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Review

Therapeutic intervention with complement and β-glucan in cancer

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Abstract

Complement (C) has two major effector systems available for host defense. The membrane attack complex (MAC) generated from components C5–C9 can form membrane-penetrating lesions that lead to cell death by causing a rapid loss of cytoplasmic components. The MAC is only effective against pathogens with outer phospholipid membranes, and cannot kill Gram-positive bacteria or yeast whose membranes are protected by cell walls. The most important effector mechanism of C is the opsonization of microbial pathogens with the serum protein C3 that leads to their high avidity attachment to the C3-receptors of phagocytic cells. Pathogens that activate complement are first coated with the C3b fragment of C3, which is rapidly proteolyzed into the iC3b fragment by serum factor I. These iC3b fragments serve to promote the high avidity attachment of the 'iC3b-opsonized' pathogens to the iC3b-receptors (CR3, CD11b/CD18) of phagocytic cells and natural killer (NK) cells, stimulating phagocytosis and/or cytotoxic degranulation. Host cells, including neoplastic tumor cells, have been endowed with natural mechanisms for self-protection against both the MAC and the cytotoxic activation of CR3. This review discusses a novel type of immunotherapy for cancer that uses soluble yeast β-glucan to override the normal resistance of iC3b-opsonized tumor cells to the cytotoxic activation of phagocyte and NK cell CR3, allowing this important effector mechanism of the C system to function against tumor cells in the same way that it normally functions against bacteria and yeast. Moreover, the cytotoxic activation of β-glucan-primed NK cell CR3 by iC3b-opsonized tumors is shown to be accompanied by a tumor-localized secretion of the cytokines TNFα, IFNα, IFNγ, and IL-6.

Keywords: Complement; Immunomodulators; Tumor immunity; Immunotherapy; Cytokines

1. Introduction

1.1. Tumor immunology and the immunotherapy of cancer

The field of tumor immunology has had a checkered history where periods of great enthusiasm were dashed by failures in the clinic. In the modern era, tumor Ags were dismissed as fetal Ags, and the hope of rIFNγ and rIL-2 was met with toxicity and mini-

Abbreviations: BRM, biological response modifier; C, complement; C3b and iC3b, fragments of C3, the third component of C; CR3, C-receptor type 3, also known as Mac-1, CD11b/CD18, or $\alpha_M\beta_2$-integrin; CTL, cytotoxic T lymphocyte; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule 1; LPS, lipopolysaccharide or endotoxin; MAC, membrane attack complex of complement; MIDAS, metal ion-dependent adhesion site; NK cell, natural killer cell; SCID, severe combined immunodeficiency

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mal success. Some of these past problems can be ascribed to the complexity of the immune system, and the remaining, to a heterogeneity of the malignant process both among different patients and in the same patient at different stages of their disease. An increasing number of tumor-specific Ags have been identified and we now have a better understanding of Ag presentation and the pathways for generating humoral vs. cellular immunity. An increasing awareness has occurred that the immune destruction of tumors requires a combination of effector mechanisms, and that a single vaccine, cytokine, or biological response modifier (BRM) is unlikely to be successful in the majority of patients. For example, vaccines may elicit immune cytotoxic T lymphocyte (CTL) cells and/or humoral Ab responses and yet both have shortcomings. Antibodies are frequently ineffective because normal host cell proteins (DAF, MCP, and CD59) inhibit complement (C)-mediated cytotoxicity (Kojima et al., 1993; Varsano et al., 1998; Venneker et al., 1998), and iC3b-opsonization of tumors does not recruit phagocytes and natural killer (NK) cells. Antibody-dependent cell-mediated immunity (ADCC) is thought to fail because the IgG density achieved on tumors is too low and FcγRIII-mediated cytotoxicity is suppressed by NK cell recognition of tumor cell MHC class I (Binstadt et al., 1996). In recent years, it was widely believed that cellular immunity could succeed where humoral immunity had failed. However, the identification of peptide epitopes that can be presented by all types of HLA molecules has proved a daunting task, and this effort could be futile if most tumors lose HLA class I as part of the metastatic process (Cordon-Cardo et al., 1991; Garrido et al., 1993; Esteban et al., 1996). A good CTL response could even function to select for class I-negative tumor cells (Khanna, 1998). A recent study of metastatic mammary carcinoma and melanoma reported that tumors from $>70\%$ of patients no longer expressed class I, and therefore, a CTL-targeted vaccine was doomed to fail in patients with breast cancer or melanoma (Porgador et al., 1997). As this study proposed, NK cells may be particularly effective against tumors that lose class I because NK cell cytotoxicity is suppressed by recognition of class I (Lanier, 1998; López-Botet et al., 1998). However, rIL-2 therapy that produces activated NK cells (i.e., LAK cells) does not specifically target the NK cells to tumors and has several toxic side effects (Kammula et al., 1998; Whiteside et al., 1998).

