Candida albicans stimulates in vivo differentiation of haematopoietic stem and progenitor cells towards macrophages by a TLR2-dependent signalling

Javier Megías,1 Victoria Maneu,2 Pedro Salvador,1 Daniel Gozalbo1 and M. Luisa Gil*1
1Departamento de Microbiología y Ecología, Universitat de València, Burjassot, Spain.
2Departamento de Óptica, Farmacología y Anatomía, Universidad de Alicante, Alicante, Spain.

Summary
Toll-like receptors (TLRs) are expressed by haematopoietic stem and progenitor cells (HSPCs), and may play a role in haematopoiesis in response to pathogens during infection. We have previously demonstrated that (i) inactivated yeasts of Candida albicans induce in vitro differentiation of HSPCs towards the myeloid lineage, and (ii) soluble TLR agonists induce in vivo their differentiation towards macrophages. In this work, using an in vivo model of HSPCs transplantation, we report for the first time that HSPCs sense C. albicans in vivo and subsequently are directed to produce macrophages by a TLR2-dependent signalling. Purified lineage-negative cells (Lin-) from bone marrow of C57BL/6 mice (CD45.2 alloantigen) were transplanted into B6Ly5.1 mice (CD45.1 alloantigen), which were then injected with viable or inactivated C. albicans yeasts. Transplanted cells were detected in the spleen and in the bone marrow of recipient mice, and they differentiate preferentially to macrophages, both in response to infection or in response to inactivated yeasts. The generation of macrophages was dependent on TLR2 but independent of TLR4, as transplanted Lin- cells from TLR2-/- mice did not give rise to macrophages, whereas Lin- cells from TLR4-/- mice generated macrophages similarly to control cells. Interestingly, the absence of TLR2, or in a minor extent TLR4, gives Lin- cells an advantage in transplantation assays, as increases the percentage of transplanted recovered cells. Our results indicate that TLR-mediated recognition of C. albicans by HSPCs may help replace and/or increase cells that constitute the first line of defence against the fungus, and suggest that TLR-mediated signalling may lead to reprogramming early progenitors to rapidly replenishing the innate immune system and generate the most necessary mature cells to deal with the pathogen.

Introduction
Candida albicans is the microorganism most frequently causing opportunistic fungal infection. Clinical manifestation range from mucosal candidiasis to life-threatening invasive infections, whose frequency has increased in the last decades, as a result of an expanding immunocompromised population (Pfaller and Diekema, 2010). Resistance to candidiasis requires the co-ordinated 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 and release several key mediators such as pro-inflammatory cytokines, which are important for protecting the host against disseminated candidiasis and for inducing a protective adaptive immune response (Hohl et al., 2006; Brown, 2011).

Phagocytic cells recognize the pathogen through a variety of pattern recognition receptors (PRRs), including TLRs and Dectin-1 (Gil and Gozalbo, 2009; Jouault et al., 2009). We have previously shown that TLR2 is the most important TLR involved in the interaction with C. albicans, triggering cytokine secretion through the MyD88 signalling pathway (Villamón et al., 2004a,b,c; 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 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).

Recent findings suggest that TLRs may play a role in haematopoiesis during infection (King and Goodell, 2011; Boiko and Borghesi, 2012). Murine haematopoietic stem cells (HSCs) and their progeny express TLRs, and upon in vitro exposure to soluble TLR2 and TLR4 ligands are...
stimulated to enter cell cycle and acquire lineage markers (Nagai et al., 2006). Signalling through TLR7/8 induces the differentiation of human bone marrow CD34+ progenitor cells along the myeloid lineage (Sioud et al., 2006), and the TLR1/2 agonist Pam3CSK4 instructs commitment of human HSCs to a myeloid cell fate (De Luca et al., 2009). Moreover, our group has previously demonstrated that inactivated yeasts of C. albicans induce the proliferation of haematopoietic stem and progenitor cells (HSPCs) and their differentiation towards the myeloid lineage in vitro. This response requires signalling through TLR2/MyD88, and gives rise to functional phagocytes that are able to internalize yeasts and secrete pro-inflammatory cytokines (Yáñez et al., 2009; 2010).

