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Letters to the Editor

Correspondence re R. Lapointe *et al.*, CD40-stimulated B Lymphocytes Pulsed with Tumor Antigens Are Effective Antigen-presenting Cells That Can Generate Specific T Cells. Cancer Res 2003;63:2836–43.

Letter

CD40-activated B cells (CD40-B cells) have been demonstrated to expand memory and prime naïve $CD8^+$ T cells in healthy individuals and cancer patients (1–6). These findings are of particular interest to the cancer vaccine field because CD40-B cells are reliably expandable from small amounts of peripheral blood without the technical limitations encountered using dendritic cells, especially in pediatric patients. A growing number of investigators have started to use CD40-B cells instead of, or in combination with, dendritic cells, and first clinical trials are under way (2, 3, 7–10). Nevertheless, it remains unclear whether B cells used as antigen-presenting cells might have significant biological limitations when used as cellular adjuvant. B cells take up and present antigen primarily and most efficiently via highly epitope-specific B-cell receptors (11, 12). Moreover, naïve B cells have been shown to induce $CD4^+$ T-cell tolerance (13). It, therefore, remained unclear whether *ex vivo*-generated polyclonal CD40-B cells, especially under long-term culture conditions, have the capacity to take up, process, and present a broad range of antigens to $CD4^+$ T-cells. This is of particular importance because $CD4^+$ T-cell responses are crucial for successful tumor rejection (14–16).

Lapointe *et al.* (17) addressed this important question in this journal and demonstrated that CD40-B cells generate tumor antigen-specific $CD4^+$ T cells in cancer patients when the B cells are pulsed with lysate from tumor cells (17). The authors conclude that these findings emphasize the role of CD40-B cells as antigen-presenting cells for immunotherapy. Melanoma antigen-specific T cells were generated from the peripheral blood mononuclear cells of two melanoma patients. Because it is most likely that these melanoma antigen-specific T cells derived from melanoma patients were expanded from a preexisting population of memory T cells, it remains to be shown whether CD40-B cells also prime naïve $CD4^+$ T cells against neoantigens as demonstrated for $CD8^+$ T-cells (2). This would also extend their potential use to neoantigens such as viral antigens or previously ignored potential tumor antigens. Most likely because of low antigen concentration in tumor lysates, Lapointe *et al.* reported a rather low efficacy of expansion of $CD4^+$ T cells after stimulation with CD40-B cells, (17), whereas antigen-specific $CD8^+$ T cell expansion was successful in more than 80% of individuals tested (2, 6). Here, we have addressed the induction of naïve $CD4^+$ T-cell responses and the efficacy of *ex vivo* $CD4^+$ T-cell expansion by stimulation with CD40-B cells.

The system used for $CD8^+$ T cells (1, 2) was modified to allow for optimal induction of antigen-specific $CD4^+$ T-cell responses to whole proteins (tetanus toxoid and keyhole limpet hemocyanin), and to the artificial promiscuous MHC class II binding peptides PADRE-AKF and PADRE-AKX, serving as peptide-neoantigens (18). T-cell expansion and cytokine production were assessed after 2–5 stimulations *in vitro* with protein- or peptide-loaded CD40-B cells in at least four T-cell lines against each of the antigens. T-cell responses against tetanus toxoid were induced in four of four T-cell lines. Similarly, two of four and three of four T-cell lines were antigen-specific to PADRE AKX and PADRE-AKF (Fig. 1, *A* and *B*). Analysis for IFN-γ or interleukin-4 production did not reveal any skewing toward a TH1 or TH2 profile. Successful generation of antigen-specific $CD4^+$ T cells in this optimized system was possible in 9 (75%) of 12 of T cell lines.

