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Glucocorticoid resistance in chronic lymphocytic leukaemia is associated with a failure of upregulated Bim/Bcl-2 complexes to activate Bax and Bak

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Glucocorticoids (GCs) represent an important component of modern treatment regimens for fludarabine-refractory or TP53-defective chronic lymphocytic leukemia (CLL). However, GC therapy is not effective in all patients. The molecular mechanisms responsible for GC-induced apoptosis and resistance were therefore investigated in primary malignant cells obtained from a cohort of 46 patients with CLL. Dexamethasone-induced apoptosis was unaffected by p53 dysfunction and more pronounced in cases with unmutated *IGHV* genes. Cross-resistance was observed between dexamethasone and other GCs but not fludarabine, indicating non-identical resistance mechanisms. GC treatment resulted in the upregulation of Bim mRNA and protein, but to comparable levels in both GC-resistant and sensitive cells. Pre-incubation with Bim siRNAs reduced GC-induced upregulation of Bim protein and conferred resistance to GC-induced apoptosis in previously GC-sensitive cells. GC-induced upregulation of Bim was associated with the activation of Bax and Bak in GC-sensitive but not -resistant CLL samples. Co-immunoprecipitation experiments showed that Bim does not interact directly with Bax or Bak, but is almost exclusively bound to Bcl-2 regardless of GC treatment. Taken together, these findings suggest that the GC-induced killing of CLL cells results from the indirect activation of Bax and Bak by upregulated Bim/Bcl-2 complexes, and that GC resistance results from the failure of such activation to occur.
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Chronic lymphocytic leukemia (CLL) results from the clonal expansion of antigen-experienced B cells with a distinctive immunophenotype.[1] Purine analogs such as fludarabine form the backbone of modern treatment regimens for CLL. However, most patients eventually become resistant to chemotherapy, a situation that is frequently associated with defects in the *TP53* tumor suppressor gene.[1]

In keeping with their p53-independent mechanism of action, glucocorticoids (GCs), either alone or in combination with other agents, have emerged as a useful and important treatment option for patients with chemoresistant or *TP53*-defective CLL. For example, high-dose methylprednisolone (HDMP) can induce objective responses in more than 50% of patients with refractory CLL regardless of *TP53* status or bulky lymphadenopathy.[2] HDMP or dexamethasone is also effective in fludarabine-refractory CLL when used in combination with rituximab.[3,4] The effectiveness of HDMP plus rituximab has been confirmed in the frontline setting where it has the theoretical advantage of delaying exposure to potentially mutagenic chemotherapy.[5] Encouraging results have also been obtained with HDMP in combination with alemtuzumab in CLL patients with *TP53* defects.[6]

Therapeutic GCs such as prednisolone, 6-methylprednisolone, hydrocortisone and dexamethasone are analogs of cortisol, a steroid hormone secreted by the adrenal cortex in response to stimulation by the pituitary adrenocorticotrophic hormone. Cortisol has a key physiological role in limiting the inflammatory response and regulating immune function, and therapeutic GCs mimic this activity. GCs pass through the cell membrane and exert their biological effects through binding to the cytoplasmic GC receptor (GR), thereby displacing it from its molecular chaperones and unmasking a nuclear localization signal.[7] Following translocation to the nucleus, the GR binds to specific DNA sequences in the promoter regions of its target genes. Co-factors are then recruited that modify chromatin structure and regulate assembly of the transcription machinery, resulting in the transcriptional activation or suppression of target genes depending on the cell type.[7] In addition to its direct effect on target genes, the GC–GR complex can also regulate gene expression indirectly by

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Abbreviations: CHAPS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate; CLL, chronic lymphocytic leukaemia; FACS, fluorescence activated cell sorting; GCs, Glucocorticoids; GR, GC receptor; HDMP, high-dose methylprednisolone; HEPES, 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid; IGHV, immunoglobulin heavy variable region; IP, immunoprecipitation; LC, lethal concentration; PBS, phosphate buffered saline; PI, propidium iodide; SDS, sodium dodecyl sulphate; TRAC assay, tumour response to anti-neoplastic compounds assay; RT-PCR, real time-polymerase chain reaction; WBC, white blood cell count
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interacting with other transcription factors, most notably NF-κB and AP-1.[7]

Among the many important biological effects mediated by GCs is the potent and selective induction of lymphoid-cell apoptosis, which underpins their therapeutic use in lymphoid malignancies. Early studies using mouse thymocytes and primary cells and cell lines derived from patients with acute lymphoblastic leukemia and multiple myeloma showed that GC-induced apoptosis is critically dependent on both the structural integrity and level of expression of the GR.[8,9] It was subsequently shown that GC-induced apoptosis requires altered gene expression.[10] Studies employing different types of lymphoid cells have shown that GCs alter the expression of a large number of genes. Although the identity of these GC-regulated genes varies widely between different studies,[11] alteration in the expression of Bcl-2 family proteins has emerged as a common theme in lymphoid cells.[12–14]

Despite the growing importance of GCs in the treatment of CLL, the exact mechanisms underlying their cytotoxicity in CLL cells are not well understood. Nor is it understood why some patients respond to GC treatment while others do not. Understanding the molecular mechanisms underlying GC cytotoxicity and resistance in CLL is important as it could provide the basis for novel therapeutic strategies.

Previous studies addressing these questions have shown that the GC-induced killing of CLL cells occurs by caspase-mediated apoptosis[15,16] and involves conformational changes of Bax and Bak before caspase activation.[17] Transcriptional activation of Bim has been implicated as a possible trigger of apoptosis via the mitochondrial death pathway[18] but proof of a causative role is lacking. Regarding GC resistance, one of the few studies to examine the GR in CLL cells did not detect any defects in the ligand-binding or DNA-binding domains in any of the 22 cases studied, although it is not clear how many of these cases were GC resistant.[19] Another study of a CLL patient with GC resistance found marked overexpression of the dominant negative GR-β splice variant but provided no experimental evidence linking the isoform to GC resistance.[20]

Therefore, major questions remain concerning exactly how GCs induce apoptosis in CLL cells and why CLL cells from some patients are resistant to such killing. The aim of this study was to address these important questions.

