IL-6-inducing Whole Yeast-based Immunotherapy Directly Controls IL-12-dependent CD8 T-cell Responses

Beth A. Tamburini, Ross M. Kedl,* and Donald Bellgrau†*

Summary: In current clinical trails, whole yeast-based immunotherapy expressing hepatitis C viral antigens demonstrated statistically significant improvement in end of treatment responses when combined with type I interferon based standard of care, even in standard of care resistant patients. Although preclinical data suggest yeast vaccination, such as type I interferon, facilitates CD8 T-cell immunity, the capacity of yeast to generate immunity in patients resistant to type I interferon calls into question the mechanism(s) underpinning the efficacy of this approach. We show yeast and a Toll-like receptor exclusive agonist, Pam3Cys, differ in CD8 T-cell generation when combined with an agonistic CD40 antibody. Although both yeast and PamCys were largely Toll-like receptor dependent, the primary CD8 response generated by yeast was significantly less than Pam3Cys in wild-type hosts even in a CD4 T-cell-deficient setting. In addition, immunization of IL6$^{-/-}$ mice with yeast produced a 3-fold to 6-fold increased CD8 response while the Pam3Cys response was unaffected. The yeast but not Pam3Cys-driven CD8 response was inhibited in both wild-type and IL-6$^{-/-}$ hosts by blocking interleukin (IL)-12. In addition, IL6$^{-/-}$ mice had increased CD86 expression on their dendritic cells after yeast immunization also inhibited by IL-12 blockade. Collectively, our results indicate the CD8 T-cell response to yeast but not Pam3Cys is influenced by IL-6-mediated control of IL-12 critical for dendritic cell activation. To our knowledge this is the first demonstration that yeast directly influence IL-12-associated CD8 T-cell immunity providing an additional route whereby recombinant yeast may provide efficacy independent of type I interferon.

Key Words: whole yeast-based immunotherapy, IL-6, IL-12, dendritic cell activation, CD8 T cells

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A major goal of immunotherapy is to generate and expand antigen-specific CD8 effector T cells that can directly target tumors or pathogen infected cells. One critical step in the activation process is the recognition of pathogen associated molecular patterns by pattern recognition receptors (PRRs) such as Toll-like receptors (TLR) and C type lectin receptors on the dendritic cells (DCs).[1] These receptors signal to the DCs that pathogens and or danger exists and mobilizes molecular machinery leading to DC antigen presentation and costimulation.[1] Presumably engaging TLRs/C type lectin receptors combined with antigen can recapitulate a normal immune response and thus be a means to generate an immunotherapy. However, it is now known that such engagement seems insufficient to effectively activate CD8 T cells.[2] One way to enhance the immune response comparable to active infection is through engagement of the CD40 costimulatory receptor along with TLRs.[2–5] Previous studies have shown that the combination of an agonistic CD40 antibody and an innate stimulus dramatically augments the magnitude of the CD8$^+$ T-cell response[2–5] and bypass the need for CD4 help in regulating CD8 T-cell memory.[6–9] Indeed, these and other studies have shown that the use of either CD40 or TLR agonists alone is insufficient for the production of long-lasting cellular immunity.[2–5] Given this, the identification of novel and safe methods for the stimulation of these and other pathways is of great interest.

The yeast cell wall is composed of mannans, cell wall proteins, b-(1,6)-glucan, b-(1,3)-glucan, and chitin. These sugars are recognized by a multitude of PRRs including TLR2, TLR4, TLR6, mannose receptor, dectin 1, dectin 2, DC-SIGN, FcgR, and CR3 on the surface of the DC.[10] As such, multiple T-cell subsets and the cytokines associated with them are activated by yeast. Among these are both MyD88-dependent TLR pathways, which can induce type I interferon (IFN), tumor necrosis factor, interleukin (IL)-12, and IFN γ usually associated with T-helper type 1 (Th1) and cytotoxic T lymphocyte (CTL) activation, and Syk-dependent IL-23 and IL-6 usually associated with Th17-cell production.[10,11]

Yeast thus represent a potential source of agonists for numerous innate receptor pathways and as such, are a logical choice for use as a vaccine vector for the elicitation of antigen specific responses. Indeed, yeast vaccine vectors have already shown significant promise in the clinic, specifically in providing therapeutic efficacy against chronic hepatitis C virus (HCV) infection in patients who are insensitive to standard of care IFN/ribavirin treatment.[12] However, the mechanistic underpinnings of this clinical success remain unclear. Preclinical data suggest that the use of antigen-expressing yeast vectors has the capacity to augment cross-presentation and elicit CD8$^+$ T-cell immunity.[13–17] In contrast, yeast is well known to elicit Th17 responses,[10,18–21] an immune environment that is not necessarily conducive to the formation of Th1/CTL immunity.

Several cytokines have been shown to be important for eliciting productive immune responses. One such cytokine, IL-12, can act as a "signal 3" mediator for T-cell activation.[11,22–27] In addition, IL-12 receptor binding on the DC promotes IL-12 production in an NF-kB-dependent manner.[28] IL-12 receptor engagement on the DC has also been shown to elicit production of IFNγ,[29] though the downstream effects of this are not well understood. Although yeast can elicit IL-12 production by the DC,[30] what role if any IL-12 has on DCs or in generating a CD8 T cell response to yeast immunization is not known. Thus, closer investigation of yeast-based vaccination is needed for

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a more comprehensive understanding of the role of IL-12 and its activation of DCs, and for improving the potential of yeast-based vaccination in clinical indications.

In this study, we demonstrate that yeast-based vaccination elicits the pro-Th17 cytokine IL-6 that interferes with CD8 T-cell generation, regardless of the presence or absence of $CD4^+$ T cells. Furthermore, elimination of IL-6 enhances the CD8 T-cell response to yeast-based vaccination, an enhancement that is driven by IL-12-mediated DC activation and upregulation of CD80/86 expression. Thus, these data represent a novel role for IL-6 after yeast-based vaccination in inhibiting IL-12-mediated activation of DCs resulting in a direct effect on CD8 T-cell immunity.

