Immune effectors responsible for the elimination of hyperploid cancer cells

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ABSTRACT

The immune system avoids oncogenesis and slows down tumor progression through a mechanism called immunosurveillance. Nevertheless, some malignant cells manage to escape from immune control and form clinically detectable tumors. Tetraploidy, which consists in the intrinsically unstable duplication of the genome, is considered as a (pre)-cancerous event that can result in aneuploidy and contribute to oncogenesis. We previously described the fact that tetraploid cells can be eliminated by the immune system. Here, we investigate the role of different innate and acquired immune effectors by inoculating hyperploid cancer cells into wild type or mice bearing different immunodeficient genotypes (Cd1d\textsuperscript{−/−}, FcγRIγ\textsuperscript{−/−}, Flt3\textsuperscript{−/−}, Foxn1\textsuperscript{nu/nu}, MyD88\textsuperscript{−/−}, Nramp3\textsuperscript{−/−}, Igκ\textsuperscript{−/−}IκB\textsuperscript{−/−}, Rag2\textsuperscript{−/−}), followed by the monitoring of tumor incidence, growth and final ploidy status. Our results suggest that multiple different immune effectors including B, NK, NKT and T cells, as well as innate immune responses involving the interleukine-1 receptor and the Toll-like receptor systems participate to the immunoselection against hyperploid cells. Hence, optimal anticancer immunosurveillance likely involves the contribution of multiple arms of the immune system.

Introduction

The immunosurveillance concept was defined by Sir Macfarlane Burnet in 1970\textsuperscript{1} as a mechanism through which the immune system eliminates or inactivates potentially dangerous cells. Liver fibrosis, ischemic damage to various tissues (such in the brain, heart, liver or kidneys), as well as neurodegenerative disorders (Alzheimer, Parkinson and Huntington disease) are subjected to immunosurveillance. However, the role of the immune system is best studied in the context of the suppression of oncogenesis and the control of tumor progression.\textsuperscript{2}

Anticancer immunosurveillance can be didactically divided into three phases, namely (i) elimination, (ii) equilibrium and (iii) escape. During the elimination phase, potentially oncogenic, pre-malignant cells are destroyed and no tumors are detectable. During the equilibrium state, a smoldering inflammatory/immune response occurs in subclinical tumors, confronting malignant cells and leukocytes in a continuous battle. It is only upon escape of cancer cells from immune control that clinically detectable and potentially lethal cancers develop.\textsuperscript{3}

Both genomic instability and immune evasion are hallmarks of cancer.\textsuperscript{4} One cause of genomic instability is tetraploidy (cells with a duplicated set of chromosomes), which favors aberrant mitoses leading to asymmetric cell divisions with the consequent generation of aneuploid cells (cells with abnormal numbers of chromosomes), as they are characteristic of malignant cancers.\textsuperscript{5} An ever-expanding literature places tetraploidy at the early steps of carcinogenesis,\textsuperscript{6-14} coinciding with the elimination step of the cancer immunosurveillance process.\textsuperscript{3,15,16} As a matter of fact, immunosurveillance failure leads to the accumulation of hyperploid cells in different murine carcinogenesis models as well as in patients with breast cancer that fail to respond to neoadjuvant chemotherapy.\textsuperscript{17} The mechanism through which hyperploid cells are recognized by the immune system implies constitutive endoplasmic reticulum stress resulting in the exposure of the ‘eat-me’ signal calreticulin (CALR) at the cell surface. Thus, hyperploid cells fail to form tumors or form slowly-growing cancers in mice, unless such animals bear a severely immunocompromised phenotype. Immunelected (but not unselected) tumors forming from initially hyperploid cells exhibit a reduced DNA content and a diminished CALR exposure\textsuperscript{17-19} thus losing their immunogenicity.\textsuperscript{17} Along the same lines, it has been recently reported that aneuploidy correlates with immune evasion markers in melanoma patients treated with anti-cytotoxic T lymphocyte – associated protein 4 (CTLA-4) therapy.\textsuperscript{20}

It is known that CD4\textsuperscript{+} and CD8\textsuperscript{+} T lymphocytes, the interferon system\textsuperscript{17} and NK cells\textsuperscript{21} are involved in the control of hyperploid and (pre)-malignant cells.\textsuperscript{22-25} Here, we investigated the role of a wide range of immunodeficiencies to shed the...
light on the complete immune cell mechanism involved in the recognition and elimination of hyperploid cancer cells.

