Signalling through TLR2/MyD88 induces differentiation of murine bone marrow stem and progenitor cells to functional phagocytes in response to Candida albicans

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Summary

We have previously demonstrated that inactivated yeasts and hyphae of Candida albicans induce in vitro the proliferation of murine haematopoietic stem and progenitor cells (HSPCs, sorted as LKS cells: Lin-c-Kit+Sca-1+) as well as their differentiation to lineage-positive cells, through a MyD88-dependent pathway. In this work, we have found that this process is mainly mediated by TLR2, and that expanding cells express myeloid and not lymphoid markers. Incubation of long-term repopulating HSCs (Lin-CD105+ and Sca-1+) with C. albicans yeasts resulted in their proliferation and upregulation of the common myeloid progenitors (CMPs) markers, CD34 and FcγR II/III, by a TLR2/MyD88-dependent signalling pathway. In addition, this TLR2/MyD88 signalling promotes the differentiation of CMPs and granulocyte and macrophage progenitors (GMPs) into cells with the morphology of macrophages and neutrophils, characterized by an increase in the expression of CD11b, F4/80 and Ly6G, independently of the presence of growth and differentiation factors. These differentiated cells were able to phagocytose C. albicans yeasts and to produce proinflammatory cytokines. In conclusion, C. albicans may be sensed by TLRs on haematopoietic stem and progenitor cells to promote the host capability for rapidly replenishing myeloid cells that constitute the first line of defence against C. albicans.

Introduction

The fungal species Candida albicans is both a commensal and an opportunistic pathogen in humans. Depending on the underlying host defect, this microorganism is able to cause a variety of infections that range from mucosal candidiasis to life-threatening invasive infections. The frequency of the latter has increased in the last decades as a result of an expanding immunocompromised population (Pfaller and Diekema, 2007).

Resistance to candidiasis requires the coordinated action of innate and adaptive immune defences. Phagocytes, such as neutrophils and macrophages, are crucial to these processes since they can eliminate the pathogen via phagocytosis. Furthermore, macrophage activation leads to the release of several key mediators such as proinflammatory cytokines, which are important for protecting the host against disseminated candidiasis, and for inducing a T helper type 1 (Th1) immune response that activates fungicidal effector mechanisms, and helps in the generation of a protective antibody response (Romani, 2004). Phagocytic cells recognize the pathogen through a variety of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs) (Zelante et al., 2007; Jouault et al., 2009). We have previously shown that TLR2 is the most important TLR involved in interaction with C. albicans, both yeasts and hyphae, triggering cytokine secretion through the MyD88 (myeloid differentiation factor 88) signalling pathway (Villamón et al., 2004a,b, Gil and Gozalbo, 2006). Other authors have also described the involvement of TLR4 in C. albicans recognition, and it is now accepted that both TLR2, which forms TLR2/TLR6 heterodimers, and TLR4 are the main TLRs involved in the signalling induced by C. albicans (Goodridge and Underhill, 2008; Gil and Gozalbo, 2009; Jouault et al., 2009).

Since cells responsible for innate immunity have a limited life span, replenishment of these cells is essential during infection. All leucocytes arise from a common...
The prevailing definition for murine HSPCs is the common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), shared by all TLRs except TLR3. Our results suggested that MyD88 is the universal TLR adaptor molecule. MyD88 promotes the differentiation of HSCs towards the myeloid lineage-positive cells, and that this response requires the exogenous differentiation factors. As a combination of proliferation and differentiation data, we calculated the amount of Lin+ cells recovered per input progenitor from TLR2−/−, TLR4−/− and wild-type mice (Fig. 1C). As expected, when LKS cells from C57BL/6 mice were incubated in presence of C. albicans yeasts or hyphae, the amount of Lin+ cells recovered per input progenitor significantly increased as compared with the negative control (cells incubated in medium without exogenous stimuli), according to previous observations (Yáñez et al., 2009). The amount of Lin+ cells recovered per input progenitor from TLR2−/− mice slightly, but significantly, increased in response to hyphae, but not in response to yeasts, whereas the amount of Lin+ cells

Results and discussion

Role of TLR2 and TLR4 in the C. albicans induced proliferation and differentiation of LKS cells

