

AWARD NUMBER: W81XWH-19-1-0167

TITLE: Therapeutic Targeting of Immune Dysfunction in Langerhans Cell Histiocytosis

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REPORT DATE: Oct 2020

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Development Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
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REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188	
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1. REPORT DATE Oct 2020		2. REPORT TYPE Annual Technical Report		3. DATES COVERED 09/01/2019 - 08/31/2020	
4. TITLE AND SUBTITLE Therapeutic Targeting of Immune Dysfunction in Langerhans Cell Histiocytosis				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-19-1-0167	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Rikhia Chaudhury Chakraborty E-Mail: rxchakra@txch.org				5d. PROJECT NUMBER 0011338275-0001	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Baylor College of Medicine One Baylor Plaza Houston, TX 77030				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Langerhans cell histiocytosis (LCH) is a myeloproliferative disorder with similar incidence and overall survival as pediatric Hodgkin's Lymphoma. The current therapeutic standard of care fails in over 50% of patients, and treatment failure is associated with significant long-term morbidity and mortality. Translational research resources and efforts to study LCH have been relatively sparse due to challenging samples and the lack of reliable <i>pre-clinical</i> models. To address this issue the Histiocytosis Program at Texas Children's Cancer Center has developed an unprecedented research resource of ~1000 viably preserved LCH lesions and paired peripheral blood mononuclear cells, along with associated clinical data. In addition, we generated the first mouse models of LCH-like disease, allowing us to characterize the molecular mechanisms that drive LCH. LCH lesions histology reveals a universal feature of accumulation of Langerin-positive dendritic cells along with a large inflammatory infiltrate. To improve outcomes for patients, we not only need to define the mechanisms driving LCH, but also to translate these discoveries into improved therapeutic strategies. The objective of this project is to develop pre-clinical models to determine the most effective therapeutic strategies for patients with LCH. We hypothesize that combination therapeutic strategies targeting MAPK activation and dysfunctional T cells can overcome the immune suppressive microenvironment in LCH, leading to better clinical outcomes than chemotherapy or MAPK inhibition alone. Aim 1: To define the impact of specific MAPK pathway mutations on initial treatment failure and immune evasion in patients with LCH; Aim 2: To test novel therapeutic salvage strategies in mutation-specific in vitro and in vivo models of LCH. In the first year, we have obtained the HRPO and ACURO approval to complete the proposed studies. In ongoing studies, analysis of the immune infiltrate in LCH lesions identified the most prominent immune cells as T lymphocytes. Both CD8+ and CD4+ T cells exhibited 'exhausted' phenotypes with high expression of the immune checkpoint receptors. LCH DCs showed robust expression of ligands to checkpoint receptors.					
15. SUBJECT TERMS Langerhans cell histiocytosis, LCH, MAPK, T cells, exhausted T cells, dysfunctional T cells, somatic mutation, immunotherapy, MAPK inhibition, combination therapy, novel therapeutics					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified		19b. TELEPHONE NUMBER (include area code)

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1. INTRODUCTION: *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

Langerhans cell histiocytosis (LCH) is a myeloproliferative disorder with similar incidence and overall survival as pediatric Hodgkin's Lymphoma. The current therapeutic standard of care for patients with multi-focal disease remains empiric chemotherapy which fails in over 50% of patients, and treatment failure is associated with significant long-term morbidity and mortality. Translational research resources and efforts to study LCH have been relatively sparse due to challenging samples and the lack of reliable *pre-clinical* models. To address this issue the Histiocytosis Program at Texas Children's Cancer Center has developed an unprecedented research resource of ~1000 viably preserved LCH lesions and paired peripheral blood mononuclear cells, along with associated clinical data. In addition, we generated the first mouse models of LCH-like disease, allowing us to characterize the molecular mechanisms that drive LCH. LCH lesions histology reveals a universal feature of accumulation of Langerin-positive dendritic cells along with a large inflammatory infiltrate. Upon immune cell phenotyping of LCH microenvironment we found T lymphocytes are most abundant but are dysfunctional and express exhaustion markers on their surface. To improve outcomes for patients, we not only need to more completely define the mechanisms driving LCH, but also to translate these discoveries into improved therapeutic strategies. The **purpose** of this proposal is to develop pre-clinical models to determine the most effective therapeutic strategies for patients with LCH. We hypothesize that combination therapeutic strategies targeting MAPK activation and dysfunctional T cells can overcome the immune suppressive microenvironment in LCH, leading to better clinical outcomes than chemotherapy or MAPK inhibition alone. Aim 1: To define the impact of specific MAPK pathway mutations on initial treatment failure and immune evasion in patients with LCH; Aim 2: To test novel therapeutic salvage strategies in mutation-specific in vitro and in vivo models of LCH. The goal for the experiments in this proposal is to develop a rational approach to therapy for pediatric patients with LCH. We are using preclinical data to determine which patients are at highest risk for relapse based on mutation and cell of origin. Further, these experiments will provide the platform from which to consider and test specific targeted agents in LCH patients with specific mutations.

2. KEYWORDS: *Provide a brief list of keywords (limit to 20 words).*

Langerhans cell histiocytosis, LCH, MAPK, T cells, exhausted T cells, dysfunctional T cells, somatic mutation, immunotherapy, MAPK inhibition, combination therapy, novel therapeutics

3. ACCOMPLISHMENTS:

What were the major goals of the project?.

A. Career Development-Specific Tasks:

Major Task 1: Training and educational development in pre-clinical and clinical translational research

- **Subtask 1:** Participate and present research at the weekly Histiocytosis Program meetings at the Texas Medical Center (Months 1-36).
- **Subtask 2:** Participate in weekly meetings and present (once annually) at the Center for Cell and Gene Therapy, Baylor College of Medicine (Months 1-36).
- **Subtask 3:** Participate in weekly meetings with Dr. Carl E Allen (Months 1-36).
- **Subtask 4:** Organize and attend quarterly meetings with mentoring team (comprising Dr. Miriam Merad, Dr. Kenneth L McClain, Dr. Helen Heslop, and Dr. Carl E Allen) to discuss progress of proposed research and career development plans. I also plan to add, in consultation with the mentoring team, a Cancer Pharmacologist to the mentoring team within the first six months of the grant period (Months 1-36).
- **Subtask 5:** Attend scientific grant writing and other relevant workshops, which are routinely offered by the Career Development Center at Baylor College of Medicine (Months 1-24).
- **Subtask 6:** Attend and present research findings at annual national and international conferences, including the (i) Annual Meeting of the American Society of Hematology, (ii) Histiocyte Society Meeting, and (iii) relevant Keystone Symposia and Gordon Research Conferences (Months 1-36).

B. Research-Specific Tasks:

Specific Aim 1: To define the impact of specific MAPK pathway mutations on initial treatment failure and immune evasion in patients with LCH.

Major Task 1: To determine the clinical significance of specific recurrent mutations in LCH.

- **IRB/HRPO Approval** (Months 0-3)
- **Subtask 1:** Analysis of LCH specimens (lesions, correlated clinical data) from the Histiocytosis Program at Texas Children's Cancer Center (TXCC). Recurrent mutations will be analyzed with respect to clinical variables including age of onset of disease, gender, single vs multifocal lesions, and low-risk vs high risk lesions (Months 4-6).
- **Subtask 2:** Association between each characteristic and mutation status will be determined by calculating crude odds ratios (OR) and 95% confidence intervals using unconditional logistic regression analysis. Develop adjusted models based on variables identified in Subtask 1 with a significance level of $P < 0.05$ (Months 4-6).
- **Subtask 3:** Kaplan-Meier estimates will be used to calculate probability of treatment failure (event-free survival) and overall mortality for each mutation group (Months 7-8).
- **Subtask 4:** Analyze differences in clinical outcomes using Cox proportional hazards models to adjust for relevant covariates, including: age, gender, race/ethnicity, and treatment (Months 7-8).

- **Milestone(s) Achieved:** Define how specific activating MAPK gene pathway mutations affect LCH patients' response to initial therapy, the results of which will be incorporated into prioritization of agents/strategies tested in Aim 2.

Major Task 2: To determine the impact of LCH-specific mutations on immune evasion.

- **Subtask 1:** Perform mass cytometric analysis on a larger cohort with ~ 15 samples per mutation type) of LCH lesions harboring different MAPK pathway somatic mutations. Analyze inhibitory receptors' (PD-1, TIM-3, LAG-3) expression on CD3+ T cells, corresponding ligands for the inhibitory receptors on CD207+ cells (Months 3-12).
- **Subtask 2:** Perform transcriptomic analysis to identify the factors and signaling mechanisms that are promoting differential immune cell exhaustion marker expression in LCH based on specific MAPK pathway mutations (Months 9-15).

Specific Aim 2: To test novel therapeutic salvage strategies in mutation-specific in vitro and in vivo models of LCH.

Major Task 3: Test the relative efficacy of myeloid-directed chemotherapy, MAPK pathway inhibition, and other targeted therapies on tumor progression and survival in mouse models of LCH.

- **IACUC/ACURO approval** (Months 15-18)

What was accomplished under these goals?

ACCOMPLISHMENTS AND PROGRESS

A. Career Development-Specific Tasks:

Major Task 1: Training and educational development in pre-clinical and clinical translational research

- **Subtask 1:** I have regularly participated in weekly Histiocytosis Program meetings at the Texas Medical Center.
- **Subtask 2:** I have participated in the weekly meeting of the Center for Cell and Gene Therapy, Baylor College of Medicine.
- **Subtask 3:** Dr. Allen and me have multiple meetings every week including a dedicated one-hour meeting where we discuss progress and expectations.
- **Subtask 4:** I had the quarterly meetings with Dr. Allena and Dr. Merad. Dr. McClain could attend only one of the meetings while Dr. Heslop could not attend these meetings as her workload as considerably increased over the last year. I have also added Dr. Greg M. Delgoffe, a world renowned expert in tumor microenvironment, to my collaboration team. I have also discussed my goals and progress with Dr. Martin Matzuk, Interim Chair of The Department of Pharmacology and Chemical Biology at Baylor College of Medicine and plan to formally request him to attend my subsequent meetings/presentations. I have also been added to the NACHO-LIBRE (North American Consortium for Histiocytosis-Registry Study of LCH and Related Disorders: Inhibition of MAPK Pathway Activation [RAS, BRAF, MEK, and ERK]) as a co-investigator which provides me direct access to experts in Cancer Pharmacology.
- **Subtask 5:** Professional educational development activities: Immuno-Oncology and Cancer Pathology using Mass Cytometry (October 2019), Clear Science Writing Workshop (September 2019), Grant Writing of Researchers NIH Process Workshop (January 2020).
- **Subtask 6:** I presented an invited seminar at the American Society of Hematology ASH-a-Palooza Blood Drops Session, Orlando, FL. I attended the 2019 Annual ASH meeting at Orlando, FL.

B. Research-Specific Tasks:

Specific Aim 1: To define the impact of specific MAPK pathway mutations on initial treatment failure and immune evasion in patients with LCH.

Major Task 1: To determine the clinical significance of specific recurrent mutations in LCH.

- **IRB/HRPO Approval** (Months 0-3): The HRPO approval was obtained on August 25, 2020 (HRPO Log Number E00845.1a). The delay was largely due to the requirement of having a stand-alone (pertaining to the goals of the DoD approved proposal) human subject protocol approved by the Institutional Review Board at Baylor College of Medicine. Given the COVID-19 pandemic and the closures this protocol submission, review, and resubmissions took much longer than initially anticipated.
- **Subtask 1-4:** Objectives proposed withing Subtasks 1-4 were not completed as planned due to the delay in the HRPO approval as explained above. In the time since the HRPO approval, we have concurrently begun the analyses proposed within subtasks 1-4. We plan to make-up for the lost time by finishing these studies within the next 4 months.

Major Task 2: To determine the impact of LCH-specific mutations on immune evasion.

- **Subtask 1:** Since receiving the HRPO approval we have started the optimization of the mass cytometry experiments proposed within this task. While waiting for the HRPO approval, we continued with the experiments presented as preliminary data in the DoD proposal. The results from this study, summarized below, do provide important indicators to the phenotype of the LCH lesion. These studies have helped us in optimizing the experimental conditions that will help in performing the proposed experiments in this subtask.

Phenotypic analysis of LCH lesions

Immune phenotyping using mass cytometry by time-of-flight (CyTOF) of patient biopsies (n=15; *BRAF* WT (n=6) and *BRAF*V600E (n=9) and healthy skin controls (skin CD207+ DCs (n=5)) revealed that the immune infiltrate in LCH lesions varied between 50 and 70% with CD3+ T cells and myeloid derived suppressor cells (MDSCs) (CD11b+CD14+HLA-DR^{low}/CD15-) as the most abundant populations (Fig. 1A-C). Overall, LCH lesion DCs (HLA-DR+CD1A+), T cells, and MDSCs accounted for 25.60% ± 10.72%, 28.20% ± 6.46% and 31.90% ± 4.02%, respectively. T cells were significantly more abundant in WT *BRAF* compared to *BRAF*V600E lesions, consistent with increased LCH DC (HLA-DR+CD1A+) in the *BRAF*V600E lesions (Fig. 1A, B, D). By comparison, immune infiltrate in normal skin included 5.00% ± 1.00% LCs, 3.00% ± 1.58% T cells and 13.60% ± 2.07% MDSC (Fig. 1C, D). The proportion of T cells compared to MDSC was significantly larger in the LCH lesions when compared to healthy skin (Fig. 1A-D). The T cells were further characterized by automatic gating using FlowSOM that allows multivariate clustering of cells based on the self-organized map (SOM) algorithm and categorizes cells into relevant meta-clusters based on their surface markers (Fig. 1E). Within LCH lesions, CD3+CD4+ T cells (74.00% ± 12.26%) were significantly higher than effector CD3+CD8+ T cells (21.00% ± 5.61%) (Fig. 1E, F) as compared to tonsil control (41.20% ± 7.02% CD4 and 32.00% ± 4.64% CD8) (Fig.

