High Titer, Prostate Specific Antigen-Specific Human IgG Production by *hu-PBL-SCID* Mice Immunized with Antigen-Mouse IgG2a Complex-Pulsed Autologous Dendritic Cells

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High Titer, Prostate Specific Antigen-Specific Human IgG Production by hu-PBL-SCID Mice Immunized with Antigen-Mouse IgG2a Complex-Pulsed Autologous Dendritic Cells

Marco A. Coccia and Peter Brams

We report here that immunization of human PBMC reconstituted SCID mice (hu-PBL-SCID mice) with in vitro cultured autologous dendritic cells (DC) pulsed with prostate specific antigen (PSA) complexed to a PSA-specific mouse IgG2a (PSA-IgG2a) consistently and reproducibly stimulates PSA-specific human IgG production. On day 0, female PBMC were used to reconstitute SCID mice and to generate DC in vitro. DC cultures were pulsed with PSA or PSA-IgG2a on day 6. The previously reconstituted hu-PBL-SCID mice were immunized with either PSA-pulsed DC and PSA, PSA-IgG2a-pulsed DC and PSA-IgG2a, or additional PBMC and PSA-IgG2a on day 7. Mice immunized with PSA-IgG2a-pulsed DC had, on the average, up to 31.5 times greater PSA-specific IgG serum concentrations than control mice. Competition ELISA confirmed the PSA specificity of serum IgG. Immunoblot analysis suggested that sera IgG preferentially recognized conformational epitopes on PSA. Therefore, our results represent a major step toward cloning human tumor-associated Ag-specific human mAbs from hu-PBL-SCID mice. In addition, flow cytometry showed that PSA-pulsed DC express significantly more B7.1, B7.2, CD40, and MHC class II surface molecules than mock-treated DC, but PSA-IgG2a-pulsed DC only had significantly enhanced B7.2 surface expression. Interestingly, PSA-specific IgG responses were reproducibly stimulated by DC expressing more B7.2, a molecule associated with Th2-type immune deviation, but not by those expressing more B7.1 and CD40, molecules associated with Th1-type immune deviation. Thus, our results show that stimulation with either Ag or Ag complexed to mAb yields DC with different phenotypes and APC effector functions. The Journal of Immunology, 1998, 161: 5772–5780.

Severe combined immune deficiency mice are deficient in mature lymphocytes, Ig production, and lymphocyte-mediated immune responses due to defective Ig and TCR gene rearrangement (1). SCID mice reconstituted with normal human splenocytes (hu-SPL-SCID mice) or PBL (hu-PBL-SCID mice) can be effective models of T cell-mediated, recall Ag-directed Ig production by human B cells (2–5). However, stimulation of weakly immunogenic neo-Ag, self-Ag, or tumor-associated Ag (TAA)-specific IgG production in hu-PBL-SCID mice reconstituted with normal donor PBMC and immunized with target Ag on adjuvant is difficult. We recently showed that hu-SPL-SCID mice immunized with horse ferritin on adjuvant could be stimulated to produce detectable levels of ferritin-specific IgG (2). Similarly, little or no neo-Ag-specific IgG production was stimulated in hu-PBL-SCID mice immunized with Ag complexed to highly immunogenic peptides (e.g., tetanus toxoid) on adjuvant (6). Only one case of self-Ag (CD2)-directed IgG production has been reported in hu-SCID mice. These mice were immunized with genetically modified CD2 protein (7). In addition, human tumor-specific IgG responses have only been generated in hu-SCID mice that were reconstituted with tumor-infiltrating lymphocytes present in coenrafted human tumor biopsy tissue (8).

Dendritic cells (DC) are professional APC that initiate an immune response (9, 10). Recently, several methods have been developed to generate human DC from PBMC-derived progenitor cells in vitro. These different culture methods yield DC subtypes with heterogeneous morphology, phenotype, and function. However, all these DC subtypes have been shown to be potent stimulators of naive Ag-specific T cells (11–13). This is due in large part to the fact that DC express class I and II MHC and costimulatory cell surface molecules B7.1 and B7.2 (14, 15). In addition, human DC pulsed with weakly immunogenic TAA are capable of stimulating TAA-specific CTL proliferation and cytotoxicity in vitro and in vivo, thus illustrating both their potency as APC and their potential utility as tumor-specific vaccines (16–19).

DC derived from PBMC and cultured with GM-CSF and IL-4 express both the high affinity IgG receptor FcγRI (CD64) and the low affinity IgG receptor FcγRII (CD32) at varying levels (11, 20). Both CD64 and CD32 have been shown to mediate Ag uptake by DC (20, 21). Targeting Ag to FcγR on human monocytes and DC via Ag-IgG complexes can reduce the quantity of Ag required for Ag-specific T cell activation (i.e., enhance the immunogenicity of an Ag) as much as 1000-fold (22, 23). It has been previously established that the Fc region of mouse IgG2a binds human CD64 with greater affinity than any other subtype of mouse Ig (24). Mouse IgG2a also binds human CD32 (25). Therefore, we investigated whether Ag can be targeted to FcγR on in vitro cultured
human DC by pulsing with Ag complexed to an Ag-specific mouse IgG2a.