We have previously reported that in vitro, inactivated yeasts and hyphae of C. albicans drive purified haematopoietic stem and progenitor cells (HSPCs) to proliferate and increase their output of differentiated (Lin−) cells, and that this response is MyD88 dependent (Yáñez et al., 2009). As MyD88 is the universal TLR adaptor molecule, shared by all TLRs except TLR3, our results suggested that the C. albicans effect on HSPCs is mediated by TLRs. It is well established that TLRs, mainly TLR2 and TLR4, are involved in mature host cells interactions with C. albicans and play a significant role in the development of host immune responses during candidiasis (Gil and Gozalbo, 2006; 2009; Jouault et al., 2009). On the other hand, the prevailing definition for murine HSPCs is the LKS (Lin− c-Kit+ Sca-1+) population, which contains a variety of transitional intermediates between long-term repopulating HSCs, and oligopotent progenitors (long-term repopulating HSCs, short-term repopulating HSCs and MPPs) (Iwasaki and Akashi, 2007). Therefore, in order to determine whether TLR2 and/or TLR4 may be involved in the interaction of C. albicans with HSPCs, we first tested the ability of C. albicans cells to induce proliferation of purified murine LKS cells from TLR2−/− and TLR4−/− mice. LKS cells from TLR2−/− and TLR4−/− mice were purified and stimulated in serum-free, stromal cell-free cultures with either inactivated yeasts or hyphae of C. albicans. Two cytokines, Flt-3 ligand (FL) and stem cell factor (SCF), were added to the cultures as they promote cell viability, but induce little differentiation under these conditions (Fig. 1B). After 72 h of culture, whereas this response did not occur with LKS cells from TLR2−/− mice, indicating that C. albicans delivers TLR2-dependent signals promoting LKS cell proliferation. As expected, Pam3CSK4 induced cell proliferation of LKS cells from TLR4−/− mice, and LPS induced cell proliferation of LKS cells from TLR2−/− mice.

Next, we focused on the ability of C. albicans cells to induce differentiation of LKS cells from TLR2−/− and TLR4−/− mice (Fig. 1B). After 72 h of culture of LKS cells from TLR2−/− mice, the frequency of Lin+ cells increased as a result of TLR stimulation with LPS (roughly, 20% increase) as compared with unstimulated cells, whereas stimulation with yeasts or hyphae only resulted in a slight increase of Lin+ (3% and 9% respectively). On the other hand, after 72 h of culture of LKS cells from TLR4−/− mice, the frequency of Lin+ cells increased as a result of TLR stimulation with Pam3CSK4 (roughly, 30% increase) as compared with unstimulated cells, whereas stimulation with yeasts or hyphae resulted in a slight increase of Lin+ (9% and 8% respectively).

As a combination of proliferation and differentiation data, we calculated the amount of Lin+ cells recovered per input progenitor from TLR2−/−, TLR4−/− and wild-type mice (Fig. 1C). As expected, when LKS cells from C57BL/6 mice were incubated in presence of C. albicans yeasts or hyphae, the amount of Lin+ cells recovered per input progenitor significantly increased as compared with the negative control (cells incubated in medium without exogenous stimuli), according to previous observations (Yáñez et al., 2009). The amount of Lin+ cells recovered per input progenitor from TLR2−/− mice slightly, but significantly, increased in response to hyphae, but not in response to yeasts, whereas the amount of Lin+ cells
Fig. 1. Proliferation and differentiation of LKS cells in response to C. albicans. LKS cells (10 000 cells/well) from TLR2−/−, TLR4−/− and C57BL/6 mice were cultured in the presence of FL and SCF with medium alone, ultrapure E. coli LPS (1 μg ml−1), Pam2CSK4 (0.125 μg ml−1) or inactivated yeasts or hyphae of C. albicans ATCC 26555 [1.5 μg (dry weight) of cells ml−1] for 72 h. Cells were then microscopically counted and analysed by flow cytometry for expression of lineage markers.

A. Data represent mean values of total cells with standard deviations from three independent experiments. *P < 0.05 and **P < 0.01, with respect to the unstimulated cells (medium).

B. Flow cytometry results: percentages indicate the frequency of Lin− cells; data are from one representative experiment of three.

C. The bar graph depicts yields of Lin− cells recovered per input progenitor, expressed as mean values with standard deviations of pooled data from three independent experiments. Results within each type of mice are compared with unstimulated cells (medium), *P < 0.05 and **P < 0.01; results within each stimuli are compared with control C57BL/6 cells, *P < 0.01.
recovered per input progenitor from TLR4−/− mice significantly increased in response to both yeasts and hyphae, compared with the negative controls. However, the Lin+ cells/input of LKS cells from both TLR2−/− and TLR4−/− was significantly decreased as compared with LKS cells from control mice (Fig. 1C). Taken together, the results obtained using LKS cells (Fig. 1) indicate that, although both TLR2 and TLR4 appear to be involved in the interaction of C. albicans with HSPCs, triggering differentiation to Lin+ cells, TLR2 is the most important TLR involved in this phenomenon, particularly in response to the yeast form of C. albicans; and besides TLR2 has also a major role in inducing proliferation of LKS cells in response to C. albicans both yeasts and hyphae.