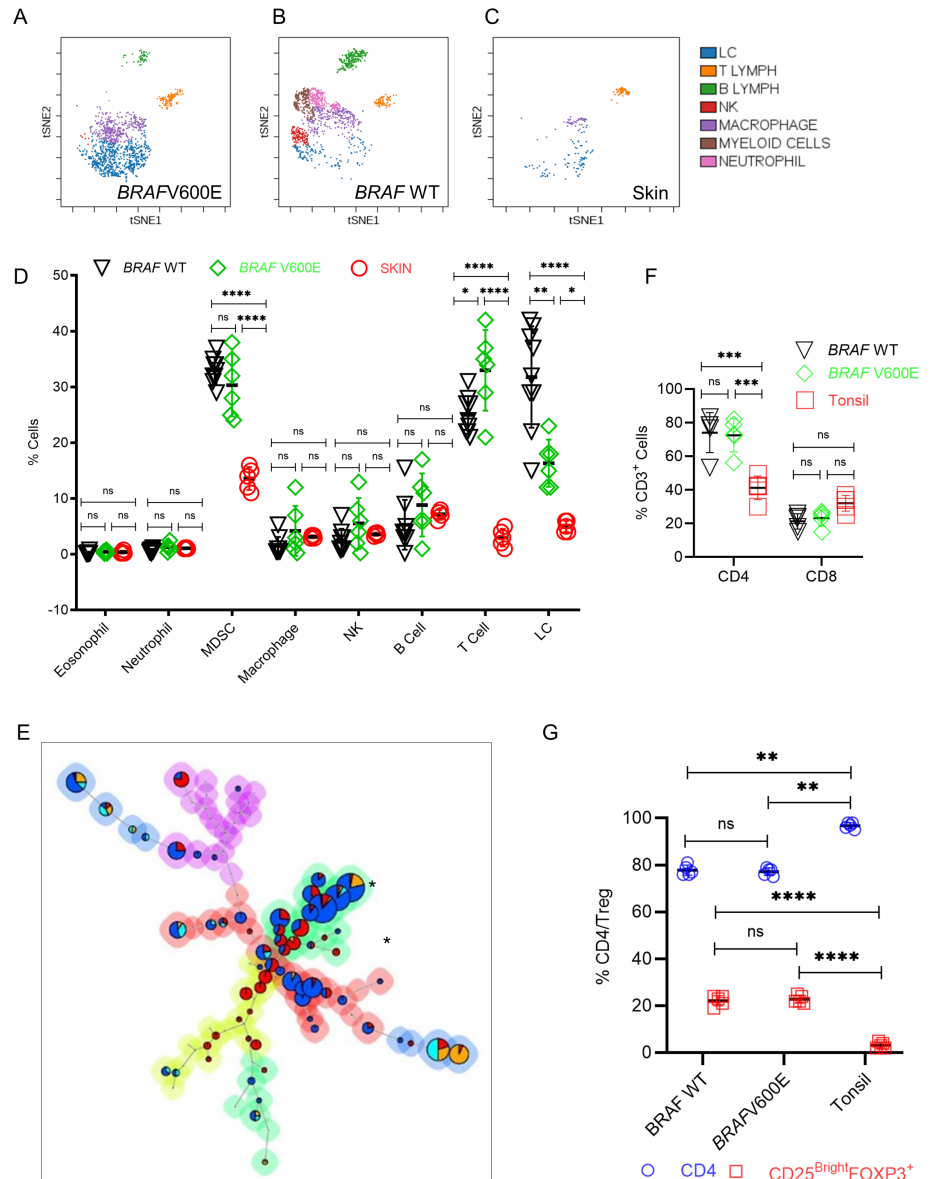


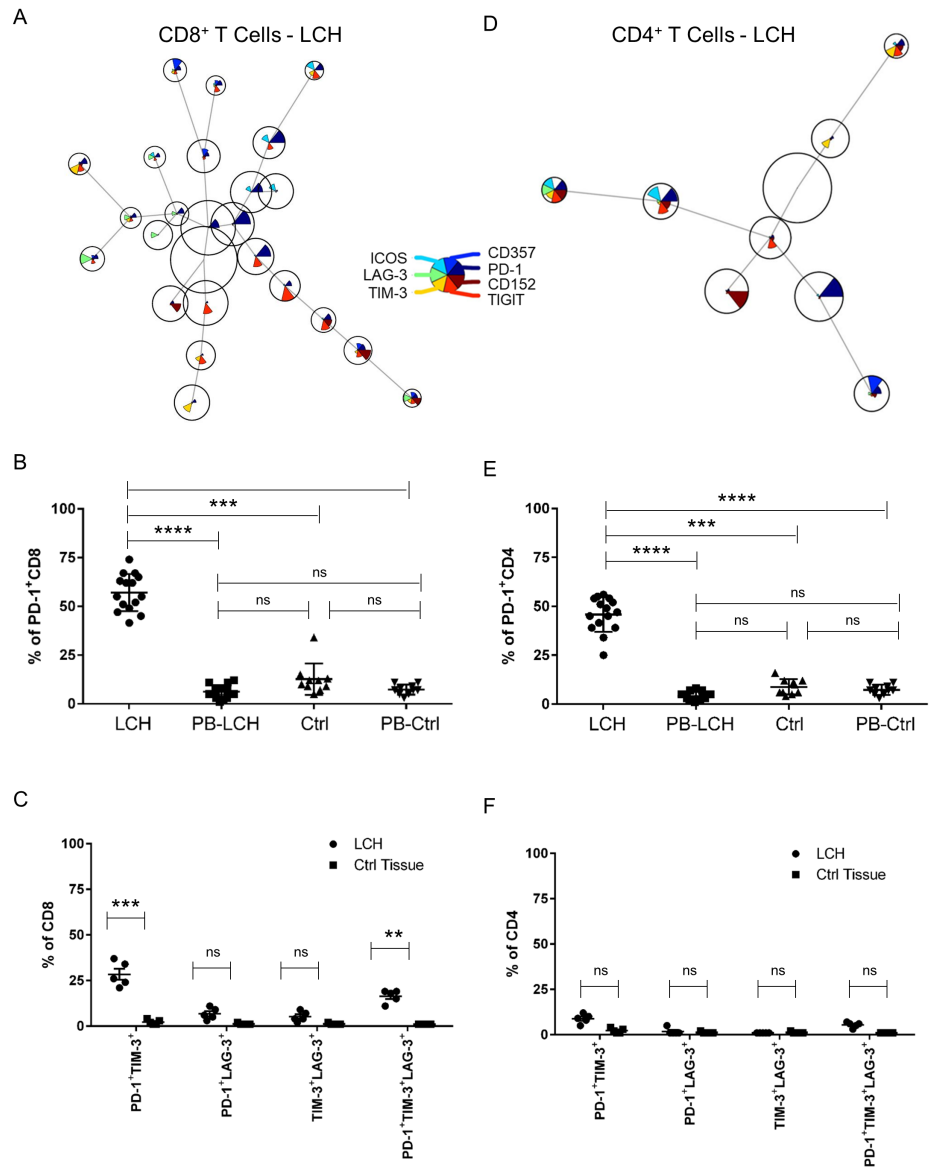
Figure 1. T cells are the major components of the immune infiltrate in LCH lesions. (A-C) viSNE plots of live cells from representative LCH patient lesion biopsy with *BRAF*V600E (A), *BRAF* WT (B), and normal skin biopsy (C). (D) Quantification of lesion-infiltrating cells shown in A-C (n=6 *BRAF* WT; n=9 *BRAF*V600E) in comparison to normal skin biopsies (n=5). (E) Relative T cell subtypes in LCH. Analysis of CyTOF data was done using self-organizing map (FlowSOM) and visualized as pie charts. Mean-marker value for indicated markers are shown in the pie charts. The background colors of the nodes result from meta-clustering corresponding to an automatic gating procedure to avoid biasedness in analysis. Size of the nodes is relative to the percentage of cells present in each cluster. Shown are representative FlowSOM minimal spanning tree (MST) plots for indicated T cell types. (F) Quantification of relative abundance of CD4 and CD8 T cells in LCH lesion infiltrate (n=5 each) or tonsil control (n=5). *P<0.05 for both CD4⁺ and CD8⁺ compared to tonsil control. (G) CD4/Treg (CD25⁺FOXP3⁺) ratios in indicated samples (n=5 each). For D, F, and G, data are mean ± SD; *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001, ns, not significant (P>0.05) – one-way ANOVA with Tukey's post hoc test. LC, Langerhans cells; NK, natural killer cell; MDSC, myeloid derived suppressor cells.

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1F). Among CD4⁺ T cells, the fraction of Tregs was significantly greater in LCH lesions than in tonsil controls (**Fig. 1G**). These results were independent of *BRAF* mutation status (**Fig. 1F, G**).

Infiltrating T cells in LCH tumor microenvironment exhibit an 'exhausted' phenotype

We next assessed expression of inhibitory receptors on CD8⁺ and CD4⁺ T cell subsets using FlowSOM. CD8⁺ T cells residing in the LCH lesions showed higher expression of the inhibitory receptors programmed death receptor-1 (PD-1) (**Fig. 2A, B**), T cell immunoglobulin- and mucin domain-containing molecule (TIM-3) (**Fig. 2A**), and lymphocyte-activation gene 3 (LAG-3) (**Fig. 2A**), but not cytotoxic T lymphocyte antigen-4 (CTLA-4) (**Fig. 2A**), compared to peripheral CD8⁺ T cells from the same patients, skin controls, or peripheral CD8⁺ T cells from controls (**Fig. 2B**). Compared to CD8⁺ T cells from healthy skin tissues, the CD8⁺ T cells from LCH lesions showed significantly higher numbers of PD-1⁺TIM-3⁺ and PD-1⁺TIM-3⁺LAG-3⁺ cells, but not PD-1⁺LAG-3⁺ or TIM-3⁺LAG-3⁺ cell (**Fig. 2C**). CD4⁺ T cells within the lesion also showed significantly higher expression of PD-1 and TIM-3 compared to peripheral CD4⁺ T cells from the same patients, skin controls, or peripheral CD4⁺ T cells from controls (**Fig. 2D, E**). However, there was no significant difference in the numbers of PD-1⁺TIM-3⁺, PD-1⁺LAG-3⁺, TIM-3⁺LAG-3⁺, or PD-1⁺TIM-3⁺LAG-3⁺ CD4⁺ T cells between LCH lesions and healthy skin tissues (**Fig. 2F**). Compared to the PD-1⁺TIM-3⁺LAG-3⁺CD8⁺ T cells, the PD-1⁺, TIM-3⁺, LAG-3⁺, PD-1⁺TIM-3⁺, and PD-1⁺TIM-3⁺LAG-3⁺ CD8⁺ T cells presented significantly decreased granzyme B and perforin secretion (**Fig. 3A**), indicating a whole-spectrum immunosuppression in these cells. The expression of PD-1 ligands PD-L1 and PD-L2 were significantly elevated in CD207⁺ DCs from LCH lesions compared to healthy skin (**Fig. 3B, C**).



These results suggest that immobilized PD-L1 or PD-L2-expressing CD207⁺ cells may engage the T cells due to their high PD-1, resulting in accumulation of exhausted T cells in the lesions. Alternatively, CD207 may induce T cells to express PD-1, in turn engaging them, and eventually preventing them from performing effector functions. Taken together, these results suggested that PD-1 blockade could influence the immune dysfunction induced by LCH DC.

- **Subtask 2:** RNA is being isolated from the proposed samples, which will then be used to perform the transcriptomic analysis.

Overall, the completion of proposed experiments within this aim was postponed due to the delay in obtaining the HRPO approval. However, I am confident that expertise of my lab personnel along with my own will allow us to complete these experiments in a timely fashion so as not to impact the overall outcome expected from the DoD approved proposal.

Specific Aim 2: To test novel therapeutic salvage strategies in mutation-specific in vitro and in vivo models of LCH.

Major Task 3: Test the relative efficacy of myeloid-directed chemotherapy, MAPK pathway inhibition, and other targeted therapies on tumor progression and survival in mouse models of LCH.

- **IACUC/ACURO approval:** Even though this was scheduled for months 15-18, we went ahead and got the ACURO approval on July 31, 2020 (ACURO Log Number CA181344.e001). This will allow us to make-up for some of the lost time in Major Tasks 1 and 2.

What opportunities for training and professional development has the project provided?

In addition to what is described above under accomplishments of Career Development Specific Tasks, this project provided me the following opportunities for training and professional development:

- Research personnel in my lab has presented posters and or talks at the 2019 Annual ASH meeting and the 2020 TXCH Symposium. My lab personnel are also scheduled to give oral presentations at the upcoming Annual Histiocyte Society meeting. These has helped me in developing my mentoring and leadership skills.
- I have also served as a peer reviewer in the American Society of Hematology Research Training Award for Fellows Study Section, the American Society of Hematology Scholar Award Study Section, and the Histiocyte Society Research Grants Program Study Section. I was appointed as an Editorial Board Member of *IUBMB Life*, the flagship journal of the International Union of Biochemistry and Molecular Biology.
- I received the mentor award under the 2020 American Society of Hematology Minority Medical Student Award Program to mentor Omar A. Ramos-Mas, first year medical student at the Universidad Central del Caribe, Bayamón, Puerto Rico.
- I was awarded (a) a competitive Baylor College of Medicine – Pilot projects on COVID-19 research, titled “T-cell activation in COVID-19 for diagnostic and therapeutic insights”, and (b) Children’s Oncology Group - Solid Malignancies - Integrated Translational Science Center (SM-ITSC) award, titled “Analytical validation of plasma microRNA biomarker in pediatric osteosarcoma”.
- Finally, I submitted my first R01 application which was scored (Percentile score – 33; Impact score -37), but not funded. I am about to resubmit the revised R01 application (November 2020).