1.2. Structure and cytotoxic function of CR3

The iC3b-receptor, CR3, known also as Mac-1 or $\alpha_M\beta_2$-integrin, has two major functions. As the Mac-1 adhesion molecule, it mediates the diapedesis of leukocytes through the endothelium via generation of a high-affinity binding site for ICAM-1 (Springer, 1994; Hogg and Berlin, 1995; Sugimori et al., 1997). As CR3, it stimulates phagocytosis and degranulation in response to microorganisms or immune complexes opsonized with iC3b (Petty and Todd, 1993; Ross and Větvička, 1993; Sutterwala et al., 1996). For these functions, the Mac-1/CR3 molecule goes through a series of 'inside-out' and/or 'outside-in' signaling steps that result in exposure of high-affinity binding sites and/or an altered linkage to the actin cytoskeleton (Brown and Hogg, 1996; Newton et al., 1997). The nature of these activation and signaling pathways has not been completely defined, and it is particularly unknown whether activation for cytotoxic responses involves a similar pathway of events as the signaling for acquisition of the high-affinity ICAM-1 binding site. Our research has focused on the cytotoxic functions of CR3 whereas the majority of research by other investigators on Mac-1 has focused on mechanisms for development of its adhesion functions.

In 1987, it was shown that neutrophil CR3-dependent phagocytosis or degranulation in response to iC3b-opsonized yeast required ligation of two distinct binding sites in CR3, one for iC3b and a second site for β-glucan (Cain et al., 1987; Ross et al., 1987). Subsequent research mapped each of these binding sites to domains within the α-chain of CR3, CD11b (Fig. 1). All protein ligands of CR3, including iC3b, bind to overlapping sites contained within the I-domain of CD11b (Diamond et al., 1993, 1995; Ueda et al., 1994; Zhou et al., 1994; Zhang and Plow, 1996; Balsam et al., 1998). Using flow cytometry with fluorescein isothiocyanate (FITC)-labeled polysaccharides and CHO cells expressing recombinant chimeras between CD11b and CD11c, the lectin site was mapped to a region of CD11b located C-terminal to the I-domain (Thornton et al., 1996).

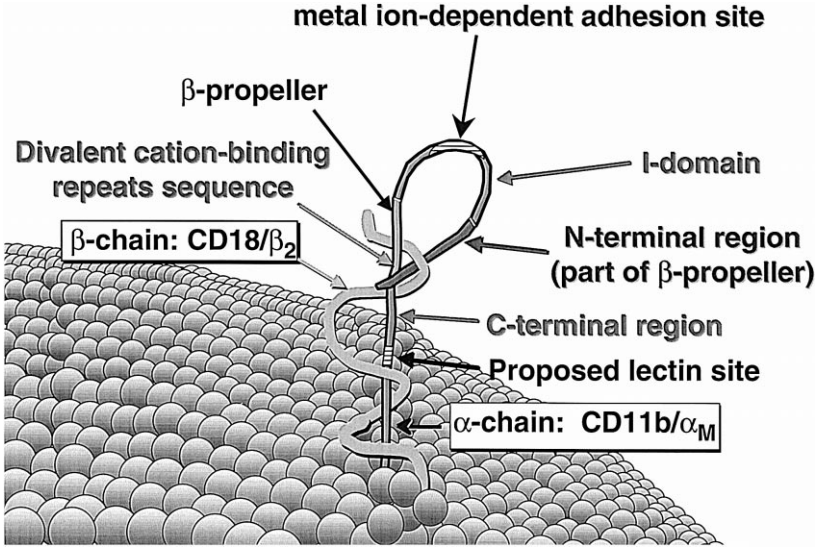

Fig. 1. Schematic representation of CR3 showing its intertwined two-chain structure and the major domains of CD11b. The exact location of the lectin site in the C-terminal domain is yet to be determined. A β-propeller domain loop has been proposed in a region of CR3 where the N-terminal region of CD11b attaches to the divalent cation-binding repeats sequence. The I-domain is inserted within the β-propeller and contains the 'metal ion-dependent adhesion site' or MIDAS for all known protein ligands of CR3.