Activation of LKS cells by C. albicans leads to myeloid cell differentiation

In order to determine whether HSPCs may be stimulated to promote myelopoiesis or lymphopoiesis in response to C. albicans, purified LKS cells from C57BL/6 mice were challenged with either yeasts or hyphae of C. albicans, as above described. As shown in Fig. 2, after the three days of culture, the expanding cells expressed the CD11b myeloid marker but not the B220 lymphoid marker. The frequency of CD11b-positive cells (Fig. 2A), as well as the amount of CD11b-positive cells recovered per input progenitor (Fig. 2B) increased significantly in response to all the stimuli tested: LPS, Pam2CSK4, yeasts and hyphae of C. albicans. As compared with unstimulated cells. These results clearly indicate that C. albicans may alter leucocyte production by promoting myelopoiesis over lymphopoiesis. These results are in line with those by Nagai et al. (2006), who showed that activation of TLRs on HSCs by LPS and Pam2CSK4, a ligand for the heterodimer TLR2/TLR1, can bias haematopoiesis towards the production of granulocytes, macrophages and dendritic cells.

C. albicans drives TLR2/MyD88-dependent proliferation and differentiation of Lin− CD105+ and Sca-1+ cells

As above indicated, the assayed LKS (Lin− c-Kit+ Sca-1+) population contains a variety of transitional intermediates between long-term repopulating HSCs, and oligopotent progenitors (long-term repopulating HSCs, short-term repopulating HSCs and MPPs) (Iwasaki and Akashi, 2007); therefore we investigated whether C. albicans may also directly activate the quiescent long-term repopulating HSC subset. Accordingly, we used endoglin (CD105), an ancillary TGF-β receptor that is differentially expressed in long-term repopulating HSCs (Chen et al., 2002; 2003) to purify these cells. Lin− cells were stained with anti-CD105 and anti-Sca-1 antibodies, and double positive cells were sorted (Fig. 3A). Lin− CD105+ and Sca-1+ cells from C57BL/6, MyD88−/−, TLR2−/− and TLR4−/− mice were purified, and stimulated in serum-free, stromal cell-free cultures, as above indicated, with LPS, Pam2CSK4 or C. albicans yeasts for 72 h.

As the number of cells was too low to perform microscopic counting, photomicrographs of the colonies after the incubation period were used to calculate their surface as an indirect measure of proliferation (Fig. 3B). Results showed that the colony of C57BL/6 cells was significantly

Fig. 2. Expression of B220 or CD11b on LKS cells in response to C. albicans. LKS cells (10 000 cells/well) from C57BL/6 mice were cultured in the presence of FL and SCF with medium alone, ultrapure E. coli LPS (1 µg ml−1), Pam2CSK4 (0.125 µg ml−1) or inactivated yeasts or hyphae of C. albicans ATCC 26555 [1.5 µg (dry weight) of cells ml−1] for 72 h. Cells were then microscopically counted and analysed by flow cytometry for expression of B220 and CD11b. (A) Percentages indicate the frequency of positive cells and (B) the bar graph depicts yields expressed as the amount of positive cells recovered per input progenitor. Results are expressed as mean values with standard deviations of pooled data from three independent experiments. *P < 0.05 and **P < 0.01, with respect to the unstimulated cells (medium).
Fig. 3. Proliferation and differentiation of Lin− CD105+ and Sca-1+ cells in response to C. albicans. Sorted cells (2000 cells per well) from C57BL/6, MyD88−/−, TLR2−/− and TLR4−/− mice were cultured in the presence of FL and SCF with medium alone, ultrapure E. coli LPS (1 μg ml−1), Pam2CSK4 (0.125 μg ml−1) or inactivated yeasts of C. albicans ATCC 26555 [1.5 μg (dry weight) of cells ml−1] for 72 h. Wells were then photomicrographed and cells analysed by flow cytometry for expression of CD34 and FcγRII/III.

A. Sort gate for isolation of double positive (CD105+ and Sca-1+) from Lin− cells. Percentage of CD105+ and Sca-1+ of total nucleated bone marrow cells is indicated.

B. Photomicrographs of Lin− CD105+ and Sca-1+ cultured colonies. The surface of each colony is indicated in μm².

C. Percentages indicate the frequency of positive cells. Results are expressed as mean values with standard deviations of pooled data from two independent experiments. Results within each type of mice are compared with unstimulated cells (medium), *P < 0.05 and **P < 0.01; results within each stimuli are compared with control C57BL/6 cells, *P < 0.05.
increased after Pam2CSK4 (64 ± 10% surface increase) or C. albicans yeasts (18 ± 6% surface increase) stimulation, as compared with colony of cells incubated without stimuli. Interestingly, this proliferative response did not occur with cells from MyD88−/− and TLR2−/− mice (no significant differences were observed), whereas cells from TLR4−/− mice proliferate in response to yeasts (21 ± 7% surface increase) similarly to control mice. These data indicate that C. albicans yeasts induce proliferation of long-term repopulating HSCs by a TLR2/MyD88 dependent pathway.