How were the results disseminated to communities of interest?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Nothing to Report.

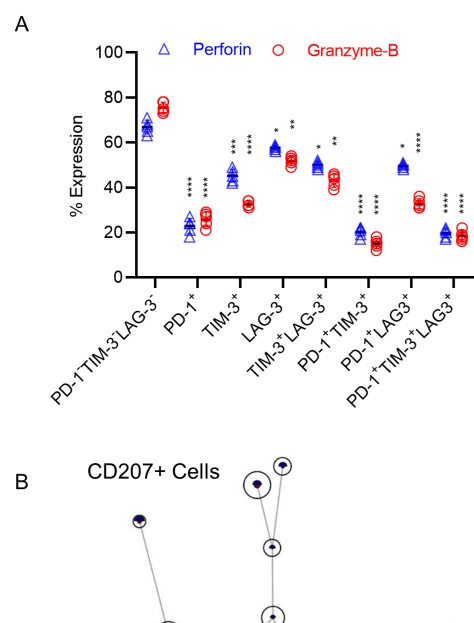


Figure 3. Infiltrating T lymphocytes in LCH tumor microenvironment exhibit exhausted phenotype. (A) Relative expression of perforin and granzyme B in LCH lesion infiltrating CD8⁺ T cells either expressing neither PD-1, TIM-3, or LAG-3, or expressing one or more of the immune checkpoint receptors (n=5). Data are mean ± SD. **(B)** Pie chart representing FlowSOM-based assessment of PD-L1 and PD-L2 expression on CD207⁺ gated cells. **(C)** Quantification of PD-L1 and PD-L2 expression on CD207⁺ gated cells obtained from LCH lesion and skin controls (n=5 each). For **A** and **C**, data are mean ± SD; *P<0.05; **P<0.01; ***P<0.001, ****P<0.0001, ns, not significant (P>0.05) – one-way ANOVA with Tukey’s post hoc test.

What do you plan to do during the next reporting period to accomplish the goals?

If this is the final report, state "Nothing to Report."

I plan to accomplish the goals set within Major Tasks 1-3 in the original Statement of Work and disseminate the information to the concerned communities through presentations, publications, and conference/symposium attendance.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

The parallel experiments allowed us to optimize the experimental conditions which will allow the quick completion of proposed experiments now that the HRPO approval has been obtained.

What was the impact on other disciplines?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

What was the impact on technology transfer?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

What was the impact on society beyond science and technology?

If there is nothing significant to report during this reporting period, state "Nothing to Report."

Nothing to Report.

- 5. CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, "Nothing to Report," if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

There is no change in approach.

Actual or anticipated problems or delays and actions or plans to resolve them

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

As mentioned above there was significant delay (8 months) to obtain the HRPO approval which hindered the planned execution of experiments proposed under Major Tasks 1 and 2. However, we used the time to obtain the ACURO approval which will make up around 3-4 months of the lost time. In addition, we are confident of expediting the proposed experiments without compromising on the scientific rigor and quality to make up for the lost time and prevent any adverse effect on the expected outcome from this project.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required, were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to report.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS: List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."

- Publications, conference papers, and presentations**

Report only the major publication(s) resulting from the work under this award.

Journal publications.

1. **Chakraborty R**, Abdel-Wahab O, Durham B.H. MAP-Kinase-Driven Hematopoietic Neoplasms: A Decade of Progress in the Molecular Age. Cold Spring Harbor Perspective in Medicine Jun 29:a034892. doi: 10.1101/cshperspect.a034892.
2. Sengal A, Velasquez J, Hahne M, Burke TM, Abhyankar H, Reyes R, Olea W, Scull B, Eckstein O, Bigenwald C, Bollard CM, Yu E, Merad M, McClain KL, Allen CE, **Chakraborty R**. Overcoming T cell exhaustion in LCH: PD-1 blockade and targeted MAPK inhibition are synergistic in a mouse model of LCH. Blood (Manuscript ID # BLD-2020-005867R2) Accepted on September 17, 2020. **In press.**

Books or other non-periodical, one-time publications.

None to Report.

Other publications, conference papers and presentations.

None to Report.

- Website(s) or other Internet site(s)**

None to Report.

- **Technologies or techniques**

None to Report.

- **Inventions, patent applications, and/or licenses**

None to Report.

- **Other Products**

None to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change".

Name: Rikhia Chaudhury Chakraborty

Project Role: PI

Nearest person month worked: 3.6

Contribution to Project: Primarily responsible for the organization and operation of the project.

Funding Support: Children's Oncology Group, BCM Pilot COVID-19 Grant, National Cancer Institute.

Name: Carl E Allen

Project Role: Mentor

Nearest person month worked: 0.12

Contribution to Project: Weekly and quarterly meeting with PI to advise on progress and goals.

Funding Support: National Cancer Institute, Leukemia and Lymphoma Society, St. Baldrick's Foundation, Cookies for Cancer Research, Children's Oncology Group.

Name: Miriam Merad

Project Role: Mentor

Nearest person month worked: 0.12

Contribution to Project: Quarterly meeting with PI to advise on progress and goals.

Funding Support: National Cancer Institute, Boehringer Ingelheim, Regeneron.

Name: Jessica Velazquez

Project Role: Research Technician

Nearest person month worked: 6.0

Contribution to Project: Jessica is responsible for sorting cell populations and preparing for transcriptomic analysis.

Funding Support: National Cancer Institute, Children's Oncology Group.

Name: Ryan Fleischman

Project Role: Research Technician

Nearest person month worked: 0.12

Contribution to Project: Ryan is responsible for mass cytometry.

Funding Support: National Cancer Institute, Children's Oncology Group.

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

New funding 05/20 – 04/21	Baylor College of Medicine – Pilot projects on COVID-19 research Role: PI Title: <i>T-cell activation in COVID-19 for diagnostic and therapeutic insights.</i> Major Goals: Characterize T cell populations and T cell function in COVID-19 patients with a range of clinical severity and compare T cells in COVID-19 patients to other diseases of pathologic hyperinflammation, namely severe sepsis and hemophagocytic lymphohistiocytosis (HLH).
10/19 – 09/20	Children's Oncology Group - Solid Malignancies - Integrated Translational Science Center (SM-ITSC) Role: PI Title: <i>Analytical validation of plasma microRNA biomarker in pediatric osteosarcoma.</i> Major Goals: Discovery and validation of non-invasive miRNA biomarkers for early detection in pediatric osteosarcoma.

What other organizations were involved as partners?
If there is nothing significant to report during this reporting period, state "Nothing to Report."

None to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

Not applicable.

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*



MAP-Kinase-Driven Hematopoietic Neoplasms: A Decade of Progress in the Molecular Age

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Mutations in members of the mitogen-activated protein kinase (MAPK) pathway are extensively studied in epithelial malignancies, with *BRAF* mutations being one of the most common alterations activating this pathway. However, *BRAF* mutations are overall quite rare in hematological malignancies. Studies over the past decade have identified high-frequency *BRAF*^{V600E}, *MAP2K1*, and other kinase alterations in two groups of MAPK-driven hematopoietic neoplasms: hairy cell leukemia (HCL) and the systemic histiocytoses. Despite HCL and histiocytoses sharing common molecular alterations, these are phenotypically distinct malignancies that differ in respect to clinical presentation and suspected cell of origin. The purpose of this review is to highlight the molecular advancements over the last decade in the histiocytic neoplasms and HCL and discuss the impact these insights have had on our understanding of the molecular pathophysiology, cellular origins, and therapy of these enigmatic diseases as well as perspectives for future research directions.

The mitogen-activated protein kinase (MAPK) pathway has a long association with human neoplasia. A key member in this pathway is the *BRAF* serine/threonine kinase belonging to the *RAF* family of serine/threonine kinases, which also includes *ARAF* and *RAF1*. *RAF* kinases transduce mitogenic signals from the cell membrane to the nucleus and regulate MEK-ERK signaling. Of the *RAF* kinases, *BRAF* is most frequently mutated in cancer with *BRAF*^{V600E} accounting for 90% of activating mutations

(Wellbrock et al. 2004). Similarly, the neoplastic cells of the systemic histiocytoses (SHs) and hairy cell leukemia (HCL) have nearly universal ERK overexpression suggesting constitutive activation of MAPK signaling in these distinct hematological neoplasms (Fig. 1; Badalian-Very et al. 2010; Tiacci et al. 2011; Haroche et al. 2012).

Although quite rare in hematological disorders overall, *BRAF* mutations are strikingly enriched in two sets of diseases: classical HCL

Editors: Michael G. Kharas, Ross L. Levine, and Ari M. Melnick

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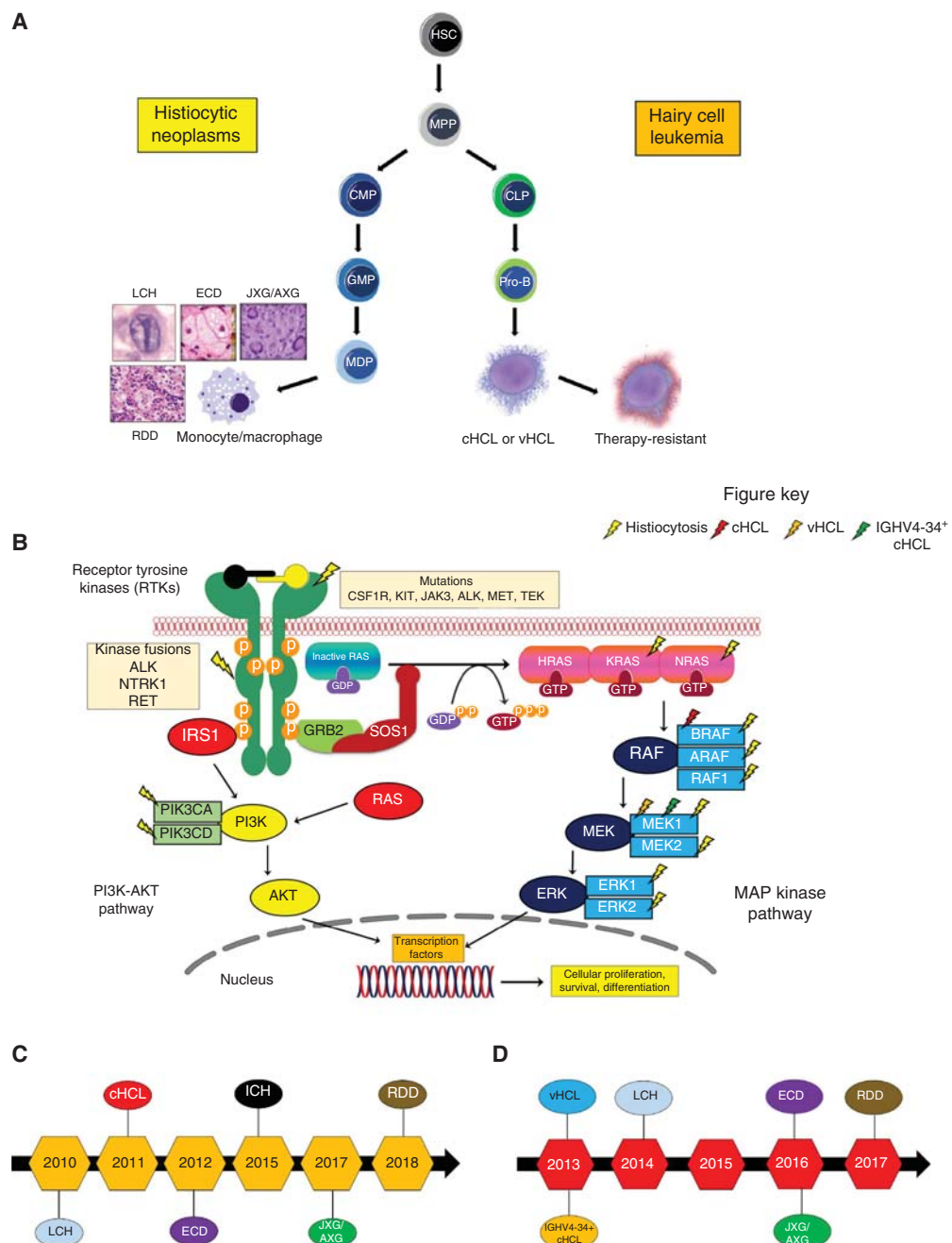


Figure 1. Overview of the mitogen-activated protein kinase (MAPK)-driven hematopoietic neoplasms with common molecular alterations but divergent phenotypes. (A) Diagram demonstrating the divergent hematopoietic development of histiocytic neoplasms and hairy cell leukemia. (B) Diagram of the MAPK and PI3K-AKT signaling pathways with description of the activation of the RAS proteins (HRAS, KRAS, and NRAS) with annotation of the signaling proteins affected by genetic alterations in the histiocytic neoplasms, classical hairy cell leukemia, IGHV4-34⁺ classical hairy cell leukemia, and hairy cell leukemia variant. (C) Timeline of the discovery of recurrent *BRAF*^{V600E} mutations in the MAPK-driven hematological neoplasms. (D) Timeline of the discovery of recurrent *MAP2K1* mutations in the MAPK-driven hematological neoplasms. LCH, Langerhans cell histiocytosis; ECD, Erdheim-Chester disease; JXG/AXG, juvenile xanthogranuloma/adult xanthogranuloma; RDD, Rosai-Dorfman-DeStombes disease; ICH, indeterminate cell histiocytosis; cHCL, classical hairy cell leukemia; vHCL, hairy cell leukemia variant; IGHV, immunoglobulin heavy chain variable; HSC, hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, granulocyte-monocyte progenitor; MDP, monocyte-dendritic cell progenitor; CLP, common lymphoid progenitor; Pro-B, pro-B-lymphocyte; RTK, receptor tyrosine kinase.