The lectin domain functions to prime CR3 for cytotoxic responses (Větvička et al., 1996). C3-opsonized microorganisms present iC3b in combination with cell wall polysaccharides, such that both of these domains of CR3 become attached to microbial pathogens, stimulating phagocytosis and cytotoxic degranulation (Fig. 2) (Cain et al., 1987). NK cell CR3 functions in a similar manner as phagocyte CR3 in mediating cytotoxic degranulation in response to dual ligation of these two CR3 binding sites. This is the mechanism used by NK cells for CR3-dependent cytotoxicity of *Candida albicans* (Forsyth and Mathews, 1996). The lack of similar CR3-binding polysaccharides on human cells explains the inability of CR3 to mediate phagocytosis or extracellular cytotoxicity of erythrocytes or tumor cells opsonized with iC3b (Perlmann et al., 1975; Newman and Johnston, 1979; Schreiber et al., 1982; Wright and Silverstein, 1982; Wright et al., 1983; Wright, 1985). Host cell membranes opsonized with iC3b engage only the I-domain of CD11b and not the lectin site. On the other hand, small soluble β(1–3)-glucan polysaccharides isolated from fungi can bind to the lectin site of CR3 with high affinity and prime the receptor for subsequent cytotoxic activation by iC3b-tumor cells that are otherwise inert in stimulating CR3-dependent cytotoxicity (Větvička et al., 1996, 1997). Polysaccharide priming of CR3 involves a Mg^{2+} and protein tyrosine kinase (PTK)-dependent conformational change in CD11b that exposes the activation epitope defined by mAb CBRM1/5, but not the high-affinity ICAM-1 reporter epitope defined by mAb 24 (Větvička et al., 1996). In addition to serving as a receptor for exogenous polysaccharides on microorganisms, the lectin site also functions to link endogenous neutrophil membrane glycoproteins to CR3. This linkage to CR3 occurs with a large family of neutrophil membrane glycoprotein receptors bearing a phosphatidylinositol glycolipid (PIG) anchor (e.g., CD14, CD16, CD59, and CD87), and the linkage to CR3 via the lectin site provides a mechanism for transmembrane signaling to receptors that have no transmembrane signaling ability of their own (nor any connection to the actin cytoskeleton for mediating particle ingestion or adhesion). For example, FcγRIIIB (CD16) binds to the lectin site of neighboring CR3, thereby acquiring through the lectin site of CR3 the ability to stimulate phagocytosis or degranulation (Zhou et al., 1993; Krauss et al., 1994; Poo et al., 1995). Like-

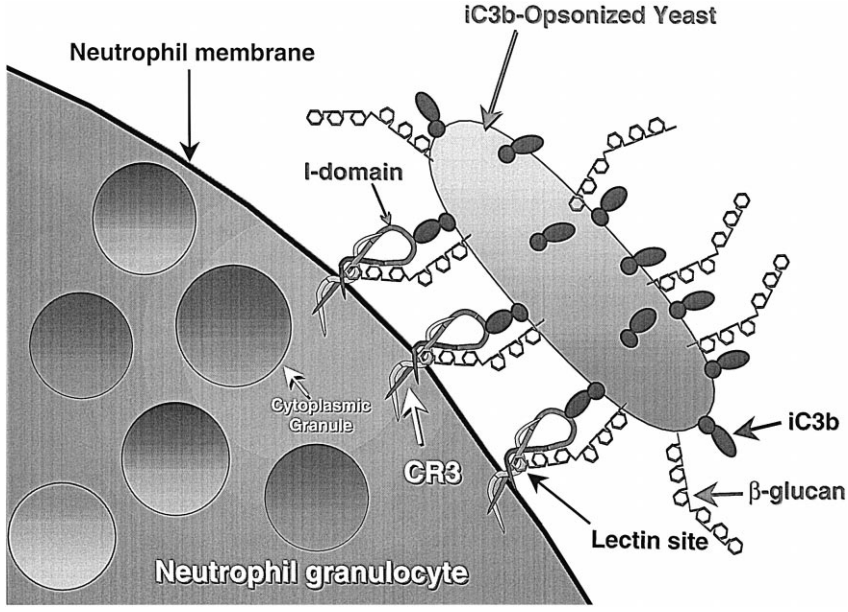

Fig. 2. Proposed mechanism for neutrophil CR3-dependent cytotoxic activation by iC3b-opsonized yeast. Based on functional data, both the lectin site and the I-domain MIDAS of CR3 must be bound simultaneously to yeast cell wall, β-glucan and iC3b, respectively, to stimulate phagocytosis, respiratory burst, and degranulation responses. With NK cells, a similar two-site stimulation of membrane CR3 results in cytotoxic degranulation and secretion of cytokines (e.g., TNFα and IFNγ, see Fig. 3).

wise, CD14 is able to mediate phagocytosis of *Escherichia coli* through attachment first to the bacteria via lipopolysaccharide (LPS), and then secondarily following CD14 attachment to the lectin site of CR3, the CD14/CR3 membrane complex is able to mediate the ingestion of *E. coli* (Zarewych et al., 1996; Ingalls et al., 1998). Recent studies have shown that CD59 and CD87 (uPAR) use the lectin site of CR3 to mediate adhesion rather than cytotoxicity. Not only is adhesion prevented or disrupted by oligosaccharides that compete with the lectin site (Gyetko et al., 1995; Sitrin et al., 1996; Cramer et al., 1998), but also CD87 knock-out mice are unable to generate the high-affinity ICAM-1 binding site in the I-domain of either CD11a or CD11b (May et al., 1998). Thus, the lectin site is not only essential for CR3-mediated cytotoxicity but also is apparently required for Mac-1-dependent adhesion. This finding, in combination with other data showing similar cell surface lectin-dependent complexes involving LFA-1 or CR4 (CD11c), suggests that homologous lectin sites may be present in the other CD11 family members and participate in similar lectin–carbohydrate complexes needed for transmembrane signaling functions (Petty and Todd, 1996; Todd and Petty, 1997).

