European Journal of Immunology

# Peer review correspondence

**Antitumor effector B cells directly kill tumor cells via the Fas/FasL pathway and are regulated by IL-10**

Huimin Tao, Lin Lu, Yang Xia, Fu Dai, Yi Wang, Yangyi Bao, Steven K. Lundy, Fumito Ito, Qin Pan, Xiaolian Zhang, Fang Zheng, Guoshun Shu, Bingmu Fang, Jinhong Jiang, Jianchuang Xia, Shiang Huang, Qiao Li and Alfred E. Chang

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| | Second editorial decision: | 13 November 2014 |
| | Second revision received: | 1 December 2014 |
| | Accepted: | 19 December 2014 |

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Handling Executive Committee member: Prof. David Gray

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 8 April 2014

Dear Prof. Li,

Manuscript ID eji.201444625 entitled "Antitumor effector B cells directly kill tumor cells via Fas/FasL and CXCL12/CXCR4 pathways and are regulated by IL-10" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.

European Journal of Immunology

You should also pay close attention to the editorial comments included below. **In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Laura Soto Vazquez

On behalf of Prof. David Gray

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European Journal of Immunology
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Reviewer: 1

Comments to the Author

Tao et al examine the role of effector B cells in the cytotoxic elimination of tumor cells. The authors show that B cells isolated from tumor draining lymph nodes secrete IL-10, reduce metastases on adoptive transfer and have cytotoxic activity in vitro. Cytotoxic activity is repressed by IL-10 and dependent on FasL. Through an undefined mechanism, antagonizing CXCR4 signaling represses cytotoxicity. Overall, the experiments are clear, but there are a number of gaps in connecting various portions of the report.

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1. Although the B cells display a convincing cytotoxic activity, it is still much less than the activity of T cells (Fig. 4). Are T cells required for the B cell anti-tumor activity? This would be important to show if the B cells are acting directly, as suggested by this report, or indirectly by inducing T cell activity.

2. Can the authors show by FACS analysis that B cells are FasL+? If IL-10+ B cells are purified, is FasL expression enriched?

3. The link between the B cell/IL-10/FasL story and the AMD3100/CXCR4 story is not very clear. Are the CD19+IL-10+ cells also CXCR4+, or is the CXCR4+ population distinct? Moreover, it is not clear how AMD3100 would affect cytotoxicity since migration in a killing assay should not be a factor. Perhaps the AMD3100/CXCR4 data could be moved to a separate report after it is developed further.

4. There are two pieces of data suggesting this system is not entirely physiological. First, a large number of B cells needs to be transferred to see the effect. It might help to know how many of the transferred B cells still survive when metastases are examined. Second, anti-IL-10 does not have any effect when administered without B cells. This would at least suggest that the proposed mechanism is not operating in endogenous B cells during normal tumor immunity. These points should be discussed further by the authors.

Reviewer: 2

Comments to the Author
I have reviewed the manuscript by Tao et al. The manuscript combines two different observations that apply to tumor-draining LN B cells: 1. A suppressory role of IL-10 producing B cells and 2. Cytotoxic killing of tumor cells by TD-LN B cells. They demonstrate that killing activity is suppressed by IL-10 production. The findings are well described and technically sound. Demonstration of in vivo effects merits attention albeit the results of the study overall are not surprising.

Major criticisms:
Fig. 1: In Fig. 1A we see a specific subset producing IL10. What are the frequencies of IL-10 producing cells in normal LN? Are these B cells increased in TD-LN? in Fig. 1C we see an overall shift (increase in MFI, not percentage of cells) that produce Il-10. This should be described and commented.
Where are these cells located (histology)?
Why did the authors look for CD25 expression?
Can the “Killer”-B cells be distinguished from the IL-10 producing B cells?
Clarify why IL-2 was injected. How does the absence of IL-2 influence the result?

Is a 95% purity sufficient to judge on the effects? Could the IL-10 act on contaminating DC or T cells?
What is the cellular composition of the TN-LD cell suspensions w/o B cells?
Please, show gating strategies and results of B cell purity.
Fig. 2: Why is the effect of IL10 depletion only seen when low cell numbers are used for transfer? Are there contaminating cells that produce IL-10? If not, then why is the result achieved with anti-IL10 better?
Did the authors check for differences in leukocyte subpopulations in TD-LN of IL10 Ko versus WT mice? Do cell numbers injected need to be adjusted? Do they represent a bias?
Did the authors check for Fas and CXCL12 expression in T cells of TD-L of WT versus IL-10 KO mice?

Open questions:
Does the IL-10 produced by B cells act in an antigen-dependent manner? Please, discuss.