Results

Characterization of CLL samples for sensitivity to dexamethasone. First, we set out to characterize a cohort of primary CLL samples obtained from different patients for their sensitivity to GC-induced killing. Cell viability was measured by propidium iodide (PI) staining and flow cytometry. Preliminary experiments were performed to identify the optimal concentration of dexamethasone and the incubation time that achieved the best compromise between minimizing spontaneous cell death and maximizing dexamethasone-induced killing (Supplementary Figure 1a). The rate of spontaneous apoptosis varied widely between different CLL samples. In some cases, it was $>50\%$ at 72 h, making it difficult to measure induced cytotoxicity. An incubation time of 48 h was considered optimal as this time point was short enough for the untreated control cells to remain sufficiently viable, yet long enough to observe significant and discriminatory dexamethasone-induced killing. The lowest concentration of dexamethasone that induced close-to-maximal killing at all time points was 100 nM. This concentration was therefore adopted as the standard for further experiments. Experiments were also performed to confirm that comparable results were obtained irrespective of whether cell death was measured by single-staining with PI or double-staining with annexin V and PI (Supplementary Figure 1b).

CLL cells from a cohort of 46 cases were then incubated with 100 nM dexamethasone for 48 h and analyzed for viability using the PI/flow method. The extent of GC-induced killing varied widely, ranging from $>80\%$ to a slight protective effect (Figure 1a). Available CLL samples from the same cohort were also incubated for 92 h with a range of concentrations of dexamethasone and analyzed for viability using the tumor response to antineoplastic compounds (TRAC) assay.[21] The latter is an improved version of the differential staining cytotoxicity assay, which has been validated against therapeutic response.[21] As expected, a strong correlation was observed between cytotoxicity due to 100 nM dexamethasone as measured by the PI/flow method and the LC_{90} values for dexamethasone obtained using the TRAC method (Figure 1b). This correlation therefore validates the use of the PI/flow method in this study. For the purposes of subsequent comparative studies, GC-sensitive and -resistant CLL samples were arbitrarily defined as those in which incubation with 100 nM dexamethasone produced $>55\%$ and $<25\%$ killing, respectively, as determined by the PI/flow method (Figure 1a).

Dexamethasone-induced killing of CLL cells is p53 independent and more pronounced in samples with unmutated *IGHV* genes. GCs are known to kill mouse thymocytes through p53-independent mechanisms.[22] To confirm that the same is true of CLL cells, CLL samples with functional impairment of the p53 pathway[23] were compared with samples that had no such dysfunction. As expected, p53 dysfunction was not associated with resistance to dexamethasone-induced killing (Figure 1c). Furthermore, dexamethasone resistance was not associated with deletion of TP53 and/or ATM (Supplementary Table 1). In agreement with previous studies,[24] CLL samples with unmutated *IGHV* genes were found to be significantly more sensitive to dexamethasone-induced killing (Figure 1d).

Dexamethasone resistance correlates with resistance to other GCs but not to fludarabine. To establish the level of cross-resistance between different therapeutic GCs, the cytotoxicity of dexamethasone, 6-methylprednisolone and hydrocortisone was compared using the TRAC assay. As is shown in Figures 2a–c, sensitivity to all 3 drugs was highly correlated, indicating shared resistance mechanisms. In contrast, there was no correlation between sensitivity to dexamethasone and fludarabine (Figure 2d). This indicates that the mechanisms responsible for GC and fludarabine resistance are not identical.

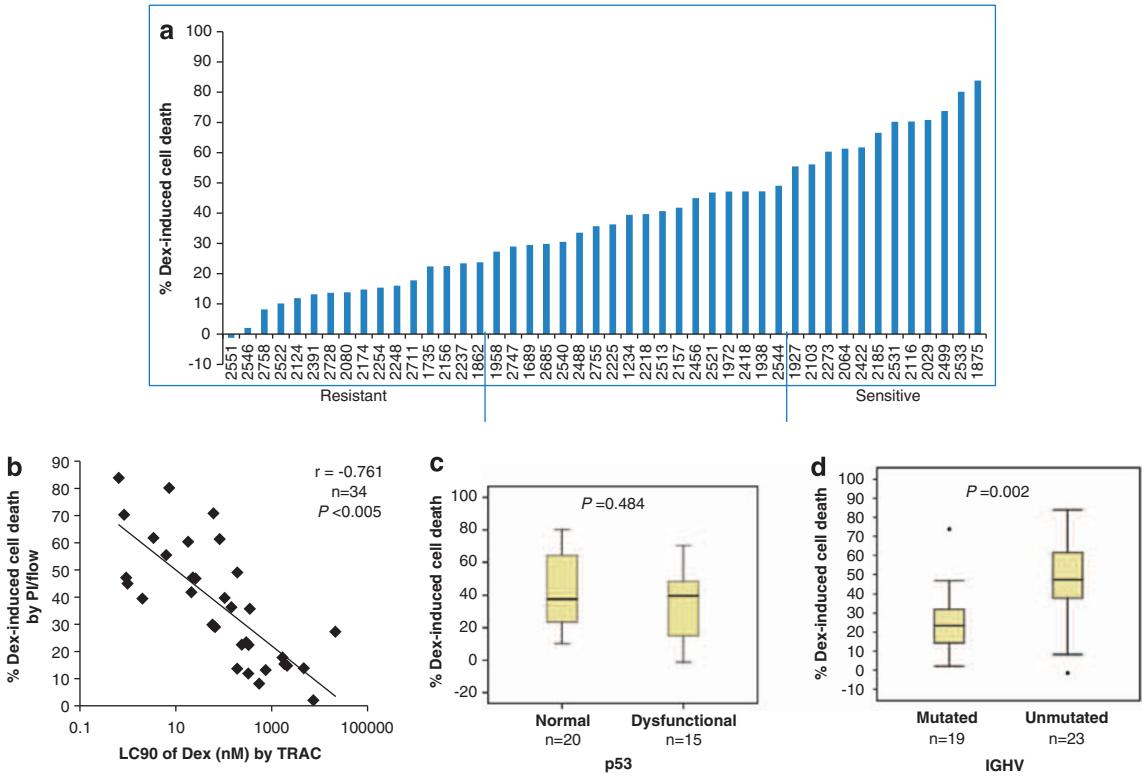

Figure 1 Variability of dexamethasone-induced killing among individual CLL samples. (**a**) CLL cells from 46 cases were cultured for 48 h in the absence or presence of 100 nM of dexamethasone (Dex) and analyzed for viability using the PI/flow method. (**b**) Correlation between Dex-induced killing as measured the by PI/flow method and LC_{90} values as determined by the TRAC assay. (**c**) Comparison of sensitivity to Dex-induced cell death among CLL samples with normal *versus* dysfunctional p53. In this and subsequent statistical analyses of the data, significance of the difference between the two groups was determined using two-tailed Mann–Whitney *U*-test. (**d**) Comparison of sensitivity to Dex-induced killing among CLL samples with mutated *versus* unmutated *IGHV* genes using a cutoff value of 2% homology with the nearest germline sequence

