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Immunization with antigen-pulsed dendritic cells significantly improves the immune response to weak self-antigens

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Abstract

Dendritic cells (DCs) are the only professional antigen-presenting cells endowed with the ability to stimulate naïve T cells and initiate a primary immune response. For this reason, DC-based immunization has been shown to be highly effective in eliciting CTL responses to viruses and tumor-associated antigens. Here we report on the use of DC immunization to enhance the B cell-mediated humoral immune response to highly conserved proteins and the application of this approach to the generation of monoclonal antibodies (mAbs) against these proteins. To illustrate the technique we describe the production of mAbs to class II transactivator (CIITA), the major histocompatibility complex (MHC) CIITA, a difficult immunogen owing to its high degree of identity among species. We show that mice immunized with a combination of an intravenous injection of DCs pulsed with recombinant fragments of CIITA followed by intraperitoneal injection of the antigen in incomplete Freund's adjuvant induced a detectable antibody response against CIITA, while sera from mice immunized using the traditional method (i.e. intraperitoneal immunization with 50 μg of protein in complete Freund's adjuvant) gave an almost undetectable response. Furthermore, a total of four fusion experiments demonstrate that immunization with Ag-pulsed DCs is necessary for the efficient generation of hybridomas and a good yield of mAbs specific for the recombinant and the native endogenous CIITA protein. Conversely, four independent fusions carried out with splenocytes from mice immunized using the traditional method failed to produce anti-CIITA hybridomas. We propose that immunization with antigen-loaded DCs should be the method of preference when attempting to raise mAbs against weak self-immunogens.
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Keywords: Class II transactivator (CIITA); Dendritic cells; Immune response; Immunization; Monoclonal antibodies

Introduction

Monoclonal antibodies (mAbs) constitute one of the most versatile and useful tools for the development of basic as well as biomedical investigation, including diagnosis and treatment of different pathologies. Since the description of this technique by Köhler and Milstein

Abbreviations: CIITA, class II transactivator; CFA, complete Freund's adjuvant; DC, dendritic cells; MHC, major histocompatibility complex; mAb, monoclonal antibody

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(1975), numerous laboratories have evaluated different strategies to make the production of mAbs a more efficient process. The generation of mAbs depends strongly on the immunization protocol. The nature of the antigen and its dose, as well as the route of immunization are considered significant aspects to attain an effective humoral immune response and in the increased likelihood to obtain the desired mAbs. Several immunization protocols have been used to improve the chances to generate mAbs; traditional immunization with proteins emulsified in complete Freund's adjuvant (CFA), coupling of proteins to KLH as an additional co-adjuvant, genetic immunization, and sophisticated protocols such as phage display libraries. However, the problem of obtaining mAbs against poorly immunogenic antigens still persists. Examples of such poor immunogens are the transactivator for the major histocompatibility complex (MHC) of class II antigens, the class II transactivator (CIITA) protein and the protein constituents of the proteasome. CIITA is an intracellular protein that is expressed at very low levels with a high homology between species (80% mouse–human), making it difficult to obtain a significant humoral immune response against it. Due to its central role in the expression of class II antigens and thus in antigen presentation, having mAbs to CIITA may be of special advantage in the studies designed at studying its trans-activating activity.

Dendritic cells (DCs) are considered the only cell type able to activate naïve CD4+ T lymphocytes and initiate cellular and humoral immune responses (Banchereau et al., 2000). Although there are some reports that DCs pulsed with certain antigens are able to induce a humoral immune response in vivo (Sornasse et al., 1992), this technology has not been applied to generate mAbs. In this study we focused on using DC immunization as a means to prepare high-quality mAbs against proteins that have shown to be refractory to this technology through the most frequently used immunization protocols. We have used DC immunization to raise mAbs against several subunits of the proteasome and the CIITA, both representatives of highly conserved proteins. Here, we exemplify the potential of this method by describing the generation of mAbs against CIITA using an immunization protocol consisting of a combination of antigen-pulsed DCs and intraperitoneal injections of the antigen in CFA. This contrasts with results using the traditional method that produced hardly detectable serum antibodies and no specific hybridomas.

