

Dendritic Cells but Not B Cells Present Antigenic Complexes to Class II-restricted T Cells after Administration of Protein in Adjuvant

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Summary

We have analyzed the relative contribution of dendritic cells (DC) and B cells in the presentation of peptide-class II complexes in an inflammatory situation *in vivo*. Draining lymph node cells from mice immunized subcutaneously with hen egg-white lysozyme (HEL) in adjuvant display HEL peptide-major histocompatibility complex class II complexes able to stimulate, in the absence of any further antigen addition, specific T hybridoma cells. The antigen-presenting capacity of three different antigen-presenting cell (APC) populations recruited in lymph nodes, DC (N418⁺, class II⁺, B220⁻, low buoyant density), large B cells (B220⁺, low buoyant density), and small B cells (B220⁺, high buoyant density), was analyzed. After immunization with HEL in adjuvant, DC are the only lymph node APC population expressing detectable HEL peptide-class II complexes. These results indicate that lymph node DC and not B cells are the APC initiating the immune response *in vivo* after administration of antigen in adjuvant.

Peptides bound to class II MHC molecules on the surface of APC are the ligands for antigen-specific receptors of CD4⁺ T cells (1). Among the different APC populations, dendritic cells (DC),¹ as compared to other class II-bearing cells such as macrophages (Mφ) and B cells, have been shown to be crucial for activation of naive T cells specific for protein antigens both *in vitro* (2–4) and *in vivo* (5). A remarkable characteristic of DC is their high expression, in addition to MHC molecules, of adhesion/costimulatory molecules (2, 6, 7). This may explain their efficiency in presenting exogenous (2, 3, 8) and endogenous (9) antigen to class II-restricted T cells.

DC appear to be critical for the initiation of the CD4⁺ T cell responses *in vivo*, whereas B cells can activate antigen-experienced but not naive CD4⁺ T cells (10, 11). This hypothesis is strongly supported by a recent study in B cell-deficient mice emphasizing the importance of DC, rather than B cells, in the priming of antigen-specific CD4⁺ or CD8⁺ T cells (12). However, based on studies in anti-μ-treated mice, it has been shown that B cells are required for the *in vivo* priming of T cell proliferative responses (13, 14). The defective T cell priming induced by anti-μ treatment could be overcome by injection of purified normal (15) or antigen-specific (16) B cells. Thus, it has been hypothesized that B cells could control the clonal expansion

of T cells and/or drive the diversification of the immune response (17).

In most of the studies mentioned above, the relative contribution of B cells as compared to professional APC such as DC, in antigen presentation *in vivo*, has been analyzed indirectly, using as read out the priming of T cell proliferative responses. CD4⁺ T cell activation depends on different factors, but the essential one is the presentation of peptide-class II complexes by APC. After intravenous administration of protein, it has been shown that splenic DC are the main cells bearing immunogenic fragments of foreign antigens (18). However, the phenotype of lymph node APC expressing antigenic complexes after subcutaneous administration of antigen in adjuvant is not known. To clarify this issue, we have analyzed the relative capacity of lymph node DC and B cells to present peptides derived from *in vivo* processing of protein antigen administered in adjuvant. This experimental model is based on our previous observation that draining lymph node cells (LNC) from mice immunized with hen egg-white lysozyme (HEL) display HEL peptide-MHC class II complexes able to stimulate, in the absence of any further antigen addition, specific T hybridoma cells (19, 20).

In the present paper, we characterize the phenotype of the lymph node APC bearing antigenic complexes by analyzing both the expression of cell surface markers and their intrinsic capacity to present an endogenously synthesized, naturally processed self-epitope, the mouse β2-microglobulin (β2M) peptide 26–39 bound to I-A^d molecules (21). Three APC populations were purified from immune lymph

¹Abbreviations used in this paper: DC, dendritic cell; Mφ, macrophage; LNC, lymph node cells; mβ2M, mouse β2 microglobulin; HEL, hen egg-white lysozyme.

node by a combination of gradient separation and selection by immunobeads; their ability to activate class II-restricted T cell hybridomas was determined. In agreement with our previous study (9), the hierarchy in the capacity to present the naturally processed self-epitopes of β 2M by lymph node APC is DC > large B cells > small B cells. After subcutaneous administration of HEL in adjuvant, the presentation of HEL peptides complexed to class II molecules is exclusively restricted to a low buoyant density APC population expressing DC markers N418 (CD11c)⁺, MHC class II^{high}, F4/80⁻, and B220⁻.

