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Research paper

Deep immune profiling by mass cytometry links human T and NK cell differentiation and cytotoxic molecule expression patterns

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ABSTRACT

The elimination of infected or tumor cells by direct lysis is a key T and NK cell effector function. T and NK cells can kill target cells by coordinated secretion of cytotoxic granules containing one or both pore-forming proteins, perforin and granulysin and combinations of granzyme (Gzm) family effector proteases (in humans: Gzm A, B, K, M and H). Understanding the pattern of expression of cytotoxic molecules and the relationship to different states of T and NK cells may have direct relevance for immune responses in autoimmunity, infectious disease and cancer. Approaches capable of simultaneously evaluating expression of multiple cytotoxic molecules with detailed information on T and NK differentiation state, however, remain limited. Here, we established a high dimensional mass cytometry approach to comprehensively interrogate single cell proteomic expression of cytotoxic programs and lymphocyte differentiation. This assay identified a coordinated expression pattern of cytotoxic molecules linked to CD8 T cell differentiation stages. Coordinated high expression of perforin, granulysin, Gzm A, Gzm B and Gzm M was associated with markers of late effector memory differentiation and expression of chemokine receptor CX3CR1. However, classical gating and dimensionality reduction approaches also identified other discordant patterns of cytotoxic molecule expression in CD8 T cells, including reduced perforin, but high Gzm A, Gzm K and Gzm M expression. When applied to non-CD8 T cells, this assay identified different patterns of cytotoxic molecule co-expression by $CD56^{hi}$ versus $CD56^{dim}$ defined NK cell developmental stages; in CD4 T cells, low expression of cytotoxic molecules was found mainly in TH1 phenotype cells, but not in Tregs or T follicular helper cells (TFH). Thus, this comprehensive, single cell, proteomic assessment of cytotoxic protein co-expression patterns demonstrates specialized cytotoxic programs in T cells and NK cells linked to their differentiation stages. Such comprehensive cytotoxic profiling may identify distinct patterns of cytotoxic potential relevant for specific infections, autoimmunity or tumor settings.

1. Introduction

In response to infections or transformation, T and NK cells can directly kill target cells. This effector function can be exerted by the ligation of death receptors or by coordinated secretion of cytotoxic granules containing pore-forming proteins (perforin) and effector proteases (e.g., granzyme (Gzm) family, granulysin) (Voskoboinik et al., 2015). These granules are delivered to the interface of the cytotoxic lymphocyte and target cell where, upon release, perforin monomers insert into the target cell membrane and polymerize to form a pore. Granule contents including the effector protease enzymes are delivered through this pore and subsequently cleave key intracellular proteins to initiate a cascade of apoptotic and non-apoptotic cell death. Although Gzm B has been studied most extensively, multiple Gzms, (A, B, K, M and H) are expressed by human cytotoxic lymphocytes. While other functions of Gzms exist and there may be non-perforin mechanisms of Gzm uptake in target cells (Wensink et al., 2015), this coordinated cytotoxic molecule pathway likely represents the canonical cytotoxic mechanism used by CD8 T and NK cells to combat infected or transformed host cells.

Expression of perforin is critical for the killing capacity of T cells and has been linked to control of HIV (Harari et al., 2009; Hersperger et al., 2010). Virus-specific T cells targeting persistent, yet controlled CMV

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infection express high levels of perforin and have high killing capacity (Harari et al., 2009). In contrast, T cells in highly viremic HIV- or HCV-infected patients express low levels of perforin, suggesting that absence of full cytotoxic capacity favors viral persistence (Appay et al., 2000; Zhang et al., 2003; Hersperger et al., 2010; Jo et al., 2012). Granulysin, a member of the saposin-like protein family, can facilitate Gzm delivery and cell death through bacterial walls (Walch et al., 2014), likely explaining its prominent role in antifungal and anti-tuberculosis responses (Stenger et al., 1998; Ma et al., 2002). Thus, T cells can employ distinct cytotoxic mechanisms to combat differing pathogens.

In addition to the role of cytotoxic cells in infection, the historical appreciation of a requirement for perforin- and cytotoxic molecule mediated killing for the elimination of cancer cells (Kagi et al., 1994; Voskoboinik et al., 2015) recently received renewed attention by the identification of a cytotoxic signature associated with outcome in cancer (Rooney et al., 2015). These studies used large genome-scale analyses of solid tissue biopsies to reveal a link between the presence of a cytolytic signature, neoepitope load, immunoediting and disease progression across various cancers (Rooney et al., 2015). Indeed, the highest expression of *GZMA* and *PRF1* in tumor biopsies was linked to favorable survival (Rooney et al., 2015). However, it remains currently unclear whether distinct cytotoxic cell types and/or specific patterns of cytotoxic molecule expression are directly responsible for the prolonged survival. For example, it remains unclear whether these signatures stem from cytotoxic CD8 T cells, cytotoxic CD4 T cells, NK cells or additional cell types. Further, how expression of the different components of the lytic machinery in cytotoxic cells is coordinated remains poorly understood.