We report here an immunization protocol that consistently and reproducibly stimulates hu-PBL-SCID mice to produce human IgG specific for the human TAA, prostate specific antigen (PSA). We show that immunizing hu-PBL-SCID mice with autologous DC pulsed with PSA complexed to a mouse IgG2a specific for human PSA (PSA-IgG2a) stimulates high titer, PSA-specific IgG production. In contrast, immunizing hu-PBL-SCID mice with PSA on alumn, PSA-pulsed autologous DC, or additional BMBC and PSA-IgG2a stimulates sporadic, low level, or no PSA-specific IgG production. Contrary to expectations, our results also show that B7.1, B7.2, CD40, and MHC class II surface expression is significantly enhanced on soluble Ag-pulsed DC, but only B7.2 expression is significantly enhanced on PSA-IgG2a-pulsed DC. Interestingly, high titer, TAA-specific IgG production was specifically stimulated by DC with enhanced B7.2 expression, an immunoregulatory surface molecule associated with enhanced Th2-type immune deviation (26–28).

Materials and Methods

DC generation in serum-free cultures

Blood donors were selected based on health, sex, and age (normal healthy female donors between 18–40 yr old). PBMC were obtained by leukopheresis or by venepuncture into heparin-coated tubes. RBC were removed from residual PBMC by hypotonic lysis in 0.13 M NH4Cl before cryopreservation in liquid N2. DC were grown as described above by Romani et al., except that Iscove’s complete medium (Iscove’s modified Dulbecco’s medium (Irvine Scientific, Santa Ana, CA) plus sodium pyruvate, minimal essential amino acids, t-glutamine (Sigma, St. Louis, MO), and gentamicin (Life Technologies, Grand Island, NY)) was supplemented with 2% Nutridoma HU (Boehringer Mannheim, Indianapolis, IN) instead of 10% FBS (11). This serum-free medium (IN2) contained mostly human serum proteins. In addition, this medium contained no cytokines, Ig, complement, or other nonspecific mitogens or immunogens heterogeneously present in FBS and human serum.

Freshly isolated and thawed PBMC were purified by Histopaque (Sigma) gradient separation, washed, and plated at 5 × 10^6 cells/ml in IN2 at 37°C for 2 h. Nonadherent cells were gently removed with the medium, additional 37°C IN2 was added, and the cells were incubated at 37°C for 5 additional min. Nonadherent cells were again gently removed, and the residual cells were cultured in IN2 supplemented with 500 U/ml IL-4 and 800 U/ml GM-CSF (Genzyme, Cambridge, MA). Cultures were fed with additional cytokines on day 3. Mouse monoclonal IgG2a specific for PSA (clone 10-P20; Fitzgerald Industries International, Concord, MA) was complexed with >99% pure PSA (Fitzgerald Industries International) at equimolar ratios and dialyzed against 1× HBSS at 4°C overnight (PSA-IgG2a). The DC-enriched cultures were pulsed with 25 μg/ml (final concentration) PSA, PSA-IgG2a in 500 μl of IN2, or an equivalent volume of IN2 on day 6. Unlike macrophages, by day 7 DC are no longer fully adherent cells. Loosely adherent and nonadherent cells were harvested on day 7.

Flow cytometric analysis

The following FITC and phycoerythrin (PE)-labeled mAbs were used: anti-HLA-DR, -DP, and -DQ (MHC class II); anti-CD1a, -CD3, -CD11c, -CD16, and -CD32w (FcγRII); anti-CD3, -CD4, -CD40, -CD45RO, and -CD64 (FcγRI); anti-CD86 (B7.2) and FITC-labeled isotype controls (PharMingen, San Diego, CA); anti-CD4, -CD14, and -CD80 (B7.1) and PE-labeled isotype control (Becton Dickinson, San Jose, CA); anti-ABC (MHC class I; Harlan Bioproducts for Science, Indianapolis, IN); and goat anti-mouse Ig F(ab′)2 (human absorbed) and goat F(ab′)2, FITC-labeled isotype control (Southern Biotechnology Associates, Birmingham, AL).

To characterize the phenotype of serum-free cultured DC, cultures were grown as described above. The cultures were harvested on day 7, washed, and then resuspended in 4°C FACS buffer (1% BSA, 1× PBS, 0.1% sodium azide, and 40 μg/ml human IgG) at 1× 10^6 cells/ml. The cells were aliquoted and stained for 45 min with FITC and PE-labeled Ab diluted to the manufacturers’ recommended concentrations. The cells were washed twice in FACS buffer, and data were acquired on a FACSscan (Becton Dickinson). Data were analyzed using LSYS1 (Becton Dickinson) or Fcapp List (Soft Flow Hungry, Pecs, Hungary) software. Analysis was performed on large, CD33+ DC cells gated by forward and side scatter.

