Rituximab capping triggers intracellular reorganization of B cells

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The antibody rituximab, which binds to the protein CD20 on the surface of B-cells, has been used to treat B-cell malignancies for several years. However, the molecular mechanisms underlying this treatment are not yet fully understood. One well-established rituximab-induced mechanism, natural killer (NK) cell mediated antibody-dependent cellular cytotoxicity (ADCC), has recently been described to involve the polarisation of bound rituximab and CD20 to one side of the B-cell. B-cells polarised this way were cleared more efficiently by NK-cells, which led us to further investigate the cellular events involved in the polarisation process. Using optical microscopy on rituximab treated cells, we have found that the rituximab/CD20-rich, polarised side accumulated mitochondria and actin, whereas the nucleus was re-organised to the opposite side of the cell. Depleting actin via different methods correlated with a decrease in rituximab, mitochondria and nucleus polarisation, suggesting polarisation to be an actin-dependent, active process that triggers intracellular rearrangement. The influence of these intracellular re-arrangements on the efficiency of NK-cell mediated clearance of B-cell malignancies remains open for future investigation.

Objective

Building on these findings, we set out to further investigate cellular changes upon rituximab polarisation since further understanding the underlying processes will ultimately allow harnessing this polarisation effect to increase rituximab treatment efficiency or to screen other monoclonal antibodies for triggering ADCC in various situations. We here aim to elucidate: 1) Organelle re-positioning upon rituximab induced polarisation. 2) Involvement of the cortical actin cytoskeleton in the rituximab induced polarisation.

Introduction

Rituximab was the first Food & Drug administration (FDA) approved monoclonal antibody for use in cancer therapy and is now used to treat some Non-Hodgkin lymphomas and rheumatoid arthritis. Further off-label use in systemic lupus erythematosus, multiple sclerosis, autoimmune haemolytic anaemia and graft versus host disease exemplifies the importance of rituximab in current medicine. Despite its long standing use, its mechanism of action is not fully understood. Rituximab is binding to the protein CD20 on the surface of B-cells and this seems to induce a combination of complement dependent cellular cytotoxicity (CDCC), "direct signalling" induced apoptosis and antibody dependent cellular cytotoxicity (ADCC) to deplete malignant or autoreactive B-cells. Recent research into the molecular basis of one of these mechanisms, ADCC, found that rituximab and CD20 polarised to one side of the target B-cell upon rituximab binding. In contrast to other forms of antibody capping, this effect was found to be cross-linking and Fc-receptor independent. Interestingly, malignant B-cells polarised in this way were more likely to be cleared by NK cells than B-cells with homogeneously bound rituximab.
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A. Representative confocal image of Raji-B-cells treated with Alexa Fluor-647 labelled rituximab (Rtx-Alexa647, red) highlighting the rituximab induced polarisation.

B. Nucleus re-positioning is dependent on rituximab polarisation, not binding. Percentage of cells showing the polarisation of nucleus, as determined from dual-color confocal images of Rtx-Alexa647 and nucleus stain (NucBlue) in live Raji cells: asymmetric nucleus re-positioning after Rtx-Alexa647 treatment, no nucleus re-positioning after Rtx-Alexa647 treatment, and asymmetric nucleus re-positioning without Rtx-Alexa647 treatment. n: number of total cells. All shown data represents pooling of at least 3 repeats.

C. Side-view of a representative 3D confocal image of a Raji cell with the polarised Rtx-Alexa647 (red), similar polarisation of mitochondria (labelled via MitoTracker Orange, yellow) and asymmetric polarisation of nucleus (NucBlue stain, blue).

D. Representative snapshots of the same kind of images of panel C for a one hour time lapse recording. Top row shows the uniform Rtx-Alexa647 binding and symmetrically distributed nucleus and mitochondria, representative of the first few minutes after addition of the antibody. Middle row shows, gradual polarisation of Rtx-Alexa647, mitochondria and nucleus, taking place at around 40 min. Bottom row shows complete Rtx-Alexa647 polarisation with re-organised mitochondria and nucleus at around one hour after adding Rtx-Alexa647.