Structural analyses of rCD11b I-domains using X-ray crystallography and mutagenesis have proposed a 'metal ion-dependent adhesion site' (MIDAS) with central Mg^{2+} whose structure allows key residues to be exposed and/or reoriented to provide binding sites of varying affinity for the protein ligands used for Mac-1 adhesion (Kamata et al., 1995; Lee et al., 1995a,b; Rieu et al., 1996; Zhang and Plow, 1997). The N-terminal domain folds back onto the divalent cation binding region, forming a loop termed as β-propeller (Fig. 1) (Lu et al., 1998). The functional contribution of regions outside the I-domain is only beginning to be explored. The C-terminal location of the lectin site of CD11b was recently confirmed in a study of rCR3 binding to *C. albicans* that suggested that ligation of *Candida* polysaccharides to the lectin site caused an increased affinity of a second binding site for *Candida* located in the I-domain (Forsyth et al., 1998). Our studies have suggested a site, located C-terminal to both the I-domain and the divalent-cation binding

repeats sequence, that became covered or hidden when mAbs were attached to distal sites in the I-domain (Thornton et al., 1996). A similar finding of lectin site blockade by a mAb to the I-domain was recently also made with mouse CR3 (Xia et al., 1999). Other studies with rCD11b expressed without CD18 by insect cells infected with recombinant baculoviruses showed that the binding to β-glucan-FITC or ^{125}I-β-glucan to rCD11b could be blocked by mAbs to the I-domain, as well as by mAbs to C-terminal domain epitopes (Xia and Ross, 1998). However, most importantly, rCD11b fragments from which the I-domain had been deleted retained lectin site activity, and this activity was blocked only by mAbs to C-terminal epitopes and not by mAbs to the I-domain. From these data, it was deduced that the lectin site was formed entirely by CD11b, and that lectin site exposure on CD11b did not require the CD11b/CD18 heterodimer.

These data suggest that occupation of the lectin site by a glycoprotein such as CD59 or CD87 can stimulate a change in the conformation of the distal I-domain (such as an increased affinity of the MIDAS for ICAM-1), and conversely, occupation of the I-domain by a mAb can change the conformation of the distal lectin site such that its binding site for soluble polysaccharides is no longer exposed. On the other hand, occupation of the lectin site by a soluble polysaccharide appears to inhibit complex formation with CD59 or CD87, thereby preventing development of the high-affinity binding site for ICAM-1, but priming CR3 for cytotoxic activation in response to ligation of the I-domain to an iC3b-opsonized target cell.

1.3. Therapeutic use of β-glucans

Biological response modifiers derived from microbial products have represented important tools for defining mechanisms of host defense. However, most BRMs have remained classified as non-specific because their exact mode of action was unknown. β-Glucan BRM were first reported 35 years ago and have been extensively investigated for both their anti-tumor and anti-infective activity. Most β-glucan BRMs are derived from yeast or fungi and have a backbone structure of linear β-1,3-linked D-glucose molecules (β-1,3-D-glucan) with β-1,6-linked side chains of β-1,3-D-glucan of varying sizes that occur at different intervals along the backbone (Bohn and BeMiller, 1995; Misaki and Kakuta, 1997). The frequency of the β-1,6-linked side chains, known as the degree of substitution or branching frequency, regulates secondary structure, solubility (Ohno et al., 1986; Maeda et al., 1988; Saito et al., 1991), and ultimately, the affinity of individual types of β-glucans for the lectin site of CR3 (Thornton et al., 1996; Ross et al., 1998a). However, our laboratory has reported the only studies that have related β-glucan receptor binding affinity to function in mediating leukocyte (neutrophil, monocyte, macrophage, NK cell) activation for tumoricidal activity or cytokine release. Over 500 papers during the past 30 years, predominantly in the Japanese pharmaceutical literature, have examined β-glucan structure only in relation to tumoricidal or bactericidal activity, and have not attempted to identify its target receptor as a way of defining optimal polysaccharide structure. These reports have shown that β-glucans, either soluble or particulate, and isolated from various natural sources, exhibit antitumor and antimicrobial activities in several animal species including mice (Diller et al., 1963; Chihara et al., 1969; Di Luzio et al., 1979; Williams et al., 1983; Ohno et al., 1984; Mimura et al., 1985; Seljelid, 1986; Kurachi et al., 1990; Kitamura et al., 1994; Sveinbjornsson et al., 1998). Some of the soluble fungal β-glucans have been applied clinically for tumor immunotherapy, such as lentinan, derived from an edible mushroom (Chihara et al., 1969), and schizophyllan (i.e., SSG or Sizofiran) isolated from the culture filtrate of *Schizophyllum commune* (Komatsu et al., 1969; Mansell et al., 1978; Nakao et al., 1983; Fujimoto et al., 1984; Wakui et al., 1986; Taguchi, 1987; Fujimoto, 1989; Chen and Hasumi, 1993; Tari et al., 1994; Nakano et al., 1996; Matsuoka et al., 1997). In vitro studies have shown that β-glucans activate macrophages, neutrophils, and NK cells to kill sensitive tumor cells (Cook et al., 1978).