Since C. albicans induces myeloid differentiation of LKS cells, the possible differentiation of Lin−/CD105− and Sca-1+ cells through the myeloid lineage was investigated, by measuring the expression of CMPs markers after 3 days of in vitro stimulation (Fig. 3C). The percentage of long-term repopulating HSCs from C57BL/6 mice that express FcγRII/III and CD34 after 72 h of culture in presence of LPS, Pam2CSK4 and yeasts increased significantly as compared with cells incubated in medium without stimuli. As expected, cells from TLR2−/− mice did not respond to Pam2CSK4, cells from TLR4−/− mice did not respond to LPS and cells from MyD88−/− mice did not respond neither to LPS nor Pam2CSK4. Notoriously, cells from MyD88−/− and TLR2−/− mice did not respond to yeasts, whereas cells from TLR4−/− mice were able to progress to CMPs similarly to cells from control mice. Nagai et al. (2006) have previously described that the long-term repopulating, stem cell-rich Flk-2+ subset express TLRs and enter into cell cycle in response to soluble TLR ligands. Here we extend this observation, for the first time, to the interaction between a microorganism and highly purified HSCs in defined in vitro cultured conditions. Overall, our results indicate that C. albicans is able to drive the quiescent long-term repopulating HSCs to enter the cell cycle and to differentiate to CMPs and that this effect is TLR2 and MyD88 dependent, whereas TLR4 does not play a significant role in this interaction.

**C. albicans drives TLR2/MyD88-dependent production of macrophages/granulocytes from committed myeloid progenitors**

The above described findings show that LKS and Lin−/CD105− and Sca-1+ cells can be stimulated by C. albicans via TLRs to induce their differentiation to myeloid lineage cells. These data prompted us to investigate whether committed myeloid cells would be also responsive to C. albicans. CMPs (Lin−/CD34+ FcγRII/III+ and GMPs (Lin−/CD34+ FcγRII/III+) from Lin− cells were sorted, as illustrated in Fig. 4A, and cultured in the same serum-free, stromal cell-free culture conditions to determine whether the lack of normal growth and differentiation factors might be overcome by the presence of fungal cells. Cells from C57BL/6, MyD88−/−, TLR2−/− and TLR4−/− mice were purified and stimulated for 72 h with LPS, Pam2CSK4, yeasts of C. albicans, M-CSF or GM-CSF, and the recovered cells were evaluated for the expression of the myeloid marker CD11b, the mature monocyte and macrophage marker F4/80 and the granulocyte marker Ly6G (Fig. 4B). C57BL/6 CMPs clearly responded to C. albicans yeasts and acquired CD11b expression (measured both as percentage of positive cells and as the fluorescence mean intensity), and this response was similar to those obtained when the same population was stimulated with Pam2CSK4 or CSFs. The response to Pam2CSK4 and C. albicans was dependent on MyD88 and TLR2 but independent of TLR4, whereas the responses to CSFs were, as expected, independent of MyD88, TLR2 and TLR4. When C57BL/6 GMPs were assayed in the same conditions, it was found that, although unstimulated cells (medium) express the CD11b marker, they still were able to respond to TLR ligands, CSFs and C. albicans yeasts by increasing CD11b expression. Again, the response to yeasts was TLR2 and MyD88 dependent, but TLR4 independent.

The TLR2 ligand (Pam2CSK4) upregulated the expression of F4/80, but not Ly6G, both on CMPs and GMPs from control mice. However, C. albicans yeasts induced the expression of F4/80, but also the expression of Ly6G particularly on GMPs. The upregulation of F4/80, in response to yeasts, was completely dependent on MyD88 and TLR2, and partially dependent on TLR4, both on CMPs and GMPs, whereas the expression of Ly6G was dependent on MyD88 and TLR2 but independent of TLR4. However, it should be indicated that surprisingly the expression of Ly6G was upregulated in response to Pam2CSK4 in cells from TLR4−/− mice but not in cells from C57BL/6 mice, in addition, C57BL/6 GMPs downregulate expression of Ly6G in response to Pam2CSK4. Also the upregulation of CD11b and F4/80 in response to Pam2CSK4 (a pure TLR2 ligand) was higher in TLR4−/− mice than in control mice. The increased response to a TLR2 ligand in conditions of TLR4 deficiency, as well as the downregulation of Ly6G in control cells in response to a TLR2 ligand, are interesting but still unexplained issues. In any case, as the response to Pam2CSK4 and yeasts are largely different it can be suggested that C. albicans may signal in committed myeloid progenitors, not only through TLR2 but also through TLR4 or other PRRs present in these progenitors that collaborate with TLR2. This differentially sensing could explain that yeasts cells promote differentiation to macrophage and granulocyte cells whereas Pam2CSK4 appears to promote differentiation to macrophages.

