Collapse of the CD27+ B-Cell Compartment Associated with Systemic Plasmacytosis in Patients with Advanced Melanoma and Other Cancers

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Abstract

Purpose: Disturbed peripheral blood B-cell homeostasis complicates certain infections and autoimmune diseases, such as HIV and systemic lupus erythematosus, but has not been reported in cancer. This study aimed to investigate whether B-cell physiology was altered in the presence of melanoma and other cancers.

Experimental Design: Flow cytometry was used to identify phenotypic differences in B cells from patients with melanoma and normal donors. In vitro stimulated B cells were assessed for responsiveness and also used as stimulators of allogeneic T cells in mixed lymphocyte reactions.

Results: We show B-cell dysregulation in patients with advanced melanoma (n = 26) and other solid tumors (n = 13), marked by a relative and absolute loss of CD27+ (memory) B cells and associated with an aberrant systemic plasmacytosis. Functionally, B cells from patients with melanoma inefficiently up-regulated immunoregulatory molecules and weakly secreted cytokines in response to CD40 and toll-like receptor 9 agonists. Stimulated B cells from patients induced proliferation of alloreactive CD4+ T cells, but these T cells poorly secreted IFN-γ and interleukin-2. These effects were recapitulated by using purified normal donor CD27neg B cells in these same assays, linking the predominance of CD27neg B cells in patients with the observed functional hyporesponsiveness. Indeed, B-cell dysfunction in patients strongly correlated with the extent of loss of CD27+ B cells in peripheral blood.

Conclusions: Disturbed B-cell homeostasis is a previously unrecognized feature of patients with advanced melanoma and other cancers and may represent an unanticipated mechanism of immune incompetence in cancer.

In humans, aberrant circulating B-cell populations are an increasingly appreciated consequence of infectious and inflammatory disease, associated with B-cell dysfunctions that extend beyond antibody abnormalities (1, 2). For patients with HIV/AIDS, recent studies show reduced circulating CD27+ memory B cells and elevated CD10+ immature/transitional B cells and CD27bright plasmablasts. Moreover, B cells from HIV-viremic patients are hyporesponse to stimulation (3–6), suboptimally stimulate allogeneic T cells in vitro (7), and exhibit decreased survival (8). These alterations are reversed by antiretroviral therapy and reduction of viral load (9). Similarly, for patients with hepatitis C infection, CD27neg B cells are overrepresented among B cells in the peripheral blood (10). In patients with systemic lupus erythematosus, there are also altered numbers of CD27+ memory B cells and increased proportions of plasma cells in peripheral blood; naive and other B-cell subsets exhibit altered activation status and expression of costimulatory molecules (11, 12). In addition, both systemic lupus erythematosus and HIV patients exhibit high proportions of recently appreciated CD27neg memory B-cell subsets that are otherwise minimally represented in peripheral blood of normal donors (6, 13). Overall, B-cell dysfunction is increasingly considered an integral aspect of HIV/AIDS and autoimmune pathogenesis, with important implications for clinical treatments (1, 2).

In cancer patients, multiple dimensions of immune dysfunction, including paralyzed T cells and dysfunctional dendritic cells (DC), are well appreciated (14), but whether B-cell physiology is altered in cancer is poorly understood. Certainly, cancer cells and the tumor microenvironment engage B cells, as...
Translational Relevance

Multiple dimensions of immune dysfunction, including paralyzed T cells and dendritic cells, have frustrated efforts to develop successful cancer immunotherapies. Whether B cells are also dysfunctional in cancer is unknown. B cells interact with cancer cells in vivo, as evidenced by antitumor antibody production and B-cell infiltration of tumors, but increasing evidence suggests that tumor immunity is enhanced in the absence of B cells. Here, we show that B cells are dysregulated and functionally inferior in patients with advanced melanoma and other solid tumors. This dysregulation manifests as a collapse in the circulating CD27+ memory B-cell compartment, the hyporesponsiveness of B cells to activation, and the inability of B cells to activate T cells, deficits that worsen with the presence of active disease. Our findings, therefore, identify B-cell dysregulation as a novel potential mechanism of immune incompetence in cancer and an important therapeutic target in the development of effective cancer therapies.

