Generation and Characterization of Antibodies Specific for IL-1RAP Antigen To Target Quiescent and Proliferating AML Leukemic Stem Cells

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ABSTRACT

We have identified several cell surface targets preferentially expressed on primary AML blast and leukemic stem cells (LSCs) that are not expressed or expressed at low levels on normal hematopoietic stem cells. We isolated and characterized AML blast and LSCs from AML patients and immunized mice to produce a family of potent, specific, monoclonal antibodies (mAbs) against Interleukin-1 Receptor Accessory Protein (IL1RAP), one of the AML LSC specific targets we identified.

mAb clones were screened, characterized and evaluated *in vitro* and *in vivo* for primary patient AML and IL1RAP$^+$ cell line binding properties. In addition, internalization after binding of several clones was investigated. These mAbs demonstrated preferential binding profiles to primary AML patients' samples and robust anti-tumor activity in cell based assays *in vitro* and a tumor xenograft engraftment model *in vivo*. Several selected clones have a high degree of specific binding and killing of primary AML patient samples (>90%) in complement-dependent cytotoxicity assays. Several IL1RAP mAb clones evaluated have shown potent inhibition of tumor engraftment and growth in an orthotopic AML xenograft tumor mode.

In summary, the binding profiles and *in vitro* and *in vivo* efficacy against patient and AML cells indicates that targeting IL1RAP is a promising therapeutic approach for the treatment of AML and has the potential for effective eradication of AML LSCs and long term remission in the clinic.. These results highlight the potential use of mAbs for generation of conjugates suitable for therapeutic use.

METHODS

Expression Analysis by Flow Cytometry: BMMC or PBMC were isolated from bone marrow aspirate or whole blood by Ficoll separation. Cells were resuspended in staining solution and blocked with either mouse, rat or human IgG prior to staining. CD34-PE and CD38-APC were added followed by addition of Dye488 conjugated antibodies. Antibodies incubated with BMMC or PBMC on ice for 30 min. Cells were washed and resuspended in 200 uL of staining solution with propidium iodide. Cells were analyzed by flow cytometry. A sample was considered positive for target if the ratio of the geometric mean fluorescence intensity of the stained sample and IgG control was greater than 1.5 and more than 5% of the cells expressed the antigen compared with the control sample.

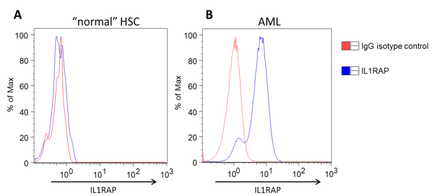
Complement-Dependent Cytotoxicity: Cells were washed and dead cells removed using Live-Dead Kit. Cells were resuspended to a final concentration of 0.8 x 10^6/mL. 50uL of the cell and 50uL of the 2X serial diluted antibody were mixed and allowed to incubate for 10 minutes at room temperature prior to addition of 10uL baby rabbit complement. Control was cell plus complement. The plate was incubated at 37°C for 2 hours before allowing to rest at room temperature for 10 minutes. 100uL of Cell Titre-Glo was added and allowed to shake for 5-10 minutes prior to reading on a luminescent plate reader.

Internalization assay: 2500 cells/50 uL/well of IL-1RAP 293 were plated in 96-well microplates. Primary antibodies and Mab-ZAP were added in a volume of 50 uL. The plates were incubated 72 h at 37°C in the presence of 5% CO_2. For each plate, 100 uL/well of Cell Titre-Glo (Promega) was added and allowed to shake for 5-10 minutes prior to reading on a luminescent plate reader. And data analyzed using GraphPad Prism. A 4-parameter curve is generated using concentration and percent of cells killing.

Efficacy study in orthotopic EOL-1 AML xenograft engraftment model: 5 x 10^6 viable EOL cells injected IV into NOD/SCID mice. mAbs dosing started 1 day prior to tumor challenge. 10mg/kg Q3d, 7 doses total N=6 mice/group.

RESULTS

Figure 1. Expression of IL1RAP on normal HSC and AML



A bone marrow sample from **(A)** a healthy volunteer and **(B)** an AML patient sample were stained with CD34, CD38 and IL1RAP antibodies. Shown are histograms of the IL1RAP expression (blue line) in direct comparison to an appropriate isotype control antibody (red line).