Materials and Methods

Mice, Antigens, and Immunization. 2–3-month-old BALB/c and C3H mice (Charles River Italy, Calco, Italy) of either sex were used. HEL, recrystallized three times and bovine RNase were obtained from Sigma Chemical Co. (St. Louis, MO). Mice were immunized subcutaneously into the hind footpads with the indicated amount of antigen emulsified in IFA or CFA containing H37Ra mycobacteria (Difco Laboratories, Detroit, MI).

Assay for Antigen-presenting Activity of LNC from HEL-primed Mice. The antigen-presenting activity of LNC from HEL-primed mice was assessed as previously described (19) using the following T cell hybridomas: 1C5.1 (I-A^k, HEL46-61) (19), 3B11.1 (I-A^k, HEL34-45) (22), 1H11.3 (I-E^d, HEL108-116) (23), and 2G12.1 (I-A^d, β 2M26-39) (21). Briefly, mice were immunized into the hind footpads with the indicated amount of antigen emulsified in IFA or CFA. 5–6 d after immunization, the draining popliteal lymph nodes were removed, and APC prepared as described below. Lymph node APC were then cultured in duplicate or triplicate at the indicated cell doses with appropriate HEL-specific T cell hybridomas (5×10^4 cells/well) in 96-well culture plates (Costar Corp., Cambridge, MA). Culture medium was RPMI 1640 (Gibco, Basel, Switzerland) supplemented with 2 mM L-glutamine, 50 μ M 2-ME, 50 μ g/ml gentamicin (Sigma Chemical Co.), and 10% FCS (Gibco). After 24 h of culture, 50- μ l aliquots of supernatants were transferred to microculture wells containing 10^4 CTLL cells and, after an additional 24-h incubation, the presence of T cell growth factors, mainly IL-2, was assessed by [³H]TdR incorporation during the last 5 h of culture.

Cytokine Assays. IL-2 concentration was also determined using a two-site sandwich ELISA with paired mAb purchased from PharMingen (San Diego, CA). For capture, the mAb was JES6-1A12 (rat anti-mouse IL-2). Samples were titrated in test solution (PBS containing 5% FCS and 1 g/liter phenol) and incubated overnight at 4°C. To detect bound cytokines, plates were then incubated with the biotinylated mAb JES6-5H4 (rat anti-mouse IL-2) in PBS containing 0.1% Tween 20 and 1% BSA. After washing, the bound biotinylated antibodies were revealed by an additional 30-min incubation with alkaline phosphatase-conjugated streptavidin (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) diluted 1/10,000. The plates were washed again and incubated with the developing substrate *p*-nitrophenylphosphate disodium (Sigma Chemical Co.) in diethanolamine buffer, pH 9.6 (100 μ l/well). The reaction was stopped by adding 50 μ l/well NaOH 3N and absorbance was read at 405 nm. IL-2 was quantified from two to three titration points using standard curves generated by purified recombinant mouse IL-2; results were expressed as cytokine concentration in pg/ml. The detection limit was 10 pg/ml.

APC Preparation. Immune LNC were depleted of T cells by cytotoxic elimination with HO-13-4 anti-Thy 1.2 mAb (TIB 99) followed by rabbit complement (low-tox M[®]; Cedarlane Laboratories, Ltd., Hornby, ON, Canada). Low and high buoyant density APC were prepared from T cell-depleted LNC by centrifugation over a discontinuous Percoll gradient (Pharmacia LKB, Uppsala, Sweden) containing 55–60% and 70% layers. Cells at the medium/55–60% and 60/70 % interface were collected separately and referred to as low and high buoyant density APC, respectively. Cells from the low density population were incubated with B220-coated microbeads and then separated into B220⁺ and B220⁻ fractions on MiniMACS[®] separation columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). Similarly, N418⁺ cells were sorted by incubating T cell-depleted LNC with biotinylated N418 mAb and streptavidin-coated microbeads before enrichment by passage on a MiniMACS[®] separation column. N418 is a hamster mAb specific for mouse splenic DC recognizing the p150/90 leukocyte integrin, likely the mouse CD11c molecule (24, 25).

