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Targeting Weak Antigens to CD64 Elicits Potent Humoral Responses in Human CD64 Transgenic Mice

Tibor Keler,* Paul M. Guyre,† Laura A. Vitale,* Karuna Sundarapandian,* Jan G. J. van de Winkel,§ Yashwant M. Deo,* and Robert F. Graziano*

Previous studies have documented that targeting foreign Ags to IgG FcγR leads to enhanced Ag-specific responses in vitro and in vivo. However, the ability to overcome immunologic nonresponsiveness by targeting poorly immunogenic Ags to FcγR has not been investigated. To address this question in a simple model, we immunized transgenic mice expressing human CD64 (FcγRI) and their nontransgenic littermates with Fab′ derived from the murine anti-human CD64 mAb m22. The m22 Fab′ served as both the targeting molecule and the Ag. We found that only CD64-expressing mice developed anti-Id titers to m22. Furthermore, chemically linked multimers of m22 Fab′, which mediated efficient internalization of the human CD64, were significantly more potent than monomeric m22 F(ab′)2 at inducing anti-Id responses. In all cases, the humoral responses were specific for m22 Id and did not react with other murine IgG1 Fab′ fragments. Chemical addition of a second murine Fab′ (520C9 anti-human HER2/neu) to m22 Fab′ multimers demonstrated that IgG1 and IgG2a anti-Id titers could be generated to 520C9 only in the CD64-expressing mice. These results show that targeting to CD64 can overcome immunological nonresponsiveness to a weak immunogen. Therefore, targeting to CD64 may be an effective method to enhance the activity of nonimmunogenic tumor vaccines.

Materials and Methods

Preparation of F(ab′)2 and F(ab′)3, Ag

F(ab′)2 of the anti-FcγRI mAb m22 (IgG1) (20) and the murine anti-human HER2/neu 520C9 (21) were prepared by pepsin digestion and purified by flowing through a protein A column followed by Superdex 200 gel filtration chromatography. To make multimeric F(ab′)3, molecules, m22 F(ab′)2 was treated with a 20-fold molar excess of sulfo-succinimidyl 4-N-maleimidomethyl-cyclohexane-1-carboxylate (Pierce, Rockford, IL). Separately, m22 F(ab′)2 or 520C9 F(ab′)2 was reduced to Fab′ with 2-ME amine. The Fab′ were then added to m22 Fab′ or 520C9 Fab′, respectively, and incubated for 2 h at ambient temperature. Purification of the multimers was performed by Superdex 200 gel filtration chromatography. To make the m22 × 520C9 Fab′ multis, equimolar amounts of m22 Fab′ and 520C9 Fab′ were added to the m22 Fab′-maleimide. The control Abs m32.2, anti-human CD64 (murine IgG1), A77; anti-human CD89 (murine IgG1), H22; and anti-human CD64 (humanized IgG1), were purified from supernatant of hybridomas or transfected myeloma cells.

Modulation of CD64

Peritoneal macrophages were elicited in human CD64 transgenic and nontransgenic littermates with 1 ml of 3% Brewer’s thioglycolate (Difco, Detroit, MI) for 4 days before recovery by peritoneal lavage. The macrophages were incubated in polypropylene microtiter plates with m22 Fab′ or m22 F(ab′)3 at varying concentrations for 2 h at 37°C (active group) or at 4°C (control group). Cells were harvested after being placed on ice for

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15 min and then washed and incubated on ice with 32.2 anti-CD64 Ab conjugated to FITC, which binds to a site on CD64 that is distinct from the m22 epitope (20). Samples were washed, and fluorescence was measured using a FACScalibur (Becton Dickinson, San Jose, CA).

**Immunization of mice**

The mice employed for these studies were 5- to 12-wk-old females from the human CD64-transgenic FVB/N line 1852 previously described by Heinen et al. (11, 19). We used F1 mice from heterozygous mating such that approximately 50% of each litter expressed human CD64. For each experiment human CD64-expressing mice were matched with their nontransgenic littermates as controls. All immunizations were performed i.p. using the Ribi Adjuvant System (Sigma, St. Louis, MO) in a final volume of 200 μl/mouse. Mice were immunized with 25 μg of Ag three or four times at approximately 2-wk intervals. Blood was drawn for analysis 1 wk after each immunization.

