Cross-linking tumor cells with effector cells via CD55 with a bispecific mAb induces β-glucan-dependent CR3-dependent cellular cytotoxicity

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Complement (C) regulatory proteins decrease the effectiveness of immunotherapeutic anti-cancer antibodies. Bispecific mAb (bi-mAb) that target a tumor antigen and simultaneously inhibit a C regulator increase the effectiveness of such a therapy. Here we investigated the mechanism by which bi-mAb increase tumor cell lysis. Apart from C-dependent cytotoxicity, C activation can lead to complement receptor 3 (CR3)-dependent cellular cytotoxicity (CR3-DCC) by CR3-positive effector cells in the presence of β-glucan. Here we show that an anti-Ep-CAM*anti-CD55 bi-mAb induced more than threefold higher CR3-DCC (71%) of human colorectal cancer cells compared with anti-Ep-CAM alone (20%). This CR3-DCC was dependent on the binding of the anti-CD55 arm of tumor-bound anti-Ep-CAM*anti-CD55 bi-mAb to effector cell CD55, CR3 priming by β-glucan and the presence of iC3b on the target cell. Comparable lysis could be obtained in the absence of iC3b, when CR3 and CD55 were cross-linked on the effector cells, suggesting cooperation between CD55 and CR3 in signal transduction. Tumor cells with low antigen expression were effectively lysed via this mechanism in contrast to direct C-dependent cytotoxicity. These data imply that the effectiveness of mAb immunotherapy can be improved using anti-tumor antigen*anti-CD55 bi-mAb and β-glucan, thereby initiating CR3-DCC as an additional effector mechanism that is efficient for eradication of tumor cells with lower antigen expression.

Introduction

Several different effector mechanisms may be involved in the elimination of cancer cells that are opsonized with therapeutic mAb. When bound to tumor cells, mAb can initiate antibody-dependent cellular cytotoxicity (ADCC) and activate the complement (C) system. C activation can lead to formation of the membrane attack complex, inducing direct lysis of target cells (C-dependent cytotoxicity, CDC). In addition, iC3b generated at the tumor cell surface is a ligand for complement receptor 3 (CR3, CD11b/CD18, Mac-1) and CR3-iC3b interactions may either enhance ADCC [1, 2] or mediate CR3-dependent cellular cytotoxicity (CR3-DCC) by CR3-positive effector cells [3, 4]. CR3-enhanced ADCC is thought to result from the increased affinity between tumor and effector cell due to the additional interaction between CR3 and iC3b. To initiate CR3-DCC, CR3 needs to be in its high-affinity state for iC3b [5]. This state is...
established by binding of the microbial surface polysaccharides containing β-glucan to the C-terminal lectin domain of CD11b [6, 7]. To utilize this CR3-dependent mechanism for eradication of tumor cells, exogenously added soluble β-glucan can be used to prime CR3, resulting in an efficient cytotoxic degranulation response after it has also bound to deposited iC3b via the N-terminal I-domain of CD11b [3]. Soluble β-glucan has been shown to increase the cytotoxic potential of several CR3-positive effector cells in vitro [3, 7, 8]. In addition, oral administration of soluble β-glucan increased the efficacy of mAb immunotherapy in tumor-bearing mice [9–11].

CDC, C3b deposition, and the subsequent generation of iC3b, induced by immunotherapeutic mAb are hampered by over-expression of membrane-bound C-regulatory proteins (mCRP) CD46, CD55 and CD59 on tumor cells and therefore limit therapeutic efficacy of the different effector mechanisms [1, 12]. Previously, we have shown that bispecific mAb (bi-mAb) directed against Ep-CAM and CD55 (anti-Ep-CAM*anti-CD55) increase C3b deposition on colorectal cancer cells, due to functional blocking of CD55 [13]. Additionally, in a syngeneic rat colorectal cancer model, we have shown that bi-mAb that block a major mCRP (Crry) can prevent tumor outgrowth [14]. In the present study, we have followed up these studies and investigated the mechanism by which bi-mAb, directed against a tumor-associated antigen (Ep-CAM) and the major mCRP CD55, increase lysis of colorectal cancer cells compared to normal anti-tumor mAb.