Material and methods

Production and purification of CIITA fusion proteins

The regions coding amino acids 465–795 (CIITA$^{465–795}$) and 977–1130 (CIITA$^{977–1130}$) were cloned into the pRSET-c expression vector which contains an N-terminal polyhistidine region to allow further purification of fusion proteins (Invitrogen, Carlsbad, CA, USA). The right orientation of each insert was confirmed by PCR and sequencing. The recombinant vectors were used to transform BL21(DE3)pLysS *Esterischia coli* (Invitrogen, USA). The expression of histidine-CIITA fragments was induced with 1 mM isopropyl-β-D(–)-thiogalactopyranoside (IPTG) for 2–3 h at 37 °C and purified under denaturing conditions using the Xpress^{TM} System Protein Purification kit (Invitrogen, USA), following the manufacturer's instructions. Protein concentration was measured using the Bradford assay (Bio-Rad, Hercules, CA, USA). The purity and size of the fusion proteins was confirmed by SDS–PAGE and staining with Coomasie blue.

Purification of dendritic cells from BALB/c mice

DCs were isolated from the spleen of BALB/c mice following a previously described protocol (Ruedl et al., 1996; Iwasaki and Kelsall, 1999; Mora et al., 2003). Briefly, mice between 8–12 weeks old were injected s.c. with 1×10^7 murine B16 melanoma cells transfected with the gene for the Flt3-L cytokine (generously provided by Dr. Hide Ploegh, Harvard Medical School, Boston, USA), in order to obtain the homogenous expansion of DCs (Pulendran et al., 1997). Two weeks later DCs were purified from splenocytes by positive selection using anti-CD11c antibody conjugated to MACS microspheres (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions.

Flow cytometry

The purity of isolated DCs was confirmed by FACS (Becton Dickinson, San Jose, CA, USA) in a double direct immunofluorescence assay using PE-labeled mouse anti-CD11c and FITC-labeled IA/IE antibodies (BD Pharmingen, San Diego, CA, USA) or isotype controls (Sauma et al., 2004). Cells plus antibodies were incubated for 30 min at 4 °C in the dark. After washing the cells once, they were resuspended in PBS + 2% FBS + propidium iodide (1 μg/ml) to gate only live cells. Analysis was done with the CellQuest program (Becton Dickinson, San Jose, CA, USA).

Immunization

DC immunization: BALB/c mice were immunized i.v. with 2×10^6 purified DC previously pulsed for 3 h at 37 °C with 2.5 μM of a mixture of CIITA$^{465–795}$ and CIITA$^{977–1130}$ or with a CIITA peptide (^{726}GEIKDKELPQYLALTR^{741}) coupled to Blue Carrier. At the same time,

50 μg of a mixture of $CIITA^{465–795}$ and $CIITA^{977–1130}$ emulsified in CFA (Sigma, USA) or of the CIITA peptide (^{726}GEIKDKELPQYLALTR^{741}) coupled to Blue Carrier (see below) (Biosonda, Santiago, Chile) emulsified in CFA were injected i.p. Finally, the mice were boosted i.p. with the appropriate antigen emulsified in incomplete Freund's adjuvant 1 and 2 weeks after the first immunization. To generate hybridomas, 3 days before the fusion procedure mice were injected i.v. with 25 μg of the appropriate antigen diluted in PBS.

Conventional immunization: BALB/c mice were immunized with 50 μg of fusion proteins $CIITA^{465–795}$ or $CIITA^{977–1130}$ or a CIITA peptide (^{726}GEIKDKELPQYLALTR^{741}) coupled to Blue Carrier and emulsified in CFA. Finally, the mice were boosted i.p. with the appropriate antigen emulsified in CFA 1 and 2 weeks after the first immunization. Three days before fusion, 25 μg of the corresponding antigen diluted in PBS, were injected i.v.