MATERIALS AND METHODS

Mice

Wild-type (WT), $IL6^{-/-}$, $IL12Rb2^{-/-}$, $MyD88^{-/-}$, $tbet^{-/-}$, MHC class $II^{-/-}$, $p19^{-/-}$, and OT1 mice were used on a C57Bl/6 background. Mice were purchased from the Jackson laboratory or National Cancer Institute and bred in house. $P19^{-/-}$ mice were obtained from Genentech (San Francisco, CA). OT1 mice are a TCR transgenic mouse, which only express CD8 T cells specific to the ovalbumin 257-264 in the context of H-2Kb. Mice were housed at the Biological Resource Center at National Jewish Health. All animal procedures were approved by the Institutional Animal Care and Use Committee at National Jewish Health.

Flow Cytometry

Tetramer

Cells were harvested from the spleen and peripheral blood taken from the tail vein or abdominal aorta and ACK lysed (BioSource, Rockville MD). The resulting cells were washed with RPMI 1640 medium containing 2.5% fetal calf serum, β-mercapthoethanol, L-glutamine, nonessential amino acids, HEPES, sodium pyruvate, penicillin, and streptomycin (complete media). Approximately $2 \times 10\hat{}6$ cells were stained with phycoerythrin-conjugated ovalbumin 257-264 H-2Kb tetramer for 1.5 hours in complete media at 37°C followed by 30 minutes with antibodies to CD8 conjugated to allophycocynin-Cy7 (APC-Cy7), B220 conjugated to peridinin chlorophyll protein (PerCP), and CD44 conjugated to Pacific Blue (Biolegend). Stained cells were washed with fluorescence-activated cell-sorting (FACS) buffer and run on Cyan ADP flow cytometer (DakoCytomation, Ft. Collins, CO).

DC Harvesting and Staining

DCs were harvested from spleens 1 to 2 hours for IL-12 staining or 24 hours for CD86 staining after immunization. Spleens were placed into EHAA media (Invitrogen Carlsbad, CA) containing DNAse (Worthington, Lakewood, NJ) and Collagenase D (Roche Diagnostic Systems, Somerville, NJ). Spleens were digested for 40 minutes at 37° C. Enzymatic digestion was stopped with 0.1M EDTA and washed with 5 mM EDTA in Click's media. After the final wash, cells were resuspended in complete media. Approximately 3×10^6 cells were stained in FACS buffer with 10% supernatant from 2.4G2 cell culture (B-cell hybridoma-blocking FcγRs) with CD11c APC-Cy7, CD86 FITC, CD11b Pacific Blue, CD8 PerCp for 30 minutes at 4°C. For IL-12 intracellular cytokine-staining cells were incubated in brefeldin A (ebioscience, San Diego, CA) for 3 hours before staining with surface markers CD11c APC-Cy7, CD8 APC, CD11b Pacific Blue, CD86 PerCP, and B220 FITC. Cells were then washed and fixed with 1% paraformaldehyde/3% sucrose. After fixing, cells were washed with FACS buffer and stained for IL-12 phycoerythrin in perm buffer (BD, Franklin Lakes, NJ) overnight. Cells were then washed with perm buffer followed by FACs buffer before running on a Cyan ADP flow cytometer and acquired using Summit acquisition software.

Flow cytometry data were analyzed using FlowJo software (Tree Star, Ashland, OR) and statistical analysis was done using the Student *t* test in prism4 (GraphPad, San Diego, CA).

Injections

Immunizations

Yeast were used as described in the study by Stubbs et al.[17] For yeast synergy immunization 2×10^7 yeast cells were injected intravenously with 100 μg of ovalbumin and 50 μg of αCD40 (clone FGK4.5, BioXCell, West Lebanon, NH) unless otherwise stated. Pam3Cys {N-palmitoyl-S-2,3-bis(palmitoyloxy)-(2R,S)-propyl]-(R)-cysteine-(S)serine-(S)lysine 4} (InvivoGen, San Diego, CA) was used at 25 μg in combination with 50 μg of αCD40 and 100 μg of whole ovalbumin protein (Pam3Cys synergy). Ovalbumin (Sigma-Aldrich, St Louis, MO) was decontaminated of lipopolysaccharide (LPS) using a Triton X-114 LPS detoxification method.[3] Mice were killed 7 days after immunization and cells were visualized by flow cytometry on a Cyan ADP as described above.

Blocking and Depleting Antibodies

Five hundred micrograms of the IL-12p70-blocking antibody, C17.8 (BioXcell, West Lebanon, NH), was administered by intraperitoneal injection 18 to 24 hours and 2 hours before immunization. Five hundred micrograms of the CD4-depleting antibody, GK1.5 (BioXcell, West Lebanon, NH), was administered by intraperitoneal injection 3 days prior and 4 days after immunization. Animals were stained with anti-CD4 PerCP (clone RM4-5) (Biolegend, San Diego, CA) to verify depletion in all samples.

RESULTS

Generation of Primary, Antigen-specific CD8-cell-mediated Immunity With Whole Yeast-based Immunization

We published previously that immunization of mice with intact yeast engineered to express ovalbumin lead to the generation of cell-mediated destruction of ovalbumin expressing tumors.[17] Efficient tumor destruction in vivo required CD8 T cells and was generated after at least 2 immunizations.[17] Using enhanced immunization procedures, we determined we could generate detectable primary, antigen-specific CD8-cell-mediated immune responses.

To assist in antigen presentation, we used an agonistic CD40 antibody,[2,5] which can bypass the need for CD4 help in engaging CD40 on the DC. Indeed this approach, wherein αCD40 is combined with antigen and an innate immune activator (TLR, type I IFN, a-galactoceramide),[3–5] leads to an exponential increase in tetramer-positive CD8 T cells when compared with either agonist alone.[2,5] Given the broad array of innate immune-activating molecules

possessed by yeast, we anticipated that antigenic immunization with combined yeast/αCD40 would likely produce the same exponential impact on $CD8^+$ T-cell expansion. However, although immunization with yeast and αCD40 (yeast synergy) generated CD8 responses that were increased over that seen in response to either yeast or αCD40 alone, similar immunizations with the TLR1,2 agonist Pam3Cys and αCD40 (Pam3Cys synergy) generated a 3-fold increase in antigen-specific CD8 T cells relative to yeast synergy (Figs. 1A, B). This difference in the magnitude of the T-cell responses between the 2 vaccinations exists despite the fact that both are primarily dependent on TLR signaling, demonstrated by the dramatically reduced frequency of antigen-specific CD8 T cells in MyD88 knockout mice challenged with either vaccination (Fig. 1C).