E. Actin disruption leads to decreased rituximab binding. Shown are absolute values of fluorescence intensity for surface bound, non-polarised Rtx-Alexa647 after Latrunculin B or Cytochalasin D mediated actin disruption in Raji cells, compared to cells with intact actin (untreated). Fluorescence intensity values (arbitrary unit) were taken from confocal microscopy images and correlate with surface concentration of bound Rtx-Alexa647.

F. Actin disruption decreases rituximab polarisation. Shown is the likelihood of rituximab polarisation upon rituximab binding. This decreases as actin is disturbed by the LatB or CytD. Raji cells were pre-incubated with the LatB or CytD for 30 min before adding the Rtx-Alexa647. Data determined from confocal microscopy images.

G. Representative confocal microscopy images of giant plasma membrane vesicles (GPMVs) from Raji cells treated with the Rtx-Alexa647, showing no rituximab induced polarisation. The GPMVs have no intact actin cytoskeleton.

H. Representative dual-color confocal image of Rtx-Alexa647 (red) and PIP2 (green, labelled via PH-PLC-GFP) in live Raji-cell, showing that PIP2 co-localises with Rtx-Alexa647. PIP2 plays a role in actin polymerisation and tethering, and is usually distributed evenly in the plasma membrane. (Scale bars are 20 µm. Error bars are standard deviation of the mean. Statistical significance is unpaired t-test.)
Results & Discussion

Rituximab polarisation induces asymmetric nucleus re-positioning:
We first validated rituximab-induced polarisation of B-cells by taking confocal images of Raji cells (an EBV-transformed Burkitt’s lymphoma B-cell line) treated with AlexaFluor 647 labelled rituximab (Rtx-Alexa647) (Figure 1A). Nucleus staining with NucBlue showed that, this polarisation in turn triggered asymmetric positioning of the nucleus (Figure 1B). While 62% of Rtx-Alexa647 polarised Raji-cells (n=172) showed nuclear asymmetry, on Rtx-Alexa647 bound but non-polarised Raji-cells only 20% (n=168) showed nuclear asymmetry. Rtx-Alexa647 untreated control cells showed nucleus polarisation only in 9% of all cases (n=539) (Figure 1B). Nuclear asymmetry is therefore linked to rituximab-triggered polarisation, rather than just rituximab binding.

Rituximab polarisation actively re-organises mitochondria:
Nuclear movement likely affects other organelles. Since mitochondria plays a key role in apoptosis-related phenomena, we have investigated potential re-positioning of this organelle. 3-color confocal imaging of Raji cells incubated with Rtx-Alexa647, NucBlue for nuclei staining and MitroTracker Orange for mitochondria staining showed a clearly correlated re-organization (Figure 1C). While the nucleus polarised to the opposite side of the Rtx-Alexa647 cap, the mitochondria localised towards the Rtx-Alexa647 cap. A detailed analysis showed that 90 ± 5% (n=106) of rituximab binding cells in which the nucleus was polarised also had their mitochondria polarised towards the rituximab-rich cap. In contrast, rituximab polarised cells without asymmetric positioning of the nucleus were found to polarise their mitochondria in only 14 ± 6% of all cases (n=66). The few cells that were not polarised by rituximab, yet showing asymmetric nuclear positioning, showed a similar likelihood of mitochondrial polarisation as did cells with polarised organisation of rituximab and nucleus, i.e. polarisation of mitochondria is observed whenever the cell nucleus polarises, independent of rituximab capping. This suggests that rituximab triggers asymmetric positioning of the nucleus and this subsequently induces polarisation of mitochondria. We further questioned whether Raji cells would show nucleus and mitochondrial pre-polarisation which could favor rituximab binding and polarisation. Time-lapse 3-color confocal microscopy of mitochondria (MitoTracker) and nucleus (NucBlue) in Raji cells upon addition of Rtx-Alexa647 showed that rituximab first bound uniformly, then polarised towards one side of the target cell which subsequently led to the re-organisation of nucleus and mitochondria (Figure 1D). This sequence of events, taking place over an hour, indicates that rituximab actively triggers the rearrangement of cellular organelles upon polarisation.

