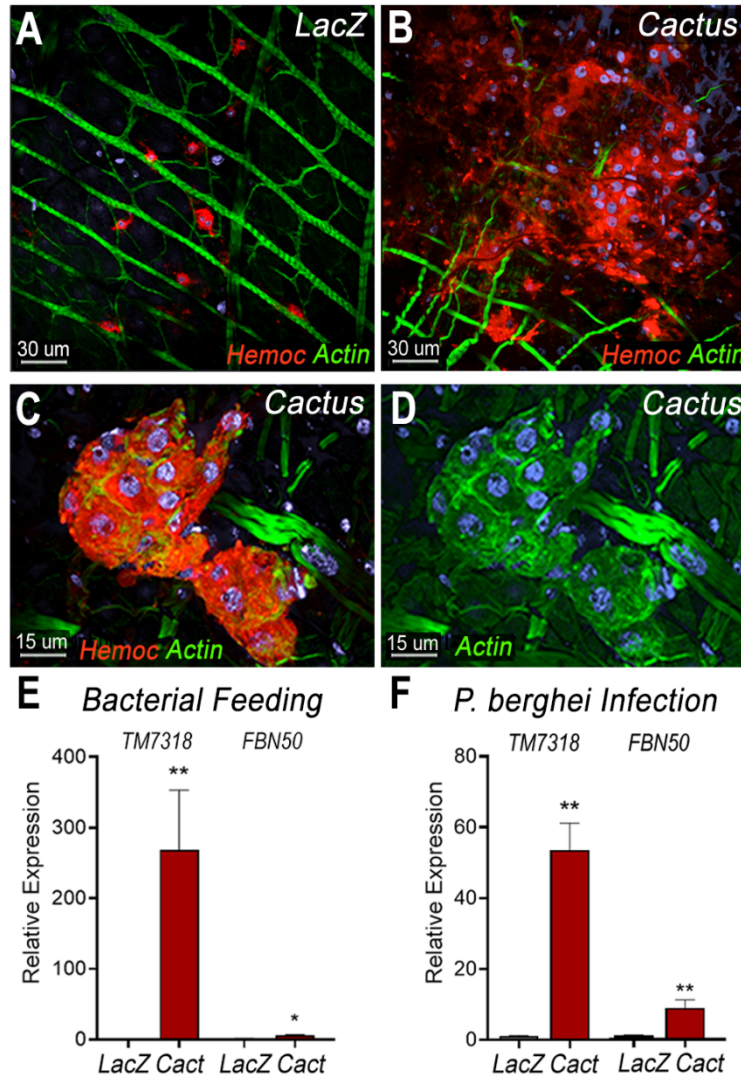
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345 **Fig. 5: Bacterial feeding and *Plasmodium berghei* infection increase megacyte association to**
346 **the midgut basal surface.** (A) Effect of bacterial feeding in LacZ-injected controls on hemocytes
347 associated to the midgut basal surface. (B) Effect of Cactus silencing on the hemocytes associated
348 to the basal surface of the midgut 4 hours post bacterial feeding. (A) and (B) Scale Bar: 30um. (C)
349 and (D) Hemocyte cluster attached to the midgut surface in Cactus-silenced mosquitoes 4 hours
350 post bacterial feeding. Scale bar: 15um. (A-D) Midgut actin is showing in green (phalloidin),
351 hemocytes (stained with Vybrant CM-DiI) in red and nuclei in blue (Hoechst). (E) Relative mRNA
352 levels of effector hemocyte markers in the midgut 4 hours after bacterial feeding in LacZ and
353 Cactus-silenced mosquitoes. Scale bar: 15um. (F) Relative mRNA levels of effector hemocyte
354 markers in the midgut 26 h post *P.berghei* infection (post-invasion) in LacZ and Cactus-silenced
355 mosquitoes. TM7318, as a megacyte marker and FBN50, as an antimicrobial granulocyte. Error
356 bars in (E) and (F) represent mean \pm SEM. Unpaired t-test, * $P \leq 0.05$, ** $P \leq 0.01$.

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359 Discussion

360 The dramatic phenotype of Toll pathway overactivation by silencing Cactus expression,
361 which results in complete elimination of *P. berghei* parasites by the mosquito complement system,
362 was documented more than fifteen years ago (Frolet et al., 2006). Furthermore, the observations
363 that the transfer of dsCactus hemocytes recapitulated the phenotype in recipient mosquitoes
364 (Ramirez et al., 2014) and that Cactus silencing greatly increased in HdMv release in response to
365 *Plasmodium* midgut invasion (Castillo et al., 2017), clearly indicated that hemocytes are key
366 players of this enhanced antiplasmodial response. However, the mechanism by which Toll
367 signaling enhanced hemocyte responses to *Plasmodium* infection remained a mystery.

368 Transcriptomic analysis indicated that Cactus silencing increased the proportion of
369 circulating megacytes, at the expense of a reduction in regular granulocytes (Fig. 2). This was
370 confirmed by morphological analysis, *in situ* hybridization and mRNA quantitation of hemocyte-
371 specific markers, TM7318 (megacytes) and FBN11228 (regular granulocytes). We provide direct
372 evidence that, besides being larger, megacytes also have higher plasticity and can greatly extend
373 their cytoplasm and flatten their nucleus as they spread on a glass surface (Fig. 1C).

374 The lack of DNA replication and the concomitant reduction in the proportion of regular
375 granulocytes, indicates that Toll activation increases the proportion of circulating megacytes by
376 promoting final differentiation of granulocytes to the megacyte lineage. Besides the dramatic
377 increase in circulating megacytes, Cactus silencing also results in megacytes that are even larger
378 and more plastic than megacytes from dsLacZ controls. Co-silencing Cactus and the transcription
379 factor LL3 partially reduced megacyte differentiation, suggesting that this transcription factor
380 participates in the differentiation process, but there may be other LL3-independent mechanisms
381 involved.