To detect PSA-IgG2a binding to PSA-IgG2a-pulsed DC, cultures were grown from thawed donor 3 PBMC in six-well plates, pulsed with 25 μg/ml PSA-IgG2a or PSA or mock treated on day 6, and harvested on day 7. The day 7 DC were labeled with goat anti-mouse Ig F(ab′)2 (human absorbed) and goat F(ab′)2, FITC-labeled isotype control, washed, and analyzed as described above.

In experiments comparing the expression of immunoregulatory surface molecule on Ag-pulsed and naive DC, cultures were grown from three donors as described above, except these cultures were grown in three triplicate sets and in six-well plates. On day 6, one set of the three triplicate cultures generated from donors 1, 2, and 3 was pulsed with either 25 μg/ml PSA or 25 μg/ml PSA-IgG2a or was mock treated. DC were harvested on day 7, and the triplicate cultures were pooled before analysis. The cultures were harvested on day 7, Ab labeled, and analyzed individually, as described above. Specific reactivity data, shown as the mean fluorescence intensity (MFI), were calculated as follows: MFI of FITC- or PE-labeled specific mAb = MFI of isotype- and fluorochrome-matched mAb control. Student’s paired t test was used to determine the statistical differences between Ag-stimulated and mock-treated naive DC. A value of p < 0.05 was considered statistically significant.

SCID mouse engraftment and immunization

PBMC were obtained from healthy random female donors by leukopheresis. RBC were removed from hypotonic lysis, as described above. Residual PBMC were frozen and stored as described above. Four- to six-week-old male Fox Chase ICR SCID mice (Taconic, Germantown, NY) were kept in ventilated microisolator cages and maintained according to National Institute of Health guidelines described in the Guide for Care and Use of Laboratory Animals. All engraftments and immunizations were performed i.p.

All mice were reconstituted with 1× 10^6 PBMC on day 0 and immunized as shown in Fig. 1, with the specific exceptions noted in Results. Human Ig serum concentrations were determined by ELISA as described below. Residual PBMC were frozen and stored as described above. Four- to six-week-old male Fox Chase ICR SCID mice (Taconic, Germantown, NY) were kept in ventilated microisolator cages and maintained according to National Institute of Health guidelines described in the Guide for Care and Use of Laboratory Animals. All engraftments and immunizations were performed i.p.

Serum Ig quantiative ELISA

Human Ig serum concentrations were assayed by quantitative ELISAs. ELISAs were performed in 96-well Immulon 2 U-bottom ELISA plates (Dynatech, Chantilly, VA). Human IgG and IgM ELISA plates were coated with 2 μg/ml polyclonal goat anti-human IgG or goat anti-human IgM (Southern Biotechnology Associates) in 50 mM bicarbonate buffer (pH 9.3) overnight. PSA-specific IgG plates were coated with >99% pure PSA at 4 μg/ml in bicarbonate buffer. PSA-specific IgG was quantitated using a mouse monoclonal IgG1 specific for PSA (clone ERPR8, ICN, Costa Mesa, CA) as a standard. Incubations were performed at room temperature.
in serially diluted duplicate wells. Binding of Ig was detected by horse-radish peroxidase (HRP)-conjugated polyclonal goat anti-human IgM-HRP, polyclonal goat anti-human IgG-HRP, or polyclonal goat anti-mouse IgG-HRP secondary Ab (Southern Biotechnology Associates) followed by enzymatic development of o-phenylenediamine dihydrochloride (OPD: Sigma) substrate. Reactions were quenched with 4 N HCl, and the plates were read on an ELISA plate reader at OD_{490}. The concentration of human Ig in hu-PBL-SCID serum was quantitated by comparison of OD_{490} values with serially diluted human Ig (Zymed, South San Francisco, CA) standard curves. Data for all ELISAs were acquired on a Thermomax plate reader (Molecular Devices, Sunnyvale, CA) and analyzed using SOFTmax 2.35 software (Molecular Devices).

**Immunoblot analysis**

Prestained m.w. standards and 2.5-μg PSA samples were boiled in 1× nonreducing Laemmli sample buffer for 5 min and then separated by SDS-PAGE using 10% polyacrylamide gels (Novex, San Diego, CA). Two replicates gels were simultaneously transferred onto a single nitrocellulose membrane at 25 V for 2 h in transfer buffer (10 mM Tris-glycine (pH 8.3), 19% methanol, and 0.01% SDS). The blot was blocked in 5% goat serum, 1× PBS, 0.05% Tween-20 (GS-PBST) for 1 h at room temperature and washed briefly in PBST, and the two replica blots were separated. One blot was stained with pooled sera from four group C mice diluted 1/10 (85 μg/ml total IgG final concentration) or 1/20 (85 μg/ml total IgG final concentration) into triplicate wells containing serially diluted concentrations of PSA. Background binding was assayed using control human IgG (Zymed) diluted into triplicate wells (85 μg/ml final concentration) containing serially diluted concentrations of PSA. Soluble PSA inhibition of PSA-specific binding by group C serum IgG and background human IgG binding were detected using polyclonal goat anti-human IgG-HRP and OPD substrate, as described above.

