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CARRIER-INDUCED, HAPTEN-SPECIFIC SUPPRESSION: A PROBLEM OF ANTIGEN PRESENTATION?

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ABSTRACT

Prior immunity against a carrier protein has been shown to modulate the serologic response to injected haptens attached to the same carrier. In particular, a carrier/hapten-carrier immunization protocol induces marked suppression for IgG2a anti-hapten Ab production but does not interfere with anti-carrier Ab responses. Although the phenomenon of epitopic suppression has been amply demonstrated, the mechanism underlying the suppression remains unknown. The selective deficiency in IgG2a secretion suggests that IFN-')'-producing Th1 cells are not properly activated. We and others have shown that the nature of the APCs present during the first encounter with the Ag influences the development of selected Th populations in vivo; dendritic cells (DCs) seem to be required for the induction of primary, Th1-type responses. Since carrier priming induces the clonal expansion of specific B cells that appear to efficiently capture the Ag, we hypothesized that the hapten-carrier conjugate may be presented by B cells in preimmunized animals. Therefore, we immunized mice to the conjugate by injecting syngeneic DCs pulsed in vitro with the Ag. Our data show that an injection of DCs and IL-12 prevents epitopic suppression, suggesting that it may result from defective Ag presentation.

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Since its discovery by Herzenberg and coworkers in 1980 (1), the hapten-specific suppression that is induced following carrier-priming has remained unexplained. The phenomenon relies on the observation that the production of haptenspecific Abs is reduced in mice that have been primed previously against the carrier. This inhibition was unexpected, since it was shown earlier that Th lymphocytes that were primed to a carrier protein clearly augmented B cell anti-DNP responses in adoptive recipients stimulated with hapten-carrier conjugates (2).

The induction and maintenance of suppression varies according to individual Ig isotype. IgM responses show no evidence of suppression, whereas hapten-specific IgG2a, IgG2b, and IgG3, and to a lesser extent IgG1, responses are significantly suppressed in carrier-primed animals (3).

The analysis of functional phenotype and lymphokine secretion pattern has recently led to the identification of two discrete subsets of Th cells (4) that appear to reciprocally regulate the expression of Ig isotypes during a humoral response. In particular, the optimal production of IgG2a is induced by the activation of Th1 cells that secrete IFN-**γ** (5, 6). It is noteworthy that the anti-hapten response of mice undergoing carrier-induced suppression resembles the response mediated by Th2 cells in the absence of Th1 cells.

We and others have shown that the nature of the APC influences the isotype profile of the immune response. There is some evidence that dendritic cells (DCs)[3] are required for the optimal activation of Th1 cells, whereas B cells and macrophages may preferentially induce the differentiation of Th2 cells (7–10). Therefore, we hypothesized that the selective deficiency in the anti-hapten Th1-like response could be due to the presentation of hapten by APCs, which are not competent to induce the development of Th1 cells. Hapten-carrier conjugates would be captured, processed, and presented by B lymphocytes specific to the carrier in preimmunized animals. In naive animals, hapten-carrier would be presented by DCs, which are described as the accessory cells of the primary response.

Based on this assumption, we attempted to overcome carrier-induced immune suppression by immunizing mice with hapten-carrier pulsed on syngeneic DCs. Our data show that coinjecting conjugate-pulsed DCs and IL-12 induces the synthesis of high levels of hapten IgG2a Abs and prevents epitopic suppression.

Materials and Methods

MICE

We purchased 6 to 8-wk-old female BALB/c mice (H-2^d) and DBA/2 mice (H-2^d) from Charles River Wiga (Sulzfeld, Germany). These mice were maintained in our own pathogen-free facility.

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AGS, IL, AND CULTURE MEDIA

The carrier used in this study was keyhole limpet hemocyanin (KLH), which was obtained from Calbiochem-Novabiochem (San Diego, CA). Trinitrophenylated hemocyanin (TNP_{10}-KLH) was prepared as described previously (11). Briefly, TNP coupling was performed by adding 38 μg of trinitrobenzene sulfonic acid (Sigma, St. Louis, MO) to 1 mg of KLH in borate-buffered saline. The reaction was allowed to proceed for2h at room temperature with stirring. The solution was dialyzed extensively against PBS at 4°C and stored at -20°C. The concentration of TNP-KLH and the degree of substitution were measured by UV absorbance at 280 nM and 340 nM. An average of 10 molecules of TNP for 1 molecule of KLH was determined.