Materials and Methods

Human peripheral blood and lymphocyte isolation. Peripheral blood was obtained after signed, informed consent from adult cancer patients (Supplementary Tables S1 and S2) and normal donors of comparable ages using protocols approved by the institutional review board of the hospital at University of Pennsylvania or Philadelphia Veterans Affairs Medical Center. Absolute lymphocyte count was obtained from a complete blood count and differential, as measured by an accredited clinical laboratory. Peripheral blood mononuclear cells were obtained by Ficoll centrifugation (Amersham Pharmacia Biotech). CD19+ B cells were isolated from peripheral blood mononuclear cells using MACS magnetic column and B-cell isolation kit II human (Miltenyi Biotec), and purity was >95%; importantly, contaminating DC were usually undetectable, as judged by expression of CD123 or CD11c and always <0.2% of cells in the isolated B-cell population. When noted, B cells were further purified into CD19+ CD27+ or CD19+ CD27−/hi subsets using CD27 microbeads (Miltenyi). Purified (>95%) CD4+ T cells were obtained using the CD4+ T-cell isolation kit human (Miltenyi) and labeled with 5 μmol/L CFSE (Molecular Probes) in PBS at a concentration of 10^7/mL.

Media and reagents. X-VIVO 15 (Lonza) supplemented with 10% heat-inactivated (56°C, 30 min) human AB serum, 2 mmol/L L-glutamine, 15 μg/mL gentamicin (Invitrogen Corp.), and 20 mmol/L HEPES (Invitrogen) was used. Lyophilized CpG oligonucleotide (ODN) 2006 and ODN 2006 control were obtained from InvivoGen and resuspended in endotoxin-free sterile water. Agonistic CD40 monoclonal antibody CP-870,893 (fully human IgG2c, clinical grade, endotoxin-free; ref. 29) was kindly provided by Pfizer Corp., and human IgG2c (hlgG2c; Chemicon International) was used as a negative control.

B-cell culture and activation. Total CD19+ B cells, CD19+ CD27+ B cells, or CD19+ CD27−/hi B cells were incubated in complex media in a 5% CO2 incubator at 37°C in 96-well round-bottomed plates at a concentration of 1 x 10^5 per 100 μL in the presence of either CP-870,893 (1 μg/mL) plus CpG ODN (1 μg/mL) or hlgG2 (1 μg/mL) plus ODN 2006 (1 μg/mL) negative controls. The optimal concentration of each stimulating reagent was determined by titration experiments using normal donor peripheral blood mononuclear cells (data not shown). After 48 h, undiluted culture supernatant was collected and preserved at -80°C until analysis for the presence of cytokines using BD Cytometric Bead Array Human Inflammatory Cytokine kit (BD Biosciences). Cells were then harvested and washed, and either surface stained or used as stimulators in mixed lymphocyte reaction (MLR) experiments.

Flow cytometry. Flow cytometry was done using a FACSCan cytometer and FASCDiva software (BD Biosciences). Cell surface molecule expression on unstimulated or 48 h stimulated B cells was done using isotype monoclonal antibody controls and monoclonal antibody obtained from AbD Serotec [CD40 (LOB 7/6), IgM (M 15/8)], R&D Systems [IL10Ra (37607), IL10Rb (90220), CCRI (150503)], and BD Biosciences [CD19 (HB19), CD14 (McP9), CD3 (SK7), CD86 (2331, FLN-1), CD95 (DX2), CD54 (HAS8), HLA-A,B,C (G4-2.6), HLA-DR (L243, G4-6), CD25 (M-A251), CD70 (Ki-24), CD10 (H10a), CD27 (M-T271), IgG (IgE-2), TACI (1A1-K21-M22), BR-3 (11C1), CD38 (HIT2), PD-1 (MH4), PD-L1 (MH1), CD11c (B-liy6), CD123(F5)]. The CD40 monoclonal antibody used for immunophe- notyping binds to an epitope distinct from the CD40- binding site and is not blocked by preincubation of B cells with CP-870,893 (data not shown).