Genetic immunizations: Three groups of mice (2 mice/group/plasmid) were immunized i.m. with 50 μg of plasmids pIRES (Clontech, Palo Alto, CA, USA), pCMV Tag2a (Stratagene, La Jolla, CA, USA) or pcDNA 3.1 His-B (Invitrogen, USA) vectors containing the CIITA gene. In order to enhance the response against the CIITA plasmids mice were inoculated with 50 μg of a plasmid containing the *GM-CSF* gene (a gift from Dr. Rosario Billetta, ICBM, Faculty of Medicine, University of Chile, Santiago, Chile). A week later the mice were boosted with the proper plasmid, and a second boost was done a week later.

In all cases sera from mice were collected before and after every immunization and tested by ELISA assay.

Cell fusion

Isolated spleen cells were treated with a red blood cell lysis solution (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.2–7.4) and about 2×10^8 cells were subjected to fusion procedure with NSO/2 myeloma cells at a ratio of 5:1 in the presence of 50% polyethylene glycol solution (PEG 4000 Merck) as described by Kohler and Milstein (1975). The screening of the hybridomas was done by ELISA and Western blot as described below.

ELISA

Immunoplates (Nunc, Naperville, IL, EE.UU.) were coated with 50 μl of purified histidine-$CIITA^{465–795}$ or $CIITA^{977–1130}$ proteins (0.6 μg/ml each) or the peptide (^{726}GEIKDKELPQYLALTR^{741}) coupled to BSA (10 μg/ml) in a coating buffer (0.33 M $NaHCO_3$, 0.66 M Na_2CO_3, pH 9.5) overnight at 4 °C. After blocking with a solution of 0.1% Tween 20/PBS (PBST) for 1 h at room temperature, test samples (hybridomas supernatants or sera) were added in serial dilution to each well and incubated for 1 h at room temperature. After washing unbound antibodies with PBST, 100 μl/well of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulins (1:1000 in PBST) (PharMingen, CA, USA) were added and incubated for 1 h at room temperature. After washing with PBST and two times with distilled water, the reaction was developed by using tetramethylbenzidine (TMB) Substrate Reagent Kit (PharMingen, CA, USA). The enzymatic reaction was stopped by adding 50 μl/well of 1 M H_3PO_4. Color development was measured by reading the absorbance at 450 nm in an ELISA reader (Emax Precision Microplate Reader, Molecular Devices. Sunnyvale, CA, USA). Positive supernatants against $CIITA^{465–795}$ or $CIITA^{977–1130}$, but not to the fusion proteins were selected as positive hybridomas. Selected hybridomas were subsequently sub-cloned and ascitic fluid produced by standard procedures.

Transfections

COS7 cells were transfected with 30 μg of a plasmid containing hCIITA (pCMV2 FLAG-CIITA) or an empty vector (pCMV2 FLAG) as control (Stratagene, USA). Cells were electroporated with a Bio-Rad apparatus (Gene pulser II) using 960 μF, 300 V and 200 Ω conditions. After 2 days, expressed protein was assayed by Western blot and immunoprecipitation.

Cell culture

Human epithelial carcinoma cell line (Hela7A) was cultured in DMEM (Gibco BRL, USA) with 10% fetal calf serum (FCS) (Gibco BRL, USA), 10% CO_2, antibiotics and 2 mM glutamine under standard conditions. Hela7A cell line was incubated for 16 h with human gamma interferon (250 U/ml) to induce CIITA expression (Barbieri et al., 2002). Human B cell lines BLS-2 and RJ2.2.5 belonging to Bare Lymphocyte Syndrome group A mutated in the *CIITA* gene (Mach et al., 1996) were cultured in RPMI plus 10% FCS and 5% CO_2.

Protein extraction

Cells were lysed by incubating for 30 min at 4 °C in a solution containing 10 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$, 5% glycerol, 0.5% Nonidet P-40, leupeptin 1 μg/ml and a cocktail of protease inhibitors (Amersham-Pharmacia, Piscataway, USA) and centrifuged at 12,000 rpm for 30 min at 4 °C. The pellet was discarded and the protein concentration of the supernatants measured with the DC Protein Assay (Bio-Rad, USA).

