Characterization of a Channel Catfish, *Ictalurus punctatus*, Cytotoxic Cell Marker, CC41

Mohadetheh Moulana, Melanie Wilson, Norman W. Miller and Eva Bengtén
Department of Microbiology, University of Mississippi Medical Center
Jackson, MS

Abstract

Although NK-like clonal cells in teleost have been partially characterized, no cell surface marker that definitively defines fish NK-like cells has been identified. To this end, a monoclonal antibody (mAb) against the catfish NK-like cytotoxic cell TS.10.1 was generated. The mAb was designated CC41 and was produced by immunizing Balb/c mice with TS.10.1 cells and screening the resultant hybridomas for their ability to produce antibody reactive with catfish NK clones and peripheral blood leukocytes (PBL). CC41 mAb differentially reacts with catfish lymphocytes and appears to identify a subpopulation of catfish spontaneous cytotoxic cells from PBL. Cell separation studies revealed that this CC41 reactive PBL subpopulation exhibited high levels of nonspecific allocytotoxic activity. Also catfish leukocytes treated *in vitro* with lipo-polysaccharide (LPS) exhibited an increase of CC41 staining 7-10 days after stimulation. When these cultures were analyzed by RT-PCR, TCR α, β, γ, and δ messages were amplified, showing that T cells were present. Similarly, chromium release assays revealed that the LPS stimulated cultures had cytotoxic activity. However, CC41 blocking experiments suggested that CC41 ligand is not involved in either cell activation or cytotoxic events. Interestingly, immunoprecipitation assays revealed that the CC41 ligand was approximately 150 KD in size, a molecular weight, which is similar to the traditional mammalian NK cell marker (CD56). Attempts are currently directed towards definitely identifying the CC41 ligand by mass spectrometry sequencing of CC41 immunoprecipitated protein.

Supported by United States Department of Agriculture (2006-35204-16880) "US Veterinary Immune Reagent Network".

Catfish TS10.1 NK-like Cells

- Spontaneously kill some, but not all allogeneic targets
- Negative for T cell receptor messages (TCR α, β, γ, and δ)
- Negative for Sudan Black staining (neutrophil marker)
- Negative for non-specific esterase (mono/macrophage marker)
- Do not express Igμ message, but express IgM on their surface via an FcμR

To identify catfish cytotoxic cell populations it is necessary to identify a unique cell surface marker.

Production of a Monoclonal Antibody

Protocol:

- Mice were immunized with catfish TS.10.1 NK-like cells.
- Hybridomas were screened for their ability to differentially stain catfish PBL.
- A potentially useful monoclonal antibody (designated CC41) was found.
- CC41 reacts with TS10.1 cells and a subpopulation of spontaneous cytotoxic cells in PBL.

Do Catfish Clonal Cell Lines Express CC41 Ligand on Their Surface?

Expression of CC41 ligand on various catfish cell lines

Cell lines	autonomous	Cell type	Reactivity with mAb CC41[α]
3H9	–	NK-like	++
4G4	–	NK-like	++
10.1	–	NK-like	++
2E12	–	NK-like	++
3B6	–	NK-like	++
2E10	–	NK-like	++
TS32.15	–	CTL (strict specificity)	+
TS32.17	–	CTL (broad specifity)	+
TS32.43	–	CTL (broad specifity)	+
28S	+	Noncytotoxic T cells	+/–
G14D	+	Noncytotoxic T cells	+/–
1G8	+	B cells	+/–
3B11	+	B cells	+/–

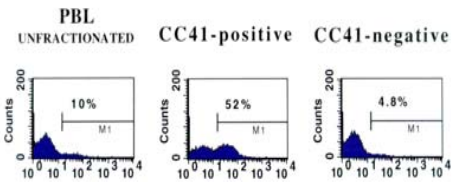
[α] Antigen expression was determined by immunofluorescent staining of the cells: ++, bright fluorescence; +, intermediate to low fluorescence; +/–, dim fluorescence.

Does CC41 React with Cytotoxic Cells?

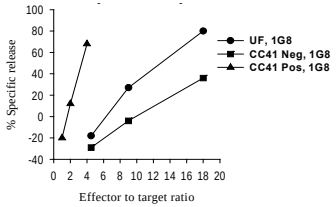
Protocol:

- PBL were sorted into CC41$^+$ and CC41$^-$ populations by Magnetic Activated Cell Sorting (MACS).
- Sorted cells were assayed for spontaneous cytotoxic activity against allogeneic target cells by ^{51}Cr-release.