The resulting condensation of mitochondria and enhanced proximity between mitochondria and the plasma membrane may favor Fas-ligand or granzyme mediated apoptosis. Such hypothesis is in line with reports of rituximab polarised B-cells being cleared more efficiently by NK cells (Rudnicka 2013[5]). This is open for future research which may test the dependency between the efficiency and time-course of NK-cell or complement mediated lysis and mitochondria re-organisation.

Rituximab polarisation is actin-dependent and leads to actin re-organisation:
The involvement of the actin cytoskeleton in these polarisation events is very likely, since actin is known to interact with both the plasma membrane and intracellular organelles (Gundersen 2013[4] Rangamani 2011[3]). To investigate a possible correlation between rituximab binding and the actin cytoskeleton, we used two different actin polymerisation inhibitors, Latrunculin B (LatB) and Cytochalasin D (CytD). Confocal microscopy of Raji cells treated with either LatB or CytD in the presence of Rtx-Alexa647 decreased both i) rituximab binding (>6-fold decrease in cell-bound intensity, Figure 1E) and ii) rituximab polarisation (reduction from around 43% (n=420) Rtx-polarised cells to 12 ± 8% (n=479) and 18 ± 5% (n=557) after LatB or CytD treatment, respectively; Figure 1F). To confirm the role of actin in rituximab induced polarisation, we moved to giant plasma membrane vesicles (GPMVs), which are derived from living cells through vesiculation agents (Sezgin 2012[6]). While GPMVs have similar membrane composition as their parent cells, they differ in lacking an intact actin cytoskeleton and cellular organelles. Confocal images of GPMVs derived from Raji cells and treated with Rtx-Alexa647 showed binding of rituximab to the vesicles but no polarisation in any of the investigated vesicles (n=589) (Figure 1G). While these observations confirms the correlation between rituximab induced polarisation of CD20 and functional actin cytoskeleton, one has to keep in mind that GPMVs differ from their parent cells in multiple aspects in addition to the absence of an actin cytoskeleton. Most importantly, GPMVs are in thermal equilibrium, which may alter membrane properties and subsequently the conformation of CD20 and thus binding kinetics of rituximab. We therefore investigated the re-organisation of the lipid PIP2 upon rituximab binding. Plasma-membrane integrated PIP2 is a strong binding partner of actin, it is known to play a major role in actin polymerisation as well as in tethering the cytoskeleton to the membrane (Yin 2003[15]) and it is therefore a candidate to orchestrate actin polarisation during CD20 capping. Dual-colour confocal images of Rtx-Alexa647 and PH-PLC-GFP (a marker for PIP2) highlighted that PIP2 was enriched in the rituximab rich side of the Raji cells, which suggests that actin is also presumably enriched in that region. Our observations suggests that rituximab-mediated CD20 polarisation is actively supported by the actin cytoskeleton and not a passive event within the plasma membrane. Polarisation may for example be mediated by actin-dependent signalling or actin-induced changes of the membrane organisation. Our findings are important in the light of recent chemotherapy approaches which target the actin cytoskeleton, especially tropomyosin fibres that form actin filaments (Stehn 2013[8]). Disruption of the actin cytoskeleton in this therapy may thus counteract the impact of rituximab treatments, since our findings suggests that actin disruption correlates with decreased rituximab polarisation and consequentially decreased NK-cell killing efficiency (Rudnicka 2013[5]).
Conclusions
CD20-mediated binding Rituximab-induced polarisation of CD20 in B-cells leads to a re-positioning of their nuclei towards the CD20-depleted and of mitochondria and actin towards the CD20-rich side. This active actin-dependent process augments effective NK-cell killing.

Limitations
Experiments above were carried out using B-cell lines. The use of primary B-cells may provide further insights.

Conjectures
Given the role of mitochondria in apoptosis, it is plausible that mitochondrial accumulation towards one side of the cell may accelerate apoptosis. This is open for future research which may look at the efficiency and time-course of NK-cell or complement mediated lysis of mitochondria in polarised versus non-polarised B-cells. Related antibodies may be screened for similar properties. Further research into actin organisation may provide insights on how to improve rituximab polarisation which may in turn improve clinical treatments.
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Additional Information

Methods and Supplementary Material
Please see https://sciencematters.io/articles/201612000001.

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Ethics Statement
Not applicable.

Citations