382 Fine ultrastructural analysis revealed that the cytoplasm of megacytes exhibits extensive
383 large vacuolar structures filled with amorphous material, as well as small vesicles and
384 mitochondria-like structures that are secreted from the cell membrane. In vertebrates,
385 mitochondrial extrusion has been recently documented as a trigger of inflammation. Activated
386 platelets release their mitochondria, both within microparticles or as free organelles; and secreted
387 phospholipase A2 IIA can hydrolyze the membrane, releasing inflammatory mediators, such as
388 lysophospholipids, fatty acids, and mitochondrial DNA, that promote leukocyte activation.
389 Furthermore, extracellular mitochondria also interact directly with neutrophils *in vivo*, and
390 trigger their adhesion to the endothelial wall (Boudreau et al., 2014). Activated monocytes
391 release mitochondria, and their proinflammatory effect on endothelial cells is determined by the
392 activation status of the monocytes that released them. It has been proposed that free
393 mitochondria could be important mediators of cardiovascular disease by inducing activation of
394 type I IFN and TNF signaling (Puhm et al., 2019).

395 Large numbers of megacytes are recruited to the midgut of dsCactus females in response
396 to bacterial feeding, forming extensive clusters of cells that are in close contact with each other,
397 indicating that Toll activation also results in functional differences in the way these cells interact.
398 *Plasmodium* midgut invasion triggered strong recruitment of megacytes to the basal surface of the
399 midgut, in agreement with the documented increase in HdMv release in proximity to epithelial
400 cells invaded by ookinetes (Castillo et al., 2017). We recently described specific subsets of
401 mosquito granulocytes based on single-cell transcriptomic analysis (Raddi et al., 2020). Here we
402 present a functional characterization of megacytes, a newly described subpopulation of final
403 effector granulocytes and provide direct evidence of their recruitment to the basal surface of the
404 mosquito midgut when bacteria are allowed to come in contact with epithelial cells. We propose

405 that Toll signaling promotes hemocyte differentiation into the megacyte lineage, and that the
406 dramatic increase in the proportion of circulating megacytes mediates the observed increase in
407 HdMv (Castillo et al., 2017), resulting in enhanced complement activation that ultimately
408 eliminates *P. berghei* ookinetes. Activation of Toll signaling in mosquitoes appears to also trigger
409 the release of free mitochondria-like structures by megacytes, suggesting that these could be an
410 ancient systemic danger signal that promotes immune activation.

411

412 **Data Availability**

413 The raw data and detailed information on individual experiments and number of replicates are
414 available at Supplementary tables file.

415

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421

422 **Author Contributions**

423 Experiments were designed by A.B.F.B., N.T., B.S., G.R. and C.B.M., carried out by A.B.F.B.
424 B.S., N.T., and analyzed by A.B.F.B., N.T., B.S., G.R. and C.B.M. A.B.F.B. and C.B.M. wrote
425 the paper.

426

427

428 **Declaration of Interests**

429 The authors declare no competing financial interests.

430

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482

483 **Material and Methods**

484 *Mosquitoes and mouse feeding*

485 *Anopheles gambiae* mosquitoes (G3 strain – CDC) were reared at 28°C, 80% humidity under a
486 12h light/ dark cycle and kept with 10% Karo syrup solution during adult stages. For mosquito
487 infections with *Plasmodium berghei*, we used the transgenic GFP *P.berghei* parasites (ANKA 2.34
488 strain) kept by serial passages into 3-4 weeks old female BALB/c mice (Charles River,
489 Wilmington, MA) starting from frozen stocks. Mouse infectivity was evaluated before feeding by
490 parasitemia levels from Giemsa-stained thin blood films and *in vitro* microgamete exflagellation
491 counting. Briefly, one microliter of tail blood was mixed with 9ul of gametocyte activating
492 medium (RPMI 1640 with 25mM HEPES + 2mM glutamine, Sodium Bicarbonate 2g/L, 100uM
493 xanthurenic acid, 50ug/ml hypoxanthine). After 10 minutes of incubation exflagellations were
494 quantified using a 40X objective by phase contrast. Four to five-day old mosquitoes were fed when
495 mice reached 3-5% parasitemia and 2-3 exflagellation per field. To feed blood-fed control
496 mosquitoes, three- to four-week-old uninfected mice were used. Following feeding, both control
497 and infected mosquitoes were maintained at 19°C, 80% humidity and 12h light/dark cycle until
498 the day of dissection.

499 *Ethics statement*

500 Public Health Service Animal Welfare Assurance #A4149-01 guidelines were followed according
501 to the National Institutes of Health Animal (NIH) Office of Animal Care and Use (OACU). These
502 studies were done according to the NIH animal study protocol (ASP) approved by the NIH Animal
503 Care and User Committee (ACUC), with approval ID ASP-LMVR5.

504 *Perfused hemocytes live imaging*

505 Three-day-old adult females were injected with Vybrant DiI (1:10 water diluted, ThermoFisher
506 Scientific, Waltham, MA, USA) on one side of the thorax. The next day, mosquitoes were injected
507 with 69 nL of either dsCactus or dsLacZ at 3 $\mu\text{g}/\mu\text{L}$ on the other side of the thorax. After 4 days,
508 hemocytes were ready for perfusion or mosquitoes were used for in vivo live imaging as described
509 below. Mosquitoes were cold-anesthetized and, using forceps, a small cut was made in the
510 abdomen. Transfer buffer (95% Schneider media + 5% citrate buffer) was injected at the thorax
511 and 10-15 μL of hemolymph was harvested at the cut-site. This was repeated for 5-7 mosquitoes
512 and collected in a microcentrifuge tube stored on ice. To stain the plasma membrane of hemocytes
513 we used CellMask green plasma membrane stain stock solution (C37608, Invitrogen, Waltham,
514 MA, USA) and for the nuclei we used the Hoechst 33342 Solution (20mM) (ThermoFisher
515 Scientific, Waltham, MA, USA). Two microliters of fluorescent label solution (58 μL H₂O + 1
516 μL Cell Mask stock + 1 μL Hoechst stock) was added for every 20 μL of perfusion and 100 μL of
517 this mixture was mounted on an ibidi μ -Slide 18 Well Glass Bottom slide. Cells were allowed to
518 settle for 30 minutes then imaged. Images were taken on a Leica SP5 confocal microscope using
519 a 63x 1.4 NA oil objective with 405 nm wavelength laser (at 3% transmission) for Hoechst, 488
520 nm (5%) for Cell Mask, and 561 nm (3%) for DiI. Pinhole was set to 1 AU and frame average was
521 12. Z-intervals of 1-2 μm encompassing the full cell height was taken every 5 minutes for 2 hours.