**Analysis of anti-Id responses**

Anti-Id responses were evaluated by sandwich immunoassays. Microtiter plates were coated with F(ab’)2 from relevant or control Abs. Nonspecific adsorption was blocked with 5% BSA in PBS. Samples of plasma from mice were diluted in PBS and incubated on the plates for 1–2 h at 37°C. After extensive washing, the plates were incubated with either goat anti-murine IgG Fc-specific or isotype-specific alkaline phosphatase-conjugated antibodies. The alkaline phosphatase substrate, p-nitrophenylphosphate, was added to the plates, and absorbance was read at 405–650 nm. Titers were calculated using the Ribi Adjuvant System (Sigma, St. Louis, MO) in a final volume of 200 μl/mouse. Mice were immunized with 25 μg of Ag three or four times at approximately 2-wk intervals. Blood was drawn for analysis 1 wk after each immunization.

**Results**

**m22 F(ab’)2 elicits anti-Id IgG in human CD64 transgenic mice**

The Id of the surface Ig expressed in B cell lymphoma have been well established as a unique target for effective immunotherapy in animal models (15–18), and anti-Id Abs can induce prolonged remissions in non-Hodgkin’s lymphoma (22). Idiotypic determinants contain unique sequences, yet they are poorly immunogenic and do not mediate significant protection against a tumor challenge when administered as unmodified vaccines (17, 18). However, Id-based vaccines can induce protective responses in animal models when linked to immunogenic carrier proteins (15–18) and have recently been shown to induce anti-tumor responses in patients with follicular lymphoma when combined with GM-CSF (23). We reasoned that the Id of the anti-CD64 Ab m22 (m22-Id) could serve as a model of a weakly immunogenic tumor Ag while providing specific targeting to the human FcγRI (CD64) on APCs in transgenic mice. Furthermore, mAb 22 efficiently binds CD64 in vivo due to its specificity for an epitope that is distinct from the Fc binding site.

Transgenic mice expressing human CD64 as well as their nontransgenic littermates were immunized with F(ab’)2 of the m22. The mice were bled 7 days after receiving biweekly immunizations of 25 μg of m22 F(ab’)2 mixed in Ribi adjuvant. After the fourth immunization, three of the six transgenic mice, but none of the six nontransgenic littermates, exhibited significant m22-Id specific increase in OD) above nonimmune pooled FVB/N sera. Pooled sera from immunized mice for 75 min at 4°C. The cells were washed and analyzed on a FACScalibur instrument with CellQuest software (Becton Dickinson).

**m22 Fab’ was linked to m22 F(ab’)2, using the bifunctional linker succinimidyl 4-N-maleimidomethyl-cyclohexane-1-carboxylate.** The data in Fig. 2A depict a nonreducing SDS-PAGE gel comparing m22 F(ab’)2 (lane 1) and the m22 Fab’ multimer (designated m22 F(ab’)3, lane 2). The multimer consisted of several molecular species representing F(ab’)3, (F(ab’)2)x F(ab’)2, F(ab’)3, and some higher m.w. forms.

To determine the ability of m22 F(ab’)3, compared with m22 F(ab’)2 to mediate internalization of CD64, we assessed the ability

**FIGURE 1.** Anti-Id response to m22 F(ab’)2, immunizations. Human CD64-expressing mice and their nontransgenic littermates were given four i.p. injections of m22 F(ab’)2 (25 μg/mouse/dose) in Ribi adjuvant. Blood was collected from each animal 1 wk after the last immunization, and the plasma was tested for anti-m22-Id IgG by ELISA. Each circle represents an individual mouse.

**FIGURE 2.** SDS-PAGE analysis and modulation of CD64 with multimeric m22 F(ab’)3. A, Comparison of the electrophoretic mobility profile for m22 F(ab’)3 and multimeric m22 F(ab’)3, performed under nonreducing conditions on a 3–12% gradient gel. The proteins were visualized with Coomassie blue stain, and the M₆ standards (kilodaltons) are indicated. B, The modulation of surface human CD64 on peritoneal exudate macrophages from transgenic mice was determined after a 2-h incubation with m22 F(ab’)2 or m22 F(ab’)3, at 37 or 4°C. The surface level of human CD64 was determined by flow cytometry using anti-CD64 mAb 32.2. The histograms of internalizing samples showed a single peak shift of the entire population. The data are representative of two independent experiments.
of each to reduce surface CD64 expression on transgenic macrophages. Peritoneal macrophages were incubated with varying concentrations of m22 F(ab’)_2 or the F(ab’)_2 multimers for 2 h before flow cytometric analysis of CD64 surface expression as detected by the noncompeting FITC-labeled anti-CD64 mAb m32.2. As expected, incubation with m22 F(ab’)_2 did not result in significant reduction of CD64 from the surface of the macrophages. However, incubation with the F(ab’)_3 at 37°C led to >50% reduction in CD64 expression in a dose-dependent fashion. Incubation with F(ab’)_3 at 4°C did not lead to a reduction in CD64 expression, demonstrating the temperature dependence of the modulation. Similarly, human CD64 expression on peripheral blood monocytes of CD64 transgenic mice was significantly reduced in vivo within 1 h after injection of m22 F(ab’)_3, but not with the F(ab’)_2 (data not shown). Since previous reports have documented that CD64 cross-linking leads to internalization (24, 25), and Wallace et al. have shown that modulation of CD64 surface expression with the humanized version of m22 correlated with internalization (26), it is likely that the reduction of surface CD64 expression represents internalization of the receptor along with the F(ab’)_3 multimers.