However, even in the presence of anti-Ep-CAM*anti-CD55 bi-mAb and a blocking anti-CD59 mAb, no noteworthy CDC of CaCo-2 cells was measured. In the presence of blocking anti-CD59 mAb and anti-Ep-CAM*anti-CD55 bi-mAb lysis of SW837 was slightly further increased compared with anti-Ep-CAM*anti-CD55 bi-mAb alone (data not shown).

Next, we investigated the ability of bi-mAb to induce CR3-DCC. Effector cells were obtained from the Ficoll interface, consisting of lymphocytes (that do not express CR3), and monocytes/macrophages. The amount of CR3-DCC induced by anti-Ep-CAM*anti-CD55 was compared with that induced by both parental mAb (anti-CD55 mAb and anti-Ep-CAM mAb) or anti-Ep-CAM mAb alone. Anti-CD55 mAb (IgG1) alone did not induce C activation or ADCC (data not shown) [13]. For CR3-DCC and CR3-enhanced ADCC experiments, C6-depleted human serum was used to prevent CDC. High levels of CR3-DCC-mediated lysis of SW837 (71%) and CaCo-2 (53%) were observed after opsonization with anti-Ep-CAM*anti-CD55 (Fig. 1b). Significant lower levels of CR3-DCC were induced by anti-Ep-CAM mAb alone (SW837: 20%, p=0.001; CaCo-2: 9%, p=0.005). Surprisingly, the combination of both parental mAb also resulted in significantly lower levels of CR3-DCC as compared to bi-mAb (SW837: 32%, p=0.005; CaCo-2: 40%, p=0.049).

**Increased CR3-DCC by bi-mAb is not caused by increased amount of ADCC or iC3b deposition**

We investigated whether the increased bi-mAb-mediated CR3-DCC of SW837 and CaCo-2 was due to an increased number of bi-mAb binding to the target cells. Higher amounts of bi-mAb were shown to bind to tumor cells compared to both parental mAb or anti-Ep-CAM mAb alone (Table 1). Subsequently, we studied whether higher level of bi-mAb binding to target cells resulted in increased ADCC or in increased CR3-enhanced ADCC. In the absence of serum (ADCC; Fig. 1c), no significant differences in lysis induced by either bi-mAb, both parental mAb or anti-Ep-CAM mAb alone were observed. ADCC levels were low, but statistically significantly higher than control values without mAb (p=0.041). Addition of β-glucan had no effect on ADCC levels (data not shown).

To examine whether increased tumor cell lysis by CR3-DCC was due to increased levels of C activation, iC3b deposition on target cells was measured by flow cytometry. However, we found similar levels of iC3b deposition induced by opsonization with anti-Ep-CAM*anti-CD55 or a combination of both parental mAb (Fig. 2). Normal human serum (NHS) and C6-depleted serum resulted in similar amounts of iC3b deposition. The levels of C activation were two times

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**Results**

**Opsonization with anti-Ep-CAM*anti-CD55 bi-mAb increases CDC and CR3-DCC**

We investigated the ability of anti-Ep-CAM*anti-CD55 bi-mAb to induce CDC of colorectal cancer cell lines. Two colorectal cancer cell lines were used; SW837, expressing high levels of Ep-CAM and low levels of CD55, and CaCo-2, expressing low levels of Ep-CAM and high levels of CD55 (Table 1) [13].

First we determined the ability of bi-mAb to induce CDC via the membrane attack complex. It was observed that bi-mAb induced significantly higher levels of CDC of SW837 cells as compared to anti-Ep-CAM mAb alone (57% versus 38%; p=0.049) (Fig. 1a). No statistical significant difference between CDC induced by bi-mAb or a combination of both parental mAb was observed. In contrast, CaCo-2 cells were hardly lysed via CDC induced by either bi-mAb or parental mAb. To prevent inhibition of CDC by CD59, CDC was also studied after addition of anti-CD59 mAb in combination with bi-mAb.