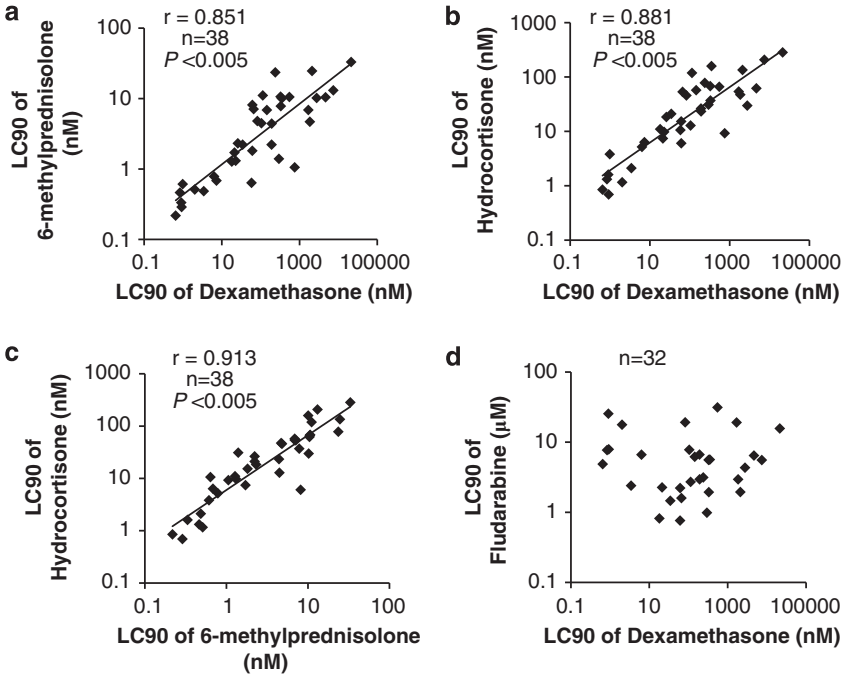

Figure 2 Cross-resistance between dexamethasone and other GCs but not fludarabine. Available CLL samples from among the 46 cases shown in Figure 1a were cultured in the absence or presence of a range of concentrations of dexamethasone, 6-methylprednisolone, hydrocortisone or fludarabine at the indicated concentrations for 92 h. Cell killing was then measured using the TRAC assay and LC_{90} values calculated. (**a**) Comparison of sensitivity to dexamethasone *versus* 6-methylprednisolone. (**b**) Comparison of sensitivity to dexamethasone *versus* hydrocortisone. (**c**) Comparison of sensitivity to hydrocortisone *versus* 6-methylprednisolone. (**d**) Comparison of sensitivity to dexamethasone *versus* fludarabine