### m22 F(ab’)₃⁺ elicits potent anti-Id IgG responses in human CD64 transgenic mice

We next examined the ability of m22 F(ab’)₃⁺, multimers to generate an m22 anti-Id response in human CD64 transgenic and nontransgenic littermates. Using the same protocol as that employed for F(ab’)₂ immunizations, a markedly stronger anti-Id response was observed (Fig. 3A). After only three immunizations with the multimer, high titers of m22-specific IgG were generated in all the CD64 transgenic mice (n = 12), and no significant anti-Id IgG was generated in any of the nontransgenic littermates (n = 10). Contrary to what we observed with the F(ab’)₁ immunizations, most of the transgenic mice developed anti-Id responses following just two immunizations of multimers (data not shown). To determine whether the enhanced efficiency of the multimer to generate anti-Id responses was due to an increased persistence in vivo, we immunized human CD64 transgenic mice with various doses of the multimer. Surprisingly, we found that three immunizations with as little as 0.25 µg/dose of the multimer were sufficient to generate a significant anti-Id response (Fig. 3B).

### Specificity and activity of the anti-Id IgG

The specificity of the anti-Id response is demonstrated in Fig. 4. Pooled sera from immunized transgenic mice reacted specifically with m22 F(ab’)₂ and not with isotype- and allotype-matched control F(ab’)₂ fragments (Fig. 4A). In addition, the sera from immune transgenic mice bound to F(ab’)₂ of the humanized 22 (H22), which contains only the complementarity-determining regions of m22 (27). Sera from immunized nontransgenic mice did not bind to any of the F(ab’)₂ (data not shown). Similarly, immune sera from the human CD64-expressing mice efficiently blocked m22 binding to CD64 on U-937 cells in a specific manner (Fig. 4B). The same sera did not interfere with m32.2 binding to CD64, and sera from nontransgenic immunized mice did not inhibit m22 binding. The data clearly show that the immune serum was m22-Id specific and blocked the activity of m22 to bind CD64.

### Targeting a model Id elicits anti-Id IgG in human CD64 transgenic mice

To determine whether an immune response could be generated against a different Fab’ by targeting to human CD64, we generated multimers of the m22 Fab’ linked to the Fab’ of the murine anti-HER2/neu mAb, 520C9 (21). One millimole each of 520C9 and m22 Fab’ were chemically coupled to 1 mmol of m22 F(ab’)₂. Similar to the m22 F(ab’)₃⁺, these multimers predominantly contained species of F(ab’)₁ to F(ab’)₆ with an estimated 3:1 ratio of m22 Fab’ to 520C9 Fab’. Human CD64 transgenic and nontransgenic littermates were immunized with three 25-µg doses of 22-520C9 Fab’₃⁺, and assessed for both 520C9 and m22 anti-Id IgG titers. The data in Fig. 5 show that seven of eight immunized CD64 transgenic mice developed high levels of IgG specific for both 520C9-Id and m22-Id. None of the seven nontransgenic mice developed measurable titers to either 520C9-Id or m22-Id. These data demonstrate that targeting a weakly immunogenic tumor Ag to CD64, such as lymphoma Id, in a manner that results in internalization by CD64⁺ APCs leads to a potent immune response to the Ag. Interestingly, in individual mice the anti-520C9-Id titers were generally 1 log greater than the anti-m22-Id titers.

### Persistence and isotype of anti-Id IgG responses

The quality of the immune response to a given Ag, particularly a tumor-associated or viral Ag, can be as important as the quantity of the response. To address these issues, we determined the duration and isotype specificity of the 520C9 anti-Id response in transgenic mice immunized with 22-520C9 F(ab’)₃⁺. Anti-Id responses were determined in the same mice at 1 wk, 4 mo, or 7 mo after the third immunization with 22-520C9 F(ab’)₃⁺ (Fig. 6A). These data illustrate that the mice maintained significant 520C9 Id-specific IgG titers for at least 7 mo, demonstrating a lasting response to the immunization. In addition, both a strong IgG1 as well as a strong IgG2a Id-specific titer were elicited, suggesting that targeting to CD64 can activate both a Th1 and a Th2 response (Fig. 6B).