(cHCL) (Tiacci et al. 2011; Arcaini et al. 2012; Swerdlow et al. 2017) and SH—Langerhans cell histiocytosis (LCH) (Badalian-Very et al. 2010) and Erdheim–Chester disease (ECD) (Haroche et al. 2012). Furthermore, additional sequencing efforts identified recurrent mutations in *MAP2K1* (MEK1) in variant HCL (vHCL) (Waterfall et al. 2014), LCH (Brown et al. 2014; Chakraborty et al. 2014), ECD, and other non-LCH neoplasms (Diamond et al. 2016; Durham et al. 2016). Interestingly, *BRAF*^{V600E} is frequently present in cHCL (~100%) (Tiacci et al. 2011) and LCH and ECD (50%–60%) (Badalian-Very et al. 2010; Haroche et al. 2012); meanwhile, *MAP2K1* mutations are present in vHCL (~50%) (Waterfall et al. 2014; Durham et al. 2017a) and SH (~25%) (Figs. 1–5; Brown et al. 2014; Chakraborty et al. 2014; Diamond et al. 2016; Durham et al. 2016). However, despite their common molecular alterations, these are distinct malignancies with different clinical presentations and biology. Nonetheless, the discovery of recurrent *BRAF*^{V600E} and *MAP2K1* mutations in both malignancies has guided new therapeutic approaches, as well as an opportunity to explore how a common genetic event gives rise to these enigmatic diseases (Fig. 1; Haroche et al. 2013; Hyman et al. 2015; Tiacci et al. 2015; Diamond et al. 2016, 2018, 2019; Durham et al. 2019). This review discusses the amalgamation of diverse kinase alterations uncovered in SH and HCL during the last decade and underscore how new insights have refined our understanding of these disorders as clonal neoplasms with constitutive MAPK and PI3K-AKT activation. We will also highlight how our concepts of the cellular origins of the MAPK-driven hematological neoplasms and molecular therapeutics have started evolving.

OVERVIEW OF HISTIOCYTIC NEOPLASMS

Histiocytic neoplasms are a heterogeneous group of disorders broadly classified as LCH and non-LCH that share the common pathological features of infiltration and accumulation of neoplastic histiocytes in tissues with nearly universal ERK activation and an accompanying inflammatory milieu (Badalian-Very et al. 2010;

Haroche et al. 2012; Swerdlow et al. 2017; Ozkaya et al. 2018). However, a revised classification recategorized LCH and non-LCH into the following: “L” Langerhans group [LCH, ECD, disseminated juvenile/adult xanthogranuloma (JXG/AXG), and indeterminate cell histiocytosis (ICH)]; “C” group (cutaneous JXG/AXG and Rosai–Dorfman–Destombes disease [RDD]); “R” group (noncutaneous RDD) (Table 1; Emile et al. 2016).

LANGERHANS CELL HISTIOCYTOSIS

Historical Perspective

The first historical descriptions of LCH patients occurred in case series. Hippocrates reported a patient with a nonfatal disease with painful skull lesions ~400 BC, a presentation that could be consistent with LCH (Donadieu and Pritchard 1999). Later, Hand–Schüller–Christian described patients with rash, lytic bone lesions, and diabetes insipidus (DI) and Letterer–Siwe discussed a fatal disseminated disease (Hand 1893; Schüller 1915; Christian 1919; Letterer 1924; Siwe 1933). Afterward, Farber characterized single lytic bone lesions as “eosinophilic granulomas” (Farber 1941). However, Lichtenstein noted a similar histology in these clinically diverse descriptions and posited they constitute a common syndrome he called “histiocytosis X,” with the “X” indicating uncertain cellular origin (Lichtenstein 1953). Eventually, Nezelof utilized electron microscopy and reported Birbeck granules in both LCH lesions and epidermal Langerhans cells (LCs), leading to the hypothesis that LCH arises from pathologically activated LCs (Nezelof et al. 1973; Lampert 1998; Arceci 1999).

Clinical Presentation

LCH has diverse manifestations from self-resolving, single-organ lesions to multi-organ disease, which is associated with 10%–20% mortality (Arceci 1999; McClain et al. 2004). Bone (75%) and skin (34%) are the most commonly involved organs with lytic bone lesions frequently involving the skull (Table 1; Guyot-Goubin

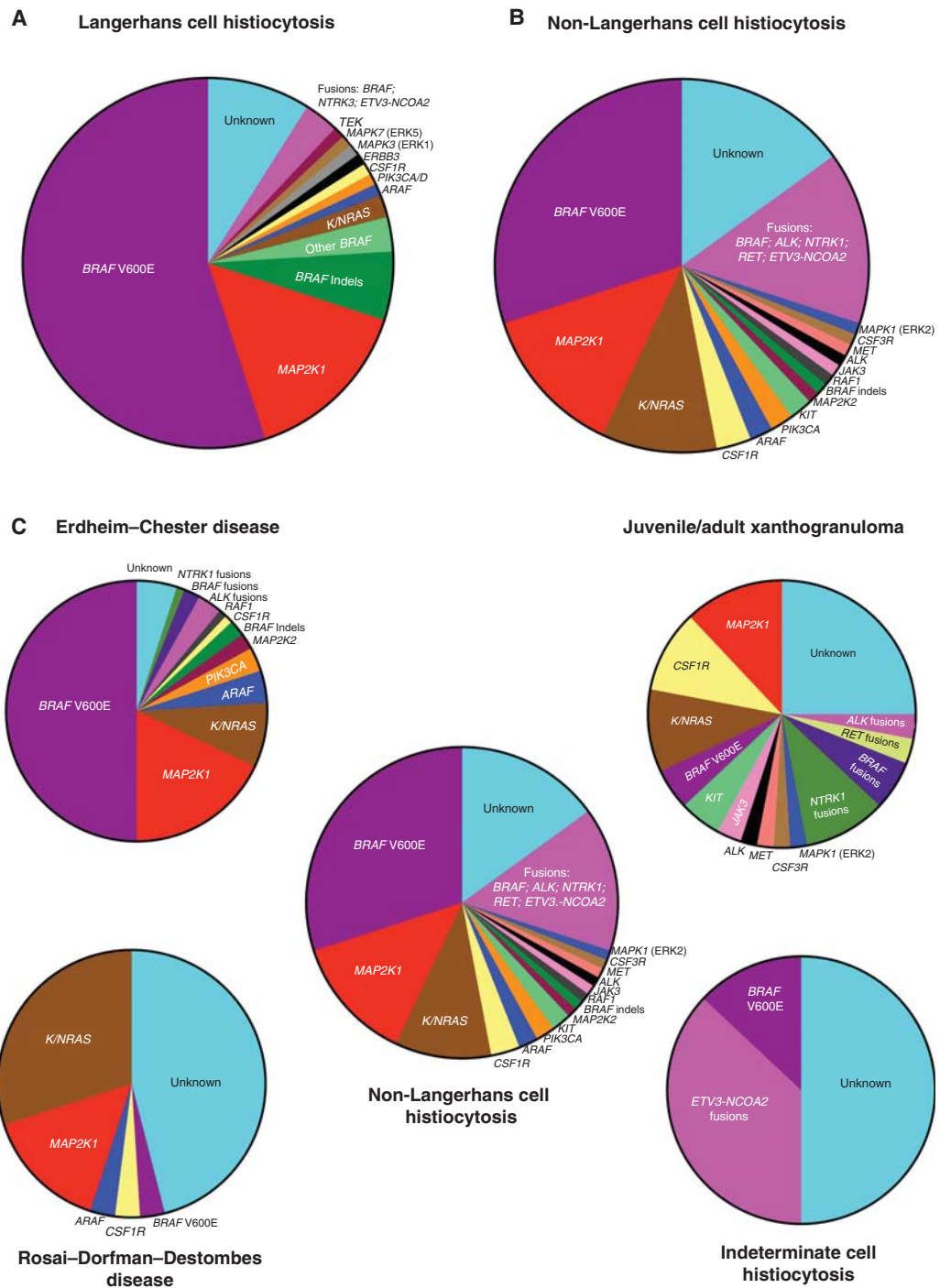


Figure 2. Summary of diverse kinase alterations discovered in the histiocytic neoplasms. (A) Pie chart illustrating a composite of the known kinase alterations in Langerhans cell histiocytosis. (B) Pie chart showing a composite of the known kinase alterations in non-Langerhans cell histiocytoses. (C) Pie charts demonstrating the published kinase alterations in the four discussed subcategories of the non-Langerhans cell histiocytic neoplasms.

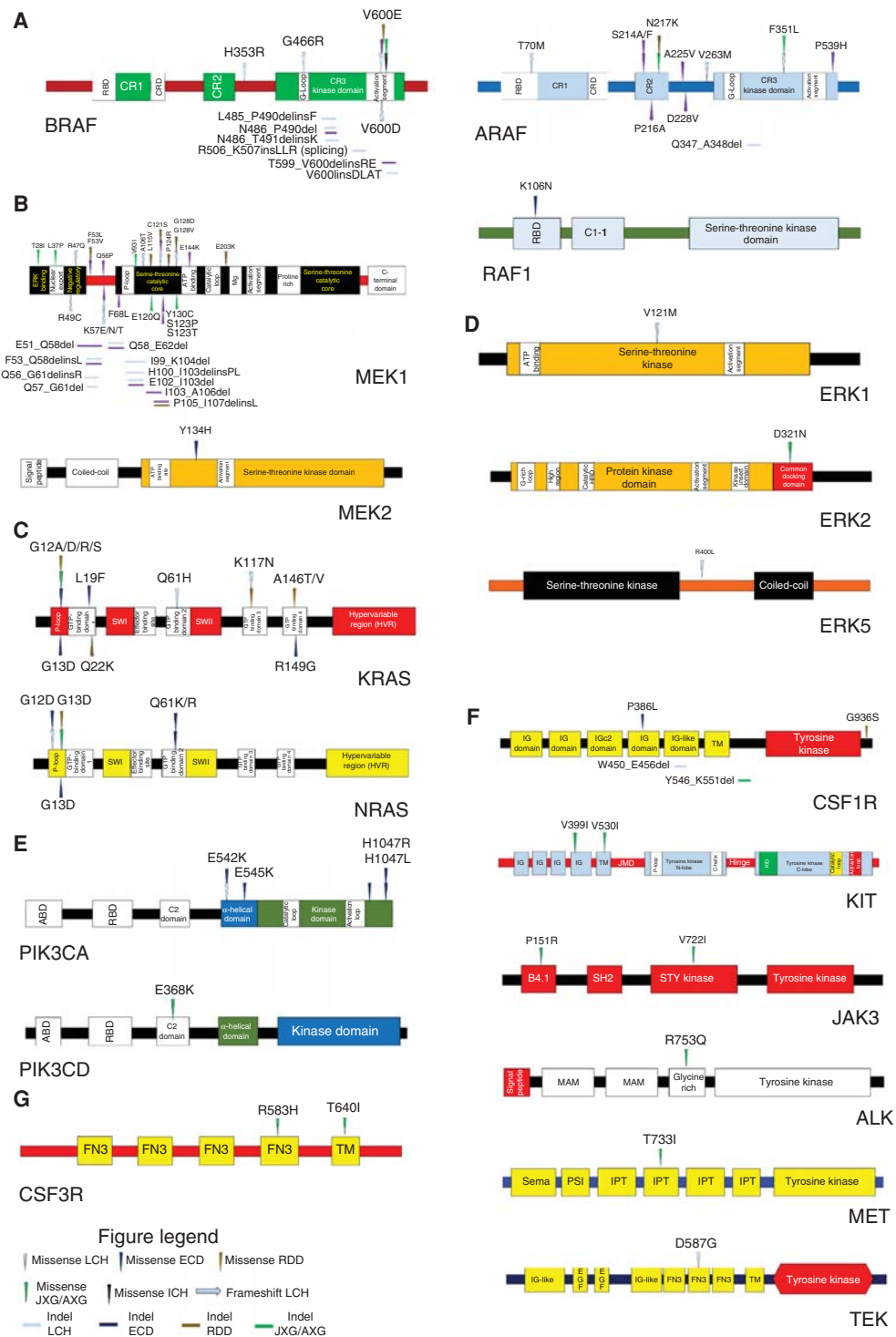


Figure 3. Summary of the diverse kinase mutations in histiocytic neoplasms. (A) Protein diagrams cataloging the published somatic mutations described in the RAF isoforms (BRAF, ARAF, and RAF1 [CRAF]). (B) Protein diagrams cataloging the somatic mutations discovered in MEK1 and MEK2. (C) Protein diagrams cataloging the somatic mutations uncovered in the RAS isoforms (KRAS and NRAS). (D) Protein diagrams cataloging somatic mutations described in ERK1, ERK2, and ERK5. (E) Protein diagrams cataloging somatic mutations involving the PI3K isoforms (PIK3CA and PIK3CD). (F) Protein diagrams documenting somatic mutations recently discovered in the receptor tyrosine kinases. (G) Protein diagram cataloging somatic mutations in CSF3R. LCH, Langerhans cell histiocytosis; ECD, Erdheim–Chester disease; JXG/AXG, juvenile xanthogranuloma/adult xanthogranuloma; RDD, Rosai–Dorfman–Destombes disease; ICH, indeterminate cell histiocytosis.