Murine rIL-12 was provided by Genetics Institute (Cambridge, MA). The culture medium used for the isolation and Ag-pulsing of APCs was RPMI 1640 (Seromed Biochem KG, Berlin, Germany) supplemented with 10% FCS (Byosis S.A., Compiègne, France), penicillin, streptomycin, non-essential amino acids, sodium pyruvate, 2-ME, and L-glutamine (Flow ICN Biomedicals, Buckinghamshire, U.K.).

PURIFICATION AND PULSING OF DCS

Spleens were digested with collagenase (CLSIII; Worthington Biochemical, Freehold, NJ) and separated into low and high-density fractions on a BSA gradient (Bovuminar Cohn fraction V powder; Armour Pharmaceutical, Tarrytown, NY) according to a procedure described by Crowley et al. (12). For DC pulsing, the adherent cells of the low-density fraction were cultured overnight in complete medium containing 100 μg/ml of TNP_{10} -KLH. Nonadherent cells contained at ≥90% of DCs after overnight culture (as assessed by morphology and specific staining using the anti-CD11c mAb, N418). TNP_{10}-KLH-pulsed DCs were washed in RPMI 1640 and administered at a dose of 3 X 10^5 cells in a volume of 200 μl.

IMMUNIZATION PROTOCOLS

KLH priming: Mice received an i.p. injection of 100 μg of KLH adsorbed onto 1 mg of Al(OH)3 adjuvant (alum) on day -14.

TNP-KLH priming: The mice were injected i.p. on day 0 with 100 μg of TNP_{10}-KLH adsorbed onto 1 mg of Al(OH)3 or injected i.v. with 3 X 10^5 conjugate-pulsed DCs (see above) and 5 μg TNP-$_{10}$KLH 5 days later. Treatment with IL-12: Some groups of mice received four daily i.p. injections of 0.2 μg rIL-12 on days 0, 1, 2, and 3, beginning at the same time as the injection of TNP-KLH.

ENZYME-LINKED IMMUNOSORBENT ASSAYS

Mice were bled at 21 days after primary immunization with TNP-KLH. Each mouse was bled and analyzed individually. The serum levels of Agspecific Abs were determined by ELISA according to standard procedures using polyclonal goat anti-mouse Ig reagent (Boehringer Mannheim Biochemicals, Mannheim, Germany) or isotype-specific rat mAbs. Anti-TNP and anti-KLH Abs were titrated using TNP_{12} human –**γ** globulin (5 μg/ml) or KLH (5 μg/ml). Ab concentrations were calculated based on a linear regression analysis of the ODs. The results are expressed as micrograms per milliliter and were determined using the midpoint of the titration curves relative to an internal standard run in each assay. The concentration of the Abs of all isotypes, as well as of the IgG1 or IgG2a isotypes, was calculated in the reference relative to the binding of

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purified IgG1 or IgG2a mAbs on TNP-or rat-anti-K-coated plates and was revealed by rabbit antimouse, rat anti-mouse IgG1, or rat anti-mouse IgG2a, respectively. All sera were tested on days 14 and 21 of the primary response and on day 8 of the secondary response with similar differences between the groups (data not shown).

STATISTICAL ANALYSIS

The data were analyzed for significance using the ANOVA test.

Results

DCS PULSED WITH HAPTEN-CARRIER COMPLEXES INDUCE A HUMORAL RESPONSE IN VIVO THAT IS SPECIFIC FOR HAPTEN AND CARRIER

We have shown previously that syngeneic DCs that have been pulsed in vitro with protein Ag induce a strong humoral response in syngeneic mice which is characterized by high levels of IgG2a Abs (7, 8). In preliminary experiments designed to determine whether conjugate-pulsed DCs would induce a humoral response to both hapten and carrier, we analyzed the immune response of mice that were primed by an injection of DCs that had been pulsed in vitro with TNP_{10}-KLH, as previously described. Some DC-primed animals were injected i.v. with 5 μg TNP_{10}-KLH in saline at 5 days after DC injection to sensitize B lymphocytes that were specific for native (unprocessed) Ag. Control groups included mice injected with soluble conjugate only and untreated animals. The data in Figure 1 show that all groups secreted high levels of Abs specific for KLH (Fig. 1A), although the level of Abs was significantly lower in mice injected with conjugate-pulsed DCs alone. By contrast, the humoral response that was specific for hapten varied among the groups, and the highest levels of Abs were induced by administrating conjugate-pulsed DCs and soluble Ag (Fig. 1B). Thus, an injection of conjugate-pulsed DCs and soluble Ag activates a humoral response that is specific for hapten and carrier.