A.



B.



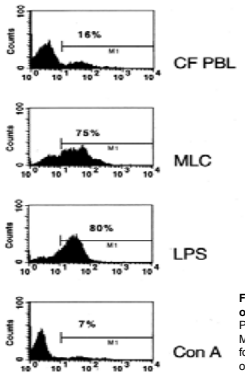
Identification of a population of nonspecific cytotoxic cells from PBL by mAb CC41. (A) Freshly isolated PBL were stained by mAb CC41 and separated into CC41 positive and CC41 negative cells by MACS. (B) Unfractionated PBL, CC41 positive and CC41 negative cells were analyzed for their cytotoxicity against ^{51}Cr labeled allogeneic 1G8 targets. Each Effector to Target condition was performed in triplicate.

CC41 positive cells show 5-6 fold increase in spontaneous cytotoxic activity Compared to unfractionated PBL.

Does Mitogen Stimulation Up-regulate CC41 Ligand Expression?

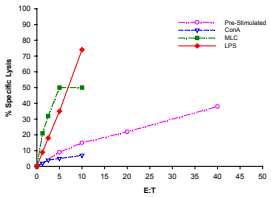
Protocol:

- Freshly isolated PBL were stimulated *in vitro* with:
  - alloantigen (3B11 or 1G8 cells)
  - LPS (200 μg /ml) at a cell density of 5 x10^5
  - Con A (50 μg /ml) at a cell density of 5 x10^5
- Analyzed with flow cytometry for CC41 staining on day 7.



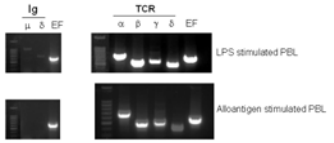
Flow cytometric analysis of CC41 ligand on activated catfish PBL. Freshly isolated PBL were isolated and stimulated *in vitro* by MLC (alloantigen = 1G8 cells), LPS or ConA for 7 days. Data shown is one representative out of five fish.

Do Catfish Mitogen Stimulated PBL have Cytotoxic Activity?



PBL stimulated by MLC or LPS exhibit high cytotoxic activity. PBL were stimulated by MLC (irradiated 3B11), ConA or LPS. On day 7, the cells were washed and tested for their ability to kill ^{51}Cr-labled 3B11 targets. Effectors (E) and targets (T) were incubated at various ratios for 4 hours. Each E:T condition was performed in triplicate.

Do Catfish Mitogen Stimulated PBL Express message for TCR or IgM (μ)?

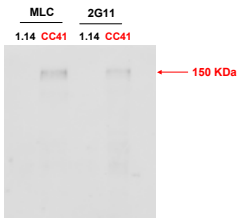


Expression of IgM (μ), IgD (δ), TCR α, β, γ, and δ transcripts in catfish PBL stimulated by LPS or MLC (irradiated 3B11B cells). RT-PCR was performed using gene specific primers. Amplification of Elongation factor 1α (EF) was used as a positive control. Product sizes: Igμ, 951bp; Igδ, 628bp; TCRα, 663 bp; TCRβ, 461 bp; TCRγ, 484 bp; TCRδ, 400bp; and EF1a: 561 bp.

What is CC41 Ligand?

Protocol:

- Catfish alloantigen-stimulated NK-like cells were surface biotinylated and lysed in 1% NP40.
- Lysates were immunoselected by either mAb CC41 or 1.14 (isotype control).
- Immunoselected proteins were separated by reducing SDS-PAGE, transferred to a nitrocellulose membrane, and visualized by chemiluminescence utilizing HRP-conjugated streptavidin.



CC41 mAb identified a ~150 KDa surface molecule in both MLC and the 2G11 NK-like cell line.

Conclusions

- CC41 mAb appears to identify NK and cytotoxic T cells in catfish.
- The CC41 ligand is up-regulated by *in vitro* stimulation of PBL with both alloantigen and LPS.
- CC41 mAb recognizes a 150 KD surface molecule.
- Future studies include identifying the CC41 ligand by mass spectrometry.