In addition, this newly described mechanism, which may represent a potential means for pathogen products to signal the rapid generation of innate immune cells, has been explored in some in vivo infectious models; production of dendritic cells from murine lymphoid precursors during herpes infection is TLR9-dependent (Welner et al., 2008), expansion of HSCs during vaccinia virus infection is MyD88-dependent (Singh et al., 2008), TLR-mediated signals play an essential role in monocyte expansion during systemic Listeria monocytogenes infection (Serbina et al., 2009), and HSPCs increase markedly following Mycobacterium tuberculosis infection in a TLR2/MyD88-dependent manner (Megías et al., 2011). Moreover, in an experimental model of invasive candidiasis in mice, HSPCs are rapidly expanded and new populations of monocyte derived dendritic cells and inflammatory macrophages are generated in the spleen, in a TLR2-dependent manner (Yáñez et al., 2011).

All these results indicate that TLRs control haematopoiesis during infection, and raise the question of whether this is due to the direct recognition of pathogens by progenitor cells and/or to secondary effects due to pathophysiological changes during infection. To deal with this issue we recently performed a new experimental in vivo approach (Megías et al., 2012). Purified HSPCs from bone marrow of B6Ly5.1 mice (CD45.1 alloantigen) were transplanted into TLR2−/−, TLR4−/− or MyD88−/− mice (CD45.2 alloantigen), which were then injected with soluble TLR ligands (Pam3CSK4, LPS or ODN respectively). As recipient mouse cells do not recognize the TLR ligands injected, interference by soluble mediators secreted by recipient cells is negligible. Transplanted cells were detected in the spleen and bone marrow of recipient mice, and cells differentiated to macrophages in response to soluble TLR ligands (Megías et al., 2012).

Here, we used a similar in vivo model of HSPCs transplantation in order to study the possible effect of C. albicans on transplanted (control, TLR2−/− or TLR4−/−) cells in wild-type (B6Ly5.1) mice. We report for the first time that HSPCs sense C. albicans in vivo and subsequently are directed to produce macrophages by a TLR2-dependent signalling. These results suggest that self/non-self-discrimination also occur at the level of HSPCs where TLR-mediated signalling may lead to reprogramming early progenitors to rapidly replenishing the innate immune system and generate the most necessary mature cells to deal with the pathogen.

**Results**

**Effect of C. albicans infection on distribution of CD45.2 Lin− cells transplanted in CD45.1 mice**

Using a model of HSPCs transplantation, we have previously reported that direct TLR mediated stimulation of HSPCs occurs in vivo and promotes differentiation towards macrophages (Megías et al., 2012). Here, we used a similar approach in order to investigate the in vivo differentiation of transplanted HSPCs in response to C. albicans infection (Fig. 1A). Purified Lin− progenitor cells from bone marrow of C57BL/6 mice (CD45.2 alloantigen) were transplanted into B6Ly5.1 mice (CD45.1 alloantigen), and 24 h after transplantation mice were infected by intravenous injection of $0.4 \times 10^6$, $0.6 \times 10^6$ or $0.8 \times 10^6$ yeasts per mouse. Three days after transplantation, bone marrow and spleen cells were enriched for CD45.2 cells by depletion of CD45.1 cells and analysed by multicolour fluorescence and flow cytometry. Analysis of the expression of CD45.2 allowed the detection of donor-derived cells (Fig. 1B). In uninfected mice, 3.8% and 1.0% of the Lin− transplanted cells were detected in spleen and bone marrow respectively. In addition, to assess the tissue outgrowth of the microorganism in infected mice, the fungal burden in kidney, the target organ in this invasive model of candidiasis, was determined. The three doses of yeasts injected resulted, as expected, in a low, medium and high level of colony-forming units (cfu) recovered from the kidney (Fig. 1C). Interestingly, a significant increase in CD45.2 cells was detected in the spleen and in the bone marrow of the low-infected animals (Fig. 1C). These results indicate that at low degree of infection, C. albicans is sensed by the transplanted progenitors and induces their proliferation and/or improves their survival in vivo. Higher rates of infection (medium and high) did not trigger any increase in CD45.2 cells, probably indicating that progenitors are being consumed by fighting the infection.

**Transplanted CD45.2 Lin− cells respond to C. albicans infection and are directed to produce macrophages**

Next, the expression of different markers on CD45.2 cells was analysed in order to determine whether the transplanted cells differentiated in vivo (Fig. 2). In uninfected mice, some CD45.2 cells still expressed markers of stem
cells (4.8% cells were c-Kit$^+$ in the spleen, and 6.1% cells were c-Kit$^+$ in the bone marrow), indicating that most of the Lin$^-$ cells were able to differentiate towards more mature cells. These cells did not express CD19 and B220 lymphoid markers either (data not shown), indicating that lymphopoiesis is not promoted in these conditions. Finally, we analysed the expression of myeloid markers and found that transplanted cells differentiated towards this lineage, as significant percentages of CD11b$^+$ cells were detected (Fig. 2A). Minor cell populations expressed markers of mature pDCs, cDCs or macrophages, but most of cells expressed markers of monocytes (Fig. 2B).