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higher compared with those induced by anti-Ep-CAM mAb alone, due to functional inhibition of CD55. Although iC3b deposition on CaCo-2 was also increased when opsonized with bi-mAb or both parental mAb as compared to anti-Ep-CAM mAb alone, the iC3b levels were very low, most likely due to limited C activation as a result of low Ep-CAM expression [13]. Subsequently, we investigated whether this higher level of bi-mAb binding to target cells led to increased CR3-enhanced ADCC. Opsonization of target cells with bi-mAb or parental mAb in the presence of C6-depleted human serum, but without β-glucan, resulted in comparable levels of tumor cell lysis (Fig. 1d). These results suggest that neither higher amounts of bi-mAb binding nor an increase in iC3b deposition accounted for the observed CR3-DCC.

CD55 on effector cells is required for bi-mAb-mediated CR3-DCC

To search for an alternative explanation for the differences in CR3-DCC induced by bi-mAb or parental mAb, we investigated whether bi-mAb bound to tumor cells was able to simultaneously bind CD55 on effector cells, thereby increasing tumor cell lysis by CR3-DCC. When tumor cells express a high amount of Ep-CAM compared to CD55 (Table 1), a large number of free CD55 binding sites of tumor-bound bi-mAb would be available to bind CD55 on the effector cell. PBMC were treated with phosphatidylinositol-specific phospholipase C to remove CD55 and tumor cell lysis was measured by ADCC. The results showed that ADCC was decreased in the absence of CD55 on effector cells, indicating that CD55 is required for bi-mAb-mediated CR3-DCC.
Pase C (PI-PLC) to remove glycosylphosphatidylinositol (GPI)-linked CD55, or pre-incubated with anti-CD55 F(ab')₂ to prevent binding of tumor-bound bi-mAb to CD55. PI-PLC treatment reduced CD55 expression levels on the PBMC by 80% on average (Fig. 3). Both removal of CD55 and blocking CD55 with anti-CD55 F(ab')₂ fragments reduced CR3-DCC to CR3-enhanced ADCC levels (Fig. 4). This indicates that binding of bi-mAb to CD55 on PBMC is responsible for the increase in CR3-DCC. When both parental mAb are present, only a limited number of free CD55 binding sites of tumor cell-bound anti-CD55 mAb will be available, due to the relative low expression of CD55 on the tumor cells. These free CD55 binding sites can subsequently bind to CD55 on the PBMC, resulting in the modest increase in CR3-DCC (Fig. 1b). To investigate whether such binding of bi-mAb to PBMC CD55 alone could induce cytolysis or whether the interaction of CR3 was also required, CR3-DCC experiments were performed with PBMC pre-treated with anti-CR3 mAb (clone LMP19c) to block iC3b binding to CR3. The LMP19c mAb has previously been shown to block the interaction between iC3b and CR3 [5, 15]. Inhibition of CR3-iC3b interaction by this mAb resulted in a decrease of lysis to a level comparable with ADCC (Fig. 4), indicating that CR3-iC3b interaction was indeed also necessary for CR3-DCC.

Bi-mAb induces association between CD55 and CR3 on PBMC

Binding of tumor-bound bi-mAb to CD55 on effector cells might lead to CR3-DCC by either increased binding affinity between the tumor and effector cells, or by inducing activation signals inside the effector cell. To investigate which of these mechanisms occur during CR3-DCC, immobilization of CD55 and CR3 on the effector cells was mimicked by cross-linking these proteins with mAb. Subsequently, it was determined if under these conditions CR3-DCC could be induced with anti-Ep-CAM mAb or both parental mAb and iC3b were then exposed to these PBMC. The amount of ⁵¹Cr release from the tumor cells was determined as described in the Materials and methods and depicted on the y-axis. The mean and SD of three independent experiments are shown.