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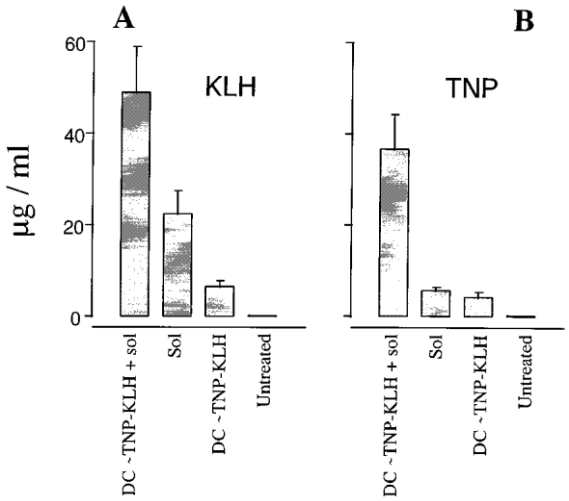
Figure 1. *Injection of TNP-KLH-pulsed DCs induces a primary humoral response to hapten and carrier. DBA/2 mice were injected with 3 X 10^5 DCs pulsed in vitro with TNP_{10} -KLH. One-half of the animals were boosted with 5 μg of conjugate in saline (sol) after 5 days. Control groups included both untreated mice and animals injected with 5 μg TNP-KLH in saline (sol). All mice were bled at 21 days after TNP-KLH immunization, and Ag-specific Abs were measured in individual sera using a goat antimouse Ig reagent. Data are shown as mean ± SD (95% confidence, n = 3).*



HAPTEN PRIMING ON DCS IN THE PRESENCE OF IL-12 PREVENTS EPITOPIC SUPPRESSION

Next, we tested whether targeting the hapten-carrier conjugate on DCs would prevent epitopic suppression. Carrier-primed DBA/2 mice were immunized either with TNP-KLH in alum or with a single injection of hapten-carrier pulsed on DCs, followed by an

i.v. Ag boost 5 days later. Some groups received four daily injections of 0.2 μg IL-12, which has been shown to promote Th1 development (13–15). All mice were bled at 21 days after the initiation of treatment, and levels of specific Abs were measured in individual sera. The data in Figure 2 show that, as expected, the production of the TNP-specific IgG2a Abs elicited by TNP-KLH in alum was suppressed by preexisting immunity to KLH (Fig. 2A). It is worth noting that the group of animals injected with Ag-pulsed DCs secreted significant levels of IgG2a specific for hapten when preimmunized against the carrier, albeit at lower levels than those produced by unprimed mice (Fig. 2B). An injection of IL-12 resulted in a similar increase in TNP-specific IgG2a Abs in mice injected with TNP-KLH in alum, regardless of whether they had been preimmunized against the carrier (Fig. 2C); therefore, IL-12 did not abrogate the suppression. Interestingly, an injection of conjugate-pulsed DCs and IL-12 completely prevented the epitopic suppression and resulted in the production of comparable amounts of TNP-specific Abs of IgG2a isotype in unprimed or carrier-primed mice (Fig. 2D).

Figure 3 summarizes the humoral responses of BALB/c mice tested individually in five independent experiments and shows an inverse correlation between KLH priming and the levels of TNP-specific IgG2a Abs in most groups. The injection of TNP-KLH in alum (group A) induced significantly lower levels of hapten-specific IgG2a in carrier-primed recipients (closed symbols) compared with unprimed animals (open symbols; $p < 0.001$). By contrast, the injection of conjugate-pulsed DCs and exogenous IL-12 (group C), but not of DCs or IL-12 alone (groups B, D,

