Highly efficient, In-vivo Fas-mediated Apoptosis of B-cell Lymphoma by Hexameric CTLA4-FasL

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Abstract

Non-Hodgkin lymphomas (NHLs) account for 4% of all malignancies. 5-year survival rate increased to 50% with new treatment modalities, however there is need for new effective treatment for the more aggressive, relapsing forms. Recently, CTLA4-FasL, that can bind to B7 and Fas receptor (Fas), was shown to induce robust apoptosis of cell lines originating from B cell lymphomas expressing both B7 and Fas, by activating pro-apoptotic signals in parallel to abrogating anti-apoptotic ones. The present study focuses on the unique properties of CTLA4-FasL as a potent apoptosis inducer of malignant cells in-vitro and in a xenograft model. CTLA4-FasL was found to naturally form a stable homo-hexamer. CTLA4-FasL induces robust apoptosis of a large variety of malignant cells while relatively sparing non-malignant ones, being more efficient when both receptors (B7 and Fas) are expressed on target cells. Even in non-B7 expressing cells, CTLA4-FasL exhibited better apoptotic activity than its parts, alone or in combination, however, only in B7 expressing cells apoptosis occurs at low concentrations and CTLA4-FasL induces activation of apoptotic signals and reduces anti-apoptotic ones. Importantly, CTLA4-FasL efficiently inhibited the growth of human B cell lineage tumors in a xenograft model, by provoking tumor cells’ apoptosis. Thus, CTLA4-FasL, a natural homo-hexamer protein, induces robust apoptosis of malignant cells, in-vitro and in-vivo. In B-cell lymphoma, its potency stems from the combination of its synergistic effect of activating the caspases while abrogating the anti-apoptotic signaling, with its unique hexameric structure, making CTLA4-FasL a promising candidate for aggressive B cell lymphomas treatment.

Introduction

Non-Hodgkin lymphomas (NHLs), as a disease set, is among the ten most malignant tumors, accounting for approximately 4% of all malignancies in both men and women [1]. NHLs are of B or T-lymphocytes lineage with most (80-90%) of them being of B-cell origin [2,3]. Though prognosis and treatment depend on specific type and stage, irradiation and chemotherapy have been proven effective in many NHL patients. New protein-based therapeutics, such as anti-CD20, have been recently added to the treatment toolbox [4]. The overall 5-year survival rate has increased to approximately 50%, but there is still need for new effective treatment for the more aggressive and relapsing forms of the disease [5]. Activated B-cells are known to express high levels of B7 receptors, also known as CD80 (B7.1) and CD86 (B7.2), which are required for T-cell activation as part of a co-stimulatory signal between the T-cell CD28 receptor and the B7 receptors on antigen-presenting cells including B lymphocytes [6]. Similarly to activated B-lymphocytes, B-cell lymphoma cells also express high levels of B7 molecules [7]. CTLA4 (Cytotoxic T-Lymphocyte Antigen 4), also known as CD152, is a Type-I membrane protein that down-regulates the immune response. CTLA4 is similar to CD28 in that they both bind to B7 in 28 that they both bind to B7, however, whereas CD28 transmits a positive T-cell activation stimulatory signal, CTLA4 does not. The membrane-bound CTLA4-4 is known to function as a homodimer, interconnected by a disulfide bond [6]. CTLA4’s strong binding affinity to B7 led to the design of protein-based therapeutics, linking the CTLA4 extracellular domain to an antibody Fc domain (CTLA4-Fc), that is already approved for use in autoimmune diseases and transplantation [8]. In these chimeric constructs, both the CTLA4 and the Fc domains form a natural homo-dimer [8]. FasL is a Type-II membrane

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protein that naturally binds and activates Fas-receptors (Fas), resulting in cellular apoptosis [9,10]. Fasl and Fas belong to the tumor necrosis factor (TNF) family. FasL:Fas interactions play a cardinal role in immune response modulation, as well as tumor growth and progression [11]. FasL, like other TNF super-family members, functions as a homo-trimer that binds to and signals through a trimerized Fas [12,13]. Upon FasL trimer binding and trimerization of Fas, a death-inducing signaling complex (DISC) is formed within the target cell, followed by activation of the caspases cascade and subsequent apoptosis [14]. Importantly, studies have shown that two adjacent trimeric FasL are required for optimal Fas signaling and the formation of DISC [15]. Also, hexameric recombinant form of FasL, termed as MegaFasL, was shown to induce robust, caspase-dependent apoptosis in Fas bearing cells [16,17]. Moreover, hexameric FasL was found to recruit more Fas into lipid rafts than the trimeric form of FasL, in agreement with its higher efficacy [18].

Signal-Converting-Proteins (SCP) are a novel type of bi-functional fusion proteins that are formed by directly linking an extracellular domain of a type I membrane protein (extracellular amino-terminus), to the extracellular domain of a type II membrane protein (extracellular carboxyl-terminus), creating a fusion protein with two active sides. CTLA4-FasL is one such SCP, in which the N-terminal side is the extracellular domain of CTLA-4 and the C-terminal side is composed of the extracellular domain of Fas-ligand (FasL) [19]. Since CTLA4-FasL has the ability to bind to B7 molecules and to Fas, and in doing so, concurrently, to inhibit co-stimulation and induce apoptosis. CTLA4-FasL has been shown to efficiently induce apoptosis of activated T-cells [20] and to function as a strong immunomodulator in multiple autoimmune and transplantation animal models [21]. Recently, we have shown that CTLA4-FasL can induce robust apoptosis of B cell lymphoma cell lines by activating pro-apoptotic signals in parallel to abrogating anti-apoptotic ones [22].

The first study describing CTLA4-FasL identified it as a homo-trimer, but since CTLA4 naturally forms a homo-dimer, while FasL naturally forms a homo-trimer, the authors raised the possibility that CTLA4-FasL can form a homo-hexamer on the surface of the target cell when anchored to the B7 molecules through the CTLA4 moiety [19]. In the present study, we present data suggesting that CTLA4-FasL naturally forms a stable and soluble homo-hexamer and propose a unique mode-of-action model for the treatment of B cell lymphoma. We also demonstrate that CTLA4-FasL confers a specific and highly effective killing of tumor cells of the B cell lineage, both in-vitro and in-vivo, indicating that CTLA4-FasL might have a possible role as a new anti-cancer agent.