Figure 3. The effect of PI-PLC treatment of PBMC on CD55 and CR3 expression levels. PI-PLC-treated PBMC were measured for CD55 and CR3 expression by flow cytometry. PBMC were stained with anti-CD55 mAb or anti-CR3 mAb, and detected with FITC-conjugated GaM secondary mAb. The histograms show (a) CD55 and (b) CR3 expression after PBS (solid line) or after PI-PLC treatment (dark dashed line) as compared with secondary mAb staining alone (light dashed line; control). A representative experiment is shown. The table in (c) shows means and SD of four experiments, corrected for the control value.

Figure 4. CD55 and CR3 on PBMC are essential for CR3-DCC. PBMC were treated with PI-PLC (light gray bars), anti-CD55 F(ab')₂ fragments (dark gray bars), or anti-CD11b mAb (black bars). As a control, PBMC were incubated in PBS alone (white bars). SW837 cells opsonized with anti-Ep-CAM*anti-CD55 bi-mAb or both parental mAb and iC3b were then exposed to these PBMC. The amount of ⁵¹Cr release from the tumor cells was determined as described in the Materials and methods and depicted on the y-axis. The mean and SD of three independent experiments are shown.

Figure 5. Cross-linking CD55 and CR3 on PBMC induces enhanced CR3-DCC. PBMC were incubated with anti-CD11b, anti-CD55 F(ab')₂, or both, and were cross-linked with RaM IgG mAb. SW837 cells opsonized with anti-Ep-CAM mAb (white bars) or PBS (black bars) as a control were used as target cells. The amount of CR3-DCC was determined with a ⁵¹Cr-release assay. The amount of released ⁵¹Cr is depicted on the y-axis. The mean and SD of three independent experiments are shown.
amount of lysis was comparable to the CR3-DCC levels observed with bi-mAb (71%; Fig. 1a). Cross-linking CR3 alone did not result in increased levels of lysis, whereas cross-linking of CD55 resulted in intermediate levels of lysis (48%). This latter observation might be explained by direct activation of the effector cells by cross-linking CD55 [16]. These results indicate that the observed induced CR3-DCC by bi-mAb is due to increased activation by co-ligation of CD55 and CR3.

**Discussion**

In the present study, we have investigated the mechanisms by which anti-Ep-CAM*anti-CD55 bi-mAb increase lysis of tumor cells. Tumor cells expressing high levels of Ep-CAM, i.e., SW837, were efficiently lysed via CDC, while cells with lower expression, i.e., CaCo-2, were hardly killed. This might be due to high expression of CD59, a common feature of tumor cells [17, 18], which inhibits direct C-mediated lysis. CR3-enhanced ADCC and CR3-DCC are not inhibited by CD59. These mechanisms are therefore more promising for mediating tumor cell lysis in vivo than CDC. Here, we show that anti-Ep-CAM*anti-CD55 bi-mAb opsonized tumor cells indeed induced high levels of CR3-DCC as compared with tumor cells opsonized with a combination of the parental mAb. In contrast, opsonization of tumor cells with either mAb or bi-mAb resulted in low levels of ADCC and CR3-enhanced ADCC. The observation that CaCo-2 cells were efficiently lysed via CR3-DCC, but not via CDC, implies that tumor cells, even with low antigen levels and subsequent low levels of mAb binding and iC3b deposition, can be killed via CR3-DCC when opsonized with appropriate bi-mAb.