MLR. Purified CD19+ B cells were activated with CP-870,893 (1 μg/mL) plus CpG ODN (1 μg/mL) plus hlgG2 (1 μg/mL) plus ODN 2006 control (1 μg/mL) for 48 h, harvested, washed, irradiated at 3000 rad, and replated at 1 x 10^5/100 μL in the presence of purified, allogeneic, CFSE-labeled CD4+ T cells at various B cell/T cell ratios. Five days later, culture supernatant was collected and preserved at -80°C until analysis for the presence of cytokines using Cytometric Bead Array Th1/Th2 Cytokine kit II (BD Biosciences). T-cell proliferation was evaluated by using flow cytometry to measure the proportion of CD4+ 7-amino-actinomycin D-negative CFSEhi cells on day 5.

Statistical analysis. Descriptive statistics were generated to examine the distribution of each variable, including mean, SD, minimum, and maximum. Student’s t test was used to compare the mean values of a variable between two blood donor groups. Student’s paired t test was used to compare the mean value of a variable measured on two distinct B-cell populations within the same blood donor (e.g., CD27+CD19− and CD27hi/CD19+ B cells from normal donors). ANOVA was used to compare the mean values of a variable among the three blood donor groups [e.g., normal donors (ND), no evidence of disease (NED) patients, AD patients]. Once a significant difference among the donor groups was established, pairwise post-hoc comparisons were done.
Disturbed Peripheral Blood B Cells in Cancer

Using Scheffe’s test at a $P < 0.05$ significance level, which controls for multiple paired testing. The magnitude of linear correlation between two variables was assessed by Pearson’s correlation coefficient. Before testing, the normality assumption was assessed for each variable using a normality test. If a variable was not considered to be normally distributed, then a natural log transformation was applied. The magnitude of linear correlation between two variables was assessed by Pearson’s correlation coefficient. Before testing, the normality assumption was assessed for each variable using a normality test. If a variable was not considered to be normally distributed, then a natural log transformation was applied. Statistics were calculated using STATA v10.0 software (StataCorp).

### Results

**Comparative analysis of circulating B cells from healthy donors and patients with melanoma.** To explore our hypothesis that cancer patients exhibit altered B-cell physiology, we analyzed peripheral blood CD19+ B cells from three groups: (a) normal donors ($n = 20$), (b) stage III or stage IV melanoma patients with AD ($n = 12$), and (c) stage IV melanoma patients with AD ($n = 12$). Patient characteristics are described in Supplementary Table S1. Using CD19 expression as a marker, we calculated the percentage and absolute count (cells/μL) of B cells among peripheral blood lymphocytes and then used multicolor flow cytometry to assess B-cell expression of 22 different cell surface molecules, including B-cell subset markers, activation markers, MHC molecules, costimulatory and inhibitory molecules, cytokine and survival signal receptors, adhesion and trafficking molecules, and surface immunoglobulin (Table 1). Endpoints were mean fluorescence intensity (MFI) for parameters for which B cells are uniformly positive (e.g., MHC class I) or percentage of positivity and absolute numbers (i.e., cell/μL for B-cell subset markers (e.g., CD27). We then compared the distributions of each parameter among the three groups of donors.

### Table 1. Comparative analysis of circulating B cells from healthy donors and patients with melanoma

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<th>Category</th>
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<th>NED versus AD</th>
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### Abbreviations:
- ND, normal donor
- ALC, absolute lymphocyte count
- NED, no evidence of disease
- AD, active disease

*Counts calculated by multiplying percentage positive by ALC.

1 CD27+ excludes CD27bright.
an average of 35% CD27+ CD19+ B cells in normal donors. We also found that the absolute number of CD27+ B cells in AD patients was significantly lower than the other groups and nearly undetectable in several patients (Fig. 1B), although AD patients did not have B-cell lymphopenia (Table 1). In contrast to normal donor peripheral blood B cells, for which the ratio of CD27neg B cells to CD27+ B cells was 2:1, CD27neg B cells outnumbered CD27+ B cells by ratios of 3.3:1 in NED patients and 10.1:1 in AD patients. These results suggest that patients with advanced melanoma experience a profound dysregulation of the circulating CD27+ B-cell compartment, most pronounced in the setting of active tumor burden.