Although somewhat controversial (Czop and Kay, 1991; Zimmerman et al., 1998), recent data suggest that CR3 serves as the major, if not the only receptor for β-glucans with human (Thornton et al., 1996) or mouse (Xia et al., 1999) leukocytes, and therefore, may be responsible for all reported functions of β-glucans in vitro and in vivo. Unlike other 'non-

specific' BRMs, β-glucan specifically targets macrophages, neutrophils, and NK cells to tumors that are opsonized with Ab and C3, and therefore, β-glucan has the same specificity as the tumor-opsonizing Ab. This research has particularly shown the therapeutic value in mice of small soluble β-glucans (5–20 kDa) that bind to CR3 with high affinity and prime the receptor for subsequent cytotoxic activation if, and only if, CR3 subsequently comes in contact with an iC3b-opsonized target cell. Particulate β-glucan and high molecular weight (m.w.) soluble β-glucans such as lentinan and schizophyllan (> 500 kDa) that have been used for patient therapy in Japan have been shown to be large enough to cross-link membrane CR3 of neutrophils and monocytes, triggering respiratory bursts, degranulation, and cytokine release in the absence of target cells (Ross et al., 1987; Doita et al., 1991; Ohno et al., 1993; Ross and Větvička, 1996; Větvička et al., 1996; Ljungman et al., 1998). Several studies have shown the safety of soluble β-glucans and the absence of undesirable side effects (Williams et al., 1988, 1991). The only problems reported have occurred with high m.w. soluble or particulate β-glucans (Maeda et al., 1996; Yoshioka et al., 1998). By comparison, Betafectin, a relatively low m.w. soluble β-glucan (~ 150 kDa), does not stimulate cytokine release (Bleicher and Mackin, 1995), probably because it is too small to cross-link membrane CR3.

The targets for β-glucan-primed CR3 appear to be any iC3b-opsonized host cell or microbial pathogen, and perhaps also tumor cells or parasites bearing endogenous ligands for CR3, although such CR3 ligands have only been detected on K562 cells (Větvička et al., 1996) and certain leishmania species (Russell and Wright, 1988). Tumors appear to be opsonized frequently with Ab and C3 as the result of an ineffective humoral response, and this could be enhanced therapeutically through either vaccines or mAbs to tumor Ags. Virus-infected cells or cells infected with intracellular bacteria also frequently activate C, either because they have become spontaneous activators of the alternative pathway or through Abs that activate the classical pathway of C. This common feature of target cell-bound iC3b appears to explain the wide range of diseases that respond to therapy with β-glucans. Thus, it proposed that resistance to β-glucan therapy corresponds to the absence of tumor cell- or microbe-bound iC3b, and that the success of β-glucan therapy can be enhanced by agents such as vaccines that enhance the target cell density of bound Ab and iC3b.

2. Mammary carcinoma as a target for β-glucan / CR3-mediated therapy

2.1. Research on human leukocytes and tumor cells in vitro

Previous reports had suggested that malignant cells frequently generated a humoral response that was ineffective in tumor destruction. Immunohistochemical staining of excised tumors for Ig and C3, as well as circulating tumor-reactive Abs, have been noted in patients with mammary carcinoma and cancers of the lung and colon (Irie et al., 1974; Seegal et al., 1976; Niculescu et al., 1992; Kotera et al., 1994). This natural humoral immune attack on tumors does not appear to prevent tumor growth, although it may reduce metastases. Our research focused on mammary carcinoma because of a relatively recent report that tumor cells from 48 patients bore Ab and C, including C3, that were detectable by immunohistochemical staining techniques (Niculescu et al., 1992). Using flow cytometry analysis of cell suspensions prepared from the mammary tumors of six patients, it was shown that the majority of malignant cells identified by double-staining for MUC1 tumor Ag also stained for IgM, IgG, and C3 (Větvička et al., 1997). Although tumors from all six patients contained malignant cells that stained for C3, a subset of the $MUC1^+$ malignant cells within individual tumors from some patients exhibited little or no C3 staining. Normal sera from AB^+ volunteers were shown to contain IgM and IgG Abs reactive with several breast tumor cell lines, but titers of the IgG Abs reactive with breast cancer cell lines were significantly increased in sera from patients with breast cancer. Breast tumor lines incubated in serum from AB^+ donors activated the classical, but not the alternative pathway of C, and became coated with C3. Despite exhibiting membrane-bound C3, serum-opsonized breast tumor lines were not killed by CR3-bearing NK cells. Priming of NK cell CR3 with

2.0 μg/ml of soluble yeast β-glucan enabled CR3-dependent killing of these same C3-bearing tumor cells. Cells from freshly excised mammary tumors also bore a sufficient C3 surface density for cytotoxic recognition by NK cells bearing polysaccharide-primed CR3, whereas they were largely resistant to NK cells bearing unprimed CR3. This study demonstrated the potential utility of naturally occurring opsonic C3 on tumor cells for specific immunotherapeutic targeting by NK cells and phagocytes bearing polysaccharide-primed CR3 (Větvička et al., 1997). However, the absence of detectable C3 on some tumor cells, as well as the resistance of some tumor cells to CR3-dependent cytotoxicity, suggest that complete remission may require combined therapy with vaccines or passively administered mAbs to tumor Ags that enhance the tumor cell surface density of C3.