522 *Bacterial artificial feeding*

523 We used a bacterial mixture obtained from the midguts of the *Anopheles gambiae* G3 from our
524 colony (Barletta et al., 2019). A pre-inoculum was set up in LB media from the frozen stocks
525 containing the bacterial mixture and allow to grow overnight at 28°C, 250rpm in a shaker
526 incubator. At the day of the experiment, the pre-inoculum was diluted in fresh LB media and
527 allowed to grow for 2 hours in the same condition described above. Briefly, after 2 hours of growth,

528 bacteria were washed with sterile PBS to remove toxins and the concentration of the culture was
529 estimated based on the Optical Density (OD) of the culture. At 600nm, 1OD was considered the
530 equivalent of 10^9 bacteria/mL. Three-to-four day mosquitos were fed a sterile 10% sucrose
531 solution containing antibiotics (Penicillin, 100U/mL and Streptomycin, 100ug/mL) for 2 days
532 prior the bacterial feeding. Control group was fed with a sterile 10% Bovine Serum Albumin
533 (BSA) solution in HBSS without calcium and magnesium and the bacteria group was fed with the
534 same solution containing 4×10^9 bacteria per feeder. Mosquitoes were dissected 6 hours post
535 feeding for visualization of hemocytes attached to the midgut basal surface.

536 *Hemocyte collection, morphology staining and quantification*

537 Hemocytes were collected by perfusion using anticoagulant buffer (60% Schneider medium, 30%
538 citrate buffer, pH 4.5 and 10% FBS), pH was adjusted to 7-7.2 after mixing all the components.
539 After perfusion, hemocytes were placed in a μ -slide angiogenesis chamber (ibidi GmbH,
540 Gräfelfing, Germany) and were allowed to settle for 15 minutes. Cells were fixed for an hour at
541 room temperature by adding 16% paraformaldehyde (PFA) solution in anticoagulant buffer to a
542 final concentration of 4%. Following fixation cells were washed with PBS 0.1% Triton and
543 incubated for 30 minutes at room temperature with 1U of phalloidin (Alexa Fluor 488, Molecular
544 Probes, ThermoFisher Scientific, Waltham, MA, USA) and 20 μ M Hoechst 33342 (405, Molecular
545 Probes, ThermoFisher Scientific, Waltham, MA, USA), both diluted in PBS 0.1% Triton. Cells
546 were then placed in mounting media for storage by adding 2 drops of Prolong Gold Antifade
547 Mountant (Molecular Probes, ThermoFisher Scientific, Waltham, MA, USA). For determination
548 of proportion of megacytes upon Cactus silencing, the hemocytes were imaged, the diameter of
549 every cell was measured and classified as granulocytes (cell diameter $>12.5-25 \mu$ m) or megacytes
550 (cell diameter $>25 \mu$ m) as mentioned before. The total number of granulocytes and megacytes

551 obtained from hemolymph pooled from 16-20 mosquitoes was noted and the percentage of
552 megacytes amongst granulocytes was determined for each sample. Data from three independent
553 biological replicates were used to plot the graphs.

554 *Measurement and categorization of the hemocytes by size*

555 The mosquito hemolymph was collected and the hemocytes were allowed to attach on a coated
556 well of 15µm chamber slide. For each well 8-10 mosquitoes were bled and for every sample,
557 bleeding was done in two wells with a total of 16-20 mosquitoes. Post attachment, the hemocytes
558 were fixed with 4% p-formaldehyde and stained with Phalloidin and DAPI to visualize the
559 morphology. Images were taken for at least 10 random fields for each well and the images were
560 used to measure the cell diameter using Imaris software. Using the “Pairs” option of “Measurement
561 points” tool in the software, the largest diameter of every cell was determined. For categorizing
562 the hemocytes into different subtypes, the following size reference was followed for every image
563 analysis. Cells with diameter ranging from 4-7.5µm were classified as prohemocytes, >7.5 µm-
564 12.5 µm as oenocytoids, >12.5-25 µm as granulocytes and >25 µm as megacytes.

565 *dsRNA synthesis*

566 Three-to-four day old female *An.gambiae* females were cold-anesthetized and injected with 69nl
567 of a 3ug/ul dsCactus or dsLacZ control. Double-stranded RNA for *Cactus* (AGAP007938) was
568 synthesized by *in vitro* transcription using the MEGAscript RNAi kit (Ambion, ThermoFisher
569 Scientific, Waltham, MA, USA). DNA templates were obtained by PCR using *An.gambiae* cDNA
570 extracted from whole body sugar-fed females. A 280-bp fragment was amplified with primers
571 containing T7 promoters (F-TAATACGACTCACTATAGGGTAACACTGCGCTTCATTTGG
572 and R- TAATACGACTCACTATAGGGGCCCTTTTCAATGCTGATGT), using an annealing
573 temperature of 58°C. Double-stranded RNA for LacZ was synthesized by amplifying a 218-bp

574 fragment from LacZ gene clones into pCRII-TOPO vector using M13 primers to generate a dsRNA
575 control as previously described (Molina-Cruz et al., 2012).