**Materials and methods**

**Reagents**

Unless otherwise indicated, media and supplements for cell culture were purchased from Gibco-Invitrogen (Carlsbad, CA, USA), plasticware from Corning B.V. Life Sciences (Schiphol-Rijk, The Netherlands), and chemicals from Sigma-Aldrich (St Louis, MO, USA).

**Cell lines and culture conditions**

All cell lines were cultured at 37°C under 5% of CO₂, in the appropriate medium containing 10% fetal bovine serum (FBS) and 100 U mL⁻¹ penicillin sodium and 100 μg mL⁻¹ streptomycin sulfate. Cell type-specific culture conditions include: RPMI 1640 medium supplemented as above plus 1 mM sodium pyruvate and 1 mM HEPES buffer for murine fibrosarcoma MCA205 cells; RPMI 1640 medium supplemented with 10% FBS, 100 U mL⁻¹ penicillin sodium, 1 mM sodium pyruvate, 1 mM HEPES buffer and 1 mM non-essential amino acids for murine lymphoma EL4 cell line.

**Generation of hyperploid clones**

Parental MCA and EL4 cells were treated for 48 h with 100 nM nocodazole and then cultured for 2 weeks in drug-free culture medium to generate hyperploid clones. Parental MCA and EL4 cells were treated for 48 h with 100 nM nocodazole and then cultured for 2 weeks in drug-free culture medium to generate hyperploid clones.

**Mice**

Mice were maintained in pathogen-free conditions, and in specific pathogen-free conditions in a temperature-controlled environment with 12 h light, 12 h dark cycles and received food and water ad libitum. Experiments followed the Federation of European Laboratory Animal Science Association (FELASA) guidelines. Protocol n° APAFIS#5251-2016050409384642v2 for animal experimentation followed the EU Directive 63/2010 and was approved by the Ethical Committee of the Gustave Roussy Institute, France.

**Histology and immunohistochemistry**

Samples from recovered tumors were fixed with 4% PFA for 4 h and then embedded into paraffin. Sections of 5 μm were stained with the Discovery Ultra automated IHC/ISH research slide staining system (Ventana Medical Systems – Roche Group, Tucson, AZ, USA). Heat-induced antigen retrieval in EDTA buffer (pH 8.0) for 32 min at 95°C and then a counterstain with Hematoxylin II for 12 min followed by Bluing Reagent for 8 min (Ventana Medical Systems – Roche Group, Tucson, AZ, USA). After staining, images were acquired with a Virtual Slides microscope VS120-SP (Olympus, Tokyo, Japan), 20X air objective (0.75 NA). VSI-code images were converted to the TIFF file format and analyzed by means of a morphometric analysis as previously described.

**Statistical analysis**

Tumor incidence was analyzed by one-tailed Barnard’s test at the end point: parental vs hyperploid (P vs H) with “no lower tumor incidence in H” as H₀; hyperploid into immunodeficient vs WT mice (H vs WT) with “no higher tumor incidence in immunodeficient mice” as H₀. Tumor growth modeling was carried by linear mixed effect modeling on log pre-processed tumor surfaces and reported p values are obtained from Wald’s test analysis for P vs H, parental into immunodeficient vs WT mice (P vs WT) and H vs WT. Statistical comparison of nuclear area was applied on the full set of distributions (Supplementary Figs. 2–5). Considering that the log₁₀ of nuclear area depends on the DNA content of injected cells (parental / hyperploid cells), the mouse strain (WT, Rag2⁻/⁻, FcRn⁻/⁻, Foxn1nu/nu, MyD88⁻/⁻, CD1d⁻/⁻, Ighm⁻/-h, Nlpr3⁻/- or Flt3l⁻/-) and the mouse replicate, we applied the following linear mixed model: (LogArea ~ DNA content / Mouse, random = ~ 1 | Mouse_Replicate), by using the ‘lme’ function of ‘nlme’ package in R (https://CRAN.R-project.org/package = nlme.), p-values are the ones associated with the mouse dependent ‘DNA content’ coefficients.