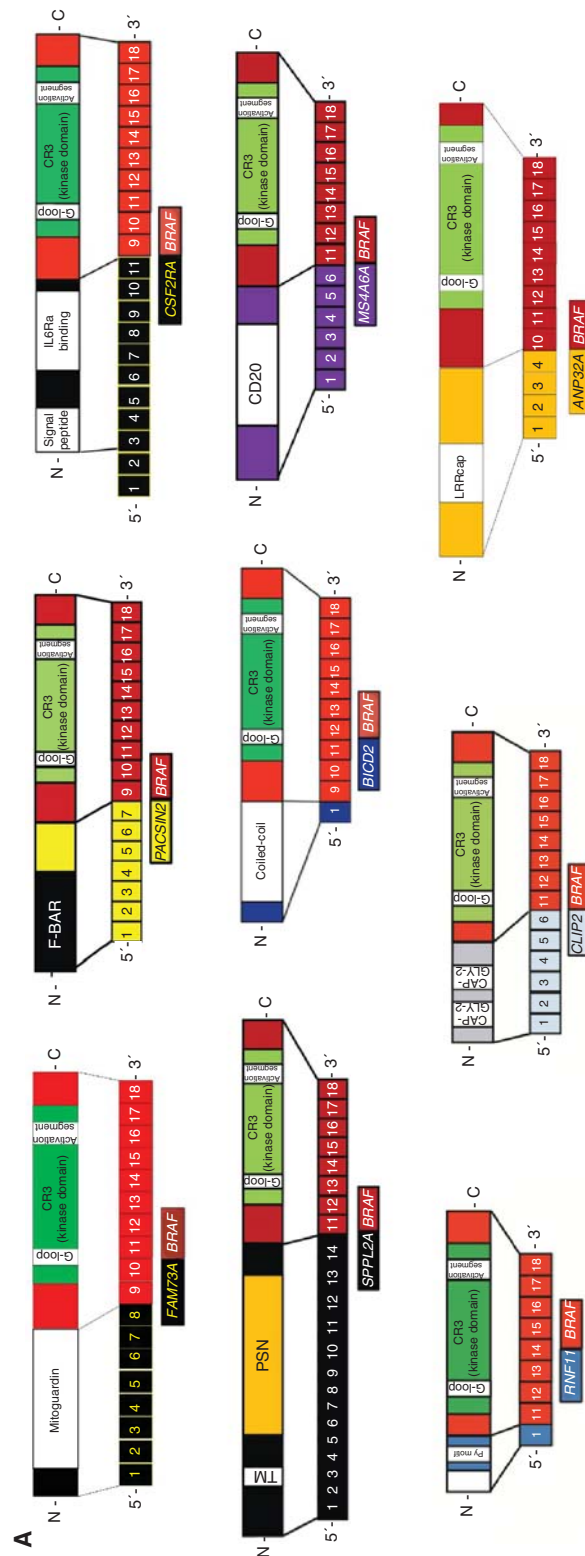


Figure 4. Summary of the diverse kinase fusions in histiocytic neoplasms. (A) Illustrations of recurrent *BRAF* fusions discovered in the histiocytic neoplasms. (B) Illustrations of the recurrent *NTRK1* fusions uncovered in non-Langerhans cell histiocytoses and an *NTRK3* fusion in Langerhans cell histiocytosis. (C) Illustrations of recurrent *ALK* fusions described in the non-Langerhans cell histiocytic neoplasms. (D) Illustration of the recurrent *ETV3-NCOA2* fusion discovered in both Langerhans cell histiocytosis and non-Langerhans cell histiocytosis. (E) Illustration of the recurrent *NCOA4-RET* fusion recently discovered in non-Langerhans cell histiocytosis.

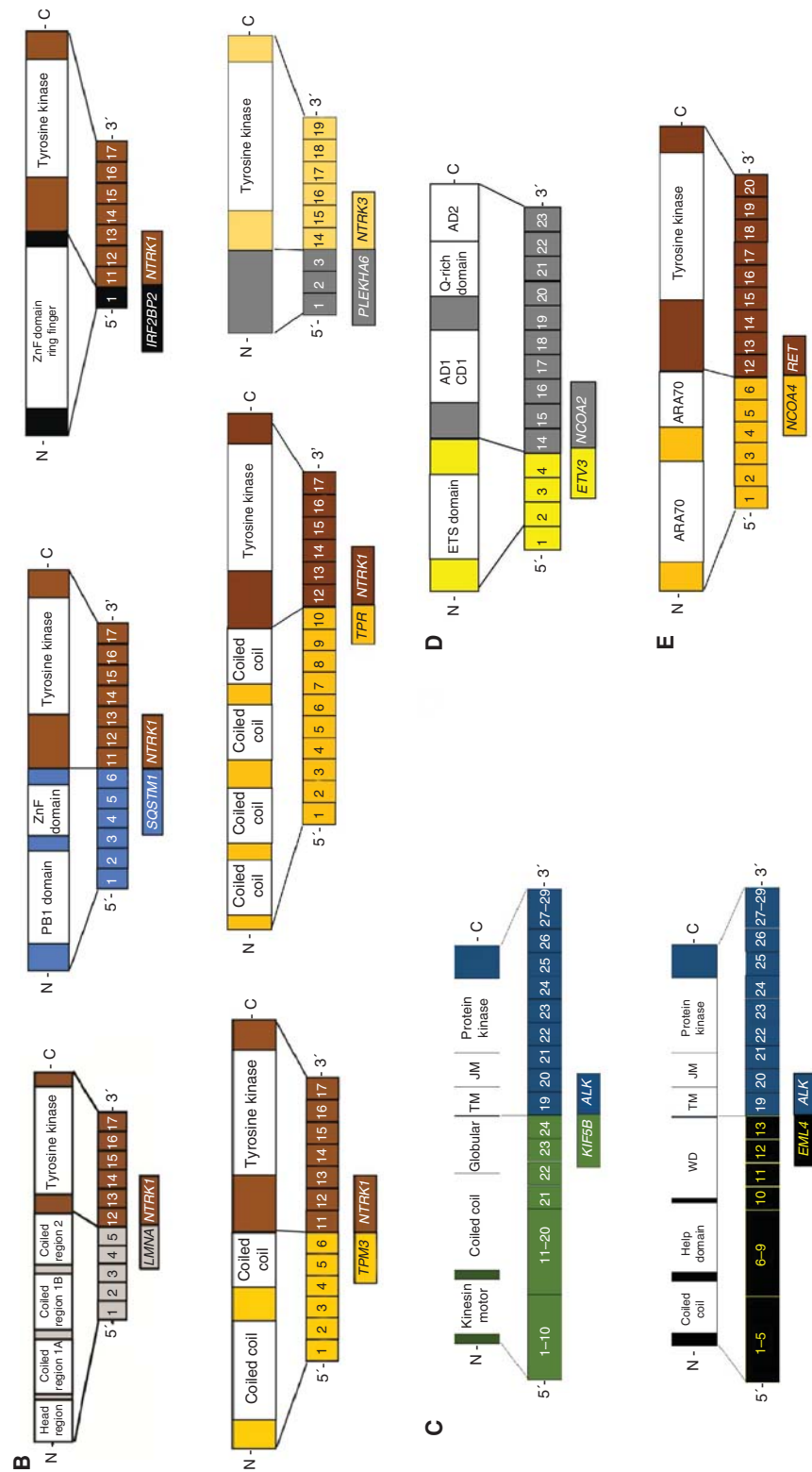


Figure 4. (Continued.)

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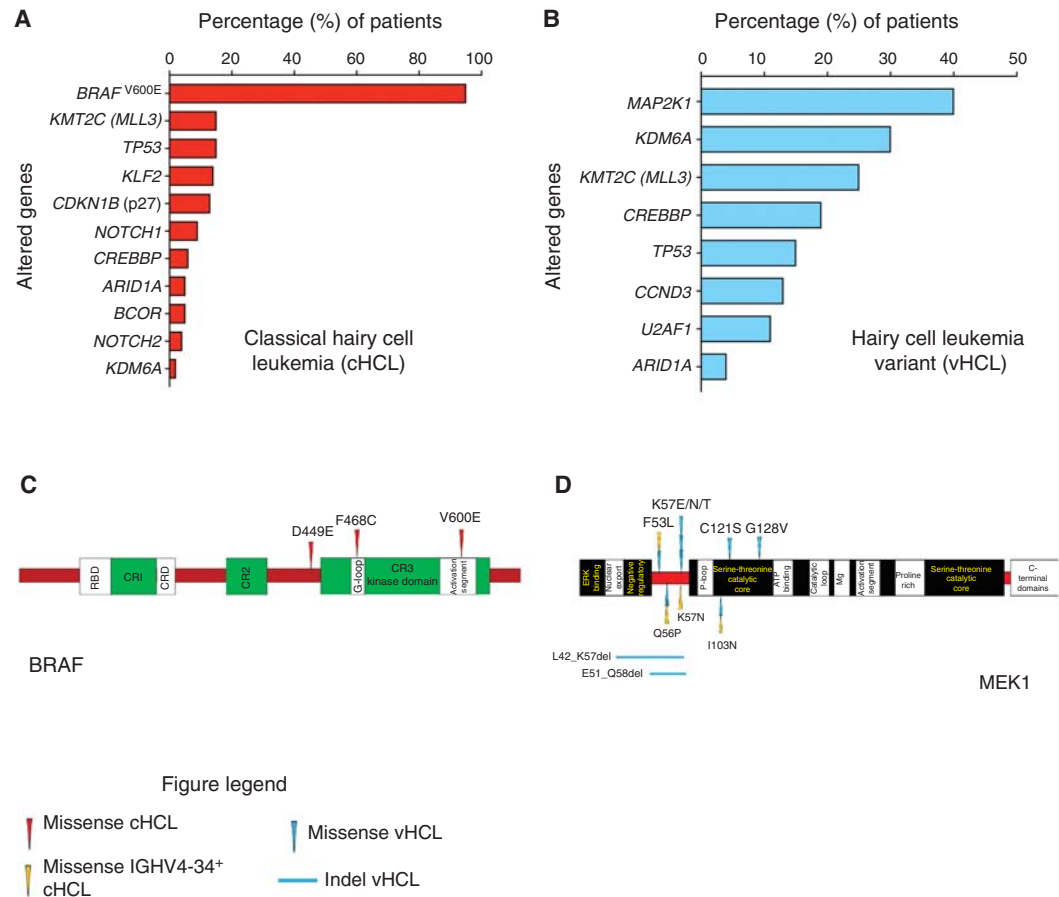


Figure 5. Summary of genetic alterations in hairy cell leukemia. (A) Histogram of driver and co-occurring mutations reported in classical hairy cell leukemia. (B) Histogram of driver and co-occurring mutations described in hairy cell leukemia variant. (C) Protein diagram mapping somatic mutations involving BRAF that have been uncovered in classical hairy cell leukemia. (D) Protein diagram mapping somatic mutations in MEK1 discovered in hairy cell leukemia variant and *IGHV4-34⁺* classical hairy cell leukemia. CHCL, Classical hairy cell leukemia; vHCL, hairy cell leukemia variant; *IGHV*, immunoglobulin heavy chain variable.

et al. 2008; Stålemark et al. 2008). Besides skin, LCH may arise in any mucosal tissue (gingiva, gastrointestinal tract) (Broadbent et al. 1994; Guyot-Goubin et al. 2008). “High-risk” LCH includes diffuse infiltration or focal lesions of spleen, liver, or bone marrow with a 5-yr survival rate of 84% compared to 99% in “low-risk” LCH (Gadner et al. 2013). LCH may also involve the central nervous system (LCH-CNS), presenting with mass lesions, diabetes insipidus, or progressive neurodegenerative symptoms (LCH-ND) arising decades after initial presentation (Grois et al. 1998, 2010; Héritier et al. 2018).

Pathologically, LCH is characterized by lesions composed of clonal, pathological “histiocytes” with reniform (coffee-bean-shaped) nuclei and abundant, pink cytoplasm with immunoreactivity for CD1a and langerin (CD207) and pathognomonic Birbeck granules (Table 1; Nezelof et al. 1973; Favara et al. 1997; Chikwava and Jaffe 2004; Swerdlow et al. 2017). Histology also shows a milieu of pathologic dendritic cells (DCs) and recruited inflammatory cells (lymphocytes, eosinophils, and macrophages) (Laman et al. 2003; Senechal et al. 2007; Allen et al. 2010; Berres et al. 2014).

Table 1. Summary of the classification, pathological, and radiological features of the histiocytic neoplasms.

Disease	LCH	ECD	JXG/AXG	RDD	ICH
Broad classification	LCH	Non-LCH	Non-LCH	Non-LCH	Non-LCH
Revised classification groupings	L	L	L (extracutaneous) and C (cutaneous)	C (cutaneous) and R (other RDD)	L
Immunophenotypic features					
CD68	+	++	++	++	+
CD163	–	++	++	++	–
CD14	–	++	++	++	–
CD1a	++	–	–	–	++
CD207 (Langerin)	++	–	–	–	–
S100	+	–/+	–/+	+	+
Factor XIIIa	–	+	+	+	–
CD45	+	+	+	+	+
Histological features					
Birbeck granules	Yes	No	No	No	No
Xanthomatous histiocytes	No	Yes	Yes	No	No
Touton giant cells	No	Yes	Yes	No	No
Emperipolesis (intracytoplasmic lymphocytes)	No	Occasional	Occasional	Abundant	No
Radiological features					
Bilateral, symmetric osteosclerosis involving meta-diaphysis of femur, tibia, and fibula	Rare	Frequent, pathognomonic	No	No	No
Lytic, “punched-out” lesions of skull and axial skeleton	Frequent	Rare	No	No	No
Infiltrative, perinephric soft tissue thickening (“hairy kidney”)	No	Frequent	Rare	No	No

LCH, Langerhans cell histiocytosis; ECD, Erdheim–Chester disease; JXG, juvenile xanthogranuloma; AXG, adult xanthogranuloma; RDD, Rosai–Dorfman–Destombes disease; ICH, indeterminate cell histiocytosis; L group, Langerhans-related; C group, cutaneous and mucocutaneous; R group, Rosai–Dorfman–Destombes disease; +, low expression; ++, high expression; –, no expression.