Western blotting

Western blot was carried out with 20 μg of total cell protein lysates from COS7 cells transiently transfected with CIITA cloned in pCMV2-FLAG vector (Sigma, USA) or with the empty vector. The samples were separated in 6% SDS–PAGE and transferred to a nitrocellulose membrane (Bio-Rad, USA). After blocking overnight at 4 °C with PBST containing 5% non-fat dried milk, membranes were incubated with supernatants or primary antibody solution for 1 h at room temperature. Membranes were washed three times with PBST for 10 min and incubated for 1 h at room temperature with HRP-labeled sheep anti mouse immunoglobulin (Amersham, USA) diluted 1:10,000 in PBST containing 0.2% non-fat dried milk. The nitrocellulose membranes were washed three times for 10 min with PBST and immunoreactive bands were visualized with ECL-plus (Amersham, USA) and autoradiographic film according to the manufacturer's instructions.

Immunoprecipitation

Three hundred microliters of cell lysates were pre-cleared for 2 h with 30 μl of a 50% suspension of Protein G-Sepharose in PBS and 3 μl of a rabbit preimmune serum. The clarified lysate was then incubated overnight at 4 °C with 5 μl of a rabbit immune serum anti-CIITA$^{465–795}$, anti-CIITA$^{977–1130}$ proteins or mAb 7G8 (not shown). The CIITA–immunoglobulin immune complexes were incubated for 30 min with 25 μl of a 50% suspension of Protein G-Sepharose in PBS and centrifuged at 8000 rpm at 4 °C for 3 min. The pellet was washed three times with a solution containing 10 mM HEPES, pH 7.9, 0.4 M NaCl, 1.5 mM $MgCl_2$ and 0.5% Nonidet P-40 and once with PBS. Proteins were eluted by incubating with 5 μl of sample buffer 3 × (156 mM Tris–HCl, pH 6.8, 6.25% SDS, 12.5% 2-mercaptoethanol, 25% glycerol and 0.05% bromophenol blue) and boiled at 100 °C for 5 min. The supernatant obtained after centrifugation was used for Western blotting.

Coupling of CIITA peptides to BSA or Blue Carrier

A mixture of 1 mg of CIITA peptide (^{726}GEIKDKELPQYLALTR^{741}) and 1 mg of BSA or Blue Carrier were dissolved in 2 ml of 0.05 M sodium citrate buffer, pH 6.5 and glutaraldehyde added to a final concentration of 0.1%. This mixture was incubated for 1 h at room temperature in the dark and dialyzed for 1 h at 4 °C against four changes of 1 l each of PBS.

Animal handling

Male and female BALB/c mice were purchased from Jackson Laboratories and were bred and maintained in our animal facilities. All experimental protocols were approved by the institutional animal care committee and procedures were carried out according to institutional guidelines.

Results

We have made several attempts to raise mAbs against the MHC CIITA using conventional protocols with no success. To improve the immune response against this protein, recombinant fragments and peptides of CIITA were loaded into DCs. These cells were used to immunize BALB/c mice and the effectiveness of this protocol was compared to conventional protocols of immunization and to genetic immunization.

First, to overcome the difficulty caused by the low amount of DCs present in normal tissue, mice were implanted subcutaneously with B16 melanoma cells that secrete Flt3-L, a cytokine that induces the general expansion of DCs. In general, one out of four BALB/c mice developed a tumor when injected with the B16 melanoma cells. DCs were isolated 2 weeks later from the spleens of BALB/c mice and purified by immunomagnetic positive selection using anti-CD11c MACS microbeads (see Material and methods) yielding DCs with a purity of 95% (see Fig. 1). The DC population was