After C. albicans infection, the immune phenotype of CD45.2 cells changed drastically. CD45.2 cells no longer expressed c-Kit (data not shown), and increased expression of CD11b and the F4/80 macrophage marker (at medium and high level of infection), both in the spleen and in the bone marrow; it should be noted that the percentage of CD11b and F4/80 double positive cells was higher in the spleen than in the bone marrow of infected mice (Fig. 2A). In the spleen of mice with medium and high level of infection, the generation of a significant percentage of CD45.2 macrophages (CD11b$^+$ CD11c$^-$/Ly6C$^-$/F4/80$^+$) was detected, and this increase in macrophages correlated with a decreased in monocytes, in heavily infected mice. A strong decrease in monocytes and a discrete increase in macrophages were detected in bone marrow of heavily infected mice, indicating that the differentiation towards macrophages plus probably emigration of monocytes to peripheral infected tissues did occur (Fig. 2B). It should be noted that the low level of infection resulted in an increase of recovered transplanted cells, as above mentioned, but no significant changes in differentiation were observed as compared with uninfected mice, probably because the infection is rapidly overcome.

Overall, these results demonstrate that HSPCs differentiate in vivo in response to C. albicans infection, and that, in these experimental conditions, give rise preferentially to macrophages.

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Effect of inactivated yeasts on distribution of TLR2− and TLR4− CD45.2 Lin− cells transplanted in CD45.1 mice

The above described findings indicate that C. albicans infection induces in vivo the differentiation of Lin− progenitors into macrophages. However, in the in vivo model of infection, the percentage of recovered transplanted cells is very low, especially in animals infected with medium or high doses of yeasts, indicating that probably the rate of dead cells is high. Therefore, we wondered whether a high number of transplanted derived cells would be produced in response to inactivated C. albicans. For this, purified Lin− progenitor cells from bone marrow of C57BL/6 mice (CD45.2 alloantigen) were transplanted into B6Ly5.1 mice (CD45.1 alloantigen), which were then injected with one daily dose of 10×10⁶ inactivated yeasts, for 3 days. Three days after transplantation, bone marrow and spleen cells were enriched for CD45.2 cells by depletion of CD45.1 cells and analysed by multicolour

Fig. 2. In vivo differentiation of CD45.2 Lin− transplanted progenitor cells in response to C. albicans infection. C57BL/6 CD45.2 Lin− progenitor cells were transplanted into CD45.1 control mice. Twenty-four hours after transplantation, CD45.1 control mice were injected with PBS alone or infected with increasing amounts of C. albicans viable yeasts. Three days after transplantation, recipient mice were slaughtered and CD45.2 cells were recovered from the spleen and bone marrow, labelled with antibodies and analysed by flow cytometry.

A. The CD45.2 population was gated, shown in CD11b versus F4/80 contour plots, and subgated as CD11b+ cells (green gate) and double positive CD11b+ F4/80+ cells (red gate). The indicated percentages refer to total CD45.2 analysed cells. At least 10 000 CD45.2 events were analysed in each spleen sample and 2500 in each bone marrow sample. Each contour plot is representative of three independent experiments.

B. The CD45.2 population was gated and cells in this gate were identified as pDCs (CD11b+ CD11c+ mPDCA+), cDCs (pre-cDCs or cDCs: CD11b+ CD11c− Ly6C− F4/80−), Mc (CD11b+ CD11c− Ly6C− F4/80−) and Mph (CD11b+ CD11c− Ly6C− F4/80−). Percentages refer to the total analysed CD45.2 population. At least 10 000 CD45.2 events were analysed in each spleen sample and 2500 in each bone marrow sample. Data represent means ± SD from three experiments. **P < 0.01; *P < 0.05 with respect to CD45.2 cells within each cell type recovered from transplanted non-infected mice. pDCs: plasmacytoid dendritic cells, cDCs: classic dendritic cells; Mc: monocytes; Mph: macrophages.