Role of IL-6 in the Generation of Antigen Specific CD8 T Cells

As budding yeast are in the fungal family, we expected they would generate IL-6.[10] We have previously reported the generation of both Th1 cytokines and IL-6 to yeast alone.[14] IL-6 has been linked to the generation of CD4 T cells including a more recently described subset termed Th17.[31] To assess the contribution of IL-6 to the antigen-specific CD8 T-cell response, we examined the ability of Pam3Cys and yeast synergy to generate antigen-specific CD8 T cells in IL-6 knockout mice (Fig. 2). Interestingly, the frequency of antigen-specific CD8 T cells was increased 3- fold to 6-fold in the IL-6 knockout mouse as compared with the WT. The marked increase in antigen-specific CD8 T cells observed with yeast synergy was not due to an

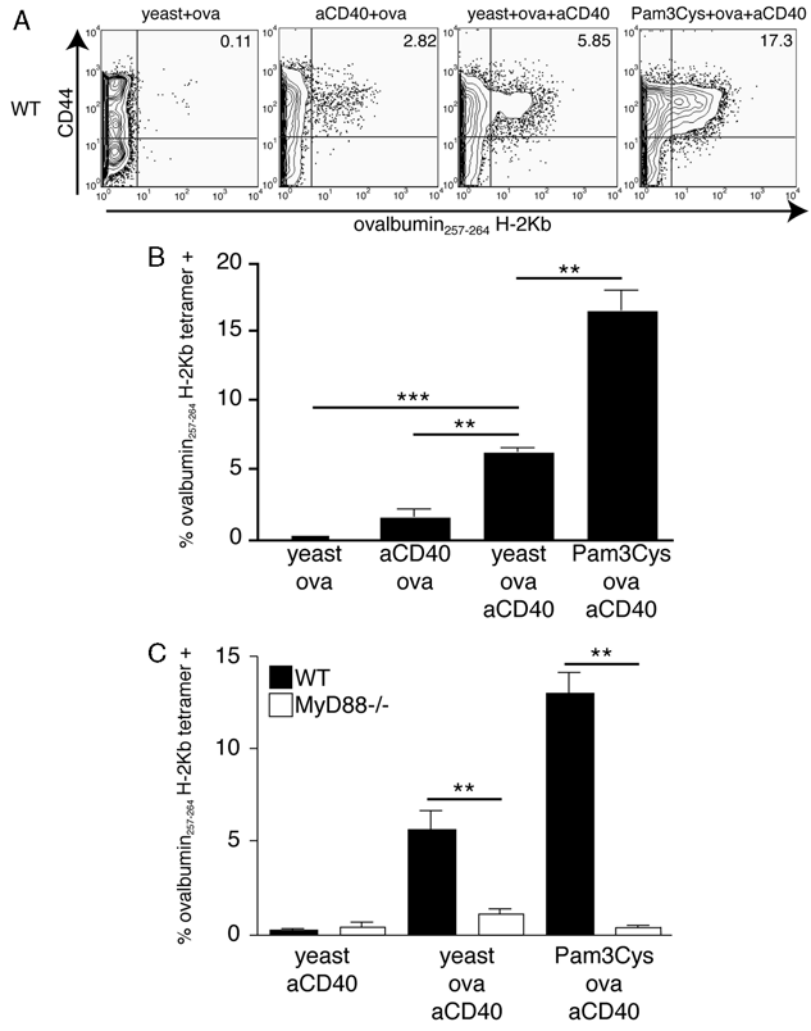

FIGURE 1. Addition of αCD40 antibody to the immunization synergistically enhances endogenous CD8 T-cell responses. Wild-type (WT) mice were immunized intravenously with 100 μg of ovalbumin (ova) and either 2×10^7 heat killed yeast, 50 μg of anti-CD40 (αCD40) (FGK4.5), both yeast and anti-CD40, or 25 μg of Pam3Cys and anti-CD40. Mice were killed 7 days after immunization and peripheral blood and spleens were taken. Peripheral blood data are shown. A, Percentages shown are live cells that are $CD8^+$, $B220^-$, CD44 high, tetramer-positive cells assessed by flow cytometric analysis of peripheral blood from a representative sample. B, Data quantified from experiment shown in part A. C, The CD8 T-cell response to yeast synergy is primarily dependent on MyD88 signaling. Bar graph representing WT (black bars) or MyD88 knockout mice (white bars) antigen-specific CD8 T-cell response to shown immunizations. Error bars represent the standard deviation. *Statistically significant values as determined by the Student *t* test (* = $P < 0.05$, ** = $P < 0.001$, *** = $P < 0.0001$). Experiments were performed at least twice with 3 mice per group.