**Results and discussion**

**Comparison of the growth of parental and hyperploid cancer cells in WT and Rag2⁻/⁻ mice**

Parental or hyperploid fibrosarcoma MCA205 cells were inoculated subcutaneously (s.c.) into adult female wild type (WT) C57BL/6 and Rag2⁻/⁻ mice. As to be expected, parental cells developed tumors in all mice to 12 days after injection, irrespective of the genotype of the recipients. Hyperploid cells readily developed into tumors when inoculated into Rag2⁻/⁻ mice, which lack mature B or T lymphocytes. In Rag2⁻/⁻ mice, no difference could be detected in the growth of parental and hyperploid MCA205 tumors. In sharp contrast, hyperploid MCA205 cells failed to form tumors in 3 out of 5

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0.5 mg mL⁻¹ in 0.2 mL of PBS) by intraperitoneal injection once per week. Tumor growth was routinely assessed by means of a caliper. Animals bearing neoplastic lesions that exceeded 20–25% of their body mass were euthanized.

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Figure 1. Tumor development characteristics of parental versus hyperploid MCA205 cells in WT and Rag2−/− mice. Parental and hyperploid MCA205 cells were inoculated into C57BL/6 or Rag2−/− mice. Tumor incidence (A) and tumor growth (B, independent mice, and C, average of all mice) were routinely monitored. Histological sections from parental and hyperploid tumors recovered from C57BL/6 mice, Rag2−/− mice were submitted to histochemical staining for the detection of nuclear area (D). Scale bar, 10 μm. Inserts in D show representative pictures. Box plots represent median values of nuclear areas (median applied to each mouse, normalized by the average of the parental median value). Tumor incidence was compared by one-tailed Barnard’s test, tumor growth curves were analyzed with Wald test and nuclear area were compared by means of a linear mixed model (see Statistical Analysis in Materials and Methods). P < 0.05 P vs H; **P < 0.01 P vs H; ***P < 0.05 P vs WT; ****P < 0.01 H vs WT; *****P < 0.001 H vs WT. Error bars represent SEM over time.
Figure 2. Tumor development characteristics of parental versus hyperploid EL4 cells in WT and Rag2<sup>−/−</sup> mice. Parental and hyperploid EL4 cells were inoculated into C57BL/6 or Rag2<sup>−/−</sup> mice. Tumor incidence (A) and tumor growth (B, independent mice, and C, average of all mice) were routinely monitored. Histological sections from parental and hyperploid tumors recovered from C57BL/6 mice, Rag2<sup>−/−</sup> mice were submitted to histochemical staining for the detection of nuclear area (D). Scale bar, 10 μm. Inserts in D show representative pictures. Box plots represent median values of nuclear areas (median applied to each mouse, normalized by the average of the parental median value). Tumor incidence was compared by one-tailed Barnard’s test, tumor growth curves were analyzed with Wald test and normalized nuclear area were compared by means of a linear mixed model (see Statistical Analysis in Materials and Methods). *p < 0.05 P vs H; **p < 0.001 P vs H; ***p < 0.001 H vs WT; ###p < 0.001 H vs WT. Error bars represent SEM over time.
immunocompetent C57BL/6 mice (Fig. 1A). In the two animals in which tumors developed from hyperploid MCA205 cells, tumors developed late, with a latency of more than 40 days and grew slowly (Fig. 1B, C). All tumors that arose in WT or Rag2−/− mice were excised and embedded into paraffin to determine the nuclear area as a proxy of the ploidy status. Tumors arising from hyperploid MCA205 cells had larger nuclei than cancers arising from parental MCA205 cells. This apparent difference in ploidy was not observed for tumors arising from parental and hyperploid MCA205 cells in immunocompetent C57BL/6 mice (Fig. 1D).