Minor details
Check phrase “immunofluorescence assay” on page 13.
Fig. 4: symbols are hard to distinguish in black and white
Material and methods:
Please, specifiy source and include citations for
- IL-10 KO mice and transgene used
- Cell lines: 4T1, Renca, TSA
- FGK45 mAb ascites

First revision – authors’ response – 30 October 2014

**Reviewer: 1**

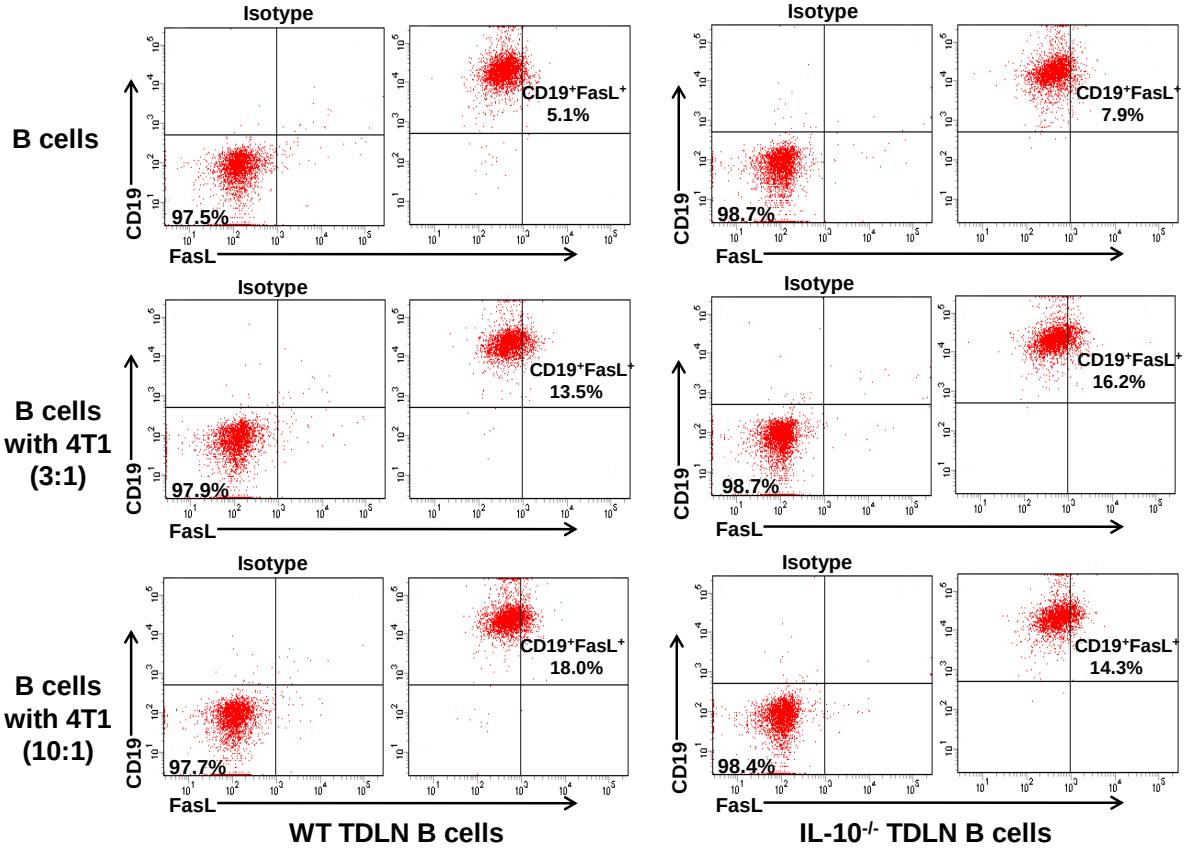
**1. Although the B cells display a convincing cytotoxic activity, it is still much less than the activity of T cells (Fig. 4). Are T cells required for the B cell anti-tumor activity? This would be important to show if the B cells are acting directly, as suggested by this report, or indirectly by inducing T cell activity.**

T cells are not required for B cell anti-tumor reactivity *in vivo*. In our previous report (JI 2009 Ref 7) we demonstrated that the adoptive transfer of purified effector B cells were highly effective in mediating tumor regression of established subcutaneous tumors in hosts that had been preconditioned with total body irradiation (500 cGy) which eliminated host T cells. This clearly indicates that B cells can act directly in causing tumor destruction *in vivo*. We have added this point in the Discussion. The reviewer is also correct that adoptively transferred effector B cells can also induce host T cell activity which was reported in a subsequent study that we published in *Clin Cancer Res* 2011 (Ref 8).