Flow Cytometry. Cells were double stained by incubating them with optimal concentrations of FITC-labeled mAb and biotin-conjugated N418 mAb (anti-CD11c) for 30 min at 4°C in PBS containing 5% FCS, 0.1% sodium azide, and 1% normal rat serum to inhibit binding to FcR. The following FITC-conjugated mAb were used: SF1-1.1 (anti-K^d), 14.4.4S (anti-I-E), 6B2 (anti-B220), and Mac-1 (anti-CD11b), all purchased from PharMingen, and F4/80 (Serotec Ltd., Oxford, UK). Biotinylated mAb were revealed using PE-streptavidin (Southern Biotechnology Associates Inc., Birmingham, AL). Analysis was performed on a FACScan[®] flow cytometer (Becton Dickinson & Co., Mountain View, CA). Data were collected on 5,000–10,000 viable cells as determined by forward light scatter intensity and propidium iodide exclusion, and were analyzed using Lysis II software (Becton Dickinson & Co.).

Results

The Antigen-presenting Activity in Immune LNC Is Restricted to the Low Buoyant Density Population. We have previously shown that APC in draining lymph nodes from mice immunized subcutaneously with HEL in adjuvant display antigenic complexes formed in vivo between peptides derived from HEL processing and class II molecules (19, 20). After immunization with HEL in IFA or CFA, antigenic complexes are readily detectable on lymph node APC from day 2 to 14 after immunization (not shown). To analyze the phenotype of lymph node APC bearing HEL peptide-class II complexes, we first separated T cell-depleted LNC in low and high buoyant density populations. Results in Fig. 1 show that all the antigen-presenting activity is recovered in the low density fraction, enriched in large B cells and DC/M ϕ . Lymph node APC from HEL-primed C3H mice were monitored for their capacity to present complexes formed between two dominant HEL epitopes, HEL34-45 and HEL46-61 bound to I-A^k molecules (Fig. 1, A and B). For both determinants, a 30–100-fold enrichment in antigen-presenting activity is displayed by the low density population, as compared to unseparated T cell-depleted LNC. Similar results are obtained by monitoring the formation of HEL108-116/E^d complexes in BALB/c