JUVENILE/ADULT XANTHOGRANULOMA

Clinical Presentation

JXG/AXG was originally described in the early 1900s and was believed to be endothelium derived and was named “nevooxanthoendothelioma” (McDonagh and McDonagh 1912). JXG/AXG is usually self-limiting with dermal lesions in most patients; but 4% present with disseminated

disease (Weitzman and Jaffe 2005; Allen and Parsons 2015). Histologically, JXG/AXG shows xanthomatous histiocytes with admixed multinucleated and Touton giant cells that are immunoreactive for CD68, CD163, CD14, fascin, and Factor XIIIa with variable positivity for S100 and no immunoreactivity for CD1a or CD207 (Table 1; Weitzman and Jaffe 2005; Diamond et al. 2014; Haroche and Ablu 2015; Swerdlow et al. 2017).

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ERDHEIM-CHESTER DISEASE

Clinical Presentation

ECD is a rare, systemic, non-LCH disease with around 800 reported cases in the literature that was first described as a “lipoid granulomatosis” in 1930 by Erdheim and Chester. ECD has diverse clinical manifestations ranging from localized presentations (bone-only disease) to multisystem disease that are extensively discussed elsewhere (Chester 1930; Weitzman and Jaffe 2005; Diamond et al. 2014; Haroche and Ablu 2015; Estrada-Veras et al. 2017; Haroche et al. 2017; Cohen-Aubart et al. 2018). The diagnosis of ECD requires combining the histological criteria and the appropriate clinical and radiological setting (Weitzman and Jaffe 2005; Diamond et al. 2014; Haroche and Ablu 2015; Swerdlow et al. 2017). Radiographically, bilateral and symmetric diaphyseal and metaphyseal osteosclerosis of the legs is observed in most patients. Histologically, ECD shows xanthomatous histiocytes with surrounding fibrosis, as well as admixed multinucleated giant cells with immunoreactivity for CD68, CD163, CD14, fascin, and Factor XIIIa and negativity for CD1a and CD207 (Table 1; Weitzman and Jaffe 2005; Diamond et al. 2014; Haroche and Ablu 2015; Swerdlow et al. 2017).

ROSAI-DORFMAN-DESTOMBES DISEASE

Clinical Presentation

RDD is a rare, non-LCH hematological disorder known as “sinus histiocytosis with massive lymphadenopathy” that was first described by Destombes, Rosai, and Dorfman (Destombes 1965; Rosai and Dorfman 1969, 1972; Haroche and Ablu 2015). RDD has heterogeneous clinical manifestations and can occur as an isolated disorder or in association with hereditary, autoimmune, or neoplastic conditions. The majority of RDD patients present with classical (nodal) RDD that primarily manifests as bilateral, massive, and painless cervical lymphadenopathy with or without fever, night sweats, and weight loss. However, 43% of patients develop extranodal RDD with 19% showing multisystem RDD, and prognosis has been correlated with the

number of extranodal systems affected with a detailed clinical discussion reviewed elsewhere (Haroche and Ablu 2015; Ablu et al. 2018). Histologically, the abnormal, xanthomatous histiocytes of RDD demonstrate abundant emperipolesis of erythrocytes, lymphocytes, and plasma cells. The abnormal histiocytes are immunoreactive for CD14, CD68, CD163, and S100 with negativity for CD1a and CD207 (Table 1; Weitzman and Jaffe 2005; Haroche and Ablu 2015; Swerdlow et al. 2017; Durham 2019).

INDETERMINANT CELL HISTIOCYTOSIS

Clinical Presentation

ICH is a rare, non-LCH neoplasm first described in 1985 that predominantly involves the skin and is characterized by the presence of dendritic cells that are morphologically and immunophenotypically like LCH. However, ICH shows a dense dermal infiltration of neoplastic histiocytes admixed with lymphocytes without significant eosinophilic infiltration and is immunoreactive for CD68, S100, and CD1a but not for CD163 and CD207. Therefore, unlike LCH, ICH lacks CD207 and Birbeck granules (Table 1; Wood et al. 1985; Rezk et al. 2008).

MOLECULAR PATHOPHYSIOLOGY OF HISTIOCYTIC NEOPLASMS

Prior to the molecular era, the determination of whether SHs were reactive or neoplastic was unclear and constituted a historical debate (Arceci et al. 1998; Degar and Rollins 2009). Furthermore, the cellular heterogeneity of histiocytoses and the limitations of molecular technology precluded classification of SHs as neoplasms (Merad et al. 2002; Senechal et al. 2007; da Costa et al. 2009). However, the dawning of the molecular era revealed a series of activating kinase alterations involved in MAPK, PI3K-AKT, and RTK signaling within the SHs (Figs. 2–4).

RAF Isoforms

The discovery of *BRAF* mutations in the histiocytoses occurred after *BRAF*^{V600E} was reported



in 57% of LCH (Badalian-Very et al. 2010) and 54% of ECD (Haroche et al. 2012). Later studies uncovered *BRAF*^{V600E} in JXG/AXG, RDD, and ICH but were not prevalent in SHs other than LCH and ECD (O'Malley et al. 2015; Techavichit et al. 2017; Fatobene et al. 2018; Durham et al. 2019). Besides *BRAF*^{V600E}, SH case reports have revealed other activation segment *BRAF* mutations (*BRAF*^{V600D}; *BRAF*^{V600insDLAT}) (Satoh et al. 2012; Kansal et al. 2013). Additionally, a *BRAF* splicing mutation (*BRAF* c.1511_1517+2 duplication) was reported (Héritier et al. 2017). Furthermore, activating, in-frame deletions in *BRAF* exon 12 (encodes the β3-αC loop critical for kinase activation) and numerous *BRAF* fusions have been described in SHs (Figs. 2, 3A, 4A; Chakraborty et al. 2016; Lee et al. 2017; Zarnegar et al. 2018; Durham et al. 2019). Other whole-exome sequencing (WES) studies have revealed activating *ARAF* mutations in LCH (Nelson et al. 2014) and non-LCH (ECD; JXG/AXG; RDD) along with *RAF1* mutations in ECD (Figs. 2, 3A; Diamond et al. 2016, 2019; Durham et al. 2019).

MAP2K1/MAP2K2

In *BRAF*^{V600E}-negative histiocytoses, NGS studies found *MAP2K1* to be a second recurrently mutated gene locus in LCH (Brown et al. 2014; Chakraborty et al. 2014) and non-LCH (ECD, JXG/AXG, and RDD) (Diamond et al. 2016; Garces et al. 2017). Functionally, the *MAP2K1* mutations occurred within mutational hotspots and clustered in the amino-terminal regulatory domain (exon 2) and amino-terminal kinase domain (exon 3) resulting in MAPK activation (Chakraborty et al. 2014; Nelson et al. 2015; Diamond et al. 2016; Garces et al. 2017). Additionally, ECD sequencing found recurrent *MAP2K2*^{Y134H} in the MEK2 kinase domain that activated MAPK signaling (Figs. 2, 3B; Diamond et al. 2019; Durham et al. 2019).

RAS Isoforms

The RAS isoforms encode small GTPases that regulate the MAPK and PI3K-AKT signaling pathways. First, *NRAS* mutations were found

in single cases of LCH and ECD (Ozono et al. 2011; Diamond et al. 2013). Afterward, studies confirmed that *NRAS/KRAS* mutations are recurrent in SH and affected the GTP-binding domains leading to constitutive MAPK activation (Figs. 2, 3C; Emile et al. 2014; Diamond et al. 2016; Shanmugam et al. 2016; Garces et al. 2017; Lee et al. 2017; Durham et al. 2019).

Extracellular-Signal-Regulated Kinases (ERK) Isoforms

As the molecular age continued to interrogate *BRAF*^{V600}-negative histiocytoses, rare mutations started to emerge in the ERK isoforms. An activating *MAPK1*^{D321N} affecting the ERK2 carboxy-terminal-docking domain surfaced in JXG and showed in vitro sensitivity to ERK inhibition but not RAF or MEK inhibition (Chakraborty et al. 2017). Another WES study of LCH found *MAPK3*^{V121M} in the ERK1 kinase domain and *MAPK7*^{R400L} affecting the ERK5 carboxy-terminal domain with both mutations influencing MAPK signaling (Figs. 2, 3D; Durham et al. 2019).

PI3K Isoforms

The PI3K isoforms include phosphatidylinositol-4,5-bisphosphate-3-kinase catalytic subunit alpha (*PIK3CA*) and catalytic subunit delta (*PIK3CD*), members of the PI3K-AKT signaling pathway. Recurrent *PIK3CA* mutations were first revealed in ECD (Emile et al. 2014) and then in LCH (Héritier et al. 2015). Later studies primarily identified *PIK3CA* mutations as recurrent events in ECD with rare *PIK3CD* mutations in JXG and LCH. *PIK3CA* mutations clustered in the α-helical and kinase domains leading to PI3K-AKT activation (Figs. 2, 3E; Chakraborty et al. 2014; Diamond et al. 2016; Durham et al. 2019).

Receptor Tyrosine Kinases

Continued sequencing of histiocytoses implicated the RTKs. A WES study found a case of *ERBB3*-mutated LCH (Chakraborty et al. 2014). Later studies uncovered recurrent *ALK* and

NTRK1 fusions in ECD and JXG/AXG (Diamond et al. 2016; Lee et al. 2017). Then, a large WES/NGS study evaluated 270 histiocytoses patients and discovered recurrent, activating mutations in *CSF1R*, the RTK critical for monocyte and macrophage development, which was enriched in JXG/AXG but found across histiocytoses; and this study was really the first time that activating *CSF1R* mutations have been implicated in cancer. Additionally, other RTK alterations were uncovered in JXG/AXG (*KIT*, *JAK3*, *ALK*, *MET*, and *CSF3R*) and in LCH (*TEK*), as well as the first *RET* and *NTRK3* fusions in the histiocytoses (Figs. 2, 3F–G, 4; Cai et al. 2019; Durham et al. 2019).

ETV3-NCOA2 Fusions

ETV3-NCOA2 fusions were described in ICH and then reported in one LCH case (Brown et al. 2015; Lee et al. 2017). These fusions involve exons 1–4 of *ETV3* and exons 14–23 of *NCOA2*. This leads to the preservation and fusion of the carboxy-terminal transcriptional activation domains of *NCOA2* to the amino-terminal ETS domain of *ETV3* (Fig. 4D; Wang et al. 2012; Mesquita et al. 2013; Brown et al. 2015), and prior studies of *NCOA2* gene fusions have demonstrated that the AD1 and CID domains in the carboxyl terminus are required for the transformation of *NCOA2* fusion proteins (Figs. 2, 4D; Carapeti et al. 1998; Deguchi et al. 2003; Strehl et al. 2008; Sumegi et al. 2010; Wang et al. 2012; Brown et al. 2015; Durham 2019). However, extensive functional characterization of the role of the *ETV3-NCOA2* fusion in SH pathogenesis is warranted.

Overall, the molecular age demonstrates most SH patients harbor diverse alterations in MAPK, PI3K-AKT, and RTK pathway genes supporting that histiocytoses are clonal, hematopoietic neoplasms with many molecularly directed therapeutic targets.

CELLULAR-ORIGIN STUDIES IN HISTIOCYTOSSES

Identification of MAPK mutations has provided a molecular etiology for the SHs and a tool to

trace potential precursor cells in these neoplasms. Thus, there has been an accumulation of evidence building on Nezelof's historical proposal of a pathological hematopoietic precursor for LCH ("pathological LCs") that has initiated new cellular origin studies into the SHs (Nezelof and Basset 2001). Gene expression studies in LCH and non-LCH have documented SH lesions have expression profiles of myeloid-derived precursors and not epidermal LCs (Allen et al. 2010; Diamond et al. 2016). Additionally, *BRAF*^{V600E} was traced to CD34⁺ hematopoietic stem/progenitor cells (HSPCs) in studies of high-risk pediatric and adult multisystem LCH and ECD patients but not in patients with single-system or low-risk, multifocal LCH (Berres et al. 2014; Milne et al. 2017; Durham et al. 2017b).

Furthermore, several groups have generated murine models with enforced *BRAF*^{V600E}-expression in langerin⁺ cells resulting in formation of localized LCH-like lesions and in CD11c⁺ cells resulting in a more aggressive phenotype similar to high-risk LCH (Berres et al. 2014; Mass et al. 2017). Notably, xenotransplantation studies using CD34⁺ HSPCs from histiocytosis patients gave rise to genetically and phenotypically accurate xenografts, which provided functional evidence of the self-renewal capacity of kinase-altered HSPCs in SH patients (Durham et al. 2017b). Cumulatively, current evidence led to the proposal of a revised model of histiocytosis pathogenesis, the misguided myeloid differentiation model, in which the developmental stage at which an ERK-activating alteration arises determines the clinical manifestations. Thus, the cell of origin of at least a proportion of SH patients resides in the HSPC compartment prior to committed monocyte or dendritic cell differentiation (Milne et al. 2017; Durham et al. 2017b).

Additional murine studies into alternate cells of origin demonstrated that mosaic expression of *Braf*^{V600E} in yolk sac-derived, erythromyeloid progenitors (EMPs), the cell of origin of murine tissue-resident macrophages (e.g., microglia and Kupffer cells), led to clonal expansion and the accumulation of ERK-activated microglia in the CNS of these models. These

mice developed a severe, late-onset neurodegenerative disorder but lacked the systemic accumulation of histiocytes outside the CNS as was seen in models with *Braf*^{V600E} expression in HSC-derived cells. Therefore, these results illustrate yolk sac–derived EMPs can be a potential cell of origin in the histiocytoses (Gomez Perdiguero et al. 2015; Mass et al. 2017). Thus, more than one immediate cellular precursor and the potential for alternate cells-of-origin exist for SHs. However, more investigation is required.

MOLECULARLY BASED HISTIOCYTOSIS THERAPY

Over the past 5 years, SH therapy has dramatically changed with the emergence of targeted therapy and the first U.S. Food and Drug Administration (FDA)-approved SH treatment (Fig. 6A,B) resulting in an improved prognosis for ECD (5-yr survival of 43% in 1996 but 83% currently) (Veyssier-Belot et al. 1996; Cohen-Aubart et al. 2018).