We performed a similar analysis on EL4 lymphoma cells that were left in their normal state or rendered hyperploid and injected into immunocompetent WT or T and B lymphocyte-deficient Rag2−/− mice. Hyperploid EL4 cells led to the generation of s.c. tumors in all (20 out of 20) Rag2−/− mice, and these tumors formed at the same speed and proliferated at the same rate as parental EL4 cells. In contrast, hyperploid EL4 cells only formed tumors in 71% (30 out of 42) of WT mice, while parental EL4 cells generated tumors in 100% (23 out of 23) of WT mice (Fig. 2A). Again, hyperploid EL4 cells formed delayed and slowly growing tumors in WT mice (Fig. 2A-C). Histological examination of EL4 tumors arising in Rag2−/− mice revealed the persistence of the difference in ploidy for parental versus hyperploid EL4 cells. This difference in the normalized nuclear area was lost for EL4 tumors arising from parental and hyperploid EL4 cells in immunocompetent WT mice (Fig. 2D).

Altogether, these results support the notion that hyperploid cells are recognized by the adaptive cellular immune system, thus reducing the probability that cancers can be formed by such cells. Moreover, when tumors formed under the selection pressure of the immune system, only cells that reduce their chromosomal content can survive and form tumors.

**Immunoselection of hyperploid cancer cells in the context of different immunodeficiencies**

Prompted by the aforementioned results, we investigated the fate of hyperploid cancer cells in the context of different types of immunodeficiency affecting the innate or adaptive immune system. Parental and hyperploid MCA205 cells were inoculated into CD1d−/− mice lacking the glycoprotein CD1d expressed at the surface of NKT cells; Ighm−/− mice missing the heavy chain of immunoglobin (Ig) M antibody μ chain, causing the absence of B cells; FcRn−/− mice deficient in the Fc receptor for IgG antibodies; or Myd88−/− mice lacking the toll-like receptors (TLRs)-signaling adaptor MyD88. Moreover, parental and hyperploid EL4 cells were injected into athymic Foxn1−/− mice (commonly known as nude) mice that lack mature T lymphocytes and are characterized by an excessive NK cell function; Flt3l−/− mice lacking the ligand for the receptor tyrosine kinase 3 (Flt3l) which show reduced numbers of hematopoietic myeloid and lymphoid progenitors, dendritic cells and NK cells; WT mice treated with the IL1R antagonist Anakinra modulating interleukin 1 (IL-1), or Nlrp3−/− mice deficient for the NACHT, LRR and PYD domains-containing protein 3 (NLRP3), also called cryopyrin, which is part of the NLRP3 inflammasome complex and hence in the generation of mature IL-1β and IL-18. Hyperploid MCA205 cells showed no significant difference in tumor incidence compared to their parental counterparts when inoculated into CD1d−/− and Ighm−/− mice, while in 60% and 90% of FcRn−/− and Myd88−/− mice, respectively, hyperploid MCA205 cells were not able to proliferate (Fig. 3A). Intriguingly, no difference was found during the first 12 days between parental and hyperploid MCA205 tumor incidence in CD1d−/−, Ighm−/− and Myd88−/−. However, later a fraction of tumors spontaneously regressed in CD1d−/−, Ighm−/− and Myd88−/− mice (Fig. 3A). With respect to tumor growth, hyperploid MCA205 cells appeared later and grew more slowly than their parental counterparts in CD1d−/−, FcRn−/− and Myd88−/− mice. There was also a tendency towards slower growth of hyperploid MCA205 cells in Ighm−/− mice (Fig. 3B-C). Nonetheless, the immune systems of CD1d−/−, Ighm−/− and Myd88−/− mice was apparently able to eliminate hyperploid tumor cells because the difference in nuclear size between parental and hyperploid cells disappeared after passage of cancers in such mice. In contrast, this difference in ploidy persisted for tumors arising from parental and hyperploid cells in FcRn−/− (Fig. 3D).

In nu/nu mice, hyperploid EL4 cells developed cancer only in 50% (3 out of 6) cases (Fig. 4A), and such tumors developed with delayed and reduced growth kinetics as compared to wild type controls (Fig. 4B, C). Moreover, the difference in ploidy between initially hyperploid and parental cells was lost after passage through Foxn1−/− mice (Fig. 4D), pleading in favor of an intact immunosurveillance system. Hyperploid EL4 cells developed close-to-always tumors in Flt3l−/−, WT mice treated with Anakinra, as well as in Nlrp3−/− mice, contrasting with a delayed tumor manifestation and growth (compared to parental EL4 cells) in such mice (Fig. 4B-C). Histological examination of parental and hyperploid EL4 tumors arising in Flt3l−/− mice, WT mice treated with Anakinra or Nlrp3−/− mice revealed the persistence of the difference in ploidy (Fig. 4D).