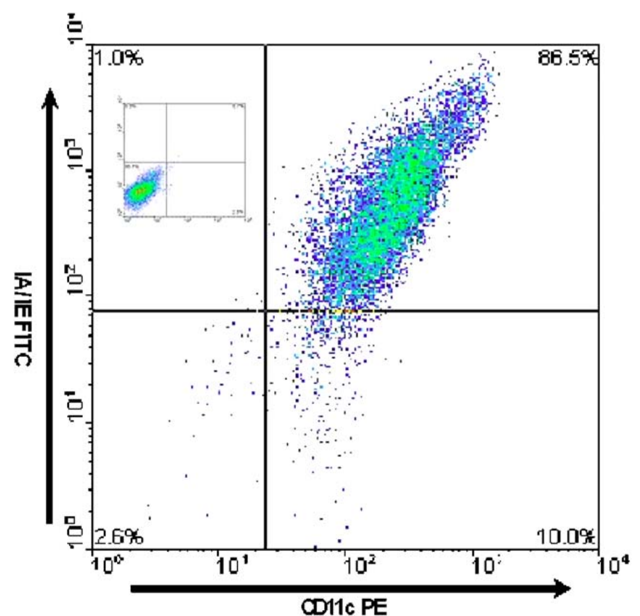

Fig. 1. Flow cytometry analysis of dendritic cells isolated from spleen of BALB/c mice implanted subcutaneously with B16 cells secreting Flt3-L. DCs were purified from the spleens by immunomagnetic positive selection using anti-CD11c antibodies (see Methods). Purified DCs have a heterogeneous maturity phenotype as seen by their differences in MHC-class II expression (I–A/I–E). This is a representative sample of nine independent DC purifications. Insert corresponds to the isotype control.

approximately 48% $CD8\alpha^+$ and 52% $CD8\alpha^-$ with 86% presenting a mature phenotype (CD11c+/MHC-II^{hi}) (Fig. 1). Before peptide loading these cells had a low expression of the co-stimulatory molecules CD80, CD86 and CD40, expression that increased after stimulation (data not shown). Purified DCs were pulsed for 3 h at 37 °C with 2.5 μM of different recombinant proteins or a CIITA-derived peptide coupled to Blue Carrier and injected intravenously into BALB/c mice. These mice were simultaneously injected with 50 μg of the same antigen emulsified in CFA. We will refer to this protocol as DC immunization as compared with a conventional protocol that only used antigen emulsified in CFA. We also used genetic immunization for comparison purposes.

DC cell immunization and antibody response

Since endogenous intracellular expression of CIITA is very low, immune sera were analyzed by Western blot against lysates of COS7 cells transiently transfected with pFlag-CIITA. To ensure that the antibodies recognized the CIITA protein and not the poly-his motif, the antibodies were tested against a pFlag-CIITA construct to discriminate for a specific humoral immune response against CIITA. As is shown in Fig. 2B, only sera from mice immunized with DCs recognized CIITA.

Only mice that underwent DC immunization with a mixture of CIITA recombinant protein ($CIITA^{977–1130}$ and $CIITA^{465–795}$) gave a significant titer against CIITA (Fig. 2A). On the other hand, mice immunized using a conventional protocol (see Methods) showed a very low or undetectable response against this protein.

Next we investigated if mice immunized with peptides coupled to Blue Carrier, a potent inducer of humoral immune responses, can reach similar levels of antibody titers compared to DC immunization. A group of 3 mice was immunized with DCs pulsed with the CIITA-peptide ^{726}GEIKDKELPQYLALTR^{741} and an i.p. injection of the same peptide coupled to Blue Carrier emulsified in CFA. Another group of three mice was immunized with the same peptide coupled to Blue Carrier emulsified in CFA only. Both groups were boosted as indicated in Material and methods. ELISA assays were carried out using BSA-coupled peptide. Again, only mice immunized with peptide-pulsed DCs were able to generate a humoral immune response with good titers against the CIITA-peptide (Fig. 3). Moreover, no humoral immune response against this antigen could be detected even when mice immunized by the conventional method received two additional boosts of the peptide coupled to Blue Carrier (data not shown). Additionally, DC immunization specifically increased serum concentration for IgG1 (4–8 fold), IgG2a (2.5–4 fold) and IgG2b (3.5–5 fold) isotypes as compared to the