Results

CTLA4-FasL purification

To start, we looked at the purified CTLA4-FasL using SDS-PAGE. Although the predicted molecular weight of CTLA4-FasL is approximately 31kD, the fusion protein migrates in reduced conditions as a protein of approximately 43kD (Figure 1A). Treating the production media samples with the “Peptide N-Glycosidase F”

Figure 1 CTLA4-FasL molecular structure. (A) SDS-PAGE analysis of the CTLA4-FasL under reducing conditions, coomassie G-250 stain. (B) Western blot analysis using anti CTLA4 antibody following enzymatic removal of the N-glycan chains from the protein. (C) Iso-Electric Focusing analysis of CTLA4-FasL at pH3-7 and pH3-10.
enzyme that removes N-glycan chains from the protein, resulted in a shift in molecular weight (MW) from ~45 kDa to ~33 kDa (Figure 1B), indicating that the apparent difference in MW is due to protein glycosylation. In addition, by using Iso-Electric-Focusing analysis, we found that the actual iso-electric point (pl) of CTLA4-FasL is approximately 4.7-5.2, while its theoretical pl is 6.59, supporting the notion that CTLA4-FasL is glycosylated (Figure 1C).

As mentioned in the material and methods section, utilizing the glycosylation of CTLA4-FasL, a preliminary purification process was developed, in which Concanavalin-A (Con-A) chromatography was used as the main capture step. This was followed by two successive size-exclusion chromatography (SEC), yielding CTLA4-FasL at over 90% purity as measured by SDS-PAGE (Figure 1A).

CTLA4-FasL forms a hexamer

To further study the higher-order structure of CTLA4-FasL, purified CTLA4-FasL was initially analyzed by gel-filtration chromatography. The protein peak of CTLA4-Fasl fractionated at a volume similar to that of Catalase, with MW of 232kD; indicating that most of the CTLA4-Fasl protein migrates as a peak of approximately 250kD (Figure 2A). Since this observed product size of about 250kD was significantly larger than the predicted homo-trimer suggested previously (e.g., ~130kD) [19], analytical Size-Exclusion High-performance Liquid Chromatography (SE-HPLC) and native-PAGE were used to study the actual product size at higher resolution. Surprisingly, by using SE-HPLC we found that roughly 90% of the fusion protein migrates as a peak of approximately 250kD, which is consistent with the size of a homo-

![Figure 2 Higher order structure of the CTLA4-FasL. (A) Gel filtration chromatography. The retention volumes of the reference proteins are presented in the table below. (B) Analytical SEC-HPLC. The retention times of the reference proteins are presented in the lower panel. (C) Native-PAGE analysis. (D) SEC-HPLC analysis of the concentrated CHO-S cells harvest (left panel, black, as compared to the purified CTLA4-Fasl profile, blue), followed by a Gyrolab analysis of CTLA4-Fasl content in the fractions (right panel, red dots, as compared to the purified CTLA4-Fasl profile, green dots).](http://www.jhoonline.org/content/7/1/64)
hexamer, while the rest of the protein (~5-10%) was found mostly as a higher-molecular-weight (HMW) peak (Figure 2B). When the samples were analyzed by Native-PAGE, an identical pattern was found (Figure 2C), with most of the protein migrating as a 250kD band and a minor band of approximately twice that size, i.e., 500kD.

To test if the CTLA4-FasL homo-hexamer structure is formed only after purification, at the highly concentrated preparations of the protein, a similar SE-HPLC analysis was performed on harvested production media, before any purification was carried out, and the amount of CTLA4-FasL in the SE-HPLC fractions was quantified by CTLA4-FasL Gyrolab analysis. As can be seen in Figure 2D, most of the CTLA4-FasL in the harvest media (based on Gyrolab analysis) corresponds to a large SE-HPLC peak with retention-time identical to that of the CTLA4-FasL homo-hexamer, suggesting that the vast majority of the CTLA4-FasL fusion protein is in the form of a homo-hexamer structure already at the concentrated harvest media, before any purification took place.

**CTLA4-FasL induced apoptosis is correlated with relevant receptors expression patterns on target cells**

The unique structure of the CTLA4-FasL chimera, predicts Fas-related apoptotic activity coupled to B7 targeting. To assess if these indeed is the case, we first measured the in-vitro killing activity of purified CTLA4-FasL on 13 different malignant and non-malignant human cell-lines. CTLA4-FasL was found to induce a significant, dose dependent killing effect in seven out of the ten cancer cell-lines we assessed, while it had almost no killing effect on the three non-malignant lines tested (Table 1).

As predicated, enhanced CTLA4-FasL killing effect was observed when human B cell lymphoma cancer cell-lines were cultured in the presence of the protein (Table 1). Of note, no viable cells of the B lineage could be detected in cultures were CTLA4-FasL was added at 30 ng/ml and above whereas in other, B7 negative cell lines this maximal effect of CTLA4-FasL could be seen only at concentrations above 300 ng/ml or not at all (Additional file 1: Figure S1 and not shown). This is of particular importance since these B-cells are known to express B7 receptors, suggesting a correlation between activity and specific receptor expression. To study this hypothesis, we used FACS analysis to quantify the expression of the three target receptors of CTLA4-FasL, namely CD80 (B7.1), CD86 (B7.2) and CD95 (Fas), on the different human cancer cell lines. As can be seen in Figure 3, the APL HL60 Human Leukemia cell line, found to be CTLA4-FasL resistant by the bioassay, expresses very low levels of surface CD86 and undetectable CD80 and Fas levels. Similarly, the multiple myeloma cell line, RPMI8226 also found to be CTLA4-FasL resistant, expresses only low surface levels of Fas and CD86, with no CD80. In contrast, the JY and Raji B cell lymphoma cell lines, shown to be highly sensitive to CTLA4-FasL, express high levels of surface Fas and CD86, with no CD80. Importantly, we previously tested another fusion protein, CD40-FasL, that cannot bind to B7 molecules [22]. CD40-FasL was much less potent in inducing apoptosis of the B cell lineage