**Concluding remarks**

In this paper, we compared the effects of a complete absence of T/B-mediated immune response (induced by the Rag2−/− genotype) with other immunodeficiencies linked to other genotypes (CD1d−/−, FcRn−/−, Flt3l−/−, Foxn1−/−, Ighm−/−, Myd88−/−, Nlrp3−/−) or long-term administration of the IL-1 antagonist anakinra on the immunoselection against hyperploid cancer cells. In Rag2−/− mice there were no signs of immunoselection against hyperploidy in the sense that hyperploid and parental cancer cells formed cancers at the same speed after inoculation, grew at the same rate and conserved their initial ploidy status. Similarly, in Flt3l−/−, Nlrp3−/− and Anakinra-treated WT mice, hyperploid cancers formed and remained hyperploid until large tumors were formed, although there was a delay in the growth of tumors arising from hyperploid compared to parental cells. Hence, the absence of Flt3l, Nlrp3 or the neutralization of IL-1 largely abolished the immunosurveillance against hyperploid cells, though not completely (because there was a still a delay in the growth of hyperploid tumors). The absence of CD1d, Ighm, FcRn and Myd88 also partially
compromised immunosurveillance against hyperploidy, either because tumors first developed and then regressed (a phenomenon not seen in fully immunocompetent mice, but seen in Cd1d<sup>−/−</sup>, I<sub>ghm</sub><sup>tm1Cgn</sup>, FcR<sub>n</sub><sup>−/−</sup> or Myd88<sup>−/−</sup> mice) or because cancer cells maintained their hyperploid status (as this is the case for FcR<sub>n</sub><sup>−/−</sup> mice). Nonetheless, the nuclear size of initially hyperploid cancer cells was reduced after passage through Cd1d<sup>−/−</sup>, I<sub>ghm</sub><sup>tm1Cgn</sup>, FcR<sub>n</sub><sup>−/−</sup> or Myd88<sup>−/−</sup> mice and tumors formed less frequently in FcR<sub>n</sub><sup>−/−</sup> mice, pleading in favor of some (though suboptimal) immunosurveillance against hyperploidy (Table 1). Interestingly, Foxn1<sup>nu/nu</sup> mice maintained characteristics of fully intact immunosurveillance suggesting that the NK system (or residual T cell functions) sufficed to assure the elimination of hyperploid cells.

Considering tumor incidence as cell recognition and normalized nuclear area as cellular elimination and looking at the parameters characterizing tumor development after inoculation of parental and hyperploid MCA205 or EL4 cell lines (Table 1), we observed that Myd88<sup>−/−</sup> and Foxn1<sup>nu/nu</sup> mice behaved as WT mice meaning that their corresponding deficiencies were not involved in the recognition and elimination of hyperploid cells. Despite of their immunodeficiency, FcR<sub>n</sub><sup>−/−</sup> mice seemed to recognize hyperploid cells but they failed to eliminate them all. Mice treated with Anakinra and, Flt3l<sup>−/−</sup> and Nlrp3<sup>−/−</sup> mice were not able to recognize nor to eliminate hyperploid cells similarly to Rag2<sup>−/−</sup> mice. Finally, Cd1d<sup>−/−</sup> and I<sub>ghm</sub><sup>tm1Cgn</sup> mice showed an intermediated behavior in the tumor incidence but eliminating the hyperploid cells.