To further determine the effect of C. albicans on the GMPs differentiation process, we wondered whether if
Fig. 4. Differentiation of CMPs (Lin<sup>-</sup>CD34<sup>+</sup> and FcγRII/III<sup>lo</sup>) and GMPs (Lin<sup>-</sup>CD34<sup>+</sup> and FcγRII/III<sup>hi</sup>) in response to C. albicans. Sorted cells (10 000 cells/well) from C57BL/6, MyD88<sup>-/-</sup>, TLR2<sup>-/-</sup> and TLR4<sup>-/-</sup> were cultured in the presence of FL and SCF with medium alone, ultrapure E. coli LPS (1 μg ml<sup>-1</sup>), Pam<sub>2</sub>CSK<sub>4</sub> (0.125 μg ml<sup>-1</sup>), M-CSF (20 ng ml<sup>-1</sup>), GM-CSF (20 ng ml<sup>-1</sup>) or inactivated yeasts of C. albicans ATCC 26555 [1.5 μg (dry weight) of cells ml<sup>-1</sup>] for 72 h. Cells were then analysed by flow cytometry for expression of CD11b, F4/80 and Ly6G.

A. Sort gates for isolation of CMPs and GMPs from Lin<sup>-</sup> cells. Percentages of GMPs and CMPs of total nucleated bone marrow cells are indicated.

B. Flow cytometry analysis: percentages indicate the frequencies of positive cells and FMI indicates fluorescence mean intensity; data are from one representative experiment of two.

C. Sorted GMPs cultured in the presence of FL and SCF with medium alone (medium) or inactivated yeasts of C. albicans ATCC 26555 [1.5 μg (dry weight) of cells ml<sup>-1</sup>] (yeast) for 72 h, were stained with Rapid Panoptic. Arrows indicate internalized yeasts.
C. albicans activates stem cells through TLR2/MyD88

**B**

### CMPs

|               | Medium | Pam2CSK₄ | M-CSF | GM-CSF | Yeast | C57BL/6 | MyD88-/ | TLR2-/ | TLR4-/ |
|---------------|--------|----------|-------|--------|-------|---------|---------|---------|---------|
| **Events**    |        |          |       |        |       |         |         |         |         |
| C57BL/6       |        |          |       |        |       |         |         |         |         |
| MyD88-/       |        |          |       |        |       |         |         |         |         |
| TLR2-/        |        |          |       |        |       |         |         |         |         |
| TLR4-/        |        |          |       |        |       |         |         |         |         |

**Fig. 4. cont.**

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Fig. 4. cont.
normal mature myeloid cells would be produced in response to *C. albicans*. Therefore, the morphology of GMPs after the 3 days’ incubation period in presence of yeasts, in comparison with cells incubated in medium without stimuli was observed (Fig. 4C). Control cells exhibited the morphology of promyelocytes and myelocytes whereas yeast-treated cells, although less homogeneous in appearance, presented more mature cell forms of monocytes/macrophages and granulocytes. In some cells it could be detected internalized yeasts, suggesting that phagocytosis has been occurred during the assay. Overall, these results clearly show that *C. albicans*, under the defined conditions of culture used, may stimulate myeloid progenitors through a TLR2/MyD88-dependent pathway, without requirement of exogenous classical growth and differentiation factors upregulating both F4/80 and Ly6G. This pattern of differentiation is similar to the response of CMPs and GMPs to GM-CSF.
Although endogenous levels of some cytokines such as TNF-α and IL-6 were undetectable (see below), the possible induction of endogenous putative growth factors can not be completely ruled out.

**The mature cells produced from committed myeloid progenitors, in response to C. albicans are functional phagocytes**

The above described findings indicate that C. albicans yeasts induce the differentiation of committed progenitors into cells that express the markers of mature monocytes and granulocytes and that exhibit the morphology of mature cells. These results prompted us to investigate whether these differentiated cells are functional phagocytes. For this, a purified mixed population of CMPs and GMPs from C57BL/6 mice was cultured in serum-free, stromal cell-free cultures for 72 h either in presence or absence of C. albicans yeasts to induce the differentiation process, and then cells were challenged with inactivated yeasts for 4 or 6 h in complete cell culture medium, in order to determine the production of proinflammatory cytokines (Fig. 5) and the phagocytosis of yeasts (Fig. 6). As shown in Fig. 5, production of TNF-α and IL-6 by cells differentiated in response to yeasts was significantly higher than the production by control cells (incubated 72 h in medium without stimuli), both after 4 and 6 h of challenge. The levels of cytokines in the supernatants of cells after 72 h of incubation (either in presence or absence of yeasts), prior to challenge, were undetectable; also the cytokine levels were not detectable when cells were further incubated for 4 and 6 h, in complete cell culture medium, but without the yeast challenge.