The cytotoxic function of NK cell CR3 has also been shown to be suppressed by NK cell recognition of tumor cell MHC class I molecules (Větvička et al., 1999). This finding was anticipated because CR3 activation is sensitive to the tyrosine kinase inhibitors, genistein and Herbimycin A (Větvička et al., 1996), and the suppression of NK cell cytotoxicity that occurs following KIR and CD94/NKG2 recognition of MHC class I is known to involve recruitment of the tyrosine phosphatases SHP-1 and SHP-2 (Lanier, 1998). However, the absence of MHC class I from many metastatic tumors (Hicklin et al., 1998; Khanna, 1998) would allow NK cells with β-glucan-primed CR3 to be especially effective at a stage of disease when recognition by CTL is no longer possible (Porgador et al., 1997).

2.2. Stimulation of tumor-localized cytokine secretion by β-glucan priming of CR3 (CD11b/CD18) for cytotoxic recognition of tumor-restricted iC3b

The functions of NK cells in mediating host defense include both direct cytotoxicity of tumor cells and the secretion of cytokines such as TNFα and IFNγ that can potentially regulate immune responses and recruit tumoricidal macrophages. Although the direct cytotoxicity of tumors by NK cells had been shown to be mediated by the activation of CR3, additional studies were carried out to investigate whether this same CR3 activation event might also trigger cytokine secretion. In Fig. 3, the ability of particulate β-glucan vs. both high and low m.w. soluble β-glucans to stimulate NK cell cytokine release was compared. These data showed that NK cell secretion of cytokines occurred in parallel to CR3 activation for cytotoxicity. Particulate β-glucan, that triggers a vigorous CR3-dependent neutrophil superoxide burst (Ross et al., 1987), likewise triggered NK cell CR3-dependent release of TNFα, IFNα, and IL-6 (but not IFNγ). However, as found previously with the neutrophil respiratory burst and degranulation (Větvička et al., 1996), cytokine secretion did not occur with the initial CR3 priming step that occurs with the binding of small soluble β-glucans to CR3, and occurred only secondarily with the CR3 activation step triggered by cross-linking of the β-glucan-primed CR3 to an iC3b-opsonized target cell. For these studies, sheep erythrocytes opsonized with iC3b (EC3bi) were used as model iC3b-opsonized target cells. Incubation of NK cells with EC3bi in medium alone, that does not stimulate NK cell lysis of the EC3bi (Větvička et al., 1996), also did not trigger cytokine secretion. However, when EC3bi was added after priming of NK cell CR3 with soluble (or particulate) β-glucan, then the secretion of TNFα, IFNα, IFNγ, and IL-6 was detected by enzyme-linked immunosorbent assay (ELISA). Such cytokine release was CR3-dependent because it was blocked when an anti-CD11b mAb was added at the same time as the target EC3bi. The large (> 500 kDa) soluble β-glucan, grifolan (Ohno et al., 1986), resembled particulate β-glucan in its ability to stimulate the secretion of TNFα and IL-6 in the absence of EC3bi, indicating that the grifolan molecule is large enough to cross-link and activate CR3 in the same way as particulate β-glucan. Other studies with FITC-labeled grifolan (not shown) indicated that neutrophil fluorescence staining was inhibited both by various anti-CD11b mAbs as well as by competing unlabeled soluble β-glucans from baker's yeast or seaweed (laminarin).

These data suggest a further explanation for the successful use of β-glucans in cancer immunotherapy. In addition to the cytotoxicity triggered when a β-glucan-primed NK cell enters a tumor opsonized with iC3b, the same localized cytotoxicity stimulated by the iC3b-opsonized tumor cells would be accom-