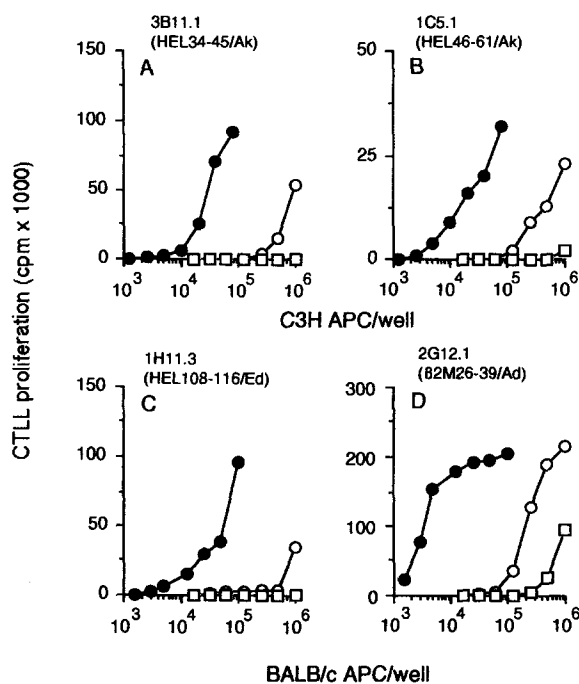


Figure 1. Presentation of exogenous HEL peptide–class II complexes and naturally processed self β 2M epitopes by low buoyant density lymph node APC. C3H (A and B) or BALB/c (C and D) mice were immunized into the hind footpads with 10 nmol HEL in IFA. 7 d later, draining popliteal LNC from five mice/group were pooled and T cells depleted by cytotoxic elimination with anti-Thy-1 mAb + C'. Cells were then separated in low and high buoyant density by centrifugation on 55% Percoll solution. The indicated numbers of T cell–depleted (○), low buoyant density T cell–depleted (●), or high density T cell–depleted (□) LNC were then cultured with the indicated T cell hybridomas. APC were not irradiated before culture. After 24 h, antigen-specific IL-2 production was determined by adding 50- μ l aliquots of culture supernatant to 10^4 CTLL cells for an additional 24 h. [3 H]Thymidine (1 μ Ci/well) was added during the last 5 h of culture. Data are presented as mean thymidine incorporation (cpm) from duplicate cultures. Background thymidine incorporation of CTLL was usually <1,000 cpm. The following number of cells was recovered after each step: T cell–depleted (80×10^6 ; 35×10^6); high density (36×10^6 ; 9.5×10^6) and low density (1.2×10^6 ; 0.98×10^6) from BALB/c and C3H mice, respectively. Results are from one representative experiment out of five performed with similar results.

mice (Fig. 1 C). In this mouse strain we have developed a system to analyze presentation by I-A^d molecules of naturally processed self- β 2M peptides corresponding to the sequence 26–39 (21). Results in Fig. 1 D show that, as for presentation of complexes derived from processing of exogenous HEL, a similar hierarchy of β 2M presentation by different APC is observed using as read out the β 2M26–39–specific, I-A^d–restricted T cell hybridoma 2G12.1 (21). Similar results were obtained using as read out β 2M–specific, I-E^d–restricted T hybridoma cells (not shown).

Antigenic Complexes Are Detectable only on DC-like APC in Immune Lymph Node. In steady state mouse spleen, DC are the most efficient in presenting endogenous naturally processed β 2M epitopes (9), suggesting that most of the HEL-presenting activity in immune lymph node APC could also be restricted to this cell population. Therefore, by a combination of Percoll gradient centrifugation and magnetic cell

sorting, we separated lymph node APC into three populations (Fig. 2). These populations were characterized by their expression of MHC class II, B220, and N418 molecules. The N418 hamster mAb recognizes CD11c, an integrin expressed on DC and at low level on M ϕ in normal lymphoid tissues (24). As shown in Fig. 2, the high density population from T cell–depleted LNC contains mainly B220⁺ I-E⁺ cells. Conversely, the low density population (90% class II⁺) is composed by a mixture of N418⁺ I-E⁺ (30%) and B220⁺ I-E⁺ (58%) cells. Results in Fig. 3 show the double staining analysis of low density lymph node APC. N418⁺ gated cells are negative for the M ϕ marker F4/80, whereas 49% are positive for Mac-1. They display a heterogeneous expression of MHC class II molecules (25% low, 75% high) and are negative for B220 molecules. Therefore, the low density population was labeled with B220-coated microbeads and then sorted in B220⁺ and B220⁻ cells. The population positively selected by B220-coated microbeads contains mostly class II⁺ B cells (97%), whereas the negatively sorted one is enriched (60–70%) in DC-like cells expressing high levels of CD11c and MHC class II molecules. Similar results were obtained using LNC from mice primed with HEL either in IFA or CFA, or with IFA only (not shown). These different APC populations were then tested for their capacity to activate T cell hybridomas specific for self β 2M26–39/A^d and exogenous HEL108–116/E^d complexes. As shown in Fig. 4, the hierarchy of endogenously synthesized self- β 2M presentation is similar to that previously observed with splenic APC (9): DC-like > large B cells > small B cells for both APC from IFA or CFA primed mice. It is interesting to note that complexes derived from exogenous HEL are only detectable on the DC-like population, whereas B cells, although able to present endogenous peptide, fail to present exogenous antigen. APC from IFA- or CFA-primed mice have a comparable capacity to present the self- β 2M peptide, but presentation of HEL-derived peptide is much more efficient using DC from HEL-IFA- as compared to HEL-CFA-primed mice.