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fluorescence and flow cytometry. Analysis of the expression of CD45.2 allowed the detection of donor-derived cells (Fig. 3B); in unstimulated mice, 3.2% and 1.9% of the control Lin- transplanted cells were detected in the spleen and in the bone marrow respectively. A significant increase in CD45.2 (C57BL/6) cells was detected both, in the spleen (19.8%) and in the bone marrow (4.7%) of C. albicans stimulated animals (Fig. 3C). These results indicate that inactivated C. albicans yeasts are sensed by the transplanted progenitors and induce their proliferation and/or improve their survival in vivo.

These results prompted us to investigate whether TLR signalling is playing a role in our transplantation in vivo model, using inactivated yeasts as stimuli. For this, Lin- progenitor cells from bone marrow of TLR2-/- or TLR4-/- mice (CD45.2 alloantigen) were transplanted into B6Ly5.1 mice (CD45.1 alloantigen), which were then injected with inactivated yeasts as above described. Using this experimental approach (Fig. 3A), the possible differences in CD45.2-derived cells from TLR2-/- and TLR4-/- mice in comparison with cells from C57BL/6 control mice, may only be due to a defective TLR signalling on transplanted progenitor cells. First, we analysed the distribution of TLR2-/-/ and TLR4-/-/CD45.2 Lin- cells transplanted in C57BL/6 CD45.1 mice and detection of CD45.2 cells in spleen and bone marrow after in vivo exposure to inactivated C. albicans yeasts.

A. Schematic protocol of cell transplantation and stimulation with inactivated yeasts (as described in Experimental procedures).

B. Three days after transplantation, donor-derived CD45.2 cells were detected in the spleen and bone marrow of CD45.1 control mice (transplanted with C57BL/6 CD45.2, TLR2-/-/CD45.2 or TLR4-/-/CD45.2 Lin- cells, but not challenged with inactivated yeasts). Dot plots show FSC against CD45.2 expression of the purified spleen and bone marrow cells. Percentage of recovered CD45.2 cells is calculated as follows: \( \frac{\text{total number of recovered cells} \times 100}{\text{total number of transplanted cells}} \). The indicated percentages are the mean ± SD of three mice. **P < 0.01 with respect to CD45.2 cells recovered from transplanted unstimulated control mice.

C. Percentages of recovered CD45.2 cells from the spleen and bone marrow of CD45.1 control mice transplanted with C57BL/6 CD45.2, TLR2-/-/CD45.2 or TLR4-/-/CD45.2 Lin- progenitor cells and stimulated daily with \( 1 \times 10^6 \) inactivated yeasts per day, for 3 days. Data represent means ± SD from three experiments. **P < 0.01; *P < 0.05 with respect to CD45.2 cells recovered from transplanted unstimulated control mice.

FSC: forward scatter; Lin-: lineage-negative cells.
Transplanted CD45.2 Lin- cells respond to inactivated C. albicans yeasts and are directed to produce macrophages by a TLR2-dependent signalling

Next, we analysed the expression of different markers on the CD45.2 cells in order to determine whether the transplanted cells differentiated in response to C. albicans (Fig. 4). As above described, control Lin- CD45.2 cells transplanted in unstimulated mice, differentiated towards the myeloid lineage and most of cells expressed markers of monocytes (Fig. 4). However, after injection of inactivated C. albicans yeasts, the immune phenotype of CD45.2 cells changed significantly. CD45.2 cells no longer expressed c-Kit (data not shown), and increased the expression of CD11b and F4/80, both in the spleen and in the bone marrow (Fig. 4A). In the spleen and in the bone marrow of these mice, the generation of a significant percentage of CD45.2 macrophages (CD11b+ CD11c- Ly6C+ F4/80-) was detected, although this change was not accompanied by a decrease of monocytes (Fig. 4B).

The generation of macrophages was dependent on TLR2 but independent of TLR4, as transplanted Lin- cells from TLR2-/- mice did not give rise to macrophages, whereas Lin- cells from TLR4-/- mice generated macrophages similarly to control cells, both in the spleen and in the bone marrow (Fig. 4B). Accordingly, the percentage of CD11b and F4/80 double positive CD45.2 cells was higher in the spleen and in the bone marrow of mice transplanted with control and TLR4-/- cells than in mice transplanted with TLR2-/- progenitors (Fig. 4A). These results demonstrate that HSPCs are stimulated by C. albicans via TLR2 and subsequently are directed to produce macrophages.