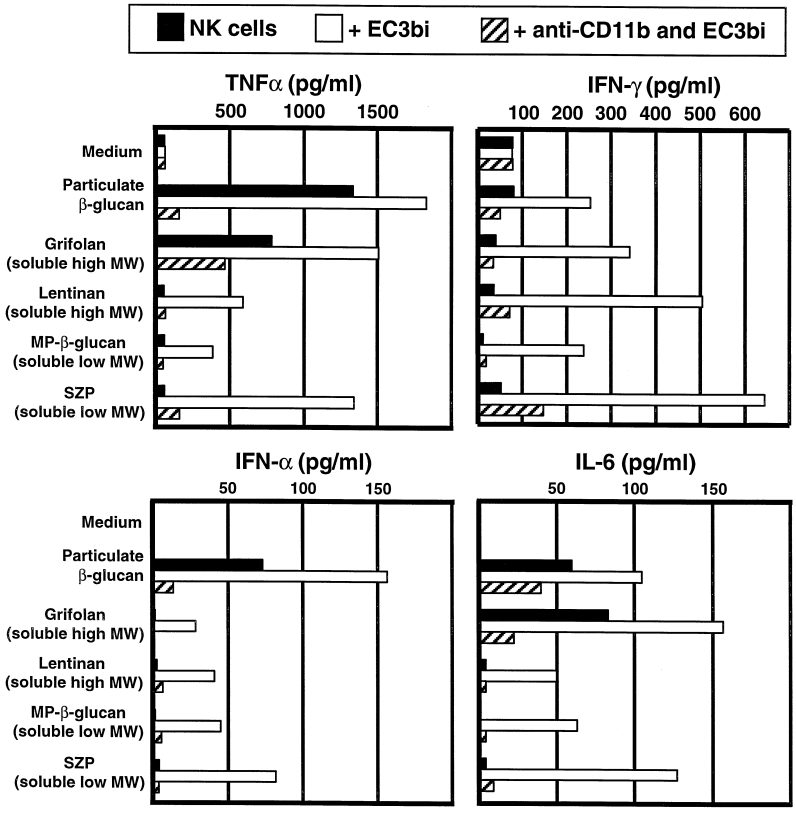

Fig. 3. CR3-dependent stimulation of NK cell cytokine secretion. Human blood NK cells, purified by positive selection with mAb-coated magnetic beads ($\geq$ 95% $CD56^+$), were cultured with either particulate yeast β-glucan (Sigma) or soluble CR3-binding polysaccharides for 18 h at 37°C. Harvested culture supernatants were analyzed for cytokines by ELISA using recombinant cytokines to establish protein concentrations. Particulate yeast β-glucan (2 μg/ml) and grifolan ($\geq$ 500 kDa soluble β-glucan from *Grifola frondosa*, 2 μg/ml, kindly provided by Dr. Naohito Ohno, Tokyo University of Pharmacy and Life Science, Japan) are able to bind and cross-link the lectin sites of surface CR3 molecules, causing cellular activation and the secretion of both TNFα and IL-6. By contrast, the small (20 kDa) soluble yeast β-glucan from Molecular Probes ('MP β-glucan;' 2.0 μg/ml) and SZP (soluble zymosan polysaccharide preparation containing β-oligomannan and/or β-glucan; 2.0 μg/ml) bind only to individual CR3 molecules and did not trigger cytokine release in the absence of target cells (black bars show cytokine secretion by polysaccharides in the absence of target cells). As with NK cell CR3-dependent cytotoxicity, binding of small β-glucans to CR3 resulted in receptor priming for subsequent cytokine release triggered by ligation to an iC3b-opsonized target cell (sheep erythrocytes opsonized with iC3b, 'EC3bi;' white bars). The EC3bi targets did not trigger NK cell cytokine release in the absence of such polysaccharide priming (white bars in medium control). After polysaccharide priming of CR3, ligation to an iC3b-target cell resulted in secretion of all four cytokines. Addition of 5 μg/ml of an anti-CD11b mAb (OKM1) blocked the secretion of all four cytokines from NK cells. Anti-CR3 blocks both β-glucan binding to CR3, as well as the binding of primed CR3 to iC3b on the EC3bi target cells (Thornton et al., 1996; Větvička et al., 1996).

panied by a local, rather than systemic, release of cytokines. This localized release of cytokines within tumors may be responsible for the known adjuvant effect of β-glucans in promoting recognition of cellular Ags by T cells (Dennert and Tucker, 1973; Hamuro et al., 1978; Suzuki et al., 1993). In addition to these studies with β-glucan-dependent cytokine secretion by NK cells, numerous studies in the literature have shown that similar triggering of macrophage and neutrophil CR3 with high m.w. or particulate β-glucans stimulates the secretion of IL-1 and IL-8 in addition to the TNFα and IL-6 shown here

(Rasmussen et al., 1987; Rankin et al., 1990; Abel and Czop, 1992; Au et al., 1994; Nemoto et al., 1994; Ljungman et al., 1998).