**Systemic plasmacytosis in patients with advanced melanoma.**
Further inspection of the CD27 staining profile in patients with advanced melanoma revealed, in some cases, a distinct subset of CD27+ cells with very bright expression of CD27 nearly 1 log brighter in MFI than the standard CD27+ CD19+ B-cell population (Fig. 1A; patient AD211). Although differences in the percentages and absolute numbers of CD27bright CD19+ cells were just beyond the cutoff of statistical significance in our analyses (P = 0.021 and P = 0.084, respectively), a clear subgroup of both NED and particularly AD patients exhibited unusually high percentages and numbers of such cells (Fig. 1C). Given that high-level expression of CD27 on human B cells...
typically distinguishes plasmablasts from memory B cells (11),
we therefore explored the hypothesis that CD27bright CD19+
cells in melanoma patients represented an aberrant appearance
of circulating plasmablasts. When costained for other cell
surface markers, CD27bright CD19+ cells were positive for CD38
and CD19 expression was lower compared with other B cells
(Fig. 1D), consistent with a known plasmablast phenotype.
Moreover, these CD19dim cells expressed high levels of CD86
(Fig. 1D), and CD27bright CD38+ cells were also shifted higher
on both forward and side scatter profiles, suggesting a blast
phenotype (data not shown). Overall, these results show a
systemic plasmacytosis not previously observed in patients with
solid tumor malignancies.

**Phenotypic differences of circulating B cells from healthy
donors and patients.** Our analysis also revealed several other
phenotypic differences among normal donors and melanoma

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**Fig. 2.** Responses of B cells from normal donors and melanoma patients to stimulation with CD40 and TLR9 agonists. CD19+ B cells were purified from peripheral blood mononuclear cells by negative selection and then stimulated in the presence of either hIgG2 plus negative control ODN (neg control) or a combination of agonist anti-CD40 and CpG 2006 ODN (αCD40 + CpG). After 48 h, supernatant was collected for cytokine analysis and cells were stained for flow cytometry. **A,** poststimulation CD19+B cell expression of CD86, CD70, CD40, HLA-ABC, and HLA-DR. Each symbol represents an individual donor and bars represent means. ND, n = 13 or 14 donors; NED, n = 8 or 9 donors; AD, n = 7 or 8 donors. **B,** IL-6 and IL-10 secretion by CD19+B cells after stimulation. ND, n = 11 donors; NED, n = 9 or 10 donors; AD, n = 7 or 8 donors. *, P < 0.05; **, P < 0.01.
patients, which may reflect underlying alterations in CD27+ B-cell subsets. For example, the percentage of CD19+ cells expressing CD25 was significantly lower in AD patients compared with normal donors (Table 1), consistent with recent observations that circulating CD27+ memory B cells include a higher percentage of CD25+ cells than naive CD27neg B cells or CD27bright plasmablasts (35). We also observed a significantly higher expression of CD54 for AD patients compared with NED patients or normal donors (Table 1). In contrast, all other variables studied, including CD40, which critically regulates memory B-cell formation (36), were not significantly different among CD19+ cells from donors in the three groups (Table 1).

Altered CD27+ B-cell compartment in patients with other advanced cancers. To determine whether dysregulation of CD27+ B-cell subsets in the blood of cancer patients was specific for patients with advanced melanoma or a general feature of patients with advanced solid tumor malignancies, we examined the CD19+ B cells from patients with other advanced tumors. Tumor types were stage IV breast cancer (n = 8 patients, ages 36-70 years, who had either not received chemotherapy for metastatic disease or who had discontinued chemotherapy for at least 2 months), high-grade glioma (n = 3 patients with glioblastoma multiforme or anaplastic oligoastrocytoma, ages 36-55 years, who had been off chemotherapy for at least 6 months), and stage IV pancreatic cancer (n = 2, ages 49 and 57 years, chemotherapy naive; summarized in Supplementary Table S2). For each patient, similar abnormalities in the circulating B-cell compartment were observed, including low percentages of CD27+ B cells and, in some patients, increased CD27bright CD38+ CD19+ plasmablasts (Supplementary Fig. S1; Supplementary Table S3). Among all patients analyzed, the percentage CD27+ ranged from 0.9% to 20.7% with 5 of 13 patients having <5.0% CD27+ cells among total CD19+ B cells. The mean percentage of CD27+ for all patients was 8.7%, and this was significantly lower than the mean calculated for normal donors (n = 20; P < 0.001). Absolute counts of CD27+ B cells were also relatively low with a mean of 10/μL, ranging from 1 to 18/μL compared with a mean of 52/μL for normal donors (P < 0.001). As was also true for the melanoma patients, a distinct subgroup of patients had a high percentage and absolute number of CD27bright B cells, which also costained brightly for CD38 (Supplementary Fig. S1; Supplementary Table S3). Two of eight breast cancer patients, all three high-grade glioma patients, and one of two pancreatic adenocarcinoma patients had ≥2.0% CD27bright plasmablasts among CD19+ B cells. The mean percentage of CD27bright among patients (2.4%) was significantly higher than the mean...
Fig. 4. Functional comparison of CD27+ CD19+ versus CD27^-neg CD19+ B cells from normal donors. Isolated CD19+ normal donor B cells were further purified into CD27+ and CD27^-neg B cells and stimulated for 48 h with anti-CD40 and CpG 2006 ODN, as described in Fig. 2. After 48 h, supernatant was collected for cytokine analysis and cells were stained for flow cytometry or used as stimulators in allogeneic MLR.

