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# Activation of complement by monoclonal antibodies that target cell-associated $\beta_2$-microglobulin: Implications for cancer immunotherapy

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ABSTRACT

$\beta_2$-Microglobulin (β2M), the light chain of the class I major histocompatibilty complex (MHC-I), is a promising tumor target for monoclonal antibodies (mAbs) in cancer immunotherapy. Several reports indicate that chelation of cell-associated β2M by specific mouse mAbs promotes tumor cell destruction by inducing apoptosis or other cytotoxic signaling pathways. Human mAbs employed in cancer therapy are usually IgG1, which mediates cell-killing by effector mechanisms including complement dependent cytotoxicity (CDC). The analogous mouse IgG2a and IgG2b isotypes are similarly effective in activating complement. Therefore, we examined the complement-activating properties of anti-β2M mouse mAbs 1B749 (IgG2a) and HB28 (IgG2b) when either mAb was bound to tumor cell lines or normal cells; we compared these β2M-specific mAbs with mouse mAb W6/32 (IgG2a), specific for human leukocyte antigens in the MHC-I heavy chain. All three mAbs bind to most human cell lines and normal cells in approximately equal amounts, consistent with a 1:1 stoichiometry for the HLA heavy chain in association with β2M. The three mAbs promote rapid C3b deposition and substantial CDC of human cell lines, and mAbs 1B749 and W6/32 have robust cytotoxic activity on reaction with normal mononuclear cells and platelets. Curiously, mAb HB28 induces modest C3b deposition and little CDC of normal cells, and its weaker complement-fixing activity was confirmed by ELISA. Based on these findings, we suggest that human IgG mAbs that target β2M for cancer immunotherapy be selected or engineered so as not to activate complement, thus eliminating the potential adverse effects of complement-mediated lysis of normal cells.



## 1. Introduction

There is considerable interest in using mAbs to target $\beta_2$-microglobulin (β2M) in the immunotherapy of cancer (Cao et al., 2011; Huang et al., 2010; Josson et al., 2011; Yang et al., 2006, 2007, 2009; Yang and Yi, 2010). This protein is non-covalently associated with the HLA heavy chains of the MHC class I (Natarajan et al., 1999), and is up-regulated on tumor cells, thus making it an attractive potential target in several forms of cancer including multiple myeloma and prostate cancer. Results to date have demonstrated that several anti-β2M mAbs can directly kill tumor cells by inducing cell death by mechanisms that include blocking the action of key signaling pathways mediated by mitogen-activated protein kinases and by the sterol regulatory element binding protein-1 (Huang et al., 2010; Josson et al., 2011; Yang et al., 2006, 2007, 2009; Yang and Yi, 2010). These mechanisms do not require immunologic effector functions such as complement activation or antibody-dependent cell-mediated cytotoxicity (ADCC). Moreover, in vitro experiments and results in mouse models have suggested that anti-β2M mAbs have little cytotoxic activity against normal human cells (Cao et al., 2011; Yang et al., 2007, 2009).

The selection of the isotype and subclass of a tumor-specific mAb that will be used for the immunotherapy of cancer is influenced by numerous factors, but most mAbs in clinical use are human IgG1 (Carter, 2006; Overdijk et al., 2012; Ruuls et al., 2008; Taylor and Lindorfer, 2008; Weiner, 2010). This subclass of IgG can recruit effector functions to kill opsonized targeted cells: it activates complement, thus mediating C3b deposition and complement dependent cytotoxicity (CDC), and it can also interact with and cross-link Fc receptors on NK cells and monocyte/macrophages, thereby promoting ADCC and phagocytosis (Bowles et al., 2006; Golay and Introna, 2012; Lindorfer et al., 2012; Minard-Colin et al., 2008; Overdijk et al., 2012; Nimmerjahn and Ravetch, 2007; Weiner, 2010). To our knowledge, the questions of CDC, ADCC or

*Abbreviations:* Al, Alexa; APC, allophycocyanin; β2M, $\beta_2$-microglobulin; CDC, complement dependent cytotoxicity; HBMT, Hepes buffered modified Tyrode's; HI, heat-inactivated; mAb, monoclonal antibody; MESF, molecules of equivalent soluble fluorochrome; RT, room temperature; NHS, normal human serum; NHS-EDTA, 50% NHS supplemented with 10 mM EDTA; SSC, side scattering.