**2. Can the authors show by FACS analysis that B cells are FasL+? If IL-10+ B cells are purified, is FasL expression enriched?**

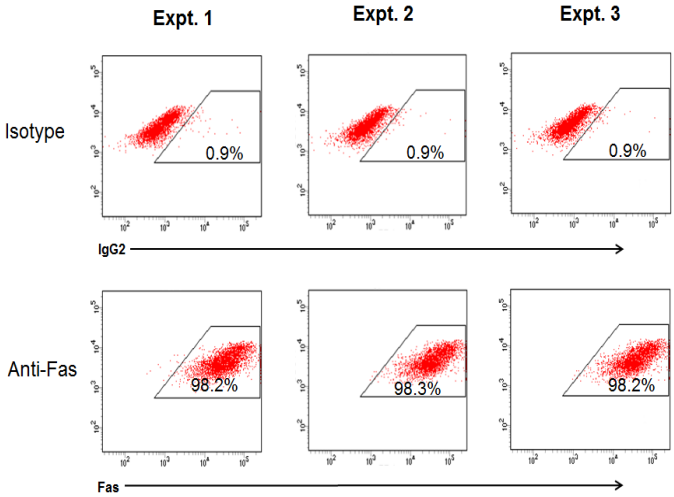
We appreciate these constructive comments, and examined the expression of FasL on B cells by FACS analysis. Because of technical difficulties to purify IL-$10^{+}$ B cells, we alternatively used TDLN B cells isolated from IL-$10^{-/-}$ knockout mice and compared their FasL expression with WT TDLN B cells.

Figure 5B (new)




As shown in **Fig. 5B (new)**, approximately 5% of the purified and anti-CD40/LPS activated/expanded (A/E) WT TDLN B cells expressed FasL. These are the effector cells we used for adoptive transfer; *in vitro* killing assays, and anti-FasL blockade throughout the study. As also observed in **Fig. 5B (new)**, there is a similar percentage (~8%) of the IL-$10^{-/-}$ TDLN B cells expressing FasL, showing no significant difference between these two types of B cells. Interestingly, we found that when these purified and activated/expanded 4T1 TDLN B cells were co-cultured *in vitro* with 4T1 tumor cells, the FasL expression was increased on the B cells. As revealed on **Fig. 5B (new)**, after the effector WT TDLN B cells were cultured with the target 4T1 cells at the ratio

**New Figure 5C:** Fas expression on 4T1 tumor cells



of 3:1 and 10:1 for 24 hours, the FasL expression on the B cells increased from 5.1% to 13.5% and 18.0% respectively. We observed similar increase of FasL expression on the IL-10$^{-/-}$ TBLN B cells after their co-culturing with 4T1 tumor cells in **Fig. 5B (new).** It is of note that data from **Figs 2A and B** respectively revealed that IL-10$^{-/-}$ B cells are more potent antitumor effector cells than WT B cells (at low doses) both *in vivo* and *in vitro*. This would suggest that B cell-mediated antitumor immunity involves other signaling pathways in addition to Fas/FasL. For this, we have added two more references in this revision (new refs #27 and #34).

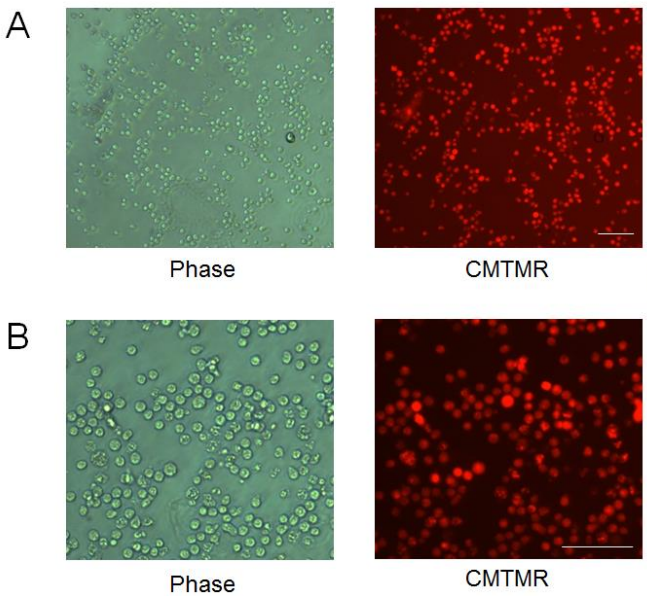
Furthermore, we detected Fas expression in target 4T1 tumor cells, and found that in 3 of the 3 experiments performed, a very high percentage (~100%) of the 4T1 tumor cells expressed Fas (**new Fig. 5C**). 4T1 expression of Fas provides a target for activated TDLN B cells to induce cell death through the Fas/FasL pathway. We have added this in the Materials and Methods, Results, Discussion, and Figure Legends sections.

**3. The link between the B cell/IL-10/FasL story and the AMD3100/CXCR4 story is not very clear. Are the CD19+IL-10+ cells also CXCR4+, or is the CXCR4+ population distinct? Moreover, it is not clear how AMD3100 would affect cytotoxicity since migration in a killing assay should not be a factor. Perhaps the AMD3100/CXCR4 data could be moved to a separate report after it is developed further.**

We agree with this suggestion of the reviewer, and deleted the AMD3100/CXCR4 data (original Figs. 5B, C and D) in this revision.