| Cell-line type                  | Cell-line | Killing effect | IC50 (nM) | Incubation time (h) |
|--------------------------------|-----------|----------------|-----------|---------------------|
| Human liver cancer             | Hep-G2    | Positive       | 1.0       | 24                  |
| SK-Hep1                        | Positive  | 1.5            | 24        |
| Huh-7                          | Negative  | >>120          | 48        |
| Human liver cells (non-malignant)| FH-B      | Negative       | 100       | 24                  |
| A498                           | Positive  | 1.0            | 24        |
| Human kidney cancer            | Caki-1    | Positive       | 2.0       | 24                  |
| 786-0                          | Positive  | 1.0            | 24        |
| Human kidney cells (non-malignant)| PCS-400-010 | Negative     | 100       | 24                  |
| PCS-400-011                     | Negative  | 100            | 24        |
| Raji                           | Positive  | 0.02           | 24        |
| JY                             | Positive  | 0.04           | 24        |
| Human multiple myeloma         | RPMI 8226 | Negative       | >120      | 24                  |
| Human leukemia                 | HL-60     | Negative       | >>120     | 24                  |

As indicated, human cell lines were incubated with different concentrations of CTLA4-FasL for 24 or 48 h. Cell viability was tested using the MTS assay.
cell lines expressing both B7 and Fas than CTLA4-FasL, but was extremely effective in causing apoptosis of CD40L and Fas expressing cells. As we now find that CTLA4-FasL is a hexamer, we performed a gel filtration of the CD40-FasL conditioned medium to test whether it is also a natural hexamer. Gel filtration fractions were loaded on SDS-PAGE and subjected to Western blot analysis using anti FasL Ab (Additional file 2: Figure S2B) or analyzed by CD40 ELISA (Additional file 2: Figure S2C). CD40-FasL was found mainly in fractions corresponding to ~300 – 500 kDa indicating a hexameric structure. As both proteins are hexamers, the fact that CD40-FasL is extremely effective in inducing apoptosis in CD40L and Fas expressing cells [22], but has much lower activity on B7 and Fas expressing cells when compared to CTLA4-FasL, supports the importance of the CTLA4 binding to the B7 molecules for inducing the robust apoptotic effect of CTLA4-FasL on B7 expressing cells.

CTLA4-FasL apoptosis-based effect is greater when compared to its two subunits or their combination
We have shown in the past that his6-CTLA4-FasL induces efficient apoptosis of lymphatic cancer cells by utilizing a dual signaling pathway that includes Fas-mediated apoptosis of CD95 expressing cells, coupled to the abrogation of cFLIP expression in cells that express B7 as well [22]. Also, we have previously shown that CTLA4-FasL inhibitory effect on T lymphocytes activation is mediated by apoptosis induction, through the caspases cascade [20]. To further investigate CTLA4-FasL mode-of-action in cancer cell line, we studied if CTLA4-FasL cytotoxic effect can be abrogated by the pan-caspase inhibitor (Z-VAD), caspase 8 inhibitor (Z-IETD-FMK) and caspase 9 inhibitor (Z-LEHD-FMK) on malignant cell lines positive for Fas only. As can be seen in Figure 4A, the pan caspase-inhibitor resulted in full inhibition of CTLA4-FasL killing effect of the Sk-Hep1 and A498 cell lines. The inhibitors of caspase 8 and 9.
Figure 4 (See legend on next page.)
resulted in partial inhibition, supporting the assumption that CTLA4-FasL activity is mediated by both the intrinsic and the extrinsic apoptotic pathways. Of note, caspase 8 inhibitor was more potent than the caspase 9 inhibitor.

SCP chimeras have been shown to confer superior activity over their parts, separately or in combination [19,22]. However, this was tested previously only in target cells that express binding molecules to both SCP sides [19,22]. As the hepatocellular carcinoma (HCC) cell lines SK-Hep1 and HEPG2, do not express B7 molecules (Figure 3 and not shown), and therefore can bind to the FasL only, we wanted to test if this superior activity will still be evident. For that, cells were incubated in the presence or absence of soluble CTLA4 (CTLA4-Fc), soluble FasL (FLAG-FasL) or the combination of the latter two for 24 h, and cell viability was measured by the MTS assay. The results represent the average of three independent experiments. +/- SE (*p ≤ 0.05). (B) SK-Hep1 cells were incubated with CTLA4-FasL, sFasL, CTLA4-Ig or combination of the later two for 24 hours. Cell viability was tested by the MTS assay. The results represent the average of four independent experiments. +/- SE (*p ≤ 0.05). (C) CTLA4-FasL, effects on the expression of apoptotic and anti-apoptotic proteins in B cell lymphoma cell lines (left) and RCC (right). Raji and A498 cell lines were incubated with indicated concentrations of CTLA4-FasL, sFasL, CTLA4-Ig or the combination of the later two for 2 h. Whole cell lysates were analyzed by Western blot. These are representative results of the three independent experiments. (D) Effect of B7 blockade on CTLA4-FasL’s effect on pro and anti apoptotic signals – Raji cells were incubated for 1 h with 1 μg/ml of anti CD80, anti CD86 or both prior to the addition of CTLA4-FasL (50 ng/ml). Cells were collected after 2 h. Whole cell lysates were analyzed by Western blot. (E) CTLA4-FasL effect on NFκB pathway - A498 (lower panel) and Raji (upper panel) cell lines were incubated with indicated concentrations of CTLA4-FasL, sFasL, CTLA4-Ig or the combination of the later two for 2 h (Raji) or 6 h (A498). Whole cell lysates were analyzed by Western blot.