Discussion

Under physiological conditions, the process of HSC self-renewal, as well as their conversion into lineage-committed progenitors, is tightly controlled to maintain daily blood cell production. Many cytokines and transcription factors fine-tune the proliferation of HSPCs and their differentiation into mature myeloid and lymphoid cells (Iwasaki and Akashi, 2007). However, haematopoiesis can be dramatically altered during infection, which influences numbers and types of cells that are produced. During most bacterial, viral and fungal infections, myelopoiesis becomes predominant with inhibition of other lineage (lymphoid and erythroid) development, and this is accompanied by alterations of the cellular composition and/or phenotype of bone marrow HSPCs (Baldridge et al., 2011; Boiko and Borghesi, 2012).

Additional perspective on haematopoiesis during infection has come from the discovery that HSPCs express functional TLRs, and that TLR signals provoke cell cycle entry and myeloid differentiation in vitro (Nagai et al., 2006; Sioud et al., 2006; De Luca et al., 2009; Yáñez et al., 2009; 2010; 2011). HSPCs expansion and alterations in haematopoiesis during infection have been described in several models of bacterial, viral and fungal infection, although the contribution of TLR signalling to this phenomenon is still a matter of discussion (Baldridge et al., 2011; Boiko and Borghesi, 2012).

Although most of the infection models demonstrate that TLR-mediated signals play an essential role in the control of HSPC expansion (Singh et al., 2008; Welner et al., 2008; Serbina et al., 2009; Yáñez et al., 2011), other authors (Scumpia et al., 2010) have described that the expansion of HSPCs following bacterial infection occurs in the absence of TLR signalling. It is important to notice that the interpretation of in vivo results is difficult as TLR1+/MyD88+ mice are more susceptible to most of the infections. Therefore, comparison of haematopoiesis between control and knockout mice during infection may reflect different tissue invasion by the microorganism, and consequently, differences in secretion of cytokines that can regulate haematopoiesis, a response to the pathogen which is also TLR/MycD88 mediated. Thus, in conventional in vivo models, the alterations in haematopoiesis and in the HSPC populations during infection can be explained by at least two mechanisms: (i) microorganism-associated molecular patterns (MAMPs) may directly induce HSPCs proliferation and differentiation, as suggested by the in vitro results, or alternatively

![Fig. 4. In vivo differentiation of TLR2-/- or TLR4-/- CD45.2 Lin- transplanted progenitor cells in response to stimulation with C. albicans inactivated yeasts. C57BL/6 CD45.2, TLR2-/- CD45.2 or TLR4-/- CD45.2 Lin- progenitor cells were transplanted into CD45.1 control mice. After transplantation, recipient mice were stimulated daily with 10 x 10^6 inactivated yeasts per day, for 3 days. Afterwards, recovered cells from the spleen and bone marrow of mice were labelled with antibodies and analysed by flow cytometry. A. The CD45.2 population was gated, shown in CD11b versus F4/80 contour plots, and sub gated as CD11b+ cells (green gate) and double positive CD11b+ F4/80+ cells (red gate). The indicated percentages refer to total CD45.2 analysed cells. At least 10 000 CD45.2 events were analysed in each spleen sample and 2500 in each bone marrow sample. Each contour plot is representative of three independent experiments. B. The C57BL/6 CD45.2, TLR2-/- CD45.2 and TLR4-/- CD45.2 populations were gated and cells in this gate were identified as pDCs (CD11b- CD11c+ mPDCA+), cDCs (pre-cDCs or cDCs: CD11b+ CD11c- Ly6C- F4/80+), Mc (CD11b+ CD11c- Ly6C- F4/80-) and Mph (CD11b+ CD11c- Ly6C- F4/80+). Percentages refer to the total analysed CD45.2 population. At least 10 000 CD45.2 events were analysed in each spleen sample and 2500 in each bone marrow sample. Data represent means ± SD from three experiments. *P < 0.05; **P < 0.01 with respect to CD45.2 cells within each cell type recovered from transplanted unstimulated mice. pDCs: plasmacytoid dendritic cells; cDCs: classic dendritic cells; Mc: monocytes; Mph: macrophages; TLR: Toll-like receptors.](https://www.cellmicrobiol.com/content/15/8/1143.full)
C. albicans activates in vivo HSPCs through TLRs

A

Spleen

Bone Marrow

C57BL6

TLR2−/-

TLR4−/-

F4/80

CD11b

F4/80

CD11b

Unstimulated

Inactivated yeasts

Unstimulated

Inactivated yeasts

B

Spleen

Bone Marrow

C57BL6

TLR2−/-

TLR4−/-

% Cells

pDCs
dDCs
Mφ
Mφ

pDCs
dDCs
Mφ
Mφ

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(ii) the alterations may be caused by an indirect effect, due to pathophysiological changes during infection. These possibilities are not mutually exclusive, and both of them may involve TLR recognition of the pathogen. In order to investigate the possible direct interaction of soluble MAMPs and TLRs on HSPCs in vivo, we have recently designed a new experimental approach: using a model of HSPCs transplantation, we reported that HSPCs may be directly stimulated by TLR2, TLR4 and TLR9 soluble agonists in vivo, and that the engagement of these receptors induces differentiation towards macrophages (Megías et al., 2012).