**4. There are two pieces of data suggesting this system is not entirely physiological. First, a large number of B cells needs to be transferred to see the effect. It might help to know how many of the transferred B cells still survive when metastases are examined. Second, anti-IL-10 does not have any effect when administered without B cells. This would at least suggest that the proposed mechanism is not operating in endogenous B cells during normal tumor immunity. These points should be discussed further by the authors.**

**New Figure 6A-B:** Labeling of B cells with CMTMR

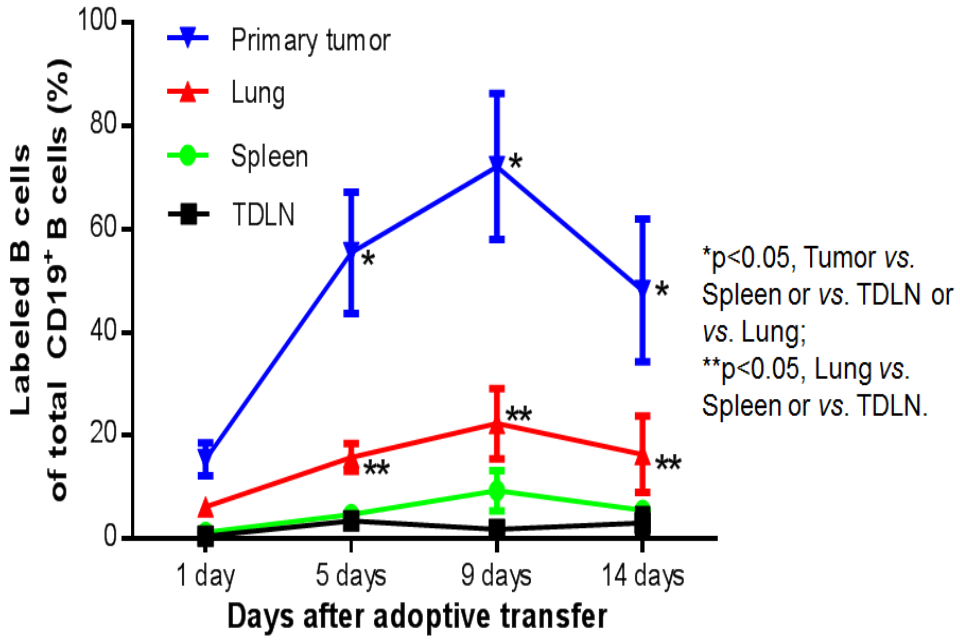



Scale bars represent 5 μm.
A, Original magnification × 100.
B, Original magnification × 200.

Thank you for these constructive comments. To address the first concern regarding how many of the transferred B cells still ***survive*** when metastases are examined, we conducted an

extensive series of additional experiments. In these new experiments, before adoptive transfer, we labeled the activated/expanded TDLN B cells with 10 μM Cell Tracker™ Orange CMTMR (5-(and-6)-(((4-chloromethyl) benzoyl) amino) tetramethyl rhodamine) (*Life Technology, Grand Island, NY* ) at 37°C for 45 minutes in the dark. When observed under flurorescence microscopy, all the B cells were labeled successfully (**new Fig. 6A, B**). After adoptive transfer, spleens, TDLNs, lungs and primary tumors were harvested at different time points to detect the labeled live B cells in these tissues by flow cytometry. **New Fig. 6C** shows the percentage of the labeled and adoptively transferred B cells among the total $CD19^+$ B cells detected in different tissues on different days; including day 14 after B cell transfer when metastases were examined. The results show that low percentage of transferred TDLN B cells were found in the spleen and TDLNs. However, high percentage of transferred B cells was found alive in the lungs and in the primary tumor. These data are associated with the observed inhibition of lung metastases from the primary tumor after B cell adoptive transfer in this study (**Fig 2A, 3, and new Fig 7A**).

**New Figure 6C:** The percentages of labeled B cells found among all the $CD19^+$ B cells detected in different sites of tumor-bearing BALB/c mice




In addition, **New Table 1** shows the absolute number of the labeled and adoptively transferred B cells detected in different tissues on different days as indicated. Clearly, larger numbers of the transferred B cells were found in the spleen and TDLN from day 1 to day 14 after B cell adoptive transfer.

**Tab. 1:** Numbers of transferred B cells detected in tumor-bearing BALB/c mice on days after B cell adoptive transfer as indicated (Mean ± SE)

| | Primary tumor ($\times 10^4$) | Lung ($\times 10^4$) | Spleen ($\times 10^4$) | TDLN ($\times 10^4$) |
|---|---|---|---|---|
| 1 day | 3.67 ± 0.38 | 1.97 ± 0.34 | 7.44 ± 0.97 | 5.33 ± 1.04[a] |
| 5 days | 14.23 ± 1.39 | 5.87 ± 0.77 | 24.15 ± 5.24 | 35.90 ± 2.33[b] |
| 9 days | 17.34 ± 0.58 | 13.41 ± 0.95 | 43.34 ± 4.34 | 28.37 ± 3.03[c] |
| 14 days | 16.18 ± 2.16 | 14.71 ± 1.91 | 42.06 ± 9.55 | 26.87 ± 5.56 |

[a] $p<0.05$, TDLN *vs.* Lung.