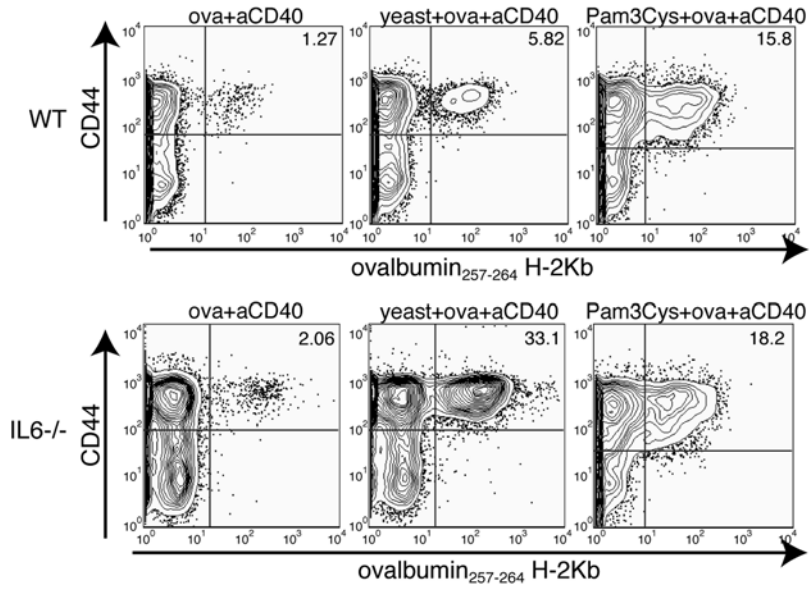

FIGURE 2. The magnitude of the CD8 T-cell response induced by yeast but not Pam3Cys is negatively influenced by IL-6. Immunization analysis of wild-type (WT) or IL6$^{-/-}$ mice was done exactly as described in the legend of Figure 1. At least 4 experiments were performed with 2 to 4 mice per group per experiment.

intrinsic enhancement of the IL-6 knockout mouse to generate antigen-specific CD8 T cells as Pam3Cys synergy responses were not significantly altered by the absence of IL-6 (Fig. 2). Therefore, it seems IL-6 is negatively influencing the generation of CD8 T cells in WT yeast synergy immunized animals.

CD8 T-cell Response is Independent of CD4 T Cells in Both WT and IL6$-/-$ mice

Fungal infection and immunization have been well documented as effective approaches to elicit CD4 Th1 and Th17 responses (reviewed in Refs. 10, 20). Although the combined use of αCD40 in combination with a TLR agonist can elicit a CD8$^+$ response in the absence of CD4 cells,[6] CD4 cells, when present, may contribute to a response after yeast/αCD40 immunization. As such, it remained possible that the reduced CD8 response could be attributed in its entirety to a CD4 T-cell subset such as Th17 that competes with and hinders a Th1-dependent CD8 T-cell response. To exclude this possibility, we used a CD4-depleting antibody before challenge with yeast synergy and examined its impact on the subsequent CD8$^+$ T-cell response. Depletion of CD4 T cells through a CD4-targeting antibody did not significantly affect the CD8 T-cell responses in either WT or IL6$^{-/-}$ mice to either Pam3Cys$^-$ or yeast synergy vaccination (Fig. 3). Similar results were obtained in MHC class II$^{-/-}$ hosts, which have a constitutive deficit in CD4$^+$ T cells (data not shown). These data indicate that (i) the addition of a CD40 agonistic antibody can produce a CD8 T-cell response to yeast and Pam3Cys synergy immunization independent of CD4 T cells and (ii) an IL-6 dependent control of the CD8$^+$ T-cell response to yeast synergy relative to Pam3Cys synergy can also occur independent of CD4 T-cell involvement.

IL-12-dependent, IL-6-influenced Regulation of Yeast Synergy

The apparent lack of necessity for CD4 T-cell-mediated involvement in the modulation of the CD8$^+$ T-cell response after immunization in the presence of αCD40 antibody implicates a direct role for IL-6. One logical candidate factor is IL-12 whose transcription is negatively regulated by the IL-6-Stat3 pathway.[32,33] Indeed, although IL-12 p70 blockade had little impact on the CD8$^+$ T-cell response to Pam3Cys synergy, IL-12 p70 blockade significantly and almost completely inhibited the

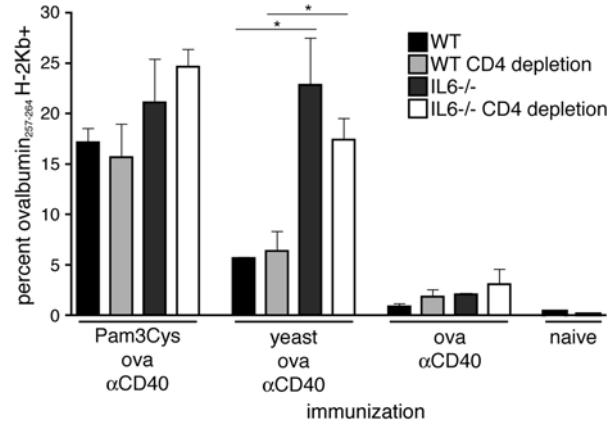

FIGURE 3. CD8 T-cell response to yeast is independent of CD4 T cells in both wild-type (WT) and IL6$^{-/-}$ mice. WT (black bars) or IL6$^{-/-}$ (dark gray bars) mice were depleted of CD4 T cells (WT CD4 depleted—light gray bars, IL6$^{-/-}$ CD4 depleted—white bars) 3 days prior and 4 days after immunization with either Pam3Cys synergy (Pam3Cys-ova-aCD40), yeast synergy (yeast-ova-aCD40), anti-CD40 (ova-aCD40), or nothing (naive) using the CD4-depleting antibody GK1.5. There were no detectable CD4 T cells after CD4 depletion in the blood or spleen as measured by flow cytometry. Bars represent percentage of SIINFEKL-specific class I tetramer in peripheral blood. Error bars and asterisks denote significance as described in the legend of Figure 1. Experiments were performed twice with 3 mice per group.

yeast synergy response in both WT and IL-6$^{-/-}$ mice (Fig. 4A). As the IL-12 p70 antibody is able to block both IL-12 and IL-23, it was entirely possible that the effect we were seeing was dependent on IL-23. To determine the role of IL23, we used the p19$^{-/-}$ mouse to measure the antigen-specific CD8 T-cell response to either Pam3Cys or yeast synergy. Neither Pam3Cys nor yeast synergy was significantly different in p19$^{-/-}$ mice when compared with WT (Fig. 4B). Therefore, the CD8 T-cell response to yeast but not Pam3Cys is highly dependent on IL-12.