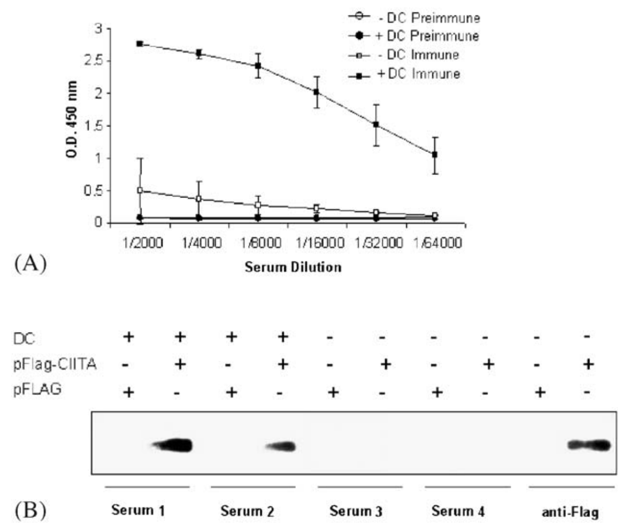

Fig. 2. Reactivity against recombinant CIITA of the sera obtained from mice immunized using the conventional or the DC protocols. (A) Antibody titer of sera obtained from mice immunized with pulsed-DC (+DC) or the conventional method (−DC), as detected by ELISA. Pre-immune sera from each mouse are included. Data are average of four mice for each one of the immunization procedures. (B) Western blot analysis of protein extracts from COS7 cells transiently transfected with pFlag-CIITA. Twenty μg of protein was subjected to SDS–PAGE on 6% gels and Western blot. Blots were revealed with sera from mice immunized with pulsed-DC (serum 1 and 2) or by the conventional method (serum 3 and 4). Negative controls were COS7 cells transiently transfected with the empty vector (pCMV2 FLAG). Mice sera were diluted 1:2000 while a commercial antibody against Flag (1:10,000) was used to confirm CIITA expression. Blots were revealed as indicated in Material and methods.

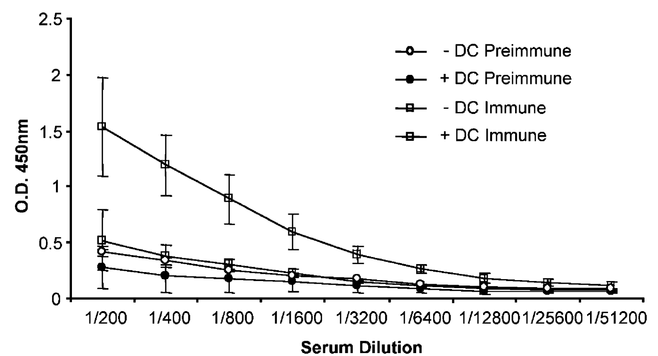

Fig. 3. Titration of antibodies generated against a CIITA peptide coupled to Blue Carrier. ELISA plates coated with the peptide ^{726}GEIKDKELPQYLALTR^{741} coupled to BSA (10 μg/ml) were used in these assays. Sera were from mice immunized with DCs pulsed with the CIITA peptide ^{726}GEIKDKELPQYLALTR^{741} coupled to Blue Carrier and boosted twice with the same immunogen emulsified in CFA (+DC) and from mice immunized with the same antigen emulsified in CFA (−DC). Pre-immune sera from each mice are included in the assay. Data correspond to the average of three mice in each condition.

pre-immune sera (data not shown). Additional experiments demonstrated that genetic immunization with several plasmids (pIRES, pCMVTag2a and pcDNA3.1-His-B) containing CIITA did not generate detectable amounts of antibodies against the recombinant CIITA protein, even after four boosts with the constructs (data not shown).

DC immunization enhances the likelihood of generating hybridomas against the endogenous form of the antigen

Table 1 shows the results of four fusion experiments of mice immunized with DCs and four with mice immunized using the conventional protocol. In both cases standard protocols were used for cell fusion, plating and hybridoma selection. As seen in Table 1, both procedures produced similar amounts of hybridomas. However, specific anti-CIITA hybridomas were obtained only from fusions done with splenocytes from animals immunized with DCs. All CIITA-positive hybridomas were cloned by limiting dilution, expanded and established as stable clones. The established mAbs were examined by Western blot of the antigen immunoprecipitated from extracts obtained from different cell lines with a specific rabbit polyclonal antibody. Fig. 4 shows a representative result obtained with one of the mAbs (clone 7G8). As we have previously described (Barbieri et al., 2002), we observe different endogenous isoforms of the CIITA protein. On the other hand, these mAbs were able to immunoprecipitate pFlag-CIITA-transiently transfected in COS7 cells (data not shown), although we could not immunoprecipitate CIITA from cell lines, probably due to the low amount of protein expressed normally in these cells.