[b] $p<0.05$, TDLN *vs.* Primary tumor or *vs.* Lung.

[c] $p<0.05$, TDLN *vs.* Primary tumor or *vs.* Lung or *vs.* Spleen.

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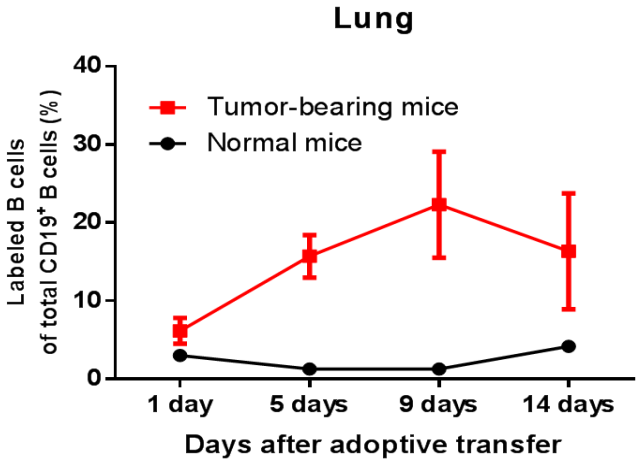
In the above experiments (**new Fig 6C**), we also used normal mice transferred with the similarly labeled TDLN B cells, and found very few transferred B cells in the lung of the normal mice (**new Fig. 6D**) as well as in the spleen and in the LN of the normal mice (data not shown). These results imply that the adoptively transferred B cells traffic to the tumor and the metastasis sites *in vivo*. Data are representative of two experiments performed. We have added these in the Materials and Methods, Results, Discussion, and Figure Legend sections.

**New Figure 6D:** Comparison of detected labeled B cells in the lungs of tumor-bearing mice *vs.* normal mice.




For the second concern, **anti-IL-10 does not have any effect when administered without B cells. This would at least suggest that the proposed mechanism is not operating in endogenous B cells during normal tumor immunity. These points should be discussed further by the authors.**

In addressing the second point, that anti-IL-10 mAb does not impact on endogenous B cells is correct in this setting. We have previously reported (*Journal of Immunology, 1997, 159: 664-673*----we have added this reference in this revision as new ref #32) that in the 3-day established pulmonary metastatic model the *iv* administration of neutralizing IL-10 mAb does not impact on the number of pulmonary metastases compared to untreated mice. This would indicate that the endogenous T and B cell host responses are not sufficiently activated to mediate tumor regression when IL-10 is neutralized. In that same study, we found that adoptive transfer of activated T cells mediated tumor regression that was enhanced by IL-10 neutralization. This latter study plus our current data indicate that the adoptive transfer of either T or B effector cells are necessary to see an effect of IL-10 neutralization.

**Reviewer: 2**

Major criticisms:

1. **In Fig. 1A we see a specific subset producing IL10. What are the frequencies of IL-10 producing cells in normal LN? Are these B cells increased in TD-LN?**

To answer this question, we performed additional experiments, and summarized the data in **new Fig. 1. E-H**. There are almost no IL-10 producing B cells in normal LN (<1% before A/E, **new Fig. 1E**; <2% post A/E, **new Fig. 1G**). However, these IL-10$^+$ B cells are significantly increased in TDLNs (~3% before A/E, **new Fig. 1F**; ~10% post A/E, **new Fig. 1H**). Data are representative of two experiments performed. We have added this in the Materials and Methods, Results, Discussion, and Figure Legend sections.