Antigen uptake by CD40-B cells was mainly due to fluid-phase uptake as demonstrated by Lucifer yellow incorporation and comparable with immature dendritic cells. As expected, mannose receptor-mediated antigen uptake (Dextran-FITC loading) was low compared with immature dendritic cells. (Fig. 1*C*). Even for molecules larger than Lucifer yellow, the MHC class II pathway of antigen uptake, processing and presentation is fully functional in CD40-B cells, as demonstrated by reduced T-cell activation by keyhole limpet hemocyanin and tetanus toxoid when using agents interfering with endocytosis (2-deoxyglucose, NaN_3), early and late lyso-/endosomal transport (chloroquine; bafilomycin A, data not shown), recycling (primaquine), trafficking of newly synthesized molecules (brefeldin A), loading (cathepsin inhibitor), and presentation by MHC class II molecules (MHC class II monoclonal antibody; Ref. 19; (Fig. 1*D*).

With this in mind, we extended the initial findings by Lapointe *et al.* (17) and clearly demonstrated that CD40-B cells efficiently process and present antigen not only to memory but also to naïve $CD4^+$ T cells and that it is possible to reliably expand antigen-specific T cells with CD40-B cells as antigen-presenting cells. These findings significantly widen the potential of CD40-B cells for immunotherapy.

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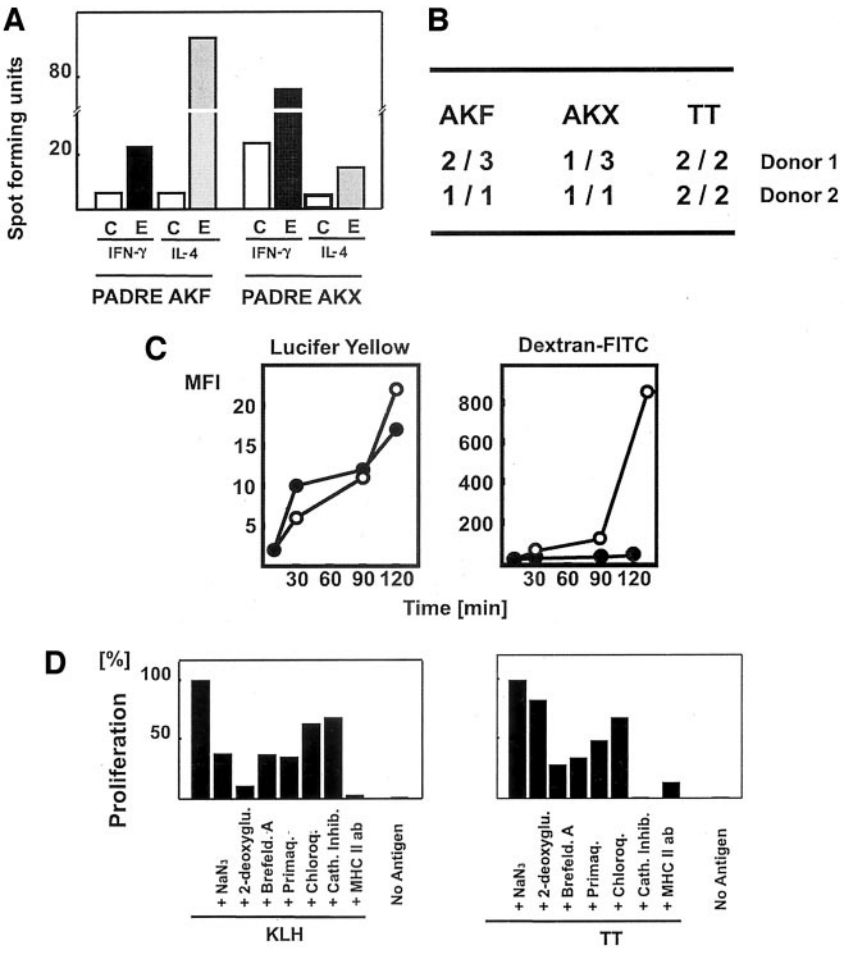