Figure 2A. Several anti-IL1RAP antibody clones bind AML patient AML samples of all subtypes
Figure 2B. Several lead clones selected based on AMP sample binding

A

FAB subtypes:								
Total	M1	M2	M4	M5	Other	NA	BM	PBMC
70/74	18/19	16/18	16/16	9/9	5/6	6/6	4/4	66/70

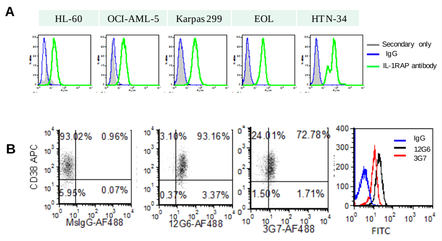
NCCN cytogenetic risk category:			
Good	Inter	Poor	NA
8/8	33/35	20/23	8/8

B

Clone	42.3G6	46.1D2	50.3G7	52.8E5	56.7A9	46.2C2	46.8G1	41.10C2	42.4D11
# of Positive	14/24	16/23	15/15	8/11	6/13	7/14	13/22	13/29	14/21

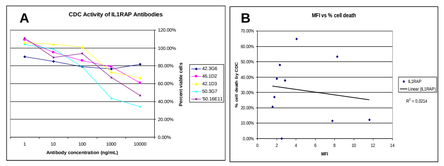
(A) Aggregated data from IL1RAP mAb staining of AML patient samples segmented by FAB subtypes and NCCN cytogenetic risk category (B) Several anti-IL1RAP clones were screened for binding AML patient samples

Figure 3A. Clone 12G6 binds IL1RAP$^+$ AMP and T lymphoma cell lines
Figure 3B. AML FACS staining profile for clones 12G6 and 3G7



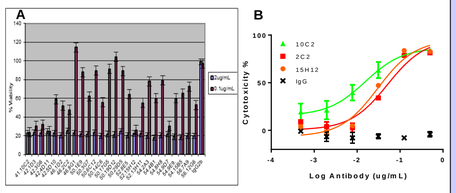
(A) Fluorescence intensity of AML cell lines stained with clone 12G6 (green line) or isotype control (blue line). (B) Representative flow cytometry dot plots and histograms showing binding of IL-1RAP clones 12G6 and 3G7 on AML PBMC.

Figure 4. CDC activity of IL1RAP antibodies against primary AML blasts



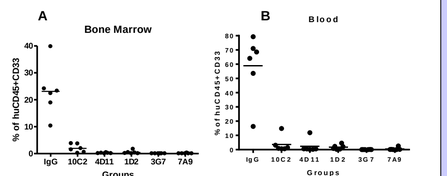
(A) CDC induced by mAbs, AML PBMC were incubated with increasing concentrations of mAbs in the presence of baby rabbit complement. (B)the plot of IL1RAP mean fluorescence index vs. CDC potency. A trendline was plotted to determine if a correlation between MFI vs % death could be identified

Figure 5. Anti-IL1RAP antibodies internalization



(A) Twenty-six mAbs were tested for internalization activity by using mAbZAP as a conjugated secondary reagent. The blue bar represents mAb concentration at 2ug/mL and purple bar represents mAb concentration at 0.1ug/mL (B) dose titration of three mAbs' internalization

Figure 6. In Vivo Activity of IL1RAP mAb clones in the orthotopic EOL-1 AML xenograft engraftment Model



A total of five mAbs were selected that bind to various epitopes and tested for in vivo activity in bone marrow (A), spleen and blood (B) of animals transplanted with an aggressively growing AML cell line EOL-1.

Conclusion

We have isolated and characterized AML blast cells and leukemic stem cells (LSCs) enriched from AML patients and identified cell surface targets selectively expressed on AML blast cells and LSCs and not their normal hematopoietic stem cell (Fig. 1).

Candidate mAb clones were screened on 74 AML patient samples and 70 (94.6%) of the samples were bound by at least one clone (Fig. 2A). The pool of candidate clones did not favor any specific FAB subtype or any cytogenetic risk category (Fig. 2B).

Clones 12G6 and 3G7 were further evaluated for binding to several AML cell lines (Fig. 3A) and additional AMP patient samples (Fig. 3B).

Preliminary results from complement dependent cytotoxicity experiments suggest variability of efficacy between various mAb clones. Clone 50.3G7 exhibited a strong dose-dependent killing of primary AML cells in addition to near complete detection of IL1RAP surface expression on blasts (Fig. 4A). Our initial results suggest IL1RAP targeted killing of primary AML blasts is not correlated with IL1RAP expression levels (Fig. 4B).

Candidate clones were tested for internalization on IL1RAP$^+$ expressing 293 cells at 2 ug/mL concentration and several were shown to be efficiently internalized (Fig. 5A). Additionally, three clones showed promising *in vitro* cell killing using a piggyback saporin conjugated goat anti-mouse secondary reagent. For these three clones tested, the internalization and killing was dose dependent (Fig. 5B).

A total of five clones were selected that bound to various epitopes on IL1RAP and tested for *in vivo* inhibition of tumor engraftment activity. All antibody clones showed *in vivo* activity with more than 90% inhibition of tumor engraftment in bone marrow, spleen and blood of animals injected with an aggressively growing AML cell line (Figure 6).

SUMMARY

We have demonstrated that IL1RAP is a promising therapuetic target on AML LSCs. An initial family of antibodies generated against IL1RAP have demonstrated specific binding and CDC-mediated killing of AML cells from patients. Additionally, these antibodies have shown inhibition of AML tumor cell line engraftment and outgrowth in an orthotopic xenograft model. These data support further development of IL1RAP targeting antibodies for the treatment of AML patients.