Table 1. Generation of mAbs-producing hybridomas after DC immunization and traditional immunization

	DC immunization	Traditional immunization
Number of fusions	4	4
Average hybridomas/ fusion	60	34
Average titer[a]	1/32,000	1/8000
Average OD[b] at titer dilution	1.4	0.3
Number of anti-CIITA mAbs	8	0

[a]Specific antibody titers of sera were determined 3 days after the second boost. Titers were determined by ELISA with recombinant proteins CIITA$^{977-1130}$ and CIITA$^{465-795}$as described in Fig. 2.

[b]ODs were obtained from the respective ELISA.

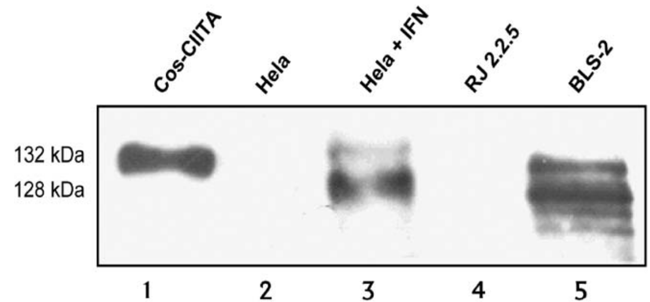

Fig. 4. Western blot analysis of mAbs against endogenous CIITA. Total protein extracts from different cell lines were immunoprecipitated with a rabbit anti-CIITA serum and visualized by Western blot with mAb 7G8. Protein extracts were obtained from: lane 1, COS7 cells transiently transfected with pFlag-CIITA (150 μg); lane 2, Hela7A cells (5 mg); lane 3, Hela7A cells treated for 16 h with IFN-γ (250 U/ml) to induce the expression of CIITA (5 mg); lane 4, RJ 2.2.5 cells that do not express CIITA due to a genomic chromosomal deletion (5 mg); and lane 5, BLS-2 cell line which expresses constitutively a mutant form of CIITA (2 mg). Other mAbs obtained against CIITA fragments presented similar reactivity (data not shown). Molecular weights are indicated on the left.

Discussion

We have succeeded in producing mAbs against a highly conserved intracellular protein using an immunization protocol with DCs. Antibody production to protein antigens depends strongly on the ability of T lymphocytes to provide help for the differentiation and proliferation of antigen-specific B cells. Also, it is now widely accepted that DCs are the only antigen-presenting cells competent for the initial activation of naïve T lymphocytes. Even though the role of DCs in initiating the immune response is well known, thus far these cells have not been used for the production of mAbs. Here we show that the delivery of an antigen by means of DCs elicit in mice an early and high specific immune response against the antigen, indicating that specific helper T lymphocytes have been effectively stimulated to provide appropriate help to B lymphocytes. In the example presented here, of a highly conserved protein, this response could not be obtained if DCs were omitted.

To illustrate the effectiveness of DCs in the immunization procedure and in the production of specific hybridomas, we choose as a model antigen the CIITA, a highly conserved transcription factor that is normally expressed in very low amounts in antigen-presenting cells. CIITA plays an essential role in antigen presentation, since expression of MHC class II molecules fully depends on CIITA expression. In spite of its importance in the adaptive immune response, very little is known on the physiological role of the CIITA protein, mainly due to the lack of commercially available mAbs that could

be used as tools for deciphering its function. Thus, having such a reagent may be of great help in clarifying the role of CIITA in immune response. Our experience, as well as that of others, indicated that it is difficult to produce mAbs to CIITA. Based on the known capacity of DCs to induce strong immune responses against difficult antigens (i.e. tumor-associated antigens) we chose to test if immunization with antigen-loaded DCs could improve the possibility to obtain hybridomas against this protein. Results obtained from mice immunized using antigen-loaded DCs were compared with results from mice immunized with a standard, conventional protocol or after genetic immunization.