Because 50%–60% of SH patients harbor *BRAF*^{V600E} (Badalian-Very et al. 2010; Haroche et al. 2012), a vemurafenib phase II clinical trial studied *BRAF*^{V600}-mutated ECD and LCH and demonstrated a nearly 100% metabolic response rate, as did several case series. As a result, the U.S. FDA–approved vemurafenib for use in *BRAF*^{V600}-mutated ECD in November 2017 (Fig. 6A; Haroche et al. 2013, 2015; Cohen-Aubart et al. 2014; Hyman et al. 2015; Borys et al. 2016; Bhatia et al. 2018; Diamond et al. 2018). Although BRAF inhibition generally achieves robust and durable responses in *BRAF*^{V600}-mutated histiocytoses, the LOVE study showed 75% of patients who discontinued vemurafenib relapsed in 6 mo but were able to recapture their prior responses when restarted on BRAF inhibitors (vemurafenib; dabrafenib) (Cohen Aubart et al. 2017; Vaglio and Diamond 2017). Additionally, BRAF inhibitor resistance in the SH is rare and has only been reported in one instance where a dabrafenib-treated *BRAF*^{V600E}-mutated ECD patient acquired a *KRAS* mutation, which responded to trametinib (Nordmann et al. 2017).

It is important to note that the extreme sensitivity and durability of response of SH to vemurafenib are quite unique across cancers. Although *BRAF*^{V600E} mutations are seen in ~8% of all cancer types overall, very few cancers have been shown to have high response rates to BRAF inhibitors as single agents as has been seen in SHs (for review, see Holderfield et al. 2014; Crispo et al. 2019). The largest experience with BRAF inhibition has been in the setting of *BRAF*^{V600E}-mutant metastatic melanoma in which BRAF inhibition is most commonly combined with MEK inhibition to improve efficacy and durability of response and reduce side effects from single-agent BRAF inhibition. But even with this approach, although 63%–76% of all patients with advanced *BRAF*^{V600E}-mutant melanoma derive clinical benefit from combined BRAF/MEK inhibition, median progression-free survival lasts only about 9 mo and 90% of patients develop resistance within 1 yr (for review, see Crispo et al. 2019). Currently, BRAF inhibitors are FDA-approved for the therapy of *BRAF*^{V600E}-mutant metastatic melanoma, either alone or in combination with MEK inhibitors, and there are three BRAF/MEK inhibitors approved for this setting (vemurafenib/dabrafenib, dabrafenib and trametinib, and encorafenib plus binimetinib). In addition, BRAF inhibitors are approved in three specific settings for thyroid cancers: (1) vemurafenib for *BRAF*^{V600E}-mutant advanced radioactive iodine-refractory thyroid cancer, (2) dabrafenib for *BRAF*^{V600E}-mutated metastatic papillary thyroid cancer, and (3) dabrafenib plus trametinib for the treatment of locally advanced or metastatic anaplastic thyroid cancer with a *BRAF*^{V600E} mutation and no satisfactory locoregional treatment options.

Accumulating molecular knowledge in *BRAF*^{V600}-negative SH led to investigations into MEK inhibitors (cobimetinib; trametinib) (Diamond et al. 2016, 2019; Cohen Aubart et al. 2017, 2018) with the cobimetinib phase II clinical trial showing an 89% overall response rate (ORR) by positron emission tomography (PET)-computed tomography (CT) with fluorodeoxyglucose (FDG) tracer irrespective of disease site. As a result, the U.S. FDA granted

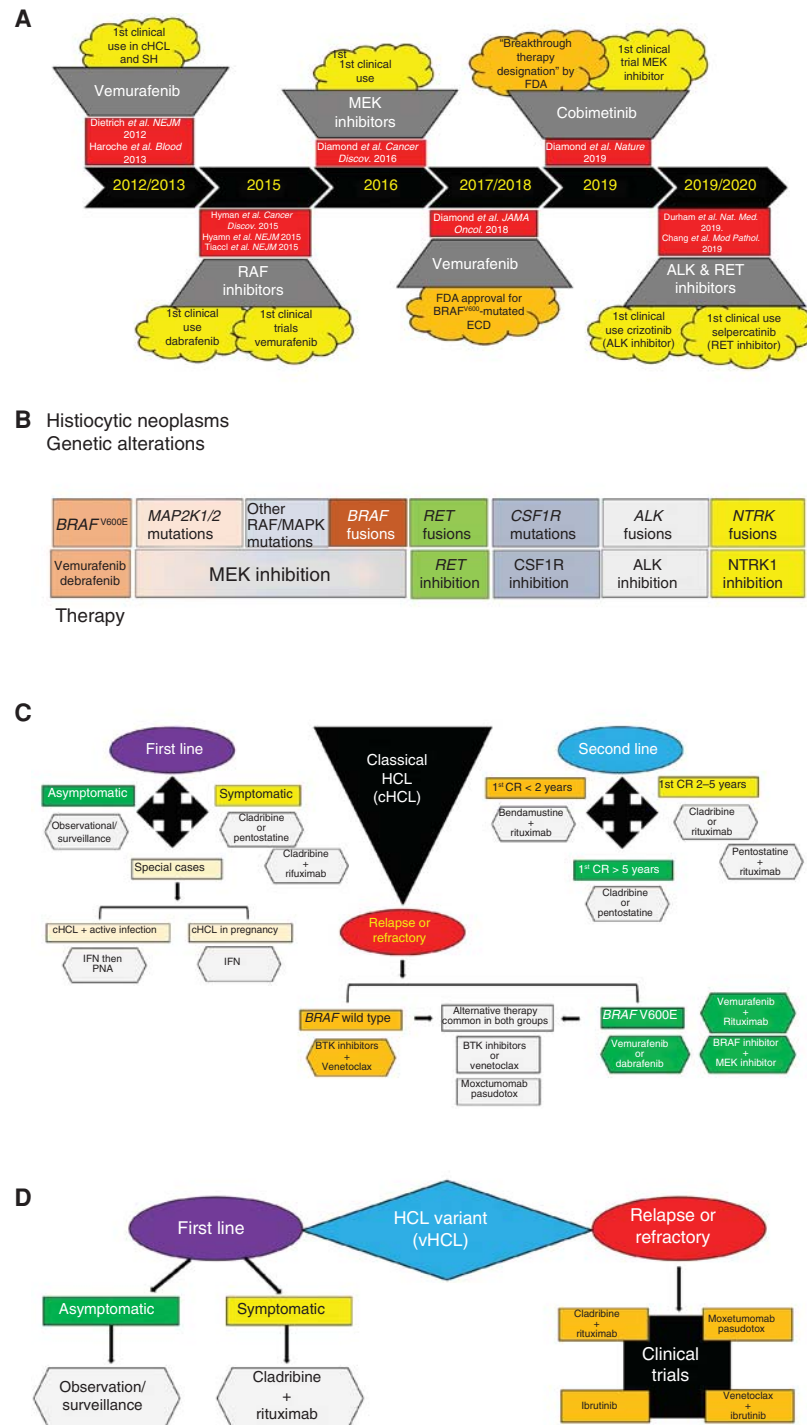


Figure 6. Therapeutic advancements achieved during a progressive molecular age for MAPK-driven hematopoietic neoplasms. (A) Timeline documenting the targeted therapeutic achievements in the histiocytic neoplasms and hairy cell leukemia over the past decade. (B) Diagram summarizing the molecular targeted therapies that have or may demonstrate clinical efficacy in the histiocytic neoplasms. (C) Diagram of a composite therapeutic algorithm for the first-line, second-line, and relapsed/refractory treatment of classical hairy cell leukemia based on current advancements during the molecular age. (D) Diagram of a composite therapeutic algorithm for the first-line and relapsed/refractory treatment of hairy cell leukemia variant founded on current molecular progress. SH, Systemic histiocytoses; ECD, Erdheim–Chester disease; cHCL, classical hairy cell leukemia; vHCL, hairy cell leukemia variant; FDA, Food and Drug Administration; PNA, purine analog; IFN, interferon; BTK, Bruton tyrosine kinase; CR, complete response.

“Breakthrough Therapy Designation” to cobimetinib in the treatment of $BRAF^{V600}$ -negative histiocytoses (Fig. 6A; Diamond et al. 2019).

Moreover, molecular discovery of *ALK*, *NTRK1*, and *RET* fusions, as well as *CSF1R* mutations in diverse histiocytoses, have stimulated investigations into other targeted therapies. These studies have provided in vitro functional data or clinical reports supporting the use of *ALK* inhibitors (crizotinib; alectinib), the *RET*-specific inhibitor selpercatinib, *NTRK* inhibitors, and *CSF1R*-inhibitors (pexidartinib) in SHs (Fig. 6B; Diamond et al. 2016; Lee et al. 2017; Taylor et al. 2018; Chang et al. 2019; Durham et al. 2019). Therefore, the molecular age has provided many exciting therapeutic options for SH patients, but molecularly targeted therapies beyond *BRAF* and *MEK* inhibitors need to be scrutinized in future clinical trials. Also, questions about the optimal dosing, treatment duration, and therapy response assessments require further study, especially in pediatric SH patients.

HAIRY CELL LEUKEMIA

Clinical Presentation

HCL was originally named “leukemic reticuloendotheliosis” and described based on the existence of numerous “hairy” surface projections (Bouroncle et al. 1958). Classical HCL is a rare, mature B-lymphocytic neoplasm comprising 2% of lymphoid leukemias. Meanwhile, vHCL is a similar mature B-lymphocytic neoplasm with variant clinical, cytological, and immunophenotypical features and is 10% as common as cHCL (Swerdlow et al. 2017; Maitre et al. 2019).

Patients with HCL commonly present with weakness, fatigue, bleeding, and fever, and the immunophenotypical profile has emerged as the key component to distinguish cHCL and vHCL. Therefore, HCL is a group of hematological malignancies consisting of cHCL, *IGHV4-34*⁺ cHCL, and vHCL that are morphologically similar with subtle pathological differences but have distinct clinical and laboratory features, immunophenotypes, and molecular characteristics that are compared in Table 2 (Tiacci et al.

2011; Waterfall et al. 2014; Dietrich et al. 2015; Falini et al. 2016; Swerdlow et al. 2017; Thompson and Ravandi 2017; Durham et al. 2017a; Maitre et al. 2019). Furthermore, newly discovered molecular features (Fig. 5; Table 2) assist in further characterization of the pathophysiology and therapeutic options for cHCL and vHCL and have been correlated with other HCL risk stratification parameters as detailed in Table 3 (Arons et al. 2009; Forconi et al. 2009; Xi et al. 2012; Poret et al. 2015; Falini et al. 2016; Swerdlow et al. 2017; Durham et al. 2017a; Maitre et al. 2019).

Pathophysiology

Genomic Profiling

In 2011, WES of cHCL uncovered $BRAF^{V600E}$ as the driving genetic event in ~97% of cHCL (Tiacci et al. 2011; Waterfall et al. 2014; Dietrich et al. 2015; Durham et al. 2017a). Occasional $BRAF^{V600E}$ -negative cHCL patients were shown to have *BRAF* exon 11 mutations ($BRAF^{D449E}$; $BRAF^{F468C}$) (Figs. 1C, 5) that were predicted but not proven to be activating (Tschernitz et al. 2014). Meanwhile, another WES study evaluated vHCL and *IGHV4-34*⁺ cHCL and found activating *MAP2K1* mutations in ~50% of these cases (Figs. 1D, 5; Waterfall et al. 2014; Dietrich et al. 2015; Durham et al. 2017a). Therefore, through MAPK pathway activation by *BRAF* and *MAP2K1* mutations, the neoplastic hairy cells demonstrate increased proliferation and survival (Tiacci et al. 2011; Waterfall et al. 2014; Dietrich et al. 2015; Durham et al. 2017a).

Recently, the discovery of co-occurring genetic alterations that may cooperate with $BRAF^{V600E}$ in cHCL and *MAP2K1* mutations in vHCL have provided further insights into their pathogenesis. Alterations in genes involved in cell cycle regulation (*CCND1*; *CDKN1B/p27*; *CCND3*), NF- κ B pathway and B-lymphocyte differentiation (*KLF2*), the spliceosome (*U2AF1*), and epigenetic regulation (*KMT2C/MLL3*, *KDM6A*, *CREBBP*, *ARID1A*) are potentially key players in the pathophysiology of cHCL or vHCL but will require further study to elucidate their mechanistic roles (Fig. 5A,B;

Table 2. Summary of the characteristic features of classical hairy cell leukemia and hairy cell leukemia variant

Characteristic Features	IGHV4-34- cHCL	IGHV4-34 ⁺ cHCL	vHCL
Laboratory parameters			
Pancytopenia	Present	Absent	Absent
Monocytopenia	Present	Absent	Absent
Absolute neutrophil count	Decreased	Normal	Normal
Leukocytosis	Absent	Present	Present
Soluble IL2 receptor (plasma)	Elevated	Unknown	Normal
Bone marrow aspiration	Dry tap	Easily aspirated	Easily aspirated
Immunophenotypic profile			
CD19	++	++	++
CD20	++	++	++
CD22	++	++	++
FMC7	+	+	+
CD11c	++	++	++
CD103	+	+	+
CD25	+	+	--
CD123	+	+	--
CD200	+	+	--
Annexin A1	+	+	--
Histological features			
Nucleus (Wright–Giemsa)	Oval/kidney-shaped	Oval/kidney-shaped	Round/oval
Nucleolus (Wright–Giemsa)	Absent	Absent	Prominent
Cytoplasm (Wright–Giemsa)	Abundant, pale	Abundant, pale	Abundant, pale
Cellular surface	Thin, often long circumferential projections	Thin, often long circumferential projections	Thin, often long circumferential projections
Infiltration pattern (bone marrow)	Diffuse and/or interstitial	Diffuse and/or interstitial	Diffuse, intrasinusoidal, and/or interstitial
Infiltration pattern (spleen)	Red pulp with effacement of white pulp	Red pulp with effacement of white pulp	Red pulp
TRAP immunocytochemistry	+	+	--
Annexin A1 immunohistochemistry	+	+	--
<i>BRAF</i> ^{V600E} immunohistochemistry	+	--	--
Molecular features			
<i>BRAF</i> ^{V600E} mutations	Present (~97%)	Absent	Absent
<i>MAP2K1</i> alterations	Absent	Present	Present (~50%)
<i>IGHV</i> somatic hypermutation	Frequent	Absent	Rare
<i>IGHV4-34</i> usage	Absent	Present	Frequent
<i>CDKN1B</i> (p27) co-occurring mutations	Present	Unknown	Absent
<i>KLF2</i> co-occurring mutations	Present	Unknown	Absent

Continued



Table 2. *Continued*

Characteristic Features	IGHV4-34- cHCL	IGHV4-34 ⁺ cHCL	vHCL
<i>NOTCH1</i> co-occurring mutations	Rare	Unknown	Absent
<i>NOTCH2</i> co-occurring mutations	Rare	Unknown	Absent
<i>BCOR</i> co-occurring mutations	Rare	Unknown	Absent
<i>KDM6A</i> co-occurring mutations	Rare	Unknown	Frequent
<i>CREBBP</i> co-occurring mutations	Rare	Unknown	Frequent
<i>CCND3</i> co-occurring mutations	Absent	Unknown	Present
<i>U2AF1</i> co-occurring mutations	Absent	Unknown	Present

cHCL, Classical hairy cell leukemia; vHCL, hairy cell leukemia variant; IL2, interleukin 2; CD, cluster of differentiation; IGHV, immunoglobulin heavy chain variable; +, low expression; ++, high expression; –, not detected.