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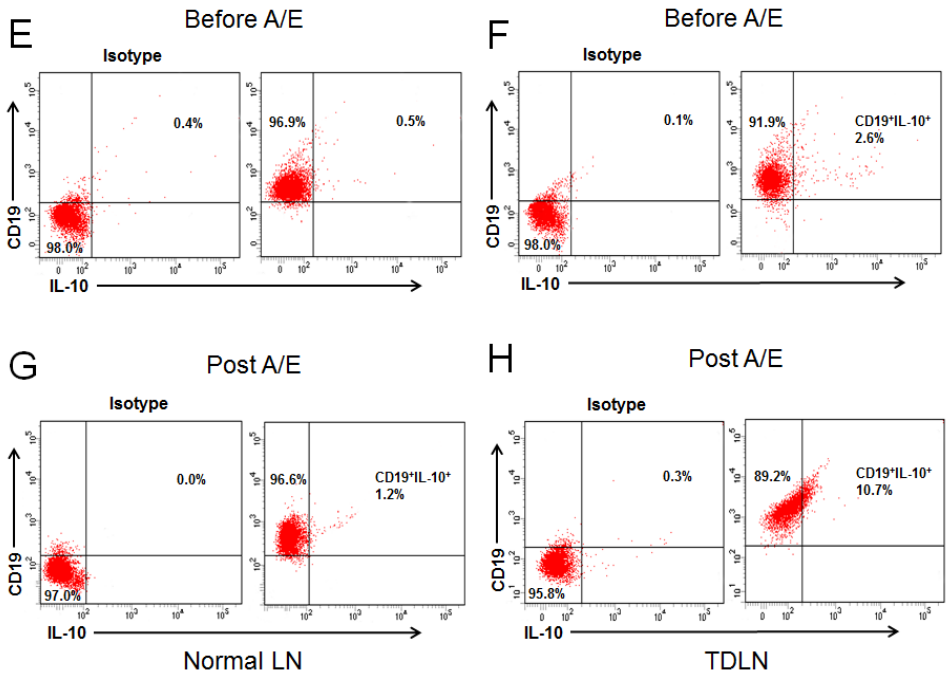
**New Figure 1E-H:** TDLN contains significantly more IL-10-producing cells than normal LN, and its number increases post A/E




**2. In Fig. 1C we see an overall shift (increase in MFI, not percentage of cells) that produce Il-10. This should be described and commented.**

We adopted this suggestion and measured the MFIs. Based on the original data in **Fig. 1C,** the MFI of sample IL-10 is 95 which is higher than the isotope control MFI (14). Similarly, the MFI of sample CD19 is 544 which is obviously higher than isotope control MFI (13).

**3. Where are these cells located (histology)?**

This is a very important issue, and was also raised by **Reviewer #1**. Please see our responses to **Item 4 of Reviewer 1** above.

**4. Why did the authors look for CD25 expression?**

This question is related to question 6 raised by this same reviewer. Please see our responses below to question 6.

**5. Can the "Killer"-B cells be distinguished from the IL-10 producing B cells?**

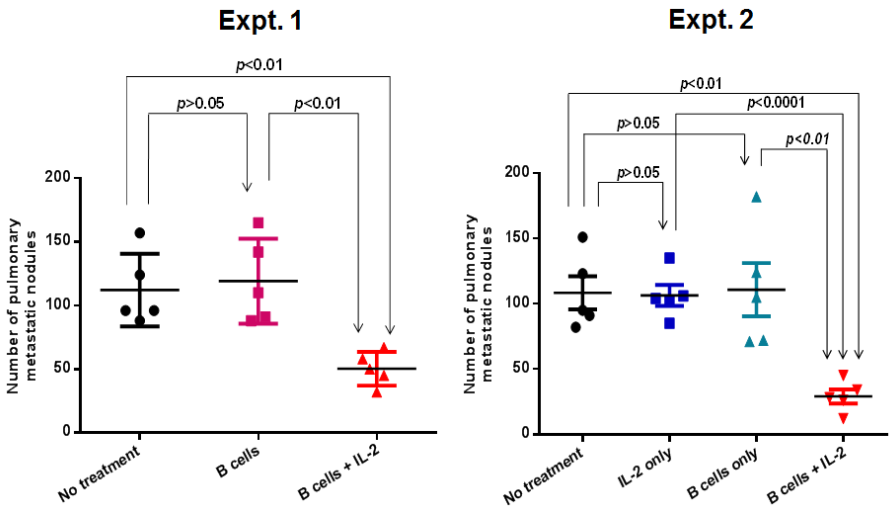
We performed extensive flow cytometry on the TDLN B cells to determine their surface phenotypes and whether co-expression of FasL and IL-10 was apparent. The surface marker expression of the TDLN B cells was very uniform before and after A/E in that almost all B cells (>98%) were of a classical follicular B cell phenotype ($IgM^{low}CD23^{+}CD21^{low}CD5^{neg}CD1d^{low}$). This is very different than previously described phenotypes of mouse killer B cells ($IgM^{high}CD5^{+}$)(Ref 35) or the major IL-10 producing regulatory B cell subsets ($CD5^{+}CD1d^{high}$ B10 cells)(Ref 24) or ($CD21^{high}CD23^{high}$ T2-MZP cells)(Ref 30). We conclude that the TDLN B cells have atypical killer or IL-10 producing regulatory B cells.

Similar to what has previously been shown (Ref 35); the co-staining of IL-10 and FasL could not be demonstrated in TDLN B cells in this study. This is a technical difficulty because the stimulation required to induce IL-10 protein levels measurable by intracellular staining simultaneously results in the loss of FasL expression. From the previous study, it was also demonstrated that sorted $FasL^{+}$ B cells were no better or worse at producing IL-10 than $FasL^{neg}$ B cells, suggesting that the two molecules do not necessarily come from the same B cell.