Figure 4: CTLA4-FasL effect on pro and anti apoptotic signals. (A) SK-Hep1 (left) and A498 (right) cell lines were pre-incubated with or without caspase inhibitors (Z-VAD-FMK (general), Z-LEHD-FMK (caspase 9), Z-IETD-FMK (caspase 8)) for 1 hour followed by incubation with his6-CTLA4-FasL at different concentrations for 24 hours. Cells’ viability was tested by the MTS assay. The results represent the average of three independent experiments. +/- SE (*p ≤ 0.05). (B) SK-Hep1 cells were incubated with CTLA4-FasL, sFasL, CTLA4-Ig or combination of the later two for 24 hours. Cell viability was tested by the MTS assay. The results represent the average of four independent experiments. +/- SE (*p ≤ 0.05). (C) CTLA4-FasL, effects on the expression of apoptotic and anti-apoptotic proteins in B cell lymphoma cell lines (left) and RCC (right). Raji and A498 cell lines were incubated with indicated concentrations of CTLA4-FasL, sFasL, CTLA4-Ig or the combination of the later two for 2 h. Whole cell lysates were analyzed by Western blot. These are representative results of the three independent experiments. (D) Effect of B7 blockade on CTLA4-FasL’s effect on pro and anti apoptotic signals – Raji cells were incubated for 1 h with 1 μg/ml of anti CD80, anti CD86 or both prior to the addition of CTLA4-FasL (50 ng/ml). Cells were collected after 2 h. Whole cell lysates were analyzed by Western blot. (E) CTLA4-FasL effect on NFκB pathway - A498 (lower panel) and Raji (upper panel) cell lines were incubated with indicated concentrations of CTLA4-FasL, sFasL, CTLA4-Ig or the combination of the later two for 2 h (Raji) or 6 h (A498). Whole cell lysates were analyzed by Western blot.

CTLA4-FasL inhibits tumor growth and improves mice survival in a B-cell lymphoma xenograft model

Prior to initiation of studies in a mouse disease model, we measured the basic pharmacokinetic (PK) parameters of CTLA4-FasL in mice. The protein serum levels were quantified by a CTLA4 commercial ELISA at specific time points following subcutaneous (sc) injections. CTLA4-FasL levels were shown to reach the highest values approximately 2 hours post injection with T1/2 of approximately 4-5 hours post injection (Figure 5). Similar results were obtained in both Balb/C and NUDE mice (not shown).

For exploring CTLA4-FasL efficacy in-vivo, NUDE mice were injected (sc) with JY cells and followed daily...
for tumor growth. When tumors were palpable they were treated based on the PK results, with twice-daily sc injections of various CTLA4-FasL dosages or vehicle for 4 consecutive days. As illustrated in Figure 6, treatment with both 50 µg and 20 µg daily dosages of CTLA4-FasL for 4 days, significantly inhibited the growth of human JY xenograft tumors (Figure 6A) and significantly improved survival of the treated mice (Figure 6B). Since the 20 µg/day dose was found to be as effective as the 50µg dose, we next tested the effect of lower dosages. In a second experiment we found that five days administration of 10 µg/day significantly inhibited tumor growth, with a significant effect lasting to ~20 days, while a low dose treatment of 4 µg/day for 4 consecutive days, which was repeated for 4 weeks, seems to keep tumor volumes at a stable reduced state (Figure 7A).

In agreement with tumor volume and the survival indexes, the high efficacy of CTLA4-FasL treatment of JY xenograft tumors was further illustrated by the immuno-staining of tumors removed from the mice, with anti-cleaved caspase 3. As seen in Figure 7B, almost all tumor cells in CTLA4-FasL treated mice undergo apoptosis, while only very few tumor cells from vehicle treated mice stained positive to anti cleaved caspase 3. As FasL and agonistic anti-Fas Abs were previously described to be significantly hepatotoxic [32,33], at another experiment mice from vehicle or 100 microgram/day CTLA4-FasL treated groups were sacrificed 8-30 days after last injection. Representative harvested livers stained with hematoxilin eosine are seen in Figure 7C. In Livers harvested from mice treated with 100 µcg a day for 4 days no significant liver damage was observed. Of note, at higher doses, liver toxicity was evident,

Figure 5 Pharmacokinetic analysis of CTLA4-FasL. CTLA4-FasL at different doses was injected s.c. to mice at a total volume of 150 µl per mouse. Mice were sacrificed at various time points (0-24 h) post injection. CTLA4-FasL level in plasma was quantified by Human Soluble CTLA-4 ELISA kit.

Figure 6 CTLA4-FasL inhibits tumor growth in vivo. Ex vivo JY cells were injected subcutaneously to irradiated NUDE mice. At day 5 after JY injection, tumor volume was calculated and mice were treated daily with subcutaneous injections of CTLA4-FasL or PBS for 5 days. (A) Tumor volume. Results represent the mean +/- SE volume of tumors, (n = 10 for each group). *P < 0.05 between PBS group and 10 µg*2 or PBS vs 25 µg*2. **P <0.01 respectively. (B) The survival curve of the experimental groups and control.
especially when higher molecular weight forms of CTLA4-FasL as dodecamers were present (not shown).

**Discussion**

In the present study we investigated the unique properties of the signal converter protein CTLA4-FasL as a potent apoptosis inducer of malignant cells. The main findings are: 1. CTLA4-FasL naturally forms a stable homo-hexamer; 2. CTLA4-FasL induces robust apoptosis of malignant cell lines while relatively sparing non-malignant ones; 3. The CTLA4-FasL killing effect is more efficient when both relevant receptors (e.g. B7 and Fas) are expressed on target cells; 4. Even in non-B7 expressing cells, CTLA4-FasL exhibited significantly higher apoptotic activity than its parts, alone or in combination; 5. In B7 expressing cell CTLA4-FasL is highly efficient in activating apoptotic signals while diminishing the anti-apoptotic ones, and 6. CTLA4-FasL efficiently inhibited the growth of human B cell lineage tumors in a xenograft model.