The cytotoxic potential of CD8 T cells is low in naïve T cells and induced during priming and differentiation to effector cells. Whereas all Gzms are thought to be able to induce cell death based on high-dose in vitro killing studies, in vivo functions of distinct Gzms may differ (Joeckel and Bird, 2014). For example, different Gzms have been reported to be involved in degrading extracellular matrix, modulating proinflammatory cytokines (Wensink et al., 2015), or exerting direct antiviral effects (van Domselaar et al., 2010), suggesting that Gzm family members may have evolved for specialized functional roles. In this context, it is interesting to note that T cells may also express different combinations of Gzms. For example, while Gzm expression is lacking in naïve T cells, the majority of memory T cells co-express either Gzm A and Gzm K or Gzm A and Gzm B, with a low number of CD8 T cells simultaneously expressing Gzm A, Gzm B and Gzm K (Bratke et al., 2005). In some settings, the co-expression patterns of these Gzms were linked to the expression of memory or effector markers of CD8 T cells, (Bratke et al., 2005; Harari et al., 2009). In other studies, expression of Gzm M and granulysin was associated with antigen-experienced differentiated T cell subsets (Bruns et al., 2009; de Koning et al., 2010). In addition to studies analyzing the relationship of differentiation markers to cytotoxic molecule expression, expression of the fractalkine receptor CX3CR1 was recently described as a useful marker of cytotoxic memory T cells in humans (Bottcher et al., 2015). However, in that study, analysis of protein expression of cytotoxic molecules was limited to Gzm B and perforin. These reports suggest that T cells may co-express different sets of cytotoxic molecules and that these patterns may reflect distinct specialized cytotoxic programs related to the state of T cell differentiation. However, in these studies, use of conventional flow cytometry limited the number of cytotoxic and differentiation molecules that could be simultaneously examined, and prevented comprehensive analysis of cytotoxic molecule and differentiation marker expression patterns. Further, it remains unclear if cytotoxic programs are shared across different lineages of cytotoxic cells (e.g., NK cells, CD8 and CD4 T cells), and how cytotoxic programs influence disease.

Addressing these questions requires the concurrent analysis of multiple cytotoxic molecules, lineage and differentiation markers. The use of mass cytometry allows the simultaneous analysis of >40 proteins on single cells providing high-dimensional data necessary for insights into complex cellular expression patterns (Spitzer and Nolan, 2016). In this work, we developed a mass cytometry panel suitable for the analysis of combinatorial cytotoxic molecule expression across NK and T cell lineages. The comprehensive, single cell, proteomic assessment of cytotoxic protein co-expression patterns demonstrates specialized cytotoxic programs in T cells and NK cells linked to their differentiation stages. Cytotoxic profiling has implications for the understanding of T cell function in infection, autoimmunity and tumor immunology.

2. Methods

2.1. Reagents

Mass cytometry antibodies were obtained as pre-conjugated metal-tagged antibodies (abs) from Fluidigm or generated in-house by conjugating unlabeled purified abs to isotope-loaded polymers using MAXPAR kits (Fluidigm). Based on titration performance, antibodies were diluted in antibody stabilization buffer (Candor Bioscience). A detailed list of the antibodies used in this study is provided in Table 1. Antibody staining performance for Gzm H was not satisfactory and hence was not interrogated in detail. ^{113}In, ^{115}In and ^{139}La isotopes not available through Fluidigm were obtained from Trace Sciences, Inc. For viability discrimination, maleimido-mono-amine-DOTA (Macrocyclics) was dissolved in MAXPAR L-Buffer (Fluidigm) and mixed with ^{139}La (L/D-139) for a 0.5 mM final concentration.

Table 1
Antibodies and panel information.

Isotope channel	Antibody/reagent name	Clone	Source	Category
89 Y	CD45	HI30	Fluidigm	Lineage
113 In	CD45RO	UCHL1	BD	Differentiation
115 In	CD57	TB01	Ebioscience	Differentiation
139 La	L/D MM-DOTA		In-house	Viability
140 etc.	Beads		Fluidigm	QC
141 Pr	CD3	UCHT1	Biolegend	Lineage
142 Nd				
143 Nd	CD4	RPA-T4	Biolegend	Lineage
144 Nd				
145 Nd	Granulysin	DH2	Biolegend	Cytotox
146 Nd	CD8	RPA-T8	Biolegend	Lineage
147 Sm	CD45RA	H100	BD	Differentiation
148 Nd	CD95	DX2	Biolegend	Differentiation
149 Sm	CD14	M5E2	Biolegend	Lineage
150 Nd	CD127	HIL-7R-M21	BD	Differentiation
151 Eu				
152 Sm	Granzyme B	CLB-GB11	Novus	Cytotox
153 Eu				
154 Sm	Granzyme K	GM6C3	Santa Cruz	Cytotox
155 Gd	CD27	L128	Fluidigm	Differentiation
156 Gd				
158 Gd	PD-1	EH12.2H7	Fluidigm	IR
159 Tb	CCR7	G043H7	Fluidigm	Differentiation
160 Gd	Tbet	4B10	Fluidigm	Differentiation
161 Dy	CD28	CD28.2	Biolegend	Differentiation
162 Dy	Foxp3	PCH101	Fluidigm	Treg
163 Dy				
164 Dy				
165 Ho	Eomes	WD1928	Ebioscience	Differentiation
166 Er	Perforin	B-D48	Abcam	Cytotox
167 Er				
168 Er	CX3CR1	2A9-1	Biolegend	Cytotox
169 Tm	TIGIT	MBSA43	Ebioscience	IR
170 Er	CXCR5	RF8B2	BD	TFH
171 Yb	2B4	C1.7	Biolegend	IR
172 Yb	Granzyme A	CD9	Biolegend	Cytotox
173 Yb	Granzyme M	4B2G4	Bovenschen	Cytotox
174 Yb				
175 Lu	Granzyme H	ARP42686_T100	Aviva pAb	failed QC
176 Yb	CD56	HCD56	Fluidigm	Lineage
191/193	Iridium			DNA
209 Bi	CD16	3G8	Fluidigm	Lineage

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