**6. Clarify why IL-2 was injected. How does the absence of IL-2 influence the result?**

This is a very interesting question. While IL-2 was originally produced as a "T cell growth factor", we have found that it can significantly enhance the antitumor immunity of our B effector cells in adoptive therapy. To investigate the role of IL-2 in adoptive immunotherapy of cancer using effector B cells, we compared the therapeutic efficacy of adoptively transferred WT TDLN B cells with *vs.* without IL-2 administration. Two weeks after 4T1 tumor cells were injected into the mammary fat pad; tumor-bearing WT BALB/c mice were treated with activated TDLN B cells or TDLN B cells plus IL-2 respectively. Another two weeks later, mice lungs were collected to enumerate pulmonary metastases. As shown in **new Fig 7A Expt. 1**, a suboptimal does of WT 4T1 TDLN B cells alone showed no efficacy, but adoptively transferred B cells with IL-2 administration *i.p.* significantly inhibited the metastasis of 4T1 tumor cells from the injection site (mammary fat pad) to the lung. However, IL-2 alone resulted in no significant reduction in pulmonary metastases compared with PBS-treated controls (**new Fig 7A Expt. 2)**. This indicated that exogenous IL-2 administration enhanced the antitumor reactivity of adoptively transferred effector B cells.

**New Figure 7A:** Adoptively transferred TDLN B cells inhibited pulmonary metastasis with IL-2 administration




To understand if the IL-2 is acting upon B cell directly or indirectly, we tested IL-2R (CD25) expression on 4T1 TDLN B cells. **Figs. 7B and C** (was Fig. 1E and F) show that expression of IL-2R on freshly purified TDLN B cells from WT and $IL\text{-}10^{-/-}$ mice was similar (about 10%). Post activation and expansion *in vitro*, expression of IL-2R were increased both on WT and $IL\text{-}10^{-/-}$ TDLN B cells to a

similar level (16-18%) (**Fig.7D, E**, was Fig.1G, H). These results suggest that IL-2 may act on TDLN B cells directly. Data are representative of two experiments performed. We have added these in the Materials and Methods, Results, Discussion, and Figure Legend sections.

**7. Is a 95% purity sufficient to judge on the effects? Could the IL-10 act on contaminating DC or T cells? What is the cellular composition of the TN-LD cell suspensions w/o B cells?**

Using the same protocol to generate effector B cells from 4T1 TDLN, we reported a 98% purity of B cells after activation with LPS/anti-CD40 in a previous report (CCR 2011 Ref 8) as well as in this study (**Fig. 1)**. We do not feel that any contaminating cells within the remaining 2% of cells could contribute to the enhancing effect of the IL-10 neutralization. This would assume that LPS/anti-CD40 culture of contaminating DC or T cells (at 2% of the infused cells) would have an antitumor effect. We are unaware if such activated DC or T cells mediate antitumor reactivity in adoptive therapy.

**8. Please, show gating strategies and results of B cell purity.**

B cells were stained with anti-CD19, and an aliquot of 10,000 cells was analyzed using flow cytometry. Cells were initially gated on forward and side scatter to remove debris and calculated by quadrant dot plot. With this gating strategy, B cell purity is higher than 95% consistently. This shows that the method we used to isolate TDLN B cells is effective. We have added this in the Materials and Methods and **Figure 1 & 5** Legends. An example of the flow analysis has been previously reported by us (CCR 2011 Ref 8).

**9. Fig. 2: Why is the effect of IL10 depletion only seen when low cell numbers are used for transfer? Are there contaminating cells that produce IL-10? If not, then why is the result achieved with anti-IL10 better?**

The effect of IL-10 deletion was seen in the group that received lower numbers of B cells, because that dose of cells in the WT group was sub-therapeutic, thus allowing us to detect an improvement with the B cells obtained from the IL-$10^{-/-}$ knockout mice. At the higher dose of cells, we were observing a maximal therapeutic effect; hence, the deletion of IL-10 was not expected to show a significant enhancing effect.

**10. Did the authors check for differences in leukocyte subpopulations in TD-LN of IL10 Ko versus WT mice? Do cell numbers injected need to be adjusted? Do they represent a bias?**

We did not check the differences in leukocyte subpopulations between WT *vs.* IL-$10^{-/-}$ knockout mice. The B cells used for all experiments were purified from the TDLN of these two groups and subsequently

activated. As illustrated in **Fig 1 A-D**, the percentages of CD19 B cells before and after activation approximated 100% for both groups. We do not believe any adjustment needed to be made between the two groups when it came to *in vitro* and *in vivo* experiments.

**11. Did the authors check for Fas expression in T cells of TD-L of WT versus IL-10 KO mice?**

In this revision, we checked Fas expression on WT and IL-$10^{-/-}$ TDLN T cells. Before A/E, about 70% T cells are $Fas^{+}$ (WT and IL-$10^{-/-}$ TDLN T cells are similar). Post A/E, $Fas^{+}$ TDLN T cells increased and nearly all of the T cells are $Fas^{+}$ (both WT and IL-$10^{-/-}$ TDLN T cells). Considering our focus in this study on the integration of antitumor effector B cells with target tumor cells, we did not include this data in the revision of this manuscript.