Antigen-specific CD8 T-cell Response to Yeast Synergy Requires IL-12-mediated Activation of DCD11c$^+$ DCs

We have previously published the capacity of Pam3Cys/αCD40 immunization[5] and numerous other innate/αCD40 combinations[3,4] to potently activate DCs in vivo. We therefore wished to address whether there were differences between the DC activation elicited in response to Pam3Cys synergy and yeast synergy. To test this, we looked at CD11c$^+$ DCs from mice immunized with either Pam3Cys synergy or yeast synergy. Splenic CD8$^+$ and CD11b$^+$ DCs were analyzed at 24 hours after immunization for their expression of various activation markers. As previously published,[5] DCs upregulated the primary costimulatory markers, CD80 and CD86, in response to the Pam3Cys synergy.[5] However, although Pam3Cys synergy induced a similar degree of CD86 upregulation between the WT and IL6$^{-/-}$ mice, DCs from WT yeast synergy immunized mice showed a significant reduction in the expression of both CD80 (not shown) and CD86 (Figs. 5A, B), a reduction that was largely corrected in the IL-6$^{-/-}$ hosts. From these data, one could surmise that yeast activate DCs differently from Pam3Cys, for example, modulating the expression of costimulatory molecules such as CD86, thus contributing to the control of antigen-specific CD8 T cells after yeast immunization.

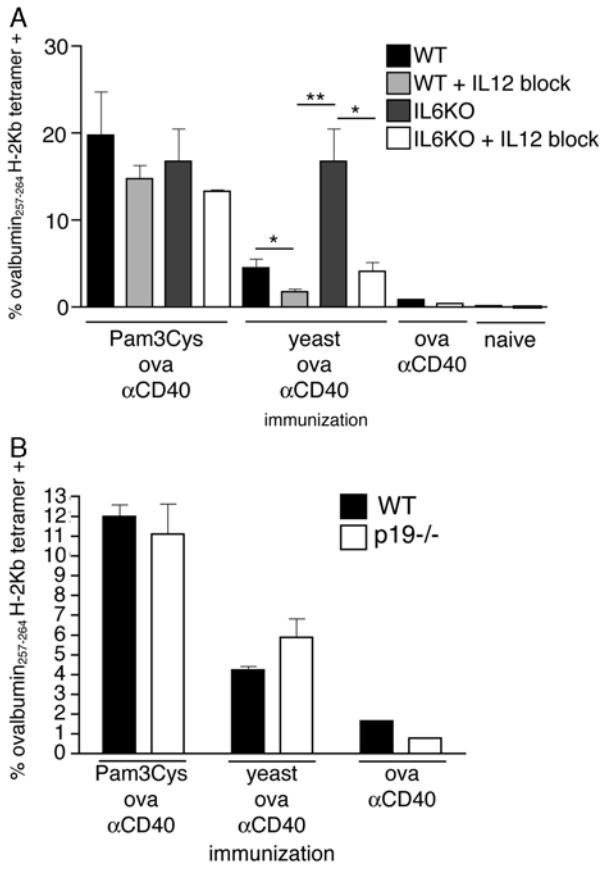

FIGURE 4. CD8 T-cell response requires IL-12 signaling. A, Mice were immunized and analyzed exactly as described in Figure 3. The IL-12p70-blocking antibody C17.8 was administered 18 and 2 hours before immunization in wild-type (WT) (light gray bars) or IL6$^{-/-}$ (white bars) mice, or not at all in WT (black bars) or IL6$^{-/-}$ (dark gray bars) mice. B, Immunization and analysis of WT (black bars) and p19 knockout mice (IL-23) (white bars) was done exactly as described in the legend of Figure 1. Statistical analysis was done as described in Figure 1. Experiments were done twice with 3 mice per experimental group.

Central Role for IL-12 in the Activation of CD11c$^+$ DC After Yeast Immunization

Thus far, we have observed that the response to yeast synergy is dependent on IL-12 and is reduced by some activity of IL-6 for inhibiting DC activation. To understand whether these 2 observations were related through the action of IL-12, we used the IL-12 p70-blocking antibody (C17.8) to block IL-12 activity during yeast immunization. Interestingly, we saw a significant decrease in the activation of both CD8$^+$ and CD11b$^+$ DC subsets, by CD80 and CD86 levels, when IL-12 was blocked (Figs. 5C, D and data not shown). This decrease was seen in both WT and IL6$^{-/-}$ mice, suggesting IL-12 is responsible for DC activation leading to CD8 generation after yeast synergy immunization.

The role of IL-12 as a so-called "signal 3" cytokine for the purposes of CD8$^+$ T-cell expansion, effector function, and memory formation is well documented.[11,22–27] Thus, we anticipated that the dependency of the yeast-elicited CD8$^+$ T-cell response on IL-12 involved a direct stimulation of CD8$^+$ T cells. However, the data described above suggested that the major role for IL-12 in this setting might be also related to the stimulation of the DCs rather than a direct action on the T cells themselves. All prior experimentation used an anti-IL-12 blockade, therefore it was not possible to determine differential roles for IL-12 on the T cells or the DCs. As similar results were seen in the IL-12Rb2$^{-/-}$ mice, we used an adoptive transfer system in which WT OT-1 TCR transgenic T cells were transferred into IL-12Rb2$^{-/-}$ mice, and the recipient was subsequently immunized with antigen in the context of Pam3Cys/αCD40, yeast/αCD40 or αCD40 alone. In this model system, the transferred T cells retain a fully functional response to IL-12 whereas the endogenous DCs do not. The OT1 T cells in the IL-12Rb2$^{-/-}$ mice responded poorly to yeast immunization as compared with the response in recipient WT hosts (Fig. 6). These data indicate that the expression of IL-12R on the antigen-specific T cells does not rescue the IL-12 dependency of the response to yeast synergy vaccination. Although this cannot completely rule out any role of IL-12 on the T cells, it is consistent with the conclusion that a major role of IL-12 in response to yeast synergy vaccination is the activation of DCs.