Direct Correlation between Enrichment of N418⁺ Cells and Increase in Antigen Presentation. Cell sorting by the N418 mAb has been successfully used to enrich DC from mouse spleen (25). To confirm the role of DC in our model, we tested whether isolation of the N418⁺ cell subset by positive selection using magnetic cell sorting would result in an enrichment of HEL-presenting activity (Fig. 5). T cell–depleted LNC from mice primed with HEL in IFA were labeled with biotinylated N418 mAb followed by streptavidin-conjugated microbeads before separation on a MiniMACS[®] column. The yield of sorted N418⁺ cells (70% enriched) at the end of the procedure is \sim 1% of the total cells recovered in the negatively selected population (see legend to Fig. 5). N418-sorted cells expressed Mac-1 but not the F4/80 antigen, as in Fig. 3, indicating that M ϕ were not selected. Morphologically, after overnight culture, N418⁺ cells were highly enriched in nonadherent cells exhibiting the large veils characteristic of DC (not shown). When HEL peptide–class II complexes expressed by these APC populations are monitored, N418-sorted cells display, as

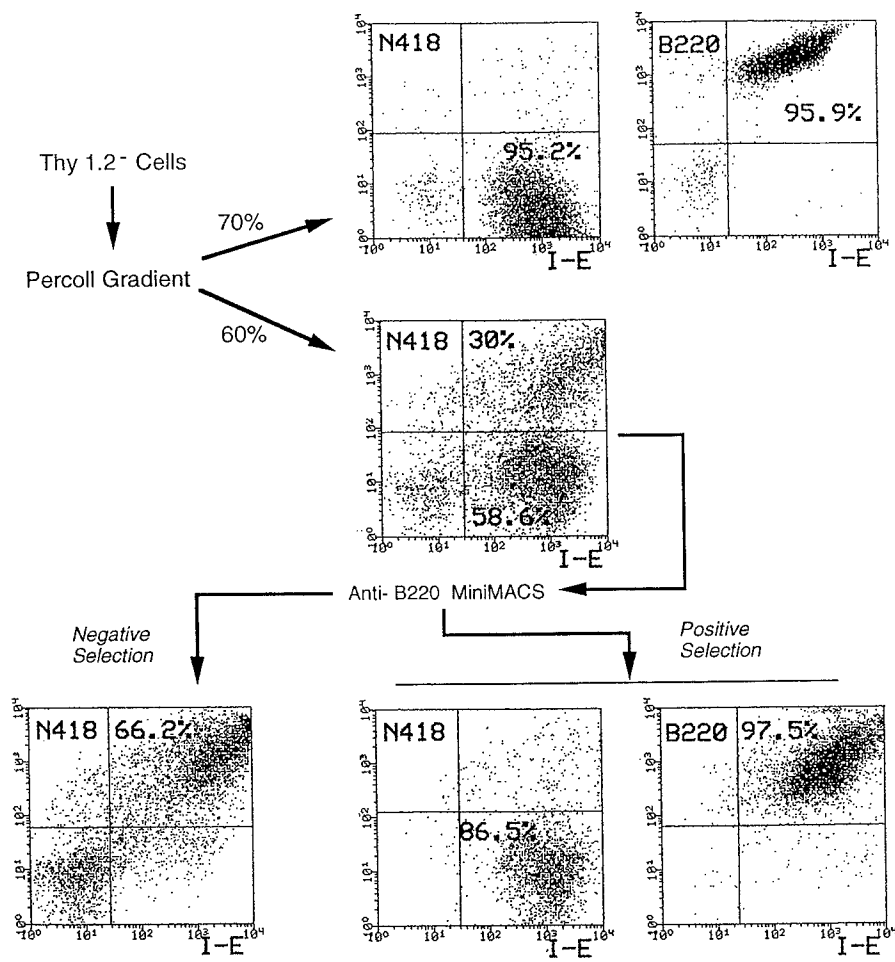


Figure 2. Separation of lymph node APC. BALB/c mice were immunized with 10 nmol HEL in IFA. 5 d later, LNC from five mice were pooled, depleted of T cells, and separated in low and high density population by centrifugation on Percoll. Cells were double stained for N418/I-E and B220/I-E expression. Low density APC were further separated by magnetic cell sorting on a MiniMACS® column using B220-conjugated microbeads. Both positively and negatively selected populations are analyzed for cell surface expression of the indicated molecules. The yield of cells recovered after each separation step (mean percentage of total LNC ± SD from nine experiments) was: T cell-depleted (42 ± 3.7); high density (17.7 ± 9.6); low density (2.43 ± 1.35); low density B220⁺ (0.96 ± 0.56) and low density B220⁻ (0.56 ± 0.25).