Fig. 1. Antigen processing and generation of antigen-specific $CD4^+$ T cells. *A*, priming of naive $CD4^+$ T cells. Secretion of IFN-γ (*IFN-*)and interleukin 4 (*IL-4*) by antigen-specific $CD4^+$ T cells on recognition of the peptides PADRE AKX or PADRE AKF). CD40-activated B cells (CD40-B cells) were pulsed overnight with irrelevant control (*C*) antigen or relevant antigen (*E*, experiment). Secretion of cytokines was measured by enzyme-linked immunospot assay (ELISPOT). The spot-forming units indicate the number of cytokine secreting $CD4^+$ T-cells out of 10^5 cells. *B*, expansion of antigen-specific $CD4^+$ T cells. The *table* shows the number of antigen-specific T cell lines out of total number of primary T cell lines stimulated with loaded-pulsed CD40-B cells; *TT*, tetanus toxoid. Antigen specificity was assessed after 2–5 restimulations. Antigen- reactivity was assessed by either antigen-specific proliferation or cytokine production (by ELISPOT analysis). If no antigen-specific proliferation or cytokine production (three SD over background) was detectable, T-cell lines were judged as nonspecific. *C*, antigen uptake. CD40-B cells (●) and monocyte-derived immature dendritic cells (○) were loaded with Lucifer yellow (*left panel*) or with Dextran-FITC (*right panel*) at 37°C, to exclude unspecific binding at 4°C (data not shown). Antigen uptake was quantified at various time points using flow cytometry; *MFI*, mean fluorescent intensity. (One representative experiment out of three.) *D*, antigen processing. CD40-B cells were loaded with keyhole limpet hemocyanin (*KLH*, *left panel*) or TT (*right panel*) for 18 h and cells were incubated with a KLH- or a TT-specific autologous T-cell line generated by weekly stimulations of purified $CD4^+$ T cells with antigen-pulsed CD40-B cells. Chemical compounds and antibodies were used to inhibit antigen processing at pivotal steps; *2-deoxyglu.*, deoxyglucose; *Brefeld. A*, brefeldin A; *Primaq.*, primaquine; *Chloroq.*, chloroquine; *Cath. Inhib.*, cathepsin inhibitor; *ab*, antibody. Proliferation (measured by thymidine incorporation; proliferation with TT or KLH alone set to 100%) above background (no antigen) is shown. (Two representative experiments out of five.)

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Reply

Antigen-specific B lymphocytes have the capacity to uptake antigens with surface antibody and present epitopes to specific $CD4^+$ T cells. The process involves cross-communication between B and T lymphocytes involving CD40L expression and cytokine secretion from T lymphocytes, which will then modify B-cell biology (1). This is central and critical in the development of humoral responses. Recently, CD40-activated B cells were exploited as a source of antigen-presenting cells in the *in vitro* generation of $CD8^+$ T lymphocytes specific to viral (2) and tumor antigens (3–5). Several strategies were exploited to load antigens in CD40-activated B cells, including peptide pulsing (3), RNA transfection (6), and retroviral transduction (2). Interestingly, CD40-activated B cells were found to be comparable with dendritic cells in their capacity to raise antigen-specific $CD8^+$ T cells (3, 6). In addition, it has been demonstrated that the culture of CD40-activated B cells is technically easier compared with that of dendritic cells, and requires only small numbers of peripheral blood mononuclear cells (3).

We have reported recently in this journal that CD40-activated B cells also have the capacity to present exogenous tumor antigens to $CD4^+$ T cells in an immunostimulatory fashion (7). We appreciate the positive comments and additional data provided by von Bergwelt-Baildon *et al.* (8) supporting our claims. Indeed, they previously succeeded in generating antigen-specific naïve and memory $CD8^+$ T cells against different antigens (5, 8). On several occasions, von Bergwelt-Baildon and collaborators generated specific T cells in a high proportion of individuals, using purified peptides or proteins as a source of antigen. This contrasts with our strategy of preparing a crude lysate from tumor cells. Although the use of lysates is advantageous in that multiple antigens, including undescribed antigens, may be represented, the concentration of any