A, poststimulation expression of CD86, CD70, CD40, HLA-ABC, and HLA-DR. Lines connect cells from the same normal donors (n = 11).

B, IL-6 and IL-10 secretion (n = 9).

C, T-cell proliferation (C) and cytokine secretion (D) by allogeneic normal donor CD4+ T cells after 5 d of incubation with CD40/TLR9-stimulated, irradiated allogeneic CD27+ versus CD27^-neg B cells (n = 8).

*, P < 0.03; **, P < 0.01.
for normal donors (0.5%; \( P = 0.006 \)), although the means of the absolute count of CD27\(^{bright} \) among CD19\(^+ \) B cells for patients compared with normal donors did not reach significance (\( P = 0.109 \)). The overall phenotype of B cells in these patients was nearly identical to that of melanoma patients with advanced disease, suggesting a common B-cell dysregulation associated with multiple forms of advanced human cancer.

**Hyporesponsiveness of patient CD19+ B cells to stimulation.** To understand the functional consequences of altered B-cell subsets in patients with advanced cancer, we compared the *in vitro* performance of CD19\(^+ \) B cells from melanoma patients and normal donors following combined stimulation with agonists of CD40 and TLR9, two critical and synergistic pathways for stimulation of human B cells (37, 38). To do this, purified CD19\(^+ \) B cells were cultured for 48 hours in the presence of the agonist anti-CD40 monoclonal antibody CP-870,893 plus the TLR9 ligand CpG 2006 ODN (versus human IgG2 plus ODN negative controls), and expression of CD86, CD70, CD40, and MHC class I and class II molecules was measured. CD40/TLR9 stimulation up-regulated each parameter on B cells isolated from each donor group (Fig. 2A). Although there was no significant difference in the extent of up-regulation of CD86 or MHC class I (HLA-ABC) among donor groups, up-regulation of CD70 was significantly inferior for AD patients compared with either normal donors or NED patients and up-regulation of HLA-DR was significantly inferior for AD or NED patients compared with normal donors (Fig. 2A). Likewise, CD40 up-regulation was significantly inferior for AD patients compared with normal donors.

We then examined cytokine production by purified B cells in response to *in vitro* stimulation with CD40/TLR9 (Fig. 2B). For interleukin-6 (IL-6) and IL-10, two key immunoregulatory B-cell cytokines, there was no detectable cytokine secretion after negative control stimulation in any group. CD40/TLR9 stimulation markedly induced both IL-6 and IL-10 secretion from normal donor B cells, although with substantial variations in effect, but significantly less so for B cells from NED and AD patients. Among the other cytokines evaluated in this assay [tumor necrosis factor \( \alpha \) (TNF\( \alpha \)), IL-1\( \beta \), IL-12p70], cytokine production was poor (<30 pg/mL) or undetectable in response to CD40/TLR9 stimulation and there were no significant differences detected among donor groups (data not shown).