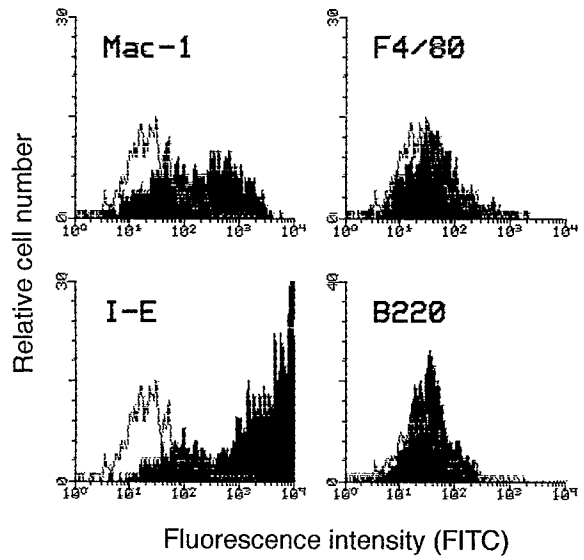


Figure 3. Cytofluorimetric analysis of lymph node DC. Low density, T cell-depleted LNC were obtained as in Fig. 2 from LNC of BALB/c mice primed with 10 nmol HEL in IFA 6 d earlier. Cells were double stained by FITC-conjugated mAb specific for the indicated molecules and by N418-biotin followed by streptavidin-PE. Cell surface expression of the indicated molecules (closed histograms) was analyzed on the N418⁺-gated population. Control staining (open histograms) represents cells stained without FITC-mAb.

compared to T cell-depleted LNC, a 100-fold increase in the HEL peptide-presenting activity which correlates with the enrichment of this cell population with ~0.7 to 70% (Fig. 5). Again, the antigen-presenting activity was higher for N418⁺ cells from IFA- as compared to CFA-primed mice. A two- to threefold decrease in antigen-presenting activity is observed in the negatively selected cells. The remaining antigen-presenting activity could be explained by residual N418⁺ cells that were not positively selected.

Collectively, these results indicate that after subcutaneous administration of protein in adjuvant, antigenic complexes are only detectable on a DC-like population expressing the N418 marker, whereas B cells are devoid of antigen-presenting activity.

Discussion

We have studied the relative capacity of lymph node DC and B cells to present in vivo to class II-restricted T cells peptides derived from the processing of protein antigen administered in adjuvant. By monitoring HEL108-116/E^d complexes after subcutaneous administration of HEL in adjuvant, peptide-class II complexes are exclusively detected on the low density N418⁺ APC subset. Unlike B cells, immature DC, such as Langerhans cells in the skin, are present