576 *RNA extraction and bulk RNAseq library preparation*

577 Hemocytes were collected as previously described above. In short, *An.gambiae* females were
578 perfused using anticoagulant buffer and immediately transferred to a glass tube for attachment.
579 After one hour, hemocytes that did not attach to the glass tube were collected and transferred to a
580 1.5 ml microcentrifuge containing 800ul of TRIZOL LS reagent (Invitrogen, Waltham, MA,
581 USA), that correspond to the unbound fraction enriched mainly by prohemocytes and oenocytoids.
582 Hemocytes that attached to the glass surface were washed twice with PBS and resuspended in 1mL
583 of TRIZOL LS reagent (Invitrogen, Waltham, MA, USA), this corresponds to the bound fraction,
584 mainly enriched by granulocytes. Hemocytes were then lysed in TRIZOL reagent for 15-30
585 minutes at room temperature to allow for full dissociation, then stored at 4°C overnight and then
586 at -20C until RNA extraction. The homogenate of hemocyte samples were transferred to Phase
587 Lock Gel Heavy 2 mL tubes (QuantaBio, Beverly, MA, USA) that had been pre-spun for 1500
588 RCF for 1 minute, and allowed to incubate for 5 minutes at room temperature. 100 uL of
589 chloroform (200 uL per 1 mL TRIZOL or TRIZOL plus media) was added, the tubes capped, and
590 then vigorously shaken for 15 seconds. Samples were then centrifuged for 12,000 RCF, 10
591 minutes, 4°C. If the clear, aqueous phase was still mixed with TRIZOL matrix then 100 uL more
592 of chloroform was added, and the samples again mixed vigorously and spun as before. The aqueous
593 phase was then transferred to a fresh 1.5 mL Eppendorf tube and the RNA precipitated by adding
594 0.25 mL of isopropyl alcohol (500 mL per 1 mL TRIZOL reagent used). 20 uL of glycogen (5 mg
595 / mL) were also added to aid in precipitation and pelleting. Samples were mixed by repeated
596 inversion 10 times, incubated for 10 minutes at room temperature, and then spun at 12,000 RCF,

597 10 minutes, 4°C. All the supernatant was removed, and the RNA pellets washed twice with 75%
598 ethanol (minimum 1 mL of ethanol per 1 mL of TRIZOL used). Each time the samples were mixed
599 by vortexing and centrifuged 7,500 RCF, 5 minutes, 4C. At the end, the supernatant was removed
600 and samples air-dried until almost dry, but not completely (still translucent). RNA was
601 resuspended with 30 uL of RNase free water, pipetting a few times to homogenize and then
602 incubating at 55°C for 10 minutes to completely resuspend. Samples were then stored at -20C until
603 library preparation by Bespoke Low-Throughput Team at the Wellcome Sanger institute. Total
604 RNA quantity was assessed on a Bioanalyser and ranged from 300 ng to 39 ng. mRNA was then
605 isolated with the NEBNext Poly(A) mRNA magnetic isolation module. RNA-seq libraries were
606 prepared from mRNA using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina
607 (New England Biolabs) as by manufacturer instructions, except that a proprietary Sanger UDI
608 (Unique Dual Indexes) adapters / primer system was used. Furthermore, Kapa Hifi polymerase
609 rather than NEB Q5 was employed. For bulk RNAseq sequencing samples libraries were run on
610 the Illumina HiSeq 4000 instrument with standard protocols using a 150-cycle kit set to a 75bp
611 paired-end configuration. Libraries supplied at 2.8 nM and loaded with a loading concentration of
612 280 pM.

613 *Bulk RNA-seq bioinformatic analysis*

614 Sequencing reads in CRAM format were fed into a personal BASH pipeline to convert cram files
615 to fastq using biobam's bamtofastq program (Version 0.0.191) (Raddi et al., 2020). Forward and
616 reverse fastq reads in paired mode were aligned to the *A. gambiae* AgamP4.3 reference genome
617 using hisat2 (Version 2.0.4) and featureCounts (Version 1.5.1) with recommended settings. Count
618 matrices were combined before downstream data processing and analysis within R version 3.5.3
619 (RStudio version 1.0.153). Downstream normalization, differential expression analysis and

620 visualization were done with DESeq2 R package (Version 1.18.1) (Love et al., 2014). Base factor
621 was defined as the LacZ, unbound condition. Data was normalized by making a scaling factor for
622 each sample. First the $\log(e)$ of all the expression values were taken, then all rows (genes) were
623 averaged (geometric average). Genes with zero counts in one or more samples were filtered out
624 and the average log value from $\log(\text{counts})$ for all genes was subtracted. Finally, the median of
625 the ratios calculated as above for each sample was computed and raised to the e to make the scaling
626 factor. Original read counts were divided by the scaling factor for each sample to get normalized
627 counts. Then, the dispersion for each gene was estimated, and a negative binomial generalized
628 linear model fitted. P values for the differential expression analysis were adjusted for multiple
629 testing using the Bonferroni correction. Genes were considered as differentially expressed in
630 Cactus knockdown compared to LacZ control if they had an adjusted P value < 0.001 (Wald T-
631 test) and a \log_2 fold change > 2 . Gene lists with vectorbase IDs were converted to gene annotations
632 with g:Profiler (Raudvere et al., 2019). g:Profiler utilizes Ensembl as its primary data source and
633 is anchored to its quarterly release cycle. g:GOST was used to perform functional enrichment
634 analysis on input gene lists to map the data onto enriched biological processes or pathways. In
635 addition to Ensembl, also KEGG, Reactome, WikiPathways, miRTarBase, and TRANSFAC
636 databases were used. Functional enrichment is evaluated with a cumulative hypergeometric test
637 with g:SCS (Set Counts and Sizes) multiple testing correction (adjusted P value reported only $<$
638 0.05). Gene lists were ordered on log-fold changes.