In this work, we used a similar in vivo model of HSPCs transplantation to investigate their possible differentiation in response to C. albicans infection. Purified Lin- cells (containing stem and all the progenitor cells) from the bone marrow of C57BL/6 mice (CD45.2 alloantigen) were transplanted into B6Ly5.1 mice (CD45.1 alloantigen), which were then injected with different doses of viable C. albicans yeasts, in order to induce low, medium or high invasive infection. In these in vivo conditions and in the absence of infection, the HSPCs differentiated towards the myeloid lineage, as most of the cells had lost stem cell markers, did not express lymphoid markers, but most of them expressed the markers of mature monocytes. This result is in accordance with Massberg et al. (2007) who showed that migratory HSPCs give rise to myeloid cells in peripheral tissues.

However, when mice were infected with C. albicans, the emergence of a new population of macrophages was found. At low levels of infection, an increase in CD45.2 cell number was observed; at medium level of infection, we detected a new macrophage population in the spleen and an increase of monocytes in the bone marrow, and at high level of infection we detected a new population of macrophages both in the spleen and in the bone marrow, which correlates with a significant decrease in the monocyte population, probably due to their maturation towards macrophages, their emigration to infected tissues and their consumption by fighting the infection. These results clearly indicate that HSPCs sense the infection and that in response to C. albicans the progenitors are directed to produce macrophages. These data are in accordance with our previous results showing that (i) in an in vivo model of candidiasis, a new population of inflammatory macrophages and monocyte derived dendritic cells was generated in the spleen (Yáñez et al., 2011), and (ii) in the in vivo model of HSPCs transplantation, progenitors differentiated to macrophages in response to TLR ligands (Megías et al., 2012).

It should be noted that in the in vivo model of HSPCs transplantation and infection, the recipient mice recognize yeasts through several PRRs, and therefore it may be expected the secretion of cytokines and soluble mediators by surrounding cells. Although our in vivo model of HSPCs transplantation and differentiation in response to soluble TLR ligands (Megías et al., 2012) and the previous in vitro results (Yáñez et al., 2009; 2010; 2011) suggest a direct interaction of HSPCs with the microorganism, it cannot be excluded the contribution of an indirect mechanism due to pathophysiological changes during infection.

To go further with this issue, we wondered whether TLR signalling may play a role in the HSPCs differentiation in response to C. albicans in vivo. For this, a new experimental approach was used: progenitor cells from bone marrow of C57BL/6, TLR2+/− or TLR4+/− mice (CD45.2 alloantigen) were transplanted into B6Ly5.1 mice (CD45.1 alloantigen), which were then injected with three doses of inactivated yeasts (Fig. 3A). In these experiments we decided to use high amount of inactivated stimuli instead of viable yeasts. Infection with viable yeasts results in (i) tissue damage and generation of endogenous host ligands for TLRs such as TLR4, and (ii) most importantly, active infection generates a strong inflammatory response. Both factors may also induce HSPCs differentiation by TLR-dependent and/or TLR-independent (cytokines or even other PRRs) manner and therefore may mask the detection of the role of individual receptors such as TLR2 in this process. The use of inactivated yeasts may significantly avoid both facts (generation of endogenous ligands and inflammatory response), allowing us to more easily detect a role for individual TLRs in the differentiation process of transplanted cells.