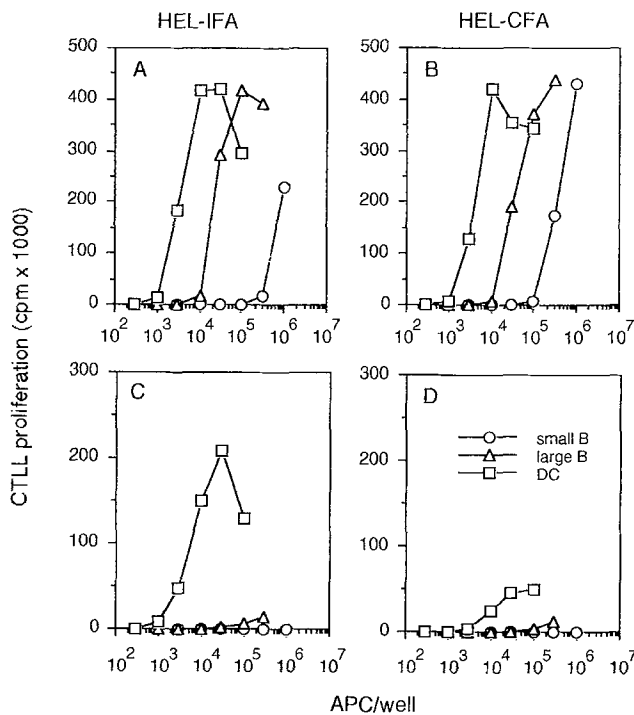


Figure 4. Antigenic complexes are expressed only by lymph node DC after administration of protein antigen in adjuvant. BALB/c mice were immunized with 10 nmol HEL in IFA (A and C) or CFA (B and D). LNC from five mice per group were pooled and APC populations separated as in Fig. 2. The self β 2M-specific, I-E^d-restricted 2G12.1, and the HEL-specific I-E^d-restricted 1H11.3 T cell hybridomas (top and bottom, respectively) were cultured (5×10^4 cells/well) with graded numbers of enriched APC populations. After 24 h of culture, IL-2 production was determined as in Fig. 1. Results are from one representative experiment out of five performed with similar results.

at the injection site where they exert a sentinel function (2, 26). It is therefore likely that protein has been endocytosed by resident immature DC, then processed and presented efficiently by DC recruited in the lymph node, where priming of naive T cells may occur. Several lines of evidence indicate that lymph node N418⁺ cells belong to the dendritic cell system. First, in addition to the CD11c (N418) marker, they express high levels of MHC class II molecules and are positive for CD11b (Mac-1) but negative for the M ϕ marker F4/80. Second, after sorting by N418 mAb and overnight culture, this population is highly enriched in nonadherent cells exhibiting the large veils characteristic of DC. Third, they are the most efficient in presenting endogenous self- β 2M peptide-class II complexes, as we have recently shown for thymic and splenic DC (9).

It is interesting to note that all three APC populations tested, DC, large and small B cells, are recruited to the draining lymph node independently of the presence of *Mycobacterium* or antigen in the adjuvant. This is in agreement with the hypothesis that tissue injury, induced in our system by subcutaneous administration of adjuvant, is sufficient to induce the migration of APC, including DC, to lymphoid organs (27). Immunization by antigen emulsified