Bi-specific and multi-specific biological drugs are believed to develop into the “next generation” of protein-based drugs. Mostly combining functional units of two known biological targets, this drug-development field is currently lead by bi-specific antibodies [34,35], while other bi-specific technologies, such as Signal Converter Proteins, are being assessed as well [20,22,36,37]. As we have shown in this study and previous ones, the main advantage of bi-specific biological drugs over existing biological drugs, that comprise only one target, is a significant synergistic effect which cannot be obtained by simply administrating the functional activity units alone or in combination [22]. These synergistic effects have been mainly suggested to stem from the ability of bi-functional molecules to influence two or more biological pathways concomitantly [38]. Notably, the efficient apoptotic activity induced by CTLA4-FasL is highly specific for human B cell lymphoma cells that express both a functional Fas receptor and B7 receptors, supporting the notion that more than one biological signaling pathway are involved. Indeed, in B7 expressing cells, CTLA4-FasL provoked activation of the caspases cascade and abrogated anti-apoptotic signals at very low concentrations, a phenomena that could not be mimicked by CTLA4-Fc, sFasL or their combination. Most interestingly, abolishment of the c-IAP2 protein expression was seen only when B7-expressing cells were incubated with CTLA4-FasL and not with sFasL, even when the later was used at much higher concentrations,

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**Figure 7** CTLA-4-FasL inhibits tumor growth in vivo. (A) Ex vivo JY cells were injected subcutaneously to irradiated NUDE mice. At day 5 after JY injection, tumor volume was calculated and mice were treated daily with subcutaneous injections of CTLA4-FasL (10 μg twice a day for 5 days for one treatment course or 2 μg twice a day for 4 days for four consecutive weeks). Control animals received similar volume of PBS. The results represent the mean +/- SE volume of tumors (n = 10 for each group). * - P <0.05, **P <0.01 between PBS group and treatment group (B) Five days post s.c. injection of human JY tumor cells to irradiated-NUDE mice the mice were treated with 10 μg CTLA4-FasL or PBS twice a day for 5 consecutive days. Tumors were harvested one hour after last injection, fixed and embedded in paraffin, and tissue sections were processed and stained with anti cleaved caspase 3 antibody. (C) Eight days following last CTLA4-FasL (100 μg a day, 4 days), or vehicle injection mice livers were harvested, fixed and embedded in paraffin, and stained with H&E.
suggesting that effective Fas activation is not solely responsible for the effect observed, and that the CTLA4-B7 interaction of the fusion protein might play a separate significant role. Of note, cIAP and RIP have been implicated before as responsible for some tumors’ resistance to FasL or TRAIL mediated apoptosis [39,40], and c-IAP antagonists have been shown to sensitize cancer cells to TRAIL-induced apoptosis [41]. Significantly, in B7 negative cells this dual effect of CTLA4-FasL could not be elicited, though at higher concentration of CTLA4-FasL, effective activation of the caspases was observed. Importantly, this also suggests that measuring the expression of Fas, CD80 and CD86 in patient tumor samples may be used as a biomarker for patient that might benefit from this treatment.

Intriguingly, CTLA4-FasL potency was higher than that of trimeric FasL, CTLA4-Fc or their combination even when incubated with non-B7 expressing cells, making other explanations for its robust potency plausible. In this study we present data suggesting that a hexameric, higher-order CTLA4-FasL structures may play a significant role in the activity and potency of these novel bi-specific drugs, as has been shown for FasL [15,23].

As reported for other TNF-super family members, activation of the Fas apoptosis pathway requires trimerization of Fas receptors upon binding of FasL trimers [12]. Moreover, it was previously shown that efficient Fas activation requires two adjacent such trimerization events [15] and that hexameric forms of FasL are highly effective in apoptosis induction [19]. Therefore, the finding that the natural stochiometry of soluble CTLA4-FasL is a homo-hexamer is of great significance for understanding its unique, robust apoptotic capabilities. Being a hexamer, CTLA4-FasL is capable of presenting two functional trimers of FasL to their relevant receptors, resulting in optimal initiation of the apoptosis signaling pathway to the malignant cells.

The formation of a membrane bound CTLA4-FasL homo-hexamer was suggested previously [19]. Since only homo-trimers were identified at that earlier study, the authors suggested that two CTLA4-FasL trimers may form a homo-hexamer on target cell’s surface when anchored to B7 molecules, thereby inducing an extremely efficient apoptotic effect that would explain the high efficacy of CTLA4-FasL observed in that report. Here we present data suggesting that CTLA4-FasL naturally form a soluble and stable homo-hexamer as early as it is produced and that this structure maintains its stability through a purification process that includes harsh conditions and multiple freeze/thaw cycles (not shown). The stable hexameric structure can be explained by the fact that CTLA4 naturally forms a disulfide-linked dimer, while FasL naturally forms a stable trimer, thus, as suggested in Figure 8, a CTLA4-FasL trimer would possess an “open cysteine” that could link one such trimer to a second trimer, forming a stable CTLA4-FasL homo-hexamer.

Using a xenograft human-mouse disease model we show that CTLA4-FasL has the ability to inhibit the growth of tumors originating from B lymphocytes lineage, and to provide a significant beneficial effect on mice survival, in a dose dependent manner and at very low dosages. We show that this in-vivo effect is mediated by activation of the caspases cascade, as can be seen by the increased cleaved caspase 3 in immunohistichemistry of the tumors.

Conclusions

In summary, in this study we present data that the fusion protein, CTLA4-FasL induces effective apoptosis of B lymphoblastoid cells, in-vitro and in-vivo, in a highly efficient way. Also, in the case of B7 expressing cells, its potency stems from the combination of its synergistic effect of activating the caspases cascade while abrogating the anti-apoptotic signaling, with its unique natural hexameric structure. We believe that this combination of properties, make CTLA4-FasL an extremely potent apoptosis inducer of B7 expressing tumors, such as B cell lymphomas.

Figure 8 Schematic model of the CTLA4-FasL homo-hexamer. Monomer represents one domain of the CTLA-4 connected to one domain of the FasL. Trimer is composed of the FasL trimer and three domains of the CTLA-4, while two of them are connected by a disulfide bridge. Two CTLA4-FasL trimers may form a homo-hexamer, made of dimer of trimmers or trimer of dimers, resulting in a shift towards a small fraction of a dodecamer state.
Materials and methods

Protein production and purification

The DNA encoding for CTLA4-FasL was synthesized at GENEART (Germany) based on the amino-acid sequence indicated in Figure 9A, and cloned into a UCOE expression vector (Cobra Biologics, Figure 9B).