Using this model, a significant increase in CD45.2 cells in mice transplanted with both TLR2+/− and TLR4+/− cells, as compared with mice transplanted with control cells, was observed, indicating that the absence of TLR2 or TLR4 gives Lin- cells an advantage in transplantation assays. Our results are not unprecedented, as it has been described that the absence of TLR4, TLR9 or MyD88 gives HSCs an improvement in transplantation in a model using irradiated recipients (Ichii et al., 2010). In that situation a possible explanation is that HSCs could be vulnerable to certain TLR ligands in the damaged environment of irradiated mice. This possibility does not apply to our model of transplantation as we used non-irradiated mice. Another possibility is that commensal microbiota and/or endogenous TLR ligands function to limit numbers of stem and progenitor cells in some situations. This possibility agrees with the results of Esplin et al. (2011) showing that chronic exposure to TLR ligands injures haematopoietic stem cells. These observations suggest that TLR signalling may induce differentiation of HSCs but impair their repopulating potential. Whether this property is already present in transplanted cells as a consequence of continuous signalling during the live span of donor mice, and/or to the transplantation into host mice, remains
to be determined. Further experiments are needed to go deeper in this issue. Regardless, our results show that TLRs are functional on HSPCs under physiological conditions.

Injection of inactivated C. albicans yeasts caused: (i) an increase in CD45.2 cell number that was dependent both on TLR2 and on TLR4, and (ii) the generation of a significant percentage of CD45.2 macrophages. It should be noted that despite using two different approaches: injection of inactivated yeasts or infection with viable yeasts that can form hyphae during infection, basically similar results were obtained. The generation of macrophages was dependent on TLR2 but independent of TLR4, as transplanted Lin- cells from TLR2-/- mice did not give rise to macrophages, whereas Lin- cells from TLR4-/- mice generated macrophages similarly to control cells. The differences in maturation of cells from TLR2-/- mice in comparison with cells from control mice, in response to C. albicans, may only be due to a defective TLR signalling on transplanted progenitors. Therefore, our results clearly indicate that (i) HSPCs sense C. albicans in vivo via TLR2 and TLR4 and (ii) TLR2 is essential for differentiation of HSPCs to macrophages, in response to C. albicans in vivo.

The ability of stem and progenitor cells to sense pathogens may be protective, allowing rapid mobilization and generation of cells of the innate immune system, in a pathogen-specific manner. Moreover, in the case of local infections, migratory HSPCs that sense microbial danger signals in peripheral tissues can proliferate within that pathogen-challenged location and contribute to the supply of effector cells (Mazo et al., 2011). Our results indicate that TLR-mediated recognition of C. albicans by HSPCs may help replace and/or increase cells that constitute the first line of defence against the fungus. This new mechanism provides new insights into TLR role on haematopoiesis, as well as in host–fungus interactions during infection, which may reveal a new potential target for anti-infection intervention.

**Experimental procedures**

**Mice**

TLR2-/- and TLR4-/- 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; wild-type CD45.1-positive allotype mice [B6.SJL-PtprcPepc/BoyCrl strain, also known as C57BL/6-Ly5.1 (Charles River Laboratories, Wilmington, MA, USA)] were used as recipient mice of Lin- progenitor cells from TLR2-/-, TLR4-/- and C57BL/6 transplant donors. Mice of both sexes between 8 and 12 weeks old were used. This study was carried out in strict accordance with the recommendations in the ‘Real Decreto 1201/2005, BOE 252’ for the Care and Use of Laboratory Animals of the ‘Ministerio de la Presidencia’, Spain. The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Valencia (Permit Number: A1264596506468). All efforts were made to minimize suffering.

**Purification of Lin- cells**

Lin- cells were purified as previously described (Yáñez et al., 2011; Megias et al., 2012). Briefly, murine bone marrow was obtained by flushing the femurs and tibia; cells were depleted of lineage-positive cells by immunomagnetic cell sorting using MicroBeads (Miltenyi Biotec, Madrid, Spain); 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. Purity of the sorted cells was assessed by labelling with anti-Lin cocktail and by flow cytometry analysis, and no Lin- cells were detected.

**Preparation of fungal stimuli**

Both viable and inactivated C. albicans ATCC 26555 yeasts were used in in vivo assays. Yeasts were obtained as previously reported (Villamón et al., 2004c; Murciano et al., 2007). Briefly, starved yeast cells were inoculated [200 µg (dry weight) of cells per ml] in a minimal synthetic medium and incubated for 3 h at 28°C to obtain yeasts. Only yeast cells without germ tubes were observed at 28°C. Viable yeasts were resuspended in PBS and used at different concentrations in infection experiments (see below). For inactivation, fungal cells were resuspended (20 x 10⁶ cells ml⁻¹) in 4% paraformaldehyde (fixation buffer, eBioscience, San Diego, CA) 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 PBS. All procedures were performed under conditions designed to minimize endotoxin contamination as described elsewhere (Villamón et al., 2004c; Murciano et al., 2007).