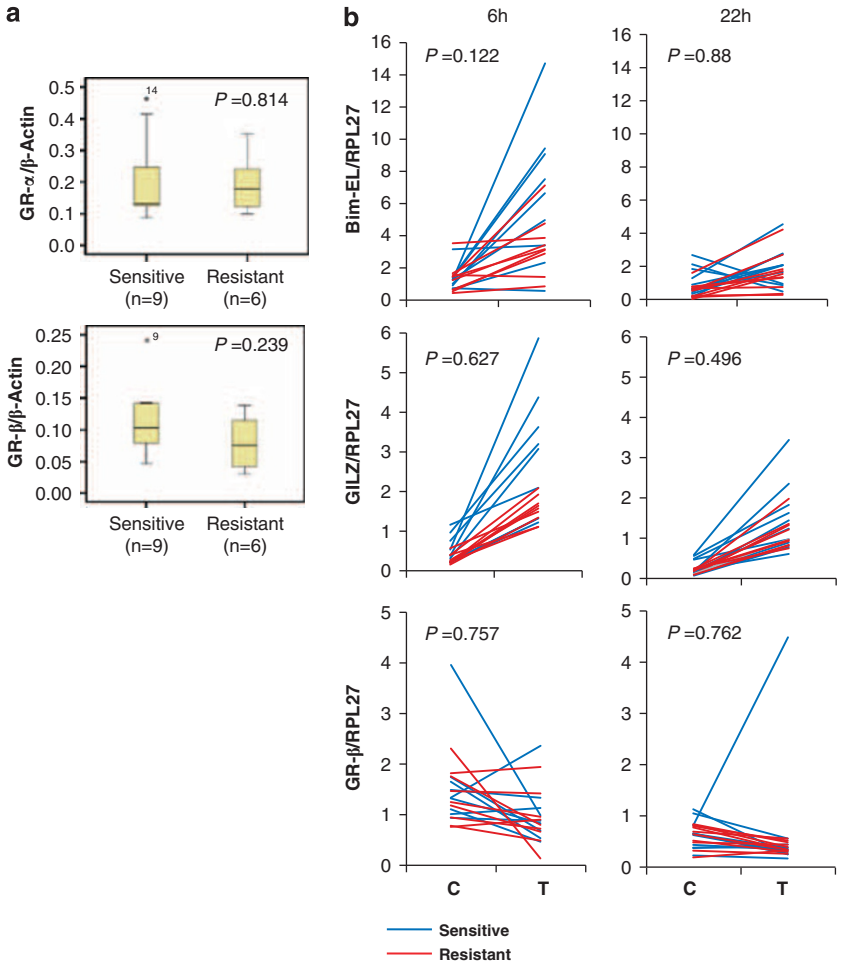

Figure 3 GC resistance is not associated with quantitative or functional defects in the GR. (**a**) Comparison of baseline expression of GR-α and GR-β proteins as determined by western blotting in GC-sensitive (#1875, #2116, #2029, #2422, #2273, #2185, #2499, #2533, #2531) *versus* resistant (#2551, #2080, #2522, #2391, #2124, #2546) CLL-cell samples. Levels of the two GR isoforms were quantified by densitometry relative to levels of β-Actin. (**b**) CLL cells from GC-sensitive (#1927, #2029, #2103, #2116, #2185, #2273, #2422, #2499 and #2533) and resistant (#2080, #2124, #2174, #2248, #2254, #2546, #2551, #2711 and #2758) samples were cultured in the presence (T) or absence (C) of 100 nM dexamethasone for 6 and 22 h. Levels of mRNAs encoding respective Bim-EL, GILZ, and GR-α were measured by qRT-PCR and expressed relative to the levels of mRNA corresponding to the house-keeping gene *RPL27*. Difference in the fold increase in each transcript between GC-resistant and sensitive cases were statistically analyzed as described in Figure 1

GC receptor expression and function are similar in GC-sensitive and resistant CLL cells. We next sought to elucidate the mechanisms responsible for GC resistance. As overexpression of the inhibitory GR-β has previously been implicated as a cause of GC resistance in CLL cells,[20] levels of GR-α and GR-β were measured by western blotting and densitometry. Both GR isoforms were expressed at similar levels in GC-sensitive and -resistant cases (Figure 3a), indicating that the differences in GR expression do not account for GC resistance in CLL.

Next, the ability of the GR to regulate gene expression was compared in GC-sensitive and -resistant CLL samples. To do this, mRNAs encoding GILZ, GR-α and Bim were measured by quantitative RT-PCR after 6 h and 22 h incubation with or without dexamethasone. These genes have been consistently reported to be upregulated by GCs in various lymphoid cells.[11] As expected, dexamethasone induced a marked increase in Bim and GILZ transcripts as early as 6 h (Figure 3b). However, the fold increase in these transcripts was similar between GC-resistant and sensitive cases. Contrary to expectations, GR-α mRNA expression decreased following dexamethasone treatment (Figure 3b). However, the fold-change was comparable in both resistant and sensitive samples. Taken together with our analysis of GR expression, these findings indicate that GC resistance in CLL cells is unlikely to reflect altered GR expression or function.

Constitutive NF-κB activity is not increased in GC-resistant CLL cells. NF-κB activity is thought to have an important role in maintaining the survival of CLL cells and varies widely among different cases.[25] We therefore postulated that GC resistance might be mediated by high constitutive NF-κB activity. To test this hypothesis, GC-sensitive and -resistant CLL samples were compared for basal NF-κB activity. This was first measured as binding of the two main subunits of NF-κB (p65 and p50) to its consensus target DNA sequence using an ELISA-based method.[25] Using this assay, basal NF-κB activity was found

to be no higher in resistant samples (Supplementary Figure 2a). In fact, the DNA-binding activity of the p50 subunit was higher in the GC-sensitive samples. We next measured Bcl-XL mRNA expression by quantitative RT-PCR as a functional read-out of NF-κB transcriptional regulatory activity. No difference was observed in basal Bcl-XL mRNA levels between GC-resistant and sensitive CLL samples (Supplementary Figure 2a). These findings indicate that GC resistance is unlikely to result from high constitutive NF-κB activity.

The GR can inhibit NF-κB through either direct protein-protein interaction[26] or by transactivation of I-κBα, which binds to NF-κB and sequesters it in the cytoplasm.[27] We therefore speculated that GCs might kill CLL cells by repressing constitutive NF-κB activity, either directly or indirectly via I-κBα, and that GC resistance might result from the failure of such repression to take place. To test this hypothesis, GC-sensitive and resistant CLL samples were incubated with or without dexamethasone for 22 h and analyzed for NF-κB activity as measured by sequence-specific DNA binding and Bcl-XL mRNA levels. Samples were also analyzed for I-κBα mRNA levels. Dexamethasone reduced NF-κB DNA-binding activity and Bcl-XL mRNA expression, but did not increase I-κBα expression (Supplementary Figure 2b). These results suggest that GC treatment suppresses NF-κB function in CLL cells via direct mechanisms. However, no significant differences were observed between GC-sensitive and -resistant cases, indicating that GC resistance is unlikely to result from failure of the GR to repress endogenous NF-κB activity.

GC treatment upregulates Bim protein to comparable levels in GC-sensitive and resistant CLL samples. The results described above indicate that GC resistance in CLL cannot be explained by differences in GR expression and function, or by altered NF-κB signaling. We therefore turned our attention to Bim owing to its established role in the GC-induced killing of normal mouse B cells[28] and its implied role in the GC-induced killing of CLL cells.[18] Our earlier experiments investigating the functional integrity of the GR showed that incubation with dexamethasone for 6 or 22 h increased the expression of Bim mRNA in both GC-sensitive and -resistant CLL samples (Figure 3b). However, Bim protein expression can also be regulated by altered proteasomal degradation.[29] It therefore seemed possible that GC resistance might result from impaired accumulation of Bim protein. To test this idea, GC-sensitive and -resistant CLL samples were treated with dexamethasone and analyzed by western blotting for Bim protein expression. However, dexamethasone increased the expression of Bim protein to comparable levels in both GC-sensitive and -resistant samples (Figures 4a and b).