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phagocytosis induced by mAbs specific for β2M have not been addressed, and in this report we have focused on a critical examination of the ability of mAbs specific for β2M to activate complement upon binding to tumor cell lines or to normal cells.

One of the anti-β2M mAbs that is being investigated, mAb HB28, is mouse IgG2b isotype (Brodsky et al., 1979). In analogy to human IgG1, this mouse isotype, as well as mouse IgG2a, is capable of activating human complement and has the potential to kill tumor cells by CDC (Brodsky et al., 1979; Dangl et al., 1988; Klaus et al., 1979; Neuberger and Rajewsky, 1981; Oi et al., 1984; da Silveira et al., 2002). Therefore, in order to generate key and essential information for potential clinical applications, we have investigated, in the presence of normal human serum (NHS) as a source of complement, the binding and interaction of mAb HB28, as well as mouse IgG2a anti-β2M mAb 1B749, with several tumor cell lines as well as with normal cells found in the circulation, including PBMC, neutrophils, platelets, and erythrocytes.

In order to provide a more general immunologic context and make use of a well-established positive control for this work, we have conducted comprehensive parallel studies with mouse IgG2a mAb W6/32, specific for the HLA heavy chains A, B, and C (Blok et al., 1998; Clayton et al., 2003; Meli et al., 2012). We find that there is approximately equivalent binding of mAbs HB28, 1B749 and W6/32 to most types of cells, which is consistent with a 1:1 stoichiometry for the HLA heavy chain in association with β2M (Brodsky et al., 1979; Natarajan et al., 1999). All three mAbs promote C3b deposition on and CDC of tumor cells. However, IgG2a mAbs 1B749 and W6/32 are considerably more effective at activating complement and promoting tumor cell killing. Moreover, although C3b deposition and CDC on *normal cells* opsonized with mAbs 1B749 and W6/32 is substantial, the results for normal cells opsonized with mAb HB28 indicate a considerably lower level of complement-mediated cytotoxic activity. These findings suggest that if tumor cells were to be targeted with a complement-fixing human IgG1 anti-β2M mAb, there could be substantial C3b deposition and complement-mediated damage to normal cells as well. Therefore, selection of a human IgG anti-β2M mAb to be used in the immunotherapy of cancer would require a human IgG isotype that does not activate complement in order to prevent this potential adverse effect.

## 2. Materials and methods

### 2.1. Cell lines and primary normal cells

ARH77, Daudi, Raji and Ramos cells (B cell lines), Molt-4 and Jurkat cells (T cell lines), THP-1 cells (monocytic cell line), Z138 cells (mantle cell lymphoma line) and U266 cells (multiple myeloma cell line), were cultured in RPMI 1640 with 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate and 2 mM glutamine (Beum et al., 2006, 2008b, 2008c; Craig et al., 2002; Estrov et al., 1998; Kennedy et al., 2003, 2004). Blood was obtained from normal volunteers in protocols approved by the University of Virginia Institutional Review Board. Mononuclear cells were isolated with Leucosep tubes (Greiner Bio-One) according to the manufacturer's directions. The isolated PBMC were re-suspended in 5 ml PBS and the cell density and viability (always ≥95%) were determined. NHS consisted of pooled sera obtained from four healthy donors.