**Immunoblot analysis**

Pooled sera from four group C mice were diluted 1/10 (113 μg/ml total IgG final concentration) or 1/20 (85 μg/ml total IgG final concentration) into triplicate wells containing serially diluted concentrations of PSA. Background binding was assayed using control human IgG (Zymed) diluted into triplicate wells (85 μg/ml final concentration) containing serially diluted concentrations of PSA. Soluble PSA inhibition of PSA-specific binding by group C serum IgG and background human IgG binding were detected using polyclonal goat anti-human IgG-HRP and OPD substrate, as described above.

**IgG4-specific ELISA**

ELISAs were performed in 96-well Immulon 2 U-bottom ELISA plates (Dynatech Laboratories). Equal volumes of day 25 sera from the four best responding mice in groups A–C and day 28 sera from all four mice in groups D–F were pooled before analysis. Human IgG4 ELISA plates were coated with 1 μg/ml monoclonal mouse anti-human IgG4 (clone G17-4; PharMingen) in 50 mM bicarbonate buffer (pH 9.3) overnight. Incubations were performed at room temperature in serially diluted duplicate (groups A–C) or triplicate (groups D–F) wells. Binding of IgG4 was detected by mouse monoclonal anti-human IgG-HRP (clone G18-145; PharMingen) followed by enzymatic development of OPD substrate. Reactions were quenched with 4 N HCl, and the plates were read on an ELISA plate reader at OD_{490}. The concentrations of human IgG4 in pooled hu-PBL-SCID sera were quantitated by comparison of OD_{490} values with serially diluted human myeloma generated IgG4 (The Binding Site, San Diego, CA) standard curves, respectively.

**Results**

**Serum-free cultured DC**

Human DC were generated from PBMC using low protein, serum-free medium (IN2) supplemented with GM-CSF and IL-4 as described in Materials and Methods. The developmental characteristics of cultures generated in IN2 were similar to what was described by Romani et al. and others for DC generation in medium containing serum (11–13). Briefly, the cells selected for adherence to plastic began aggregating into large clusters on day 2. These clusters increased in size and became increasingly adherent until day 7, when most of the cells with dendritic morphology were loosely adherent or nonadherent to the culture flask. Cultures generated from 10 individual PBMC donors were between 50–85% (typically 75%) large MHC class I+/MHC class II+/CD11chigh/CD32−/CD33+/CD14−/CD40−/CD64−/B7.1+/B7.2− cells with dendritic morphology (Fig. 2). The remaining cells were mostly T cells and some B cells. DC generated from different donors were heterogeneous for CD1a, CD4, CD14, and CD64 expression (results not shown).

To certify that PSA-IgG2a bound to human DC, cultures were grown from donor 3 PBMC, pulsed with either PSA or PSA-IgG2a or mock treated on day 6, and harvested on day 7. DC were stained with anti-CD64 and FITC-labeled, human Ig-absorbed goat F(ab')2 specific for mouse IgG, as described in Materials and Methods. The data showed that donor 3 DC were heterogeneous for CD64 expression and that PSA-IgG2a-pulsed DC had detectable quantities of mouse Ig (PSA-IgG2a) on their surface (Fig. 3B).

**PSA-specific IgG production in hu-PBL-SCID mice**

Similar to what has been previously reported for other weakly immunogenic neo-Ag, our preliminary experiments confirmed that immunization of hu-PBL-SCID mice with PSA on adjuvant did not stimulate detectable PSA-specific human IgG responses (data not shown) (1, 29). This suggested that successful generation of PSA-specific IgG responses required an immunization protocol that enhanced the immunogenicity of PSA. We therefore analyzed...
whether PSA-specific IgG responses could be generated by 1) targeting PSA to FcγR on endogenous APC by immunization of hu-SCID mice with PSA-IgG2a, 2) targeting PSA directly to autologous DC by pulsing in vitro cultured DC with soluble PSA, or 3) combining these immunogenicity enhancing strategies. To test these possibilities, three groups of hu-PBL-SCID mice (eight mice per group) were engrafted with 1 × 10^6 donor 1 PBMC on day 0 (Fig. 1). Autologous DC cultures were simultaneously initiated on day 0, pulsed with either PSA or PSA-IgG2a on day 6, and harvested on day 7 as described in Materials and Methods. Group A mice were immunized with 7.5 × 10^6 thawed autologous PBMC on day 7; 25 μg of PSA-IgG2a on days 0, 7 and 14; and 25 μg of PSA on day 21. Group B mice were immunized with 7 × 10^6 PSA-pulsed DC cultures on day 7 and 25 μg of PSA weekly starting on day 0. Group C mice were immunized with 7.5 × 10^6 PSA-IgG2a-pulsed DC cultures on day 7; 25 μg of PSA-IgG2a on days 0, 7, and 14; and 25 μg PSA on day 21. Serum was collected on days 14, 25, and 28. DC cultures generated from donor 1 were 65% CD1a^{low}/CD14^{low} /CD32^{hi} /CD64^{lo} DC (Fig. 2). Mice in all three groups had similar total and PSA-specific IgM serum concentrations on day 14 (Fig. 4, A and B). In contrast, on day 28 PSA-specific IgG concentrations in group A, B, and C sera averaged 2.3, 2.2, and 70.8 μg/ml, respectively (Fig. 4C). Therefore, on day 28 the average PSA-specific IgG concentration in group C serum was approximately 31.5-fold greater than the average PSA-specific IgG concentrations in control mice. On day 28, the average IgG concentrations in group A, B, and C sera were 0.3, 0.6, and 1.5 mg/ml, respectively (Fig. 4D). Therefore, the percentage of PSA-specific IgG in group A, B, and C sera averaged 0.5, 0.6, and 4.2%, respectively (Fig. 4E). In summary, group C mice averaged both significantly higher PSA-specific IgG serum concentrations (31.5-fold; p = 2.5 × 10^{-5}) and percent PSA-specific IgG serum concentrations (9.7-fold; p = 0.0002) than either control group.