Bim is required for the GC-induced killing of CLL cells. In order to establish the functional importance of Bim in the

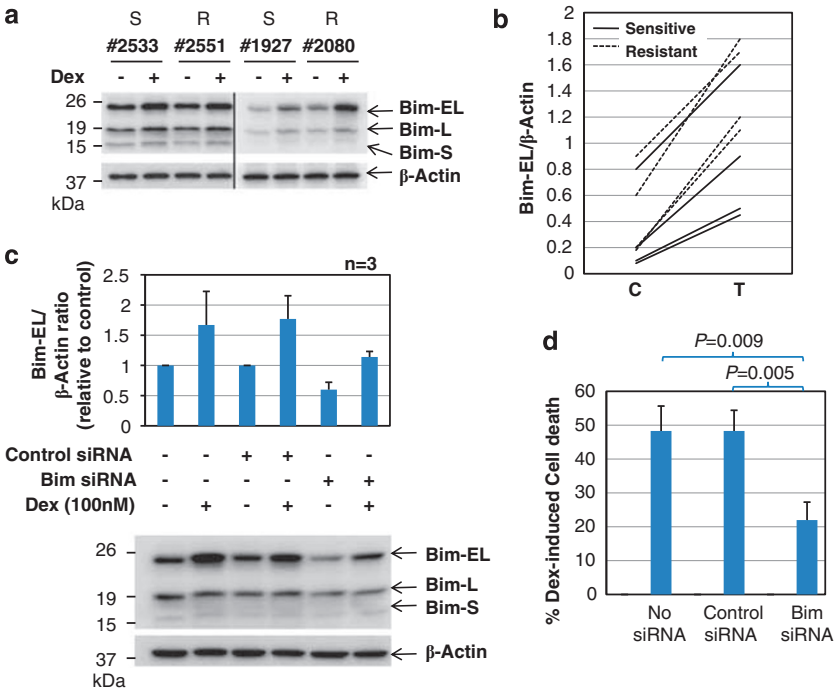

Figure 4 Pivotal role of Bim in the killing of CLL cells by dexamethasone. CLL cells from GC-sensitive (S) and GC-resistant (R) cases were cultured for 48 hours in the absence or presence of 100 nM dexamethasone. (**a**) Bim protein expression was examined by western blotting using β-Actin as a loading control. Representative examples are shown from among the four GC-sensitive (#1927, #2273, #2422 and #2533) and four resistant (#2080, #2124, #2522 and #2551) cases examined. (**b**) Quantification by densitometry of Bim-EL relative to β-Actin in the four resistant and four sensitive samples as described in (**a**) following incubation without (C for control) or with (T for treatment) dexamethasone. (**c**) Effect of Bim-specific or control siRNA on Bim protein levels in GC-sensitive CLL samples (#1927, #2273 and #2422). (**d**) Effect of Bim-specific or control siRNA on Dex-induced killing as measured by the PI/flow method in the same CLL samples as described in (**c**). A two-tailed, paired *t*-test was performed to determine the statistical significance of the difference between the two groups of data

GC-induced killing of CLL cells, we used siRNA to prevent GC-induced Bim upregulation. As expected, pre-incubation of GC-sensitive CLL cells with Bim siRNA reduced baseline levels of Bim protein and inhibited its upregulation following dexamethasone treatment by approximately 50% (Figure 4c). Bim siRNA also rendered the CLL cells significantly more resistant to dexamethasone-induced killing (Figure 4d). These findings provide direct evidence that Bim has a major role in mediating the cytotoxicity of GCs in CLL cells.

GC resistance is associated with impaired Bax/Bak activation. We next addressed the question of why upregulated Bim does not kill CLL cells in GC-resistant cases. Bim requires the pro-apoptotic multi-domain Bcl-2 family proteins Bax and/or Bak to mediate its apoptotic effects.[30] It was therefore important to establish whether GC resistance resulted from the failure of Bax/Bak activation, or failure of activated Bax/Bak to induce downstream apoptotic events. To address this question, GC-sensitive and -resistant CLL samples were compared for the activation status of Bax and Bak following GC treatment.

Bax is a soluble cytoplasmic protein that undergoes conformational changes including the opening up of its N-terminal domain to facilitate oligomerization and mitochondrial localization during the early stage of apoptosis.[31,32] The monoclonal antibody 6A7, raised against the peptide comprising amino acids 12–24 in the N-terminus of Bax, does not bind the soluble form of Bax in healthy cells but recognizes Bax after it undergoes the conformational change associated with the induction of apoptosis.[33] We therefore used this antibody to immunoprecipitate activated Bax from cell lysates prepared from GC-treated or untreated CLL cells. As shown in Figure 5a, a significant quantity of activated Bax was pulled down from GC-sensitive CLL samples. In contrast, very little activated Bax was pulled down from GC-resistant samples (Figure 5a) despite comparable Bim upregulation (Figure 4a). These findings suggest that upregulated Bim fails to activate Bax in GC-resistant CLL cells.

Next, we examined Bak activation using a similar method. As with Bax, the N-terminus of Bak is also concealed in healthy cells and becomes exposed following the induction of apoptosis.[34] The monoclonal antibody TC-100, raised against the peptide sequence corresponding to amino acids 1 to 52 of Bak, recognizes Bak only when in its apoptotic conformation.[34,35] As shown in Figure 5b, a significant quantity of activated Bak was pulled down by the TC-100 antibody from GC-sensitive CLL cells after GC treatment, while very little activated Bak was pulled down from resistant cells despite comparable Bim upregulation (Figure 4a). These findings indicate that upregulated Bim fails to activate Bak in GC-resistant CLL samples. Taken together, our results suggest that GC resistance results from a blockade in the mitochondrial death pathway between Bim upregulation and Bax/Bak activation.

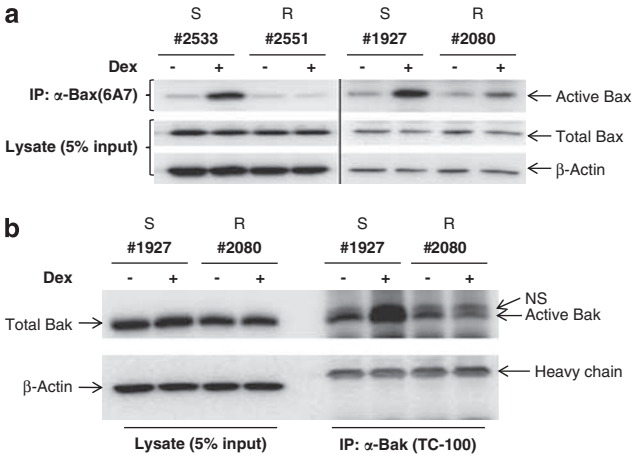

Figure 5 Failure of Bax/Bak activation in GC-resistant CLL samples. CLL cells from GC-sensitive (S) and -resistant (R) cases were cultured for 48 h in the absence or presence of 100 nM dexamethasone and then lysed in buffer containing 1% CHAPS. (**a**) Immunoprecipitation (IP) with an antibody specific for the activated form of Bax (clone 6A7) followed by western blotting for total Bax. The un-manipulated cell lysates (5% input for IP) was also probed for total Bax by western blotting. Four representative examples are shown from among the 4 GC-sensitive and 4 GC-resistant cases examined as described in Figure 4a. (**b**) Immunoprecipitation with an antibody specific for the activated form of Bak (clone TC-100) followed by western blotting for total Bak. The un-manipulated cell lysates (5% input for IP) was also probed for total Bak by western blotting. Two representative examples are shown from among the 2 GC-sensitive (#1927 and #2272) and 2 GC-resistant (#2080 and #2551) cases examined. NS denotes a non-specific band