Next, in order to determine the ability of these cells to internalize the yeasts, we performed the same assay using pHrodo-labelled yeasts to distinguish the internalized yeasts from cell surface and adherent microorganisms, by measuring the increase in fluorescence emission of the fluorogenic dye at the pH acidic conditions in the phagosome, after 4 h of challenge. As shown in Fig. 6, the percentage of phagocytosis (measured as cells that contain at least one internalized yeast) was significantly higher in differentiated cells than in control cells. These results indicate that cells differentiated in the presence of yeasts are able to internalize the microorganism and secrete proinflammatory cytokines, suggesting that these cells are functional, as they have acquired not only markers of differentiation but also these important antifungal functions.

**Modification in B lineage, monocyte/macrophage and granulocyte cells in C. albicans infected mice**

The observed in vitro effect of C. albicans on HSPCs activation may be of biological relevance in vivo. When C57BL/6 mice were intravenously infected with 1.5 × 10⁶ C. albicans PCA2 yeasts, it was found that fungal cells reach the bone marrow and that the number of yeasts recovered from this site increases with time (roughly 90 colony-forming units (cfu) at 2 h, 150 cfu at 6 h and 600 cfu at 24 h in bone marrow obtained by flushing the femurs and tibias of one animal), suggesting that C. albicans may grow at this location. Therefore, during invasive candidiasis fungal cells or soluble MAMPs (microorganism-associated molecular patterns) may rapidly reach the bone marrow cavity and engage the receptors of stem and progenitor cells. Analysis of the

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**Fig. 5.** Cytokine production by myeloid progenitors differentiated in response to C. albicans. A mixed population of sorted CMPs and GMPs cells (10 000 cells/well) from C57BL/6 mice were cultured in the presence of FL and SCF with medium alone (medium) or inactivated yeasts of C. albicans ATCC 26555 [1.5 μg (dry weight) of cells ml⁻¹] (yeast) for 72 h. Afterwards, cells were plated at a density of 400 000 cells in 200 μl of complete cell culture medium and challenged with inactivated yeasts at a 1:5 ratio (progenitor : yeasts) for 4 or 6 h. Concentration of TNF-α and IL-6 in cell-free culture supernatants was measured by ELISA. Results are expressed as means ± SD of pooled data from two experiments. *P < 0.05 and **P < 0.01, with respect to the unstimulated cells (medium).

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bone marrow cells harvested 3 or 6 days post infection (Fig. 7) showed a decrease of newly formed AA4.1+ B220+ B lineage lymphocytes concomitantly with a transitional increase in the number of CD11b+ F4/80+ monocytes and/or macrophages and a sustained increase of CD11b+ Ly6G+ granulocytes. This in vivo result correlates with the above described in vitro effect of C. albicans yeast on proliferation and differentiation of HSPCs, suggesting that this phenomenon may contribute to the increased granulopoiesis detected in mice during candidiasis. This mechanism is compatible with the results previously reported by Basu et al. (2000), showing that mice lacking both G-CSF and GM-CSF are neutropenic, but upon challenge with C. albicans, they develop a profound neutrophilia; moreover, the C. albicans induced neutrophilia in G-CSF-deficient mice is sustained and it is also accompanied by an increase in both precursor and mature neutrophils in bone marrow, indicating that it is not merely due to mobilization of a neutrophil reservoir pool, but involves de novo neutrophil production. Interestingly, there are no defects in haematopoietic maturation in MyD88 or TLR gene targeted mice, indicating that it may be functional redundancy among signals from TLR ligands and those from normal growth and differentiation factors in regulating haematopoiesis, suggesting that HSCs may use TLRs only in response to infections (McGettrick and O’Neill, 2007). In any case, these results open the question of how HSCs integrate signals from extrinsic pathogens with those from normal growth and differentiation factors, in order to replenishing the innate immune system during infection.

In conclusion, our results indicate that TLR-mediated recognition of C. albicans by haematopoietic stem and progenitor cells may help replace and/or increase cells that constitute the first line of defence against C. albicans. This new mechanism represents a potential means for the host to detect pathogen products and to signal the rapid generation of the innate immune cells within the bone marrow or other tissues containing stem cells.