**Patient B cells suboptimally activate normal donor allogeneic CD4+ T cells.** Similar to DC, B cells can function as professional antigen-presenting cells, but their capacity to do so is tightly linked to their activation status (39, 40). Given the hyporesponsiveness we observed for melanoma patient B cells to CD40/TLR9 stimulation, we examined whether B cells from melanoma patients are inferior antigen-presenting cells compared with normal donor B cells at inducing T-cell activation. To test this hypothesis, we used B cells isolated from normal donors as well as NED and AD patients in independent MLR, and measured proliferation and effector cytokine secretion by responder normal donor allogeneic T cells. Normal donor B cells stimulated with CD40/TLR9 agonists activated allogeneic CD4\(^+ \) T cells and did so more efficiently than control-treated B cells from the same donor (Fig. 3A and B). Enhanced proliferation of T cells cocultured with CD40/TLR9 stimulated B cells (versus control B cells) was also observed for AD or NED patients, with no statistically significant difference in the extent of T-cell proliferation in response to CD40/TLR9 stimulated B cells from the three donor groups (Fig. 3C).

To test whether the quality of the T-cell response triggered with CD40/TLR9 stimulated B cells from patients was comparable with that triggered by normal donor B cells, we measured CD4+ T-cell cytokine section by analyzing culture supernatant at MLR day 5. Although activated normal donor B cells induced the secretion of large amounts of IFN\( \gamma \) (mean \( \pm \) SD = 653 \( \pm \) 358 pg/mL) on average CD40/TLR9-stimulated B cells from NED patients induced less than half that amount (mean \( \pm \) SD = 246 \( \pm \) 114 pg/mL, \( P = 0.034 \); Fig. 3D). Moreover, stimulated AD patient B cells were almost completely ineffective as stimulators of T-cell IFN\( \gamma \) secretion, inducing less than one-tenth the amount as normal donor B cells (mean \( \pm \) SD = 60 \( \pm \) 26 pg/mL, \( P < 0.001 \)) and one-fourth the amount as B cells from NED patients (\( P = 0.003 \)). Similar findings were observed for T-cell IL-2 secretion, for which stimulated AD patient B cells were significantly inferior compared with normal donor (\( P = 0.022 \)) or NED patient B cells (\( P = 0.023 \); Fig. 3D). CD40/TLR9 stimulated B cells from AD patients also induced less than half of the TNF\( \alpha \) induced by stimulated normal donor B or NED B cells, but these differences did not reach statistical significance (Fig. 3D). Unstimulated B cells from AD patients were also inferior to unstimulated B cells from normal donors or NED patients with regard to IL-2 and TNF\( \alpha \) T-cell secretion. Taken together, these results suggest that the capacity of circulating patient B cells to stimulate T cells is compromised, and the severity of this deficit worsens with the presence of AD.

**CD27\(^{neg} \) B cells from normal donors are hyporesponsive to stimulation and suboptimally activate allogeneic CD4+ T cells.** To understand the mechanism responsible for patient B-cell hyporesponsiveness and inferior T-cell stimulation, we hypothesized that the CD27\(^{neg} \) CD19\(^+ \) population of B cells represents a B-cell subset inferior in this regard, whether isolated from normal donors or patients. This hypothesis predicts that the B-cell deficiencies we observed in patients would then be directly related to the abnormal predominance of this B-cell subset in patients and that the use of purified CD27\(^{neg} \) CD19\(^+ \) B cells from normal donors in our assays would recapitulate the deficits observed with unfractionated B cells from patients. CD19\(^+ \) CD27\(^+ \) and CD19\(^+ \) CD27\(^{neg} \) B-cell fractions were therefore purified from normal donors and tested in the same experimental systems described for Figs. 2 and 3. In response to CD40/TLR9 stimulation, both CD27\(^+ \) and CD27\(^{neg} \) B cells up-regulated CD86, CD70, CD40, and MHC class I and class II molecules; however, up-regulation of CD70, CD40, HLA-ABC, and HLA-DR was significantly inferior for CD27\(^{neg} \) B cells (Fig. 4A), a pattern very similar to the response of patient B cells to CD40/TLR agonists. In contrast, there was no significant difference in the up-regulation of CD86 between CD27\(^+ \) and CD27\(^{neg} \) B cells (Fig. 4A), again similar to the pattern for patient versus normal donor B cells in response to CD40/TLR agonists. Purified CD27\(^{neg} \) and CD27\(^+ \) normal donor subsets secreted similar amounts of IL-6 and IL-10 (Fig. 4B). This contrasts with the significant difference in cytokine secretion exhibited by melanoma patient versus normal donor B cells, suggesting that B cells in melanoma patients may have been subjected to an additional suppressive element *in vivo* not recapitulated by normal donor B cells in our *in vitro* stimulation system.
Finally, we determined whether CD27+ and CD27neg B cells from normal donors differentially induce T-cell activation and repeated the MLR assay using, as stimulators, purified normal donor CD27+ versus CD27neg B cells that had been incubated with the combined CD40/TLR9 agonists. Although CD27neg B cells were slightly inferior to CD27+ B cells at stimulating allogeneic T-cell proliferation (Fig. 4C; \( P = 0.026 \)), CD27neg B cells were markedly inferior to CD27+ B cells for the induction of T-cell secretion of IFN\( \gamma \) (top left), IL-2 (top right), and TNF\( \alpha \) (bottom left) in the 5-d allogeneic MLR, or to the percentage of CD70 expression at 48 h after CD40/TLR9 stimulation (bottom right). Open diamonds, NED patients; filled diamonds, AD patients. \( r \) indicates Pearson’s correlation coefficient.