2.3. Development of mouse breast tumor immunotherapy models

In order to investigate the mechanisms and potential utility of β-glucan immunotherapy in vivo, it was necessary to develop a mouse model system. First, mouse leukocyte CR3 was shown to function as a receptor for soluble and particulate β-glucans in the same way as human CR3 (Xia et al., 1999). Soluble zymosan polysaccharide (SZP) or pure β-glucans, labeled with FITC or ^{125}I, bound in a saturable and reversible manner to murine neutrophils, macrophages, and NK cells. This lectin activity was blocked by the anti-CD11b mAbs M1/70 or 5C6, and did not occur with leukocytes from CR3$^{-/-}$ (CD11b-deficient; 'knock-out') mice (Xia et al., 1999). Preparations of soluble yeast polysaccharides containing primarily mannose or glucose bound to CR3, and the binding of ^{125}I-β-glucan to CR3 were competitively inhibited by β-glucans from barley or seaweed (laminarin), but not by yeast α-mannan. Also, as with human CR3, the lectin site of mouse CR3 was inhibited by α- or β-methyl-glucoside (but not D-glucose), α- or β-methyl-mannoside, and *N*-acetyl-D-glucosamine. Phagocytosis of zymosan and serum-opsonized zymosan was partially inhibited by anti-CR3 and was reduced to $<50\%$ of normal with leukocytes from CR3$^{-/-}$ mice. Zymosan phagocytosis by CR3$^{-/-}$ macrophages was mediated exclusively by mannose receptors because it was blocked by soluble α-mannan (but not by soluble β-glucan). As with neutrophils from patients with CD18 deficiency (LAD; leukocyte adhesion deficiency) (Ross et al., 1987), neutrophils from CR3$^{-/-}$ mice exhibited no phagocytosis of particles made up of pure β-glucan (Xia et al., 1999). As noted with human leukocytes, soluble β-glucan primed murine CR3 of neutrophils, macrophages, and NK cells for cytotoxicity of iC3b-opsonized tumor cells that otherwise did not trigger killing. β-Glucan priming for cytotoxicity was inhibited by anti-CR3 and did not occur with leukocytes from CR3$^{-/-}$ mice. The primed state of macrophage and NK cell CR3 remained detectable for up to 24 h after pulsing with β-glucans. The similarity of mouse and human CR3 in response to β-glucans confirmed the utility of mouse tumor models for investigation of β-glucans (Xia et al., 1999).

As found earlier with human mammary tumors (Větvička et al., 1997), flow cytometry of syngeneic tumors removed from BALB/c, 129/J, or C57BL/6 mice 3 to 4 weeks after implantation provided evidence for in situ opsonization with IgM, IgG, and C3 (Yan et al., 1999). Analysis of normal mouse sera demonstrated naturally occurring IgM and IgG Abs that bound to syngeneic tumors and activated the classical pathway of C. This was similar to the earlier finding that normal human sera contained IgM and IgG Abs capable of opsonizing human breast tumor cell lines (Větvička et al., 1997). Young mice (<5 weeks) had lower levels of serum Abs to breast tumor Ags, and tumors removed from young mice exhibited less staining for Ig and C3 than tumors removed from older mice (Yan et al., 1998; Yan et al., 1999). As expected, no antitumor Abs were detected in sera from severe combined immunodeficiency (SCID) BALB/c mice, and tumors removed from SCID mice showed no staining for IgM, IgG, or C3 (Ross et al., 1998b). Reconstitution of SCID mice with i.v. IgM or IgG purified from normal mouse sera caused in vivo opsonization of implanted tumors. To determine whether the amount of C3 on mouse breast tumors was sufficient for in vivo cytotoxic recognition by leukocytes with β-glucan-primed CR3, BALB/c mice with established syngeneic (H-2D^{d}) mammary tumors were given daily i.v. therapy for 2 weeks with 200 μg of soluble yeast β-glucan vs. control i.v. therapy with PBS. β-Glucan therapy resulted in a 70–95% reduction in tumor weight as compared to the PBS control group. The need for natural anti-tumor Abs in β-glucan tumor therapy was shown in SCID mice in which an absent therapeutic effect was reconstituted with IgM or IgG purified from normal mouse sera. However, β-glucan therapy resulted in less tumor reduction in these SCID mice than in normal mice, despite the administration of amounts of IgM or IgG sufficient to attain the same titers of tumor-reactive natural Abs as in normal mouse sera. The tumors removed from SCID mice reconstituted with IgM or IgG also bore less C3 than tumors removed from normal

mice, and the amount of tumor-bound C3 appeared to correspond to the amount of tumor reduction obtained with β-glucan therapy. A requirement for C3 on tumors was confirmed by the failure of β-glucan therapy to reduce tumor weight in C3-deficient mice. Finally, the function of leukocyte CR3 was highlighted by the failure of β-glucan to promote tumor destruction in CD11b-deficient mice (Ross et al., 1998b; Yan et al., 1999). These studies show the feasibility of using Abs and C for tumor therapy when combined with soluble polysaccharides that prime leukocyte CR3 to mediate cytotoxicity of iC3b-opsonized tumors in the same way as CR3 normally functions in the killing of iC3b-opsonized bacteria and yeast (Yan et al., 1999). Further studies of normal mice showed that the titer of IgG Ab reactive with tumor cells increased steadily after tumor implantation, so that there was 4-fold more IgG and C3 on tumors in normal mice than on tumors in SCID mice that had been reconstituted with normal serum levels of natural IgG. This finding may explain why there was less therapeutic effect of β-glucan in IgG-reconstituted SCID mice as compared to normal mice (Yan et al., 1999).

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