### Discussion

Previous studies using human primary cultures or human CD64 transgenic mice have demonstrated CD64 as a proficient target on APCs for eliciting enhanced Ag processing and presentations to specific Ags in vitro and in vivo. In vitro, 100- to 1000-fold lower

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**FIGURE 3.** Anti-Id response to m22 F(ab’)_3⁺ immunizations. Human CD64-expressing mice and their nontransgenic littermates were given three i.p. injections of m22 F(ab’)_3⁺, in Ribi adjuvant. Blood was collected from each animal 1 wk after the last immunization, and the plasma was tested for anti-m22-Id IgG by ELISA. A, Comparative titers between transgenic and nontransgenic mice given 25 µg Ag/mouse/dose. B, Anti-m22-Id titers of transgenic mice immunized with varying doses of m22 F(ab’)_3⁺. Each circle represents an individual mouse.
concentrations of CD64-targeted Ag are required to achieve the same level of Ag-specific T cell proliferation compared with Ag alone using monocytes (4, 5, 8) or dendritic cells (7) as APCs. Similarly, human CD64 transgenic mice developed significantly greater anti-human IgG responses than their nontransgenic littermates following immunization with the humanized anti-CD64 mAb, H22 (11). In this report we have extended these studies by demonstrating that targeting human CD64 in transgenic mice can overcome immunological nonresponsiveness to murine Ab idiootypic determinants.

Consistent with reports on the lack of immunogenicity of idiootypic determinants (15–18), we found that chemically coupled Fab of the murine anti-human CD64 mAb m22 and anti-human HER2/neu mAb 520C9 did not elicit measurable IgG humoral responses in nontransgenic mice. In fact, immunization of transgenic mice with whole 520C9 mAb employing the same protocol also failed to elicit significant anti-Id titers to 520C9 (data not shown). However, strong anti-Id titers to both the coupled fragments were induced by targeting to APCs in the human CD64 transgenic mice. Importantly, the development of anti-Id IgG to the targeting moiety (m22) did not impede the ability to elicit 520C9-specific anti-Id responses. The transgene-expressed human CD64 is predominantly responsible for the observed effects, since the Fc portion of both m22 and 520C9 was removed to eliminate the potential interactions with murine FcγRs. However, given that the mice were immunized three times, it is plausible that immune complexes formed between the Ag and anti-Id IgG may have been taken up and processed by murine FcγRs.

Although strong Id-specific responses were found in the immunized transgenic mice, due to technical limitations these studies were unable to determine whether Ab responses to constant domains of m22 or 520C9 Fab were elicited. Any Abs to murine Fab constant domains would bind the large excess of murine IgG in serum and not be detected unless overwhelming responses were elicited. None of the immune mice displayed symptoms of adverse reactions up to 7 mo postimmunization.
Comparison of the anti-Id responses from m22 F(ab′)2 vs m22 F(ab′)3, immunized transgenic mice suggests that CD64 cross-linking is important for eliciting more rapid and consistent responses. Polyclonal binding of the m22 F(ab′)3 multimers to CD64 improved the efficiency of eliciting anti-Id responses, most likely by enhanced internalization of Ag-receptor complexes. The multimeric form of m22 Fab′ also may have enhanced immune responses by mechanisms other than superior internalization. Cross-linking of FcγRs has profound effects on macrophage and dendritic cell activity, resulting in the secretion of cytokines and up-regulation of costimulatory and adhesion molecules (28–30). Although we were unable to demonstrate consistent up-regulation of CD40, CD80, or CD86 on peritoneal macrophages treated with m22 F(ab′)3, (data not shown), it is feasible that activation of macrophages or dendritic cells via CD64 cross-linking contributed to potent vaccine effects of the multimer.

Previously, several investigators have shown that developing immunity specific for the idiotypic determinants expressed by surface Ig on B cell lymphoma cells can lead to potent tumor cell-specific immunity (15–18, 22). Our experiments demonstrated that a specific anti-520C9 Id response can be readily elicited by directing the 520C9-Id to human CD64, and we are currently developing a syngeneic tumor model in human CD64 transgenic mice to determine whether this approach can be used as a viable tumor vaccine. The high levels and persistence of the anti-Id IgG responses indicate that these vaccines may have therapeutic effects in an appropriate model. In addition, both IgG1 and IgG2a anti-Id isotypes were elicited, suggesting that CD64-targeted vaccines can enhance both humoral (Th2) and cellular (Th1) immunity to the Id.

References