IL-12 is Increased After Yeast Immunization in CD11c$^+$ CD8$^+$ DC

Our data indicate that IL-12 is central to DC activation in response yeast synergy. This, coupled with the observation that the loss of IL-6 enhances DC

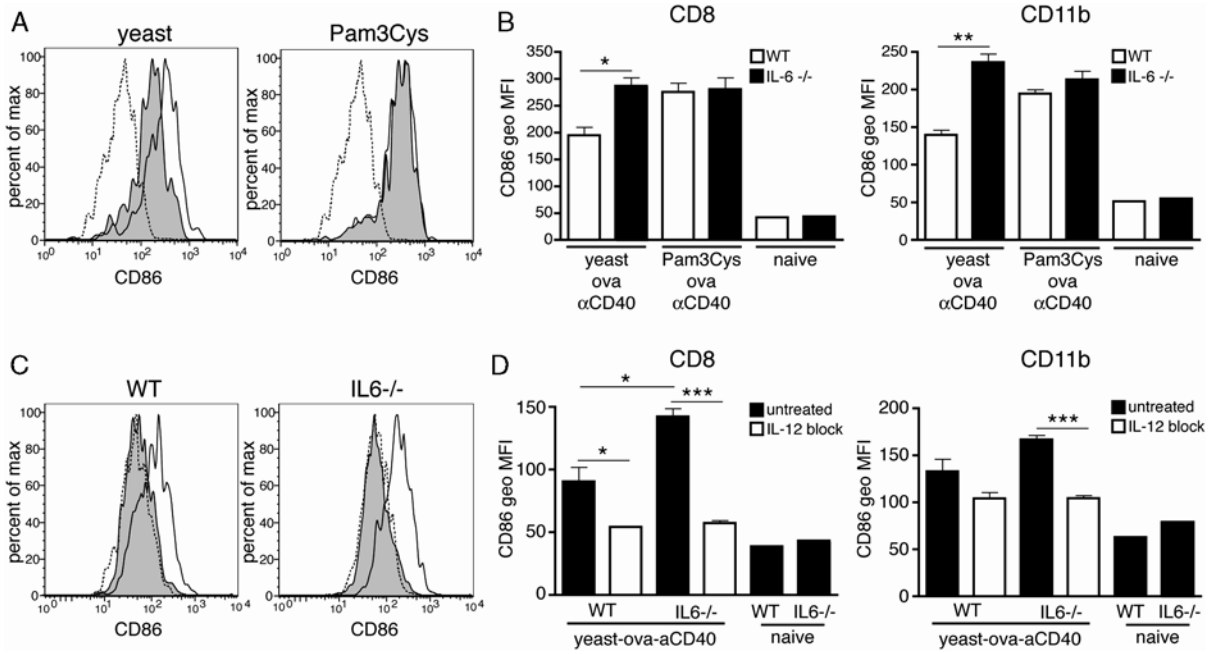

FIGURE 5. Dendritic cell activation is rescued by removal of IL-6 and dependent on IL-12. A, Dendritic cell (DC) activation marker CD86 is decreased in wild-type (WT) mice in response to yeast. WT or IL6$^{-/-}$ mice were immunized with yeast+ova+aCD40, Pam3Cys+ova+aCD40, or nothing (naive) 24 hours before harvesting splenic DCs. DCs shown are CD11c^{+}. Shown is a representative sample of splenic CD8^{+} DCs in WT (gray shaded histogram) and IL6$^{-/-}$ (open histogram), or WT naive (dashed line open histogram) immunized as indicated above each graph. B, Bar graph of data shown in (A) of WT (black bars) and IL6$^{-/-}$ (white bars) mice. Shown is the geometric mean fluorescence intensity of CD86 for both CD8^{+} and CD11b^{+} DCs. C, CD86 is reduced after IL-12 block. Shown is a representative sample of CD8^{+} DCs in WT and IL6$^{-/-}$ mice were treated with IL-12-blocking antibody (gray shaded histogram) 18 and 2 hours before yeast immunization or left untreated (open histogram). Naive mice are shown with a dashed line. D, Bar graph of data shown in (C). CD86 expression was calculated as in (A). Statistical analysis was done as described in Figure 4. Statistical analysis was done as described in Figure 1. Experiments were done twice with 3 mice per group.

activation in response to yeast synergy, lead us to predict that IL-6$^{-/-}$ mice might demonstrate increased production of IL-12 in response to yeast synergy relative to WT mice. To investigate this, we examined IL-12 production in CD8^{+} DCs, isolated from WT or IL-6 hosts, in response to yeast synergy. CD8^{+} DCs are well documented to produce IL-12 to a variety of stimuli,[34–38] specifically in response to the use of an innate stimulus in combination with αCD40.[38] Consistent with our prediction, we found that CD8^{+} DCs produced significantly more IL-12 after yeast immunization in IL6$^{-/-}$ mice as compared with WT hosts (Fig. 7). In addition, consistent with the published data,[34–38] this seems to be specific to CD8 DCs. Thus, yeast immunization increases IL-12 production by DCs and this is enhanced in IL6$^{-/-}$ mice.

DISCUSSION

The use of yeast as an immunogen has been recently reported to provide clinically relevant efficacy in human clinical trials.[12] Although the clinical application of TLR agonists has been heavily pursued,[39] success outside the topical application of TLR7 agonists has not been generally observed.[40] It is now well established that vaccination with the combined stimulus of an innate receptor[3–5] and a CD40 agonist induces a magnitude of CD8^{+} T-cell expansion that is exponentially larger than that seen in response to the use of either stimulus alone. Given that yeast possess potential agonists for multiple innate pathways (TLR, C-type lectins, NLRs), we speculated that vaccination in the context of whole yeast and αCD40 (yeast synergy) might further augment the level of CD8^{+} T-cell expansion. Surprisingly, given the multiple innate pathways used by yeast, the antigen-specific CD8 T-cell response to yeast synergy was on the order of one-third that of Pam3Cys in WT mice (Fig. 1). This reduced CD8 response by yeast versus Pam3Cys was not due to an intrinsic deficiency in the ability of yeast to generate CD8 responses as the response to yeast was improved to at least the same level as that of Pam3Cys/CD40 in the IL-6$^{-/-}$ host.