To establish the reproducibility of our hu-PBL-SCID immunization method, additional SCID mice were reconstituted with donor 3 PBMC and immunized by similar methods. Specifically, mice in groups D, E, and F (four mice per group) were engrafted with 1 × 10^8 PBMC on day 0. Group D mice were immunized with 25 μg of PSA-IgG2a weekly starting on day 0 and with 6.9 × 10^6 thawed autologous PBMC on day 7. Group E mice were immunized with 25 μg of PSA weekly starting on day 0 and with 6.9 × 10^6 PSA-pulsed DC cultures on day 7. Group F mice were immunized with 25 μg of PSA-IgG2a weekly starting on day 0 and with 6.8 × 10^6 PSA-IgG2a-pulsed DC cultures on day 7. DC cultures generated from donor 1 were 70% CD1a^{low}/CD14^{low}/CD32^{hi}/CD64^{lo} DC. Similar to the results obtained with donor 1 mice, all three groups had similar total and PSA-specific IgM serum concentrations on day 14 (Fig. 5, A and B). On day 28 group F mice averaged both significantly higher PSA-specific IgG serum...
concentrations (3.5-fold; $p = 0.04$) and percent PSA-specific IgG serum concentrations (2.3-fold; $p = 0.04$) than either control group (Fig. 5, C–H).

When the day 28 data generated from the two random donors are analyzed together, it is clear that immunization with PSA-IgG2a-pulsed DC and PSA-IgG2a produced both the highest and the most consistent PSA-specific IgG responses. Only 3 of 24 control mice (12.5%) produced $>5 \mu g/ml$ PSA-specific IgG. The best responding control mouse produced $5.7 \mu g/ml$ PSA-specific IgG, and that represented only 1.7% of the total response. In addition, only 2 of 24 group A, B, D, and E mice (8%) produced 1.5% or more PSA-specific IgG. In contrast, all 12 group C and F mice (100%) produced $>5 \mu g/ml$ PSA-specific IgG, and 11 of 12 mice (96%) produced 1.5% or more PSA-specific IgG.

To confirm the reproducibility of this immunization protocol, group G mice were generated from random donor 4 and immunized as described for group F mice, except group G mice were immunized with $3.5 \times 10^6$ PSA-IgG2a pulsed DC cultures on day 7. Group G mice were bled on days 14 and 21. Of the three donors tested, donor 4 PBMC produced both the highest PSA-specific IgG serum concentrations and the highest percent PSA-specific IgG serum concentrations. On day 14, PSA-specific IgG concentrations ranged between 1.1–17.8 $\mu g/ml$ (Fig. 6 A). PSA-specific IgG serum concentrations increased dramatically in some mice. By day 21, PSA-specific IgG serum concentrations ranged between 4.2–196.5 $\mu g/ml$. In addition, group G mice produced between 1.9–59.4% PSA-specific IgG by day 21 (Fig. 6 B).

In summary, DC cultures pulsed with PSA-IgG2a consistently stimulated PSA-specific IgG production in hu-PBL-SCID mice generated from all female donors tested. Control mice immunized with PSA-pulsed DC cultures or PSA-IgG2a in combination with an equivalent number of additional autologous PBMC stimulated inconsistent and low level PSA-specific human IgG production.