Experimental procedures

Mice

MyD88−/−, TLR4−/− and TLR2−/− mice (C57BL/6 background) provided by Dr Shizuo Akira (Osaka University, Osaka, Japan) were bred and maintained at the animal production service facilities (University of Valencia); wild-type C57BL/6 mice (Harlan Ibérica, Barcelona, Spain) were used as controls. Mice of both sexes between 8 and 12 weeks old were used and all assays involving mice were approved by the Institutional Animal Care and Use Committee.
Isolation of haematopoietic stem and progenitor cells

Murine bone marrow was obtained by flushing the femurs and tibias. Cells were first depleted of lineage-positive cells by immunomagnetic cell sorting using MicroBeads (Miltenyi Biotec, Madrid, Spain). Briefly, bone marrow cells were labelled with a cocktail of antibodies against a panel of lineage antigens [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4, and Ter-119], and then cells were purified by negative selection according to the manufacturer’s instructions (Lin- cells). LKS cells were purified from Lin- cells by immunomagnetic positive selection using anti-Sca-1 MicroBeads (Miltenyi Biotec). Purity of the sorted cells was assessed by labelling with PE-labelled anti-c-Kit monoclonal antibody (3C1, Miltenyi Biotec) and anti-Lin cocktail, and flow cytometry analysis with an EPICS XL-MCL flow cytometer (Beckman Coulter). A homogeneous c-Kit+ Lin- cell population was purified.

For isolation of long-term repopulating HSCs (Lin- CD105+ and Sca-1+), Lin- cells were further stained with PE-labelled anti-CD105 (MJ7/18, Miltenyi Biotec) and FITC-conjugated anti-Sca-1 (D7, Miltenyi Biotec), and double positive cells were sorted using a MoFlo high-speed sorter (Beckman Coulter); the sort gate for long-term repopulating HSCs is illustrated in Fig. 3A. For isolation of CMPs (Lin- CD34+ and FcγRII/II/III+) and GMPs (Lin- CD34+ and FcγRII/II/III+), Lin- cells were further stained with Alexa Fluor 647-

Fig. 7. B lineage cells, monocytes/macrophages and neutrophils in the bone marrow of C. albicans infected mice. C57BL/6 mice were intravenously infected with 1.5 × 10⁶ C. albicans PCA2 cells per mouse. At day 3 or 6 post infection total bone marrow cells were stained with mAbs to the indicated markers and analysed by flow cytometry. Percentages of positive cells are indicated. Data are from one representative experiment of two.
labelled anti-CD34 (RAM34, eBioscience, San Diego, CA, USA) and FITC-conjugated anti-FcγRII/III (2.4G2, BD Pharmingen, San Diego, CA, USA) and sorted as illustrated in Fig. 4A. Propidium iodide (Sigma Aldrich, Madrid, Spain) was added to Lin- population to exclude dead cells before cell isolation using the MoFlo cell sorter. Purity of the sorted cells (long-term repopulating HSCs, CMPs and GMPs) was assessed by postsorting analyses on the MoFlo cell sorter with the same gates used for purification. The frequency of purified long-term repopulating HSCs was 0.015% of the total nucleated bone marrow cells, and these cells were negative for CD34 and FcγRII/III. The frequency of both sorted CMPs and GMPs was 0.2% of the total nucleated bone marrow cells, and these cells were positive for c-kit and negative for Sca-1.

**Serum-free, stromal cell-free cultures**

Sorted cells were immediately cultured in a serum-free, stromal cell-free medium (StemPro-34 SFM medium) containing 2 mM L-glutamine, 1% penicillin-streptomycin stock solution (Gibco, Barcelona, Spain) and two cytokines: SCF (20 ng ml⁻¹) and FL (100 ng ml⁻¹) (Peprotech, Rocky Hill, NJ, USA) as previously described (Yañez et al., 2009). Cells were cultured in round-bottom 96-well plates at a density of 10 000 (for LKS, CMPs and GMPs) or 2000 (for long-term repopulating HSCs) cells per well in 0.2 ml and challenged for 72 h with the indicated stimuli.

**Soluble stimuli used and preparation of fungal stimuli**

The stimuli used were Ultrapure *E. coli* LPS (1 μg ml⁻¹, Invivogen, San Diego, CA, USA), Pam3CSK4 (0.125 μg ml⁻¹, Invivogen), M-CSF (20 ng ml⁻¹, Peprotech), GM-CSF (20 ng ml⁻¹, Peprotech), and two inactivated *C. albicans* ATCC 26555 forms, yeast and hypha [1.5 μg (dry weight) of cells ml⁻¹], obtained as previously reported (Villamón et al., 2004b; Murciano et al., 2007). Briefly, starved yeast cells were inoculated [200 μg (dry weight) of cells ml⁻¹] in a minimal synthetic medium and incubated for 3 h at 28°C to obtain yeasts or at 37°C to obtain hyphae; more than 90% of the cells exhibited well-defined germ tubes (true hyphae) at 37°C, whereas only yeast cells were observed at 28°C. For inactivation, fungal cells were resuspended (20 × 10⁶ cells ml⁻¹) in 4% paraformaldehyde (fixation buffer, eBioscience) and incubated for 1 h at room temperature. After treatment, fungal cells were extensively washed in PBS and brought to the desired cell density in cell culture medium. All procedures were performed under conditions designed to minimize endotoxin contamination as described elsewhere (Villamón et al., 2004b; Murciano et al., 2007).