Mice were immunized with recombinant fragments of the CIITA protein or with a CIITA-derived peptide coupled to Blue Carrier, a potent adjuvant. We also performed genetic immunization with several constructs of the *CIITA* gene, together with *GM-CSF* as a coadjuvant. Our results show that serum antibodies specific for CIITA could only be detected when the immunization included DCs pulsed with the respective antigen (see Figs. 2 and 3). More significant is the fact that hybridomas specific for the antigen could only be produced when DCs were used in the immunization protocol (Table 1). The mAbs produced recognized not only the immunogen but more importantly they also recognized the endogenous CIITA protein from different cell lines (Fig. 4) and immunoprecipitate CIITA from COS7 cells transiently transfected with CIITA (not shown). Altogether these results point out the efficacy of DCs as antigen-presenting cells for the production of mAbs compared to the conventional methods, which probably use macrophages recruited from the peritoneum by the action of the adjuvant as antigen-presenting cells. Also, DCs infused into the blood stream through the tail vein home directly to the spleen, where they initiate a robust immune response.

The method developed here could be the preferred strategy to generate mAbs against poor immunogenic proteins or peptides. In another set of experiments that will be published elsewhere, we have used DC immunization to generate mAbs against the intracellular proteases Cathepsin S and L, two well-conserved intracellular proteins. This corroborates the efficiency and the potential of DC immunization to generate an immune response.

Our results also indicate that immunization with spleen-derived DCs does not induce a bias towards a specific IgG class since the method produced antibodies of the IgG1, IgG2a and IgG2b classes (not shown). We have recently reported that Peyer's patch DCs are an essential element in the selective imprinting of T cell homing to the gut (Mora et al., 2003). Given the central role of T lymphocytes in providing the appropriate help for the activation of specific B cells, one can speculate that the use of DCs isolated from different lymphoid organs could induce different classes of Ig. If this proves to be the case, the protocol described here could be used to "tailor" mAb production toward a specific Ig class.

The ability of DCs to activate naive CD4+ helper T cells and subsequently produce an efficient activation of B lymphocytes is well known. More recently, however it has been reported that DCs are able to directly activate B lymphocytes, independently of CD4+ T cell help (Dubois et al., 1997, 1998). These unique characteristics of DCs might explain their ability as an effective means to improve antibody production.

The main problem in using DCs as adjuvant is their low frequency in tissues (0.2–0.5% of the total nucleated cells in the spleen). This made it necessary to expand their numbers before the purification step to get enough cells for the primary immunization. We used a method described by Iwasaki and Kelsall (1999) that uses the cytokine Flt3-L to expand DCs uniformly in the different lymphoid secondary organs, without altering their composition and function (Maraskovsky et al., 1996, 2000; Pulendran et al., 1997, 1998, 1999; Maldonado-Lopez et al., 1999). Starting from a single spleen, the one-step immunomagnetic purification procedure used here yielded DCs with a purity of 95%, enough for the immunization of at least 20 mice. These cells consist mainly of DCs in a mature stage (CD11c+/MHC-II+) (Fig. 1), with 10% being immature DCs. Current data indicate that mature DCs, although highly efficient as antigen-presenting cells, are ineffective at capturing and processing antigen, a property characteristic of the more immature phenotype. In the experiments presented here it appears that the fraction of immature DCs present in our purified population is sufficient to generate a strong humoral immune response (Klagge and Schneider-Schaulies, 1999).

Recent evidence indicates that DCs pulsed ex vivo with antigens have a significant advantage compared to in vivo cell presentation, since they do not induce T cell tolerance, that may constrain the development of a potential humoral immune response (Viney et al., 1998). We propose that primary immunization with antigen-loaded DCs could become a preferred method to improve the production of mAbs, specifically to more difficult antigens.

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