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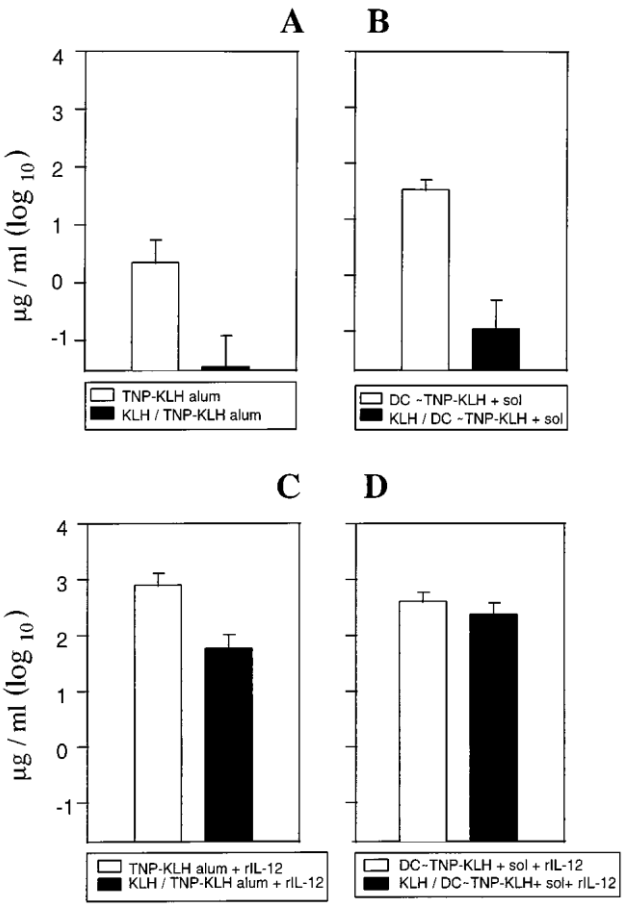
and E), induced similar levels of IgG2a Abs in animals that had been primed with the carrier on day 21 of the primary response or left unprimed ($p = 0.23$ for group C; $p < 0.001$ for groups B, D, and E). The production of anti-TNP Abs of all isotypes and of the IgG1 isotype was also significantly reduced in some carrier-primed mice (groups A and B), but was not reduced in mice injected with conjugate-pulsed DCs and IL-12 (group C). Of note, all mice primed with the carrier displayed increased levels of IgG2a anti-KLH (data not shown), whereas most groups (A, B, D, and E) showed decreased levels of IgG2a anti-TNP compared with unprimed animals.

Figure 2. *Injection of TNP-KLH-pulsed DCs and rIL-12 prevents carrier-induced epitopic suppression. DBA/2 mice were either untreated (open bars) or injected with 100 µg KLH in alum (closed bars). After 2 wk, the mice were divided into four groups that were injected either with 100 µg TNP_{10}-KLH on alum i.p. (A and C) or with 3 X 10^5 TNP-KLH-pulsed DCs i.v. and 5 µg TNP-KLH (sol) 5 days later (B and D). C and D received four daily injections of 0.2 µg murine rIL-12. All mice were bled at 21 days after TNP-KLH immunization, and hapten-specific Abs of IgG2a isotype were measured in individual sera. Data are shown as mean ± SD (95% confidence, n = 5).*



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Discussion

Herzenberg and coworkers have noted that mice primed with KLH and subsequently vaccinated with KLH-DNP conjugates showed reduced rather than augmented anti-DNP IgG responses compared with unprimed mice (1). More recently, evidence was found of apparent epitopic suppression in humans, as significant dose-dependent negative correlations were observed between prevaccination Ab responses to the carrier tetanus toxoid and postvaccination Ab responses to the haptenic Ag (16). Therefore, prior exposure of the target population to a carrier protein may render this protein unsuitable as a carrier; this phenomenon raises important questions concerning strategies for vaccine development.

The selective deficiency in the TNP-specific IgG2a response suggests that IFN -**γ** producing cells (Th1 cells) may not be properly activated in carrier-primed animals. All attempts to compare the Th1- and Th2-type cytokines that are produced following hapten restimulation in vitro by cells from control and suppressed mice were inconclusive, probably because of the coexistence of carrier- and hapten-specific Th cells (see below).