**Analysis of hu-PBL-SCID serum IgG for PSA specificity**

To confirm that hu-PBL-SCID serum IgG was specific for PSA, pooled group C serum IgG (92 $\mu g/ml$ PSA-specific IgG) was compared with mouse A.1 serum IgG ($<1 \mu g/ml$ PSA-specific IgG) and purified polyclonal control IgG in a PSA-specific ELISA. Group C serum IgG bound PSA 10 times greater than background binding generated by equivalent concentrations of either mouse A.1 serum IgG or control human IgG (Fig. 7 A). The specificity of group C serum IgG for PSA was further confirmed using soluble PSA as a competitor for group C serum IgG binding to PSA immobilized on a surface. Soluble PSA inhibited group C serum IgG binding in a dose-dependent manner (Fig. 7 B). Also, 20 $\mu g/ml$ of soluble PSA reduced pooled group C serum IgG binding to background titers. **FIGURE 5.** Ig production in hu-PBL-SCID mice generated from donor 3. The hu-PBL-SCID mice were reconstituted with $1 \times 10^6$ donor 2 PBMC and immunized as described in Materials and Methods. Serum was collected on days 14 and 28. Total and PSA-specific human Ig were quantitated by ELISA. A, B, and F–H show results for individual mice. Symbols representing individual mice remain constant for all graphs. C–E show averaged data $\pm$ SEM for group D (closed circles), group E (open circles), and group F (inverted triangles) on days 14 and 28. The relative quantity of PSA-specific IgG was calculated as follows: PSA-specific IgG/total IgG $\times 100$. A, PSA-specific IgG serum concentrations. B, IgG serum concentrations. C, Percent PSA-specific IgG serum concentrations. The data show that group F mice produced high serum concentrations of PSA-specific IgG.

**FIGURE 6.** IgG production in hu-PBL-SCID mice generated from donor 4. The hu-PBL-SCID mice were reconstituted with $1 \times 10^6$ donor 4 PBMC and immunized as described in Materials and Methods. Serum was collected on days 14 and 21. Total and PSA-specific human Ig were quantitated by ELISA. The data show results for individual mice. Symbols representing individual mice remain constant for all graphs. The relative quantity of PSA-specific IgG was calculated as follows: PSA-specific IgG/total IgG $\times 100$. A, PSA-specific IgG serum concentrations. B, IgG serum concentrations. C, Percent PSA-specific IgG serum concentrations. The data show that group G mice produced high serum concentrations of PSA-specific IgG.
IgG (Fig. 8). IgG binding to PSA was observed on blots probed with control fragments between 12–34 kDa, but with less intensity. No human IgG bound strongly to a 37-kDa PSA isoform and to a 25-kDa fragment (Fig. 8). Group C serum IgG also bound to the other PSA isoforms and to immobilized PSA in a concentration-dependent manner.

**Immunoblot analysis of hu-PBL-SCID sera**

Immunoblot analysis of pooled group C sera was performed to determine whether PSA-specific IgG recognized conformational epitopes on PSA. Replica blots were generated from 2.5 μg of PSA following SDS-PAGE under nonreducing conditions, as described in Materials and Methods. Group C serum IgG bound most strongly to a 37-kDa PSA isoform and to a 25-kDa fragment (Fig. 8A). Group C serum IgG also bound to the other PSA isoforms and fragments between 12–34 kDa, but with less intensity. No human IgG binding to PSA was observed on blots probed with control IgG (Fig. 8B). Group C serum IgG also bound PSA on immunoblots generated from SDS-PAGE under reducing conditions, but with reduced intensity (data not shown). Apparently, Ab generated from donor 1 responded most intensely to an epitope(s) that is associated with secondary structures formed by one of the several cysteine disulfide bonds present in PSA (30).

**IgG4 isotype analysis of hu-PBL-SCID sera**

IgG4 isotype-specific ELISA were performed on pooled sera from groups A–F as described in Materials and Methods. Sera from donor 1 mice (groups A, B, and C) were collected on day 25. Sera from donor 3 mice (groups D, E, and F) were collected on day 28. Total IgG concentrations in day 25 pooled group A, B, and C sera were 0.43, 0.51, and 1.12 mg/ml, respectively (data not shown). Total IgG concentrations in day 28 pooled group D, E, and F sera were 322, 577, and 463 μg/ml, respectively (Fig. 9). Error bars show ±SD for replicate wells. The percent IgG4 concentrations were determined as follows: IgG4/total IgG × 100. A, IgG4 concentration in mouse serum generated from donor 1. B, Ratio of IgG4 to total IgG in mouse serum generated from donor 1. C, IgG4 concentrations in mouse serum generated from donor 3. D, Ratio of IgG4 to total IgG in mouse serum generated from donor 3. The data show that IgG4 concentrations were up to 6.5-fold elevated in sera of mice immunized with PSA-IgG2a-pulsed DC and up to 3.1-fold enhanced relative to IgG concentrations.