Bi-mAb-induced CR3-DCC appeared to be dependent on several conditions: (1) binding of a ligand (CR3-iC3b interaction); (2) priming of CR3 (β-glucan); and (3) binding of CD55 on effector cells. Firstly, CR3-DCC was shown to require CR3-iC3b interaction, since this effect was abolished when the interaction was blocked by an inhibiting anti-CR3 mAb. Although ICAM-1 has also been described as a ligand for CR3 [19] and is expressed on these colorectal cancer cells (data not shown), no CR3-DCC was observed in absence of iC3b, indicating that CR3-ICAM-1 interactions did not mediate the observed effect. Secondly, as shown previously, β-glucan is required for enhanced CR3-DCC [3, 4, 8]. Without β-glucan, low levels of CR3-enhanced ADCC were observed. In this case, there was no difference in lysis between with bi-mAb or parental mAb opsonized cells. Both iC3b and β-glucan are essential for efficient CR3-DCC of tumor cells [1, 5, 8]. Although CR3-DCC is a primary mechanism for disposing of iC3b-opsonized microorganisms that bear β-glucan in their cell walls, e.g., fungi or yeast, addition of soluble β-glucan also makes IgG opsonized tumor cells, when coated with sufficient levels of iC3b, targets for CR3-DCC [5].

Thirdly, the immobilization of CD55 on the effector cells by the free anti-CD55 arm of anti-Ep-CAM*anti-CD55 bi-mAb opsonized tumor cells was required to induce enhanced CR3-DCC, as demonstrated by inhibition of CR3-DCC after blocking or removing CD55 on the effector cells. Bi-mAb-mediated interactions between tumor cells and effector cells via other molecules have been shown to be effective in inducing tumor cytotoxicity. Recruiting effector cells with anti-CD20*anti-CD89 (FcγR) on neutrophils [20] or with anti-Ep-CAM*anti-CD3 on T cells [21] increased lysis by an improved interaction between tumor and effector cells. In the present study, anti-Ep-CAM*anti-CD55 bi-mAb induced signal transduction via CD55 on the effector cell, and thereby stimulated CR3-DCC. Our results are in agreement with previous studies that demonstrated signaling via CD55 [22, 23] or increased cytolytic capacity of the effector cells upon forced cross-linking of only CD55 [16]. In our experiments the highest levels of CR3-DCC were induced if CR3 was co-cross-linked with CD55. It is likely that upon association of CD55 with CR3, increased activation is induced via CR3. Although GPI-linked proteins normally cannot initiate signal transduction, association with CR3 may provide CD55 with signaling capacity. It has been demonstrated by several groups that CR3 indeed recruits GPI-linked proteins, such as CD16 on neutrophils, within membrane rafts, after which GPI-linked molecules obtained signal capacity [24]. Co-capping experiments have shown that neutrophil CD16 is constitutively associated with CR3, and that this is required for FcγRIII-mediated ADCC [25]. Our suggestion that CR3 and CD55 may have a similar interaction is supported by the observation that CD55 on monocytes is a unit of the LPS-induced receptor complex, and that LPS induces clustering of CR3 with CD55 [26]. Furthermore, it has been shown that effective CR3-mediated phagocytosis of *Mycobacterium kansasii* requires co-ligation of CR3 with GPI-linked molecules. Several GPI-linked proteins were demonstrated to be able to fulfill this role, amongst these CD16 and CD55 [27]. These observations indicate that GPI-anchored molecules can activate CR3 via inside-out signaling, comparable to the inside-out signaling of β-glucan [5]. Association of GPI-linked CD16 with CR3 has been described to enhance affinity of CR3 for iC3b [25]. This suggests that upon immobilization of the CD55 of effector cells by the anti-CD55 arm of the bi-mAb bound to its tumor antigen, an association between CD55 and CR3 is facilitated on PBMC, comparable to the association of CR3-CD16 in neutrophils. This association induces or enhances signal transduction and activation of the
effector cell, leading to increased cytotoxicity. Since the effector cell population used in our experiments lacked neutrophils and only contained monocytes and NK cells as CR3-positive cells, it is likely that these cells exert the observed effect.