One candidate for the control of yeast-based generation of antigen-specific CD8 T cells was CD4^{+} Th17 T cells. Th17 are induced by IL-6 provoked by DC engagement with extracellular pathogens such as fungi.[19,41–43] The balance between Th1 and Th17 cells has been well described, thus it was possible this balance was, at least in part, responsible for the immune response generated by yeast. IL-6 knockout mice generated a much greater CD8 T-cell response than WT animals (Fig. 2), perhaps due to the skewing of the CD4 T-cell response from Th17 to Th1. To focus on aspects of the yeast-derived immune response independent of the contribution of CD4 T cells, we used CD4-deficient mice where we observed no significant change in the percent of CD8 T cells found in the blood after CD4

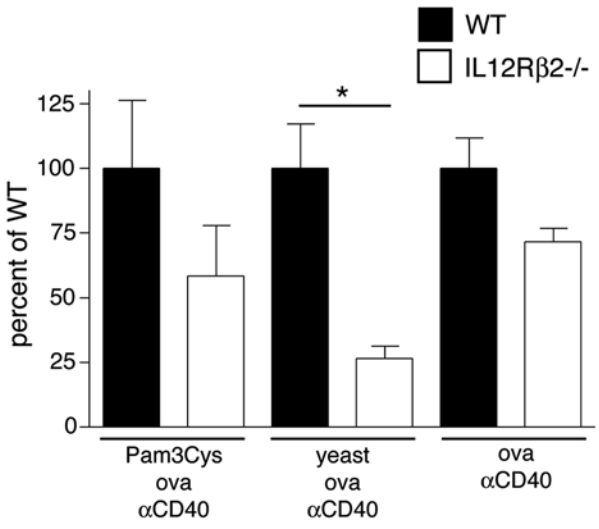

FIGURE 6. IL-12 is necessary to the dendritic cell. Wild-type (WT) TCR transgenic OT-1 CD8 T cells were adoptively transferred into WT (black bars) or IL12Rb2$^{-/-}$ (white bars) mice and immunized with either yeast-ova-aCD40 or ova-aCD40 24 hours after transfer. Total numbers of expanded OT-1 T cells in the spleen were counted and percent expansion based on the WT mouse was calculated. Statistical analysis was done as described in Figure 1. This experiment was performed twice with 3 mice per group.

depletion even in the IL-6 knockout mice (Fig. 3). As this rescue occurred even under CD4-depleting conditions, this implicated a direct effect of IL-6 on the CD8$^+$ T-cell response.

There is good experimental evidence that engaging an IL-6-inducing pathway such as dectin 1 can interfere with the IL-12 pathway.[41] A previous report by Dennehy et al[41] outlined a role for dectin 1 in suppressing IL-12 while concomitantly activating IL-23. However, yeast synergy-induced CD8 frequencies were unchanged in a dectin 1 knockout mouse (unpublished observation) perhaps due in part to the myriad of pattern-recognition receptors that yeast are able to bind,[10] including dectin 2,[44] which are also able to induce IL-6. As such, cytokines produced by yeast immunization, namely IL-6, may directly or indirectly influence IL-12 signaling.

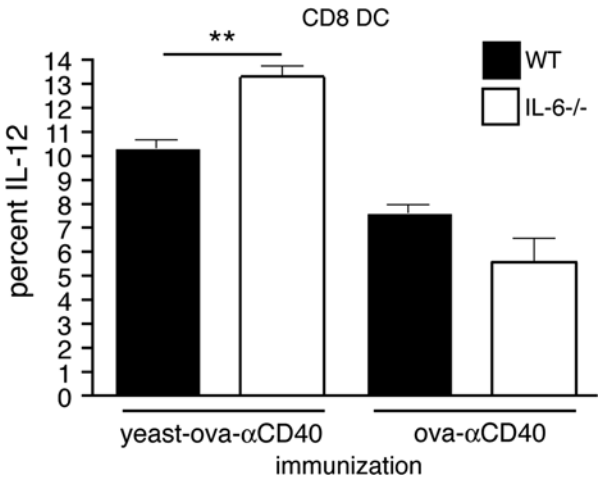

FIGURE 7. IL-12 production is increased in CD8$^+$ DCs of IL6$^{-/-}$ mice in response to yeast synergy. Wild-type (WT) (black bars) or IL6$^{-/-}$ (white bars) were immunized with either yeast-ova-aCD40 or ova-aCD40. Splenic dendritic cells (DCs) were harvested 1 to 2 hours after immunization and stained for intracellular IL-12. DCs are CD11c$^+$, B220$^-$, CD8$^+$, IL-12$^+$. Statistical analysis was done as described in Figure 1. This experiment was performed twice with 3 mice per group.

Previous work has shown that IL-12 can act directly on the CD8 T cells as a signal 3 to enhance CD8 T-cell proliferation.[11] To get a better understanding of the role of IL-12 in the increased CD8 T-cell response after yeast synergy in IL-6 knockout mice, we used an IL-12-blocking antibody and evaluated the CD8 T-cell response to yeast synergy. Although the CD8 T-cell response remained unchanged in Pam3Cys immunized mice (Fig. 4), the CD8 T-cell response was decreased significantly in both WT and IL-6 knockout mice treated with the IL-12-blocking antibody. These data suggest that IL-12 is largely, if not wholly, responsible for the CD8 T-cell response in WT mice and is also implicated for the majority of the increase seen in IL-6 knockout mice immunized with yeast synergy. Since previously IL-12 has been shown to act as a signal 3 to the T cells to provide a productive immune response we believed that IL-12 was acting in this role. However, unpublished data in our lab and previous data regarding other types of TLR/CD40 combinations showed that B7.1/B7.2 knockout mice[2] were unable to mount a CD8 T-cell response leading us to hypothesize that CD80/86 signaling—signal 2—might also be altered after yeast synergy. To address this question, we looked at DCs 24 hours after yeast immunization. Indeed, CD80/86 expression on the DCs was strikingly higher in IL6$^{-/-}$ mice (Figs. 5A, B). Interestingly, CD86 levels, in response to yeast, in IL6$^{-/-}$ mice rose to the same level as mice immunized with Pam3Cys mirroring the antigen-specific CD8 T-cell increase. Thus, IL-12, typically thought to act as a signal 3 to the T cell,[11] also seemed to be acting on the DC (Figs. 5C, D). Indeed, an IL-12-blocking antibody caused CD86 to decrease on the DC, suggesting IL-12 is necessary for CD86 upregulation and activation leading to increased CD8 T-cell responses to yeast synergy. To further understand the role of the DCs in response to yeast synergy, we used a mouse deficient for IL-12Rβ2 to assess the role of IL12 on the DC. Transferring WT OT-1 TCR transgenic T cells, we were able to evaluate whether IL-12 was necessary to the DC. We found a significant defect in CD8 T-cell expansion when the IL12 receptor was absent only on the DC, suggesting an important role for IL-12 on the DC in mediating the CD8 T-cell response (Fig. 6).