Figure 3. Characteristics of tumor development of parental versus hyperploid MCA205 cells in different immunodeficient mice. Parental and hyperploid MCA205 cells were inoculated into Cd1d<sup>−/−</sup>, I<sub>ghm</sub><sup>tm1Cgn</sup>, FcR<sub>n</sub><sup>−/−</sup> or Myd88<sup>−/−</sup> mice. Tumor incidence (A) and tumor growth (B, independent mice, and C, average of all mice) were routinely monitored. Histological sections from parental and hyperploid tumors recovered from Cd1d<sup>−/−</sup>, I<sub>ghm</sub><sup>tm1Cgn</sup>, FcR<sub>n</sub><sup>−/−</sup> and Myd88<sup>−/−</sup> mice were submitted to histochemical staining for the detection of nuclear area (D). Box plots represent median values of nuclear areas (median applied to each mouse, normalized by the average of the parental median value). Tumor incidence was compared by one-tailed Barnard’s test, tumor growth curves were analyzed with Wald test and normalized nuclear area were compared by means of a linear mixed model (see Statistical Analysis in Materials and Methods). *p < 0.05 P vs H; **p < 0.01 P vs H; ***p < 0.001 P vs H. Error bars represent SEM over time.
In conclusion, the present results support the notion that multiple distinct innate and cognate immune effectors including myeloid cells, B, T, NK and NKT cells may contribute to the immunosurveillance against hyperploid cancer cells. Moreover, Toll-like receptors and/or interleukin-1 receptors (downstream of MyD88) and the interleukin-1 system (activated by NLRP3, neutralized by anakinra) apparently play some role in this immunosurveillance system, be it at the level of tumor growth and tumor incidence.

**Table 1.** Summary of results (*p*-values).

|                     | MCA205 fibrosarcoma | EL4 lymphoma |
|---------------------|---------------------|--------------|
| Tumor incidence     |                     |              |
| P vs H              | 0.03                | 0.01         |
| H vs WT             | 0.33                | 0.35         |
| Tumor growth P vs H | 0.05                | 0.42         |
| H vs WT             | 0.12                | 0.46         |
| Nuclear area        |                     |              |
| P vs H              | 0.36                | 0.25         |
| H vs WT             | 0.0004              | 0.31         |

*Figure 4.* Characteristics of tumor development of parental versus hyperploid EL4 cells in mice with different immunodeficiencies. Parental and hyperploid EL4 cells were inoculated into Foxn1<sup>nu/nu</sup>, Flt3l<sup>−/−</sup>, Nlrp3<sup>−/−</sup> mice or mice treated with the IL-1 receptor antagonist Anakinra. Tumor incidence (A) and tumor growth (B, independent mice, and C, average of all mice) were routinely monitored. Histological sections from parental and hyperploid tumors recovered from Foxn1<sup>nu/nu</sup>, Flt3l<sup>−/−</sup>, Nlrp3<sup>−/−</sup> mice or mice treated with Anakinra were submitted to histochemical staining for the detection of nuclear area (D). Box plots represent median values of nuclear areas (median applied to each mouse, normalized by the average of the parental median value). Tumor incidence was compared by one-tailed Barnard’s test, tumor growth curves were analyzed with Wald test and normalized nuclear area were compared by means of a linear mixed model (see Statistical Analysis in Materials and Methods). *p < 0.05 P vs H; **p < 0.001 P vs H; ***p < 0.001 H vs WT. Error bars represent SEM over time.
of the configuration of a fully functional immune system, be it at the level of effector mechanisms that come into action when hyperploid cancer cells are inoculated into the organism. Altogether, these findings underscore the complexity of the immune system with respect to its functions in suppressing oncogenesis and controlling tumor progression.

**Abbreviations**

CALR: calreticulin  
CTLA-4: cytotoxic T lymphocyte – associated protein 4  
DC: dendritic cell  
FcRn: neonatal Fc receptor  
FELASA: Federation of European Laboratory Animal Science Association  
Flt3l: ligand for the receptor tyrosine kinase 3  
FBS: fetal bovine serum  
Ig: immunoglobulin  
IL-1: interleukin 1  
IL-1Ra: interleukin 1 receptor antagonist  
IL-18: interleukin 18  
IL-2R: interleukin 2 receptor  
IL-4: interleukin 4  
IFN-γ: interferon gamma  
MHC: major histocompatibility complex  
NK: natural killer cell  
NKT: natural killer T cell  
NLRP3: NACHT, LRR and PYD domains-containing protein 3  
RAG-2: recombinase activating gene 2  
PBS: phosphate-buffered saline  
PFA: paraformaldehyde  
Th: T helper type  
T cells: CD4+ T helper cells expressing IFN-γ  
TLR: toll-like receptors  
WT: wild type  
γc: cytokine receptor γ chain

**Disclosure of potential conflicts of interest**

No potential conflicts of interest were disclosed.

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