We hypothesized that epitopic suppression may result from defective hapten presentation by carrier-specific B lymphocytes that would efficiently capture the TNP-KLH conjugate (17, 18) and favor the development of Th2 cells. Therefore, we immunized mice by an injection of DCs that had been pulsed extracorporeally with the conjugate. The data clearly show that priming with DCs and daily injections of IL-12 prevented the suppression. Although the injection of conjugate pulsed on DCs or the administration of conjugate in alum with IL-12 induced increased levels of TNP- specific IgG2a Abs, it did not prevent epitopic suppression. Thus, the induction of an optimal Th1-type response is not sufficient to bypass the suppression. Our results support the hypothesis that epitopic suppression may involve a defective hapten presentation, as targeting the conjugate on DCs is required to prevent the suppression. The requirement for exogenous IL-12 may be related to the consumption of IL-12 by carrier-specific T cells that are present in the same environment or to the very low amounts of IL-12p70 (the bioactive form) secreted by DCs (19). Interestingly, activated B cells have been shown to down-regulate the expression of CD40 ligand/CD154 by $CD4^+$ T cells (20). Since IL-12 secretion is strongly enhanced in DCs upon interaction with CD40 ligand-expressing T cells (19), the inhibition of CD40 ligand/CD154 expression on T cells by KLH-specific B cells in carrier-primed animals would lead to the secretion of lower levels of IL-12 as compared with mice that were not primed to the carrier.

Hapten-specific suppression has been shown to strongly affect the IgG2a response and mildly affect the IgG1 response. Similarly, our data show a significant decrease in the production of TNP- specific IgG1 Abs in groups injected with TNP-KLH in alum and preimmunized with KLH (Fig. 3, group A). There is evidence that the synthesis of IgG1 could result from the activation of either Th1 or Th2 cells, as the IgG1 response to T cell-dependent Ag remained significant in the absence of IL-4 (21, 22). Therefore, as the synthesis of IgG2a strictly depends upon Th1 cells, whereas the production of IgG1 depends upon the activation of either Th cell population, a selective deficiency in Th1 cells would result in a strong decrease in IgG2a and only a mild decrease in IgG1 production.

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Of note, several reports have shown that immunization with the hapten-carrier conjugate in the presence of pertussis toxin or *Bordetella pertussis* LPS prevented epitopic suppression (23). Since bacteria and derivatives have been shown to induce the maturation of DCs in vitro and in vivo (24 –25) as well the migration of DCs to T cell areas (25), it is tempting to speculate that heat-killed bacteria or bacterial components favor the presentation of hapten-carrier by fully competent, mature DCs.

Several observations may be compatible with the role of carrier-specific B cells in inducing the preferential development of Th2 cells in carrier-primed animals: 1) priming hapten-specific B cells before carrier/hapten-carrier immunization abrogates the suppression (16); 2) epitopic suppression does not require carrier-primed $CD4^+$ or $CD8^+$ cells for its expression (26); 3) carrier- (diphtheria or tetanus toxoid) induced suppression can be circumvented by the use of peptide as carrier (27, 28); 4) the increase in epitope density of hapten-carrier conjugates decreases their ability to induce epitopic suppression (16); and 5) KLH priming of mice subsequently immunized with TNP-KLH does not interfere with the development of normal numbers of TNP-specific memory B cells (29).

Several reports indicate that B cells may favor the differentiation of Th2 cells. Th2 clones were found to proliferate preferentially in vitro in response to Ag presented by B cells (9). Ag-primed lymph node cells have been shown to produce more IL-4 when Ag is presented by B cells than when it is presented by splenic adherent cells (30). Polyclonal activation of the murine immune system in vivo by Abs to mouse IgD has been shown to stimulate T cells to secrete IL-4 (31, 32). More recently, Macaulay and coworkers have clearly shown that B cells activated by IgR-mediated endocytosis of Ag induced both naive and Ag-primed $CD4^+$ T cells to produce high levels of IL-4 (33). In contrast, splenic adherent cells induced the production of very low levels of IL-4 but much higher levels of IFN-$\boldsymbol{\gamma}$ (33).

The most effective inducer of Th cell differentiation appears to be the local cytokine environment. In particular, IL-4, IL-6, and IL-10 appear to induce the polarization of naive CD4+ T cells to effector Th2 cells (34 –38). IL-6 and IL-10 are produced by a wide spectrum of cells, including macrophages and B and T lymphocytes. IL-4 is secreted by mast cells, basophils, $NK1.1^+$ cells, and T lymphocytes. Therefore, it is possible that some of these cytokines are produced during priming to KLH and cause TNP-specific naive T cells to differentiate to Th2 cells.

An alternative explanation may involve hapten-specific suppressor cells. However, no evidence has been found for suppressor cells, as the IgG response is not inhibited by the coculture of TNP-specific B cells from control mice with TNP-specific B cells from suppressed mice (29).