639 *Transmission Electron Microscopy (TEM)*

640 Hemocytes were collected by perfusion using anticoagulant buffer, described above and they were
641 allowed to settle on Thermanox™ coverslips (Ted Pella, Redding, CA) for 15 minutes at room
642 temperature then fixed 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C,

643 and then post-fixed 1hr with 1.0% osmium tetroxide/0.8% potassium ferricyanide in 0.1 M sodium
644 cacodylate buffer, washed with buffer then stained with 1% tannic acid in dH₂O for one hour.
645 After additional buffer washes, the samples were further osmicated with 2% osmium tetroxide in
646 0.1M sodium cacodylate for one hour. The samples were then washed with dH₂O and additionally
647 stained overnight with 1% uranyl acetate at 4°C, dehydrated with a graded ethanol series, and
648 embedded in Spurr's resin. Thin sections were cut with a Leica UC7 ultramicrotome (Buffalo
649 Grove,IL) prior to viewing at 120 kV on a FEI BT Tecnai transmission electron microscope
650 (Thermo fisher/FEI, Hillsboro, OR). Digital images were acquired with a Gatan Rio camera
651 (Gatan, Pleasanton, CA).

652 *Mitotracker staining*

653 Hemocytes were perfused with anticoagulant buffer, described above. Cells were incubated at
654 room temperature for 15 minutes for spreading. Then washed three times with 95% Schneider
655 media, 5% citrate buffer to remove most of the serum from the cells. Hemocytes were placed with
656 200nM Deep Red Mitotracker 644/665 which is retained after fixation (Molecular Probes,
657 ThermoFisher Scientific, Waltham, MA, USA) diluted in 95% Schneider media, 5% citrate buffer.
658 Cells were incubated for 45 minutes at room temperature in the dark, then washed with PBS and
659 fixed with 4% Paraformaldehyde in PBS for 15 minutes at room temperature. Hemocytes were
660 then counterstained with phalloidin and Hoechst as described above.

661 *TM7318 in situ hybridization (ISH)*

662 The ISH protocol includes a permeabilization step with a protease treatment, which compromises
663 the cell morphology. To evaluate the morphology of hemocytes and RNA expression by ISH, we
664 used a two-step protocol to image morphology first and then proceed to image the probes,
665 described in (Raddi et al., 2020). Hemocytes collected by perfusion four days after dsCactus

666 injection, fixed and stained with Alexa 488 phalloidin (actin) as described above. Ten random
667 fields of each well were imaged using a tile scan “mark and find” tool, where coordinates of the
668 field are recorded and can be restored to image the same cells later. Then, hemocytes were
669 subjected to ISH using RNAscope multiplex fluorescent reagent kit v2 assay (cat# 323110,
670 ACDBio, Abingdon, United Kingdom) following the manufacturer’s instructions. TSA based
671 fluorophores Opal 4- color automation IHC kit (cat # NEL801001KT, PerkinElmer, Waltham,
672 MA, USA) was used for the development of fluorescence (Opal 620 – C3). A specific RNA probe
673 for TM7318 (cat# 543201-C3; Aga-Transmembrane-C3) designed by ACDBio was used to stain
674 specifically megacytes. At the end of the ISH protocol, hemocytes were placed in prolong gold
675 and re-imaged using the “mark and find” tool to recall the positions of the morphology pictures.
676 Images were merged using Imaris 9.3.1 (Bitplane, Concord, MA, USA). Each well was imaged
677 taking 12 fields per well. Post imaging, the cell diameter of every cell was measured by Phalloidin
678 stain as described previously and the total number of granulocytes were determined for each
679 sample. Amongst the granulocytes and larger cells (cells with diameter >12.5), the number of cells
680 positive for the TM7318 probe were counted and their percentage was determined for both the
681 control and Cactus silencing.

682 *Confocal microscopy and Tile scan imaging*

683 Confocal images were captured using a Leica TCS SP8 (DM8000) confocal microscope (Leica
684 Microsystems, Wetzlar, Germany) with either a 40x or a 63x oil immersion objective equipped
685 with a photomultiplier tube/ hybrid detector. Hemocytes were visualized with a white light laser,
686 using 498-nm excitation for Alexa 488 (phalloidin); 588-nm excitation for Opal620 (TM7318
687 probe) and Vybrant DiI (hemocytes); 644-nm excitation for Deep Red Mitotracker (Mitochondria)
688 and a 405-nm diode laser for nuclei staining (Hoechst 33342). Images were taken using sequential

689 mode and variable z-steps. For combined morphology and in RNA in situ hybridization, we used
690 tile scan “mark and find” tool included in LASX software to capture the same areas of the slide
691 before and after the hybridization. Image processing and merge was performed using Imaris 9.3.1
692 (Bitplane, Concord, MA, USA) and Adobe Photoshop CC (Adobe Systems, San Jose, CA, USA).

693 *RNA extraction, cDNA synthesis and qPCR analysis*

694 *An. gambiae* hemocytes were collected as described above four days after dsRNA injection
695 (dsLacZ and dsCactus). Hemolymph pools of 20 mosquitoes (5ul/ each mosquito) were placed
696 directly into 800ul of TRIzol LS reagent (ThermoFisher Scientific, Waltham, MA, USA). For
697 midgut RNA extraction, pools of 20 midguts were homogenized directly in 1mL TRIzol reagent.
698 RNA extraction was carried out as described above in the section *RNA extraction and bulk RNAseq*
699 *library preparation*. Total extracted RNA was resuspended in nuclease free water and one
700 microgram was used for cDNA synthesis using the Quantitect reverse transcription kit (Qiagen,
701 Germantown, MD, USA) following the manufacturer’s instructions. Quantitative PCR (qPCR)
702 was used to measure FBN11228 (AGAP011228), TM7318 (AGAP007318) and FBN50
703 (AGAP005848) gene expression in hemocytes cDNA. We used the DyNamo SYBR green qPCR
704 kit (ThermoFisher Scientific, Waltham, MA, USA) with target specific primers and the assay ran
705 on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). A 139-bp
706 fragment was amplified for FBN11228 (F- CCAGCATCGGTACAACGGAA and R-
707 AAGCTCGTGTTCGTCGCTG). A 150-bp fragment was amplified for TM7318 (F-
708 AAAACATCCAGAAACACGCC and R- GGATTCCGGTTAAGTCCACC). A 92-bp fragment
709 was amplified for FBN50 (F- ATCACAAGGTTCCGGCTATG and R-
710 CGTTGGTGTAGGTGAGCAGA). Relative expression was normalized against *An. gambiae*
711 ribosomal protein S7 (RpS7) as internal standard and analyzed using the $\Delta\Delta$ Ct method (ref –

712 Livak and Schmittgen, 2001; Pfaffl, 2001). RpS7 (AGAP010592) primers sequences were: F-
713 AGAACCAGCAGACCACCATC and R – GCTGCAAACCTTCGGCTATTC. Statistical analysis
714 of the fold change was performed using Unpaired t-test (GraphPad, San Diego, CA, USA). Each
715 independent experiment was performed with three biological replicates (three pools of 20
716 mosquitoes) for each condition.