**In vivo transplantation of CD45.2 cells, infection and stimulation with inactivated yeasts**

Murine bone marrow was extracted from CD45.2 C57BL/6, CD45.2 TLR2-/- or CD45.2 TLR4-/- mice, and Lin- progenitor cells were purified as described above. Approximately 1.9 x 10⁶ Lin- cells in 100 µl of PBS (purified from four mice) were intravenously injected into one CD45.1 C57BL/6-Ly5.1 mouse. Mice transplanted with CD45.2 C57BL/6 Lin- cells were injected, 24 h after transplantation, with 0.4 x 10⁶, 0.6 x 10⁶ or 0.8 x 10⁶ viable yeasts of C. albicans in 100 µl of PBS, or with 100 µl of PBS alone. To assess the tissue outgrowth of the microorganism the fungal burden in the kidney, at day 3 after transplantation (day 2 post infection) was determined. Kidneys were weighed, and homogenized in 1 ml of PBS; dilutions of the homogenates were plated on Sabouraud dextrose agar. The cfu were counted after 28°C. Viable yeasts were resuspended in PBS and used at different concentrations in infection experiments (see below). For inactivation, fungal cells were resuspended (20 x 10⁶ cells ml⁻¹) in 4% paraformaldehyde (fixation buffer, eBioscience, San Diego, CA) 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 PBS. All procedures were performed under conditions designed to minimize endotoxin contamination as described elsewhere (Villamón et al., 2004c; Murciano et al., 2007).
injections of PBS alone (Fig. 3A). Each variable was performed with three Lin− transplanted mice.

Detection and characterization of CD45.2 transplanted cells

Each transplanted mouse was killed at day 3, and the spleen and bone marrow from femurs and tibias were removed aseptically. Total spleen cells were obtained by collagenase D treatment of the organ as previously described (Murciano et al., 2006; 2007). Erythrocytes were lysed in ammonium chloride buffer. Fc receptors were blocked with FcR Blocking Reagent (Miltenyi Biotec) and cells were depleted of CD45.1+ cells by immunomagnetic cell sorting using biotinylated anti-CD45.1 antibody and anti-biotin magnetic MicroBeads (both from Miltenyi Biotec). Recovered cells were microscopically counted, labelled with various combinations of antibodies and analysed by flow cytometry (see below).

Antibodies and flow cytometry analyses

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], PE-labelled anti-c-Kit (clone 3C1), FITC-labelled anti-CD45.2 (clone A20), APC-labelled anti-CD11c (clone N418) and APC-labelled anti-mPDCA-1 (clone JF50-1C2.4.1). The following antibodies were from eBioscience (San Diego, CA): PE-labelled anti-B220 (clone RA3-6B2), FITC-labelled anti-CD19 (clone 1D3), PE-labelled anti-CD11b (clone M1/70) and PE-Cy7-labelled anti-F4/80 (clone BM8), or from BD Pharmingen (San Jose, CA): PE-labelled anti-B220 (clone RA3-6B2), FITC-labelled anti-CD45.2 (clone A20), APC-labelled anti-CD11c (clone N418) and APC-labelled anti-mPDCA-1 (clone JF50-1C2.4.1). The following antibodies were from BD Biosciences (San Jose, CA): PerCP-Cy5.5-labelled anti-Ly6C (Ly-6G/C), 7-4 and Ter-119, PE-labelled anti-c-Kit (clone 3C1), FITC-labelled anti-CD45.2 (clone A20), APC-labelled anti-CD11c (clone N418) and APC-labelled anti-mPDCA-1 (clone JF50-1C2.4.1). The following antibodies were from eBioscience (San Diego, CA): PE-labelled anti-B220 (clone RA3-6B2), FITC-labelled anti-CD19 (clone 1D3), PE-labelled anti-CD11b (clone M1/70) and PE-Cy7-labelled anti-F4/80 (clone BM8), or from BD Pharmingen (San Jose, CA): PerCP-Cy5.5-labelled anti-Ly6C (clone AL-21). Flow cytometry analyses were performed on a FACScanto cytometer (BD Biosciences) and the data were analysed with FACSDiva and FlowJo 7.6.5. software.

Statistical analysis

Statistical differences were determined using one-way ANOVA followed by Dunnett’s test for multiple comparisons and two-tailed Student’s t-test for dual comparisons. Data are expressed as mean ± SD. Significance was accepted at *P < 0.05 and **P < 0.01 level.

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