Bcl-2 is the main binding partner for Bim. We next sought to further explore the failure of Bim to activate Bax and Bak in GC-resistant CLL cells. Reciprocal immunoprecipitation experiments were performed to establish whether Bim binds directly to Bax and Bak. First, we confirmed the ability of Bim antibody to pull-down Bim protein from cell lysates of untreated CLL cells (Supplementary Figure 3). We then immunoprecipitated Bim-containing protein complexes from GC-sensitive and -resistant CLL cells that had been incubated with or without dexamethasone, and probed the complexes for Bax and Bak by western blotting. As shown in Figure 6a, neither Bax nor Bak was detected in the precipitated complexes. To confirm these results, antibodies against activated Bax and Bak were used to pull-down the respective protein complexes, which were then probed for Bim. Bim was not detected in the protein complexes pulled down by either antibody (Figure 6b and c). Together, these results suggest that Bim does not directly bind to Bax or Bak in CLL cells and therefore most likely activates these proteins via indirect mechanisms.

We next examined the binding of Bim to the two most abundant antiapoptotic Bcl-2 family proteins expressed in CLL cells, namely Bcl-2 and Mcl-1. As is shown in Figure 6d, Bcl-2 but not Mcl-1 was co-immunoprecipitated by the Bim antibody, indicating that Bim preferentially binds to Bcl-2 in CLL cells. The physical association between Bim and Bcl-2 was confirmed in reciprocal pull-down experiments in which Bim was co-immunoprecipitated by a Bcl-2 antibody (Figure 6e). Importantly, Bim was almost undetectable in cell lysates that had been immunodepleted with the Bcl-2 antibody (Figure 6e). This observation held true for both GC-resistant and sensitive samples, with or without GC treatment. Together, these findings indicate that, irrespective of GC treatment/sensitivity, virtually all of the cellular Bim is bound to Bcl-2. This implies a pivotal role for Bim/Bcl-2 complexes in the regulation of GC-induced apoptosis in CLL.

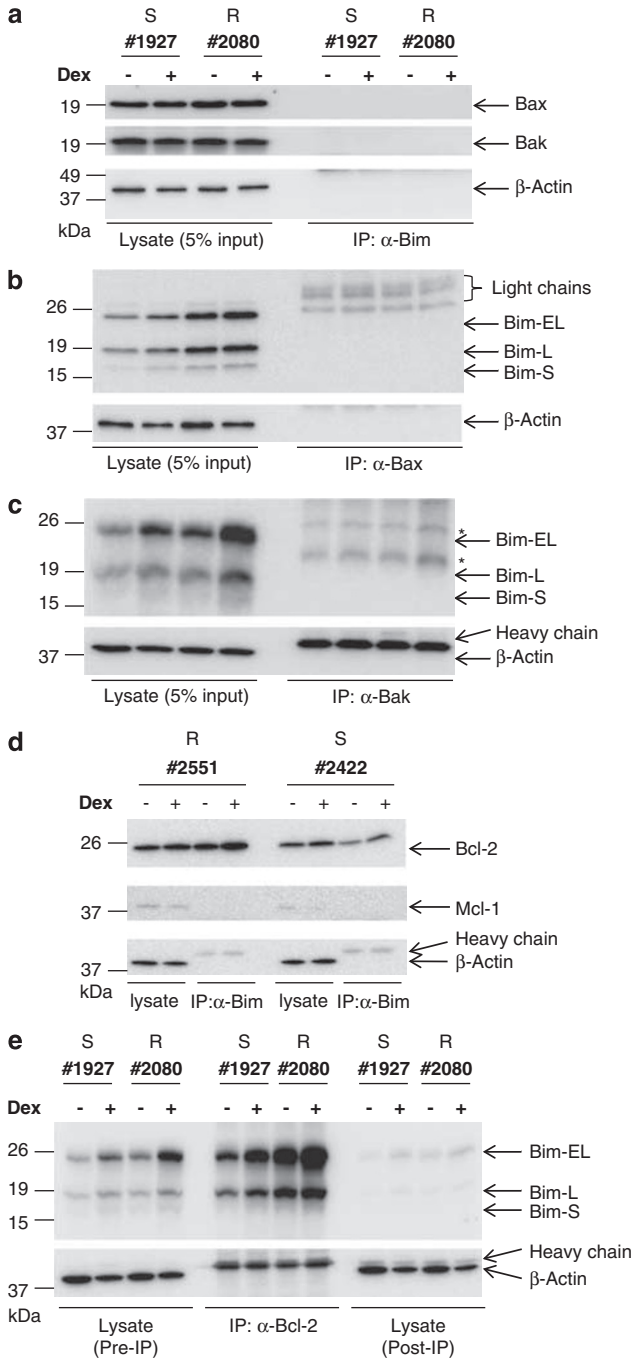

Figure 6 Bim does not interact directly with Bax or Bak, but preferentially binds to Bcl-2. CLL cells from GC-sensitive (S) and resistant (R) cases were cultured for 48 hours in the absence or presence of 100 nM dexamethasone and then lysed as described in Figure 5. Reciprocal co-immunoprecipitations were performed to examine the interaction between Bim and Bax, Bak or Bcl-2. Representative examples are shown from among the three GC-sensitive (#1927, #2273 and #2422) and three GC-resistant (#2080, #21124 and #2551) cases examined. (**a**) Immunoprecipitation of Bim followed by western blotting with antibodies against total Bax or Bak. (**b**) Immunoprecipitation of active Bax followed by western blotting for Bim. (**c**) Immunoprecipitation of active Bak followed by western blotting for Bim. Un-manipulated cell lysates (5% input for IP) were used as a positive control. * denotes unknown immunoreactive products. (**d**) Immunoprecipitation of Bim followed by western blotting for Bcl-2 and Mcl-1. Un-manipulated cell lysates were used as a positive control. (**e**) Immunoprecipitation for Bcl-2 followed by western blotting for Bim. Cell lysates were also probed for Bim before and after immunodepletion of Bcl-2