**Correlation of CD27 positivity with patient B-cell function.** If, as our data suggest, CD27neg B cells—even from normal donors—are intrinsically hyporesponsive to stimulation and suboptimally activate T cells, then, in patients, the proportion of CD27+ B cells might predict performance in our in vitro assays. As shown in Fig. 5, we observed a strong positive relationship between secretion of T-cell IFN\( \gamma \) (\( r = 0.897, P = 0.0002 \)), IL-2 (\( r = 0.673, P = 0.023 \)), and TNF\( \alpha \) (\( r = 0.581, P = 0.061 \)) and the percentage of circulating B cells expressing CD27 used as stimulators in the MLR assay. In addition, there was also a positive relationship between the extent of CD70 up-regulation from CD40/TLR9 stimulation and the percentage of patient B cells expressing CD27 (\( r = 0.611, P = 0.016 \)). Other assay end points, such as B-cell cytokine production or up-regulation of CD86, CD40, MHC class I and class II, did not correlate with CD27 positivity (\( P > 0.05 \)). The results suggest that most, but not all, of the B-cell functional deficits we observed for patients are highly related to the predominance of the CD27neg CD19+ B-cell subset in these donors.

**Discussion**

Our findings show a previously unrecognized disturbed B-cell homeostasis in patients with advanced melanoma and other solid tumors, marked by a relative and absolute loss of CD27+ B cells in peripheral blood. Among 22 phenotypic variables evaluated on peripheral B cells from advanced melanoma patients, deficiency of CD27+ B cells was the most striking abnormality, particularly in patients with AD. Predominance of a CD27neg B-cell population was associated with systemic plasmacytosis, similar to elements of B-cell dysregulation observed in HIV/AIDS and systemic lupus erythematosus (1, 2). Patients with other solid tumors exhibited similar aberrations. Effects were not treatment related because patients undergoing therapy were excluded from the study. In functional studies, B cells from patients with advanced melanoma were hyporesponsive to CD40 and TLR9 stimulation. Moreover, T cells stimulated with CD40/TLR9-activated B cells from patients poorly secreted effector cytokines compared with T cells stimulated by activated normal donor B cells. Altered B-cell function in patients strongly correlated with diminished populations of CD27+ B cells and isolated CD27neg B cells from normal donors largely recapitulated these deficits.