717 *In vivo live imaging*

718 Mosquitoes were prepared the same way for imaging of perfused hemocytes and injected with
719 Vybrant DiI cell labelling (ThermoFisher Scientific, Waltham, MA, USA) for both dsCactus and
720 dsLacZ. After 4 days, mosquitoes were starved in the morning and then fed on a BALB/c mouse
721 in the afternoon. Imaging took place the next day at 18-20 hours post-bloodmeal. Mosquitoes were
722 imaged as previously described (Trisnadi and Barillas-Mury, 2020). Briefly, 5-10 mosquitoes with
723 legs and head removed were placed between a coverslip and glass slide with craft putty as a spacer.
724 Images were taken on a Leica SP5 confocal microscope using a 40x 1.25 NA oil objective with
725 561 nm (3%) for Vybrant DiI. A z-stack with 1 μ m intervals was taken to include hemocytes
726 circulating in the hemolymph to the midgut lumen. The z-stack was taken every 1 minute for 1-2
727 hours.

728 *Visualizing hemocytes attached to the midgut basal lamina*

729 To preserve hemocyte-midgut bound, midguts were quick fixed using a higher concentration of
730 fixative injected straight into the hemolymph of the mosquito (207nl of 16% paraformaldehyde).
731 To stain hemocytes, the day before the dsRNA treatment (dsLacZ and dsCactus), three-to-4-day-
732 old mosquitoes were injected with 69nl of a 100uM solution Vybrant CM-DiI cell labelling
733 solution (ThermoFisher Scientific, Waltham, MA, USA), final concentration in the hemolymph
734 (approximately 3.5uM). Engorged mosquitoes fed with 10% BSA solution containing bacteria

735 were anesthetized and injected with 207nl of 16% paraformaldehyde, rested 40 seconds before
736 midgut dissection in 4% paraformaldehyde solution. After dissected, midguts were placed in ice-
737 cold PBS and opened longitudinally, and the bolus was removed. Clean opened tissues were then
738 fixed overnight at 4°C in 4% paraformaldehyde. The following day, midguts were washed twice
739 with PBS, blocked for 40 minutes with PBS containing 1% BSA and washed twice with the same
740 solution. For actin and nuclei staining, midguts were incubated for 30 minutes at room temperature
741 with 1U of phalloidin (Alexa Fluor 488, Molecular Probes, Waltham, MA, USA) and 20uM
742 Hoechst 33342 (405, Molecular Probes, Waltham, MA, USA), both diluted in PBS. Tissues were
743 mounted in microscope slides using Prolong Gold Antifade mounting media (Molecular Probes,
744 Waltham, MA, USA). Hemocytes were visualized by confocal microscopy and the number of
745 hemocytes per midgut in each biological condition was also analyzed.

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Supplemental Information

***Toll* signaling enhances mosquito antiplasmodial immunity by promoting differentiation of hemocytes to the Megacyte lineage**

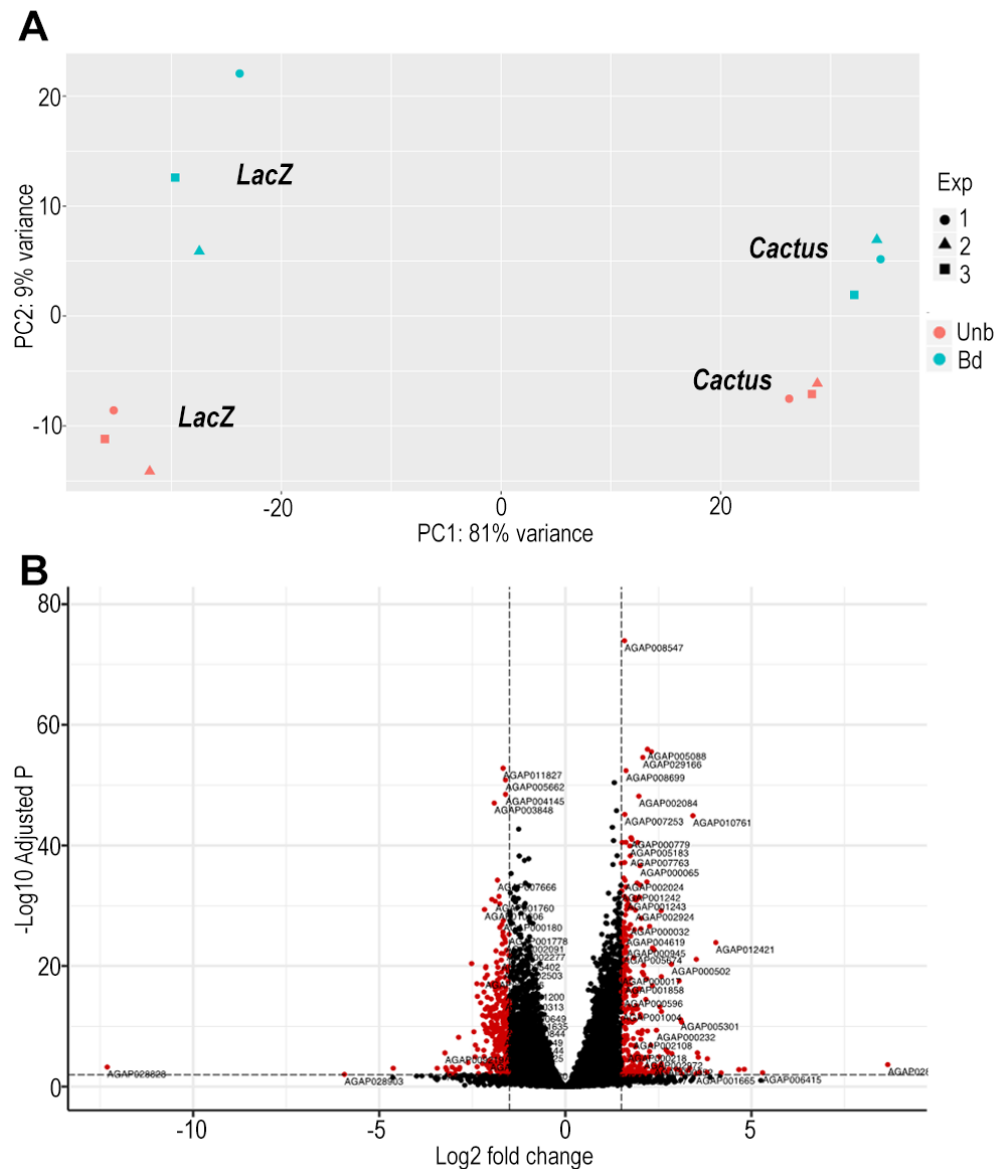
Ana Beatriz Barletta Ferreira¹, Banhisikha Saha¹, Nathanie Trisnadi^{1#}, Gianmarco Raddi^{1#}, and
Carolina Barillas-Mury^{1*}.