CHO-S cells (Life technologies GIBCO, Invitrogen Corporation, NY, USA) were grown in CD-CHO medium (Life technologies) and transfected with 30 micrograms linearised DNA using DMRIE-C (Life technologies). Purumycin (Invitrogen) at 12.5 micrograms/ml was used for selection. CTLA4-FasL in culture media was quantified by a commercial FasL ELISA kit (e-Bioscience, CA, USA). Clones with the highest expression were expanded. One clone, with highest level of expression, was selected for limiting dilution, after which a final clone was selected based on growth profile analysis and CTLA4-FasL expression levels, tested by ELISA.

The selected clone was inoculated into a 50 L single use bioreactor. Cultivation and fed batch process medium was 50% CD CHO (Invitrogen), 50% EX-CELL® CHO 5 (SAFC, SIGMA-ALDRICH), supplemented with 8 mM Glutamax and 1x HT (Hyposaxthine 0.1 mM, Thymidine 0.016 mM) (Invitrogen). The titer of CTLA4-FasL at time of harvest was 50 mg/L (Gyrolab platform immunoassay; see below).

To purify the protein, thawed production harvest was centrifuged at 5000 g, followed by 0.2 μm filtration (10 kDa cut-off cellulose centrifugal filters) (Sartorius-Stedim, Goettingen, Germany) and loaded onto a Concavalin-A (Con-A) HiTrap column (GE Healthcare, Little Chalfont, UK) at 7 mg/mL resin. The Con-A eluate loaded onto a Size-Exclusion-Chromatography (SEC) Sephacryl S-200 column (GE Healthcare). The SEC eluate was 0.2 μm filtered (Minisart syringe filter) (Sartorius-Stedim) and frozen at -70°C.

His6-tagged protein

Some of the in-vitro experiments were performed with a His6 tagged version of CTLA4-FasL [42]. The activity of the tagged His6-CTLA4-FasL was compared to that of the purified non-tagged CTLA4-FasL and found to be identical (not shown).

Cell lines

Liver adenocarcinoma Sk-Hep1 cell line [43], A498 Renal Carcinoma Cell line [44] and Raji B cell lymphoma cell line [45] were purchased from ATCC (Manassas, Virginia, USA). The JY lymphoblastoid cell line [22] was a kind gift from Prof. M.L. Tykocinski laboratory, Jefferson Medical School, PA, USA. Other cell lines were a kind gift from the Gene Therapy institute and Hepatology Unit, Hadassah Hebrew University Medical Center in Jerusalem, Israel. Attached cells were grown in DMEM (Gibco) supplemented with 10% FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/mL streptomycin, and were detached using Trypsin-EDTA solution. Suspended cells were grown in RPMI (Gibco) with the same additives. All cell lines were cultured at 37°C, 6% CO₂, and tested periodically for

Figure 9 CTLA4-FasL amino-acid sequence. (A) The amino-acid sequence of the CTLA4-FasL. The underlined sequence represents the signal peptide of the human Urokinase protein, utilized to secrete the protein out of the cell. (B) A schematic map of the CTLA4-FasL cloned in UCOE expression plasmid vector.
mycoplasma contamination using EZ-PCR mycoplasma test kit (Biological Industries, Israel).

Activity bioassay
For in-vitro examination of the CTLA4-FasL cytotoxic effect on different human cell lines, 32,000 cells per well (suspended cultures) or 8000 cells per well (attached cells) in 50 ul of complete RPMI (suspended cultures) or DMEM (attached cells) medium without Phenol Red, were seeded in triplicates, in a flat 96-wells plate (Nunc or similar), and 50 ul of CTLA-4-FasL (or his,CTLA-4-FasL) dilutions (in growth media; 3000 ng/ml-0.1 ng/ml, triplicates), or dilution media as negative control were added. Calibration curve wells contained serial dilution from 64,000 to 2000 cells per well for suspended cultures or 16,000 to-2000 cells for attached cells in triplicates. Plates were incubated for 24 hours at 37°C in 6% CO2 humidified incubator. Cell viability was quantified by a MTS kit (Promega, CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay) according to manufacturer instructions.

SDS-PAGE, western blot and native-PAGE analysis
For CTLA4-FasL and CD40-FasL, SDS-PAGE and western blots, 4-12% Bis-Tris gel (1 mm, 12 wells, NP0322BOX, Life Technologies) and “See Blue Plus 2” MW markers (LC5925, Life Technologies) were used. After blocking (skim milk) membranes (PVDF) were incubated with either goat anti-human CTLA4 antibody (AF-386-PB, R&D Systems, 1:300 dilution) or goat anti-human Fas Ligand (MAB-126, R&D systems) was used. After blocking (skim milk) membranes (PVDF) were incubated with either goat anti-human CTLA4 antibody (AF-386-PB, R&D Systems, 1:300 dilution) or goat anti-human Fas Ligand (MAB-126, R&D Systems, 1:100 dilution). The secondary antibody was a donkey anti-Sheep/Goat Immunoglobins (AB126, R&D Systems, 1:100 dilution). The secondary detection was performed with HRP-conjugated antibodies (BioRad, Hercules, CA, USA). In some experiments blocking anti CD80 and/or anti CD86 Abs (MAB126, R&D Systems, 1:100 dilution) were used.

FACS analysis
1 × 10⁶ cells were washed with PBS and re-suspended in 95 μl of staining buffer (1% BSA, 0.1% azide in PBS) and 5 μl of human Fc blocker (e-Bioscience), and incubated on ice for 5’. Cells were immunostained with PE-anti hCD95 (eBioscience), APC-anti hCD86 (BD) or FITC-anti hCD80 (BD) or matching isotype Abs (PE-mouse IgG1 kappa, APC-mouse IgG1 kappa or FITC-mouse IgG1 kappa, respectively, all from eBioscience) on ice for 30’ and 20,000 events per sample were counted using a BD™ LSR II Flow Cytometer, and data were analyzed using CellQuest software (Becton Dickinson).

Size-exclusion - HPLC
Analytical size-exclusion (SE) was performed using a Dionex HPLC instrument (Pump P580, Auto sampler ASI-100/ASI-100 T Injector, UV/VIS Detector UVD340U,
Chromatography with Tosoh Bioscience

Iso Electric Focusing (IEF)

Gel filtration chromatography

Xenograft lymphoma model

Immunohistochemistry

Additional files
viability was quantified by a MTS kit. Expression of CD80, CD86 and CD95 on Raji cells surface. Raji cells were immunostained with PE-anti hCD86, APC-anti hCD85 and FITC-anti hCD80 or matching isotype Abs. 20,000 events per sample were counted using a BD™ LSR II Flow Cytometer. Dot plots are presented. Data were analyzed using CellQuest software (Becton Dickinson).