**Open questions:**

**Does the IL-10 produced by B cells act in an antigen-dependent manner? Please, discuss.**

We did not examine this question in any experimental manner.

**Minor details**

**Check phrase "immunofluorescence assay" on page 13.**

We have used the term "flow cytometry" in this revision.

**Fig. 4: symbols are hard to distinguish in black and white Material and methods:**

**Please, specifiy source and include citations for**

- **IL-10 KO mice and transgene used**

IL-10 KO (IL-$10^{-/-}$) mice on BALB/c background are homozygous for a targeted mutation in the IL-10 gene. The *Il10*$^{tm1Cgn}$ mutation was achieved by a targeting vector designed to replace codons 5-55 of exon 1 of the targeted gene with a 24 bp linker (providing a termination codon) and a neo expression cassette, as well as introduce a termination codon into exon 3. IL-10 KO (IL-$10^{-/-}$) mice were purchased from the Jackson Laboratories, Bar Harbor, ME. We have added this to Materials and Methods.

- **Cell lines: 4T1, Renca, TSA**

4T1 is a mammary carcinoma syngeneic to Balb/c mice (provided by Dr. M. Sabel, University of Michigan). Renca is a kidney cancer cell line and TSA a highly aggressive mammary adenocarcinoma, and these cell lines are all syngeneic to Balb/c mice and used as specific controls. Renca and TSA are purchased from ATCC (American Type Culture Collection, Rockville, MD). We have added this to Materials and Methods.

- **FGK45 mAb ascites**

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FGK45 mAb ascites were generated using FGK45 hybridoma cells by the Hybridoma Core at the University of Michigan. FGK45 hybridoma cells are purchased from ATCC. We have added this to Materials and Methods.

Second Editorial Decision – 13 November 2014

Dear Prof. Li,

Thank you for submitting your revised manuscript ID eji.201444625.R1 entitled "Antitumor effector B cells directly kill tumor cells via the Fas/FasL pathway and are regulated by IL-10 and IL-2" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referee(s) are included at the bottom of this letter.

Although the referee(s) have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of the referee(s) and revise your manuscript accordingly.

You will see that reviewer 2 feels that despite the wealth of data provided the paper leads to more questions than answers. We feel that clarifying all these questions would expand your manuscript unnecessarily, since sience always opens up new questions. So we ask you to comment on each of the reviewers concerns and re open questions of mechanism in your discussion. The concerns are highlighted in the attached text file.

You should also pay close attention to the editorial comments included below.
**In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.**

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referee(s) to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology. We look forward to receiving your revision.

Yours sincerely,
Karen Chu

# Peer review correspondence

European Journal of Immunology

on behalf of Prof. David Gray

Dr. Karen Chu
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

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Reviewer: 1

Comments to the Author
The authors have adequately addressed my previous concerns and have added new data that add to the mechanistic insight of the presented studies. The manuscript is greatly improved.

Reviewer: 2

Comments to the Author
The authors present a wealth of new data. They have addressed many of the issues raised by the reviewers. However, the following questions remain unanswered:

1. Is the negative regulatory effect of IL-10 on cytotoxicity a cell-autonomous autoregulatory effect or rather an effect promoted by IL-10 secreting cells distinct from those promoting cytotoxicity?
2. How does IL-10 affect the cytotoxicity? It does not seem to influence FasL expression.
3. How exactly does IL-2 enhance cytotoxicity?

Note, that IL-2 is introduced relatively late in the manuscript. In views of the open questions and the length of teh manuscript it might be better to omit the data on IL-2 to prevent dilution of the message on Il-10.
For gating strategies the authors should not refer to previous publications.

Second revision – authors' response – 1 December 2014

Thank you for allowing us to make minor revisions to the above manuscript. We have made all the suggested changes by the Editorial staff. In addition we have made the following revisions:

1. All the figure legends have been revised to follow the Journal's standards.
2. We have deleted the data regarding IL-2R expression and enhancing effects of IL-2 administration as suggested by Reviewer 2. This has resulted in changing the title, shortening the Results section, and eliminating one of the Figures.

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3. We have responded to Reviewer 2's queries by addressing them in the Discussion. All changes in the manuscript have been highlighted in red.
4. As requested by Reviewer 2, we have described the gating strategies used for the flow analysis in the Legends of the appropriate figures, and have not referred to previous publications.
5. We have revised the References to conform to the Journal's standards.

We look forward to having this manuscript published by the Journal.

Third Editorial Decision – 11 December 2014

Dear Prof. Li,

It is a pleasure to provisionally accept your manuscript entitled "Antitumor effector B-cells directly kill tumor cells via the Fas/FasL pathway and are regulated by IL-10" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. David Gray

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