¹Laboratory of Malaria and Vector Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, MD 20852.

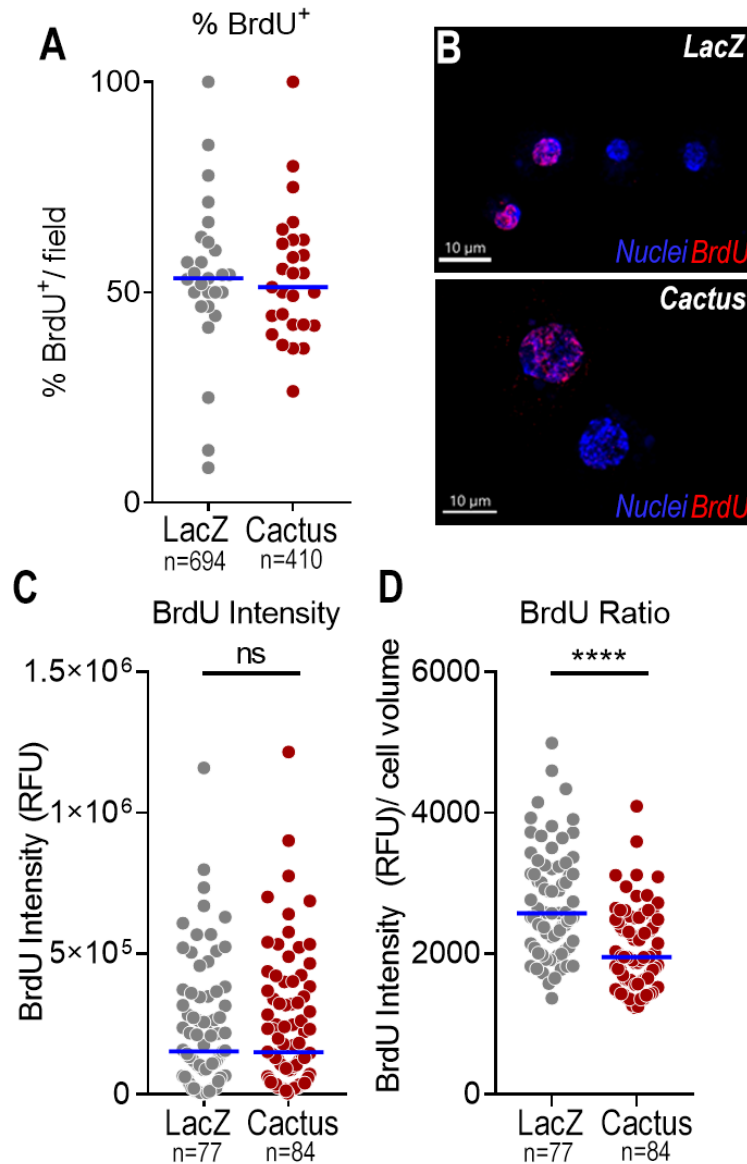
Present address: Nathanie Trisnadi, Atropos Therapeutics Inc., San Carlos, California, USA.
Gianmarco Raddi, School of Clinical Medicine, University of Cambridge, Cambridge CB2 0SP, UK, CRUK Cambridge Institute, Cambridge CB2 0RE, UK.

* Correspondence should be addressed to: cbarillas@niaid.nih.gov (CB-M)