Additional file 2: Figure S2. Gel filtration analysis of CD40-FasL containing media. A. Molecular weight standards for the gel filtration fractions. B. Western blots of fractions 4-11 from the gel filtration of the CD40-FasL containing media using anti-fas Abs. C. ELISA detecting human CD40 of fractions 5-14 from the gel filtration of the CD40-FasL containing media.

Additional file 3: Figure S3. CTLA4-FasL effects on the expression of pro-apoptotic and anti-apoptotic proteins in B cell lymphoma A) and R (H) cell lines. Raji and M98B cell lines were incubated with indicated concentrations of CTLA4-FasL, sFasL, CTLA4-Ig or the combination of the later two for 2h at 37°C. Whole cell lysates were analyzed by Western blot using the indicated Abs. Data was normalized against GAPDH by a Quantity One program (BioRad, Version 4.6.9). Data are presented as mean ± SE. P<0.05; CTLA4-FasL, a: vs Untreated (UT); b: vs sFasL, c: vs CTLA-4-Ig, d: vs sFasL + CTLA-4-Ig.

Competing interests
Amsili Shira, Kobi Tzdana, Fanny Szafir and Noam Shani are employed by KAHRI Medical LTD that owns the CTLA4-FasL patent. NS is the CEO. Per Edelbrink and Man-Ariane Rauvola are employed by Cobiologics that produces CTLA4-FasL. Michal Dranitzki Elhalel has a research grant from KAHRI Medical LTD, and consults fees.

Authors’ contributions
AA carried out in-vitro assays including MTS, FACS and WB. She also carries out animal studies and drafted the manuscript. SA carried out some of the MTS and FACS experiments, and participated in animal studies including immunohistochemistry and manuscript drafting. TB performed animal studies. RS participated in MTS and animal studies. KT participated in animal immunohistochemistry and manuscript drafting. All authors read and approved the final manuscript.

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References
1. Smedby KE, Hjalgrim H: Epidemiology and etiology of mantle cell lymphoma and other non-Hodgkin lymphoma subtypes. Semin Cancer Biol 2011, 21:293–298.
2. Alexander DD, Mink PJ, Adamo HO, Chang ET, Cole P, Mandel JS, Trichopoulos D: The non-Hodgkin lymphomas: a review of the epidemiologic literature. Int J Cancer 2007, 120:Suppl 1:3–19.
3. Donouco S, Galland C, Touitou V, Sautès-Fridman C, Fabry Z, Fisson S: Murine models of B-cell lymphomas: promising tools for designing cancer therapies. Adv Hematol 2012, 2012:701704.
4. Miy U, Hitz F, Lohri A, Pedreira S, Tavares C, Tzankov A, Meier O, Yeev K, Renner C: Diagnosis and treatment of diffuse large B-cell lymphoma, Swiss Med Wkly 2012, 142:0.
5. van Meerten T, Hagenbeek A: Novel antibodies against follicular non-Hodgkin’s lymphoma. Best Pract Res Clin Haematol 2011, 24:231–256.
6. Schultz J, Ladner WM, Grieben JG: B7-mediated costimulation and the immune response. Blood Rev 1996, 10:111–127.
7. Suvas S, Singh V, Sadhuk S, Vohra H, Agarwala JN: Distinct role of CD80 and CD86 in the regulation of the activation of B cell and cell lymphoma. J Bioi Chem 2002, 277:7716–7775.
8. Herrera-Baumont G, Martínez Calatrava MJ, Castañeda S: Abatacept mechanism of action: concordance with its clinical profile. Reumatol Clin 2012, 8:78–83.
9. Nagata S: Fas ligand-induced apoptosis. Annu Rev Genet 1999, 33:29–55.
10. Kiyokawa N, Yagita H: Metalloproteinase-mediated release of human fas ligand. Nihon Rinsho 1996, 54:1747–1752.
11. Wajant H: Fas Signaling. TX, USA: Landes Bioscience and Springer; 2006.
12. Locksley RM, Killeen N, Lenardo MJ: The TNF and TNF receptor superfamily: integrating mammalian biology. Cell 2001, 104:487–501.
13. Bodmer JL, Schneider P, Tschopp J: The molecular architecture of the TNF superfamily. Trends, Biochem Sci 2002, 27:19–26.
14. Scafﬁdi C, Fulda S, Srinivasan A, Friesen C, Li F, Tommaselli KJ, Debatin KM, Krammer PH, Peter ME: Two CD95 (APO-1/Fas) signaling pathways. EMBO J 1998, 17:1675–1687.
15. Hoffer N, Tardivel A, Kovacsovics-Bankwolski M, Hertig S, Gaide O, Martinon F, Tinel A, Depertes D, Calderara S, Schultess T, Engel J, Schneider P, Tschopp J: Two adjacent trimeric Fas ligands are required for Fas signaling and formation of a death-inducing signaling complex. Mol Cell Biol 2003, 23:1428–1440.
16. Nahinama A, Aubry D, Lagopoulos L, Greaney P, Attinger A, Demotz S, Dawson KM, Schapira M, Tschopp J, Dupuis M, Duchosal MA: A novel potent Fas agonist for selective depletion of tumor cells in vivo. J Immunol 2007, 179:1463–1469.
17. Wajant H, Gerpach J, Plenzenauer K: Engineering death receptor ligands for cancer therapy. Cancer Lett 2013, 332:163–174.
18. Lang I, Fick A, Schäfer V, Giner T, Siegmund D, Wajant H: Signaling active CD95 receptor molecules trigger co-translocation of inactive CD95 molecules into lipid rafts. J Biol Chem 2012, 287:24026–24042.
19. Huang JH, Tykocinski ML: CTLA-4-Fas ligand functions as a trans signal converter protein in bridging antigen-presenting cells and T cells. Int Immunol 2001, 13:59–59.
20. Orbach A, Rachmilewitz J, Parnas M, Huang JH, Tykocinski ML, Dranitzki-Elhalel M: CTLA-4. Fas induces early apoptosis of activated T cells by interfering with anti-apoptotic signals. J Immunol 2007, 179:7287–7329.4.
21. Zhang W, Wang F, Wang B, Zhang J, Yu Y: Intracellular gene delivery of CTLA4-FasL suppresses experimental arthritis. Int Immunol 2012, 24:379–388.
22. Orbach A, Rachmilewitz J, Shani N, Isenberg Y, Parnas M, Huang JH, Tykocinski ML, Dranitzki-Elhalel M: CD40- FasL and CTLA-4-FasL fusion proteins induce apoptosis in malignant cell lines by dual signalling. Am J Pathol 2010, 177:3159–3168.
23. Eifeldt G, Roth P, Hasenbach K, Aukwurm S, Wolpert F, Tabatabai G, Wick W, Weller M: APO010, a synthetic hexameric CD95 ligand, induces human glioma cell death in vitro and in vivo. Neuro Oncol 2011, 13:155–164.
24. Parrish AB, Freid CD, Kornbluth S: Cellular mechanisms controlling caspase activation and function. Cold Spring Harb Perspect Biol 2013, 5:a008672.
25. Li H, Zhu H, Xu CJ, Yuan J: Cleavage of Bid by caspase 8 mediates the mitochondrial damage in the Fas pathway of apoptosis. Cell 1998, 94:491–501.
26. Wurstle ML, Laussmann MA, Reinm H: The central role of initiator caspase-9 in apoptosis signal transduction and the regulation of its activation and activity on the apoptosome. Exp Cell Res 2012, 318:1213–1220.
27. Safa AR: c-FLIP, a master anti-apoptotic regulator. Exp Oncol 2012, 34:176–184.
28. Nueve A, Schmitz I, Baumann S, Krammer PH, Kirchhoff S: Cellular FLICE-inhibitory protein splice variants inhibit different steps of caspase-8 activation at the CD95 death-inducing signaling complex. J Bioi Chem 2001, 276:20633–20640.
29. de Gaaaf QA, van Krieken JH, Tonnissen E, Wissink W, van de Locht J, Overes I, Dolstra H, de Witte T, van der Reijden BA, Jansen JH: Expression of C-IAP1, C-IAP2 and SURVIVIN discriminates different types of lymphoid malignancies. Br J Haematol 2005, 130:852–859.
30. de Almarg AC, Vicug D: The inhibitor of apoptosis (IAP) proteins are critical regulators of signaling pathways and targets for anti-cancer therapy. Exp Oncol 2012, 34:200–211.
31. Röder C, Trauzold A, Kalthoff H: Impact of death receptor signaling on the malignancy of pancreatic ductal adenocarcinoma. Eur J Cell Biol 2011, 90:450–455.
32. Ogasawara J, Watanabe-Fukunaga R, Adachi M, Matsuzawa A, Kasugai T, Kitamura Y, Itoh N, Suda T, Nagata S: Lethal effect of the anti-Fas antibody in mice. Nature 1993, 364:806–809.