**Phenotype analysis of Ag-stimulated DC**

PSA-IgG2a-pulsed DC, PSA-pulsed DC, and mock-treated DC were analyzed by flow cytometry to determine whether altered immunoregulatory surface molecule expression could have contributed to the distinct PSA-specific IgG stimulatory capabilities of PSA-IgG2a-pulsed DC. Briefly, three sets of triplicate cultures were generated from three donors in three separate experiments. One set of triplicate cultures generated from each donor was pulsed with PSA, another set was pulsed with PSA-IgG2a, and the final set was mock treated on day 6. Identical triplicate cultures were pooled and analyzed on day 7. The results showed that MHC class II, CD40, B7.1, and B7.2 surface expression was enhanced on PSA-pulsed DC relative to that on mock-treated DC for all
The CD1a and CD14 expression was also included to determine whether PSA-IgG2a- or PSA-pulsed DC developed into significantly enhanced surface expression of B7.1, B7.2, CD40, and MHC class II. However, as we showed here, immunization with PSA-IgG2a-pulsed autologous DC consistently stimulated high titer, PSA-specific human IgG responses in hu-PBL-SCID mice established from all tested donors. These results represent an important first step toward generating human mAb specific for human TAA because we have previously shown that Ag-specific IgG-producing B cells isolated from hu-SCID mice with high Ag-specific IgG titers can be immortalized (2). In addition, we showed that this method generates TAA-specific IgG responses against conformational epitope(s), thus suggesting that some TAA-specific mAb immortalized from hu-PBL-SCID mice will bind to relevant native protein structures.

We suggest that one factor in the successful stimulation of PSA-specific IgG production was the intentional use of female donors. Previous in vitro experiments comparing male and female splenocytes cultures revealed that only female cells were capable of producing PSA-specific IgG (36). We had suggested that this was due to the fact that males are naturally tolerized to PSA, while females are not. In fact, PSA-specific IgG titers have been documented in a few normal females (37). Thus, PSA-specific IgG production by female leukocytes is more akin to a neo-Ag response than to a self-Ag response. It would be interesting to use this model to determine whether tolerance to self-Ag expressed during ontogeny is different from tolerance to self-Ag expressed following puberty.

Previous reports have established that murine IgG2a binds to human FcγR via the Fc region of the IgG2a heavy chain (24, 25). We suggest that one factor in the successful stimulation of PSA-specific IgG production was the intentional use of female donors. Previous in vitro experiments comparing male and female splenocytes cultures revealed that only female cells were capable of producing PSA-specific IgG (36). We had suggested that this was due to the fact that males are naturally tolerized to PSA, while females are not. In fact, PSA-specific IgG titers have been documented in a few normal females (37). Thus, PSA-specific IgG production by female leukocytes is more akin to a neo-Ag response than to a self-Ag response. It would be interesting to use this model to determine whether tolerance to self-Ag expressed during ontogeny is different from tolerance to self-Ag expressed following puberty.

Previous reports have established that murine IgG2a binds to human FcγR via the Fc region of the IgG2a heavy chain (24, 25). We showed here that PSA-IgG2a bound to human DC. Therefore, PSA-specific IgG production by hu-PBL-SCID mice was probably facilitated by targeting PSA to FcγR on human DC by the Fc region of PSA-IgG2a.

**Table I. Effects of PSA and PSA-IgG2a on DC immunoregulatory surface molecule expression**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Donor 1</th>
<th>Donor 2</th>
<th>Donor 3</th>
<th>Mean ∆MFI</th>
<th>∆MFI&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p Value&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>12.1</td>
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<td>18.7 ± 5.9</td>
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<tr>
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<td>318</td>
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<td>328.7 ± 119.4</td>
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<sup>a</sup> DC from were grown in three sets of triplicate cultures for each of the three random female donors. One set of triplicate cultures from each donor was pulsed with either 25 μg/ml PSA, 25 μg/ml PSA-IgG2a or mock treated on day 6, pooled on day 7, and subjected to flow cytometric analysis as described in Materials and Methods. Results shown are for DC gated by forward and side scatter.

<sup>b</sup> Mean ∆MFI is calculated as follows: MFI − MFI of matched isotype controls.

<sup>c</sup> ∆MFI is calculated as follows: mean ∆MFI of Ag pulsed cells − mean ∆MFI of control cells.

<sup>d</sup> The paired Student t test was used to determine the statistical significance of ∆MFI values. A p < 0.05 was considered to be statistically significant. Data shows significantly greater B7.1, B7.2, CD40, and MHC class II expression on DC pulsed with PSA compared to mock treated control DC. Only B7.2 expression was found to be significantly greater on PSA-IgG2a pulsed DC when compared to control DC.
Ingmar et al., who showed that human FcγRI transgenic mice immunized with a human FcγRI-specific mAb containing antigenic determinants had greatly enhanced target Ag-specific IgG responses compared with those of littermate controls (38).