Discussion

This study was performed in the context of the growing importance of GCs in modern CLL therapy. The key findings are that GC cytotoxicity is critically dependent on Bim upregulation, that GC resistance results from blockade of the mitochondrial death pathway between Bim upregulation and Bax/Bak activation, and that Bim does not bind directly to Bax or Bak but instead forms complexes with Bcl-2 regardless of GC treatment or GC sensitivity. Taken together, these findings suggest that the GC-induced killing of CLL cells results from the indirect activation of Bax and Bak by upregulated Bim/Bcl-2 complexes, and that GC resistance results from the failure of such activation to occur. In contrast, quantitative and functional abnormalities of the GR were not identified as a cause of GC resistance since GC-sensitive and resistant cases differed neither in the expression of the major GR isoforms nor in the transcriptional activation of key GC-regulated genes. Similarly, differences in basal and GC-induced alterations in NF-κB activity were not identified as a cause of GC resistance as NF-κB DNA-binding activity, I-κBα induction and Bcl-XL expression were similar in GC-sensitive and resistant cases irrespective of GC treatment.

The utilization of primary CLL cells for this study deserves particular mention. In addition to the paucity of representative cell lines, it would have been difficult to investigate naturally occurring drug resistance mechanisms other than by studying the primary malignant cells. The use of primary cells from multiple patients also allowed comparisons to be made between clinically important subgroups. Thus, we were able to demonstrate that GC sensitivity was independent of p53 functional status and greater in CLL samples with unmutated *IGHV* genes. We also showed that GC sensitivity did not correspond to sensitivity to fludarabine. These observations are in keeping with previous *in-vitro*[24] and clinical studies[2] and very much support the idea that GCs have a valuable role in patients with high-risk features including unmutated *IGHV* genes, TP53 defects and a history of responding poorly to prior fludarabine-based therapy.[6] From a mechanistic angle, the association between GC resistance and mutated IGVH genes is intriguing and may provide clues as to why upregulated Bim/Bcl-2 complexes fail to induce Bax/Bak activation and apoptosis in some samples. Furthermore, the lack of cross-resistance observed between dexamethasone and fludarabine suggests that the molecular mechanisms responsible for drug resistance are distinct.

The siRNA knockdown experiments proved for the first time that Bim upregulation has a major role in the GC-induced killing of CLL cells. This pivotal observation is supported by a previous study implicating Bim as an important mediator of such killing[18] and with another report showing that Bim is required for the GC-induced apoptosis of normal mouse B cells.[29] Bim has also been implicated in the GC-induced killing of malignant B cells in acute lymphoblastic leukemia[36] and Burkitt lymphoma.[37] However, in these diseases GC resistance was associated with impaired Bim upregulation rather than failure of upregulated Bim to activate Bax/Bak.

Exactly how 'activating' BH3-only proteins (such as Bim) activate Bax/Bak and how this process is regulated by antiapoptotic Bcl-2 family proteins is still unclear and remains

the subject of intense investigation.[32,38] Two working models have been proposed. In the first of these, activation of Bax and Bak occurs through their direct interaction with activating BH3-only proteins. In the second model, activation of Bax and Bak results from their displacement from preformed complexes with antiapoptotic Bcl-2 family proteins. It was beyond the scope of present study to conduct a detailed elucidation of the mitochondrial death pathway in CLL cells. However, our failure to demonstrate any binding of upregulated Bim to Bax or Bak would be more in keeping with the 'indirect activation' model.

Our demonstration that Bim is almost entirely bound to Bcl-2 is consistent with a previous study showing that Bcl-2 is the main binding partner of Bim in CLL cells[39] and with another report showing that CLL cells express significantly more Bcl-2 than Bim.[40] In contrast to its close association with Bcl-2, Bim did not interact with Mcl-1 in any significant way. These observations do not support Mcl-1 as a mediator of GC resistance. However, it is possible that some GC-sensitive CLL samples might be rendered GC resistant when stimulated by extrinsic factors in the *in-vivo* leukemic microenvironment, and that this induced resistance might be mediated by molecules distinct from those responsible for intrinsic resistance. These complex questions, together with the issue of whether upregulated Bim/Bcl-2 complexes also include Bax/Bak, are being addressed as part of an ongoing study examining interactions between Bcl-2 family members and how they are affected by drug treatment and micro-environmental stimuli.

In conclusion, the present study has established Bim/Bcl-2 complexes as a pivotal mediator of GC-induced cytotoxicity in CLL cells. It has also shown that GC resistance in CLL cells results from a blockade in the mitochondrial death pathway between upregulation of Bim/Bcl-2 complexes and Bax/Bak activation. By pinpointing the level at which GC-mediated apoptotic signaling is blocked in GC-resistant cases, our findings shed new light on the molecular mechanisms underlying such resistance and how it might be overcome.

Materials and Methods

Patients, cell preparation and culture. All samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee. The clinical details of the patients' samples used for this study are shown in Table 1. CLL cells were isolated by centrifugation of blood over Lymphoprep (Axis-Shield PoC AS, Oslo, Norway) and stored in liquid nitrogen before use. After thawing, the cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Paisley, UK).