Platelets were isolated from whole blood anti-coagulated with 0.32% sodium citrate according to standard differential centrifugation procedures (Peerschkle et al., 1993; Peerschke et al., 2006). Briefly, collection tubes were centrifuged at 280 × *g* for 15 min at 22 °C, with no brake. The plasma layer was harvested and combined with an equal volume of a buffer containing 140 mM NaCl, 2.7 mM KCl, 3.8 mM Hepes, 5 mM EGTA, pH 7.4 supplemented with 1 μM prostaglandin-1 (Sigma). This diluted platelet-rich plasma was centrifuged for 20 min at 1000 × *g* at 22 °C and the pellet was re-suspended in 134 mM NaCl, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM $Na_2HPO_4$, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4 (Hepes buffered modified Tyrode's, HBMT) (Peerschkle et al., 1993; Peerschke et al., 2006) with 2 g/l BSA to a platelet concentration of $2 \times 10^7$ platelets/ml.

### 2.2. Antibodies and reagents

The mAbs 7C12 and 3E7 (both specific for C3b/iC3b) and mAb 1H8 (specific for C3b/iC3b/C3d) have been described (Kennedy et al., 2003; Lindorfer et al., 2003). mAb W6/32 (specific for HLA-ABC) (Barnstable et al., 1978) and mAb HB28 (BBM.1, specific for β2M) were purified from hybridomas, obtained from ATCC, by protein A/G chromatography (Chang et al., 1995). Anti-β2M mAb 1B749 was obtained from MyBioSource, and supplied at a concentration of 200 μg/ml. Because we did not have access to the hybridoma, it was not used in all assays; in particular, it was not labeled with FITC. Allophycocyanin (APC) CD41, PE CD41 and PerCP CD45 were obtained from Becton Dickinson BioSource. FITC goat anti-mouse IgG (F-8771) was obtained from Sigma. MAbs were labeled with FITC (Sigma) using standard procedures or with Alexa (Al) 488 or Al 594 (Invitrogen) according to the manufacturer's directions. TO-PRO-3 and calcein AM were obtained from Invitrogen. We also purchased several additional anti-β2M mAbs from other companies, but in our hands they failed to bind to cell lines (not shown) that were chelated by mAbs HB28 and 1B479.

### 2.3. Quantitation of binding of mAbs to cells

For direct binding experiments, cells ($3.6 \times 10^6$ cells/ml) were incubated in RPMI 1640 medium containing 2 mg/ml mouse IgG (to block non-specific binding) and 10–30 μg/ml of FITC-labeled mAbs HB28 or W6/32 for 30 min at room temperature (RT) protected from light; the cells were then washed twice and analyzed by flow cytometry. Preliminary experiments demonstrated that for both mAbs, saturation of binding to all cells in media was obtained at concentrations of 10–20 μg/ml. In some experiments the FITC-labeled mAb was diluted with the identical unlabeled mAb in order to adjust fluorescent intensities for flow cytometry. In indirect binding experiments, cells were blocked with 2 mg/ml goat IgG, and then reacted with unlabeled mAbs HB28, 1B749 or mAb W6/32 (15 or 30 μg/ml), washed with PBS and probed secondarily with saturating amounts (50 or 100 μg/ml) of FITC labeled goat anti-mouse IgG (F-8771, Sigma) for 30 min at RT. The samples were washed and re-suspended in PBS. All flow cytometry was performed on a FACSCalibur flow cytometer (BD Biosciences). Mean fluorescence intensities were converted to molecules of equivalent soluble fluorochrome (MESF) using calibrated beads (Spherotech) (Kennedy et al., 2003).

In order to determine the effect of free β2M in NHS on mAb binding, binding of the mAbs (final concentrations between 66 and 150 μg/ml) to cells in either 33 or 50% NHS respectively supplemented with 10 mM EDTA (NHS-EDTA) was examined. In some binding experiments whole blood anti-coagulated with EDTA was washed 3 times with BSA/PBS, the pellet resuspended to 2/3 of the original volume and incubated for 30 min at RT with either FITC mAb HB28 or FITC mAb W6/32 along with PerCP CD45. Erythrocytes were lysed with FACSlyse (Becton Dickinson), and the remaining cells washed twice with PBS and analyzed by flow cytometry. PerCP CD45 positive neutrophils and PBMC were differentiated by side scattering (SSC) (Kennedy et al., 2003; Vikentiou et al., 2009; Williams et al., 2006).