The anti-Ep-CAM*anti-CD55 bi-mAb has a low affinity for CD55 and a high affinity for Ep-CAM. Therefore, it is expected that these bi-mAb mainly home to tumor cells with high Ep-CAM expression levels, and only minimally bind to normal cells that only express CD55. In a syngeneic rat colorectal cancer model, we have previously shown that comparable bi-mAb (anti-Ep-CAM*anti-Crry) that are directed against the major mCRP in the rat (Crry) and a tumor antigen (MG4) can prevent tumor outgrowth in vivo. These bi-mAb are safe to administer despite their reactivity with Crry, which is also expressed on normal cells [14]. In contrast to administration of bi-mAb, administration of the parental anti-Crry mAb has been shown to be lethal upon injection in rats. In humans, CD55 is a less potent mCRP than Crry in rats, and therefore administration of anti-Ep-CAM*anti-CD55 bi-mAb in patients with an Ep-CAM-expressing tumor might also be feasible.

In conclusion, we have demonstrated that bi-mAb directed against a tumor-associated antigen and CD55 induce CR3-DCC as well as CDC. Immobilization of CD55 on effector cells was required for the observed CR3-DCC, and is thought to provide an important costimulatory signal for CR3 via CD55. Even tumor cells with low antigen expression that induce no substantial ADCC or CDC can be eradicated via bi-mAb induced CR3-DCC. Bi-mAb that induce this CR3-DCC may, therefore, increase the success rate of immunotherapy in cancer patients.

Material and methods

Antibodies

The following antibodies were used: anti-Ep-CAM mAb (clone 323/A3; IgG2a) [28]; anti-CD55 mAb (clone MBC1; IgG1, MBC1 hybridoma cells were kindly provided by Prof. Dr. B.P. Morgan, University of Wales, Cardiff, UK); F(ab')2 of MBC1 mAb were produced as described previously [29]. Bric229 mAb (IgG2b; IBGRL) directed against CD59; LPM19c mAb (IgG2a, DAKO, Glostrup, Denmark) directed against CD11b; anti-ic3b (IgG2b, Quidel, San Diego, CA), FITC-conjugated goat-anti-mouse (GaM) IgG/M antibodies (DAKO) and GaM IgG2b-RPE antibodies (DAKO); anti-Ep-CAM*anti-CD55 bi-mAb: quadroma cells producing this bi-mAb (IgG2a*IgG1) were developed in our own department as described before. Bi-mAb were purified with a protein A column. The presence of bi-isotypic mAb was measured by ELISA as described before [13, 30].

Cell lines

The following colorectal carcinoma cell lines were used: SW837 and CaCo-2 (both obtained from ATCC, Rockville, MD). Cells were cultured in DMEM (Life Technologies, Rockville, MD) containing 22.5 mM HEPES, supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glucose, 50 U/ml penicillin and 50 μg/ml streptomycin (DMEM*+ medium).

Complement sources

NHS was prepared from freshly collected human AB+ serum. C6-depleted human serum was obtained from Quidel (Clindia, Leusden, The Netherlands). Sera were aliquoted and stored at −70°C until use.

Flow cytometry

Determination of antigen expression

Cells (2.5 × 10^5) were incubated with 100 μl primary antibodies diluted in PBS containing 1% BSA (30 min, 4°C). Cells were washed twice with PBS/1% BSA and incubated with 100 μl secondary antibodies (30 min, 4°C) when indicated followed, after washing, by incubation with 100 μl tertiary mAb (30 min, 4°C). After washing, the cells were resuspended in 250 μL PBS/1% BSA containing 1 μM propidium iodide (PI) to stain dead cells. Staining was measured on a FACS Calibur (Becton Dickinson, San Jose, CA). Ten thousand living cells were counted and analyzed. Fluorescence compensation was used to correct for spectral cross-talk between the fluorescent signals. Data are expressed in molecules of equivalent soluble fluorochrome (MESF) values to correct for different instrument settings, making inter-experimental values comparable [31]. MESF values were calculated on the basis of a flow cytometry standardization kit, Quantum 25 FITC (Flow Cytometry Standards Europe, Leiden, The Netherlands).