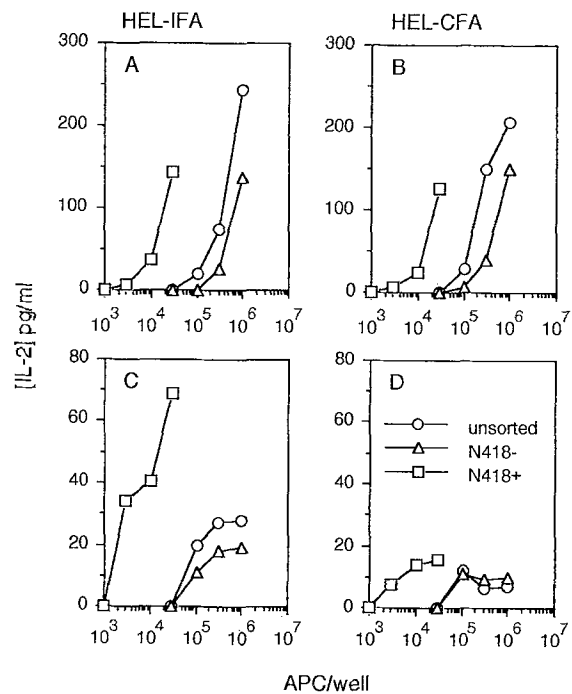


Figure 5. Direct correlation between N418⁺ cell enrichment and antigen presentation. BALB/c mice were immunized with 10 nmol HEL in IFA (A and C) or CFA (B and D). 5 d later, LNC from five mice per group were pooled and depleted of T cells. APC populations were enriched in N418⁺ cells by magnetic cell sorting on a MiniMACS[®] columns using biotin-conjugated N418 mAb and streptavidin microbeads. The indicated numbers of T cell-depleted unsorted APC (○), N418-enriched (□), or negatively selected N418 cells (△) were cultured with the self- β 2M-specific T cell hybridoma 2G12.1 (A and B), or with the HEL108-116 specific, I-E^d-restricted T cell hybridoma 1H11.3 (C and D). The N418-sorted cells represent 0.8% (IFA) and 0.7% (CFA) of the negatively selected, T cell-depleted LNC. After 24 h of culture, IL-2 concentration was determined by two-site sandwich ELISA. Results are from one experiment out of two performed with similar results.

in CFA or IFA leads to the recruitment of similar numbers of N418⁺ cells in draining lymph nodes. However, the antigen-presenting activity is higher in APC from mice primed with protein antigen emulsified in IFA rather than CFA. Several explanations could account for this difference. As we have previously shown for MHC class II-blocking peptides (19), peptides from *Mycobacterium tuberculosis* proteins present in CFA could compete with HEL-derived peptides for antigen presentation. Alternatively, components in CFA might increase the local secretion of proinflammatory cytokines such as TNF- α , which has been shown to downregulate the antigen-capturing and -processing capacity of human DC in vitro (28). However, changes in the stimulatory function of DC do not appear to play a role in our system because no differences were observed in their intrinsic capacity to activate T cell hybridomas specific for the self β 2M peptide. Therefore, competition for antigen presentation and/or induction of DC maturation, rather than changes in their immunostimulatory capacity, could explain these differences. T cell priming is usually more efficient in mice immunized with antigen in CFA than in IFA, and there-

fore it does not correlate with the antigen-presenting capacity of lymph node DC. It is possible that priming of a high frequency of Mycobacterium-specific T cells could provide substantial help to the development of HEL-specific T cells because of increased local production of IL-2. In addition, immunization in CFA might favor IL-12 production, which would result in a stronger polarization of the T cell response towards the Th1 phenotype, characterized by IFN- γ and IL-2 production associated with increased T cell proliferation (29). All these mechanisms may contribute to compensate for the decreased capacity of DC to present peptides derived from exogenous proteins administered in CFA.

The demonstration that DC are the only APC able to display antigen-presenting capacity after subcutaneous administration of antigen in adjuvant strongly supports the hypothesis that DC rather than B cells are required for priming of T cell proliferative responses in vivo (5, 10, 11, 30, 31). Direct evidence for this hypothesis has been recently provided by the demonstration of successful T cell priming in B cell-deficient mice (12). Using anti- μ -treated mice, B cells have also been implicated in the priming of T cell responses in vivo (13–16). Administration of rabbit anti- μ Ig from birth results in B cell depletion associated

with defective T cell proliferation that could be restored by B cell transfer (15, 16). Chronic stimulation of the immune system could favor the development of antigen-specific T cells with Th2 phenotype (32), which proliferate less than Th1 cells (33). The fact that B cell transfer overcomes inhibition of T cell proliferation in a MHC nonrestricted fashion (15) could be explained by the capacity of B cells to consume endogenous IL-4, which in turn would favor the development of a proliferative Th1 response.

In conclusion, lymph node DC, unlike B cells, present very efficiently antigenic peptides derived from the processing of protein antigen endocytosed at the site of inflammation. These results indicate that DC and not B cells are the initiating APC in immune lymph nodes. IL-12 administration to nonobese diabetic mice results in acceleration of autoimmune diabetes, associated with massive infiltration of lymphoid cells, including N418⁺ cells, into the pancreas (34). Considering the efficiency of DC in antigen presentation and their capacity to produce IL-12 (35, and Guéry, J.-C., F. Ria, and L. Adorini, manuscript in preparation), it is possible that pancreatic N418⁺ cells play a role in the induction of autoreactive T cells. If this is the case, targeting DC may be an interesting approach to prevent autoimmunity.

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