The phenomenon of carrier-induced epitopic suppression raises the question of the specificity of the Th cells that induce Ab se cretion by hapten-specific B cells. Indeed, the production of carrier- and hapten-specific IgG2a Abs appears inversely regulated, as carrier-primed mice produce increased levels of IgG2a specific to the carrier but decreased levels of hapten-specific IgG2a Abs (Fig. 3 and data not shown). There is some evidence that the $CD4^+$ T cell responses mainly reflect the recognition of the hapten determinant alone, and that the carrier may allow the correct positioning of TNP molecules (39, 40). Therefore, although carrier-specific Th cells have been shown to enhance the hapten-specific immune response (2), a distinct Th population may activate the differentiation of B cells producing anti-hapten Abs.

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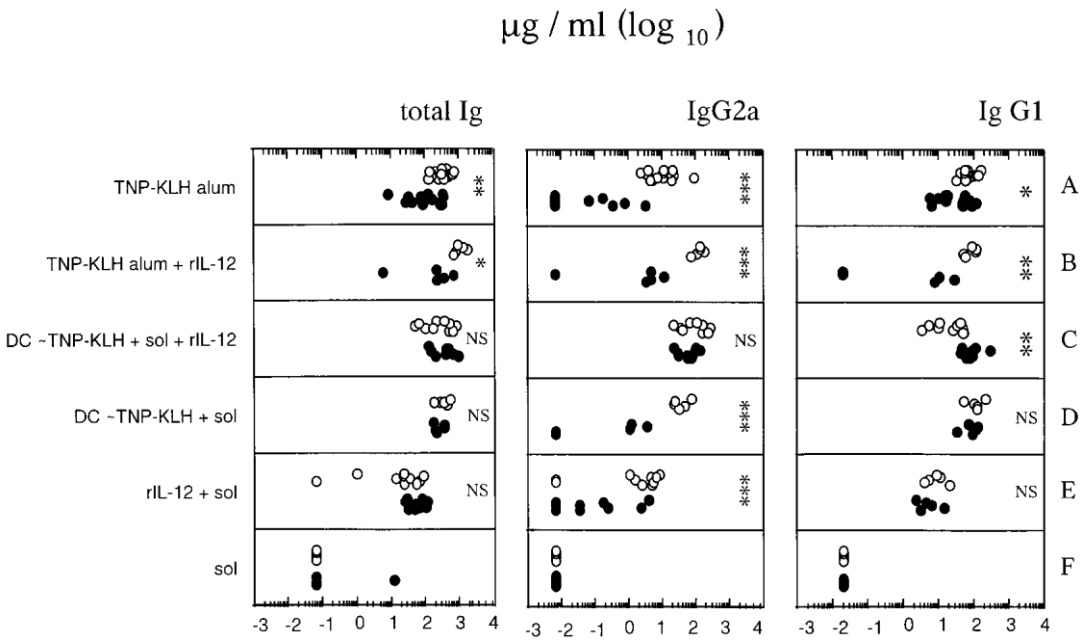
In conclusion, our data suggest that the phenomenon of carrier-induced epitopic suppression may result from deficient hapten presentation, possibly by carrier-specific B cells. Indeed, targeting the conjugate on DCs, which have been shown to optimally sensitize Th1 cells, prevents suppression in the presence of IL-12 and leads to the production of hapten-specific IgG2a Abs near control level. Additional experiments involving immunization to hapten-carrier in the absence of carrier-specific B cells will be required to directly assess the role of these cells in the phenomenon of carrier-induced, hapten-specific suppression.

Figure 3. *Isotype analysis of TNP-specific responses. BALB/c mice were either primed (closed symbols) to KLH on alum (100 μg) or left unprimed (open symbols) and further treated after 2 wk with either TNP-KLH on alum i.p., TNP-KLH-pulsed DCs i.v., and/or 5 μg TNP-KLH (sol) i.v. 5 days later. Some groups received injections of rIL-12. All mice were bled at 21 days after TNP-KLH immunization, and the Ag-specific Abs of all isotypes, as well as of IgG2a and IgG1 isotypes, were tested as described in Materials and Methods. p values were calculated for comparison of the TNP-specific response of mice preimmunized against the carrier vs unprimed animals (*p < 0.05; **p < 0.01; ***p < 0.001).*



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ABBREVIATIONS USED IN THIS PAPER: DC, dendritic cell; TNP, trinitrophenol; KLH, keyhole limpet hemocyanin; alum, Al(OH)3 adjuvant.

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