Determination of iC3b deposition

After incubation with (bi-)mAb (30 min, 4°C), cells were incubated with 10% NHS diluted in DMEM containing 2 mM CaCl_2 and 2 mM MgSO_4 (DMEM*+/Ca/Mg) (20 min, 37°C). iC3b deposition levels were assessed by flow cytometric analysis as described above. iC3b deposition was detected with anti-iC3b mAb (30 min, 4°C) and subsequently with GaM-IgG2b-RPE (30 min, 4°C). Ten thousand living cells were counted and analyzed. iC3b deposition was expressed in MESF values.

β-Glucan

(1→3),(1→4)-β-D-glucan derived from barley (Sigma) was dissolved by boiling in PBS for 10 min (50 mg in 5 mL). Samples were aliquoted and stored at −20°C. The carbohydrate concentration was measured using the phenol-sulfuric acid colorimetric method [32].
Isolation of PBMC

PBMC were isolated from buffy coats (Sanquin blood bank, Leiden, The Netherlands) by collecting the interphase after Ficoll-Hyphaque (Pharmacy of the LUMC, Leiden, The Netherlands) centrifugation. The obtained population mainly contained lymphocytes and monocytes.

$^{51}$Cr-release assays

**CDC**

All dilutions were performed in DMEM$^{++}$/Ca/Mg. Tumor cells (1 x 10$^5$) were labeled with 100 $\mu$Ci $^{51}$Cr and washed twice with DMEM$^{++}$/Ca/Mg. $^{51}$Cr-labeled target cells (25 $\mu$L, 3000 cells/well) were mixed with 50 $\mu$L (bi)-mAb in round-bottom microtiter plates and incubated for 30 min at 4°C. As a control source, 50 $\mu$L NHS was added (final concentration of 10%). Plates were incubated for 4 h at 37°C. Subsequently, 75 $\mu$L supernatant was harvested and the amount of $^{51}$Cr release was assessed. The percentage of specific $^{51}$Cr release was determined with a 1282 Compugamma CS counter (Pharma-lands) centrifugation. The obtained population mainly contained lymphocytes and monocytes.

**ADCC/CR3-enhanced ADCC/CR3-DCC**

After labeling with $^{51}$Cr, tumor cells were pre-incubated with appropriate (bi-)mAb (30 min, 4°C) and washed twice with DMEM$^{++}$/Ca/Mg medium. Opsonized tumor cells were pre-incubated with 12.5% C6-depleted human serum (Quidel); 20 min, 37°C) diluted in DMEM$^{++}$/Ca/Mg medium (CR3-enhanced ADCC and CR3-DCC experiments) or with DMEM$^{++}$/Ca/Mg medium only (ADCC experiments). After washing, cells were diluted in medium and divided over the wells of a round-bottom 96-well plate (75 $\mu$L; 2000 cells/well). PBMC (effector cells) were resuspended to the appropriate concentration in DMEM$^{++}$/Ca/Mg medium with or without 20 $\mu$g/mL $\beta$-glucan (10 $\mu$g/mL final concentration). After incubation (30 min, on ice), effector cells were added to the tumor cells (75 $\mu$L; E:T ratio 10:1, total volume 150 $\mu$L). In the experiments indicated, PBMC were pretreated with PI-PLC (described below) or pre-incubated with mAb (30 min, 4°C). After incubation (16 h, 37°C), 75 $\mu$L supernatant was harvested and the amount of $^{51}$Cr release in the supernatant was assessed. The percentage of specific $^{51}$Cr release was calculated as described under ‘CDC’.

PI-PLC treatment

Effector cells were suspended in DMEM$^{++}$ medium (4 x 10$^5$/mL). PI-PLC (Bacillus thuringiensis; ICN biomedicals B.V., Zoetermeer, The Netherlands) was added (final concentration 100 $\mu$L/mL; 1 h, 37°C). Subsequently, cells were washed twice with DMEM$^{++}$ and either used in $^{51}$Cr-release assays or flow cytometry experiments.

Statistical analysis

Data are presented as mean values ± SD and are representative for at least three independent experiments. Significant differences between group means were determined using Mann Whitney U or Student’s t-test. A value of $p<0.05$ was considered a statistically significant difference.

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