Table 1 *Generation of gp100-specific T cells from melanoma patients*

Peripheral blood mononuclear cells from *HLA-DRβ1*0701*$^+$ melanoma patients were incubated with recombinant gp100 (rgp100) for 7 days, and CD40-activated B cells were prepared concomitantly. Cultured T cells were restimulated with rgp100-pulsed CD40-activated B cells, and 60 IU/ml of interleukin-2 (IL-2) were added every 3 days. T-cell specificity was evaluated using autologous CD40-activated B cells pulsed with different concentrations of a *DRβ1*0701* epitope from gp100 ($gp100_{170-190}$) or a control peptide ($Igk_{188-201}$), or with rgp100 or recombinant NY-ESO-1 (rNY-ESO-1). Alternatively, T cells were cocultured with melanoma cell lines engineered to express MHC class II after CIITA expression or treatment with IFN-γ (except for 836mel). T-cell reactivity was assessed by IFN-γ secretion ELISA (pg/ml). Patient 2 and 888mel share *HLA-DQβ1*06*. Bold and underline values are twice the background and higher than 100 pg/ml.

			Patient 1			
	DRβ1 0701*	Class II	T-cell line A	T-cell line B	Patient 2	Patient 3
Pulsed B cells						
Medium	+	+	210	576	213	24
$gp100_{170-190}$ 50 μM	+	+	**3,368**	781	179	**1,991**
$gp100_{170-190}$ 10 μM	+	+	**1,925**	581	174	**879**
$gp100_{170-190}$ 1 μM	+	+	**1,990**	526	289	**215**
$Igk_{188-201}$	+	+	207	364	144	19
rgp100	+	+	**7,425**	**2,530**	**2,115**	**>5,000**
rNY-ESO-1	+	+	182	388	27	55
Tumors						
1087mel	+	+	172	**453**	17	39
624.38mel	+	+	**1,329**	**6,275**	**135**	**>5,000**
pt1mel/gp100	+	+	**24,500**	**48,250**	24	**>5,000**
pt1mel/GFP	+	+	122	64	14	8
836mel	+	−	173	99	NA[a]	NA
888mel	−	+	155	17	**1,328**	NA
1102mel	−	+	NA	NA	45	**228**
1088mel	−	+	NA	NA	97	8

[a] NA, not available.

particular antigen is likely to be low, perhaps limiting the efficiency of T cell generation against a specific antigen compared with the use of purified proteins or peptides. In their Letter to the Editor, von Bergwelt-Baildon *et al.* (8) convincingly present data demonstrating the efficiency of CD40-activated B cells in the generation of naïve, specific CD4$^+$ T cells against several foreign antigens with purified proteins.

Many tumor antigens that have been described are "self" antigens and may be more challenging to use, compared with foreign antigens, to generate specific immune responses because of tolerance mechanisms. To explore the ability of CD40-activated B cells pulsed with purified proteins to generate immune responses to self antigens, we performed studies with recombinant gp100 protein, an antigen expressed on melanoma cells as well as normal melanocytes. With peripheral blood mononuclear cells and CD40-activated B cells prepared from five different *HLA-DRβ1*0701*$^+$ melanoma patients, several gp100-reactive T-cell lines were successfully generated from three patients (Table 1). Interestingly, T-cell lines generated from two of the patients were specific to an *HLA-DRβ1*0701* epitope ($gp100_{170-190}$) that we had identified previously (9). Also, T-cell lines potentially specific to new gp100 epitopes were raised (Table 1; patient 1 T-cell line B, and patient 2).

Interestingly, von Bergwelt-Baildon *et al.* demonstrated the ability of these activated B cells to take-up and process exogenous antigens. We suggested that the observed down-regulation of HLA-DO is involved in the efficient antigen presentation by activated B cells (7). This is in line with recent studies by different groups elegantly showing that germinal center B lymphocytes express very little HLA-DO as compared with naïve or memory populations (10–12). The assumption that DO down-regulation plays a role in our *in vitro* systems will need to be addressed directly and for each model antigen (13).

Considering all of the data we obtained thus far, and the additional evidence presented in the Letter to the Editor from von Bergwelt-Baildon *et al.* (8), we believe CD40-activated B cells have significant potential as a source of antigen-presenting cells for the generation of CD4$^+$ T cells specific to foreign or tumor antigens.

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