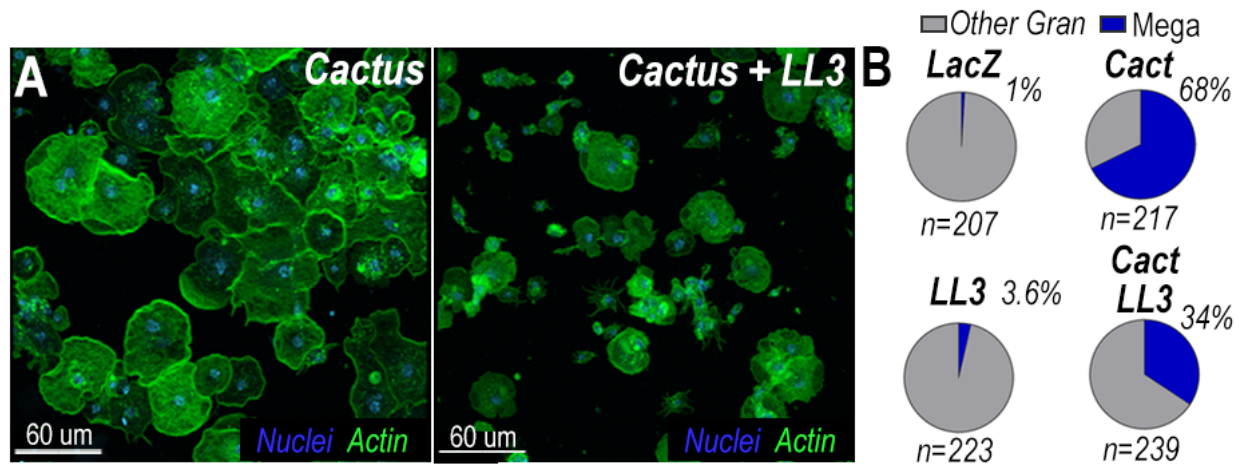
Supplementary figures



Supplementary Figure 1. Quality control of dsCactus knockdown bulk RNA seq and Differential expression between bound and unbound fractions. (A) PCA plot showing first and second principal component of dsRNA Cactus and control LacZ knock-down, for bound and unbound hemocyte fractions. Cactus and LacZ cluster separately, and so do bound and unbound hemocyte fractions. Bd = bound. UnB = unbound. One, two and three represent different biological replicates for each condition. (B) From a total of 9421 filtered genes (B) Volcano plot of DE genes between bound and unbound hemocytes filtered for log₂ fold change >1.5 and Q-value <0.01.



Supplementary Figure 2. Toll activation controls megacyte differentiation and not proliferation. (A) Percentage of BrdU⁺ hemocytes in dsLacZ and dsCactus mosquitoes 4 days after silencing. (B) Representative pictures of positive BrdU nuclei in LacZ and Cactus hemocytes. Nuclei is in Blue and BrdU is showing in Red. Scale Bar: 10um. (C) BrdU fluorescence intensity (Relative fluorescent units) in hemocytes from dsLacZ and dsCactus female mosquitoes. (D) Ratio between BrdU fluorescence intensity (Relative fluorescent units) and cell volume calculated based on the fluorescence of the nuclei (Hoechst staining). Quantification of cells was performed from 2 independent experiments. In each experiment 10 fields were collected, counted, and analyzed for BrdU staining. Blue bar in A, C and D represent medians. Mann-Whitney t-test, ****p<0.0001, ns – nonsignificant.



Supplementary Figure 3. LL3 partially mediates megacyte differentiation through Toll signaling. (A) dsCactus and double silenced dsCactus + dsLL3 hemocytes perfused 4 days after silencing. Actin is showing in green and the nuclei in blue. Scale Bar: 60 μ m. (B) Percentage of megacytes among all granulocytes in response to co-silencing of dsCactus and dsLL3. Percentages were compared using X^2 -test, $p < 0.0001$ and calculated from 2 independent experiments. In each experiment 10 fields per condition were counted.

Video S1. Front view (XY) of a regular granulocyte from *An.gambiae* mosquito female. Showing in red is the microvesicle staining and in green the plasma membrane. Scale Bar: 10 μ m. Hemocyte was imaged for 1 hour in intervals of 5 minutes.

Video S2. Side view (XZ) of a regular granulocyte from *An. gambiae* mosquito female. Showing in red is the microvesicle staining and in green the plasma membrane. Scale Bar: 5 μ m. Hemocyte was imaged for 1 hour in intervals of 5 minutes.

Video S3. Front view (XY) of a megacyte from *An. gambiae* mosquito female. Showing in red is the microvesicle staining and in green the plasma membrane. Scale Bar: 10 μ m. Hemocyte was imaged for 1 hour in intervals of 5 minutes.

Video S4. Side view (XZ) of a megacyte from *An. gambiae* mosquito females Showing in red is the microvesicle staining and in green the plasma membrane. Scale Bar: 5 μ m. Hemocyte was imaged for 1 hour in intervals of 5 minutes.

Video S5. *In vivo* hemocyte patrolling activity in dsLacZ mosquitoes. Hemocytes stained in red were imaged through the cuticle of the mosquito for 1 hour and 20 minutes. Scale Bar: 30 μ m.

Video S6. *In vivo* hemocyte patrolling activity in dsCactus mosquitoes. Hemocytes stained in red were imaged through the cuticle of the mosquito for 1 hour and 20 minutes. Scale Bar: 30 μ m.

Video S7. DsCactus megacyte dynamics *in vitro*. Perfused hemocytes from dsCactus mosquitoes. Plasma membrane is showing in green, microvesicles in red and nuclei in blue. Scale Bar: 20um.

Table S1. List of upregulated genes in Cactus silenced hemocytes

Table S2. List of downregulated genes in Cactus silenced hemocytes

AGAP010042	AGAP010042	solute carrier organic anion transporter family member	21.0389471	-2.013165455	0.3254	-6.1858	6.17889E-10	2.36631E-09
AGAP001515	AGAP001515	nan	423.014864	-2.012263574	0.2512	-8.0115	1.13334E-15	6.52239E-15
AGAP001200	AGAP001200	glycogen debranching enzyme	2019.46101	-2.008162939	0.1894	-10.605	2.82763E-26	2.77338E-25
AGAP004793	AGAP004793	ornithine--oxo-acid transaminase	92.9537762	-2.006348426	0.2092	-9.5893	8.86788E-22	7.14054E-21
AGAP005662	AGAP005662	acyl-CoA dehydrogenase	10248.125	-2.004762998	0.177	-11.326	9.73147E-30	1.12079E-28
AGAP006513	nan	nan	210.228167	-2.004080989	0.1764	-11.364	6.3446E-30	7.39758E-29
AGAP002429	CYP314A1	cytochrome P450	38.4907408	-2.003366386	0.2933	-6.8304	8.46568E-12	3.74437E-11
AGAP001550	AGAP001550	sodium-coupled monocarboxylate transporter 1	198.112491	-2.001364547	0.2249	-8.8995	5.61224E-19	3.87631E-18