**Antibodies and flow cytometry analyses**

The following antibodies used in flow cytometry analyses were purchased from eBioscience: PE-labelled anti-CD11b (clone M1/70), PE-labelled anti-B220 (clone RA3-6B2), Alexa Fluor 647-labelled anti-CD34 (clone RAM34), PE-Cy7-conjugated anti-FcγRII/III (clone 93), PE-labelled anti-F4/80 (clone BM8), PE-labelled anti-Ly6G (clone RB6–8C5), and FITC-conjugated anti-AA4.1 (clone AA4.1). The following antibodies used in flow cytometry analyses were purchased from Miltenyi Biotec (Madrid, Spain): cocktail of biotinylated anti-lineage antigens [CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7-4 and Ter-119], FITC-labelled anti-Sca-1 (clone D7), and PE-labelled anti-c-Kit (clone 3C1).

Flow cytometry analyses were performed on an EPICS XL-MCL flow cytometer (Beckman Coulter) or a MoFlo high-speed sorter (Beckman Coulter), and the data were analysed with EXPO32 and SUMMIT V4.3.01 software respectively.

**Photomicrographs**

Cultured progenitor cells were stained with Rapid Panoptic (Panreac Quimica S.A., Barcelona, Spain), mounted in 50% glycerol in PBS and cell morphology was observed on a Nikon Eclipse E800. Photomicrographs were taken with a Nikon DXM1200F camera. Long-term repopulating HSCs cultured colonies were observed with a Nikon ECLIPSE TE2000-S and the photomicrographs were taken with a Nikon DIGITAL SIGHT DS-5M camera. All the photomicrographs were analysed with MetaMorph 6.3 software.

**Measurement of in vitro cytokine production**

A mixed population of CMPs and GMPs from C57BL/6 mice was cultured in serum-free, stromal cell-free cultures for 72 h in presence or absence of inactivated *C. albicans* yeasts, as above described. Afterwards, cells were washed once with PBS and plated in flat-bottomed 96-well plates at a density of 400 000 cells in 200 μl of complete cell culture medium (RPMI 1640 medium supplemented with 5% heat-inactivated FBS and 1% penicillin-streptomycin stock solution, Gibco, Barcelona, Spain). Cells were challenged with inactivated yeasts at a 1:5 ratio (progenitor:yeasts), settled onto the cells by centrifugation, and incubated for 4 or 6 h, and supernatants were then harvested and tested for TNF-α and IL-6 release by commercial ELISA kits (eBioscience). Duplicate samples were analysed in each assay.

**C. albicans phagocytosis assay**

*Candida albicans* ATCC 26555 yeasts (30 mg, dry weight of cells) were labelled using 1 mg of pHrodo succinimidyl ester (Molecular Probes, Invitrogen, Eugene, OR, USA), according to the manufacturer’s instructions. A mixed population of CMPs and GMPs from C57BL/6 mice was cultured in serum-free, stromal cell-free conditions for 72 h in presence or absence of *C. albicans* yeasts, as above described. Afterwards, cells were washed once with PBS and plated in flat-bottomed 96-well plates at a density of 400 000 cells in 200 μl of complete cell culture medium (RPMI 1640 medium supplemented with 5% heat-inactivated FBS and 1% penicillin-streptomycin stock solution, Gibco, Barcelona, Spain), pHrodo-labelled yeasts were added at a 1:5 ratio (progenitor:yeasts), settled onto the cells by centrifugation and incubated for 4 h. For the final 45 min of incubation, cells were stained with Hoechst 33342 (Molecular Probes, Invitrogen) at 5 μg ml⁻¹. After the incubation period cells were scraped, washed with cold PBS and plated in poly D-lysine coated clear flat-bottomed 96-well black plates (BD Biosciences, San Diego, CA) at a density of 130 000 cells per well in 50 μl of cold PBS. Samples were analysed on an automated epifluorescence microscope InCell Analyzer 1000 (GE Healthcare) and image analysis.
C. albicans infection assay

Cells of *C. albicans* PCA2, a low-virulence non-germinative strain, were obtained as previously described (Villamón et al., 2004a; Murciano et al., 2006) and diluted in PBS to the appropriate cell concentration. Mice were challenged intravenously with $1.5 \times 10^6$ yeasts in a volume of 0.1 ml. Three and six days after the infection, whole bone marrow cells were harvested, as described above, and stained with mAbs to B220, AA4.1, F4/80, Ly6G and CD11b, and analysed in an EPICS XL-MCL flow cytometer (Beckman Coulter).

Statistical analysis

Statistical differences were determined using Student’s two-tailed *t*-test. Data are expressed as mean ± SD. Significance was accepted at $^*P < 0.05$ and $^{**}P < 0.01$ level.

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