B-cell dysregulation was most pronounced in patients with active tumor burden, many of whom exhibited <10% CD27+ CD19+ B cells and <10 such cells per microliter in peripheral blood. In humans, CD27 is a well-characterized marker for memory B cells, which are distinguished by mutated immunoglobulin antigen receptors and a heightened potential for mediating anamnestic antibody responses. Classically, CD27+ memory B cells have been thought to arise from germinal center reactions and involve immunoglobulin class switching and hypermutation (31). Recent data show that a subset of CD27+ B cells in blood is unswitched, retains expression of both IgM and IgD, and likely arises from the splenic marginal zone. Such B cells, nevertheless, show somatic immunoglobulin hypermutation, contribute to immunologic memory, and respond to encapsulated bacteria (34). If the loss of circulating CD27+ B cells in cancer patients (whether classic memory or marginal zone B cells) is not otherwise compensated in tissues, the physiologic effect of this dysregulation on adaptive immunity may be significant and represent a novel mechanism of tumor-related immune subversion. However, whether or not the tumor or its microenvironment directly induces this memory B-cell deficit—and how it might occur (death, differentiation to plasma cells, altered migration)—remains
unclear. IL-10 is well characterized to promote B-cell differentiation (41) and is also highly secreted by melanoma tumor cells (42). High serum IL-10 levels predict worse clinical course of melanoma (43). Although we found elevated serum IL-10 levels in some patients with AD (>10 pg/mL), most patients had low IL-10 levels and nevertheless exhibited profound B-cell deficits. Moreover, patients with other malignancies, such as breast, pancreatic, and brain tumors, exhibited similar aberrations, suggesting a common pathway in cancer not likely explained by serum IL-10 levels alone. Although chemotherapy may cause lymphopenia, the possibility that treatment effects confound our observations is unlikely because patients undergoing active therapy were excluded from the study. In fact, median time since systemic therapy for NED patients was 29 months for those having received such therapy (28% of patients had not received systemic therapy). Median time since systemic therapy for AD patients was 4 months. Importantly, each of the two AD patients, who had never received prior therapy (phlebotomy occurring at the time of initial presentation), had markedly aberrant B-cell subsets. Indeed, in preliminary analyses, we found no differences between patients with or without a history of prior systemic therapy for any significant variable in Table 1 (P > 0.40; data not shown).

In the wake of CD27− B-cell deficiency in patients, CD27− B cells predominate, outnumbering CD27+ B cells in AD patients by an average of >10:1. In humans, at steady-state, CD27− B cells are a heterogeneous population and include naive B cells, immature/transitional B cells, “anergic” B cells, and other distinct (yet rare) types of memory B cells (6, 13, 44–46). Immature/transitional B cells also express CD10 but represented only ~5% of CD19+ B cells in our study and did not vary between the normal donors and patients (Table 1). CD27− anergic B cells, recently identified as IgD− IgM− (46), represented at most 1% of CD19+ B cells in our study and also did not vary between normal donors and patients (data not shown). Recently identified CD27− CD21− tissue-like memory B cells are also rare in normal donor peripheral blood but expanded in HIV viremic patients (6). Although we observed CD27− CD21+ CD19+ cells exceeding 10% of B cells in some AD melanoma patients (data not shown), additional analysis will be necessary to determine whether this is a functionally exhausted subset with memory characteristics, as has been shown for HIV patients. We, therefore, favor the interpretation that the predominate CD27− B-cell population in melanoma patients is largely composed of naive CD21+ B cells.

Finally, in light of our findings that cancer patients exhibit disturbed B-cell homeostasis and function, it is important to consider the potential consequences of these aberrations on immune competence. Although one possibility, as noted above, is a deleterious effect on humoral immunity with the loss of circulating CD27+ memory B cells in patients, antibodies against melanocyte differentiation antigens can be detected in melanoma patients (15). B cells also function as important and professional antigen-presenting cells in the regulation of T-cell immunity, and similar to DC, their activation state critically regulates the quality and effectiveness of the immune response (2, 39, 47, 48). On one hand, fully activated B cells autonomously trigger effective and productive T-cell responses; on the other, resting B cells may drive T-cell tolerance and anergy (39, 40, 48). Here, we show that CD27− B cells, which are aberrantly overrepresented in cancer patient blood, are hyporesponsive to stimulation and poorly stimulate T-cell responses in vitro. Further experiments will help understand if the dominance of this subset in cancer and its resistance to activation translates to inferior T-cell responses in vivo, including cancer-specific responses. This notion has precedence with regard to DC insofar as DC from melanoma patients seem hyporesponsive, tolerogenic, and poorly stimulate T cells (49).

In summary, we show a novel B-cell dysregulation in patients with advanced melanoma and other solid tumors that is linked to a collapse in the circulating CD27+ memory B-cell compartment. Response to activation signals and the capacity of circulating patient B cells to stimulate T cells is compromised, and the severity of these deficits worsens with the presence of AD. Dysfunctional B cells may, therefore, represent an unanticipated mechanism of immune incompetence in cancer.

Reference:


Collapse of the CD27+ B-Cell Compartment Associated with Systemic Plasmacytosis in Patients with Advanced Melanoma and Other Cancers

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