Table 1 Summary of clinical features of 46 CLL samples studied

Sex	Male: 26 Female: 20
Age at diagnosis	Mean: 67.2 (95% CI: 64.2–70.2)
Prior therapy[a]	Yes: 21 No: 25
WBC (10^9/l)	Mean: 110.5 (95% CI: 89.5–131.5)
IGHV	Mutated: 19/42 Unmutated: 23/42
Cytogenetics	17p-: 5/27 11q-: 2/26 +12: 5/23 13q-: 22/32
p53 function[b]	Normal: 20/35 Abnormal: 15/35

Abbreviation: CLL, chronic lymphocytic leukemia
[a]Prior therapy consists of treatment with various combinations of steroid, chlorambucil, or fludarabine plus cyclophosphamide
[b]p53 function is measured by functional probing of the ATM-p53-p21 pathway flowing ionizing radiation by flow cytometry[23]

Chemicals, antibodies and other reagents. Dexamethasone, 6-methylprednisolone, hydrocortisone and fludarabine were obtained from Sigma-Aldrich (Gillingham, UK). Rabbit polyclonal antibody to Bim was from Cell Signaling Technology (New England Biolabs, Herts, UK). Rabbit polyclonal antibodies against GC receptor α and β isoforms were from Santa Cruz Biotechnology (Insight Biotechnology, Middlesex, UK) and Abcam (Cambridge, UK), respectively. Mouse monoclonal antibodies which recognize active Bax (clone 6A7) and Bak (Ab-1, clone TC-100) were from Sigma-Aldrich and Calbiochem (Merck Biosciences, Nottingham, UK), respectively. Antibodies against total Bax and Bak were from BD Biosciences (Oxford, UK) and Santa Cruz Biotechnology, respectively. Mouse monoclonal antibodies to Bcl-2 (clone 100/D5) (Abcam) and to β-Actin (clone AC-74) (Sigma-Aldrich) and rabbit polyclonal Mcl-1 antibody (Santa Cruz Biotechnology) were also employed. Other chemicals, unless otherwise stated, were obtained from Sigma-Aldrich.

Analysis of cell death by flow cytometry and the TRAC assay. CLL cells were incubated for 48 h in the presence or absence of the indicated reagents at a density of 4×10^6 cells/ml in multi-well plates. After treatment, cell death was measured by PI staining and flow cytometry as previously described.[41] Percentage of killing due to dexamethasone was calculated as: 100 $\times$ ((viability of control cells $-$ viability of treated cells)/ viability of control cells). Cell death was also evaluated using the TRAC assay as previously described.[21] In brief, CLL cells were incubated with or without drugs at the indicated concentrations in Octospot 8-well strips (TEST Laboratories, Bath, UK) for 92 h, cytocentrifuged onto microscope slides and then stained for assessment of cell death by microscopy. To ensure accuracy, duplicate slides were independently scored by different operators. The LC_{90} value was defined as the drug concentration at which 90% of CLL cells were killed. The TRAC assay incorporates a standard incubation time of 94 ± 2 h. This later time point was necessary as the assay measures LC_{90} values and was possible owing to the different culture conditions employed.[21]

Quantitative RT-PCR. Total RNA extracted from CLL-cell samples using an RNeasy mini kit (Qiagen, Crawley, UK) was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Promega, Southampton, UK) and an oligo$(dT)_{15}$ primer. For detailed information on quantitative RT-PCR and sequences of the primers, see Supplementary Information.

ELISA-based NF-κB DNA-binding assay. The binding activity of the two main subunits of NF-κB (p65 and p50) to its consensus target DNA sequence was measured using an ELISA-based method (with experimental details provided in the Supplementary Information).

Immunoprecipitation. At the end of incubation, 2×10^7 CLL cells were collected for each treatment and washed in ice-cold PBS before being lysed in 200 μl of lysis buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA, 1% CHAPS and a cocktail of protease and phosphatase inhibitors (Calbiochem) at 4 °C for 1 h. Lysate was collected from the supernatant after centrifugation at 13 000 $\times$ *g* for 15 min at 4 °C and pre-cleared by incubating with protein A or G Sepharose beads (Invitrogen) at 4 °C for 1 h. Protein concentrations were then determined using a protein assay kit (Bio-Rad Laboratories, Hertfordshire, UK), and 100 μg of protein lysate was incubated with 1 μg of the appropriate antibodies overnight at 4 °C. Protein A or G Sepharose beads were then added to pull-down the immunocomplexes. Unbound proteins were removed by washing the beads three times in ice-cold buffer containing10 mM HEPES (pH 7.4), 150 mM NaCl, 2 mM EDTA and 0.2% CHAPS. The bound proteins were retrieved from the beads after heating for 5 min at 95 °C in SDS sample buffer and resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and the identities of proteins of interest determined by western blotting.

Western blotting. Cellular proteins were separated on an SDS-polyacrylamide gel and transferred to Immobilon-P polyvinilidene difluoride membranes

(Millipore Corporation, Bedford, MA, USA), which were probed with the appropriate primary antibodies. Immunoreactivity was detected with the relevant HRP-labeled secondary antibodies which, in turn, were visualized on an Image Reader LAS-1000 (Fujifilm, Tokyo, Japan) using an enhanced chemiluminescence kit (Amersham Biosciences, Buckinghamshire, UK). For quantification of the data, the images were further analyzed on the same instrument using 2D Densitometry Aida Image Analyzer software (Fujifilm).

Transfection of Bim siRNA into CLL cells. To knockdown Bim, we used a mixture of four different siRNA duplexes (Catalog no. M-004383-02, Thermo Scientific Dharmacon, Surrey, UK) targeting all three major transcript variants of Bim (Bim-EL, Bim-L and Bim-S). As a control, non-targeting siRNA (Catalog no. D-001210-02, Thermo Scientific Dharmacon) was also used. As the transfection efficiency varied considerably between individual CLL samples, we monitored it by flow cytometry 24 h after transfecting CLL cells with plasmids expressing GFP (pMaxGFP supplied by Amaxa AG, Cologne, Germany) by electroporation. Knockdown experiments were confined to those CLL-cell samples with a transfection efficiency of $>35\%$. For detailed experimental conditions, see Supplementary Information.

Conflict of Interest

The authors declare no conflict of interest.

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Author Contributions

TM contributed to the design and execution of the study, analyzed data and contributed to the manuscript. JZ contributed to the design and execution of the study, analyzed data and wrote the manuscript. KL contributed to the design and execution of the study and reviewed the manuscript. NR performed some of the experiments. AGB supervised work using TRAC assay and reviewed the manuscript. MO collected patients' samples for the study. JS contributed to the design of the study and reviewed the manuscript. ARP conceived and supervised the study, analyzed data and wrote the manuscript.

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