How are IL-12 and IL-6 functioning to regulate the CD8 T-cell response? Our data suggest IL-6 is either directly blocking IL-12, IL-12 signaling to CD86, or the IL-12 receptor. Figure 7 predicts that IL-12 is responsible for the changes we see in IL6$^{-/-}$ mice as IL-12 is significantly higher after yeast immunization, but not anti-CD40 alone nor Pam3Cys synergy (data not shown). In addition, when we looked at IL-12Rβ2 expression on DCs we did not see any significant differences between any of the groups. In all immunizations tested, we saw an increase in IL-12Rβ2 expression, suggesting that indeed IL-12 signaling is in play (data not shown). However, based on the IL-12 levels, we think it is likely that the cytokine itself is in some way being blocked by IL-6.

Ultimately, our data are significant for its implications on mechanism of immunity underlying the success of yeast-based vaccination in the clinic. Yeast vaccine vectors have been used to successfully treat chronic HCV infection,[45] and it has been largely assumed that the success of this vaccination, based on previous mouse data, was rooted in the capacity of the vaccine to elicit CTL responses. However, we show here that yeast vaccination, even in the presence of a costimulus as robust as αCD40, results in a more rationed $CD8^+$ T-cell expansion as compared with isolated TLR-based adjuvants. Furthermore, although not measured by us, the reduced level of IL-12 elicited by yeast vaccination can only be expected to bifurcate any CD4 T-cell response into the parallel development of Th1 and Th17-based immunity. Taken together, these data and observations suggest possible ways of integrating our results with the clinical data on yeast vaccination. For example, if the efficacy of yeast vaccination is related to its generation of CTL responses, then our demonstration that other innate stimulants in combination with αCD40 produce substantially greater CD8+ T-cell responses suggest such alternative combination adjuvants could have greater efficacy and should be explored in a clinical setting.

Another scenario can be derived from the HCV clinical data demonstrating that effective treatment of a chronic viral infection such as HCV might be achievable through responses more skewed toward cytokines not associated with type I IFN dependence. Type I IFNs are being used in the clinic to not only upregulate viral resistance genes in HCV-infected individuals but also to suppress autoimmune disorders such as multiple sclerosis. In this latter setting, type I IFN has anti-inflammatory properties assumed to modulate proinflammatory Th17 responses implicated in autoimmunity, perhaps in part due to Th17-mediated interference with regulatory T cells. The administration of nonphysiologic doses of type I IFN to HCV-infected individuals may promote IFN dependent cross-presentation to CD8 T cells. Interestingly, a subset of patients seems resistant or unaffected by type I IFN therapy. This resistance is strongly linked to a genotype, which maps close to the IL-28 B gene locus. IL-28 B is a type III IFN with properties similar to type I IFN. Individuals in the HCV-infected type I IFN resistant group have been reported to make less type III IFN,[46] but how this is involved in the decreased sensitivity to type I IFN based standard of care is not yet understood. However, in the whole yeast-based clinical trial, 3 of the 5 genotypically type I IFN resistant patients were cured (the other 2 patients had favorable reduction in viral titer but discontinued therapy mid-treatment) whereas 0 of 5 patients in the standard of care arm achieved stable viral reduction/cure. Therefore, if type I IFN s suppress CD4-driven cellular immunity and whole yeast-based therapy continues to function in this setting, perhaps the type I IFN resistant population can use a CD4 independent/type I IFN independent means to generate CD8 T cells, similar to what we show in this study.

Others have documented successful tumor regression after a therapeutic intervention from IL-17-producing $CD8^+$ T cells.[47,48] Although counterintuitive with the dogma regarding the role of Th17 responses in immunity and disease, the capacity of IL-17-producing cells to elicit tissue-specific inflammation may well promote an optimal environment for the kind of localized effector response necessary for combating cancer or chronic infectious diseases. Indeed, Th17 cells can elicit protective inflammation near the tumor thereby recruiting DCs responsible for $CD8^+$ T-cell activation.[49] In the case of cancer, few rules underpinning successful therapeutic intervention have been identified over the last 20 years of attempts at immunotherapy other than the observation that the production of IFNγ by tumor-specific T cells has therapeutic and prognostic value (reviewed in Ref. 50). The overall imprecision of this conclusion coupled with the fact that cells producing IL-17 often also produce, or can produce, IFNγ[51,52] makes it tempting to speculate that our overall strategy behind immunotherapeutic intervention of cancer may be improved by altering our focus toward the generation of Th17, or Tc17, like responses. The clinical efficacy of yeast vaccination may well be an example of this and closer examination of the responses generated in yeast-vaccinated patients will provide an opportunity to clarify this in a human disease setting. To that end, a phase II clinical trial for pancreatic cancer using recombinant yeast-expressing mutant ras antigens is fully enrolled but the results are yet to be unblinded.

CONFLICTS OF INTEREST/ FINANCIAL DISCLOSURES

D. Bellgrau is a Scientific Founder of GlobeImmune Inc., a privately held biotech company. He holds founders shares that are not publicly traded, Chairs the Scientific Advisory Board and serves as a scientific consultant. R. Kedl is a founder of ImmuRx Inc., a vaccine company whose intellectual property is based on the combined TLR agonist/anti-CD40 platform. B. Tamburini has no conflicts of interest in regards to this work.

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