33. Sangwan V, Palouras GN, Cheng A, Dubé N, Tremblay ML, Park M: Protein-tyrosine phosphatase 1B deficiency protects against Fas-induced hepatic failure. J Biol Chem 2006, 281:221–228.

34. Chames P, Baby D: Bispecific antibodies for cancer therapy: the light at the end of the tunnel? MAbs 2009, 1:539–547.

35. Booy EP, Johar D, Maddika S, Pirzada H, Sahib MM, Gehrke I, Loewen S, Louis SF, Kadkhoda K, Mowat M, Loi M: Monoclonal and bispecific antibodies as novel therapeutics. Arch Immunol Ther Exp (Warsz) 2006, 54:85–101.

36. Prinz-Hadad H, Mizrachi T, Irony-Tur-Sinai M, Prigozhina TB, Aronin A, Brenner T, Dranitzki-Elhalel M: Amelioration of autoimmune neuroinflammation by the fusion molecule Fn14-TRAIL. J Neuroinflammation 2013, 10:36.

37. Gupta P, Goldenberg DM, Rossi EA, Chang CH: Multiple signaling pathways induced by hexavalent, monospecific, anti-CD20 and hexavalent, bispecific, anti-CD20/CD22 humanized antibodies correlate with enhanced toxicity to B-cell lymphomas and leukemias. Blood 2010, 116:3258–3267.

38. Notarbartolo M, Cervello M, Dusonchet L, Cusimano A, D’Alessandro N: Resistance to diverse apoptotic triggers in multidrug resistant HL60 cells and its possible relationship to the expression of P-glycoprotein, Fas and of the novel anti-apoptosis factors IAP (inhibitory of apoptosis proteins). Cancer Lett 2002, 180:91–101.

39. Wang P, Zhang J, Bellail A, Jiang W, Hugh J, Kneteman NM, Hao C: Inhibition of RIP and c-FLIP enhances TRAIL-induced apoptosis in pancreatic cancer cells. Cell Signal 2007, 19:2237–2246.

40. Finlay D, Vamos M, González-López M, Ardecky RJ, Ganji SR, Yuan H, Su Y, Cooley TR, Hauser CT, Welsh K, Reed JC, Cooldred ND, Vuori K: Small-molecule IAP antagonists sensitize cancer cells to TRAIL-induced apoptosis: roles of XIAP and cIAPs. Mol Cancer Ther 2014, 13:5–15.

41. Elhalel MD, Huang JH, Schmidt W, Rachmilewitz J, Tykociński ML: CTLA-4, FasL induces alloantigen-specific hyporesponsiveness. J Immunol 2003, 170:5842–5850.

42. di Certo MG, Faggioni A, Barile G: Redistribution and unmasking of Annexin V binding sites in apoptotic Raji cells. Cell Biol Int 2003, 27:497–502.

43. Elhalel MD, Huang JH, Schmidt W, Rachmilewitz J, Parnas M, Tykociński ML: CD40/FasL inhibits human T cells: evidence for an auto-inhibitory loop-back mechanism. Int Immunol 2007, 19:355–363.