(Waterfall et al. 2014; Clipson et al. 2015; Dietrich et al. 2015; Piva et al. 2015; Falini et al. 2016; Jallades et al. 2017; Durham et al. 2017a; Maitre et al. 2018, 2019).

Gene Expression and Methylation Profiling

The molecular era has refined epigenetic advancements, which has yielded new insights into the peculiar clinical-pathological features of cHCL, whereas few notable studies have investigated IGHV4-34⁺ cHCL or vHCL. The methylation and gene expression profiles in cHCL supported the constitutive activation of the MAPK pathway. Also, hypomethylation and overexpression of genes that inhibit matrix metalloproteinase activity and hypermethylation and underexpression of chemokine receptors critical for B-lymphocyte migration to peripheral lymphoid organ follicles result in neoplastic hairy cells homing to bone marrow, splenic red pulp, and hepatic sinusoids rather than splenic white pulp and lymph nodes. Additionally, cHCL showed hypomethylation and overexpression of genes that stimulate fibronectin production and may contribute to the bone marrow reticulin fibrosis and poor bone marrow aspiration in cHCL (Basso et al. 2004; Arribas et al. 2019). Furthermore, overexpression of

TGFB1, which stimulates neoplastic hairy cells to produce TGF- β , has been posited as a reason for the inhibition of normal hematopoiesis in cHCL (leukopenia; monocytopenia) (Basso et al. 2004; Swerdlow et al. 2017). Meanwhile, other studies support *LST1* (leukocyte transcript 1) and *ACTB* (actin β) genes are enriched in and important for the formation of actin-containing, circumferential “hairy” membrane projections characteristic of HCL (Pettirossi et al. 2015; Falini et al. 2016).

Cellular Origins of cHCL

Through the discovery of *BRAF*^{V600E} in cHCL, a biomarker for the evaluation of the cell of origin of cHCL appeared. Although epigenetic profiling suggests cHCL is derived from transformed, postgerminal center B-lymphocytes (Basso et al. 2004; Falini et al. 2016; Arribas et al. 2019), recent cellular-origin studies reported that the HSCs in cHCL harbor the *BRAF*^{V600E} mutation. Furthermore, xenografting of purified, *BRAF*^{V600E}-mutated HSCs into immunodeficient mice resulted in stable engraftment, which functionally demonstrated the self-renewal capacity of *BRAF*^{V600E}-mutant HSCs in cHCL. However, the transplanted mice did not develop the complete cHCL phenotype, and this



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Table 3. Summary of risk-stratification parameters in hairy cell leukemia that predict a poor prognosis and resistance to purine analogs

Parameters	Measurement
Clinical	
Splenomegaly	>3 cm below the costal margin
Laboratory	
Leukocytosis	$>1 \times 10^9$ cells/L
Hairy cells in peripheral blood	>5 g/L
Beta-2 microglobulin level	>2× the upper limit of normal (High)
CD38 by immunophenotype	Present
Molecular features	
<i>BRAF</i> ^{V600E}	Absent
<i>IGHV</i> mutational status	Unmutated
<i>IGHV4-34</i> rearrangement	Present
Telomere length	Short

cHCL, Classical hairy cell leukemia; vHCL, hairy cell leukemia variant; CD, cluster of differentiation; *IGHV*, immunoglobulin heavy chain variable

raises the question as to whether or not a permissive epigenetic background and acquisition of cooperating genetic alterations are required for phenotypically accurate cHCL (Chung et al. 2014; Falini et al. 2016). Therefore, more functional studies and murine modeling are required to attain a faithful cHCL animal model.

TREATMENT

HCL patients should be treated when symptomatic or when presenting with one of these hematological parameters: hemoglobin <11 g/dL; platelet count <100,000/ μ L; or ANC <1,000 cells/ μ L. Meanwhile, asymptomatic cHCL patients are to be managed with observation/surveillance (Grever et al. 2017; Andrasiak et al. 2018; Maitre et al. 2019).

Chemotherapy

First-line treatment in cHCL patients involves purine analog (PNA) monotherapy (cladribine or pentostatin) because large studies have shown 76%–83% of patients achieved a complete response (CR) and 31%–33% a partial response (PR) (Dearden et al. 2011; Cornet et al. 2014; Else et al. 2015; Maitre et al. 2019). Additionally, a phase II trial of chemoimmunotherapy with cladribine followed by rituximab demonstrated

a CR of 100% and has high efficacy as a first-line therapy. However, special cases do arise and include patients with symptomatic cHCL and a febrile infection requiring infection management prior to PNA treatment (INF- α before PNA). Also, pregnant cHCL patients should be treated with interferon (IFN) (Fig. 6C; Maitre et al. 2019).

Second-line chemotherapy is necessary for the 50% of cHCL patients who relapse during the first 5 yr following first-line chemotherapy, and the consensus on treatment options is stratified based on duration of the first CR (CR > 5 yr; CR = 2–5 yr; CR < 2 yr) (Fig. 6C; Else et al. 2011, 2015; Burotto et al. 2013; Cornet et al. 2014; Chihara et al. 2016; Sadeghi and Li 2018; Maitre et al. 2019).

First-line chemotherapy in symptomatic vHCL patients has no current consensus; however, a combination of cladribine/rituximab is the common treatment used when managing vHCL (Fig. 6D; Kreitman et al. 2013; Maitre et al. 2019).

Targeted Therapy

The molecular era spawned the discovery of *BRAF*^{V600E} in >97% of cHCL and the first molecular target in these neoplasms (Fig. 6A; Tiacci et al. 2011; Dietrich et al. 2012; Falini et al. 2016). The first clinical trials of vemurafenib in re-

lapsed/refractory cHCL showed an ORR approaching 100% with 35%–40% CRs, and the median relapse-free survival was ~19 mo (CR patients) and 6 mo (PR patients) (Tiacci et al. 2015; Falini et al. 2016; Maitre et al. 2019). Thus, the most promising therapeutic options for relapsed/refractory cHCL include targeted therapeutics: BRAF inhibitors in *BRAF*^{V600E}-mutated cHCL (Hyman et al. 2015; Tiacci et al. 2015); BRAF/MEK inhibitor combinations in *BRAF*^{V600E}-mutated cHCL; recombinant immunoconjugates targeting CD22 (moxetumomab pasudotox), which has promising preliminary results in a phase I clinical trial (91% ORR, including 59% with CRs) (Kreitman et al. 2018; Maitre et al. 2019); and the first-in-class Bruton tyrosine kinase (BTK) inhibitor ibrutinib approved for treating relapsed/refractory B-cell malignancies (e.g., chronic lymphocytic leukemia [CLL]/small lymphocytic leukemia [SLL]) (Fig. 6C; Byrd et al. 2015; Sarvaria et al. 2016; Maitre et al. 2019). Furthermore, clinical trials utilizing moxetumomab pasudotox, ibrutinib, and ibrutinib/venetoclax are suggested for the treatment of relapsed/refractory vHCL (Fig. 6D; Bohn et al. 2017; Maitre et al. 2019). Future directions for molecular treatments in HCL should include trials combining BRAF/MEK inhibitors in cHCL, MEK inhibitors in vHCL and *IGHV*4-34⁺ cHCL, CDK4/6 inhibitors in *CCND3*-mutated vHCL, and ibrutinib in both cHCL and vHCL (Maitre et al. 2019).

Mechanisms of Vemurafenib Resistance

As in metastatic melanoma, the dramatic clinical efficacy of vemurafenib in refractory/relapsed cHCL has now shown relapse, which suggests the development of resistance mechanisms (Tiacci et al. 2015; Falini et al. 2016). However, knowledge of the vemurafenib resistance mechanisms in cHCL has just begun to emerge. For example, resistance in two patients treated in a phase II cHCL clinical trial was secondary to acquired mutations in *KRAS/NRAS* following treatment with vemurafenib, which induce reactivation of the MAPK pathway through *RAF1* (Trunzer et al. 2013; Tiacci

et al. 2015; Durham et al. 2017a). Another patient experienced complete, de novo vemurafenib resistance, and genomic analysis of his pre-treatment sample revealed a gain-of-function mutation in *IRS1* (*IRS1*^{P1201S}) that functionally activated PI3K-AKT signaling. Furthermore, this patient had heterozygous deletions of *NF1* and *NF2* that functionally induced vemurafenib resistance in vitro (Whittaker et al. 2013; Shalem et al. 2014; Durham et al. 2017a). Nonetheless, more functional and sequencing studies are required to better elucidate and catalog cHCL vemurafenib-resistance mechanisms.

Frequency and Implication of BRAF Mutations in Hematologic Malignancies outside of HCL and SH

In contrast to the very high frequency of *BRAF*^{V600E} mutations in HCL and SH, BRAF mutations are far rarer in more common hematologic malignancies. Outside of HCL and SH, recurrent BRAF mutations have been reported in 4%–10% of multiple myeloma (Andrulis et al. 2013; Lohr et al. 2014), 2%–5% of patients with CLL (Jebaraj et al. 2013; Leeksa et al. 2019), and 1%–2% patients with acute myeloid leukemia (AML) (Papaemmanuil et al. 2016). To date, the only formal evaluation of the use of BRAF inhibition in BRAF-mutant hematologic malignancies outside of HCL and SH was the use of vemurafenib for nine patients with *BRAF*^{V600E}-mutant multiple myeloma as part of the VE-BASKET Study (Raje et al. 2018). The best confirmed overall response rate in this cohort was 33% with two patients achieving partial remissions. In the single published report of an AML patient treated with a BRAF inhibitor, a refractory AML patient was treated with combined dabrafenib/trametinib with only a very transient response (Wander et al. 2017). It is important to note that in CLL and myeloma, BRAF mutations are often subclonal. Moreover, in CLL, BRAF mutations frequently occur outside of the V600 residue (most commonly at the G466, D594, and K601 residues). Given the use of vemurafenib and dabrafenib for *BRAF*^{V600E}/K-mutant diseases specifically, the therapeutic implications of BRAF inhibition with approved

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BRAF inhibitors has an unclear role in CLL and has never been evaluated.

CONCLUDING REMARKS

Molecular advancements over the past decade helped unravel the molecular pathophysiology, cellular origins, and therapeutic targets in the MAPK-driven hematological neoplasms. Since the description of *BRAF*^{V600E} in SH and cHCL (Badalian-Very et al. 2010; Tiacci et al. 2011; Haroche et al. 2012), there has been an onslaught of molecular progress linking diverse kinase alterations activating MAPK, PI3K-AKT, and RTK signaling to the histiocytic neoplasms, as well as *MAP2K1* mutations in *IGHV4-34*⁺ cHCL and vHCL (Figs. 1–5). These recent discoveries have refined the current pathological understanding of the histiocytoses as clonal, myeloid neoplasms with constitutive activation of MAPK and PI3K-AKT signaling and confirm HCL belongs to the MAPK-driven, hematological neoplasms. Furthermore, molecular progress has re-imagined therapeutic options for patients with these disorders (Fig. 6). Additionally, many biomarkers are now available that have enhanced our biological understanding of the cellular pathogenesis and ontogeny of the SH and HCL. However, our functional genomic conceptualization of the molecular pathogenesis and histogenesis of SH and HCL are just emerging with many aspects still enshrouded in mystery requiring systematic dissection with studies employing single-cell molecular and epigenetic analyses, as well as pre-clinical models. Finally, as the molecular era continues to unfold, future studies to elucidate why HCL and SH share common MAPK alterations but are phenotypically distinct neoplasms with differing clinical presentations, pathophysiology, and suspected cellular origins are a desperately needed dimension of investigation.

ACKNOWLEDGMENTS

O.A.-W. is supported by 1 R01 CA201247-01A1 from the National Cancer Institute (NCI) of the National Institutes of Health (NIH). B.H.D. is supported by the Fellow Scholar Award in Basic/Translational Research from the American

Society of Hematology (ASH), a grant from the Erdheim–Chester Disease Global Alliance Foundation, and K08 CA218901 from the NCI/NIH. R.C. is supported by U.S. Department of Defense Peer Reviewed Cancer Research Program Career Development Award W81XWH1910167.

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Cold Spring Harb Perspect Med published online June 29, 2020

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