It is interesting to consider how targeting PSA to FcγR on human DC may have enhanced PSA-specific humoral responses. It is known that complexing Ag to an Ag-specific mAb decreases the quantity of Ag required for human APC to elicit half-maximal proliferation responses from Ag-specific T cells up to 1000-fold (23, 39). It has been suggested that increased immunogenicity is due to efficient Ag capture by FcγR and consequential enhanced Ag presentation by MHC molecules (39). It has also been previously established by ourselves and others that Th cell help is required for Ag-specific Ab production in similar hu-SCID models (2, 8, 40). Therefore, PSA-specific IgG production may have been due in part to an increased immunogenicity of PSA, i.e., a greater frequency of PSA-specific Th cell activation. As a result, the minimum amount of PSA-specific Th cell help needed by PSA-specific B cells may have been consistently achieved in mice immunized with PSA-IgG2a-pulsed DC, but not in control mice. However, it is well established that there is sufficient TAA capture and presentation by soluble TAA-pulsed DC to activate T cells and stimulate TAA-specific Th1-type and/or CTL responses (i.e., cellular immunity) in vitro and in vivo (12, 16–19, 41–43). Thus, these studies suggest that additional factors other than enhanced PSA presentation may have also contributed to enhanced PSA-specific humoral responses by our PSA-IgG2a-pulsed DC immunized mice.

We showed here that stimulating naive DC cultures with either soluble Ag or Ag-mAb complexes induced DC with significantly distinct phenotypes. B7.1, B7.2, CD40, and MHC class II surface expression was significantly enhanced on soluble PSA-pulsed DC relative to that on mock-treated naive DC. In addition, enhancement of B7.1 and CD40 surface expression was neither a donor-specific nor a PSA-specific occurrence. Similar results were obtained when donor 5 DC were pulsed either with the recall Ag, tetanus toxoid, or with the neo-Ag, horse ferritin (data not shown). Apparently, stimulation by soluble Ag leads to a surface molecule expression pattern that is associated with activation (14, 44, 45). It has recently been shown that human and murine DC express and presentation by surface membrane receptors produced for absorptive endocytosis and presentation of neo-glycoproteins to T cells (46–48). Perhaps Ag binding to glycosylated protein receptors on DC and/or endocytosis of glycosylated Ag by surface membrane receptors produced activation enhancing signaling events that led to the distinct phenotypic changes we characterized. Regardless, our data raise the possibility that these distinct DC had different effects on Th effector cell maturation, and that the DC phenotype induced by PSA-IgG2a pulsing may have contributed to the enhanced PSA-specific humoral immune responses. This idea is supported by recent reports that show that APC with different immunoregulatory molecule surface expression levels, particularly different B7.1 and B7.2 levels, stimulate distinct Th cell effector functions (28, 49, 50). Analysis of IL-4, IL-2, and INF-γ serum concentrations was considered as a means to determine relative differences in Th1 or Th2 activation, but previous attempts to identify these cytokines in sera generated from similar SCID models were inconclusive (P. Brams, unpublished observations). Rather, we showed here that PSA-IgG2a DC-immunized mice produced up to 3.1-fold more IgG4 relative to total IgG compared with control mice. This observation strongly suggests Th2 activation was enhanced in group C and F mice because IgG4 production is specifically associated with B cell stimulation by the Th2-derived cytokine, IL-4 (31, 32, 51). Unfortunately, we could not detect any PSA-specific IgG4 (data not shown). This result was not unexpected, since the sensitivity of our isotype-specific assays is greatly reduced compared with polyclonal targeting of both IgG heavy and light chains. Regardless, when DC phenotype and relative IgG4 expression data are evaluated together, it suggests a model with greatly enhanced B7.1 costimulation by PSA-pulsed, DC-polarized, PSA-specific Th cells to a Th1 phenotype through DC/T cell feedback maturation and differentiation (28, 52–55). Without sufficient additional Th2 activation, little or no PSA-specific IgG1 was produced by group B and E mice (51). In contrast, PSA-IgG2a-pulsed DC only had significantly enhanced B7.2 surface expression compared with naive DC. This suggests the inverse of the same model, where significantly more B7.2 may have mediated PSA-specific IgG1 production by directing more frequent Th2 activation than in control mice (28, 49). Increased Th2 activation may have subsequently stimulated enhanced PSA-specific IgG1 production by direct Th2 cytokine stimulation of B cells and through Th2 cytokine-modified Th1 help (51, 56–60). We plan to investigate whether Th1/Th2 deviation can be influenced by an Ag-Ab complex pulsed vs Ag-pulsed DC in vitro, where both cytokine production and costimulatory surface molecule expression by the responding T cells can be fully analyzed.

In summary, our results showed that DC pulsed with FcγR-binding PSA-mAb complexes consistently and reproducibly stimulated PSA-specific IgG responses in hu-PBL-SCID mice, but DC pulsed with PSA did not. Interestingly, functional IgG responses were associated with immunizing DC that had enhanced B7.2 surface expression but with B7.1, MHC class II, and CD40 surface expression levels comparable to those of naive DC. Furthermore, we previously showed that Ag-specific IgG responses can be immortalized from hu-SCID mice with Ag-specific IgG titers, our results open the possibility that this immunization strategy may be useful for generating human mAb to human TAA and other weakly immunogenic Ag (2).

Acknowledgments

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References


